LUNG EPITHELIAL CELL RESPONSES TO HOST DEFENCE PEPTIDE LL-37

by

Jelena Pistolic

B.Sc., University of British Columbia, 2004

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(MICROBIOLOGY AND IMMUNOLOGY)

UNIVERSITY OF BRITISH COLUMBIA

April 2007

Abstract

LL-37, the only cathelicidin family member of cationic host defence peptides in humans, has been shown to mediate multiple immunomodulatory effects and as such is thought to be an important component of innate immune responses. A growing body of evidence suggests that LL-37 affects lung mucosal responses to pathogens through altered regulation of cell migration, proliferation, wound healing, and cell apoptosis. The aforementioned functions are consistent with LL-37 playing a role in regulating lung epithelial inflammatory responses, a role that is, however, not yet clearly defined. The effect of LL-37 on cytokine and chemokine production and signalling regulation in airway epithelial cells were investigated here. In this report I demonstrated that LL-37 induced protein release and transcriptional up-regulation of IL-6 and GRO-α in airway epithelial cells. LL-37 stimulation activated NF-κB signalling, which was further demonstrated herein to robustly regulate production of IL-6 and GRO-α in airway epithelial cells. With respect to receptor regulation, LL-37-stimulated IL-6 release was demonstrated to be regulated by EGFR and a G-protein coupled receptor, possibly FPRL-1. Furthermore, EGFR was shown to regulate LL-37-stimulated GRO-α. MAP kinase pathways mediated partial signalling regulation of both IL-6 and GRO-α release, while PI3K did not regulate this response. The evidence presented in this report shows that LL-37 is indeed a potent regulator of lung immune responses mediated through activation of multiple signalling pathways.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AP-1	activator protein-1
ARDS	acute respiratory distress syndrome
ASF	airway surface fluid
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BALF	bronchio-alveolar lavage fluid
BzATP	benzyl-ATP
C/EBP	CCAAT/enhancer binding protein
CF	cystic fibrosis
COPD	chronic obstructive pulmonary disease
CREB	cAMP-responsive element binding protein
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ENA-78	epithelial-derived neutrophil activating protein 78
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular regulated protein kinase
FBS	foetal bovine serum
FPRL-1	formyl peptide like receptor-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GM-CSF	granulocyte macrophage-colony stimulating factor
G-CSF	granulocyte-colony stimulating factor
GRO-α	growth regulated oncogene alpha
HBD	human beta defensin
HBE	human bronchial epithelial cells
HDL	high-density lipoproteins
IκB	Inhibitor of nuclear factor-κB
IKK	IκB kinase
IL-	interleukin
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAPK	mitogen activated protein kinase
MCP-1	macrophage chemotattractant protein 1
MCP-3	monocyte chemotactic protein 3
MEM	minimum essential medium
MIP-1α	macrophage inflammatory protein alpha
MIP-1β	macrophage inflammatory protein beta
NAP-2	neutrophil activating peptide-2
NF-κB	nuclear factor κB
NF-IL-6	nuclear factor IL-6
NHBE	normal human bronchial epithelial cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PI3K	phosphatidylinositol-3-kinase

PKC	protein kinase C
PMN	polymorphonuclear cells
PMSF	phenylmethylsulfonyl fluoride
Ptx	pertussis toxin
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TBST	Tris buffered saline + 0.1% Tween 20
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha

Acknowledgements

1. Introduction

1.1   Lung innate immunity

The lung mucosal surface is constantly bombarded by microbes and particulate matter
delivered through respiration. Considering the amount of microbial stimulation, infections and
colonization of the mucosa occur relatively infrequently, due to highly evolved host defense
mechanisms (1). The primary lines of lung mucosal defense are the innate immune mechanisms
of the epithelial cell layer, and the interacting hematopoietic cells (neutrophils, macrophages,
and eosinophils, for example) (2, 3). In particular, airway epithelial responses of the conducting
airways, which will be discussed in more detail herein, have several described mechanisms of
host defence: 1) physical sequestration and removal of microorganisms; 2) recruitment of
hematopoietic cells that can modulate or trigger the inflammatory response; and 3) secretion,
into the mucus, of an array of compounds with a variety of activities (*e.g.* cytokines,
chemokines, host defence peptides, proteases) (3).

The first line of defence of respiratory epithelium is its barrier function (3). The luminal
surface of airways is lined with a pseudostratified layer of highly differentiated polarized
epithelial cells. The ciliated apical cell surface is covered with airway surface fluid (ASF),
which is composed of a periciliary serous layer and an outer viscous mucus layer (3, 4). The
majority of inhaled particulate matter and potential pathogens that become trapped in the mucus
layer are expelled through mucociliary movement and cough (1, 3, 4). The basal surface of the
polarized epithelium overlays the basement membrane, in close proximity to underlying
capillaries and various neighboring cells, including muscle cells and leukocytes in the
connective tissue matrix (4). Cell polarization is a means of constraining the availability of
receptors to an enormous dose of stimuli delivered through respiration, and represents yet

1

another method of avoiding excessive stimulation/inflammation. Furthermore, cell polarization allows for tightly controlled ion, water and cell-derived molecule flow between the compartments, which subsequently allows a coordination between luminal stimuli and mucosal responses (4).

The importance of epithelial cells in lung immunity well exceeds their role as an effective physical barrier to invading pathogens: a wealth of studies indicate that these cells are also active participants and modulators of inflammatory responses that aid to maintain the lower airways free of pathogens (2, 5, 6). Airway epithelial cells express a variety of receptors such as the Toll-like receptors (TLRs 1-9), various growth factor receptors [*e.g.* epidermal growth factor receptor (EGFR)], and an abundance of cytokine/chemokine receptors [*e.g.* tumor necrosis factor (TNF) and interleukin-1 (IL-1) receptors], through which a cornucopia of signalling pathways [such as Nuclear factor κB (NF-κB), Phosphatidylinositol-3-kinase (PI3K) and Mitogen-activated protein (MAP) kinases] can be activated. Activation of these pathways governs the course of inflammation and infection in the airway mucosa, and commonly results in production and release of a variety of compounds that modulate lung mucosal immunity. Various cytokines, chemokines, proteases, and cationic antimicrobial peptides produced by the epithelial cells, and by infiltrating leukocytes, have immunomodulatory and/or antimicrobial functions in lung immune responses (2). Thus it is clear that the function of the respiratory epithelium is diverse. It can orchestrate the onset of inflammation in response to luminal stimulation by potential pathogens and environmental irritants, and it can modulate the extent of the immune response by recruiting effector immune cells, which can in turn activate/modulate epithelial cell responses through paracrine stimulation (4).

1.2   Effectors of lung innate immunity – cationic peptides

Airway epithelial cells produce a variety of compounds with antimicrobial and immunomodulatory properties through which they modulate host defence responses. Lysozyme, lactoferrin, secretory leukoprotease inhibitor (SLPI), cytokines/chemokines, human β defensins (HBD), and LL-37 (cathelicidin), among others, regulate processes such as polymorphonuclear cell (PMN)/macrophage influx and activation, wound healing, angiogenesis, microorganism removal, and activation of adaptive immunity (3, 5, 7). The role of HBDs and cathelicidins in lung innate immunity is of particular interest since these molecules may modulate host inflammatory responses in addition to potentially participating in direct killing of pathogens under appropriate physiological circumstances (8, 9). Both HBDs and cathelicidin LL-37 are classified as cationic peptides, the structural characteristics of which are summarized below. An increase in HBD and LL-37 concentrations in the lung fluid has been described in many lung conditions such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), pneumonia, and sarcoidosis (7, 10, 11). Furthermore, it has been shown that knock-out mice deficient in mouse β-defensin 1 (mBD-1) show delayed clearance of *Haemophilus influenzae* from the lung (12), while viral vector mediated over-expression of LL-37 results in augmentation of host defences in a CF xenograft model, and in murine models of septic shock and pneumonia (7, 13, 14). Induction of these cationic peptides in various lung diseases and inflammatory conditions, as well as their ability to affect lung host defence responses, suggests the relevance of these peptides in lung immunity.

1.3   Chemokines and cytokines - role in regulation of lung responses

Cytokines and chemokines released into the lung airways are an important part of lung host defenses. Numerous lung diseases such as COPD, acute respiratory distress syndrome (ARDS), and cystic fibrosis have characteristic patterns of induced cytokines and chemokines

(11, 15-17). These small molecules, such as CXCL-8 [interleukin-8 (IL-8)], interleukin-6 (IL-6), CXCL-1 [growth-regulated oncogene α (GRO-α)], interleukin-1β (IL-1β), to name a few, are incidentally the very ones known to be potent modulators of lung innate responses. In addition to being produced by infiltrating leukocytes, cytokines and chemokines can be produced by airway epithelial cells. Lung epithelial cells have been shown to produce a variety of cytokines and chemokines such as IL-8, TNF-α, IL-1β, IL-6, CCL3 [macrophage inflammatory protein-1α (MIP-1α)], GRO-α, CXCL-5 [epithelial-derived neutrophil-activating protein-78 (ENA-78)], and granulocyte-macrophage colony-stimulating-factor (GM-CSF), among others, thus displaying an ability to regulate initiation, amplification and progression of inflammatory responses (18, 19).

Cytokines are multifunctional molecules that orchestrate various aspects of lung immunity. TNF-α and IL-1β are potent pro-inflammatory regulators of airway epithelial cells. Both have been shown to induce release of various chemokines and cytokines from airway epithelial cells, including IL-8, IL-6, GM-CSF, CCL20 [macrophage inflammatory protein-3α (MIP-3α)] and GRO-α (6, 18, 20). They also affect other processes in the lung such as apoptosis, tissue remodeling and wound healing (1, 19, 21). Another example of such molecules, IL-6, is a pleiotropic cytokine and an important player in inflammatory responses (22). Depending on other factors present at the site of infection, IL-6 has been described as having pro- and anti-inflammatory properties in the lung (23, 24). Numerous studies have shown that IL-6 can regulate neutrophil recruitment, clearance and apoptosis (24, 25), inflammatory cytokine and chemokine expression in monocytes/macrophages (26), and T cell adhesion, migration and apoptosis (27, 28), thus modifying the transition from PMN infiltration (innate response) to mononuclear cell influx (adaptive response initiation).

Chemokines are structurally-related low molecular weight peptides involved primarily in the recruitment of various effector cells to infection foci. They can be divided into two groups,

according to the position of first two conserved cysteine residues, CXC and CC chemokines. CXC chemokine family members pertinent to lung immunity include CXCL-8 (IL-8), CXCL-1 (GRO-α), CXCL-2 (GRO-β), CXCL-5 (ENA-78), CXCL-7 [neutrophil activating protein-2 (NAP-2)], among many (4, 29). These chemokines predominantly regulate, with variable potency, chemotaxis and activation of neutrophils and other granulocytes (4, 29). Furthermore, members of the ELR-CXC subgroup of CXC chemokines, of which IL-8 and GRO-α are prominent examples, have been shown to mediate angiogenesis – a shared feature of this group (6, 30). The CC family chemokines which include CCL5 (RANTES), CCL3 (MIP-1α), CCL20 (MIP-3α), and CCL2 [monocyte chemoattractant protein-1 (MCP-1)], to name a few, are mainly involved in chemoattraction of macrophages, monocytes, and T cells (4, 6, 29)

1.4   Signalling pathways that regulate airway cell responses – NF-κB

Signalling regulation of cytokine and chemokine induction is complex. It involves TLR pathway activation by microbial antigens (a common mechanism of activation of NF-κB transcription factor), growth factor receptor signalling (activation of MAP kinases, PI3 kinases, etc.), as well as signalling induced by other cytokines/chemokines themselves (such as IL-1β, TNF-α acting through their respective receptors). NF-κB activation and signalling is one of the most common signalling pathways that regulate numerous complex events of lung innate and adaptive immune responses. The NF-κB family of transcription factors consists of 5 members: p65 (RelA), Rel-B, c-Rel, p50/p105 (NF-κβ1), and p52/p100 (NF-κB2) which can form homodimers/ heterodimers that are sequestered in the cytoplasm by any of the seven members of the Inhibitor of nuclear factor-κB (IκB)-family proteins, including Iκ-Bα and Iκ-Bβ (31, 32). The best studied NFκB subunit dimer is the p65/p50 heterodimer, which is activated most commonly in lung innate immune responses. The p65/p50 heterodimer is associated with Iκ-Bα

5

in the cytosol (33). Alternatively, p50 and p52 homodimers have been shown to repress gene expression as these proteins lack transactivation domains (32, 33). Binding of the NF-κB subunits to Iκ-B proteins *via* their Rel homology domains prevents the complex from translocating to the nucleus, binding to DNA, and initiating transcription of a variety of pro- and anti-inflammatory molecules (31-33). In order to alleviate negative regulation of the pathway mediated by Iκ-Bα, these molecules need to be phosphorylated; phosphorylation of Iκ-Bα is followed by ubiquitination, and subsequent proteosomal degradation (32, 34). Once inhibition by Iκ-Bα is relieved, NF-κB dimers are free to translocate to the nucleus. Phosphorylation of Iκ-B molecules is mediated by IκB kinases (IKK), which can in turn be activated by multiple upstream pathways, including mitogen-activated protein kinase kinase kinase 1 (MEKK1), MEKK3, transforming-growth-factor-β-activated kinase (TAK1) and NF-κB-inducing kinase (NIK) (34). NF-κB signalling is an important mediator of innate immune responses in many lung diseases. Activation of NF-κB signalling has been shown in ARDS, where it potentially mediates lung injury and neutrophil infiltration (31). Furthermore, increased activation of NF-κB has been demonstrated in asthma and CF, which are characterized by an increase of pro-inflammatory cytokines/chemokines and adhesion molecules (31, 35).

1.5   Cationic peptides – structural classification and functional diversity

Cationic peptides, often referred to as antimicrobial peptides, are evolutionarily conserved innate immunity effectors ubiquitously expressed in all forms of life ranging from bacteria, fungi, plants, insects, amphibians, to mammals (3, 36). These amphiphilic peptides are between 12 to 50 amino acids in length and have a net positive charge of +2 to +9 due to an excess of positively charged lysine and arginine residues (3, 36, 37). Though very diverse in sequence, these peptides can be classified in four groups based on 3-D structures they form upon interaction with biological membranes: 1) β-sheets with 2 or 3 disulphide bonds (*e.g.* β-

6

defensins), 2) amphipatic α-helices (*e.g.* cathelicidin LL-37), 3) extended molecules (*e.g.* indolicidin), and 4) loops with a single disulphide bond (*e.g.* bactenecin) (Structures shown in Figure 1) (3, 36, 37). All cationic peptides are derived from a larger precursor molecule by proteolysis, and can include a variety of post translational processing methods (*e.g.* glycosylation), depending on the specific peptide (38). Initial studies of cationic peptide function indicated that some peptides are potent microbicidal agents, with a wide spectrum of specificity ranging from bacteria, fungi, and parasites to viruses (36, 37), although this function can be inhibited at physiological mono- and divalent cation concentrations. In addition, many of these molecules also have been shown to exhibit an amazing range of other functions, such as innate immune modulating, wound healing, anti-cancer, and chemotactic activities [See (3, 36, 37, 39) for reviews]. Due to the multitude of functions that these "antimicrobial peptides" were determined to possesses, a new, more descriptive name has been coined for these molecules – host defence peptides (8, 40). Cathelicidins comprise a well-described and diverse family of host defence peptides, and together with defensins represent the two major cationic peptide groups found in mammals (41).

1.6   Cathelicidins

Cathelicidins are expressed in many mammalian species including dogs, cattle, mice, rats, monkeys, pigs, horses, rabbits, to name a few (41-43). However, several recent studies have identified cathelicidin-related peptides in various species of fish (hagfish, trout, and salmon) and in chickens (44-48). Thus the distribution of cathelicidins is not restricted to mammalian species only, suggesting that they might be part of an ancient and highly relevant host defence mechanism.

Cathelicidins were discovered in early 1990's, and they were named based on similarity of their pro-region to a porcine peptide cathelin, a putative cysteine-proteinase inhibitor (49).

7

Members of this peptide group are synthesized as precursor molecules consisting of an N-terminal signal sequence (29-30 amino acids) and a conserved pro-region (cathelin domain – 99-114 residues), with a highly variable C-terminal sequence that is the host defence peptide (12-100 amino acids) (Figure 1.) (43, 49). These inactive pro-peptides are often stored in neutrophil granules and require proteolytic processing to release the mature, biologically active C-terminal portion of the peptide (42, 49, 50). Even though numerous bovine and porcine cathelicidins have been described, the sole member of this cationic peptide group in humans is hCAP-18/LL-37 (51-53).
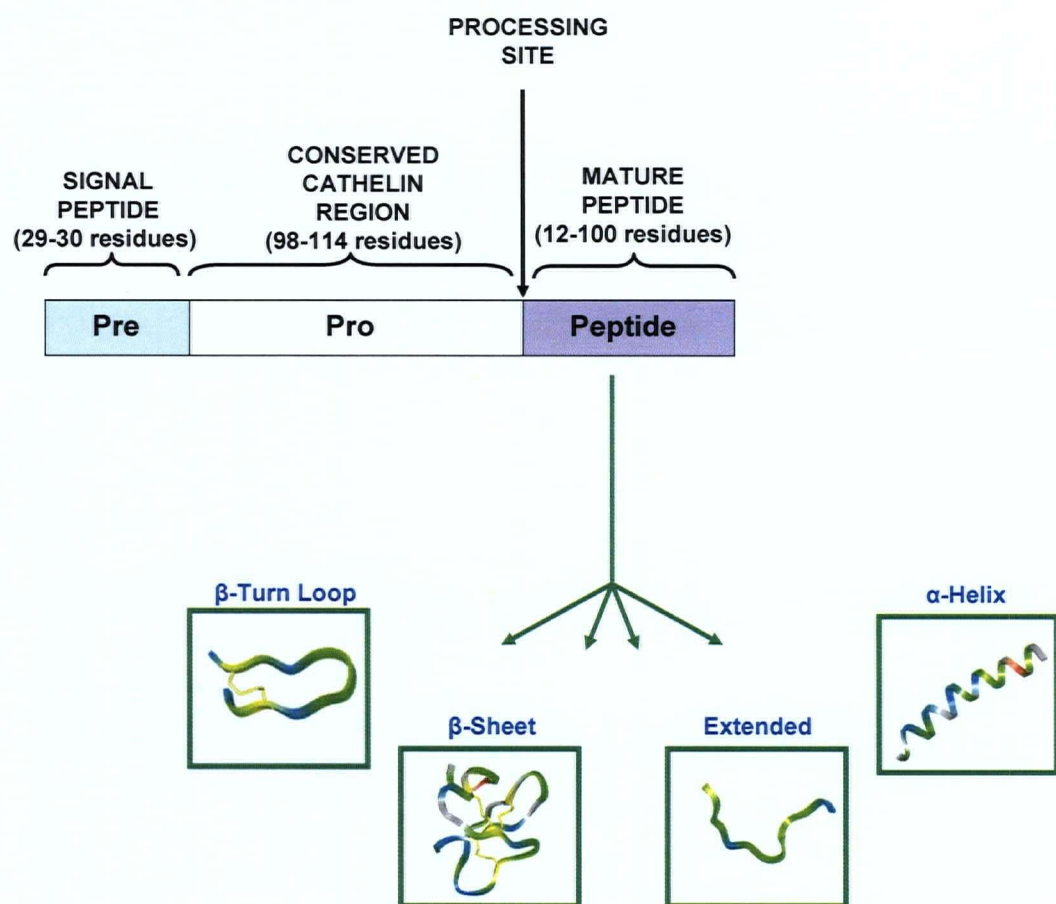
Figure 1. **Basic structures of cathelicidin peptides**
Schematic depiction of cathelicidin pre-pro-peptide sequence with proposed secondary structures that processed mature peptides can fold into. Modified from (36, 49).

1.7   hCAP-18/LL-37 – the only human cathelicidin

hCAP-18, an 18 kDa peptide, has the characteristic cathelicidin pre-pro-peptide structure described in Figure 1 (54). hCAP-18, upon exocytosis from neutrophils is processed by proteinase-3 (serine protease from azurophilic granules) to yield the biologically relevant host defence peptide LL-37 (55). LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) is a 37 amino acid peptide, predicted to form an amphiphatic helix structure (56). In addition to proteinase 3 action, other processing mechanisms of hCAP-18/LL-37 have been described. For example, in sweat, differential processing of hCAP-18 by the kallikrein-family of serine proteases results in a variety of shorter peptides (57, 58), while in seminal plasma, hCAP-18 is processed by a prostate-derived gastric acid protease gastricsin to generate the ALL-38 peptide, hypothesized to play a role in preventing post-coital infections (59).

LL-37 is expressed in a variety of cell types and tissues in the human body. It is abundant in neutrophils and was also shown to be expressed by macrophages, monocytic cells, B cells, subsets of T cells, and skin mast cells (60-64). LL-37 expression was furthermore demonstrated in gastric, colon, gut, gingival and sinus epithelial cells, as well as squamous epithelium of many sites (skin, mouth, tongue, esophagus, cervix, and vagina) under numerous inflammatory conditions (20, 52, 65-68).

In the lung, expression of LL-37 has been demonstrated in respiratory epithelial cells of proximal and distal airways, as well as the cells of submucosal glands (11, 64, 67). Under a number of pathological lung conditions such as cystic fibrosis, sarcoidosis, and chronic obstructive pulmonary disease, studies have reported an increase of LL-37 in bronchioalveolar lavage fluid (BALF) of patients (10, 11, 64, 67). Similar increases in LL-37 concentrations were also observed in BALF experiments of premature and mature infants with infections (69). The widespread distribution of this peptide, and its induction during inflammation suggest that it plays a highly relevant, though not yet fully understood role in lung immune defence

9

mechanisms.

1.8   LL-37 and modulation of innate immune responses

Although quite small in size and simple in structure, LL-37 has been shown to have multiple immunomodulatory properties. One of these widespread functions is chemotaxis, through which LL-37 exerts remarkable effects on innate and adaptive immune responses. LL-37 is directly chemotactic for neutrophils, eosinophils, monocytes, T cells, and mast cells (70-72). LL-37 has been demonstrated to be a potent inducer of chemokines in a variety of cells. For example, peptide stimulation induced IL-8 release in human primary monocytes (73), PBMCs (Yu, Mookherjee and Hancock, submitted for publication), and airway smooth muscle and epithelial cells (74, 75). Alternatively, MCP-1, MCP-3, MIP-1α, and MIP-1β gene expression upregulation has been shown in human primary monocytes (73, 76), however protein release was only demonstrated for MCP-1 (76). In addition, LL-37 has been shown to induce mast cell degranulation, and release of leukotriene B4 and histamine, which can further modulate cell influx to the site of infection by increasing vascular permeability (62, 77). Another well described function of LL-37 is the ability to neutralize endotoxin (LPS) and its TLR-4-mediated effects in both *in vivo* (rodent models) and *in vitro* studies (monocytes, macrophages, neutrophils) [(40, 78, 79); Yu *et al.*, 2007, submitted for publication]. Furthermore, LL-37 has been demonstrated to influence dendritic cell (DC) maturation and DC-mediated T cell polarization (80), while recent studies have shown that LL-37 can influence dendritic cell activation by modulating TLR activation *via* TLR ligand stimulation (81). In keratinocytes, LL-37 exerts a multitude of functions. Namely, it has been shown to induce cell migration, proliferation, and wound healing (82, 83). In addition, LL-37-stimulated keratinocytes were shown to release a variety of pro- and anti-inflammatory cytokines and chemokines, such as IL-6, IL-10, IL-18, IL-20, CCL5, IP-10, MCP-1, and MIP-3α (82, 84).

A prominent increase in peptide concentration during lung inflammation and its ability to improve clearance of pulmonary pathogens in overexpression studies, indicate that LL-37 is very likely a significant effector molecule of lung immune responses (13, 14). In *in vitro* studies, LL-37 has been shown to induce IL-8 production from bronchial epithelial cells in a dose- and time-dependent manner (73, 74, 85). Furthermore, LL-37 is able to induce proliferation, migration and wound healing in bronchial epithelial cells in a serum-dependent manner at relatively low concentrations (86). Peptide concentration and serum are important factors with respect to LL-37-mediated effects on lung epithelial cells. For example, LL-37 was shown to induce caspase-3-mediated apoptosis in bronchial epithelial cells in a serum-dependent manner (78, 86, 87). The high concentrations of LL-37 shown in these studies to induce apoptosis in epithelial cells might occur at inflammation foci due to neutrophil and macrophage degranulation. At concentrations higher than 30 μg/ml in the absence of all sera, LL-37 can induce apoptosis after 24 h in lung epithelial cell lines (87). In the presence of FBS, >50 μg/ml of peptide is required to observe this effect. Human serum, or rather the HDL component, completely protected epithelial cells from apoptosis induced by LL-37 at aforementioned concentrations (87). Human serum also inhibited IL-8 release and LL-37 uptake into lung epithelial cells, while these processes were observed in FBS or no serum conditions (87).

1.9   LL-37 – receptor-mediated immunomodulation?

All of the aforementioned LL-37 effects share a common theme with respect to signalling pathway regulation. LL-37-mediated effects on target cells have been proposed to be induced by a receptor-mediated interaction. For example, Lau and colleagues (85) have shown that LL-37 interaction with alveolar epithelial cell membranes and subsequent uptake are necessary in order to observe the peptide-mediated IL-8 induction. To date, three receptors have been proposed to mediate the effects of LL-37 on various cells, namely – epidermal factor

receptor (EGFR), a G-protein coupled formyl peptide receptor-like-1 (FPRL-1), and a purinergic P2X$_7$ receptor (72, 74, 88). However, regulation by these proposed receptors cannot account for many peptide-mediated responses. The discovery and identification of additional novel LL-37 receptor(s) is an ongoing effort in the LL-37 research community.

One of the first proposed LL-37 receptors reported to be involved in chemotactic activity of the peptide towards various hematopoietic cells was FPRL-1 (72). In addition to chemotaxis, FRPL-1 was also shown to be involved in LL-37-mediated angiogenesis and artheriogenesis by mediating the responses of endothelial cells (89). In alveolar epithelial cells, Lau and colleagues (85) demonstrated by binding assays that there are two classes of receptors with which LL-37 can interact, a high affinity receptor, and a low affinity receptor identified to be FPRL-1. FPRL-1 is a G-protein coupled receptor (GPCR), and is therefore sensitive to pertussis toxin (Ptx) inhibition; however, Ptx is not a specific inhibitor for FPRL-1, as functions of other GPCRs can also be impaired by this molecule. A number of studies show that Ptx-sensitive events induced by LL-37 are not necessarily regulated through FPRL-1. For example, mast cell chemotaxis, IL-8 production in keratinocytes, as well as lung epithelial wound closure and cell proliferation are regulated by a GPCR (Ptx sensitive) other than the FPRL-1, since a specific FPRL-1 agonist WKYMV, used as a positive control, failed to induce these effects (70, 86, 90).

Furthermore, LL-37 has been shown to regulate IL-1β processing and release in LPS-primed monocytes *via* the purinergic receptor P2X$_7$ (88). P2X$_7$ is a member of P2X family of nucleotide-gated receptors most efficiently activated by ATP and Benzyl-ATP (BzATP) (88). The potential involvement of P2X$_7$ in LL-37-mediated inhibition of neutrophil apoptosis and LL-37-induced extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation and IL-8 release in airway smooth muscle cells has also been described (75, 78, 91).

Tjabringa and colleagues (74) were the first to show LL-37-mediated trans-activation of EGFR, subsequent regulation of ERK1/2 phosphorylation and IL-8 release in bronchial

12

epithelial cells. The aforementioned activation was proposed to occur through an indirect step where the peptide activates an ADAM metalloprotease that in turn cleaves EGFR ligands from the membrane, thus allowing receptor activation. Thereafter, EGFR was proposed to regulate wound closure and proliferation in lung epithelial cells, and proliferation, migration, and IL-8 induction in keratinocytes (84, 86, 90).

1.10   Signalling pathways implicated in LL-37-stimulated effects

Another common theme in LL-37-induced responses is the activation of MAP kinases. LL-37-mediated ERK1/2 and p38 MAP kinase pathway activation has been demonstrated in mast cells, monocytes, lung epithelial cells, and keratinocytes (73, 74, 77, 82, 86). LL-37 has been demonstrated to induce phosphorylation of EGFR, as well as the ERK1/2, p38, and c-Jun N-terminal kinase (JNK) MAP kinases in bronchial epithelial cells, while inhibitor studies have shown that EGFR, ERK1/2 and p38 all regulate IL-8 release from these epithelial cells (73, 74). Similarly, ERK1/2 activation was determined to regulate IL-8 release from airway smooth muscle cells (75). As compared to p38, ERK1/2 has been demonstrated to have a more prominent role in regulation of wound healing, migration and proliferation of bronchial epithelial cells (86). Moreover, regulation of ERK1/2 and p38 has been described with respect to LL-37-mediated IL-18 release from primary human keratinocytes, and in mast cell degranulation (77, 82). Therefore, ample evidence suggests that LL-37-induced activation of MAP kinases is a central regulatory mechanism for a variety of responses in numerous cells and tissues.

In addition to MAPK regulation, other signalling pathways have been implicated in the regulation of LL-37 responses. Our laboratory (Yu, Bowdish and Hancock submitted for publication, and unpublished results) has demonstrated that LL-37 induces the transient degradation of phosphorylated Iκ-Bα, and subsequent p50 and p65 NF-κB subunit translocation

13

into the nucleus in PBMCs. Li and Hancock (unpublished results) have also shown that the NF-κB pathway is involved in regulation of chemokine production in primary human neutrophils. Alternatively, the PI3K pathway has been shown to be involved in regulation of LL-37-induced IL-8 release in human PBMCs (Yu, Mookherjee and Hancock, submitted for publication), as well as in apoptosis inhibition of human neutrophils (78). Moreover, in endothelial cells, LL-37-mediated activation of the FPRL-1 receptor required for angiogenesis was also shown to induce activation of the PI3K, MAPK, protein kinase C (PKC), and NF-κB pathways (89).

1.11   Rationale

Lung innate immunity depends heavily on cytokines and chemokines to regulate inflammation and fight infections. Bronchial epithelial cells have been shown to produce a variety of cytokines and chemokines such as IL-8, IL-6, MIP-1α, GRO-α, ENA-78, and GM-CSF, among others, consequently displaying an ability to regulate initiation, amplification and progression of inflammatory responses (18, 19). Defensins have already been shown to be potent regulators of these responses, in that they were demonstrated to regulate MCP-1, IL-8, IL-6, GM-CSF, and ENA-78 in epithelial cell lines and primary cells (92). Considering the fact that there is an increase in LL-37 and HBD production in BAL fluids of patients with inflammatory and infectious lung diseases that is frequently associated with an increase in several cytokines, and chemokines (*e.g.* IL-8, IL-6), it was of interest to investigate if LL-37 could regulate airway epithelial cell responses and induce additional cytokines or chemokines.

1.12   Hypothesis and experimental goals

The aim of this study was to further describe the effects of LL-37 on lung epithelial cell responses. LL-37 has previously been shown to induce IL-8 release from bronchial epithelial cells, but induction of other cytokines and chemokines has not been examined to date, even

though LL-37 is a potent inducer of these small effector molecules in other cell types. As these molecules are critical regulators of lung innate responses, I hypothesized that there were other chemokines and cytokines induced by LL-37 stimulation of bronchial epithelial cells. Furthermore, I wanted to determine the signalling pathways through which LL-37 induces production of these cytokines and chemokines, including the ERK1/2 and p38 MAP kinases, and the EGFR pathway (known to be regulated by LL-37 in lung epithelial cells). In addition, I aimed to examine if there are any other signalling pathways involved in cytokine/chemokine regulation, which have not to date been described to be activated in LL-37-stimulated airway epithelial cells, particularly NF-κB.

2. Materials and Methods

2.1   Cell culture

The SV40-transformed, immortalized human bronchial epithelial cell line 16HBE4o-
was a gift from Dr. D. Gruenert (University of California, San Francisco, CA) (93). 16HBE4o-
cells were cultured in Minimum Essential Medium (MEM) with Earle's salts (Life Technologies
Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life
Technologies Invitrogen) and 2 mM L-glutamine (Life Technologies Invitrogen). 16HBE4o-
cells were routinely cultured to 85-90% confluence in 100% humidity and 5% CO_2 at 37° C and
were used between passages 5 and 15.

When indicated, 16HBE4o- cells were polarized in Corning 6-well Transwell plates
(PET Membrane Clear Inserts, 0.4 µM pore size; Corning, Acton, MA). Cultures were started
with 1 x 10^5 cells, and a total of 1.5 ml of MEM with antibiotics (1% penicillin/streptomycin;
Life Technologies Invitrogen) in each Transwell insert, and with 2.6 ml of media in each well of
the 6-well Transwell plate. Media changes were done every other day, until the cells were
polarized. Cell polarization was assessed by the increase and stabilization of transepithelial
resistance (Ω) using the Milicell Electrical Resistance System (Milipore, Billerica, MA). On
average, 10-14 days were required to polarize 16HBE4o- cells, with recorded transepithelial
resistance values ranging from 210 to 260 Ω/cm^2.

Clonetics primary normal human bronchial epithelial (NHBE) cells were purchased from
Cambrex BioScience Inc. (Walkersville, MD, USA) and were cultured and maintained in BEGM
bronchial epithelial growth medium (Cambrex BioScience Inc.), according to manufacturer's
instructions. BEGM is a basal medium (Cambrex BioScience Ltd.) supplemented with bronchial
epithelial cell SingleQuots growth factors and supplements (Cambrex BioScience Ltd.), as a
serum substitute optimized for growth and appropriate differentiation of these primary cells.

SingleQuots includes human epidermal growth factor (EGF), triiodothyronine, bovine pituitary extract, epinephrine, transferrin, insulin, hydrocortisone, gentamycin/amphotericin, and retinoic acid. According to manufacturer's instructions, cells were cultured in complete BEGM media to 85-90% confluence in 100% humidity and 5% CO_2 at 37° C, and were used between passages 3 to 10.

Human tumorigenic alveolar epithelial cell line A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A549 cells were routinely cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine (Life Technologies Invitrogen, Burlington, ON) in 100% humidity and 5% CO_2 at 37° C.

2.2   Peptides, inhibitors, antibodies, and other reagents

LL-37 (sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was synthesized by *N*-(9-fluorenyl)methoxy carbonyl chemistry (F-moc) chemistry at the Nucleic Acid/Protein Synthesis Unit at the University of British Columbia. Human recombinant epidermal growth factor (EGF), and interkeukin-1β (IL-1β) were purchased from Research Diagnostics (Flanders, NJ). The EGFR inhibitor AG1478, pertussis toxin (Ptx), the NF-κB inhibitor parthenolide, and the FPRL-1 agonist WKYMV were purchased from Calbiochem (San Diego, CA, USA). The NF-κB inhibitor Bay 11-7085 was purchased from Biomol International (Plymouth Meeting, PA), while the PI3K inhibitor (LY294002) was purchased from Cell Signalling Technology, Inc. (Mississauga, ON, Canada). An inhibitor of $P2X_7$, KN-62, and the $P2X_7$ receptor agonist Benzyl-ATP (Bz-ATP) were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Mouse monoclonal antibody anti-phospho-Iκ-Bα, rabbit anti-total-Iκ-Bα, anti-p50, anti-

p65, anti-phospho CREB and HRP-conjugated goat anti-rabbit antibodies were purchased from Cell Signalling Technology Inc (Danvers, MA, USA). The mouse monoclonal anti-GAPDH antibody and anti-mouse IgG antibody were purchased from Amersham (Piscataway, NJ, USA). LPS from *Pseudomonas aeruginosa* strain H103 was purified free of proteins and lipids using the Darveau-Hancock method as previously described (94). *Salmonella typhimurium* flagellin was obtained from Cedarlane Laboratories Ltd. (Burlington, ON, Canada).

2.3   Detection of cytokines and chemokines

16HBE4o- cells were seeded in 24-well plates (Sarstedt Inc., Montreal, QC) in 10% FBS/2 mM L-glutamine MEM medium at a concentration of 1.5 x 10^5 cells/ml (1 ml per well), and cultured to confluence at 37°C and 5% CO_2 for 2 days. Unless otherwise indicated, cells were treated in MEM supplemented with 2% FBS and 2 mM L-glutamine. When confluent, cells were washed with 1 ml per well of serum-free MEM, and 1 ml of 2% FBS/2 mM L-glutamine MEM was added to each well. If chemical inhibitors were used, they were first dissolved in 2% FBS MEM, and then added to the cells after the washing step. Cells were rested for 1 hour, and were subsequently treated with the peptide/other stimuli. After the desired incubation time supernatants were collected and stored at -20° C in aliquots.

Primary NHBE cells were seeded at 5 x 10^4 cells/ml (1ml per well) in 24-well plates (Sarstedt Inc.) in complete BEGM, and were cultured for 2 days at 37° C and 5% CO_2. When confluent, cells were washed with 1 ml per well of Hank's Balanced Salt Solution (Cambrex BioScience Ltd.), and 1 ml of complete BEGM (without EGF) was added to each well. Cells were rested for 1 hour, and were subsequently treated with the peptide or other stimuli. After the desired incubation time, supernatants were collected and stored at -20° C in aliquots.

A549 cells were seeded at 1 x 10^5 cells/ml (1 ml/well) in 24-well plates (Sarstedt Inc.) in

complete DMEM, and were cultured for 24 hours. When confluent, cells were washed with 1 ml per well of serum-free media, and then 1 ml of 2% FBS/2 mM L-glutamine DMEM was added to each well. Cells were rested for 1 hour, and were subsequently treated for 6 hours. Supernatants were collected and stored at -20° C in aliquots.

The concentrations of IL-8, IL-6, GRO-α, RANTES, TNF-α, IL-1β, IP-10, CCL22, MIP-1α, and IL-10 in cell culture supernatants were measured using capture ELISA as per the manufacturer's suggestion (GRO-α, IP-10, CCL22, and Rantes ELISA from R&D Minneapolis, MN; TNF-α, IL-1β, IL-10 and IL-6 ELISA from eBioscience San Diego, CA; IL-8 and MIP-1α ELISA from BioSource International, Camarillo, CA).

2.4   RNA isolation and real-time PCR

16HBE4o- cells were seeded in 15 x 30 mm plates (Sarstedt Inc.) at 2.8×10^5 cells/ml, and cultured to sub-confluence at 37° C and 5% CO_2 and in 10%FBS/2mM L-glutamine MEM media for 2 days. On the day of treatment, media was removed from the plates, and sub-confluent cells were washed with 1 ml/plate of serum-free MEM, and then 1 ml of 2% FBS/2 mM L-glutamine supplemented MEM was added. After the medium change, cells were rested for 1 hour, and subsequently treated and incubated over a time-course of 1, 2, and 4 hours. Total RNA was isolated using the RNAeasy Mini kit (Qiagen, Maryland, USA), as per the manufacturer's instructions, and was DNAse treated using the RNAse-Free DNAse kit (Qiagen, Maryland, USA). After RNA integrity was confirmed by 1% agarose electrophoresis and spectrophotometry, 1 μg of total RNA was converted to cDNA as per the manufacturer's instructions using the SuperScript III Platinum CellsDirect Two-step qRT-PCR kit with SyberGreen (Invitrogen), with a non-template negative control for each of the treatments. Quantitative real-time PCR was done using the ABI Prism 7000 system, with a Dissociation

Curve program, at 50° C for 2 minutes, 95° C for 2 minutes, then 50 cycles at 95° C for 15 seconds and 60° C for 30 seconds. GAPDH was used as the house keeping gene control. The PCR was conducted in a 12.5 μl reaction volume containing 2.5 μl of 1/10 diluted cDNA template, and 10 μl of a master mix (0.25 μl of Rox, 6.25 μl of UDG, 0.5 μl of 10 μM primer mix, and 3 μl of nuclease-free H_2O per reaction). A melting curve was performed to ensure that any product detected was specific to the desired amplicon. Fold changes were calculated after normalization to endogenous GAPDH and using the comparative Ct method (95). PCR primers used in this study are listed in Table 1.

Table 1. Primer sequences used in this study.

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
IL-6	AATTCGGTACATCCTCGACGG	GGTTGTTTTCTGCCAGTGCC
IL-8	CACCACACTGCGCCAACAC	CTTCTCCACAACCCTCTGCAC
GRO-α (CXCL-1)	GCCAGTGCTTGCAGACCCT	GGCTATGACTTCGGTTTGGG
RANTES (CCL5)	GAGCTTCTGAGGCGCTGCT	TCTAGAGGCATGCTGACTTC
GAPDH	GAAACTGTGGCGTGATGG	GTCGCTGTTGAAGTCAGAGG

2.5   Western immunoblotting

16HBE4o- cells were seeded in 15 x 30 mm plates (Sarstedt Inc. Montreal, QC) at 2.8 x 10^5 cells/ml, and cultured to sub-confluence in MEM (with 10%FBS/2mM L-glutamine) at 37° C and 5% CO_2 for 2 days. On the day of treatment, medium was removed from the plates, and sub-confluent cells were washed with 1 ml/plate of serum-free MEM. Cells were incubated with 1ml of serum-free MEM (with 2 mM L-glutamine) for 4 hours, followed by a medium change of 1ml of 2% FBS/2 mM L-glutamine MEM and an immediate treatments of cells. Before lysis,

cells were washed with ice cold PBS, with 1mM sodium vanadate. Samples were solubilized on ice with 200 μl of NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich), scraped, and were subsequently centrifuged. The protein concentration of sample lysates was quantified using the BCA Protein Assay Kit (Pierce). Lysates were denatured at 95° C for 10 minutes, and were resolved on a 12% SDS-PAGE, followed by subsequent transfer at 100 V for 1 hour to Immuno-blot PVDF membranes (Bio-Rad).

Membranes were probed with specific antibodies at 1/1000 dilution in TBST (20 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% skimmed milk powder (TBST/milk) or 5% BSA (TBST/BSA), for incubation times specified by the manufacturer. Incubation with an appropriate secondary antibody (HRP-conjugated goat anti-mouse or anti-rabbit antibodies) was performed following the primary antibody incubation. Finally, the membranes were developed using the ECL chemiluminescence peroxidase substrate (Sigma-Aldrich), according to the manufacturer's instructions. The blots were re-probed with an anti-GAPDH antibody to ensure that equal amounts of protein were loaded

2.6   Statistical Analysis

Student's t-test was performed in order to determine the statistical significance of results, with $p \leq 0.05$ being considered statistically significant. Values shown are expressed as mean ± standard deviation or standard error as indicated in the Results section and Figure legends.

3. Results

3.1   LL-37-induced cytokines in airway epithelial cells – LL-37-mediated IL-6 release is regulated by NF-κB signalling

3.1.1   Introduction

With respect to cytokine release and regulation, a lot is known about LL-37-mediated effects in hematopoietic cells and keratinocytes (40, 84, 96). In airway epithelial cells however, LL-37 has only been shown to induce IL-8, *via* EGFR and MAPK regulation (73, 74). As discussed in the Introduction, cytokines and chemokines are potent and important effectors of lung innate immunity, therefore it is of interest to examine if LL-37 can alter regulation and affect the expression of these small molecules, other than IL-8. In this chapter, I assessed induction of cytokines by LL-37 using the human bronchial epithelial cell line 16HBE4o- and primary normal human bronchial epithelial (NHBE) cells as biological models. Regulation of particular LL-37-induced cytokines was determined with the use of specific chemical inhibitors and ELISA assays, while gene expression and signalling pathway regulation were assessed *via* qPCR and Western blotting.

3.1.2   LL-37 stimulated release of IL-6 cytokine from bronchial epithelial cells

To determine which cytokines are induced by the host defence peptide LL-37, 16HBE4o- or primary NHBE cells were stimulated with LL-37 at 50 μg/ml or 100 ng/ml of LPS for 6 hours. Cytokines released into the cell culture supernatants were assayed by ELISA. LL-37 induced a significant amount of IL-6 ($p<0.05$), without inducing detectable IL-1β, TNF-α, or IL-10 from 16HBE4o- cells (Figure 1A). LL-37 stimulation of primary NHBE cells resulted in a comparable response to that of the 16HBE4o- cell line, with IL-6 as the only measured cytokine induced by the peptide treatment ($p<0.05$) (Figure 1B).

A.

B.
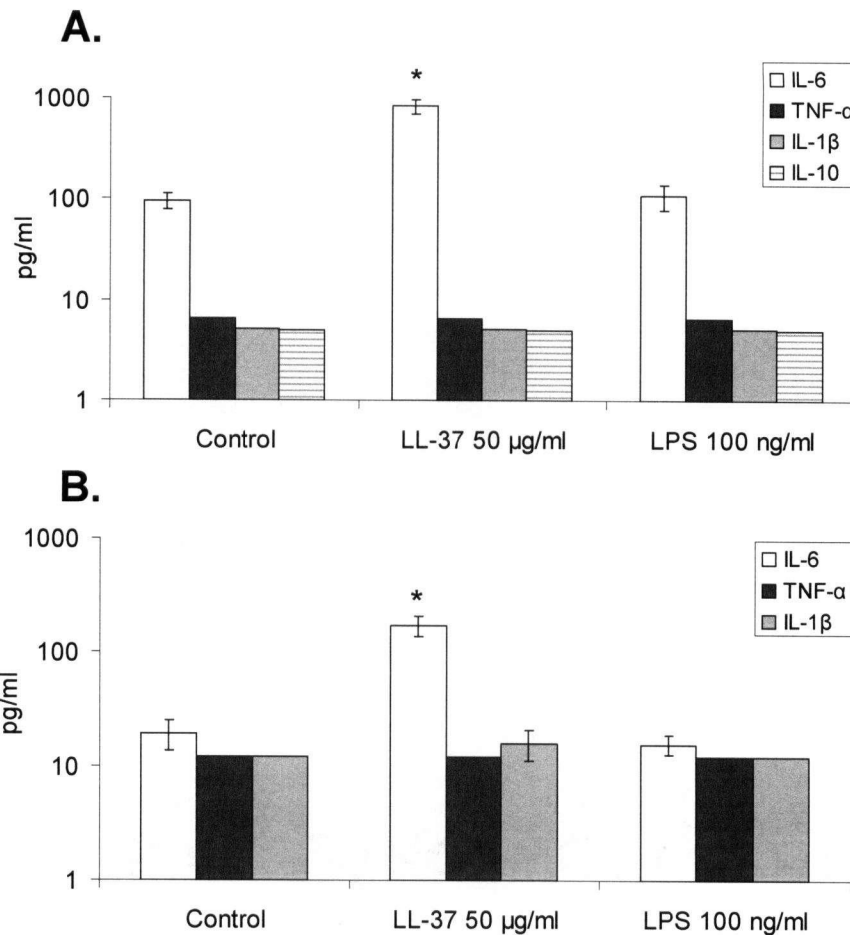
Figure 1. **LL-37 induced IL-6 release from 16HBE4o- cells and primary normal bronchial epithelial cells.**
Confluent **A.** 16HBE4o- or **B.** primary NHBE cell monolayers were treated with 50 μg/ml LL-37 or 100 ng/ml LPS for 6 hours. IL-6, TNF-α, IL-1β, and IL-10 release in cell culture supernatants was determined *via* ELISA. Results are expressed as mean values of cytokine produced (Y scale-log scale) ± standard deviation of three independent experiments. Paired sample Student's t-test analyses were used to compare LL-37 and LPS-treated samples to control treatments (* $p<0.05$ denotes significance relative to untreated controls).

Thus, LL-37-induced IL-6 release was a specific peptide-mediated response and not just a reflection of a general inflammatory response, *e.g.* due to contaminating endotoxin, nor a secondary effect of autocrine stimulation with IL-1β/TNF-α. Similar results were observed in both primary cells and a bronchial cell line, therefore corroborating the physiological relevance of obtained results.

LL-37 mediated IL-6 release from both 16HBE4o- cells and primary NHBE cells in a dose-dependent and time-dependent manner, as shown in Figure 2. A statistically significant

accumulation of released IL-6 was seen with as low as 10 µg/ml of LL-37 ($p < 0.05$), with a rather modest 1.7-fold change (Figure 2A). A more prominent increase was observed with 25 and 50 µg/ml of the peptide with, respectively, 4 and 9 fold up-regulation of IL-6 production and release. In 16HBE4o- cells, LL-37 stimulation induced IL-6 release at the earliest time point evaluated, while the peak of accumulation was observed between 6 and 10 hours (Figure 2B). Comparable results were obtained with primary normal human bronchial epithelial cells (Figure 2C, 2D), further demonstrating the physiological relevance of these results. Primary NHBE cells were even more sensitive to LL-37 stimulation with significant IL-6 release upon stimulation with as low as 2.5 µg/ml of LL-37 ($p < 0.05$), while a 17-fold increase in induction was observed with 25 µg/ml of LL-37 (Figure 2C). As described for the 16HBE4o- cell line, LL-37-stimulated primary cells released IL-6 in a time-dependent manner (Figure 2D), with significant accumulation of the cytokine as early as 6 hours post-stimulation, and a continuing build-up of IL-6 until 24 hours post-stimulation.
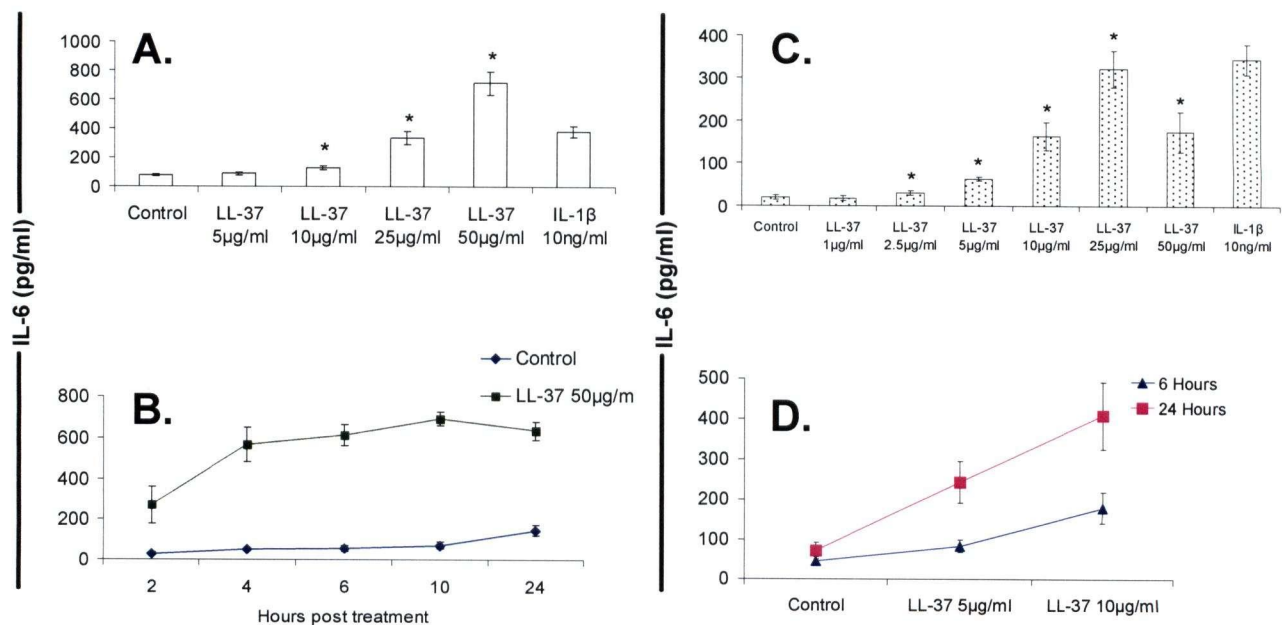
Figure 2. LL-37 induces IL-6 release from the 16HBE4o- bronchial epithelial cell line and primary NHBE cells in a dose-dependent and time-dependent manner.
Confluent 16HBE4o- monolayers were treated with **A.** LL-37 at 5, 10, 25, and 50 µg/ml concentrations, or IL-1β at 10 ng/ml for 6 hours, or **B.** 50 µg/ml of LL-37 for 2, 4, 6, 10, and 24 hours respectively. Confluent primary NHBE monolayers were treated with **C.** 1, 2.5, 5, 10, 25,

and 50 µg/ml of LL-37 or IL-1β for 6 hours, or **D.** 5 and 10 µg/ml of LL-37 for 6, 24, or 48 hours. IL-6 release in culture supernatants was monitored *via* ELISA. Results are expressed as mean values ± standard deviation of three independent experiments (* p<0.05 denotes significance relative to untreated controls).

To evaluate the effect of LL-37 on regulation of IL-6 at the transcriptional level, 16HBE4o- cells were treated with 50 µg/ml of LL-37 or 10 ng/ml of IL-1β over a time-course of 1, 2, and 4 hours. Total RNA was extracted from treated samples, and interleukin-6 transcriptional profile was subsequently assessed by qPCR (Figure 3).
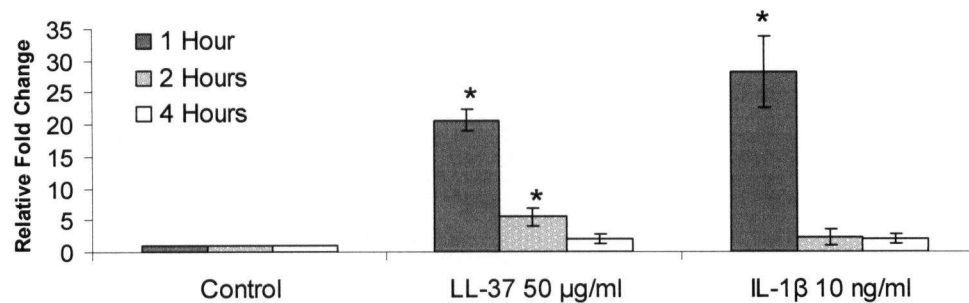
Figure 3. **LL-37 enhances IL-6 gene expression in 16HBE4o- cells.**
Sub-confluent 16HBE4o- cells were exposed to LL-37 at 50 µg/ml, or IL-1β at 10 ng/ml (as a positive control) for 1, 2 and 4 hours. Transcriptional regulation was assessed *via* quantitative RT-PCR. Fold changes (Y-axis) were normalized to GAPDH and are relative to the gene expression in un-stimulated cells (normalized to 1) using the comparative Ct method (refer to Materials and Methods for details). Results are expressed as mean values ± standard deviation of three independent experiments (* p<0.05 designates significance relative to the respective untreated control at a given time-point).

LL-37 induced IL-6 gene expression up-regulation in a time-dependent manner in 16HBE4o- cells. As shown in Figure 3, 50 µg/ml of LL-37 (the dose that induced a peak of protein release) increased IL-6 transcription by 20 ± 4.9 fold within an hour of peptide addition. This up-regulation decreased to 5.5 ± 1.4 fold at 2 hours (p<0.05), and ultimately approached near-control levels at 4 hours post-stimulation (p>0.05). A similar pattern of transcriptional up-regulation was observed with 10 ng/ml of IL-1β, used as a positive control, which induced a peak of transcription at 1 hour (28 ± 5.6 fold, p<0.05), and a subsequent decrease at 2 and 4

25

hours post-stimulation to 2.37 ± 1.26 and 1.98 ± 0.78 fold, respectively (p> 0.05).

LL-37-mediated IL-8 induction has been demonstrated in primary bronchial epithelial cells (74), bronchial epithelial cell lines (73, 74), and an alveolar epithelial cell line A549 (87). Thus, I wanted to examine if, in addition to inducing IL-6 in 16HBE4o- cell line and primary NHBE cells (Figure 2), LL-37 would induce IL-6 release from the A549 alveolar cell line as well (Figure 4). As expected, LL-37 induced a significant release of both IL-6 and IL-8 from 16HBE4o- cells, as did the positive control IL-β (Figure 4A). While LL-37 induced a significant release of IL-8 in A549 cells (Figure 4 B.), it did not induce any IL-6, however. IL-1β significantly increased both IL-6 and IL-8 from A549 cells, while LPS only affected IL-8 release. Therefore, LL-37-mediated induction of IL-6 is an event exclusive to bronchial (airway) epithelial cell, but not to cells of alveolar origin (limited to the A549 cells), which might indicate that the peptide has slightly different modes of activity depending on a particular site in the lung (alveoli *versus* bronchi).
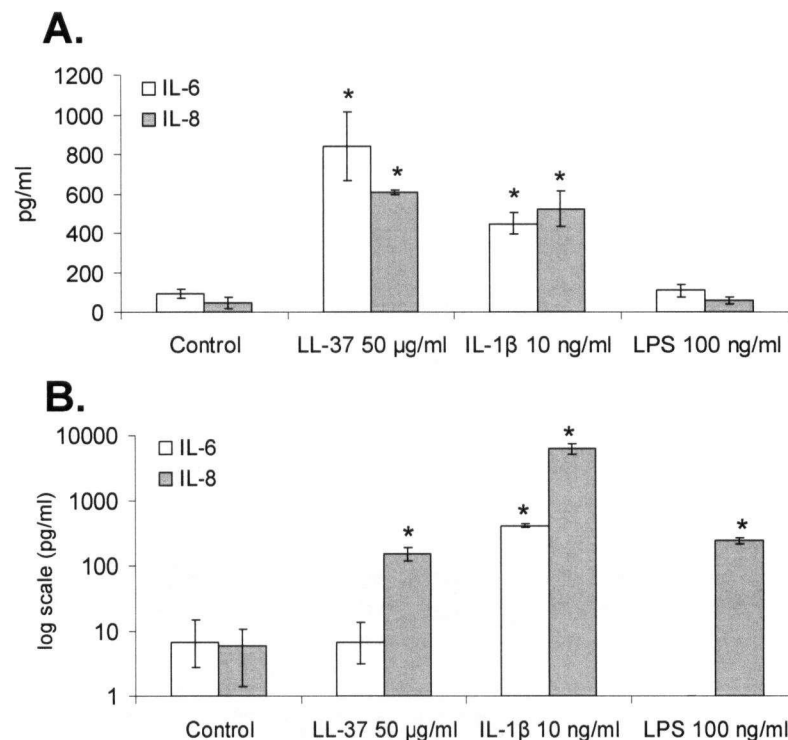
Figure 4. LL-37 indu ces both IL-6 and IL-8 in 16HBE4o- cells (bronchial epithelial cells), but does not induce IL-6 in A549 cells (alveolar epithelial cells).

Confluent monolayers of **A.** 16HBE4o- cells, or **B.** A549 cells were treated with LL-37 at 50 µg/ml, IL-1β at 10 ng/ml (as a positive control), or 100 ng/ml of LPS for 6 hours. IL-6 and IL-8 release in cell culture supernatants was monitored by ELISA. Results are expressed as mean values ± standard deviation of three independent experiments (* $p<0.05$ denotes significance relative to untreated control samples).

3.1.3 LL-37 induced biased IL-6 and IL-8 release in the apical chamber of polarized 16HBE4o- cells

A specific property of 16HBE4o- cells is that they can form polarized monolayers on semi-permeable support Transwell plates, where the cells develop tight and gap junctions upon prolonged culture, and the differentiated monolayer tightly regulates molecule traffic between distinct apical and basolateral compartments (93). *In vivo*, this structured organization of the epithelium controls water, ion and protein content of the air surface fluid (ASF), limits cell exposure to environmental irritants, and also, through compartmentalized receptor expression, regulates immune receptor activation by the vast antigen stimulation delivered in the air (4). Furthermore, a polarized release of cytokines and chemokines into either of the distinct compartments aids in the directionality of the immune response, for example: apical antigen or pathogen stimulation could cause an increase of apically secreted chemokines to attract, into the affected area, effector cells such as neutrophils and macrophages, to help limit the infection.

As such, these polarized 16HBE4o- cells should represent the cellular structure expected *in vivo* in the bronchi (93). Using polarized 16HBE4o- cells as the model, I aimed to examine the effect of stimulating the cells *via* each of the compartments, and observe how or if this differential stimulation would affect cytokine production. A polarized release of cytokines or chemokines into the apical chamber (equivalent to the bronchial luminal space) or baso-lateral chamber (equivalent to the cell basement membrane or tissue side of the monolayer), would provide information on how LL-37 acts with respect to modulating bronchial cell responses to invading pathogens delivered by inhalation.

27

Therefore, polarized 16HBE4o- cells were stimulated with 50 μg/ml of LL-37 *via* the apical or the baso-lateral chamber for 6 hours, and the levels of IL-6 and IL-8 released into the cell supernatants were determined by ELISA (Figure 5).
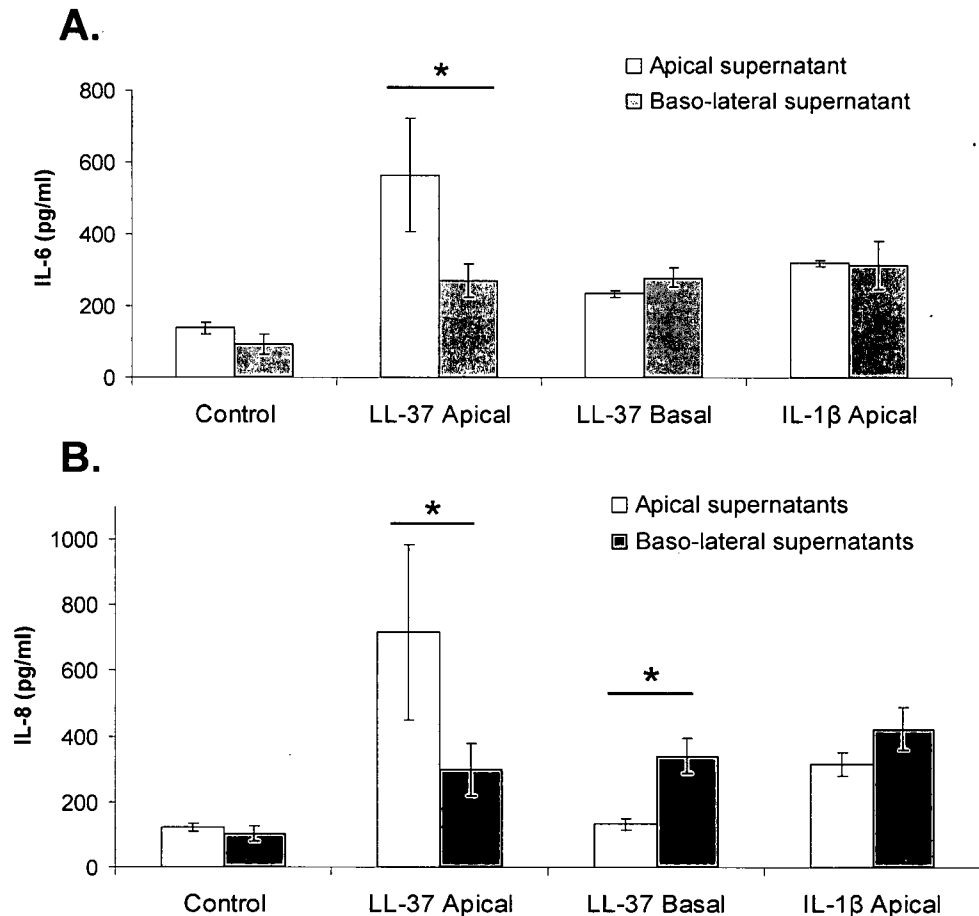
Figure 5. **LL-37 stimulation of polarized 16HBE4o- cells induced differential IL-6 and IL-8 secretion pattern in apical and baso-lateral compartments.**
Polarized 16HBE4o- cell monolayers established on Transwell plates, as described in Materials and Methods, were stimulated with 50 μg/ml of LL-37 *via* the apical or the basolateral chamber; IL-1β (10 ng/ml) treatment was applied to the apical chamber. Cells were incubated for 6 hours, and apical and baso-lateral chamber supernatants were assayed for **A.** IL-6 and **B.** IL-8 release by Elisa individually. Results are expressed as averages ± SEM of three independent experiments (* $p < 0.05$).

Peptide treatment in the apical chamber of polarized 16HBE4o- cells resulted in the maximal IL-6 release overall, with substantially more cytokine detected in the apical chamber

28

(p<0.05) (Figure 5A). Conversely, basolateral stimulation by LL-37 induced less total IL-6 release, and there was no significant difference between the chambers. Similar results were observed with IL-8 release (Figure 5B), with apical peptide stimulation inducing the most chemokine overall, while significantly more IL-8 was released into apical chamber (p<0.05). On the contrary, baso-lateral LL-37 stimulation induced an IL-8 release in the basolateral chamber only. In summary, the location of LL-37 stimulation in polarized bronchial epithelial cells determined the magnitude and the polarity of the induced cytokine/chemokine release.

3.1.4   LL-37-mediated IL-6 release is regulated by serum type and concentration

As previously described in the Introduction, serum is an important factor in LL-37-mediated effects on lung epithelial cells. As Lau and colleagues from our lab (2006) have demonstrated, the presence of human serum (10%), in contrast to FBS, can inhibit LL-37 uptake, reduce cell cytotoxicity, and abolish IL-8 release. Therefore, it was of interest to examine if serum type (pooled human serum or FBS), or serum concentration (2 or 10%) affected LL-37-induced IL-6 release from 16HBE4o- cells. To test this, 16HBE4o- cells were treated with 50 μg/ml of LL-37 or 10 ng/ml of IL-1β in medium containing either 2 or 10% FBS, or 2 or 10% pooled human serum, for 6 hours; subsequent IL-6 release in cell culture supernatants was assayed *via* ELISA (Figure 6A). The strongest IL-6 induction was observed in 2% FBS medium (8 fold, p<0.05), followed by 10% FBS (2 fold, p<0.05). Alternatively, 10% human serum prevented any significant LL-37-induced IL-6 release, while 2% human serum induced a modest 2 fold increase that was nevertheless significant (p<0.05) (Figure 6A).

To make the experimental conditions even more physiologically relevant, I aimed to examine if substituting pooled human serum for pooled human plasma would affect LL-37-induced IL-6 release from 16HBE4o- cells (Figure 6B). 16HBE4o- cells were treated as described above, in medium containing either 2 or 10% pooled human serum, or 2 or 10%

pooled human plasma for 6 hours, and released IL-6 was quantified by ELISA. A significant increase of IL-6 was observed with both 2% serum and plasma conditions (p<0.05), while with 10% of either in the medium no significant IL-6 was detected. However, no significant difference was observed when either human serum or human plasma was used. The aforementioned experiments demonstrate that conditions in which LL-37 interacts with bronchial epithelial cells can exceedingly affect the extent to which the peptide can induce IL-6 release from these cells.
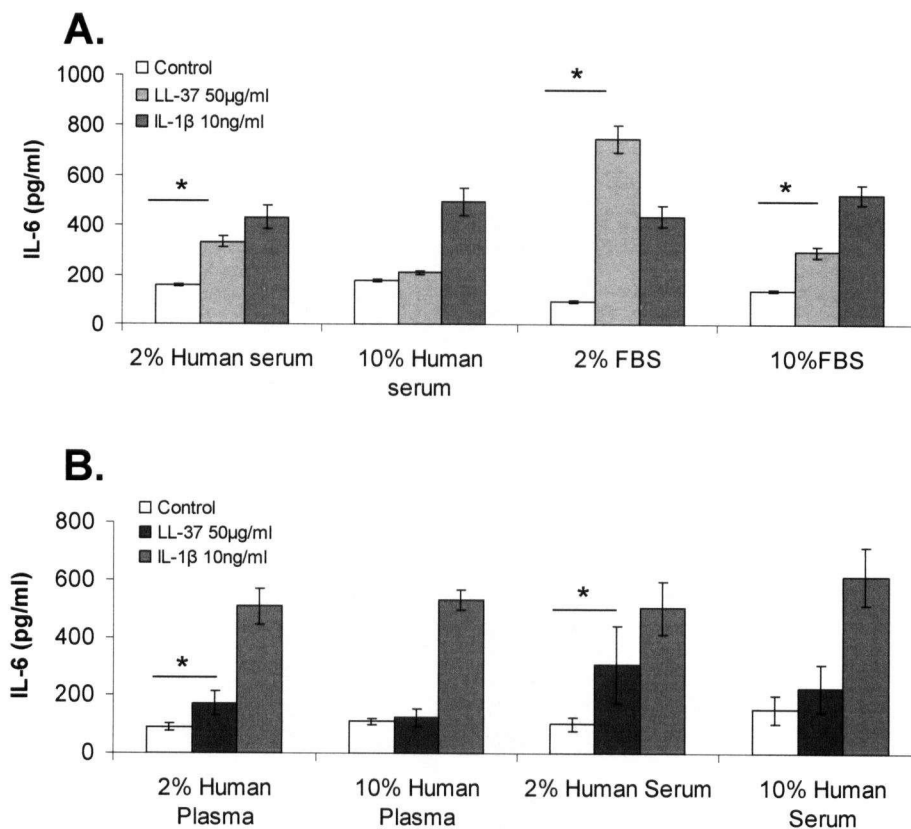
Figure 6. **LL-37-induced IL-6 release in 16HBE4o- cells is dependent on serum type and concentration.**
Confluent 16HBE4o⁻ monolayers were treated with LL-37 at 50 µg/ml or IL-1β at 10 ng/ml (as a positive control) in either **A.** 2% and 10% FBS, or 2% and 10% pooled human serum supplemented medium, or **B.** 2% and 10% pooled human serum, or 2% and 10% pooled human plasma supplemented medium for 6 hours. IL-6 release in culture supernatants was monitored *via* ELISA. Results are expressed as the mean values ± SEM of three independent experiments (* p<0.05 denotes significance relative to the untreated control).

3.1.5 LL-37-induced IL-6 release is regulated partly by EGFR and a Ptx-sensitive receptor

Even though it is not very clear whether LL-37 acts through a specific receptor(s), a variety of different receptors have been proposed to play various roles in mediating the biological activities of this host defence peptide, including EGFR (in epithelial cells) (74, 82-84), G-protein coupled FPRL-1 (epithelial cells, leukocytes) (70, 72, 85, 91), and the P2X$_7$ receptor (monocytes, neutrophils) (78, 88, 91). In epithelial cells, EGFR appears to play a significant role in regulation of a multitude of processes including IL-8 release (74), cell migration, wound healing and proliferation (83, 84, 86). Therefore, to evaluate if any of these proposed receptors play a potential role in regulation of LL-37-induced IL-6, 16HBE4o- cells were pre-treated with specific chemical inhibitors including A1478 (EGFR inhibitor), Ptx (G-protein coupled receptors, including FPRL-1), and KN-62 (P2X$_7$ inhibitor) for 1 hour. Following the inhibitor pre-treatment, cells were exposed to LL-37 and a specific receptor agonist for 6 h.
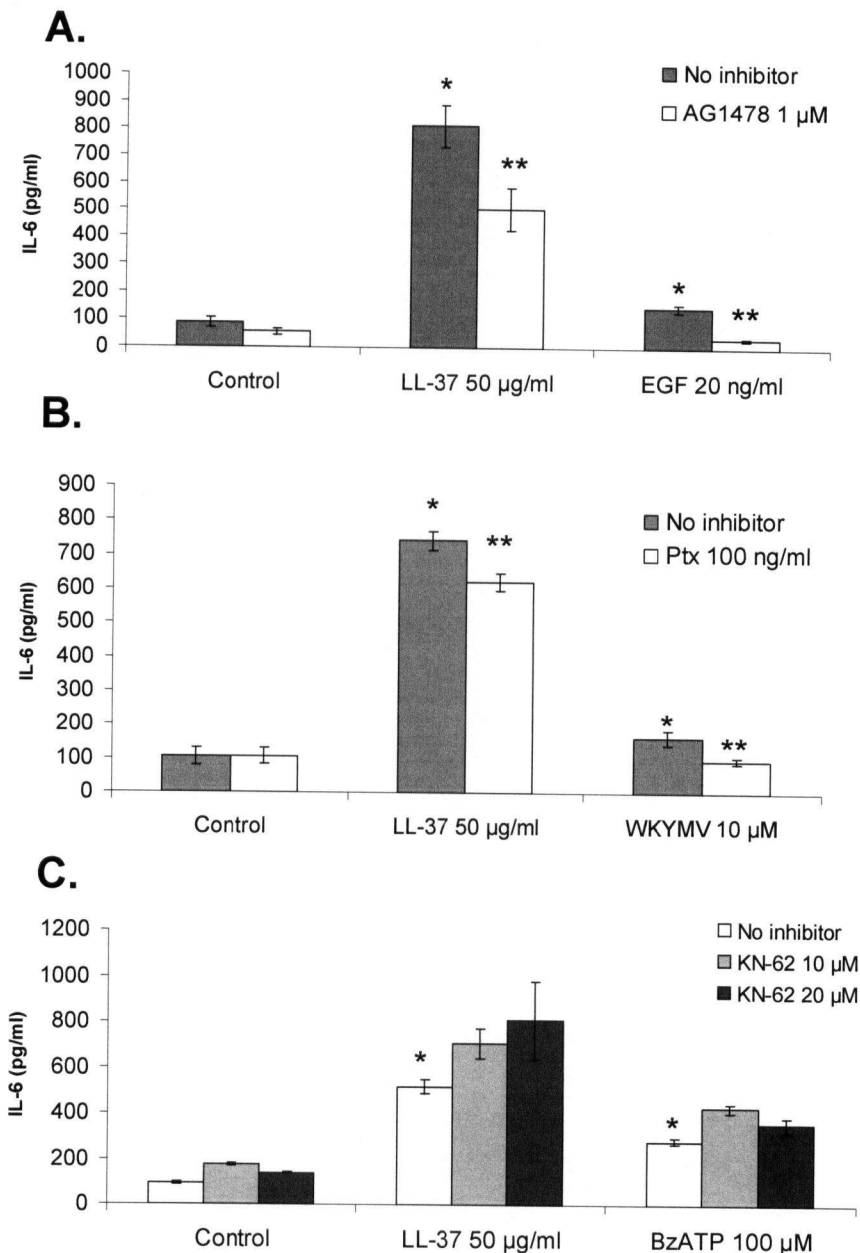
Figure 7. **LL-37-induced IL-6 release in 16HBE4o- cells is partially regulated by EGFR signalling.**

Confluent 16HBE4o- monolayers were pre-treated with **A.** AG1478 (1 µM), an EGFR inhibitor, or **B.** Pertussis toxin (Ptx) (100 ng/ml), or **C.** KN-62 at 10 and 20 µM (P2X$_7$ inhibitor) for 1 hour, prior to the stimulation with A. LL-37 at 50 µg/ml and EGF at 20 ng/ml, or B. LL-37 at 50 µg/ml and WKYMV at 10 µM, or C. LL-37 at 50 µg/ml and Benzyl-ATP (BzATP) at 100 µM. Cell culture supernatants were collected after 6 hours of incubation, and were assayed for IL-6 content *via* ELISA. Results are expressed as means ± SEM of three independent experiments (* $p < 0.05$ relative to untreated control; ** $p < 0.05$ relative to the respective treatment without the inhibitor).

Specific receptor agonists were used as positive controls for inhibitor efficiency; these were EGF (EGFR agonist), WKYMV (FPRL-1 agonist), and Benzyl-ATP (BzATP) (P2X$_7$ agonist). ELISA analysis of the resultant IL-6 release indicated that AG1478, a specific EGFR inhibitor, significantly reduced LL-37-stimulated IL-6 release by 38 ± 15 %, while epidermal growth factor-induced IL-6 was completely inhibited (p<0.05) (Figure 7A). Ptx inhibited IL-6 by a modest, but significant, 16 ± 4 %, while it completely abrogated WKYMV-induced IL-6, a process known to be dependent on Gi-coupled protein receptors (Figure 7B). KN-62 at 10 or 20 μM did not inhibit LL-37-induced IL-6 production, but rather increased its release (Figure 7C). Similarly, in my hands, KN-62 incubation failed to inhibit any BzATP-induced IL-6.

3.1.6 LL-37-mediated IL-6 release is regulated by ERK1/2 and p38 MAP kinases, but not by PI3K signalling

In addition to receptor regulation mediated by EGFR, LL-37-stimulated release of IL-8 from bronchial epithelial cells and monocytes is regulated by the ERK1/2 and p38 MAP kinases (73, 74). Given that MAP kinases have also been shown to play a significant role in regulation of other LL-37-induced lung responses including airway epithelial cell proliferation and wound closure (86), these pathways were good candidates to investigate for IL-6 regulation. To test this assumption, 16HBE4o- cells were pre-treated with specific chemical inhibitors of ERK1/2 activation (PD98059 at 12.5 μM), and p38 (SB208350 at 12.5 μM), and were subsequently stimulated with LL-37 at 50 μg/ml or IL-1β at 10 ng/ml for 6 hours. IL-6 ELISA results indicated that ERK1/2 and p38 inhibitors individually reduced LL-37-mediated IL-6 release by ~20 %, while the inhibitors in combination additively decreased IL-6 by 40 ± 10% (Figure 8A). Yu *et al.* (submitted for publication) have demonstrated that PI3K regulates LL-37-induced IL-8 production in PBMCs, while other groups have also demonstrated PI3K regulation in other LL-37-mediated responses (78, 89).

A.

Legend:
- ☐ No inhibitor
- ■ SB208350 12.5 µM
- ▨ PD98059 12.5 µM
- ☐ SB + PD

B.

Legend:
- ■ No inhibitor
- ▨ LY294002 5 µM
- ▨ LY 294002 10 µM
- ☐ LY294002 25 µM
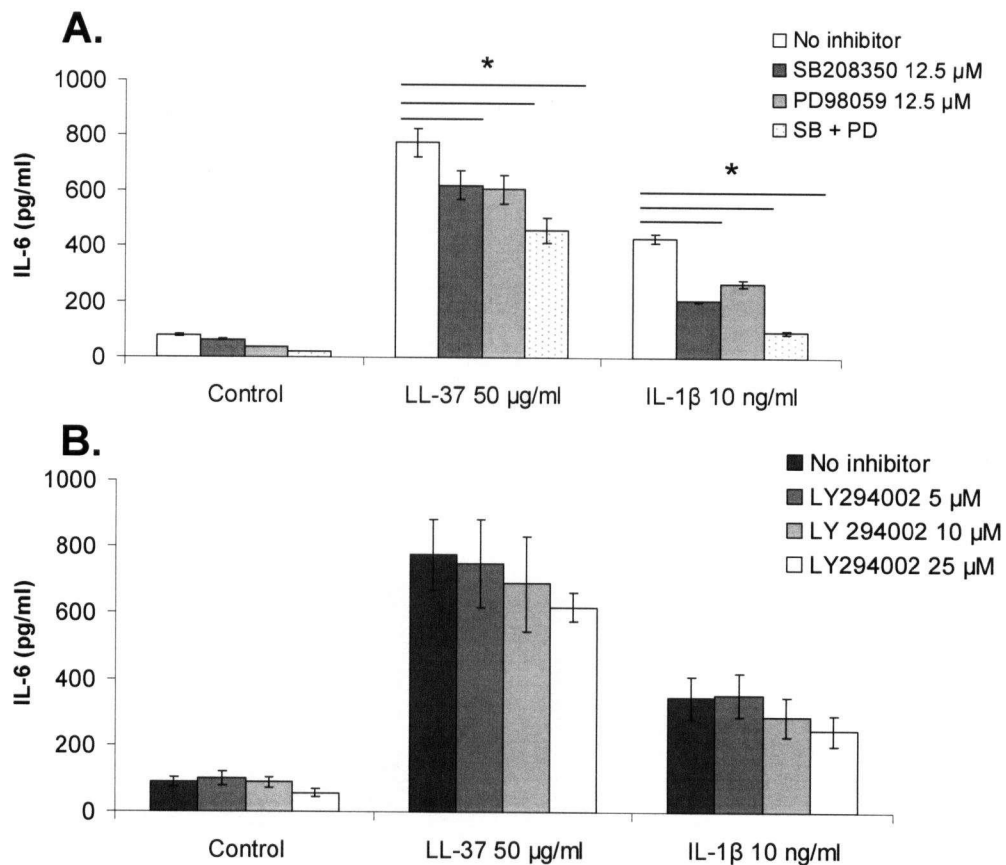
Figure 8. **LL-37-mediated IL-6 release in 16HBE4o- cells is regulated by ERK1/2 and p38 signalling, but not by PI3K pathways.**
Confluent 16HBE4o- monolayers were pre-treated with **A.** SB208350 (p38 inhibitor) at 12.5 µM alone, PD98059 (MEK/ERK1/2 inhibitor) at 12.5 µM alone, or with both inhibitors at 12.5 µM; or **B.** LY294002 (PI3K inhibitor) at 5, 10, and 25 µM for 1 hour prior to exposure to LL-37 at 50 µg/ml or IL-1β at 10 ng/ml for 6 hours. Cell culture supernatants were assayed for IL-6 release by ELISA. Results are expressed as mean values ± SEM of three independent experiments. (* $p < 0.05$ denotes significance relative to the respective treatment without the inhibitor).

Therefore, the role of PI3K was tested using a chemical inhibitor LY294002 (Figure 8B). None of the inhibitor doses tested (5, 10, 25 µM) resulted in a significant decrease of LL-37-induced IL-6 release after a 6h incubation ($p > 0.05$), as determined by ELISA.

3.1.7 NF-κB signalling is a major regulator of LL-37-induced IL-6 in 16HBE4o- cells.

LL-37-mediated induction of IL-6 in bronchial epithelial cells stems from transcriptional up-regulation (Figure 3). The literature shows that IL-6 transcription is mediated through the

transcription factors NF-κB, cAMP-responsive element binding protein (CREB), activator protein-1 (AP-1) and nuclear factor IL-6 (NF-IL-6) (97, 98). In particular, studies performed in bronchial epithelial cells show that IL-6 is NF-κB regulated (99-101), thus making this transcription factor an excellent candidate to study in LL-37-mediated IL-6 production. To examine if NF-κB activation was involved in IL-6 production in LL-37-exposed 16HBE4o- cells, I used two chemical inhibitors, Bay11-7085 and parthenolide, previously demonstrated to inhibit NF-κB pathway activation (Figure 9).
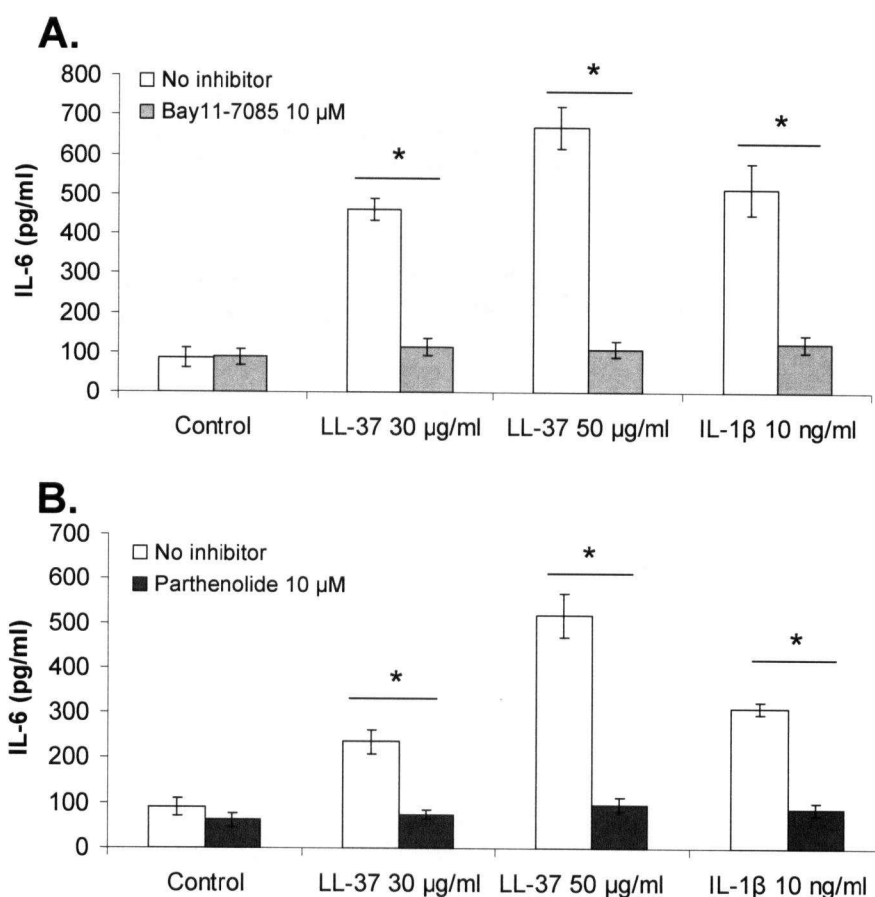
Figure 9. **LL-37-induced IL-6 release in 16HBE4o- cells is robustly regulated *via* NF-κB signalling.**
Confluent 16HBE4o- monolayers were pre-treated with A. Bay 11-7085 (Iκ-Bα phosphorylation inhibitor) at 10 μM, or B. Parthenolide (NF-κB inhibitor) at 10 μM for 1 hour, prior to stimulation with LL-37 at 30 and 50 μg/ml, or IL-1β at 10 ng/ml for 6 hours. Supernatants were assayed for IL-6 release by ELISA. Results are expressed as averages ± SEM of A. three, or B. two independent experiments (* p<0.05 relative to the respective treatment without the inhibitor).

16HBE4o- cells were incubated with Bay11-7085 or parthenolide at 10 µM for 1h prior to stimulation with LL-37 (30 and 50 µg/ml) or IL-1β (10 ng/ml) for 6h. Bay 11-7085 abrogated all IL-6 induced by both LL-37 concentrations tested, as well as the IL-1β treatment (p<0.05; Figure 9A), as did the parthenolide incubation (Figure 9B).

To further corroborate that NF-κB pathway was implicated in LL-37-mediated responses in bronchial epithelial cells, I investigated the activation of this pathway. Specifically, serum-starved 16HBE4o- cells were treated with 50 µg/ml of LL-37, or 10 ng/ml of IL-1β (as a positive control), over a time course of 15, 30 and 60 minutes, while phosphorylation and degradation levels of Iκ-Bα were examined by Western blots. LL-37 induced Iκ-Bα phosphorylation within 15 min of stimulation (Figure 10A.), and this effect was confirmed by the LL-37-induced degradation of total Iκ-Bα over a time-course of 15 to 60 min (Figure 10B.).
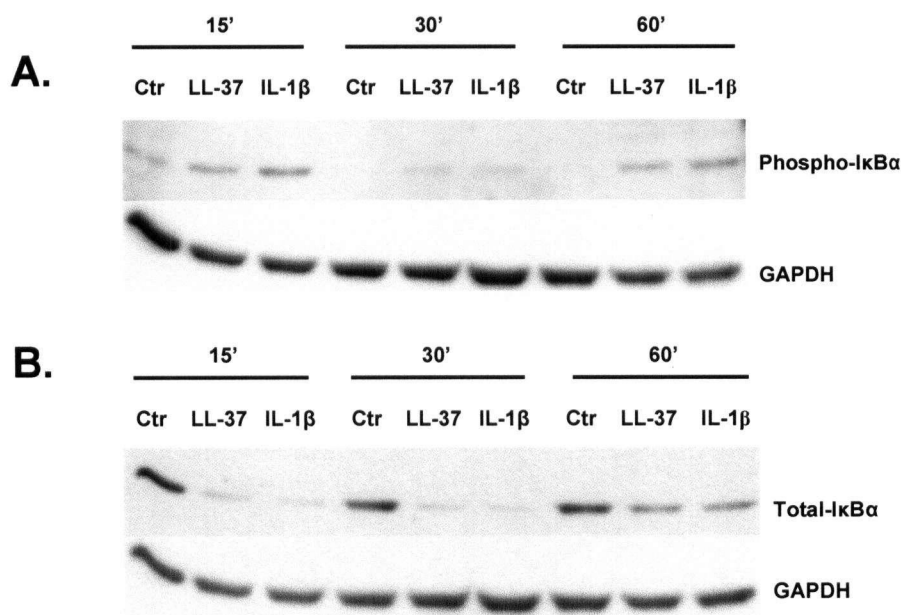
Figure 10. LL-37 induces Iκ-Bα phosphorylation and total Iκ-Bα degradation in 16HBE4o- cells.
16HBE4o- cells were treated with 50 µg/ml LL-37, 10 ng/ml IL-1β, or were left untreated (Ctr – control), over a time course of 15, 30, and 60 minutes. Whole cell protein lysates were prepared and analysed by SDS-PAGE and Immunoblotting. **A.** Immunoblot for phosphorylation of Iκ-Bα, with expression of the housekeeping protein GAPDH assessed as a loading control. **B.** Immunoblot for total Iκ-Bα, with expression of the housekeeping protein GAPDH assessed as a loading control. Results are from one experiment for each panel, representative of three experiments.

Therefore, in addition to demonstrating that LL-37 stimulation could lead to NF-κB pathway activation (Figure 10), I established that this signalling pathway is a major regulator of LL-37-induced IL-6 production in bronchial epithelial cells (Figure 9).

3.1.8 LL-37 can induce IL-6 release additively with IL-1β in bronchial epithelial cells

Studies done by Yu *et al.* (2007, submitted for publication), and Barlow *et al.* (78), have demonstrated that LL-37 (at low doses, < 20 µg/ml) often demonstrates synergistic or additive cytokine/chemokine regulation in the presence of the endogenous cytokine IL-1β in monocytes, THP-1 cells and neutrophils. IL-1β is an important mediator of lung immune responses (3, 4), and is known to be present and activating and regulating bronchial epithelial cell responses during inflammation/infection. Therefore, it was of interest to examine how LL-37 and IL-1β stimulation affected IL-6 release from bronchial epithelial cells (Figure 11).
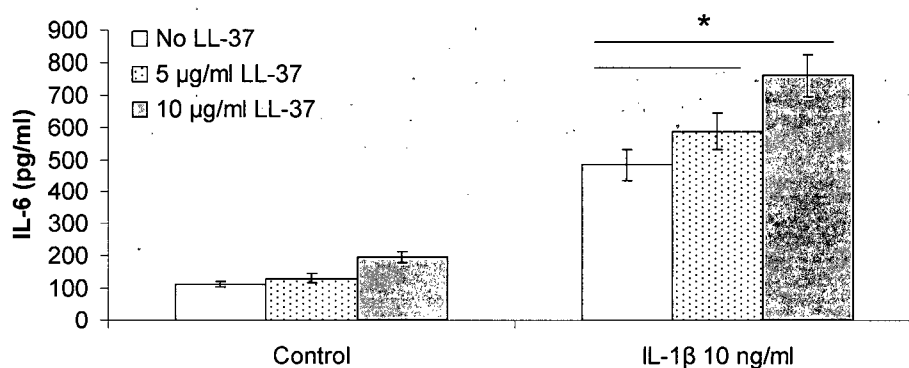
Figure 11. Low doses of LL-37 and IL-1β induce IL-6 from 16HBE4o- cells in an additive (synergistic) manner.
Confluent 16HBE4o- cells were treated with LL-37 at 5 and 10 µg/ml alone, IL-1β at 10 ng/ml alone, with IL-1β and LL-37 (at 5 and 10 µg/ml) in combination, or were left untreated (control) for 6 hours. IL-6 release in culture supernatants was monitored *via* ELISA. Results are expressed as the mean values ± SEM of three independent experiments (* $p < 0.05$ relative to the IL-1β alone treatment).

To examine if LL-37 at lower doses can additively increase IL-1β-mediated IL-6 release, 16HBE4o- cells were treated with 5 and 10 μg/ml of LL-37 and/or IL-1β at 10 ng/ml for 6 hours. Cell culture supernatants were tested for IL-6 release by ELISA. As shown in Figure 11, a statistically significant increase in IL-6 was observed when cells are treated with LL-37 in presence of IL-1β, relative to cytokine treatment alone (p<0.05). These results indicate that LL-37 can act with the pro-inflammatory cytokine IL-1β and co-stimulate bronchial epithelial cells to release IL-6 in an additive manner.

3.1.9 LL-37 increased flagellin-induced IL-6 release from bronchial epithelial cells

LPS stimulation and TLR4 expression and signalling in bronchial epithelial cells have been a subject of considerable debate to date. Many studies indicate that bronchial epithelial cells respond poorly to LPS stimulation, possibly due to low expression or the lack of the LPS receptor TLR4, or certain accessory molecules required for TLR4 pathway induction (19, 102, 103). Consistently with these studies, in bronchial epithelial cells LPS did not induce IL-8 or IL-6, which are known to be induced by the TLR4 pathway (Figure 4A). TLR5 signalling pathway expression and its role in pathogen recognition in the lung has been well described (102, 104).
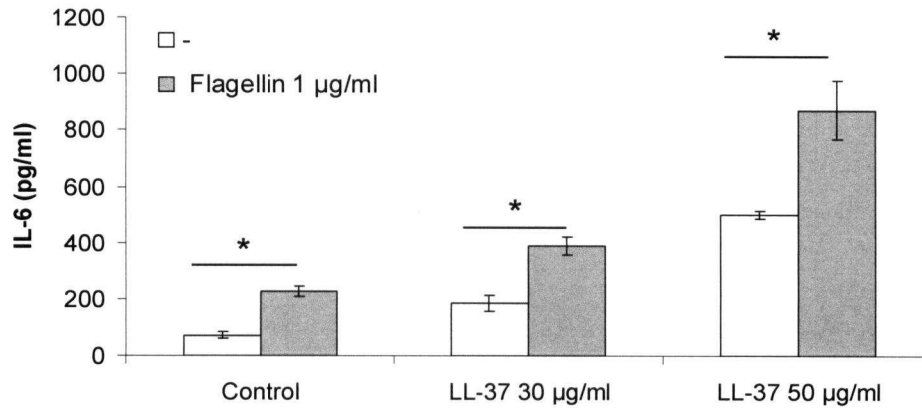
Figure 12. LL-37 increased flagellin-induced IL-6 release in 16HBE4o- cells.
Confluent 16HBE4o- cells were treated with LL-37 at 30 and 50 µg/ml alone, flagellin at 1 µg/ml alone, with flagellin and LL-37 (at two concentrations) in combination, or were left untreated (control) for 6 hours. IL-6 release in culture supernatants was monitored *via* ELISA. Results are expressed as means ± SEM of three independent experiments (* $p < 0.05$ relative to the respective treatment without flagellin).

To examine if LL-37 would have any effect on flagellin-mediated cytokine release. 16HBE4o- cells were treated with 30 and 50 µg/ml of LL-37 and/or flagellin at 1 µg/ml for 6 hours. Cell culture supernatants were tested for IL-6 release by ELISA. LL-37 at both 30 and 50 µg/ml enhanced flagellin-induced IL-6 release significantly ($p < 0.05$) (Figure 12). These results indicate that LL-37 can act as a co-stimulatory molecule to increase TLR5-mediated cytokine release in bronchial epithelial cells.

3.1.10 Summary

LL-37-induced cytokine release and signalling regulation in 16HBE4o- and primary NHBE cells were examined using ELISA, and qPCR. Among the tested cytokines, LL-37 significantly induced IL-6 in both examined cell types. This LL-37-mediated IL-6 induction was dose and time-dependent, and was dependent on serum type and concentration. Furthermore, LL-37 mediated up-regulation of IL-6 at the transcriptional level. Specific chemical inhibitors of

various pathways were used to evaluate signalling regulation of LL-37-stimulated IL-6 release. The major pathway that could be shown to robustly regulate IL-6 release was NF-κB, while regulation *via* MAPK and EGFR pathways was more modest. Western blot studies confirm NF-κB pathway activation by LL-37 by the peptide induced phosphorylation of Iκ-Bα, and degradation of total Iκ-Bα.

3.2 LL-37-induced chemokine release from human respiratory epithelial cells and signalling regulation of this LL-37-mediated response

3.2.1 Introduction

Very much like cytokines, chemokines are potent regulators of lung inflammatory processes. This large family of small molecules plays an important role in recruitment of various effector cells to the site of the infection/inflammation, and thus greatly affect its course and outcome. As previously stated, a well described LL-37 effect on airway epithelium is induction of IL-8, a potent CXCL family chemokine (73, 74). However, it would be of great importance to examine if LL-37 has the ability to induce other chemokines. In this Chapter, I tested the ability of LL-37 to induce chemokines using the human bronchial epithelial cell line 16HBE4o- and normal primary bronchial epithelial cells as biological models. LL-37-mediated induction of chemokines was determined using specific chemical inhibitors and ELISA assays, while transcriptional regulation was assessed by qPCR.

3.2.2 LL-37 induced GRO-α, RANTES and IL-8 release from bronchial epithelial cells

To investigate if any chemokines other than IL-8 were induced by LL-37 in airway epithelial cells, 16HBE4o- monolayers were treated with 50 μg/ml of the peptide, or IL-1β at 10 ng/ml, for 6 hours. Cell culture supernatants were assayed by ELISA for released chemokines. In addition to an expected, significant increase in IL-8, a significantly enhanced release of CXCL-1/GRO-α and CCL5/RANTES was observed (p<0.05) (Figure 13). However, LL-37 did not induce CCL3/MIP-1α, CCL22 or CXCL-10/IP-10. To further describe this LL-37 effect on chemokine induction in bronchial epithelial cells, I exposed monolayers of 16HBE4o- and primary NHBE cells to a range of LL-37 concentrations or IL-1β (as a positive control) for 6 hours, to determine the kinetics of the chemokine response; produced chemokines were

quantified *via* ELISA.
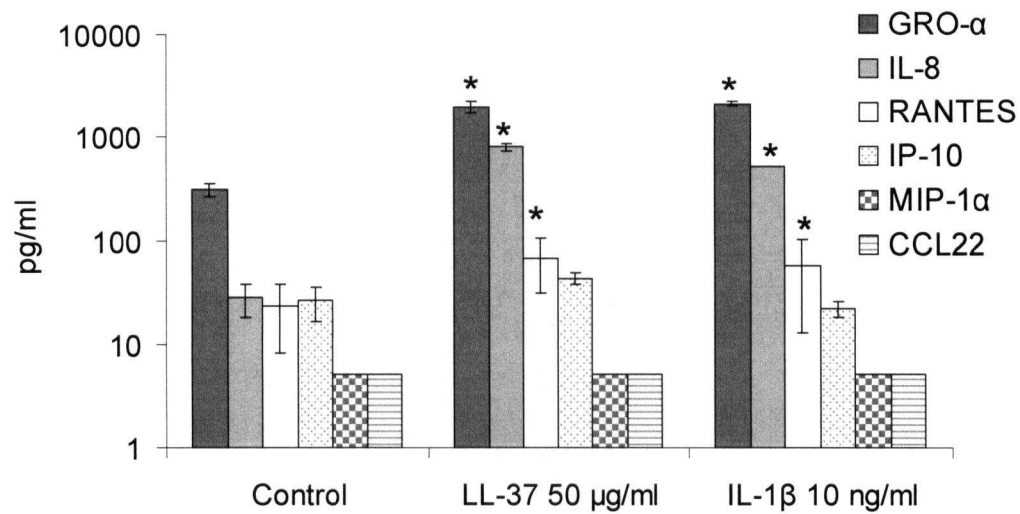
Figure 13. **LL-37 induces GRO-α, IL-8 and Rantes release from 16HBE4o- cells.**
Confluent 16HBE4o- cell monolay ers were treated with 50 µg/ml of LL-37, IL-1β at 10 ng/ml, or were left untreated (controls). Cell culture supernatants were assayed for GRO-α, IL-8, Rantes, CCL22, MIP-1α and IP-10 by ELISA. Results are expressed as the mean values of chemokine produced (Y axis – log scale) ± standard deviation of three independent experiments (* $p < 0.05$ denotes significance relative to untreated controls, as determined by Student's t-test analyses).

As presented in Figure 14, LL-37 induced GRO-α release in a dose-dependent manner in both 16HBE4o- cells (Figure 14A) and primary NHBE cells (Figure 14B). Specifically, LL-37 at 10 µg/ml induced a 2-fold increase of GRO-α release ($p < 0.05$), with the peak of chemokine induction detected with 50 µg/ml of LL-37 in 16HBE4o- cells (Figure 14A). Primary NHBE cells released significant GRO-α at concentrations as low as 2.5 µg/ml of LL-37, while the highest magnitude of the response was detected with 25 µg/ml of the peptide, at 4.8 ± 0.6 fold ($p < 0.05$) (Figure 14B). As expected, a corresponding effect was observed with respect to LL-37-mediated IL-8 release. LL-37 induced a dose-dependent release of IL-8 from 16HBE4o- and primary NHBE cells, with LL-37 inducing a significant chemokine release in the 10 to 50 µg/ml range in 16HBE4o-, and 2.5 to 50 µg/ml range in primary NHBE cells (Figure 15B).

As established in Figure 13, LL-37 induced a statistically significant increase of RANTES ($p < 0.05$), however the increase was only ~2 fold. Given that a very high dose of LL-37 (50μg/ml) mediated an unexceptional increase of RANTES, a dose-response of this induction was not warranted. A brief time-course (6 and 24 hours) with 50 μg/ml of LL-37 or IL-1β at 10 ng/ml was performed with 16HBE4o- cells. LL-37 induced a significant increase in RANTES at both 6 and 24 hours ($p < 0.05$) (Figure 16A). However, the difference in chemokine induction between the two time points was miniscule, with a slight increase from 2.4-fold at 6 hours to 2.9-fold at 24 hours. Curiously, there was no detectable RANTES released when primary NHBE cells were treated with a range of different LL-37 concentrations (from 1 to 50 μg/ml) (Figure 16B), thus suggesting that this effect may not be physiologically relevant.
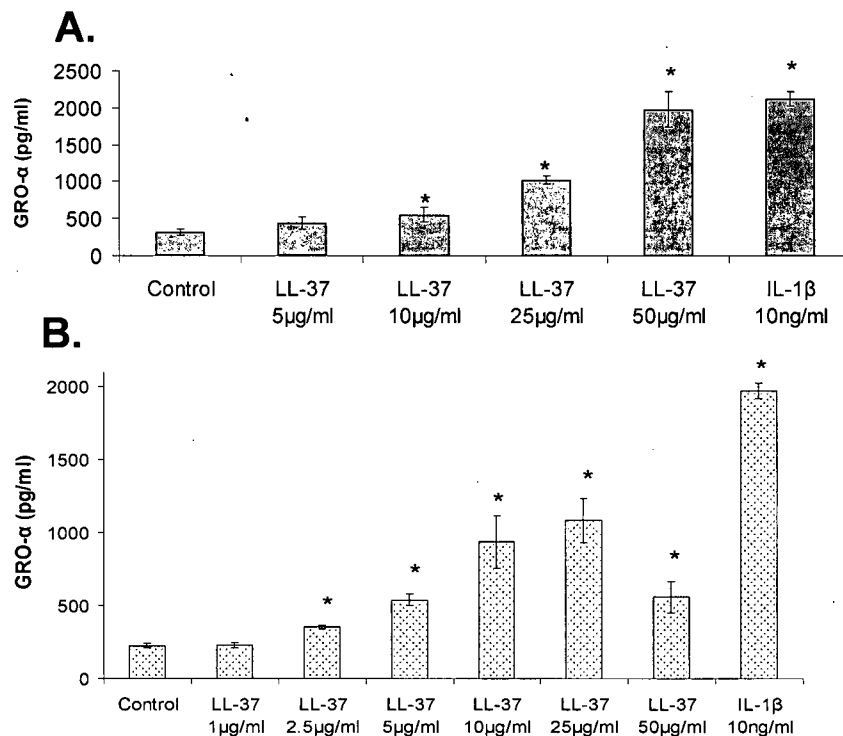
Figure 14. LL-37 induces GRO-α release from 16HBE4o- bronchial epithelial cell line and primary NHBE cells in a dose-dependent and time-dependent manner.
Confluent **A.** 16HBE4o- or **B.** Primary NHBE monolayers were stimulated with a range of LL-37 concentrations A. 5, 10, 25, and 50 μg/ml or B. 1, 2.5, 5, 10, 25, and 50 μg/ml, or IL-1β at 10 ng/ml for 6 hours. GRO-α release in culture supernatants was monitored *via* ELISA. Results are expressed as averages ± standard deviation of three independent experiments (* $p < 0.05$ denotes significance relative to untreated controls).

A.

B.
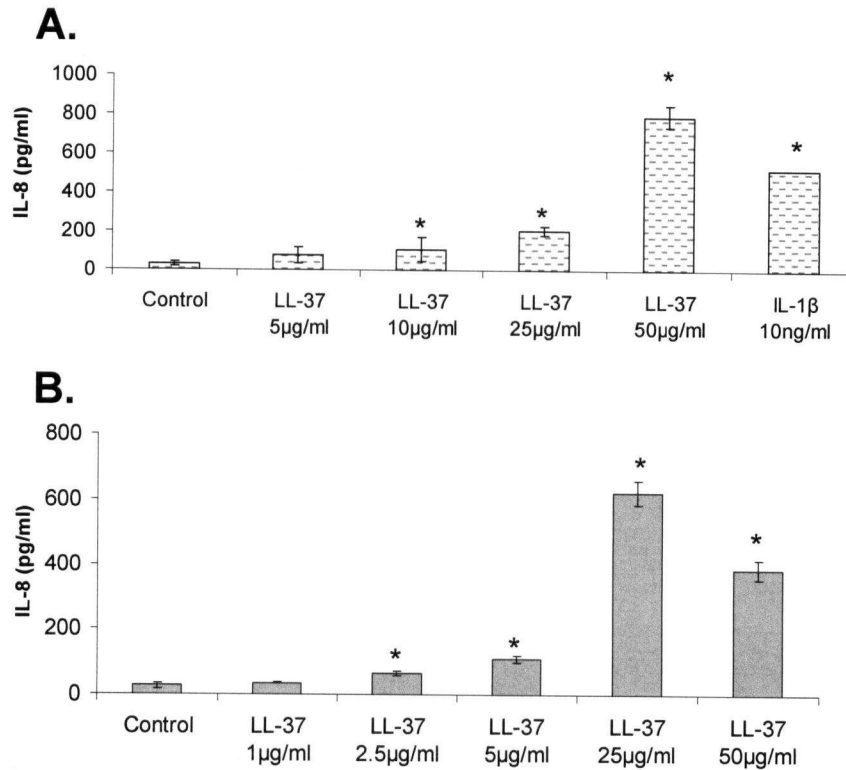
Figure 15. LL-37 induces IL-8 release from 16HBE4o- bronchial epithelial cell line and primary NHBE cells in a dose-dependent manner.
Confluent **A.** 16HBE4o- or **B.** Primary NHBE monolayers were treated with a range of LL-37 concentrations **A.** 5, 10, 25, and 50 µg/ml or 10 ng/ml of IL-1β or **B.** 1, 2.5, 5, 10, 25, and 50 µg/ml for 6 hours. IL-8 release in culture supernatants was monitored *via* ELISA. Results are expressed as mean values ± standard deviation of three independent experiments (* $p<0.05$ denotes significance relative to untreated control samples).
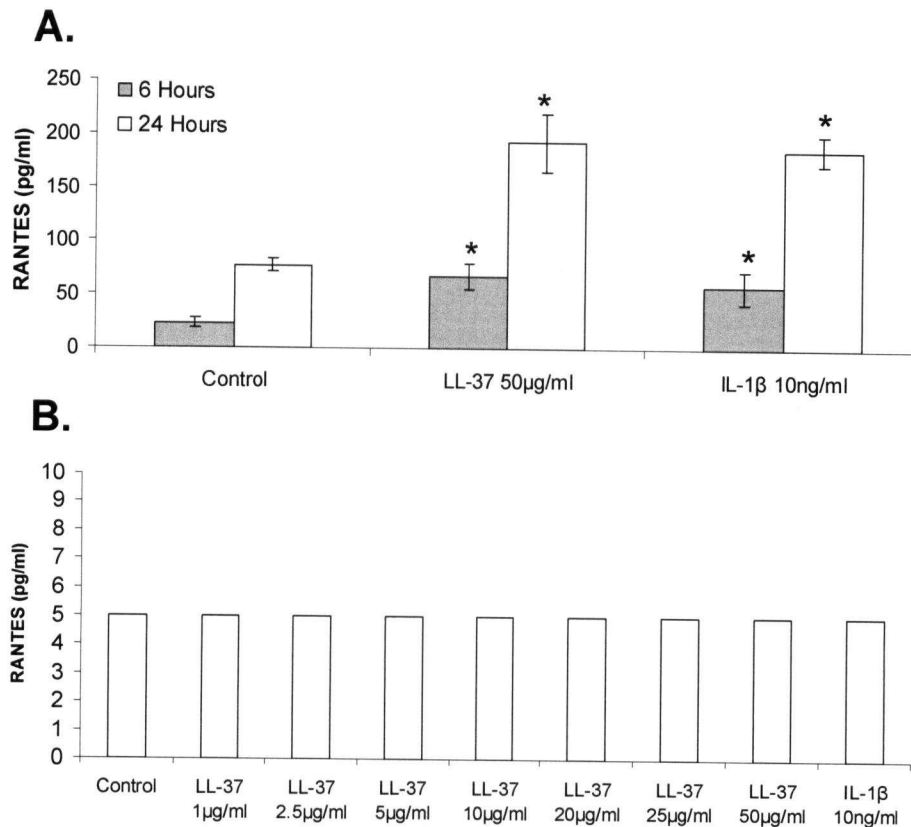
Figure 16. LL-37 induces RANTES release from 16HBE4o- bronchial epithelial cell line, but not from primary NHBE cells.

Confluent **A.** 16HBE4o- monolayers were treated with LL-37 at 50 μg/ml or IL-1β at 10 ng/ml for 6 and 24 hours. **B.** Primary NHBE monolayers were treated with a range of LL-37 concentrations (1, 2.5, 5, 10, 25, and 50 μg/ml), or IL-1β at 10 ng/ml for 6 hours. Rantes release in culture supernatants was monitored *via* ELISA. Results are expressed as averages ± standard deviation of two independent experiments (* $p < 0.05$ denotes significance relative to untreated control samples).

3.2.3 LL-37-induced chemokine production is a transcriptionally-regulated response

In addition to focusing on LL-37-mediated protein release, transcriptional regulation of GRO-α, IL-8 and Rantes was also evaluated. 16HBE4o- cells were stimulated with 50 μg/ml of LL-37, or IL-1β at 10 ng/ml, over a time-course of 1, 2, and 4 hours. Gene expression profiles were obtained through a subsequent quantitative PCR analysis (Figure 17).
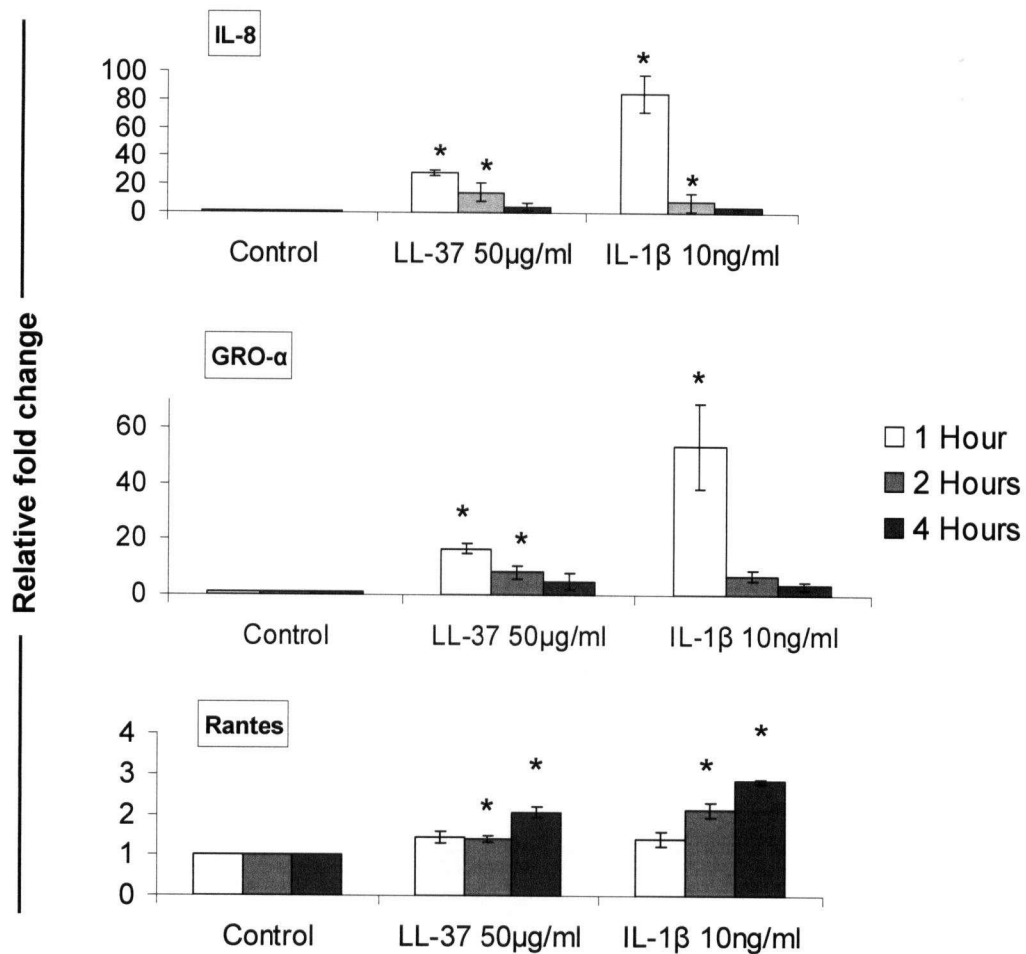
Figure 17. LL-37 up-regulates GRO-α, IL-8 and Rantes gene expression in 16HBE4o- cells.
Sub-confluent 16HBE4o- cells were stimulated with LL-37 at 50 μg/ml, or IL-1β at 10 ng/ml (as a positive control) for 1, 2 and 4 hours. Transcriptional regulation was assessed *via* quantitative RT-PCR. Fold changes (Y-axis) were normalized to GAPDH and are relative to the gene expression in un-stimulated cells (normalized to 1) using the comparative Ct method (refer to Materials and Methods for details). Results are expressed as averages ± SEM of three independent experiments (* p<0.05 designates significance relative to the respective untreated control at a given time-point).

As presented in Figure 17, all of the chemokine genes examined were transcriptionally up-regulated by LL-37. Both GRO-α and IL-8 had similar temporal regulation patterns, with the peak of gene expression observed at 1 hour post-stimulation, and with relative fold changes of

16.7 ± 1.7 and 28.4 ± 2.3 respectively (p<0.05). Moreover, LL-37-mediated transcriptional up-regulation of GRO-α and IL-8 remained statistically significant at 2 hours, with respective 8.3 ± 2.4 and 14.4 ± 2.1 fold change (p>0.05). Both subsequently reached near-control levels at 4 hours post-stimulation (p>0.05). LL-37-mediated up-regulation of RANTES expression was considerately more subtle, reaching a modest 2 fold up-regulation (p<0.05) at the 4 hour time point only.

3.2.4 Signalling regulation of LL-37-stimulated GRO-α in airway epithelial cells is mediated by EGFR, MAP kinase, and NF-κB signalling pathways

As LL-37-mediated IL-8 release and regulation in airway epithelial cells is a well described phenomenon (73, 74), and RANTES induction appeared insignificant (Figure 13, 16), the emphasis of the remainder of this study was on the peptide-induced GRO-α response. GRO-α was strongly induced at the transcriptional level upon LL-37 stimulation (Figure 17), and at the protein release level in both 16HBE4o- cell line and the primary NHBE cells (Figure 13, 14). As such, a closer evaluation of signalling regulation of this CXCL type chemokine was warranted to extend knowledge of the mechanism of action of host defence peptide LL-37 on bronchial epithelial cells.

EGFR is a potent regulator of most LL-37-mediated effects in epithelial cell types, thus I tested whether EGFR was involved in LL-37 induction of GRO-α. Confluent monolayers of 16HBE4o- cells were pre-treated with a specific EGFR kinase inhibitor AG1478 for 1 hour, and cells were subsequently stimulated with LL-37 (30 and 50 μg/ml) or EGF (20 ng/ml), a specific EGFR agonist, for 6 hours. The resulting GRO-α increase was quantified by ELISA. AG1478 significantly inhibited GRO-α release by ~ 60 % (p<0.05) for the two tested LL-37 concentrations, while it completely inhibited EGF-induced GRO-α (Figure 18). Thus, signalling through EGFR is required in part for LL-37-mediated GRO-α release, in addition to IL-6

(Figure 7A) in bronchial epithelial cells.

Figure 18. **LL-37-induced IL-6 release in 16HBE4o- cells is partially regulated by EGFR signalling.**
Confluent 16HBE4o- monolayers were pre-treated with AG1478 (1 μM), an EGFR inhibitor for 1 hour prior to treatment with LL-37 at 50 μg/ml or EGF at 20 ng/ml. Cell culture supernatants were collected after 6 hours of incubation, and were assayed for GRO-α content *via* ELISA. Results are expressed as averages ± SEM of three independent experiments (* $p < 0.05$ relative to untreated control; ** $p < 0.05$ relative to the respective treatment without the inhibitor).

Furthermore, other signalling pathways that could possibly regulate LL-37-mediated GRO-α release in airway epithelial cells were examined with the use of specific chemical inhibitors. The literature indicates that GRO-α regulation occurs through NF-κB signalling activation (105, 106), which might be mediated in part through the MAP kinases, ERK1/2, p38 and/or JNK (105, 107), or other pathways such as PI3K and PKC (108). To examine signalling regulation, 16HBE4o- cells were pre-treated with specific pathway inhibitors, namely PD98059 (MEK1/2 inhibitor of ERK1/2 activation), SB208350 (p38 inhibitor), LY294002 (PI3K inhibitor), and Bay11-7085 (NF-κB pathway inhibitor) prior to the treatment with LL-37 (50 μg/ml) or IL-1β (10 ng/ml) for 6 hours. Subsequently, released GRO-α was quantified from cell culture supernatants by ELISA assays.

The PI3K inhibitor LY294002 did not inhibit LL-37-induced GRO-α production over a range of inhibitor concentrations (5, 15, and 25 μM) (Figure 19B). Alternatively, ERK1/2 and

p38 MAP kinase inhibitors independently reduced peptide-stimulated GRO-α by ~34% and 30% respectively (Figure 19A.). The two MAPK inhibitors combined reduced GRO-α release by 55±4.1% relative to the LL-37 treatment alone ($p < 0.05$) (Figure 19A). The inhibitor of the NF-κB pathway (Bay 11-7085) reduced GRO-α to control levels in LL-37-treated cells at both 30 and 50μg/ml of the peptide ($p < 0.05$), thus suggesting NF-κB signalling is a major regulator of LL-37-induced GRO-α production in 16HBE4o- cells (Figure 20), as was previously observed for LL-37-stimulated IL-6 (Figure 9).
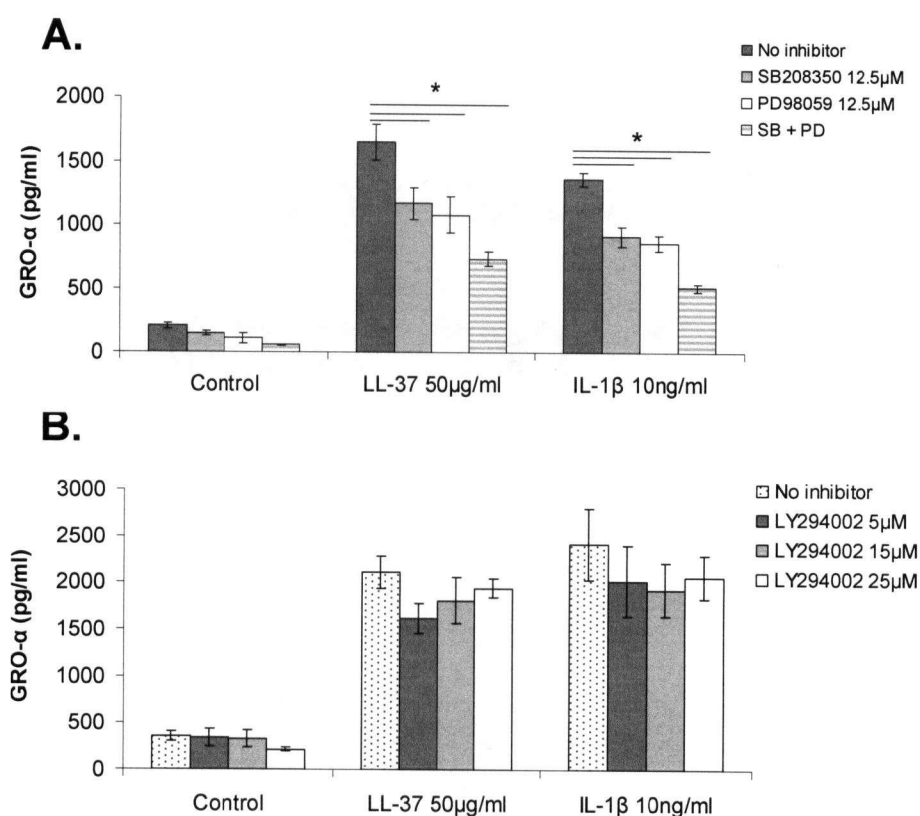
Figure 19. LL-37-induced GRO-α release in 16HBE4o- cells is regulated by ERK1/2 and p38 signalling, but not by PI3K pathways.
Confluent 16HBE4o- monolayers were pre-treated with **A.** SB208350 (p38 inhibitor) at 12.5 μM alone, PD98059 (ERK1/2 inhibitor) at 12.5μM alone, both inhibitors at 12.5 μM, or **B.** LY294002 (PI3K inhibitor) at 5, 10, and 25μM for 1 hour prior to stimulation with LL-37 at 50 μg/ml or IL-1β at 10 ng/ml for 6 hours. Supernatants were assayed for GRO-α release by ELISA. Results are expressed as averages ± SEM of A. three, and B. two independent experiments (* $p < 0.05$ relative to the respective treatment without the inhibitor).
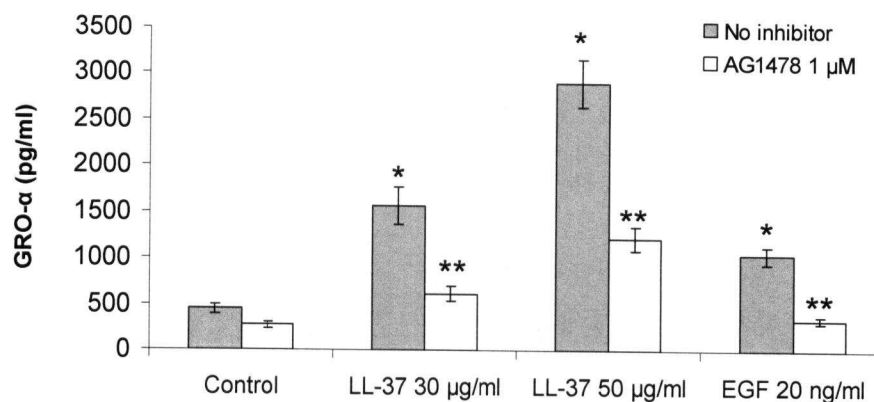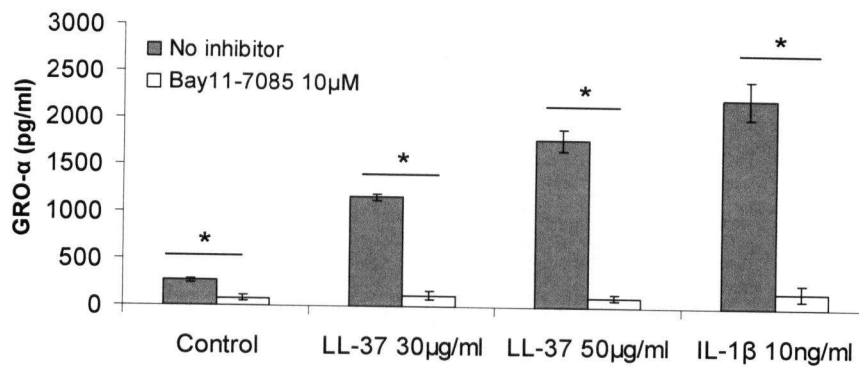
Figure 20. LL-37-induced GRO-α release in 16HBE4o- cells is robustly regulated *via* NF-κB signalling.

Confluent 16HBE4o- monolayers were pre-treated with Bay 11-7085 (Iκ-Bα phosphorylation inhibitor) at 10 μM for 1 hour, prior to stimulation with LL-37 at 30 and 50 μg/ml or IL-1β at 10 ng/ml for 6 hours. Supernatants were assayed for GRO-α release by ELISA. Results are expressed as the mean values ± SEM of three independent experiments (* p<0.05 relative to the respective treatment without the inhibitor).

3.2.5 Summary

LL-37-induced chemokine release and the signalling events involved in this response in 16HBE4o- and primary NHBE cells was examined using ELISA, and qPCR. Among the tested chemokines, LL-37 significantly induced GRO-α, IL-8 and RANTES in 16HBE4o- cells, but only GRO-α and IL-8 in primary NHBE cells. LL-37 increased GRO-α and IL-8 release in a dose-dependent manner in both cell types. In 16HBE4o- cells, gene expression of GRO-α, IL-8 and RANTES was up-regulated by LL-37 over a time-course. Specific chemical inhibitors of various pathways were used to evaluate signalling regulation of LL-37-stimulated GRO-α release. The major pathway by which LL-37 regulated GRO-α release was the NF-κB pathway, while the contribution of the MAPK and EGFR pathways was more modest. These results correspond to those obtained for LL-37-induced IL-6 regulation, thus indicating that the NF-κB pathway is a major mechanism by which LL-37 exerts its effect in bronchial epithelial cells.

4. Discussion

4.1 Introduction

As the already plentiful evidence keeps accumulating, it is becoming more apparent that cationic peptides truly are a vital component of host defence mechanisms. Host defence peptide deficiencies that have been described in humans are rare, which might be due to their crucial role in innate immune defences; one in particular has been described as an LL-37 deficiency - the morbus Kostmann disorder (109). Morbus Kostmann patients suffer from severe congenital neutropenia, and until granulocyte-colony stimulating factor (G-CSF) treatments became available, these patients were plagued by frequent infections and would die at a young age (109). G-CSF treatments have been shown to result in normal neutrophil counts in these patients, however there is still an inherent absence of LL-37 in neutrophil secondary granules (110). Even with G-CSF treatment, individuals with morbus Kostmann suffer from recurrent oral infections and chronic periodontal disease (109, 110).

LL-37, the only human cathelicidin, has been shown to mediate multiple immunomodulatory effects. Substantial increases in the local concentration of LL-37 have been described in inflammation and infection of various sites. This increase has been demonstrated in psoriatic lesions (52, 111), in subcutaneous wounds (112), in nasal secretions of patients with chronic rhinitis (15), and cystic fibrosis, pulmonary sarcoidosis and COPD (7, 10, 11, 69). Increased LL-37 at the affected site would stimulate surrounding cells to induce various effects, including modulation of LPS-stimulated responses of macrophages/monocytes (79, 113), chemotaxis of hematopoietic cells (70-72), proliferation and wound healing of different epithelial models (84, 86), regulation of cell apoptosis (78, 87, 91), and release of cytokines and chemokines (73, 74, 76, 84).

As discussed beforehand, host defence peptides, cytokines and chemokines are essential

defence components of the airway epithelium. Cationic host defence peptide LL-37 is an important effector molecule of lung immune responses, however the mechanism by which LL-37 modulates airway cell responses is not entirely clear. As previously mentioned, increased LL-37 concentrations have been observed in several lung pathologies including CF and COPD, and in other inflammatory conditions (7, 11, 69). In addition, LL-37 induction associated with these lung afflictions often correlates with cytokine/chemokine induction, which strongly suggests that these effects might be mediated by peptide stimulation of airway epithelial cells. Thus, a closer examination of this peptide's ability to induce cytokines and chemokines from airway epithelial cells was warranted.

4.2   LL-37 and IL-6 induction in airway epithelial cells – a novel peptide response

In this report I have shown that LL-37 is able to induce IL-6 release from 16HBE4o- and primary NHBE cells in a dose-dependent and time-dependent manner (Figure 2), without inducing any IL-1β or TNF-α (Figure 1). Furthermore, LL-37 was able to enhance IL-6 gene expression in a time dependent manner in 16HBE4o- cells. LL-37-mediated induction of IL-6 has previously been demonstrated in keratinocytes (84), but this is the first report of such an effect in airway epithelial cells. It is particularly significant and interesting to observe induction of IL-6, but not IL-1β and TNF-α, since this cytokine group is commonly induced as part of a general pro-inflammatory response mediated, at least in part, through the same signalling pathways, such as NF-κB. Mookherjee *et al.* (79) from our laboratory have demonstrated that LL-37 can selectively modulate gene transcription profiles in LPS-treated human primary monocytes through a complex mechanism of action. For example, LL-37 has been proposed to alter gene expression through selective modulation of NF-κB subunit translocation in endotoxin stimulated monocytes (79). Furthermore, the expression of many cytokine and chemokine genes is regulated by numerous transcription factors acting together to induce efficient transcriptional

activation. Thus, a mechanism involving differential effects on ancillary transcription factors (activated *via* multiple pathways, *e.g* LL-37 up-regulates MAP kinases) could account for the observed selective cytokine induction. In summary, the demonstrated absence of potential IL-1β/TNF-α autocrine stimulation, and absence of induction of any cytokines by LPS (ruling this out as a contaminant), indicate that the aforementioned IL-6 response is specific to LL-37 stimulation, and it is not a secondary effect.

Furthermore, LL-37-induced IL-6 was shown to be a cell-type specific response: IL-6 was induced in bronchial epithelial cells, but not in alveolar epithelial cells (Figure 4). This differential IL-6 induction indicates that perhaps LL-37 modulates cell responses in a distinctive manner depending on the site of stimulation. Van Wetering *et al.* (114) have shown a similar discrepancy with respect to chemokine release in A549 and primary bronchial epithelial cells in response to defensins.

IL-6 is a particularly interesting cytokine with respect to lung immunity. An increase in IL-6 secretion and expression has been described in several lung conditions, such as cystic fibrosis, COPD, and ARDS (15, 20, 115). The anti-inflammatory or pro-inflammatory role of IL-6 in the lung appears to depend on the stimulus and inflammation context. In an IL-6 knock-out mouse model of systemic endotoxaemia and in an endotoxic lung, results indicate that IL-6 plays a crucial anti-inflammatory role (24). This anti-inflammatory response in the endotoxic lung was mediated through negative regulation of pro-inflammatory molecules TNF-α and MIP-2, and decreased neutrophil influx. In the systemic endotoxin challenge model, IL-6 negatively regulated serum levels of proinflammatory TNF-α, MIP-2, GM-CSF, and interferon-γ (IFN-γ) (24). A similar anti-inflammatory effect was also seen in IL-6 knock-out mice in lipoteichoic acid-mediated lung inflammation, while a more pro-inflammatory response was seen in peptidoglycan-induced inflammation (23). Furthermore, results from an *in vitro* study have shown responses consistent with the anti-inflammatory role of IL-6. Schindler *et al.* (116) have

demonstrated that this cytokine can suppress production of the pro-inflammatory cytokines IL-1β and TNF-α induced by a variety of stimuli in human PBMCs through a negative feed-back transcriptional regulation; a more recent study suggested that IL-6 could mediate this negative regulation by inhibiting NF-κB binding activity (117). Aside from inflammation regulation, IL-6 also appears to be a regulator of lung injury and angiogenesis responses. In a lung injury model of IL-6 knock-out mice, it has been shown that IL-6 is essential for neovascularization (118). Furthermore, several studies have also described IL-6-mediated protection against lung injury, postulated to likely occur through inhibition of pro-inflammatory mediators (*e.g.* TNF-α, IL-1β) and signalling pathways (*e.g.* NF-κB) shown to exacerbate injury (22). Though not directly demonstrated in the lung, IL-6 has been proposed to mediate transition from the early-response neutrophilic infiltration to the late-response mononuclear cell recruitment through chemokine-directed effects on endothelial cells and neutrophils in a model of peritoneal inflammation (119).

4.3   LL-37-mediated IL-6 release in physiological conditions

Since airway epithelium *in vivo* is structured as a polarized barrier, I sought to examine how these structured epithelial monolayers would respond to differential peptide stimulation, and how this would affect IL-6 release with respect to the two distinct chambers. As shown in Figure 5A, the greatest total induction of IL-6 was observed with apical LL-37 stimulation, and there was also a greater amount of cytokine in the apical compartment. Basolateral LL-37 stimulation induced a lower total IL-6 response in both chambers equally. This polarized cytokine release would correspond to an *in vivo* situation since the majority of LL-37 would be present at the luminal surface (apical stimulation), and the apically-released IL-6 would be ideally located to stimulate the recruited effector cells at the infection site. Similarly to the IL-6 result, apical LL-37 stimulation induced the most total IL-8, with an apically skewed chemokine release (Figure 5B). This would create a chemotactic gradient to increase the influx of effector

cells to the site of LL-37 stimulation.

LL-37 effects on airway epithelial cells greatly depend on peptide concentration and serum conditions at the site of peptide challenge. LL-37 is present constitutively on pulmonary mucosal surfaces at low concentrations (up to 2.5 μg/ml), but it increases in times of pulmonary infections and in inflammatory conditions due to leukocyte degranulation and secretion from the mucosa (67). For example, BALF analyses of newborns with pulmonary infections have shown peptide concentrations up to ~30 μg/ml [(69), see (40) for a review]. However, higher peptide concentrations, up to 50 μg/ml, could be expected in areas of neutrophil/macrophage degranulation. One of the latest studies has shown that LL-37 can bind to mucin preparations *in vitro* and mucus in patient sputum samples (120). The interesting observation is that the mucus preparation from patients with severe cases of CF contained extremely high LL-37 concentrations – up to 30 mg/ml was reported (120).

In the 1-20 μg/ml concentration range, LL-37 has been demonstrated to increase bronchial epithelial cell proliferation and migration (86). Furthermore, LL-37 has been found to promote closure of mechanically created airway epithelial wounds at concentrations < 10 μg/ml, in a serum-dependent manner (86). Serum regulation was shown to be highly relevant with respect to apoptosis induction. At doses higher than 30 μg/ml, LL-37 has been shown to induce caspase-3-mediated apoptosis in lung epithelial cells in serum-free conditions within 24 hours of stimulation (87). In contrast, in 10% FBS conditions, 50 μg/ml of LL-37 was required to observe apoptosis induction, while 10% of human serum protected epithelial cells from apoptosis, even at a dose of 100 μg/ml of the peptide (87). Curiously though, the presence of human serum was also inhibitory with respect to IL-8 gene up-regulation and release (87). The human serum component responsible for the decrease of apoptosis induction, and likely responsible for negative regulation of IL-8, has been shown to be serum/plasma high density lipoprotein (HDL) (87), and not dependent on apolipoprotein A-1 as previously demonstrated

(121). Thus, considering the relevance of serum conditions to LL-37-mediated responses but also the physiological conditions in which LL-37 stimulation of airway epithelial cells would occur, I sought to examine if serum content and serum type would affect peptide mediated IL-6 release from airway epithelial cells.

In vivo, airway epithelial cells are in an environment that is considered serum-free/low serum, unlike hematopoietic cells which would reside in a plasma/serum-rich environment (87). Making conditions more physiological also implied initially using human serum, and subsequently human plasma, as compared to FBS routinely used in cell culture. As demonstrated in Figure 6A, the greatest induction of IL-6 was observed with 2% FBS, while equal human serum concentration induced a modest 2 fold IL-6 increase that was comparable to the 10% FBS condition. Alternatively, 10% of human serum ablated the peptide-mediated IL-6 response. It should be noted that the peptide response with 2% FBS was comparable to that of when no serum was used (data not shown), and therefore these conditions were used throughout this study. Furthermore, since the true physiological condition would be cell stimulation in presence of plasma, a comparison of LL-37-cell stimulation in either human serum or plasma was performed. No major difference between human plasma and serum was observed with respect to peptide-mediated IL-6 response (Figure 6B), which indicated that human serum commonly used in *in vitro* experiments is representative of *in vivo* conditions.

Thus it can be assumed that cells are most sensitive to LL-37 stimulation in organs like the healthy lung, where they would be stimulated to release the optimum dose of IL-6 in a serum-depleted environment. As the peptide concentration increases during lung inflammation/ infection, the peak production of cytokines/chemokines would occur under these conditions. If the LL-37 concentration increased sufficiently to reach the cytotoxic levels, peptide would act to initiate the onset of apoptosis in the epithelial cells, which would limit the source of cytokines and chemokines to resolve inflammation. The local serum/plasma concentration in the lung can

increase due to tissue damage in infection/inflammation state, for example in the epithelium during ARDS and COPD (15, 20). The increase in serum would subsequently promote the angiogenic function of LL-37, and would protect the epithelium from excessive apoptosis mediated by the peptide. Importantly, a serum increase would also inhibit peptide-stimulated IL-6 production, as demonstrated in this thesis, and IL-8 release, as previously shown by Lau *et al.* (85), which could act to limit the inflammatory response and prevent it from worsening the existing tissue damage. Any residual IL-6 and IL-8 left at the inflammatory site could potentially act to further promote healing, angiogenesis and return to homeostasis (6, 118). IL-6 appears to promote transition from innate to acquired immune response through differential regulation of cell recruitment and clearance from the site of the infection (119). Dysregulation of this effect could potentially lead from acute inflammatory responses to chronic inflammation. IL-6 has been implicated as a marker of several chronic inflammatory diseases, such as Crohn's disease and cystic fibrosis (11, 122), and is considered a contributor to the pathogenesis of these chronic conditions. Thus, inhibition of peptide-induced IL-6 and IL-8 by increased concentrations of human serum, which would be a characteristic of injured lungs, could be considered as a mechanism promoting a return to homeostasis.

4.4   LL-37 and NF-κB-mediated regulation of IL-6

In airway epithelial cells, TLR agonists such as flagellin, IL-1β and TNF-α have been shown to mediate NF-κB activation, most commonly through promotion of nuclear translocation and transcriptional activity of the p65/p50 heterodimer (31, 99, 100, 104). This NF-κB activation in the lung mucosa has been described to regulate a multitude of responses including cytokine regulation (IL-6, IL-8, IL-1β, TNF-α to name a few), chemokine regulation (for example IL-8, GRO-α, RANTES), adhesion molecule expression, and other processes central to inflammatory responses (31).

A role for NF-κB has already been described with respect to LL-37-mediated immunomodulation. In a monocytic cell line, LL-37 was shown to inhibit LPS-stimulated nuclear translocation of various NF-κB subunits, which was proposed to be one of the mechanisms by which LL-37 suppresses LPS-induced gene transcription and exerts an anti-endotoxin effect (79). In contrast, LL-37 stimulation alone has been described to induce the transient phosphorylation of Iκ-Bα and nuclear translocation of p50 and p65 NF-κB subunits in primary PBMCs (Yu *et al.*, 2007; submitted for publication). Furthermore, a relevant role for this transient translocation of NF-κB has been demonstrated in LL-37-stimulated production of IL-8 and other chemokines, as well as in induction of MCP-3 by the synergistic combination of LL-37 and IL-1β, through inhibitor studies in PBMCs (Yu *et al.*, 2007; submitted for publication).

In this report, I have demonstrated for the first time a contribution of NF-κB signalling in LL-37-mediated responses of airway epithelial cells. Phosphorylation of Iκ-Bα is a signal that induces ubiquitin-mediated proteasome degradation of this regulator, and this degradation allows NF-κB subunits sequestered in the cytosol to migrate to the nucleus, and activate the transcription of many genes, including IL-6 (33). In addition to demonstrating the induction by LL-37 of Iκ-Bα phosphorylation and total Iκ-Bα degradation (Figure 10), I showed through inhibitor studies that this NF-κB signalling activation is required for LL-37-stimulated IL-6 release (Figure 9). A more direct study to examine LL-37-mediated NF-κB subunit translocation into the nucleus would further affirm these conclusions, and will be conducted in the near future.

In airway epithelial cells in particular, the significance of NF-κB signalling in the production and regulation of IL-6 has been previously demonstrated (99, 100, 123). In the majority of cases, MAPK activation, in particular p38, was associated with NF-κB activation and cytokine regulation (100, 123), which is to some extent the case with LL-37-mediated IL-6

58

production. A detailed analysis of the regulatory region of the IL-6 gene shows putative binding sites for a variety of response regulators including NF-κB, AP-1, CREB, and NF-IL-6 (124-126), an observation consistent with the possibility that IL-6 transcriptional regulation requires a complex of transcriptional factors for optimal expression.
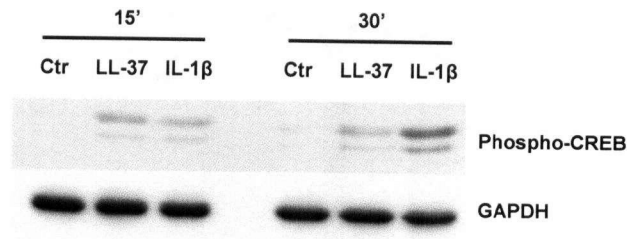
Figure 21. LL-37 induces CREB phosphorylation in 16HBE4o- cells.
16HBE4o- cells were treated with 50 µg/ml LL-37, 10ng/ml IL-1β, or were left untreated (Ctr – control), over a brief time course of 15 and 30 minutes. Whole cell protein lysates were prepared and analyzed by SDS-PAGE and Immunoblotting. Immunoblot for phosphorylation of transcriptional factor CREB, with expression of the house-keeping protein GAPDH assessed as a loading control. Results are from one experiment, representative of three experiments.

Further evidence supporting the putative involvement of multiple transcription factors in cytokine gene regulation is that LL-37 was able to induce cAMP-responsive element binding protein (CREB) phosphorylation in 16HBE4o- cells (Figure 21), in addition to activating NF-κB. Though not directly demonstrated in airway epithelial cells, LL-37 was furthermore shown to activate other transcription factors such as Elk-1 in monocytes (*via* MAPK) (73) and STAT transcription factors (*via* EGFR) in keratinocytes (83). Thus, it appears that host defence peptide LL-37 induces its immunomodulatory effects through a complex mechanism.

Activation of these multiple transcriptional factors implies activation of multiple signalling pathways (such as ERK1/2 and p38, for example) and provides an explanation as to how convergent signals might regulate IL-6 gene transcription and protein expression. Both the p38 and ERK1/2 pathways have been shown to affect NF-κB-mediated IL-6 transcription by

modulating transactivation capacity of the p65 subunit of NF-κB (127). The p38 pathway has also been implicated in IL-6 transcript stabilization, possibly through activation of a downstream effector MAPK-activated protein kinase-2 (MAPKAP-K2 or MK-2) (128). In addition, ERK1/2 signalling *via* p90 ribosomal S6 protein kinase 1 (Rsk1) activation has been shown to regulate CREB phosphorylation in airway epithelial cells (129). Therefore, even though IL-6 gene expression in the lung is usually associated with NF-κB-mediated regulation, the actual mechanism is likely to involve complexes of transcription factors that might potentially regulate gene expression in a cell-specific manner.

None of the proposed LL-37 receptors could completely account for LL-37-indced IL-6 production, indicating that there may be multiple receptors and more than one signalling pathway(s) responsible for NF-kB activation mediated by this host defence peptide. In addition to the traditionally recognized NF-κB activator receptors such as TLRs, IL-1 receptor, or the TNF receptor, receptor tyrosine kinases, such as EGFR have been implicated in NF-kB activation in human fibroblasts, proximal tubule cells, and smooth muscle cells among others (101, 130, 131). In airway epithelial cells, a study reported that stimulation by metals from ambient air particles can activate Ras, which in turn activated NF-κB and MEK to ERK1/2 signalling independently (132). Therefore, a possibility exists that LL-37 trans-activation of EGFR could participate in NF-κB activation and IL-6 regulation described in this report. My own preliminary studies indicate that there are several other receptor tyrosine kinases, in addition to EGFR, that appear to be activated in response to LL-37. This area of LL-37 research is relatively unresolved, and the identification of other receptors will provide further insight into other signalling pathways and processes mediated by the host defence peptide LL-37.

In conclusion, LL-37-mediated activation of the NF-κB signalling in airway epithelial cells demonstrated in this report is a novel effect of this host defence peptide. This NF-κB regulation proved to be the pivotal regulation mechanism with respect to selective peptide-

60

mediated cytokine and chemokine production.

4.5   LL-37 and receptor regulation of the IL-6 response

With respect to receptor regulation of LL-37-stimulated IL-6 release, the inhibitor studies performed in this thesis indicated that EGFR signalling partially regulated IL-6 release in airway epithelial cells by ~40 % (Figure 7A), a result I reproduced at a lower peptide concentration of 30 µg/ml (data not shown). Epidermal growth factor receptor has been implicated in various LL-37-mediated responses in epithelial cells. EGFR inhibitors were previously shown to completely reduce LL-37-stimulated IL-8 release in airway epithelial cells (74), and EGFR signalling was also shown to regulate airway wound closure and cell proliferation (86). Though not shown in airway epithelial cells in particular, EGFR signalling has been implicated in IL-6 regulation in thymic epithelial cells (133). In addition, EGFR has been shown to regulate MAPK activation (134), and multiple reports have described the reciprocal involvement of MAP kinases in IL-6 regulation (97, 98, 100, 106). Therefore, it seems reasonable to conclude that LL-37 induces IL-6 expression in part through transactivation of EGFR and subsequent MAPK signalling.

It appears that a Ptx sensitive receptor (Figure 7B) might be partially involved in mediating LL-37-induced IL-6 release, but it is difficult to confidently conclude whether this receptor is FPRL-1. I used a specific FPRL-1 agonist WKYMV at a concentration that was previously shown to potently induce FPRL-1-mediated responses (135); however, this concentration of WKYMV was only able to induce a quite modest, although significant, IL-6 release of ~ 1.6 fold above background.

Though $P2X_7$ has been shown to regulate LL-37-induced IL-1β processing and release in LPS-primed monocytes (88), it has not been described to mediate LL-37-induced responses in lung epithelial cells. However, $P2X_7$ agonists have been shown to stimulate IL-6 release from

various cell types, including epithelial cells (136-138), thus warranting the examination of potential involvement of $P2X_7$ in LL-37-induced IL-6 regulation. The $P2X_7$ inhibitor KN-62 used in this report did not block LL-37-induced IL-6 release. The same inhibition experiments were attempted with various newly purchased KN-62 inhibitor stocks, however identical results were obtained. Moreover, KN-62 also failed to inhibit IL-6 release induced by a specific $P2X_7$ agonist Bz-ATP (Figure 7C). As the control experiment did not work it is difficult to make definitive conclusions with respect to $P2X_7$ involvement in regulation of LL-37-mediated IL-6 release until studies with an efficient $P2X_7$ inhibitor are performed.

4.6   Other signalling regulation of LL-37-mediated IL-6

MAPK pathways have been shown to regulate numerous LL-37-mediated effects. In bronchial epithelial cells in particular, ERK1/2 and p38 MAP kinases have been show to each regulate IL-8 release to a degree, but when combined abrogated all IL-8 production (73). I showed that MAP kinases are involved in regulation of LL-37-mediated IL-6 induction (Figure 8); however, this regulation was more subtle when compared to their effects on LL-37-mediated IL-8 induction. ERK1/2, p38, and JNK MAP kinases have been described as IL-6 expression regulators in various cells and conditions. In osteoblasts and chondrocytes, TNF and IL-1β induced IL-6 regulation has been shown to occur through post-translational mRNA stabilization mediated by p38 (106), whereas transcriptional upregulation of IL-6 by TNF and IL-1β in monocytes was mediated by ERK1/2 and JNK regulation (139). While IL-1β-stimulated IL-6 release in skeletal muscle was regulated by both p38 and ERK1/2 (97), regulation of IL-6 in pulmonary epithelial cells has been demonstrated to involve either both ERK/p38 or p38 only, depending on the stimulus (100, 123). Thus, MAPK regulation of IL-6 release is a well described occurrence, and the results shown in Figure 8A describe yet another LL-37-mediated event that is significantly regulated by MAP kinases in airway epithelial cells.

As interest in LL-37-mediated immunomodulatory effects and their regulation grows, our knowledge of the range of signalling pathways that LL-37 activates or modulates is continually expanding. In addition to the well-described involvement of MAPK signalling in LL-37-mediated responses, a role for PI3 kinase is becoming more apparent. Barlow *et al.* (2006) have recently illustrated the significance of PI3K signalling as a regulator of LL-37-mediated inhibition of neutrophil apoptosis, while PI3K signalling has been shown to potently regulate endothelial cell growth and partially regulate NF-κB translocation in peptide-stimulated endothelial cells (89). Furthermore, the role of PI3K in regulation of LL-37-mediated IL-8 release, and combination IL-1β/LL-37-mediated MCP-3 release has been demonstrated in PBMCs (Yu *et al.* 2007, submitted for publication). However, PI3K signalling regulation did not affect LL-37-mediated IL-6 release in airway epithelial cells. Therefore, in addition to affecting different receptors and inducing different responses in various target cells, this host defence peptide targets regulatory mechanisms in a cell-specific manner as well.

4.7 LL-37 can augment IL-6 release mediated by pro-inflammatory mediators

As previously discussed, LL-37 can induce a variety of responses in lung epithelial cells over a range of concentrations. For example, low doses such as 2-5 µg/ml of LL-37 will mediate wound healing and proliferation, while doses higher than 50 µg/ml will induce apoptosis in various epithelial cell lines. Similarly LL-37-mediated induction of cytokines and chemokines was shown to be a dose-dependent response [Figures 2, 14, 15; (74)]. Another important factor that needs to be taken into consideration are the multiple stimuli usually present at the sites of infection and inflammation. These sites contain, in addition to LL-37, a multitude of pro- and anti- inflammatory stimuli acting simultaneously. A variety of effects are synergistically induced in human PBMCs by IL-1β and LL-37 (at a moderate peptide dose of 20 µg/ml) (Yu *et al.*, 2007

submitted for publication). Furthermore, the synergistic induction of chemokines with IL-1β and low doses of LL-37 has been demonstrated in neutrophils [Li, Y., M.Sc.thesis, UBC; (78)].

Similarly, I have shown in this report that co-stimulation of 16HBE4o- cells with IL-1β and LL-37 (low doses of 5 and 10 μg/ml) induced IL-6 release in an additive, rather than synergistic fashion (Figure 11). IL-1β is one of the major pro-inflammatory cytokines that can modulate airway epithelial cell responses. IL-1β stimulation has been shown to affect cytokine and chemokine production from the airway epithelium, and adhesion molecule expression in endothelial cells, which can directly influence epithelial layer permeability and neutrophil transmigration (140, 141). Even though LL-37 was shown to induce a significant IL-6 release over a range of concentrations, more than 10 μg/ml of peptide is needed to observe a greater than 2-fold cytokine induction. In the early stages of inflammation which are characterized by low LL-37 concentrations, the co-stimulation of cells with IL-1β and LL-37 would result in an augmented IL-6 release. Thus, the ability of low doses of LL-37 to increase IL-1β-mediated IL-6 release indicates that LL-37 can act to selectively modulate certain innate inflammatory signals relevant to normal host defence functions.

In addition to increasing IL-1β-mediated IL-6 release from airway epithelial cells, LL-37 also augmented flagellin-mediated cytokine release (Figure 12). Flagellin-mediated activation of TLR-5 signalling in lung epithelial cells has been demonstrated to induce transient cytosolic calcium increase, MAPK signalling and subsequent NF-κB activation leading to production of molecules such as IL-8 and IL-6 (102, 104). Since LL-37-mediated IL-6 production was shown to be NF-κB-regulated (Figure 9), it is possible that LL-37/flagellin stimulation results in additive increase in NF-κB stimulation. Since both flagellin and LL-37 have been shown to modulate IL-8 release from airway epithelial cells, it would be of interest to examine how co-stimulation would affect production of this chemokine.

4.8   LL-37 and chemokine regulation

In addition to the expected induction of IL-8, LL-37-mediated induction of GRO-α was demonstrated in this report. LL-37 stimulated a dose-dependent release of GRO-α from 16HBE4o- and primary bronchial epithelial cells (Figure 14). Furthermore, LL-37 induced an increase in GRO-α gene expression and protein release through EGFR, MAP kinases (ERK1/2 and p38), and NF-κB regulation. As previously described for peptide-mediated IL-6 regulation, the PI3K pathway was not found to be a significant regulator of this chemokine. Though LL-37 was shown to mediate RANTES increase in gene expression and protein release from 16HBE4o- cells, there was no detectable RANTES induced in primary bronchial epithelial cells stimulated by LL-37 (Figure 16). RANTES (CCL5) is a member of CC family of chemokines and has been shown to regulate chemotaxis of eosinophils, basophils, monocytes and memory T cells (142, 143). Though LL-37-mediated up-regulation of RANTES would be a novel and interesting finding due to the involvement of this chemokine in allergic responses and viral defences (142, 144, 145), its lack of induction in primary NHBE cells indicates that this result may not be physiologically relevant.

Even though the direct EGFR regulation of GRO-α has not been described before, ERK1/2 kinase activation by Raf-1 has been shown to be involved in regulation of this chemokine (108). The activation of Raf-1 as one of the downstream targets of EGFR signalling has been well documented (134), so it is possible to state that GRO-α is downstream of EGFR activation. The roles of MAPK regulation and NF-κB activation in GRO-α production have been demonstrated in airway epithelial and smooth muscle cells (105, 106, 108). In addition to the regulation of p65 NF-κB subunit, other transcriptional regulators such as the CCAAT/enhancer binding protein (C/EBP) elements have been described to regulate GRO-α transcription (106).

Lung epithelial cells are the first cells to encounter potential pathogens, and one of their essential roles in inflammation is to recruit effector cells to the site of pathogenic challenge. PMNs are important effector cells of lung innate responses. In the healthy lung, approximately 40% of total body's PMNs reside in the pulmonary vasculature (1). However, PMNs are rapidly and efficiently recruited to the site of infection through action of chemokines and cytokines originating from the lung epithelium. Host defence peptide LL-37 in this sense is an important factor in airway cells responses, as it can induce chemokines and cytokines at moderate concentrations and act as a direct chemoattractant at higher concentrations, and as such can affect progression of the inflammatory response. IL-8 and GRO-α shown to be regulated by LL-37 are potent modulators of PMN chemotaxis and activation.

IL-8 (CXCL-8) and GRO-α (CXCL-1) are structurally similar chemokines and members of the ELR-CXC chemokine family. IL-8 has been described as a potent neutrophil chemotactic factor, but also a neutrophil activator; it has been shown to regulate neutrophil adhesion, degranulation and respiratory burst [reviewed in (131)]. Aside from neutrophil recruitment, it may also induce chemotaxis of eosinophils, basophils, T cells, B cells, and macrophages (131). Similarly, GRO-α has been shown to induce chemotaxis, elastase exocytosis, transient cytosolic calcium increase and a modest respiratory burst, as compared to IL-8, in human neutrophils and basophils (but not eosinophils or monocytes) (12, 21). Even though IL-8 is perceived as the most potent neutrophil chemoattractant, *in vivo* studies show that IL-8 neutralization results in only partial inhibition of neutrophil infiltration, thus indicating that other CXC chemokines as GRO-α or ENA-78 are likely to be significant players (108). Furthermore, as members of the ELR-CXC family, both IL-8 and GRO-α have been proposed to mediate angiogenesis, which is likely relevant to wound repair or chronic inflammation (6).

Though neutrophil recruitment and activation are beneficial and necessary features of an inflammatory lung response, a dysregulated response can result in excessive neutrophilia and

tissue damage, characteristic of several lung inflammatory afflictions such as ARDS (17). As discussed previously, LL-37 concentration in inflammatory lung conditions would be increased. In turn, the increased LL-37 would act on airway epithelium to induce additional chemokines, which would drive the infiltration of additional leukocytes that can further the amplification of the inflammatory response through a positive feedback mechanism. However, under such circumstances, high concentrations of LL-37 (>50 µg/ml) would induce apoptosis in airway epithelial cells, which would act to reduce production of chemokines and cytokines and would help establish homeostasis of the affected site. Furthermore, a local serum increase would act to reduce excessive apoptosis and would inhibit cytokine and chemokine production.

4.9 Future areas for study

Even though a new facet of the role of host defence peptide LL-37 in lung innate immunity was described in this report, there are still many questions left unanswered. In addition to the panel of cytokines and chemokines I tested in this thesis, there are many that are relevant to lung immunity that could be potentially regulated by LL-37. A global transcriptional and/or proteomic analysis of LL-37 induced responses over a time course would be valuable as a tool for uncovering other potential processes that this host defence peptides could affect.

Chronic lung pathologies such as CF and COPD are characterized by mucus hypersecretion, which can impair gas exchange and obstruct the airways (15, 20). The differentiation of airway epithelial cells into mucus-secreting cells has been shown to occur upon stimulation with cytokines such as IL-4, IL-13, and IL-6 (146). In addition to cytokines, defensins have also been shown to affect mucin up-regulation in bronchial epithelial cells (147). Considering that LL-37 activates signalling pathways that commonly regulate mucin production, and that this peptide induces an IL-6 increase shown to potentially regulate mucin expression in airway epithelial cells, it is probable that LL-37 could affect mucin regulation and

expression, similarly to defensins.

Furthermore, a study in keratinocytes has shown that LL-37 and HBD-2,-3, and -4 induced IL-18 release synergistically (82). While cationic peptide synergy has been considered with respect to antimicrobial activity (148), it seems likely that synergy might be a more general property with respect to host defence immunomodulation. Bowdish *et al.* (2005) have examined this effect and have reported that LL-37 and indolicidin (bovine cathelicidin) synergistically reduced LPS-stimulated TNF-α release in a human monocytic cell line. They have furthermore described that LL-37 mediated endotoxin-induced TNF-α inhibition with either HBD-1 or HBD-2 in an additive manner (96). Given the important role of defensins in lung innate immunity (114), and the substantial functional similarity between LL-37 and defensins with respect to airway cells induced responses, it would be of interest to examine if these cationic peptides could synergistically mediate responses in the airway epithelium.

4. 10   Conclusions

In conclusion, I have shown in this report that the host defence peptide LL-37 induced a novel effect in airway epithelial cells – induction of IL-6 and GRO-α through regulation of NF-κB, EGFR, and MAP kinase signalling pathways. The evidence presented in this report has shown that LL-37 is indeed a potent regulator of lung immune responses mediated through multiple signalling pathways. Alone or acting with other inflammatory mediators, this host defence peptide was shown to participate in selective induction of pro-inflammatory signals required for a proper immune response, while mediating the delicate balance of beneficial inflammatory response with regulation of homeostasis.

The extensive, ever-increasing evidence of the ability of cationic peptides to modulate diverse aspects of innate and adaptive immunity alike has fuelled an interest in the design of therapeutics based on naturally occurring peptides. These synthetic derivative molecules should

efficiently modulate and boost intrinsic host defences, without causing cell toxicity characteristic of high doses of many host defence peptides. A prototype for such peptides termed innate defence regulators was recently published (Scott, M.G., E. Dullaghan, N. Mookherjee, N. Glavas, M. Waldbrook, A. Thompson, A. Wang, K. Lee, S. Doria, P. Hamill, J. Yu, Y. Li, O. Donini, M.M. Guarna, B.B. Finlay, J.R. North, and R.E.W. Hancock. 2007. Innate defence regulators as a new approach to anti-infective therapy. Nature Biotech., March 26th). The inherent ability of cationic peptides to enhance immune responses, without the adverse potential for developing resistance mechanisms, makes host defence peptides especially attractive targets for therapeutic development. However, before these synthetic analogues can be fully exploited as therapeutics, the arrays of functions and effects of their natural counterparts will require further clarification through rigorous *in vitro* and *in vivo* studies.

References

1.	Zhang, P., W. R. Summer, G. J. Bagby, and S. Nelson. 2000. Innate immunity and pulmonary host defense. *Immunological Reviews* 173:39-51.
2.	Hippenstiel, S., B. Opitz, B. Schmeck, and N. Suttorp. 2006. Lung epithelium as a sentinel and effector system in pneumonia - molecular mechanisms of pathogen recognition and signal transduction. *Respiratory Research* 7:97.
3.	Hancock, R. E. W., and G. Diamond. 2000. The role of cationic antimicrobial peptides in innate host defences. *Trends in Microbiology* 8:402-410.
4.	Thompson, A. B., R. A. Robbins, D. J. Romberger, J. H. Sisson, J. R. Spurzem, H. Teschler, and S. I. Rennard. 1995. Immunological functions of the pulmonary epithelium. *Eur Respir J* 8:127-149.
5.	Rogan, M. P., P. Geraghty, C. M. Greene, S. O. O'Neill, C. C. Taggart, and N. G. McElvaney. 2006. Antimicrobial proteins and polypeptides in pulmonary innate defence. *Respiratory Research* 7:29.
6.	Strieter, R. M., J. A. Belperio, and M. P. Keane. 2002. Cytokines in innate host defense in the lung. *Journal of Clinical Investigation* 109:699-705.
7.	Chen, C. I. U., S. Schaller-Bals, K. P. Paul, U. Wahn, and R. Bals. 2004. β-defensins and LL-37 in bronchoalveolar lavage fluid of patients with cystic fibrosis. *Journal of Cystic Fibrosis* 3:45-50.
8.	Tjabringa, S. G., K. F. Rabe, and P. S. Hiemstra. 2005. The human cathelicidin LL-37: a multifunctional peptide involved in infection and inflammation in the lung. *Pulmonary Pharmacology & Therapeutics* 18:321-327.
9.	Zhang, P., W. R. Summer, G. J. Bagby, and S. Nelson. 2000. Innate immunity and pulmonary host defense. *Immunological Reviews* 173:39-51.
10.	Agerberth, B., J. Grunewald, E. Castanos-Velez, B. Olsson, H. Jornvall, H. Wigzell, A. Eklund, and G. H. Gudmundsson. 1999. Antibacterial components in bronchoalveolar lavage fluid from healthy individuals and sarcoidosis patients. *Am. J. Respir. Crit. Care Med.* 160:283-290.
11.	Xiao, W., Y.-P. Hsu, A. Ishizaka, T. Kirikae, and R. B. Moss. 2005. Sputum cathelicidin, urokinase plasminogen activation system components, and cytokines discriminate cystic fibrosis, COPD, and asthma inflammation. *Chest* 128:2316-2326.
12.	Moser, C., D. J. Weiner, E. Lysenko, R. Bals, J. N. Weiser, and J. M. Wilson. 2002. {beta}-Defensin 1 contributes to pulmonary innate immunity in mice. *Infect. Immun.* 70:3068-3072.
13.	Bals, R., D. J. Weiner, R. L. Meegalla, and J. M. Wilson. 1999. Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model. *Journal of Clinical Investigation* 103:1113–1117.
14.	Bals, R., D. J. Weiner, A. D. Moscioni, R. L. Meegalla, and J. M. Wilson. 1999. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect. Immun.* 67:6084-6089.
15.	Kim, S. T., H. E. Cha, D. Y. Kim, G. C. Han, Y. Chung, J. L. Jae Lee Young, Y. J. Hwang, and H. Lee. 2003. Antimicrobial peptide LL-37 is upregulated in chronic nasal inflammatory disease. *Acta Oto-Laryngologica* 123:81-85.
16.	Stecenko, A. A., G. King, K. Torii, R. M. Breyer, R. Dworski, T. S. Blackwell, J. W. Christman, and K. L. Brigham. 2001. Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. *Inflammation* 25:145-155.

17. Puneet, P., S. Moochhala, and M. Bhatia. 2005. Chemokines in acute respiratory distress syndrome. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 288:3-15.

18. Cromwell, O., Q. Hamid, C. J. Corrigan, J. Barkans, Q. Meng, P. D. Collins, and A. B. Kay. 1992. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1β and tumour necrosis factor-α. *Immunology* 77:330-337.

19. Levine, S. J. 1995. Bronchial epithelial cell-cytokine interactions in airway inflammation. *J Investig Med* 43:241-249.

20. Hase, K., M. Murakami, M. Iimura, S. P. Cole, Y. Horibe, T. Ohtake, M. Obonyo, R. L. Gallo, L. Eckmann, and M. F. Kagnoff. 2003. Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against *Helicobacter pylori*. *Gastroenterology* 125:1613-1625.

21. Geiser, T., P.-H. Jarreau, K. Atabai, and M. A. Matthay. 2000. Interleukin-1beta augments in vitro alveolar epithelial repair. *Am J Physiol Lung Cell Mol Physiol* 279:L1184-1190.

22. Jones, S. A. 2005. Directing transition from innate to acquired immunity: Defining a role for IL-6. *J Immunol* 175:3463-3468.

23. Leemans, J. C., M. J. B. M. Vervoordeldonk, S. Florquin, K. P. van Kessel, and T. van der Poll. 2002. Differential role of interleukin-6 in lung inflammation induced by lipoteichoic acid and peptidoglycan from *Staphylococcus aureus*. *Am. J. Respir. Crit. Care Med.* 165:1445-1450.

24. Xing, Z., J. Gauldie, G. Cox, H. Baumann, M. Jordana, X.-F. Lei, and M. K. Achong. 1998. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J. Clin. Invest.* 101:311-320.

25. McLoughlin, R. M., J. Witowski, R. L. Robson, T. S. Wilkinson, S. M. Hurst, A. S. Williams, J. D. Williams, S. Rose-John, S. A. Jones, and N. Topley. 2003. Interplay between IFN-{gamma} and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation. *J. Clin. Invest.* 112:598-607.

26. Aderka, D., J. M. Le, and J. Vilcek. 1989. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *Journal Of Immunology.* 143:3517-3523.

27. McLoughlin, R. M., B. J. Jenkins, D. Grail, A. S. Williams, C. A. Fielding, C. R. Parker, M. Ernst, N. Topley, and S. A. Jones. 2005. IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. *PNAS* 102:9589–9594.

28. Teague, T. K., P. Marrack, J. W. Kappler, and A. T. Vella. 1997. IL-6 rescues resting mouse T cells from apoptosis. *J Immunol* 158:5791-5796.

29. Christopherson, K., II, and R. Hromas. 2001. Chemokine regulation of normal and pathologic immune responses. *Stem Cells* 19:388-396.

30. Kirikae, T., M. Hirata, H. Yamasu, F. Kirikae, H. Tamura, F. Kayama, K. Nakatsuka, T. Yokochi, and M. Nakano. 1998. Protective effects of a human 18-kilodalton cationic antimicrobial protein (CAP18)-derived peptide against murine endotoxemia. *Infect. Immun.* 66:1861-1868.

31. Christman, J. W., R. T. Sadikot, and T. S. Blackwell. 2000. The Role of Nuclear factor-{kappa} B in pulmonary diseases. *Chest* 117:1482-1487.

32. Hayden, M. S., and S. Ghosh. 2004. Signaling to NF-kB. *Genes and Development* 18:2195-2224.

33. Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-kB AND REL PROTEINS: Evolutionarily conserved mediators of immune responses. *Annual Review of Immunology* 16:225-260.

34. Liu, S. F., and A. B. Malik. 2006. NF-{kappa}B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol Lung Cell Mol Physiol* 290:L622-645.

35. Saadane, A., S. Masters, J. DiDonato, J. Li, and M. Berger. 2007. Parthenolide inhibits I{kappa} B kinase, NF-{kappa}B activation and inflammatory response in CF cells and mice. *Am. J. Respir. Cell Mol. Biol.*:2006-0323OC.

36. Hancock, R. E. W. 2001. Cationic peptides: effectors in innate immunity and novel antimicrobials. *The Lancet* 1:156-164.

37. Hancock, R. E. W., and M. G. Scott. 2000. The role of antimicrobial peptides in animal defenses. *PNAS* 97:8856-8861.

38. Zasloff, M. 2002. Antimicobial peptides of multicellular organisms. *Nature* 415:389-395.

39. Jenssen, H., H. P., and R. E. W. Hancock. 2006. Peptide antimicrobial agents. *Clinical Microbiology Reviews* 19:491-511.

40. Bowdish, D. M. E., D. J. Davidson, and R. E. W. Hancock. 2005. A Re-evaluation of the role of host defence peptides in mammalian immunity. *Current Protein & Peptide Science* 6:35-51.

41. Zanetti, M. 2004. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 75:39-48.

42. Bals, R., and J. M. Wilson. 2003. Cathelicidins - a family of multifunctional antimicrobial peptides. *Cellular and Molecular Life Sciences* V60:711-720.

43. Tomasinsig, L., and M. Zanetti. 2005. The Cathelicidins - structure, function and evolution. *Current Protein & Peptide Science* 6:23-34.

44. Chang, C., O. Pleguezuelos, Y. Zhang, J. Zou, and C. J. Secombes. 2005. Identification of a novel cathelicidin gene in Rainbow trout *Oncorhynchys mykiss. Infect. Immun.* 73:5053-5064.

45. Chang, C., Y. Zhang, J. Zou, P. Nie, and C. J. Secombes. 2006. Two cathelicidin genes are present in both Rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). *Antimicrob. Agents Chemother.* 50:185-195.

46. Lynn, D. J., R. Higgs, S. Gaines, J. Tierney, T. James, A. T. Lloyd, M. A. Fares, G. Mulcahy, and C. O'Farrelly. 2004. Bioinformatic discovery and initial chracteriazation of nine novel antimicrobial peptide genes in the chicken. *Immunogenetics* 56:170-177.

47. Uzzell, T., E. D. Stolzenberg, A. Shinnar, and M. Zasloff. 2003. Hagfish intestunal antimicobial peptides are ancient cathelicidins. *Peptides* 24:1655-1667.

48. Xiao, Y., A. L. Hughes, J. Ando, Y. Matsuda, J. Cheng, D. Skinner-Noble, and G. Zhang. 2004. A genome-wide screen identifies a single β-defensin gene cluster in the chicken: implication for the origin and evolution of mammalian defensins. *BMC Genomics* 5:56.

49. Zanetti, M., R. Gennaro, and D. Romeo. 1995. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Letters* 374:1-5.

50. Shinnar, A., K. L. Butler, and H. J. Park. 2003. Cathelicidin family of antimicrobial peptides: proteolytic processing and protease resistance. *Bioorg. Chem.* 31:425-436.

51. Borregaard, N., and J. B. Cowland. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* 89:3503-3521.

52. Frohm, M., B. Agerberth, G. Ahangari, M. Stahle-Backdahl, S. Liden, H. Wigzell, and G. H. Gudmundsson. 1997. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J. Biol. Chem.* 272:15258-15263.

53. Larrick, J. W., M. Hirata, R. F. Balint, J. Lee, J. Zhong, and S. C. Wright. 1995. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect. Immun.* 63:1291-1297.

54. Gudmundsson, G. H., B. Agerberth, J. Odeberg, T. Bergman, B. Olsson, and R. Salcedo. 1996. The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. *Eur J Biochem* 238:325-332.

55. Sorensen, O. E., P. Follin, A. H. Johnsen, J. Calafat, G. S. Tjabringa, P. S. Hiemstra, and N. Borregaard. 2001. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* 97:3951-3959.

56. Agerberth, B., H. Gunne, J. Odeberg, P. Kogner, H. G. Boman, and G. H. Gudmundsson. 1995. FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. *PNAS* 92:195-199.

57. Murakami, M., B. Lopez-Garcia, M. Braff, R. A. Dorschner, and R. L. Gallo. 2004. Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense. *J Immunol* 172:3070-3077.

58. Yamasaki, K., J. Schauber, A. Coda, H. Lin, R. A. Dorschner, N. M. Schechter, C. Bonnart, P. Descargues, A. Hovnanian, and R. L. Gallo. 2006. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *FASEB J.* 20:2068-2080.

59. Sorensen, O. E., L. Gram, A. H. Johnsen, E. Andersson, S. Bangsboll, G. S. Tjabringa, P. S. Hiemstra, J. Malm, A. Egesten, and N. Borregaard. 2003. Processing of seminal plasma hCAP-18 to ALL-38 by gastricsin: A novel mechanism of generating antimicrobial peptides in vagina. *J. Biol. Chem.* 278:28540-28546.

60. Agerberth, B., J. Charo, J. Werr, B. Olsson, F. Idali, L. Lindbom, R. Kiessling, H. Jornvall, H. Wigzell, and G. H. Gudmundsson. 2000. The human antimicrobial and chemotactic peptides LL-37 and alpha -defensins are expressed by specific lymphocyte and monocyte populations. *Blood* 96:3086-3093.

61. Cowland, J. B., A. H. Johnsen, and N. Borregaard. 1995. hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. *FEBS Letters* 368:173-176.

62. Di Nardo, A., A. Vitiello, and R. L. Gallo. 2003. Cutting Edge: Mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide. *J Immunol* 170:2274-2278.

63. Sorensen, O., K. Arnljots, J. B. Cowland, D. F. Bainton, and N. Borregaard. 1997. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood* 90:2796-2803.

64. Wah, J., A. Wellek, F. Frankenberger, P. Unterberger, U. Welsch, and R. Bals. 2006. Antimicrobial peptides are present in immune and host defense cells of the human respiratory and gastroinstestinal tracts *Cell and Tissue Research* 324:449-456.

65. Hosokawa, I., Y. Hosokawa, H. Komatsuzawa, R. B. Goncalves, N. Karimbux, M. H. Napimoga, M. Seki, K. Ouhara, M. Sugai, M. A. Taubman, and T. Kawai. 2006. Innate immune peptide LL-37 displays distinct expression pattern from beta-defensins in inflamed gingival tissue. *Clinical and Experimental Immunology* 146:218-225.

66. Nell, M. J., G. Sandra Tjabringa, M. J. Vonk, P. S. Hiemstra, and J. J. Grote. 2004. Bacterial products increase expression of the human cathelicidin hCAP-18/LL-37 in cultured human sinus epithelial cells. *FEMS Immunology and Medical Microbiology* 42:225-231.

67. Bals, R., X. Wang, M. Zasloff, and J. M. Wilson. 1998. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *PNAS* 95:9541-9546.

68. Frohm Nilsson, M., B. Sandstedt, O. Sorensen, G. Weber, N. Borregaard, and M. Stahle-Backdahl. 1999. The human cationic antimicrobial protein (hCAP18), a peptide

antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. *Infect. Immun.* 67:2561-2566.

69. Schaller-Bals, S., A. Schulze, and R. Bals. 2002. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. *Am. J. Respir. Crit. Care Med.* 165:992-995.

70. Niyonsaba, F., K. Iwabuchi, A. Someya, M. Hirata, H. Matsuda, H. Ogawa, and I. Nagaoka. 2002. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology* 106:20-26.

71. Tjabringa, G. S., D. K. Ninaber, J. W. Drijfhout, K. F. Rabe, and P. S. Hiemstra. 2006. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *International Archives of Allergy & Immunology* 140:103-112.

72. Yang, D., Q. Chen, A. P. Schmidt, G. M. Anderson, J. M. Wang, J. Wooters, J. J. Oppenheim, and O. Chertov. 2000. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J. Exp. Med.* 192:1069-1074.

73. Bowdish, D. M. E., D. J. Davidson, D. P. Speert, and R. E. W. Hancock. 2004. The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes. *J Immunol* 172:3758-3765.

74. Tjabringa, G. S., J. Aarbiou, D. K. Ninaber, J. W. Drijfhout, O. E. Sorensen, N. Borregaard, K. F. Rabe, and P. S. Hiemstra. 2003. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. *J Immunol* 171:6690-6696.

75. Zuyderduyn, S., D. K. Ninaber, P. S. Hiemstra, and K. F. Rabe. 2006. The antimicrobial peptide LL-37 enhances IL-8 release by human airway smooth muscle cells. *Journal of Allergy and Clinical Immunology* 117:1328-1335.

76. Scott, M. G., D. J. Davidson, M. R. Gold, D. Bowdish, and R. E. W. Hancock. 2002. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J Immunol* 169:3883-3891.

77. Chen, X., F. Niyonsaba, H. Ushio, I. Nagaoka, S. Ikeda, K. Okumura, and H. Ogawa. 2006. Human cathelicidin LL-37 increases vascular permeability in the skin via mast cell activation, and phosphorylates MAP kinases p38 and ERK in mast cells. *Journal of Dermatological Science* 43:63-66.

78. Barlow, P. G., Y. Li, T. S. Wilkinson, D. M. E. Bowdish, Y. E. Lau, C. Cosseau, C. Haslett, A. J. Simpson, R. E. W. Hancock, and D. J. Davidson. 2006. The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system. *J Leukoc Biol* 80:509-520.

79. Mookherjee, N., K. L. Brown, D. M. E. Bowdish, S. Doria, R. Falsafi, K. Hokamp, F. M. Roche, R. Mu, G. H. Doho, J. Pistolic, J.-P. Powers, J. Bryan, F. S. L. Brinkman, and R. E. W. Hancock. 2006. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *J Immunol* 176:2455-2464.

80. Davidson, D. J., A. J. Currie, G. S. D. Reid, D. M. E. Bowdish, K. L. MacDonald, R. C. Ma, R. E. W. Hancock, and D. P. Speert. 2004. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *J Immunol* 172:1146-1156.

81. Kandler, K., R. Shaykhiev, P. Kleemann, F. Klescz, M. Lohoff, C. Vogelmeier, and R. Bals. 2006. The anti-microbial peptide LL-37 inhibits the activation of dendritic cells by

TLR ligands. *Int. Immunol.*:dxl107.

82. Niyonsaba, F., H. Ushio, I. Nagaoka, K. Okumura, and H. Ogawa. 2005. The human {beta}-defensins (-1, -2, -3, -4) and cathelicidin LL-37 induce IL-18 secretion through p38 and ERK MAPK activation in primary human keratinocytes. *J Immunol* 175:1776-1784.

83. Tokumaru, S., K. Sayama, Y. Shirakata, H. Komatsuzawa, K. Ouhara, Y. Hanakawa, Y. Yahata, X. Dai, M. Tohyama, H. Nagai, L. Yang, S. Higashiyama, A. Yoshimura, M. Sugai, and K. Hashimoto. 2005. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *J Immunol* 175:4662-4668.

84. Niyonsaba, F., H. Ushio, N. Nakano, W. Ng, K. Sayama, K. Hashimoto, I. Nagaoka, K. Okumura, and H. Ogawa. 2006. Antimicrobial peptides human [beta]-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J Invest Dermatol.*

85. Lau, Y. E., A. Rozek, M. G. Scott, D. L. Goosney, D. J. Davidson, and R. E. W. Hancock. 2005. Interaction and cellular localization of the human host defense peptide LL-37 with lung epithelial cells. *Infect. Immun.* 73:583-591.

86. Shaykhiev, R., C. Beisswenger, K. Kandler, J. Senske, A. Puchner, T. Damm, J. Behr, and R. Bals. 2005. Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. *Am J Physiol Lung Cell Mol Physiol* 289:L842-848.

87. Lau, Y. E., D. M. E. Bowdish, C. Cosseau, R. E. W. Hancock, and D. J. Davidson. 2006. Apoptosis of airway epithelial cells: human serum sensitive induction by the cathelicidin LL-37. *Am. J. Respir. Cell Mol. Biol.* 34:399-409.

88. Elssner, A., M. Duncan, M. Gavrilin, and M. D. Wewers. 2004. A novel P2X$_7$ receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1{beta} processing and release. *J Immunol* 172:4987-4994.

89. Koczulla, R., G. von Degenfeld, C. Kupatt, F. Krotz, S. Zahler, T. Gloe, K. Issbrucker, P. Unterberger, M. Zaiou, C. Lebherz, A. Karl, P. Raake, A. Pfosser, P. Boekstegers, U. Welsch, P. S. Hiemstra, C. Vogelmeier, R. L. Gallo, M. Clauss, and R. Bals. 2003. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J. Clin. Invest.* 111:1665-1672.

90. Braff, M. H., M. i. A. Hawkins, A. D. Nardo, B. Lopez-Garcia, M. D. Howell, C. Wong, K. Lin, J. E. Streib, R. Dorschner, D. Y. M. Leung, and R. L. Gallo. 2005. Structure-function relationships among human cathelicidin peptides: Dissociation of antimicrobial properties from host immunostimulatory activities. *J Immunol* 174:4271-4278.

91. Nagaoka, I., H. Tamura, and M. Hirata. 2006. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X$_7$. *J Immunol* 176:3044-3052.

92. Wetering, S. v., S. P. G. Mannesse-Lazeroms, M. A. J. A. v. Sterkenburg, and P. S. Hiemstra. 2002. Neutrophil defensins stimulate the release of cytokines by airway epithelial cells: modulation by dexamethasone. *Inflammation Research* V51:8-15.

93. Cozens, A. L., M. J. Yezzi, K. Kunzelmann, T. Ohrui, L. Chin, K. Eng, W. E. Finkbeiner, J. H. Widdicombe, and D. C. Gruenert. 1994. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am. J. Resp. Cell Biol.* 10:38-47.

94. Darveau, R. P., and R. E. Hancock. 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *J. Bacteriol.* 155:831-838.

95. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.* 29:e45-.

96. Bowdish, D. M. E., D. J. Davidson, M. G. Scott, and R. E. W. Hancock. 2005. Immunomodulatory activities of small host defense peptides. *Antimicrob. Agents Chemother.* 49:1727-1732.

97. Luo, G., D. D. Hershko, B. W. Robb, C. J. Wray, and P.-O. Hasselgren. 2003. IL-1beta stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-kappa B. *Am J Physiol Regul Integr Comp Physiol* 284:R1249-1254.

98. Vanden Berghe, W., L. Vermeulen, G. De Wilde, K. De Bosscher, E. Boone, and G. Haegeman. 2000. Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. *Biochemical Pharmacology* 60:1185-1195.

99. Quay, J. L., W. Reed, J. Samet, and R. B. Devlin. 1998. Air pollution particles induce IL-6 gene expression in human airway epithelial cells via NF-kappa B activation. *Am. J. Respir. Cell Mol. Biol.* 19:98-106.

100. Wang, C. B., C. K. Wong, W. K. Ip, M. L. Y. Li, Y. P. Tian, and C. W. K. Lam. 2005. Induction of IL-6 in co-culture of bronchial epithelial cells and eosinophils is regulated by p38 MAPK and NF-κB. *Allergy* 60:1378-1385.

101. Habib, A. A., S. Chatterjee, S.-K. Park, R. R. Ratan, S. Lefebvre, and T. Vartanian. 2000. The epidermal growth factor receptor engages RIP (receptor interacting protein) and NIK (NF-kB inducing kinase) to activate NF-κB: Identification of a novel RTK signalosome. *J. Biol. Chem.*:M008458200.

102. Muir, A., G. Soong, S. Sokol, B. Reddy, M. I. Gomez, A. van Heeckeren, and A. Prince. 2004. Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol* 30:777-783.

103. Ritter, M., D. Mennerich, A. Weith, and P. Seither. 2005. Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. *Journal of Inflammation* 2:16.

104. Tseng, J., J. Do, J. H. Widdicombe, and T. E. Machen. 2006. Innate immune responses of human tracheal epithelium to *Pseudomonas aeruginosa* flagellin, TNF-{alpha}, and IL-1beta. *Am J Physiol Cell Physiol* 290:C678-690.

105. Issa, R., S. Xie, K.-Y. Lee, R. D. Stanbridge, P. Bhavsar, M. B. Sukkar, and K. F. Chung. 2006. GRO-{alpha} regulation in airway smooth muscle by IL-1beta and TNF-{alpha}: role of NF-{kappa}B and MAP kinases. *Am J Physiol Lung Cell Mol Physiol* 291:L66-74.

106. Patil, C., X. Zhu, C. Rossa, Y. J. Kim, and K. Kirkwood. 2004. p38 MAPK regulates IL-1β induced IL-6 expression through mRNA stability in osteoblasts. *Immunological Investigations* 33:213-233.

107. Thomas, L. H., J. S. Friedland, M. Sharland, and S. Becker. 1998. Respiratory syncytial virus-induced RANTES production from human bronchial epithelial cells is dependent on Nuclear factor-{kappa}B nuclear binding and is inhibited by adenovirus-mediated expression of inhibitor of {kappa}B{alpha}. *J Immunol* 161:1007-1016.

108. Kawaguchi, M., F. Kokubu, S. Matsukura, K. Ieki, M. Odaka, S. Watanabe, S. Suzuki, M. Adachi, and S.-K. Huang. 2003. Induction of C-X-C chemokines, growth-related oncogene {alpha} expression, and epithelial cell-derived neutrophil-activating protein-78 by ML-1 (interleukin-17F) involves activation of Raf1-mitogen-activated protein kinase kinase-extracellular signal-regulated kinase 1/2 pathway. *J Pharmacol Exp Ther* 307:1213-1220.

109. Zetterstorm, R. 2002. Kostmann disease-infantile genetic agranulocytosis: historical views and new aspects. *Acta Paediatrica* 91:1279-1281.

110. Putsep, K., G. Carlsson, H. G. Boman, and M. Andersson. 2002. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *The Lancet* 360:1144-1149.

111. Ong, P. Y., T. Ohtake, C. Brandt, I. Strickland, M. Boguniewicz, T. Ganz, R. L. Gallo, and D. Y. M. Leung. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 347:1151-1160.

112. Dorschner, R. A., V. K. Pestonjamasp, S. Tamakuwala, T. Ohtake, J. Rudisill, V. Nizet, B. Agerberth, G. H. Gudmundsson, and R. L. Gallo. 2001. Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against Group A *Streptococcus*. 117:91-97.

113. Scott, M. G., A. C. E. Vreugdenhil, W. A. Buurman, R. E. W. Hancock, and M. R. Gold. 2000. Cutting Edge: Cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) to LPS binding protein. *J Immunol* 164:549-553.

114. van Wetering, S., G. S. Tjabringa, and P. S. Hiemstra. 2005. Interactions between neutrophil-derived antimicrobial peptides and airway epithelial cells. *J Leukoc Biol* 77:444-450.

115. Arlene, A. S., K. Gayle, T. Keizo, M. B. Richard, D. Ryszard, S. B. Timothy, W. C. John, and L. B. Kenneth. 2001. Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. *Inflammation* V25:145-155.

116. Schindler, R., J. Mancilla, S. Endres, R. Ghorbani, S. C. Clark, and C. A. Dinarello. 1990. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. *Blood* 75:40-47.

117. Hegde, S., J. Pahne, and S. Smola-Hess. 2004. Novel immunosuppressive properties of interleukin-6 in dendritic cells: inhibition of NF-kappa B binding activity and CCR7 expression. *FASEB J.*:03-0969fje.

118. McClintock, J. Y., and E. M. Wagner. 2005. Role of IL-6 in systemic angiogenesis of the lung. *J Appl Physiol* 99:861-866.

119. Kaplanski, G., V. Marin, F. Montero-Julian, A. Mantovani, and C. Farnarier. 2003. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends in Immunology* 24:25-29.

120. Felgentreff, K., C. Beisswenger, M. Griese, T. Gulder, G. Bringmann, and R. Bals. 2006. The antimicrobial peptide cathelicidin interacts with airway mucus. *Peptides* 27:3100-3106.

121. Wang, Y., B. Agerberth, A. Lothgren, A. Almstedt, and J. Johansson. 1998. Apolipoprotein A-I binds and inhibits the human antibacterial/cytotoxic peptide LL-37. *J. Biol. Chem.* 273:33115-33118.

122. Jones, S. A., S. Horiuchi, N. Topley, N. Yamamoto, and G. M. Fuller. 2001. The soluble interleukin 6 receptor: mechanisms of production and implications in disease. *FASEB J.* 15:43-58.

123. Ning, W., A. M. K. Choi, and C. Li. 2005. Carbon monoxide inhibits IL-17-induced IL-6 production through the MAPK pathway in human pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 289:L268-273.

124. Baccam, M., S.-Y. Woo, C. Vinson, and G. A. Bishop. 2003. CD40-mediated transcriptional regulation of the IL-6 gene in B lymphocytes: Involvement of NF-{kappa} B, AP-1, and C/EBP. *J Immunol* 170:3099-3108.

125. Dendorfer, U., P. Oettgen, and T. A. Libermann. 1994. Multiple regulatory elements in the interleukin-6 gene mediate induction by prostaglandins, cyclic AMP, and lipopolysaccharide. *Molecular And Cellular Biology* 14:4443–4454.

126. Matsusaka, T., K. Fujikawa, Y. Nishio, N. Mukaida, K. Matsushima, T. Kishmoto, and S. Akira. 1993. Transcription factors NF-IL6 and NF-{kappa}B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *PNAS* 90:10193-10197.

127. Vanden Berghe, W., S. Plaisance, E. Boone, K. De Bosscher, M. L. Schmitz, W. Fiers, and G. Haegeman. 1998. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for Nuclear factor-kappa B p65 transactivation mediated by tumor necrosis factor. *J. Biol. Chem.* 273:3285-3290.

128. Hoffmann, E., O. Dittrich-Breiholz, H. Holtmann, and M. Kracht. 2002. Multiple control of interleukin-8 gene expression. *J Leukoc Biol* 72:847-855.

129. Song, K. S., J.-K. Seong, K. C. Chung, W.-J. Lee, C.-H. Kim, K. N. Cho, C.-D. Kang, J. S. Koo, and J.-H. Yoon. 2003. Induction of MUC8 gene expression by interleukin-1{beta} is mediated by a sequential ERK MAPK/RSK1/CREB cascade pathway in human airway epithelial cells. *J. Biol. Chem.* 278:34890-34896.

130. Haussler, U., G. von Wichert, R. M. Schmid, F. Keller, and G. Schneider. 2005. Epidermal growth factor activates nuclear factor-{kappa} B in human proximal tubule cells. *Am J Physiol Renal Physiol* 289:F808-815.

131. Lin, Z.-Q., T. Kondo, Y. Ishida, T. Takayasu, and N. Mukaida. 2003. Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *J Leukoc Biol* 73:713-721.

132. Wu, W., I. Jaspers, W. Zhang, L. M. Graves, and J. M. Samet. 2002. Role of Ras in metal-induced EGF receptor signaling and NF-kappa B activation in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 282:L1040-1048.

133. Le, P. T., S. Lazorick, L. P. Whichard, B. F. Haynes, and K. H. Singer. 1991. Regulation of cytokine production in the human thymus: epidermal growth factor and transforming growth factor alpha regulate mRNA levels of interleukin 1 alpha (IL-1 alpha), IL-1 beta, and IL-6 in human thymic epithelial cells at a post-transcriptional level. *J. Exp. Med.* 174:1147-1157.

134. Jorissen, R. N., F. Walker, N. Pouliot, T. P. J. Garrett, C. W. Ward, and A. W. Burgess. 2003. Epidermal growth factor receptor: mechanisms of activation and signalling. *Experimental Cell Research* 284:31-53.

135. Schmitz, M. L., and P. A. Baeuerle. 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *EMBO* 10:3805–3817.

136. Douillet, C. D., W. P. Robinson, III, P. M. Milano, R. C. Boucher, and P. B. Rich. 2006. Nucleotides induce IL-6 release from human airway epithelia via P2Y2 and p38 MAPK-dependent pathways. *Am J Physiol Lung Cell Mol Physiol* 291:L734-746.

137. Inoue, K., J. Hosoi, and M. Denda. 2006. Extracellular ATP has stimulatory effects on the expression and release of IL-6 via purinergic receptors in normal human epidermal keratinocytes. *J Invest Dermatol* 127:362-371.

138. Shigemoto-Mogami, Y., S. Koizumi, M. Tsuda, K. Ohsawa, S. Kohsaka, and K. Inoue. 2001. Mechanisms underlying extracellular ATP-evoked interleukin-6 release in mouse microglial cell line, MG-5. *Journal of Neurochemistry* 78:1339-1349.

139. Tuyt, L. M. L., W. H. A. Dokter, K. Birkenkamp, S. B. Koopmans, C. Lummen, W. Kruijer, and E. Vellenga. 1999. Extracellular-regulated kinase 1/2, Jun N-terminal kinase, and c-Jun are involved in NF-{kappa}B-dependent IL-6 expression in human monocytes. *J Immunol* 162:4893-4902.

140. Carolan, E. J., and T. B. Casale. 1996. Neutrophil transepithelial migration is dependent upon epithelial characteristics. *Am. J. Respir. Cell Mol. Biol.* 15:224-231.

141. Furie, M. B., and D. D. McHugh. 1989. Migration of neutrophils across endothelial

monolayers is stimulated by treatment of the monolayers with interleukin-1 or tumor necrosis factor- alpha. *J Immunol* 143:3309-3317.

142. Welling, M. M., P. S. Hiemstra, M. T. van den Barselaar, A. Paulusma-Annema, P. H. Nibbering, E. K. J. Pauwels, and W. Calame. 1998. Antibacterial activity of human neutrophil defensins in experimental infections in mice is accompanied by increased leukocyte accumulation. *J. Clin. Invest.* 102:1583-1590.

143. Schall, T. J., K. Bacon, K. J. Toy, and D. V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* 347:669-671.

144. Coffey, M. J., C. Woffendin, S. M. Phare, R. M. Strieter, and D. M. Markovitz. 1997. RANTES inhibits HIV-1 replication in human peripheral blood monocytes and alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 272:L1025-1029.

145. Manni, A., J. Kleimberg, V. Ackerman, A. Bellini, F. Patalano, and S. Mattoli. 1996. Inducibility of RANTES mRNA by IL-1[beta] in human bronchial epithelial cells is associated with increased NF-[kappa]B DNA binding activity. *Biochemical and Biophysical Research Communications* 220:120-124.

146. Chen, Y., P. Thai, Y.-H. Zhao, Y.-S. Ho, M. M. DeSouza, and R. Wu. 2003. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J. Biol. Chem.* 278:17036-17043.

147. Aarbiou, J., R. M. Verhoosel, S. van Wetering, W. I. de Boer, J. H. J. M. van Krieken, S. V. Litvinov, K. F. Rabe, and P. S. Hiemstra. 2004. Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression in vitro. *Am. J. Respir. Cell Mol. Biol.* 30:193-201.

148. Yan, H., and R. E. W. Hancock. 2001. Synergistic interactions between mammalian antimicrobial defense peptides. *Antimicrob. Agents Chemother.* 45:1558-1560.