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Cathelicidin and its Role in Defence against Bacterial Infections of Epithelial Cells

THE UNIVERSITY
of EDINBURGH

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Doctor of Philosophy
The University of Edinburgh
2014

Declaration

I declare that all work included in this thesis is my own, except where otherwise stated. No part of this work has been, or will be, submitted for any other degree or professional qualification.

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Abstract

Cathelicidins are antimicrobial peptides (AMPs) that were first discovered to have microbicidal properties but more recently to be multifunctional immunomodulators and thus important in influencing host defence against infectious disease. Whilst roles in various organs have been demonstrated, their expression patterns in health and disease in other organs are less clear and their key immunomodulatory functions remain undefined, particularly with regard to the balance of immunomodulatory properties and microbicidal activity in their ability to promote defence against infection.

I therefore set out to describe LL-37 expression (human cathelicidin) in the female reproductive tract (across the menstrual cycle) and in the lung (during specific lung diseases), to define the effects on the function of airway epithelial cells during bacterial infection and to evaluate the key *in vivo* roles of endogenous cathelicidin (using a knockout mouse model) as well as the effect of therapeutic administration of LL-37 in a pulmonary *Pseudomonas aeruginosa* infection model.

I demonstrated that cathelicidin protein and transcription shows a cyclical pattern of expression in female reproductive tissues which is maintained at high levels in decidua. LL-37 protein was also detected in hTERT endometrial epithelial cells but despite the suggestion that cathelicidin may be regulated by steroid hormones there was no direct effect of progesterone on transcription. LL-37 is barely detected in healthy airways however is well known to increase during infection or inflammation. I observed that sputum from patients with bronchiectasis showed a correlation between the level of LL-37, TNF, MPO and chronic colonisation of *Pseudomonas aeruginosa*. Patients with lung cancer expressed much less LL-37 than the bronchiectasis patients but there was a trend towards increased production post-surgery compared to pre-surgery.

LL-37 was previously shown by our lab to selectively promote BAX and caspase-dependant death of infected epithelial cells. I went on to show that this appears to be a partially caspase-1 dependent mechanism and that human bronchial epithelial (HBE) cells and A549 cell lines

both express several of the components required to form inflammasomes, a caspase-1 dependant form of inflammatory cell death.

Finally, I showed using murine models that cathelicidin enhances bacterial clearance during pulmonary infection *in vivo,* a response which is defective in mice lacking endogenous cathelicidin and that administration of exogenous, synthetic LL-37 at the time of infection can promote an early protective neutrophil influx in the absence of endogenous cathelicidin production.

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Abbreviations

ADP	Adenosine diphosphate
AEC	Airway epithelial cell
AIF	Apoptosis inducing factor
AIM2	Absent in melanoma 2
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
Bax	Bcl-2 associated X protein
Bcl2	B-cell lymphoma 2
BID	BH3-interacting domain death agonist
BIP V5	Bax inhibitory peptide
BIR	Baculovirus inhibitor of apoptosis repeat
BOS	Bronchiolitis obliterans syndrome
BSA	Bovine serum albumin
Caspase	Cysteinyl aspartate-specific proteases
CARD	Caspase activation and recruitment domain
cDNA	Complementary Deoxyribonucleic acid
CF	Cystic fibrosis
CFU	Colony forming units
CFTR	Cystic fibrosis transmembrane regulator
CHDP	Cationic host defence peptides
CLR	C-type lectin receptor
COPD	Chronic obstructive pulmonary disease
COX2	Cyclooxygenase 2
CRAMP	Cathelin-related antimicrobial peptide
Cyt c	Cytochrome c
DAB	Diaminobenzidine
DAMP	Danger-associated molecular pattern
DC	Dendritic cell

DMEM	Dulbecco's modified Eagles medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
EEPC	Endometrial epithelial cells
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
Exo S, T, U	Exoenzymes S, T, U
FBS/FCS	Foetal bovine serum/foetal calf serum
FPRL-1	Formyl peptide receptor-like 1
hCAP18	Human cationic antimicrobial peptide of 18kDa
hCG	Human chorionic gonadotropin
HBD	Human β-defensin
HBE	Human bronchial epithelial
HNP	Human neutrophil peptide
HRP	Horse radish peroxidase
htert	Human telomerase reverse transcriptase
HUMEC	Human uterus microvascular endothelial cell
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
GTP	Guanine triphosphate
IFN-γ	Interferon-gamma
IL	Interleukin
IPAF	ICE protease-activating factor
ITS	Insulin transferrin selenium
IUS	Intrauterine system
KO	Knock out
LRR	Leucine-rich repeats
LTA	Lipoteichoic acids
LPS	Lipopolysaccharide
LH	Luteinising hormone
MAPK	Mitogen-activated protein kinases
MCP 1	Monocyte chemotactic protein
mCRAMP	Mouse cathelin-related antimicrobial peptide

MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MIP	Macrophage inflammatory protein
MOI	Multiplicity of infection
Momp	Mitochondrial outer membrane permeability
MPA	Medroxyprogesterone acetate
MPO	Myeloperoxidase
NALP	NACHT domain-leucine-rich repeat-, and PYD-containing protein
NHBE	Normal human bronchial epithelium
NK	Natural killer
NLR	Nod-like receptor
NLRC	NLR family CARD-domain-containing
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing family
NOD	Nucleotide-binding oligomerisation domain
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear cell
PR	Progesterone receptor
PRR	Pathogen recognition receptor
PTK	Protein tyrosine kinase
PYD	Pyrin domain
qPCR	Quantitative Polymerase Chain Reaction
RLR	RIG-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SLPI	Serine leukocyte protease inhibitor
STOP	Surgical termination of pregnancy
TIR	Toll/IL-1 receptor
TIRAP	Toll/IL-1 receptor adaptor protein
TLR	Toll-like receptor

TNF	Tumour necrosis factor
TRAF	Tumour necrosis-factor-receptor-associated
TRAM	TRIF-related adaptor molecule
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter-inducing interferon-β
TUNEL	Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labelling
T3SS	Type 3 secretion system
XIAP	X-linked inhibitor of apoptosis protein

Chapter 1: Introduction

1.1 General Introduction

Cationic host defence peptides (CHDP) are evolutionarily conserved, small, positively charged peptide components of innate host defences. These peptides, also known as antimicrobial peptides, were originally discovered and described on the basis of their direct microbicidal properties. However, it has become increasingly clear that CHDP have an extensive range of immunomodulatory properties that can be complementary to microbicidal activity, or may even represent their major antimicrobial function. As a result of their capacity to interact with cells involved in host defence CHDP can modulate both innate inflammatory processes and interact with the generation of adaptive immunity. CHDP have been implicated in a variety of disease processes at diverse organ sites, and are attracting increasing attention as templates for the development of novel immunomodulatory antimicrobial therapeutics. One of the most extensively studied immunomodulatory CHDP is called LL-37/hCAP18.

1.2 Mammalian cationic host defence peptides

In mammals, CHDPs are represented by two main classes of peptide; the defensins and cathelicidins. The multiple different defensins are believed to share a common ancestral gene and can be subdivided into α- , β- and θ-defensins, based on the organisation of three characteristic cysteine disulphide bonds in the mature peptide fragment of the prepropeptide (reviewed in [1]). In contrast, cathelicidins are not grouped as a family on the basis of the mature peptide structure, which displays considerable diversity, but rather by the presence of an evolutionarily conserved N-terminal cathelin domain in the propeptide (reviewed in [2]). Mammals express a plethora of defensins (with humans expressing six α-defensin genes and having over forty predicted β-defensin gene [1]), and multiple cathelicidins are seen in some species. However certain species, including humans and mice, express only a single cathelicidin. Although steadily more reports detailing the immunomodulatory properties of defensins are emerging, the greater body of such studies relate to cathelicidin peptides.

The defining features of cathelicidins are an N-terminal signal sequence, a conserved cathelin domain and a variable C-terminal domain which, upon cleavage, becomes the mature functional peptide. The cathelin domain was named on the basis of its capacity as a cathepsin

L inhibitor, and the cleaved cathelin protein has been described as a cysteine protease inhibitor with some microbicidal properties in its own right [3]. The mature cathelicidin peptides range from 12 – 88 amino acids in length and take various forms including linear peptides with the capacity to form amphipathic α-helical structures, disulphide bond-stabilised β-hairpin structures, and proline rich structures [2]. The sole human cathelicidin Human Cationic Antimicrobial Peptide of 18KDa (hCAP18) generates a 37 amino acid peptide called LL-37 as its primary mature product [4] which adopts an α-helical structure in lipid membranes and in physiological ionic environments [5].

1.3 Human Cathelicidin

1.3.1 Human cathelicidin hCAP18/LL-37

hCAP18 is encoded by the *Camp* gene on chromosome 3p21.3 (*Fig 1.1*). After removal of the signal peptide, the propeptide may be stored before cleavage by proteinase 3 to form LL-37, the 4.5kDa mature peptide fragment [6, 7]. Although LL-37 is the major mature form, smaller fragments such as KS-30, KS-22, LL-29, KR-20, RK-31, LL-23, KS-27 may also be formed by serine proteases (e.g. kallikreins) in keratinocytes and sweat [8, 9] and cleavage by gastricin in the semen can lead to the formation of the ALL-38 form [10]. These alternatively processed forms have variations in the balance of microbicidal and immunomodulatory properties [11], demonstrating a mechanism of *in vivo* functional control and illustrating the therapeutic potential to modulate function through peptide sequence manipulation.

hCAP18/LL-37 is produced in highest concentrations by neutrophils (~ 630 μg/ 10^9 cells [12]) where it is stored in propeptide form in the secondary granules. However, expression can also be induced in epithelial cells, keratinocytes, monocytes, macrophages, mast cells, NK cells, γδT cells and B cells (reviewed in [13]). hCAP18/LL37 can be detected in a broad range of tissues and bodily fluids including plasma, bone marrow, airway surface fluid, skin, sweat, reproductive tract, semen, urine, breast milk and vernix (reviewed in [14])

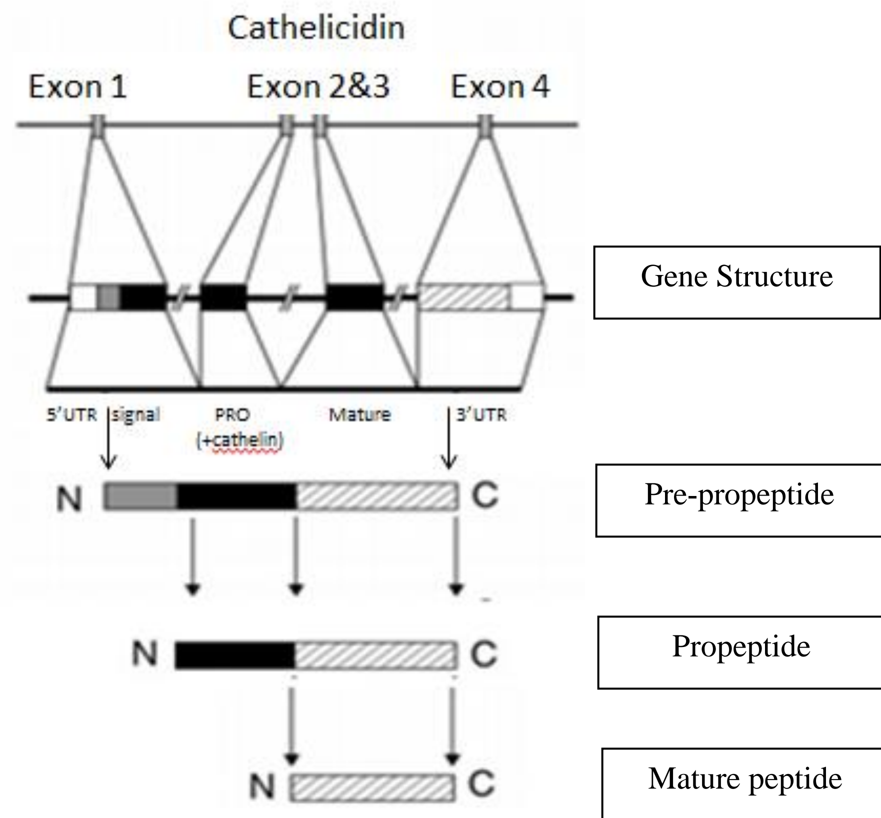

Figure 1.1 Transcription of the *Camp* gene results in a prepropeptide composed of an N-terminal signal domain, a cathelin domain and a C-terminal functional domain. The prepropeptide is processed to remove the signal peptide to leave the propeptide (hCAP18) until the mature LL-37 peptide is cleaved from the cathelin domain of hCAP18 by proteinase 3 [15]

The expression of hCAP18 is subject to complex transcriptional and post-transcriptional control, upregulated in response to inflammatory and infectious stimuli (such as lipopolysaccharide (LPS), IL-6 and IL-1α [16-18] and to wounding [19]. The precise mechanisms of regulation remain to be fully determined, however recent studies have clearly shown the importance of the active vitamin D metabolite 1,25-dihydroxyvitamin D3 (1,25 D3) as an inducer of hCAP18 expression, acting via a vitamin D response element in the *Camp* gene promoter [21-24]. The observation that expression of the hydrolase CYP27B1, which converts 25-hydroxyvitamin D3 to the active 1,25 D3, can be up-regulated by TLR2/1 stimulation [24], indicates a mechanism for vitamin D-dependent up-regulation of *Camp* expression in response to inflammatory and infectious stimuli. Other mechanisms of control include butyrate-enhanced histone acetylation at the *Camp* promoter, resulting in AP-1-mediated transcription [26], recruitment of the PU.1 transcription factor to the *Camp* promoter in response to vitamin D, butyrate or lithocolic acid [27], and the identification of a nuclear factor for interleukin-6 expression site [4].

The importance of hCAP18/LL-37 *in vivo* can be seen in patients with the rare condition morbus Kostmann, in whom neutrophils are deficient in hCAP-1/LL-37 and who are more susceptible to infection [27], and in the association between hCAP18/LL-37 levels and susceptibility to infection in dermatological pathologies (reviewed in [28]). Whereas expression is not increased in response to the inflammation in atopic dermatitis and increased susceptibility to infection is observed, pathologically high levels of hCAP18/LL-37 [29] that may contribute to the pathogenesis of psoriasis [30] are associated with a relative protection from skin infections. Increased levels of hCAP18/LL-37 have also been reported in pulmonary infections, cystic fibrosis (CF) lung disease and bronchiolitis obliterans syndrome [31-32]. Although these could represent a protective microbicidal response, the immunomodulatory effects of LL-37 might actually contribute to pathology in some cases, with the severity of CF lung disease found to correlate with increased LL-37 levels in the lung persisting between exacerbations [32]. Indeed altered post-translational processing of hCAP18, associated with an increase in stratum corneum tryptic enzyme, contributes to disease pathogenesis in acne rosacea [34], demonstrating a pathological role for the angiogenic properties of this CHDP [35].

1.3.2 Microbicidal activity

LL-37 was initially described and characterised as an antimicrobial peptide, reported to be microbicidal against a broad range of gram-positive and gram-negative bacteria, including *P. aeruginosa*, *S. aureus*, and *E. coli* [5, 45-47] , the yeast *Candida albicans* [53] and some viruses [54, 55], and to inhibit biofilm formation by *P. aeruginosa* [56]. However, the microbicidal activity of most CHDP is most potent in environments of low ionic strength and care must be taken to conduct *in vitro* MIC studies under physiologically-relevant conditions.

The microbicidal properties of CHDP have been variously attributed to three main mechanisms [57] ; a) a "barrel-stave" pore formation where hydrophobic surfaces interact with membrane lipid acyl chains while hydrophilic regions align to form a pore which may enlarge as more monomers are added, b) a "carpet model" with transient toroidal pore formation induced through CHDP-mediated membrane curvature strain at sites of high local peptide concentration, and c) a "carpet model" characterised by detergent-like bilayer disruption eventually leading to the formation of micelles at high peptide concentrations. LL-37 appears to function by toroidal pore formation, binding to the negatively charged bacterial surfaces and adopting a stable α-helical conformation at the polar/nonpolar interface, aligned parallel to the membrane surface [57]. Studies evaluating the properties of LL-37 analogues and truncated peptides have demonstrated that hydrophobicity and the propensity to form α-helices is critical to microbicidal function, but that the helical sense (using enantiomeric peptides) is not (reviewed in [58]). In addition, the core microbicidal region has been defined as amino acids 17-32 [47], and this truncated peptide has enhanced microbicidal activity in comparison to full length LL-37 (MIC ~100 μg/ml against *E. coli* K12, compared to 200 μg/ml for LL-37). The membrane defects induced by CHDP are proposed to allow leakage of intracellular contents, although whether this alone induces death, or whether subsequent intracellular translocation of the peptide to interact with internal targets [59] is also critical remains to be determined. Although microbes appear less able to develop resistance to CHDP than to conventional antibiotics, various resistance strategies have been reported. These include the production of proteases capable of cleaving LL-37 (e.g. SpeB of *Streptococcus pyogenes*, metalloproteases of *Pseudomonas aeruginosa*, gelatinose by *Enterococcus faecalis* and ZapA from *Proteus mirabilis* [60, 61], membrane modifications (e.g. *Neisseria meningitides* lipid A modifications [62], PmrA-PmrB based modification of *P. aeruginosa*

LPS structure [63]), and the capacity of *Shigella spp.* to downregulate hCAP18/LL-37 production by host cells [64].

In specific protected environments, such as leukocyte phagolysosomes, high concentrations of peptide and controlled ionic environment may be well suited for direct effects on pathogens [22, 65]. In addition, alterations to *in vitro* culture conditions, designed to more closely mimic those present in mammalian tissues by increasing carbonate concentration, can alter the sensitivity of *S. aureus* and *E. coli* to LL-37 [66]. This suggests that adaptations occurring in invading organisms may increase their susceptibility to innate microbicidal CHDP defences *in vivo*. Furthermore, LL-37 can act synergistically with other CHDP [67] and has been shown to synergise with conventional antibiotics [68]. Nevertheless, at mucosal surfaces *in vivo* the capacity of LL-37 to play a fundamentally microbicidal role seems unlikely given the expression levels of LL-37, the presence of serum proteins, DNA and f-actin and the concentrations of cations. It is at these sites that the additional bioactivities of LL-37 may prove to be of greatest significance to host defence.

As with many antimicrobial peptides, the minimum inhibitory concentrations (MIC) for LL-37 against microbes *in vitro* (in the range of 10 - 250 μg/ml [5, 45-47]) is much higher than the physiological concentrations that have been described *in vivo* at uninflammed mucosal sites (< 2 μg/ml hCAP18/LL-37, of which it is unclear what proportion is mature peptide). In addition, LL-37 can be inhibited by the presence of cations [48], serum apolipoprotein [49], DNA and f-actin [50, 51] Indeed, in the presence of concentrations of divalent cations (Ca^{2+}, Mg^{2+}) found in the human body, even 100 μg/ml LL-37 (exceeding levels observed at inflamed mucosa) was not microbicidal for *Staphylococcus aureus*, *Salmonella* Typhimurium [48], or for *P. aeruginosa* [52] against which cathelicidin-mediated *in vivo* protection has been observed [40, 44]. The question therefore arises as to how cathelicidins function as antimicrobial agents *in vivo*. While antimicrobial effects might be mediated through direct microbicidal properties at sites of localised high peptide concentrations (such as inside neutrophils following engulfment of pathogens), or through synergy with other antimicrobial agents, perhaps the most important functions are indirect immunomodulatory effects.

1.3.3 Modulation of cytokine expression

Mammalian cells respond to a range of different microbial components or pathogen-associated molecular patterns (PAMPs) via innate pattern recognition receptors (PRR) including toll-like receptors (TLR), RIG-I-like receptors (RLR) and nucleotide-binding domain leucine-rich repeat containing receptors (NLR) (reviewed in [69]). Lipopolysaccharide (LPS) and lipotechoic acid (LTA) from Gram-negative and Gram-positive bacteria respectively are powerful, well-studied pro-inflammatory PAMPs that are components of bacterial cell walls and membranes and may also be released by dying bacteria. These PAMPs can activate leukocytes and epithelial cells to promote an initially protective inflammation but can, if the infection is not cleared, induce harmful inflammation and sepsis. The properties of LL-37 appear to extend beyond pathogen killing, to include mopping up and detoxifying liberated endotoxin upon microbial death to limit the damage to host tissues. LL-37 has been shown to bind and neutralise both LPS and LTA and to modulate downstream TLR signalling, down-regulating expression of PAMP-induced genes [70-73], even when the peptide was not applied for up 90 minutes after PAMP stimulation [71]. Interestingly these effects are observed at peptide concentrations lower than those required for microbicidal activity (typically 1 – 5 μg/ml) but appear to be PRR-specific, inhibiting TLR4 and TLR2/1 agonists but not TLR2/6, TLR5, TLR7 and TLR8 agonists in peripheral blood mononuclear cells [74], and the precise points in the signalling pathways at which LL-37 functions have not yet been defined. These anti-inflammatory activities presumably account for the protective effects of LL-37 in animal models of sepsis [75, 76]. The use of analogues and truncated peptides has demonstrated that the LPS neutralizing activity of LL-37 resides primarily in the C terminal portion of the peptide and resulted in the generation of a 24 amino acid peptide derivative with similar efficacy to LL-37 in terms of LPS and LTA neutralization, but lower pro-inflammatory activity [77]. These studies highlight the potential for development of cathelicidin-based peptides as novel anti-endotoxic therapeutics.

Inflammatory responses induced by PAMPS are driven by classic pro-inflammatory cytokines (e.g. TNF-α) and by chemokine-dependent recruitment of leukocytes. Interestingly, while LL-37 can inhibit PAMP-induced TNF-α responses, it can also promote the production of chemokines (e.g. IL-8, MCP-1; [11, 71, 73, 78, 79]) and has potent chemotactic properties for neutrophils, monocytes, memory T cells and mast cells *in vitro* and *in vivo* [80-84] . In addition LL-37 can induce degranulation in mast cells, resulting in the release of histamine,

prostaglandin D2 and leukotriene B4, increasing vascular permeability and further promoting infiltration of leukocytes to the site of inflammation [85]. While optimal induction of chemokine production by monocytes, epithelial cells and keratinocytes occurs at ~25–50 μg/ml, and involves activation of MAPK pathways [78, 86], the optimal direct chemotactic activity is observed in response to 2-25 μg/ml, and functions through FPRL-1, CXCR2, and other unidentified G-protein coupled receptors [80, 81, 83, 87]. Importantly, in contrast to the microbicidal properties, the chemotactic properties of LL-37 are not inhibited by serum [80].

LL-37 has also been shown to enhance responses to IL-1β and GM-CSF in peripheral blood mononuclear cells, but antagonize the responses to IFN-γ, IL-4, or IL-12 [88], to promote caspase 1-dependent posttranslational processing and release of IL-1β by LPS-primed monocytes [89], and induce a caspase 1-independent processing of IL-18 from keratinocytes acting synergistically with β-defensins [90]. These functions all suggest that rather than being conventionally anti-inflammatory or pro-inflammatory, LL-37 can "re-balance" inflammatory responses in a concentration- and stimulus-dependent manner. Such complexity highlights the need to examine the effects of potential cathelicidin-based therapeutics in a pathogen-specific manner.

1.3.4 Leukocyte differentiation and function

The nature and extent of any inflammatory response is dictated by the functional properties of the participating innate and adaptive immune effector cells, including neutrophils, macrophages, monocytes, dendritic cells and lymphocytes. The appropriate responses of these cells, and the resolution of their responses, are critical to the successful outcome of an inflammatory response, while avoiding host damage and chronic infection. In addition to roles in the chemotaxis and cytokine responses of these effector cells, LL-37 also has the capacity to alter their differentiation and function in a number of other important ways.

Neutrophils are the key, innate immune effector cells that are the major cellular constituent of the early phase response to inflammatory stimuli. In keeping with observations in other cells types, LL-37 can both promote neutrophil IL-8 responses in a MAPK p38 and extracellular signal regulated kinase (ERK)-dependent manner [91], and inhibit cytokine responses to Toll-like receptor (TLR) agonists and whole bacteria [92]. However in addition, recent studies have

shown that exposure to 5 – 20 μg/ml of LL-37 can induce dose-dependent increases in neutrophil intracellular calcium mobilisation [88, 91], induce the generation of reactive oxygen species (ROS; [91]) and/or amplify ROS production in response to PMA or whole bacteria [92]. Significantly decreased ROS production in *Camp*$^{-/-}$ murine neutrophils underscores the role of the endogenous peptide in this process [92]. Given the importance of ROS as effector molecules in the direct microbicidal function of neutrophils, and the additional capacity of LL-37 to enhance neutrophil phagocytosis [92], these results suggest that LL-37 can prime and enhance neutrophil antimicrobial functions. Furthermore, LL-37 was shown to induce expression and release of human α-defensins (human neutrophil peptides 1–3) from live and apoptotic neutrophils [91, 93]. These α-defensins have recently been shown to also have effective anti-inflammatory properties *in vitro* and *in vivo* [94], and are likely to act in concert with the immunomodulatory effects of LL-37 to modify the responses of macrophages and other cells.

LL-37 has been clearly shown to modulate the inflammatory responses of macrophages and monocytes, as described earlier, however LL-37 is also capable of modulating macrophage differentiation [95]. While LL-37 exposure during the *in vitro* generation of human monocyte-derived macrophages (MDMs) promoted a more pro-inflammatory M1 phenotype, LL-37 could also redirect fully M2 phenotype differentiated MDMs to produce more IL-12p40 and less IL-10. This bioactivity of LL-37 was localized to the C-terminus of the peptide, and LL-37 internalisation by the cells was necessary to modulate the phenotype. In addition, the vitamin D-regulated anti-mycobacterial activity of human monocyte cells, attributed in part to the activity of CHDP [22], has recently been demonstrated to involved LL-37-mediated autophagy of the infected cells [96]. Expression of LL-37 was shown to be critical both for the infection-induced transcription of autophagy-related genes Beclin-1 and Atg5, and for the colocalization of mycobacterial phagosomes with autophagosomes. These studies demonstrate that both LL-37 expression by monocyte cells and exposure of these cells to external sources of this peptide can modulate the antimicrobial and immunomodulatory properties of these cells.

In addition to their multiple roles in innate immunity, it is becoming clear that CHDP can modulate the adaptive immune response (reviewed in [14]). Immunization of mice with a plasmid fusing LL-37 to a tumour antigen generated enhanced antigen-specific humoral and

cytotoxic responses, and prolonged survival in a tumour model *in vivo* [97]. LL-37 fusion plasmids were found to be significantly more effective than the tumour antigen plasmid alone, or co-administration of separately encoded plasmids for LL-37 and the tumour antigen, but the mechanisms remain unclear. Direct modulation of lymphocyte activity and/or proliferation, although demonstrated for defensins [98], is not a reported property of LL-37. Indirect mechanisms, such as alteration of the local cytokine environment should all be considered, but a likely explanation may be found in the effects of LL-37 on dendritic cell (DC) differentiation and function. LL-37 has been shown to modulate DC differentiation from monocyte precursors *in vitro*, with LL-37-primed DC displaying significantly up-regulated endocytic capacity, modified phagocytic receptor expression and function, up-regulated co-stimulatory molecule expression (including CD86 expression in the absence of DC maturation), and enhanced Th-1 responses *in vitro* [99], as well as enhanced Th-1 responses *in vivo* (Davidson, Schwarze, Wang unpublished data). LL-37 therefore has the capacity to induce the differentiation of immature DC "primed" to skew the nature of the adaptive response. Thus, LL-37/tumour antigen fusion proteins may function by delivering both the target for the adaptive immune response and a CHDP to generate a "primed" DC to the same cell in a temporally appropriate manner for an enhanced adaptive response. These effects of LL-37 involve signalling via an unidentified G-protein coupled receptor [99], while related DC phenotype-modulating properties have been shown to require internalisation of LL-37 by the DC [100]. In addition to the effects of LL-37 on DC differentiation, LL-37 has been shown to inhibit LPS-induced maturation of differentiated DC [101] in a manner consistent with its anti-endotoxic activities, but to promote DC activation in response to DNA and RNA [30, 102]. In the latter studies, LL-37 was demonstrated to bind non-inflammatory self-DNA and RNA and promote its uptake into DC in a manner that resulted in retention in early endocytic vesicles and activation of both plasmacytoid and myeloid DC, via TLR7, 8 and 9. These findings suggest a possible mechanism by which the excessively high levels of LL-37 found in psoriatic skin plaques might be involved in breaking self-tolerance and driving autoimmunity in psoriasis. However, the initiation of LL-37 overexpression in this disease remains unclear, as do the mechanisms by which tolerance is maintained in the context of inflammatory levels of LL-37 and dead cells in the healthy individual. These studies demonstrate the capacity of LL-37 to modulate DC differentiation and function in an inflammatory environment, and reiterate the contrasting effects of this cathelicidin on cellular responses to diverse stimuli.

It is therefore clear that by modifying the influx, functional responses and differentiation of inflammatory effector cells, LL-37 can orchestrate and modulate responses to infectious and inflammatory signals. However, in addition to these properties, recent studies have demonstrated that this peptide can also influence inflammation through effects on cell death.

1.3.5 Modulation of cell death

Although CHDP can rapidly permeabilise prokaryotic membranes, most natural peptides are relatively less toxic to eukaryotic cells, an observation proposed to relate to the essentially neutral outer surface of eukaryotic membranes and their cholesterol content (reviewed in [103]). This affords host cells a degree of protection from the lytic effects of such peptides. However, negatively-charged erythrocytes are more susceptible, presenting a challenge in the design of novel therapeutic derivatives (reviewed in [58]), and CHDP can be cytotoxic to mammalian cells in a manner specific to cell-type and its concomitant stimuli.

LL-37 has long been known to have cytotoxic effects on peripheral blood leukocytes at concentrations above 125 μg/ml, even in the presence of 10% foetal bovine serum (FBS; [5]), but it was unclear whether this death was due simply to primary necrosis resulting from peptide-induced membrane damage or an induction of programmed cell death. LL-37 can enter eukaryotic cells by an active process requiring endocytic machinery [104], and can facilitate the cellular entry of nucleic acids [105, 106] and DNA dyes [89, 107] without inducing cell lysis, suggesting temporary membrane disruption or pore opening mediated by this cathelicidin in live cells. Exposure to higher concentrations of LL-37 can induce apoptosis of airway epithelial cells in a dose-dependent manner (with substantial cell death at $\geq$ 50 μg/ml) *in vitro* and in murine airway epithelial cells *in vivo* [108, 109]. The presence of high density lipoproteins from human serum blocks entry of LL-37 into epithelial cells, inhibiting this LL-37-induced cell death and the IL-8 production by these cells [108]. LL-37 has also been shown to induce death in Jurkat T leukaemia cells, although requiring exposure to higher concentrations of peptide (50 – 200 μg/ml). This was demonstrated to be mediated via a caspase-independent and calpain- and AIF-dependent apoptosis that involved Bax activation and translocation to the mitochondria [110], but also associated with significant levels of necrosis (with propidium iodide entry into the cells) at the higher peptide concentrations in another study [111]. However, no cell death was induced in primary human lymphocytes, or

monocytes, at more physiologically relevant levels of LL-37 (up to 50 μg/ml) in the presence of 10% FBS [86, 99]. Furthermore, LL-37 has been found to protect primary keratinocytes from induction of apoptosis by camptothecin, an effect mediated by a cyclooxygenase-2-dependent mechanism involving production of inhibitor of apoptosis 2 protein [112], and to inhibit tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in intestinal epithelial cells *in vitro* [113], demonstrating the cell-type specificity of cathelicidin-mediated effects on cell death.

The extent to which direct induction of eukaryotic cell death at high peptide concentrations might modulate innate or adaptive immune responses *in vivo* remains unclear. However, a recent study performed by Dr Peter Barlow and myself in the Davidson lab has demonstrated that more physiological, inflammatory concentrations of LL-37 (10-30 μg/ml) can preferentially induce death in airway epithelial cells that have been infected with *P. aeruginosa* [52] and this will be expanded upon in chapter 5 of this thesis. Thus, LL-37 may contribute to innate defence against epithelial cell-invading microbes by inducing the death of infected, compromised epithelial cells as part of an inflammatory response, denying microbes a safe niche for replication and invasion of the host tissue.

The control of cell death is critical in maintaining homeostasis and in host responses to infection and inflammation, but also for the resolution of inflammatory responses. Despite the key roles played by neutrophils in innate immunity, uncontrolled or persistent neutrophilia is detrimental to the host. Neutrophils undergo spontaneous apoptosis and have a short half-life that can be modulated by a broad range of substances, including bacterial products (e.g. LPS), and cytokines (e.g. GM-CSF) [116]. Control of neutrophil death and the anti-inflammatory effect that apoptotic neutrophils have on phagocytosing macrophages are critical in the resolution of inflammatory responses [117]. LL-37 can antagonise the effects of LPS on neutrophil survival [93], and has been shown to modulate neutrophil death directly. Although initially proposed to be an inhibitor of neutrophil apoptosis [109, 118], the principal effect of LL-37 is the rapid induction of secondary necrosis of apoptotic neutrophils, occurring at concentrations of peptides as low as 1 μg/ml [93, 119, 121]. This property was retained by C-terminal partial peptides and was also evident for mCRAMP (the murine orthologue of LL-37). In contrast to expectation, LL-37-induced secondarily necrotic neutrophils had anti-inflammatory effects on activated macrophages [93]. The maximal anti-inflammatory effects

were observed in association with LL-37-mediated release of granule contents from the apoptotic cells, induced by exposure to higher concentrations of LL-37 (25μg/ml). These effects were independent of the anti-endotoxic activity of the peptide used to induce secondary necrosis, and may result from the release of both LL-37 and α-defensins from the apoptotic neutrophils [94]. Although other granule contents could have deleterious effects, LL-37-mediated release of CHDP from apoptotic neutrophils may enhance the apoptosis-driven resolution of inflammation.

Thus, the capacity of LL-37 to modulate the induction of cell death and modalities of death should be considered as one of the immunomodulatory properties of this cathelicidin. Interestingly these properties are complemented by peptide-mediated enhancement of cell proliferation, indicating that LL-37 has the potential to generate both protective cell death and repair in an inflammatory environment.

1.3.6 Cellular proliferation and angiogenesis

The expression of LL-37 is up-regulated at sites of wounding and has been shown to play roles in cell proliferation, wound healing and angiogenesis. hCAP18/LL-37 was found to be strongly expressed in healing skin, but absent from chronic skin ulcers, and to promote re-epithelialisation of wounds in organ-cultured human skin [122]. LL-37 has been shown to induce keratinocyte migration *in vitro* at concentrations as low as 100 ng/ml (in the absence of serum; [123, 124]), associated with MAPK and matrix metalloproteinase-dependent epidermal growth factor receptor (EGFR) activation, and to enhance re-epithelialisation at skin wound sites *in vivo* [123]. This cathelicidin can also promote fibroblast proliferation [108], but inhibits collagen production by dermal fibroblasts and may have anti-fibrotic properties in wound healing; with the degree of fibrosis in dermal keloids found to be inversely correlated with the expression of hCAP18/LL-37 [125]. Furthermore, in studies using airway epithelial cells, LL-37 promoted wound healing in a dose-dependent manner by stimulating epithelial cell migration and proliferation at concentrations as low as 1 μg/ml, but interestingly only in the presence of serum [126]. In addition to these wound healing properties, LL-37 has been shown to induce the proliferation of endothelial cells and neovascularisation *in vitro* and *in vivo*, with decreased vascularisation observed during wound repair in *Camp*$^{-/-}$ mice [36].

The capacity of LL-37 to modulate cell proliferation has stimulated a number of studies to evaluate the effects of this peptide on tumour growth and metastasis (reviewed in [127]). LL-37 derivatives have been proposed to have tumouricidal activity, via induction of apoptosis [128]. However, increased expression of hCAP18/LL-37 has been found in breast, ovarian and lung carcinomas [129-131], correlating with vascular density [130], and proposed to be mitogenic. Transfection of epithelial cell lines (HEK293 and HaCaT cells) with hCAP18 enhanced cellular proliferation *in vitro* [129]. Similarly, recombinant LL-37 stimulated proliferation of ovarian cell lines [130], although this occurred exclusively in the presence of serum and the enhanced proliferation observed at 1 μg/ml LL-37 was lost for two of the three cell lines at higher concentrations of peptide. The growth of anchorage-independent lung carcinoma cell lines *in vitro* was shown to be enhanced after the addition of ng/ml concentrations of LL-37, but significantly diminished by 20 μg/ml of peptide [131]. In addition, LL-37 has been proposed to promote ovarian tumor progression by enhancing invasion, matrix metalloproteinase expression, and the recruitment of multipotent mesenchymal stromal cells [130, 132], and tumours derived from transformed cells injected into nude mice showed significantly faster growth when engineered to over-express hCAP18 [129]. However, in contrast, exogenous LL-37 demonstrated anti-proliferative properties for gastric carcinoma cells, inducing cell cycle arrest, and had direct anticancer activity *in vivo* in a gastric cancer xenograft model [133]. Thus, although this cathelicidin can clearly impact upon tumour growth in model systems, the cell-type specificity and net effect of its properties *in vivo* remains to be determined.

1.3.7 Mechanisms of immunomodulatory activity

The pleiotropic effects of LL-37 in modulation of host defence responses raise questions about the mechanisms that could underpin such a broad array of bioactivities. At the simplest level, the anti-endotoxic properties of LL-37 are at least partly a consequence of direct, charge-based binding of LPS as discussed above, inhibiting interaction between LPS and its binding protein and/or receptor. However, even for this property, additional mechanisms are required to explain the selective LL-37-mediated inhibition of specific LPS-induced pro-inflammatory genes, without inhibition of LPS-induced genes that antagonise inflammation [73], and a variety of receptor-specific and alternative mechanisms for LL-37-mediated immunomodulation have been proposed.

A classical receptor-ligand mechanism has been proposed for LL-37, functioning through formylpeptide receptor-like 1 (FPRL1); a G protein coupled receptor (GPCR). This receptor interaction was initially identified as the mechanism for LL-37-mediated chemotaxis of leukocytes [80]. FPRL-1 has also been implicated in LL-37-mediated wound healing [123], angiogenesis [35], inhibition of neutrophil apoptosis (in one study [118]), and in activating MAPK and enhancing invasiveness of ovarian carcinoma cells [136]. However additional mechanisms occurring concomitantly have been implicated for many of these properties, and a recent study has described CXCR2 as an alternative receptor for LL-37-mediated neutrophil and monocyte chemotaxis [87]. Unidentified GPCR other than FPRL-1 have also been proposed as receptors for LL-37 (104), and implicated in LL-37-mediated modulation of DC differentiation [99] and mast cell chemotaxis [81], based on inhibition of LL-37-mediated effects by pertussis toxin. Furthermore, utilisation of GPCR by cathelicidins has been excluded in many studies, implicating alternative mechanisms and receptors, including $P2X_7R$, EGFR and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The purinergic receptor $P2X_7R$ has important roles in the regulation of inflammatory processes [135]. Activation by ATP (described as its principal ligand) reversibly opens large non-selective pores involving $P2X_7R$ and pannexin 1, which can enable ion flux across the cell membrane. The $P2X_7R$ has been identified as responsible for LL-37-mediated posttranslational modification and release of IL-1β from LPS-primed monocytes [89], experimentally implicating LL-37 as an alternative direct ligand for this receptor. $P2X_7R$ activation has also been implicated in LL-37-mediated modulation of neutrophil apoptosis [109, 118], endothelial cell stiffening [136] and the mitogenic properties of LL-37 on fibroblast proliferation [107]. However, the latter study demonstrated that LL-37 could restore pore-forming activity to a truncated $P2X_7R$, which could not itself generate the classical non-selective pore [107]. This activity, its independence from pannexin 1 and the equivalent mitogenic activity of similarly structured orthologues and the D-enantiomer of LL-37, lead to a proposal of functional interaction between $P2X_7R$ and amphipathic peptides with appropriate helix-forming propensity, mediated by binding of transmembrane segments, and opening pores through mechanisms distinct from that of ATP-stimulated $P2X_7R$. LL-37 has also been proposed to function through activation of metalloproteinases in the cell membrane, by as yet undefined mechanisms, with consequent cleavage of soluble membrane-bound EGFR ligands and transactivation of EGFR. This mechanism has been implicated in LL-37-

mediated induction of IL-8 expression [11, 78], wound healing, keratinocyte migration, and enhanced cellular proliferation [123, 124, 126]. Common to these and other studies is the activation of MAPK pathways by LL-37 [86], a downstream signalling event can also be observed following FPRL-1 ligation by LL-37 [134], and has been implicated in LL-37-mediated modulation of TLR responses [74]. The potential for LL-37 to modulate multiple signalling processes via interactions with transmembrane domains of diverse membrane-bound receptors may help to explain its pleiotropy and the apparently key nature of the amphipathicity of this peptide, irrespective of helical sense [11. 107]. However, a role for promiscuous receptors cannot be excluded and other properties of LL-37 require peptide entry into the eukaryotic cell. These include the induction of chemokine expression [104], altered MDM / DC differentiation [95, 100] and peptide-mediated cell death [108]. The identification of GAPDH as a novel intracellular receptor for LL-37 [137] may be significant in this regard, but the full extent of intracellular effects mediated by this peptide, and the mechanisms involved remain to be determined. Membrane integration of cathelicidin in the absence of peptide internalisation by the cell might also be fundamental to the cathelicidin-mediated induction of secondary necrosis in apoptotic membranes [93, 119]. Clearly the mechanisms of immunomodulation employed by LL-37 are complex and may be atypical, and elucidation will be important to furthering our understanding of these intriguing peptides.

1.3.8 Expression of Cathelicidin in health and disease

Due to the abundant quantities of hCAP18 found stored in neutrophil granules much work has looked at the role of cathelicidin in inflammatory diseases involving acute neutrophilia and the activity of LL-37 on neutrophils and inflammatory cells themselves. Epithelial cells however are another important source of cathelicidin and a poorly understood area in the field of CHDP research is the role of LL-37 at mucosal surfaces, its expression by epithelial cells at different body sites and the effect this peptide has on epithelium during infectious or non-infectious disease. These questions represented an important focus in my PhD work, with particular emphasis on the female reproductive tract and the lung.

1.4 Antimicrobial Defence and Regulation of Cathelicidin at Mucosal Surfaces: the Female Reproductive Tract

The female reproductive tract undergoes cyclical expression of many proteins due to its unique nature of continual differentiation, sloughing and renewal [120]. The tissue of the endometrium, ovaries and fallopian tubes are continually changing under the influence of ovarian and pituitary hormones (*Fig 1.2*). A great deal of tissue remodelling occurs within the reproductive tract with concomitant angiogenesis as well as apoptosis of cells in the functional layer of the endometrium. A standard menstrual cycle is 28 days long however this can vary by several days between individuals. The first day of the menstrual period (day 1) begins with menstruation when the functional layer of the endometrium is shed following the degeneration of the corpus luteum and withdrawal of the ovarian hormone progesterone. Following menstruation there is a period known as the proliferative (or follicular) phase. At this time a new ovarian follicle will grow under the influence of follicle stimulating hormone (FSH) and produce oestrogen which causes the endometrium to thicken and spiral arterioles to lengthen from the straight arteries of the endometrial basal layer to the functional layer. A mid-cycle surge of luteinising hormone (LH) results in ovulation and the formation of a progesterone-secreting corpus luteum. The secretory (or luteal) period follows and progesterone levels peak in the middle of this period. The increase in progesterone maintains the spongy, glandular endometrium and regrowth of spiral arterioles continues. Should fertilisation and implantation of the blastocyst not occur, the corpus luteum dies and progesterone levels fall which triggers menstruation. The withdrawal of progesterone results in degradation and shedding of the uterus lining and retraction and vasoconstriction of the spiral arterioles accompanied by leukocyte influx [138, 139].

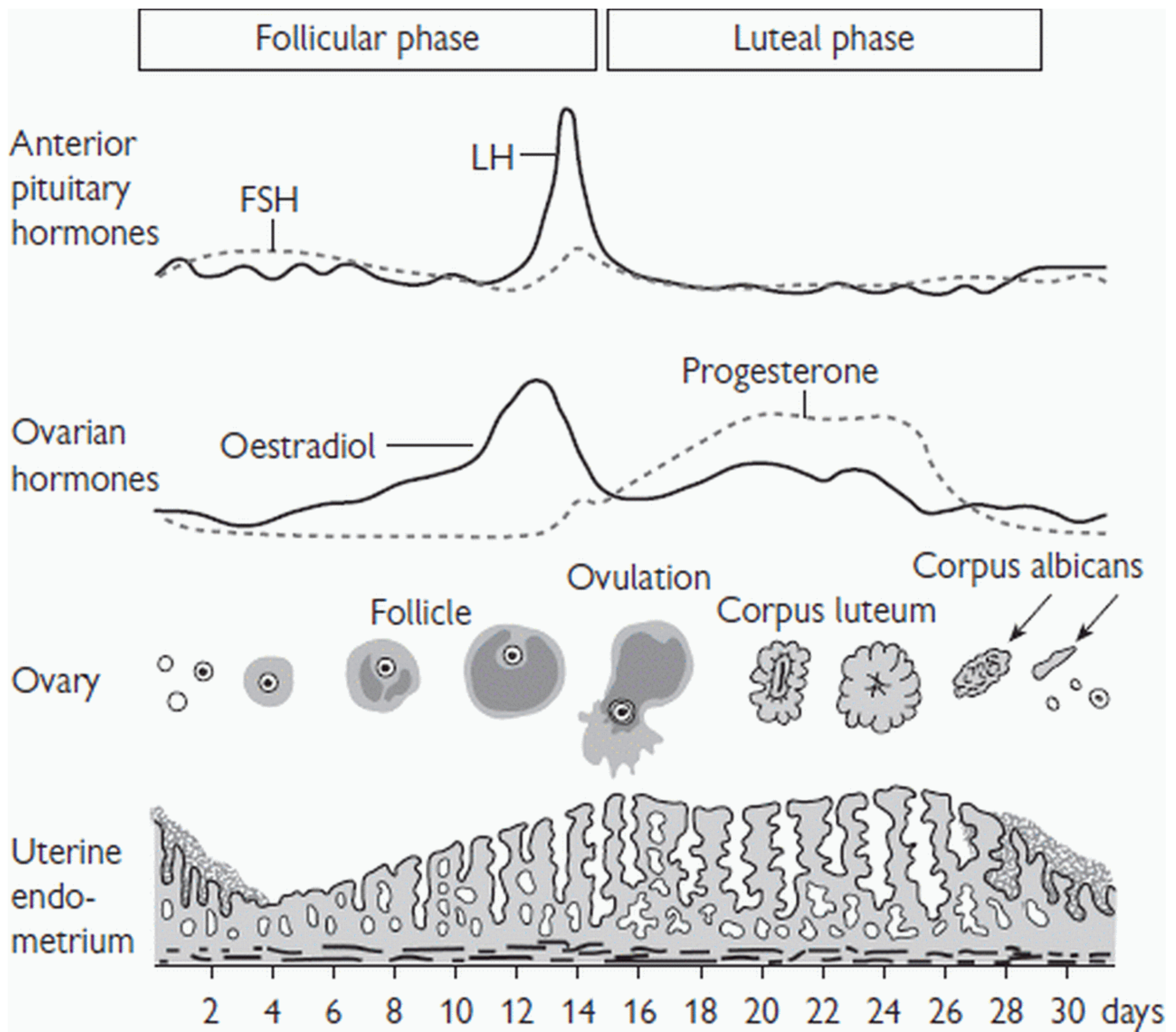

Figure 1.2 Cyclical changes in the female reproductive tract. Illustration showing the changes that occur in the tissue of the ovaries and endometrium on different days of the cycle under hormonal regulation from the ovaries and the brain. Reproduced from Sanders S, Dawson J, Datta S, *et al*. (eds) (2005). *Oxford Handbook for the Foundation Programme*. Oxford: OUP. By permission of Oxford University Press

The epithelial cells lining the vagina, endo-cervix, uterus and fallopian tubes are major sources of antimicrobial peptides and CHDP and the various ways in which these barrier cells contribute to innate immunity to infection is reviewed in [142], however, in addition to constitutive or infection-initiated expression, the presence or absence of particular proteins is not constant at all times of the menstrual cycle. Previous studies have shown the presence of antimicrobial peptides (AMPs) such as α and β defensins, SLPI and Elafin to vary in concentration throughout the menstrual cycle with distinct periods of prevalence and waning [139, 140, 143]. The antimicrobial peptide secretory leukocyte protease inhibitor (SLPI) for example has been shown to display cyclical expression which is highest at the time of blastocyst implantation and during subsequent pregnancy and it is speculated that this is in order to protect the mucosal surfaces at these crucial times [139]. Also, the serine protease inhibitor elafin, another antimicrobial protein is up-regulated during menstruation [140]. The human endometrium has been shown to express hBD1-4 [143] differentially throughout the cycle. hBD 1&3 are expressed at highest levels during the secretory stage whilst hBD4 is expressed during the proliferative and hBD 2 during menstruation. The purpose of this varying milieu of peptides is likely to include antimicrobial defence of the woman or trophoblast at times where the risk of infection is greater or during menstruation or implantation, but they may also have physiological roles in these latter processes themselves.

One host defence peptide present in the uterus and fallopian tubes for which, at the time of this PhD, there was little published description in the upper reproductive tract is Human cationic antimicrobial peptide of 18kDa (hCAP18). Due to the documented roles that LL-37 plays in other body systems, including angiogenesis [35], wound repair [122, 125], cell proliferation [107, 125, 129] and modulation of inflammatory cells [13, 147] it is tempting to speculate that LL-37 could be influential in inflammatory settings. Menstruation shares many characteristics of an acute inflammatory event with local increases in pro-inflammatory prostaglandins, cytokines and chemokines and an increase in leukocyte migration to the endometrium [261] and changes in systemic levels of C-reactive protein may also be seen, associated with low grade inflammation [262].

1.5. Host defence peptides in the lung in health and disease

The respiratory system is essential for extracting oxygen from the surrounding air and eliminating carbon dioxide waste from the blood. Inhaled air flows into the airways via the nasal cavity, pharynx, larynx, and trachea and then through left and right primary bronchi to increasingly smaller bronchi and bronchioles to terminate in alveoli, the functional unit of the lungs where the majority of gas exchange occurs.

1.5.1 Cellular composition of the lung

Human lung epithelium

The trachea and bronchi are lined by respiratory epithelium [221], a pseudostratified, tall columnar, ciliated epithelium containing numerous goblet cells for mucin secretion (*Fig 1.3*). As the airways become smaller the height of the epithelial cells decrease as does the number of goblet cells and cilia. Terminal bronchioles are lined by simple non-ciliated columnar or cuboidal cells (*Fig 1.4*) with interspersed clara cells (Non-ciliated, secretory cells). There are two lungs, enclosed within pleural sacs both of which have a lung hilum where bronchi and pulmonary arteries enter the lung and pulmonary veins leave. Normally the right lung is divided into three lobes and the left lung into two, all of which contain millions of alveoli. The alveoli are composed of type I and type II pneumocytes. Type I pneumocytes make up the simple squamous lining of the alveoli and type II or septal cells reside in the septa between alveolar lobes (*Fig 1.5*) secreting surfactant as well and acting as stem cells to replenish the type I pneumocyte population as required.

Mouse lung epithelium

Mouse lung structure varies from humans in that the left lung is undivided and the right lung is divided into 4 lobes. Mice have fewer airway generations (few if any respiratory bronchioles) and although there are few mucus-producing cells in the murine airways there are an abundance of Clara cells which are the predominant cell type [264].

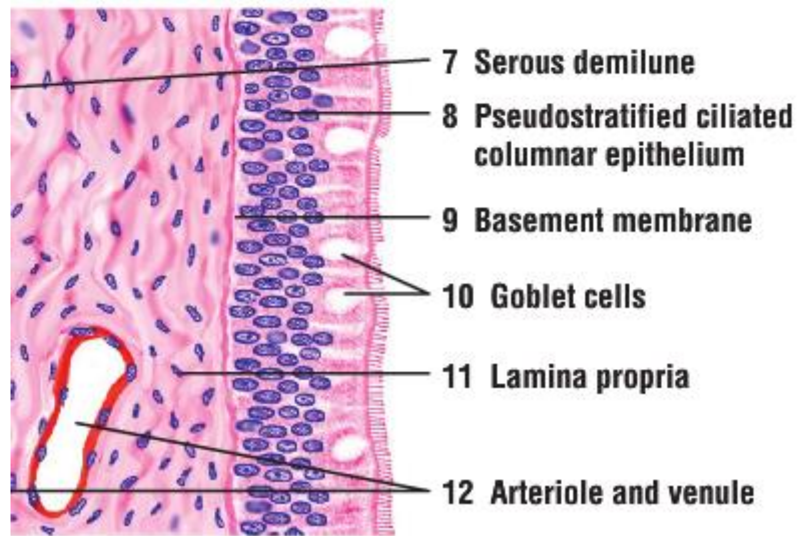

Figure 1.3 The human trachea and larger bronchi are lined with Pseudostratified columnar ciliated epithelium (respiratory epithelium) with numerous goblet cells for mucin production. With permission from Lippincott Williams and Wilkins [221].

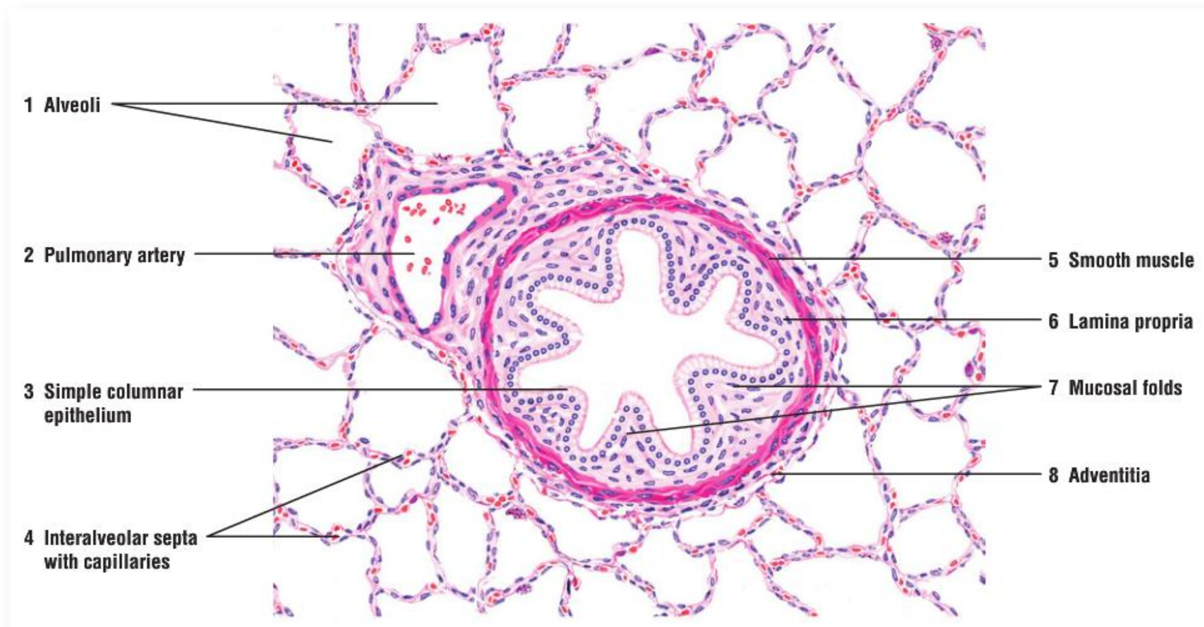

Figure 1.4 The terminal bronchiole is lined with simple cuboidal epithelium. As the airways get smaller the number of goblet cells decrease and the epithelial cells are no longer ciliated. With permission from Lippincott Williams & Wilkins [221].

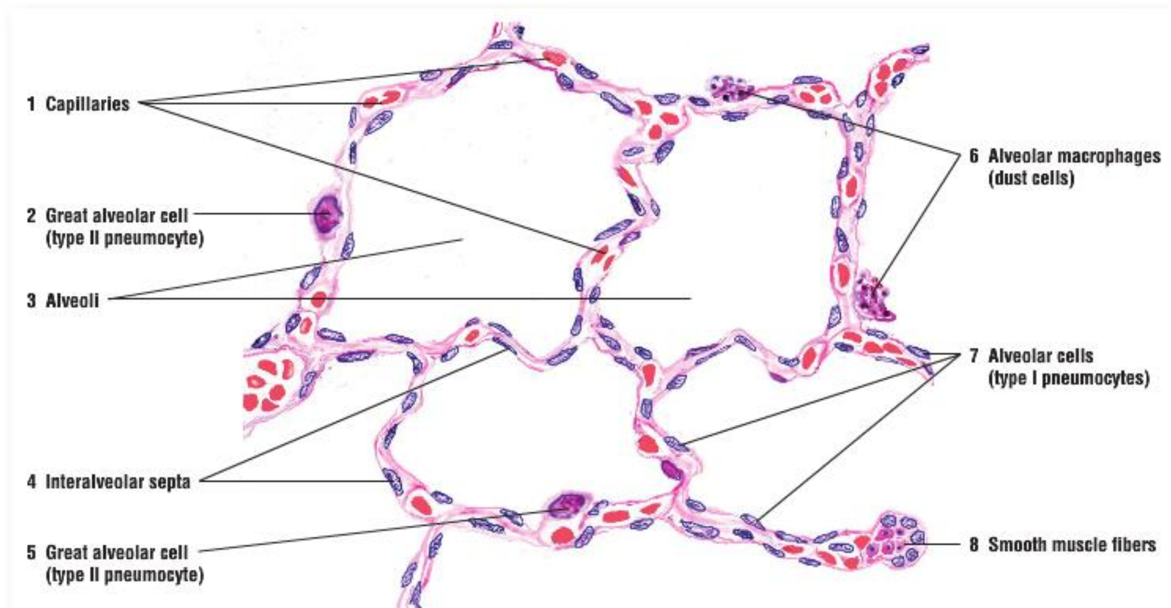

Figure 1.5 Alveolar walls and pneumocytes. The alveoli walls are composed of Type I pneumocytes and split into lobes by interalveolar septa. Type II pneumocytes are present in fewer numbers and produce surfactant. With permission from Lippincott Williams & Wilkins [221].

Inflammatory cells in the lung

Innate immune surveillance of the airways is carried out primarily by alveolar macrophages (also known as sentinel or dust cells) which are resident in the connective tissue of alveolar walls or in the alveoli themselves. These macrophages phagocytose cellular debris, microorganisms and other inhaled particles and also initiate and sustain inflammation recruiting other immune cells upon recognition of infection via the release of chemotactic mediators and activating chemokines such as IL-12. Dendritic cells found in the lung also phagocytose material but in addition to destroying microorganisms (when activated following ligation of PRRs) they migrate from the lungs to local lymph nodes to display antigens to lymphocytes. Neutrophils and natural killer cells are not normally found in the healthy lung but are quickly recruited in the presence of danger signals and chemokines that are released by macrophages and epithelial cells.

Epithelial cells, whilst not typically recognised as immune cells, play an important role in forming both a physical barrier to infection (through tight junction connections) a chemical barrier (as a source of antimicrobial peptides) and also play a part in immune surveillance releasing cytokines and chemotactic mediators upon stimulation of pathogen recognition receptors to draw other leukocytes into the area.

1.5.2 Non cellular innate host defence of the lung

The majority of inhaled particles are removed from the airways by the mucocilliary escalator. Mucin [229, 230] produced by goblet cells is the main component of mucus which contains water, immunoglobulins, defensins and other antimicrobial peptides as well as cytokines and destructive enzymes such as lysozyme to inactivate microorganisms. The cilia on respiratory epithelium beat in a co-ordinated movement that pushes mucus up towards the oropharynx where microorganism-containing mucus can be coughed up or swallowed. Organisms that escape removal by the mucocilliary escalator and reach the alveoli may be inactivated or opsonised by soluble factors such as IgG and secretory IgA [232, 233, 234], complement proteins [234 236], surfactant protein A (SPA) [237], lysozyme, anti-oxidants, lactoferrin, α1-antitrypsin [239] and CHDP such as the defensins and cathelicidin [3-5, 7].

Defensins are small, cationic microbicidal peptides [13], first discovered in 1985 that show both direct antimicrobial activity against a broad spectrum of pathogens and are also chemotactic for inflammatory leukocytes. They are also involved in opsonisation of microbes and modulation of the behaviour of recruited cells. The α defensins, also known as human neutrophil peptides (HNPs), are constitutively expressed by neutrophils and stored in azurophilic granules for intracellular pathogen killing or for degranulation onto epithelial surfaces following recruitment and activation. The β defensins [43] are produced by epithelial cells and may be constitutively expressed as in the case of hBD1 or like hBD 2, 3 and 4, inducible during infection or inflammation.

1.5.3 Cathelicidin expression in the lung

HCAP18/LL-37 secreted into the airways of healthy, uninfected individuals is estimated to be less than 5µg/ml as measured in bronchoalveolar lavage fluid (BALF) [149] however when infection is present or during the course of specific lung diseases the amount of cathelicidin present may be much greater. Comparisons between reported levels of cathelicidin in the lung are complicated by the various detection methods used. Some studies use an ELISA approach and it must be determined whether the antibodies used detect the propeptide hCAP18/LL-37 or the cleaved LL-37. Other studies use the less accurate dot blot approach, again with the same consideration for antibody specificity.

1.5.3.1 Infection

Cathelicidin concentrations are elevated in response to infection. When hCAP18/LL-37 is measured in BALF from neonates with lung infections, concentrations are closer to 30 µg/ml [149]. Levels of LL-37 in human lung disease such as sarcoidosis are also associated with increased LL-37 production alongside up-regulation of other CHDPs, and BALF from sarcoidosis patients has increased bacterial killing capacity compared to that of healthy controls [150].

1.5.3.2 Cystic Fibrosis (CF)

Cystic fibrosis is an inherited condition caused by a mutation in a chloride channel called the cystic fibrosis transmembrane regulator (CFTR). Individuals who inherit two mutated alleles have non-functional salt channels and produce thick salty mucus in the lung resulting in impaired mucocilliary clearance and are thus subject to multiple respiratory infections and reduced lung function [258] . hCAP18/LL-37 is found at higher than normal levels in the lungs of cystic fibrosis patients suffering chronic lung infections (around 15µg/ml BALF) and although increasing amounts correlate with disease severity rather than protection [154] this is likely as a correlate of neutrophil infiltration to the lungs and the damage caused by chronic inflammation.

1.5.3.3 Bronchiolitis Obliterative Syndrome (BOS)

Bronchiolitis obliterative syndrome describes a condition where there is non-reversible progressive fibrosis and inflammation of the small airways which result in obstructive lung disease. There are multiple causes of BOS but, although normally a rare condition, it is the most common cause of death amongst lung transplant recipients who survive >1yr post-transplant. Anderson and colleagues found that levels of hCAP18/LL-37 was higher in the BAL of recipients suffering from BOS as compared to stable recipients (Means of 10ng/ml-1 compared to 0.4ng/ml-1) even after taking into account a higher neutrophil influx in the BOS group [152]. The authors propose that there may be a not as yet understood contribution to the pathology of BOS by antimicrobial peptides such as cathelicidin.

1.5.3.4 Asthma

Asthma is a disease characterised by hyper-reactive airways, reversible bronchial restriction and airflow obstruction, chronic inflammation which leads to fibrosis and airway wall remodelling [153]. Patients may display wheezing, coughing, chest tightness and shortness of breath to varying degrees. The disease may be atopic or non-atopic but either type can suffer exacerbations or “attacks”

The role of cathelicidin is unclear in asthma. Some studies have reported that sputum cathelicidin is the same or reduced in asthmatics compared to non-asthmatic controls [151]

whilst others have demonstrated that allergic airway inflammatory responses suppress the expression of cathelicidin in the lung. A study in 2006 [155] showed that LL-37 via interaction with FPR is chemotactic for eosinophils and recently others looked at Cysteinyl leukotriene release from eosinophils as triggered by LL-37 interacting via the FPR2 [156]. These findings would indicate that cathelicidin may be detrimental for allergic asthma inflammation. However, the use of Budesonide, a glucocorticoid used to treat asthma decreased the expression of LL-37 resulting in an increase in experimental lung infections in a mouse model [157]. Additionally challenge with an allergen in asthmatics increased the amount of vitamin D in the lungs as well as the expression of LL-37 [158] and giving vitamin D therapeutically to asthmatics increases the amount of LL-37 correlating with better lung function and a reduction in respiratory infections according to another group [159]. The role of Vitamin D induced LL-37 expression and whether it is beneficial or detrimental in asthma remains poorly understood.

1.5.3.5 Vitamin D regulation of cathelicidin expression in the lung

7-dehydrocholesterol in the skin is converted by UVB light into inactive vitamin D3 which is then stored and converted in the liver to the active form 1, 25-dehydroxy-D3 (1, 25-D3 or calcitriol) by the enzyme 25-hydroxylase. This enzyme has been found to be expressed in the lung [23] making it possible to also increase the levels of active vitamin D locally. Vitamin D deficiency has been linked in several studies to be associated with lung disease, especially of an infective nature (reviewed in [160]). Increased expression of the vitamin D receptor (VDR) in cells during inflammation and infection coupled with the presence of 1,25D3 results in an increase in the production of VDR regulated genes including cathelicidin [20-23] and so the increased susceptibility to infection of those with vitamin D deficiency could in part be due to a lack of cathelicidin up-regulation.

1.5.4 Murine Cathelicidin mCRAMP

Additional evidence for the importance of cathelcidin *in vivo* comes from studies of mCRAMP (mouse cathelin-related antimicrobial peptide, encoded by *Camp*), the murine orthologue of hCAP18. Genetically-modified mice deficient in mCRAMP expression (*Camp*$^{-/-}$) demonstrate increased susceptibility to infections of the skin, intestinal tract, cornea and urinary tract [36-39]. At the time of starting this PhD the lung phenotype was unknown.

Interestingly, regulation of murine cathelicidin expression diverges from that observed in humans, as mCRAMP is not regulated by vitamin D [21], but has been shown to be HIF1α (hypoxia-inducible factor 1 alpha)-responsive [41]. Nevertheless, these studies show considerably more severe effects upon host defence than knockout models deficient in single β-defensins [42, 43], where there may be considerable redundancy. They also demonstrate multi-organ effects of cathelicidin deficiency *in vivo.* Additional evidence of *in vivo* antimicrobial function was demonstrated using gene-augmentation with hCAP18/LL-37 to enhance the clearance of *Pseudomonas aeruginosa* from the murine lung [44], a study that also demonstrated the therapeutic potential of these peptides. Although this research clearly indicates the importance of cathelicidins to host defence, the precise mechanisms responsible for these observations remain uncertain.

1.6 Opportunistic respiratory infections caused by *Pseudomonas aeruginosa.*

Pseudomonas aeruginosa is a ubiquitous, Gram-negative, aerobic bacterium that rarely causes disease in healthy individuals, but can adapt quickly to invade respiratory epithelium when normal immune barriers are compromised. This is especially true in critically ill patients who may develop hospital-acquired pneumonia (HAP) as a result of introducing or facilitating the movement and growth of Pseudomonas into the lower airways during intubation and mechanical ventilation.

1.6.1 Opportunistic Infections in Humans

Ventilator-associated pneumonia caused by *P. aeruginosa* is associated with a poor prognosis and mortality is high [161]. Other cohorts of patients who are highly susceptible to respiratory colonisation and infection with *P. aeruginosa* are those with cystic fibrosis. The cystic fibrosis affected airway has abnormal physiology due to the absence of an ion channel, the cystic fibrosis transmembrane conductance regulator (CFTR) on the epithelial cells lining the airways. Deregulation of salt concentration in the lumen of the respiratory tract leads to the production of thick, sticky mucus and a reduced volume of airway surface liquid. In addition, many of the antimicrobial peptides normally produced in this area are rendered non-functional as a consequence of salt levels or by other salt-independent mechanisms [162-164]. Thus, the clearance and killing of bacterial pathogens is hindered and colonisation with characteristic opportunists such as *P. aeruginosa* occurs.

Infection with this bacterium can have variable consequences for airway epithelial cells depending on the infecting strain and its associated virulence factors. Invasion, destruction and apoptosis of host cells are all potential outcomes. Pier and colleagues have long argued that the CFTR acts as a receptor for the internalisation of *P. aeruginosa* to epithelial cells [165, 166] and that following internalisation the epithelium can remove infectious organisms by way of apoptosis and shedding of infected cells. Logically if the CFTR is absent and cannot perform this function then bacteria remain in the immunocompromised mucociliary lining and are able to multiply and cause chronic infection with relative impunity to normal clearance mechanisms.

Others contest this view of the role of the CFTR in clearance of Pseudomonas and present work which indicates more a role for damaged epithelial cells presenting glycolipid receptors which *P. aeruginosa* attach to via pili [167], facilitating invasion of the mucosal surface via the activity of type III toxins.

Strain variability can clearly be seen in both environmental strains and clinical isolates of *P. aeruginosa* that express different combinations of the type III toxins; Exoenzyme S, T, U or Y. ExoS and ExoT have ADP-ribosylating activities to low molecular weight GTP-binding proteins of the Ras family [241]. The C-terminal is the site of ADP-ribosylating activity and the N-terminal functions as a Rho GAP (GTPase activating protein). When present in the host cell cytosol, ExoS and ExoT cause disruption of actin cytoskeleton. ExoU is a phospholipase cytotoxin [242] that interferes with cell membranes via lipase activity and ExoY is an adenylate cyclase [243]. Strains which are directly cytotoxic are thought to express both U and T whereas cells which are invasive and non-cytotoxic (but may induce apoptosis) express S and T. This is complicated however by observations that the potential for cytotoxicity is dependent on the type of cell infected as well as the makeup of different exotoxins [244]. Both cytotoxic and invasive bacterial infections are thought to involve protein tyrosine kinase activity (PTK). Mutation of csk, which regulates Src family PTKs reduces invasion and increases cytotoxicity of *P. aeruginosa* infections. Only 20% of clinical isolates express *exoU*. PA01 expresses *exo S*, *exoT* and *exoY* but not *exoU* [245]. Infection with non-cytotoxic strains may however still cause cell death by the induction of regulated cell death pathways [241].

1.6.2 Current Treatments

P. aeruginosa is an important opportunistic respiratory pathogen that is associated with high levels of mortality in hospitalised patients and amongst growing concerns that there is a shortage of antibiotics which are effective against multi-resistant strains alternative treatments need to be found. Drug treatment is complicated in patients with other co-morbidities and regional drug resistance information must also be taken into account. Generally for pneumonia caused by *P. aeruginosa* the recommended treatment is initial dual therapy with a beta lactam and aminoglycoside for 5 days de-escalating to monotherapy once culture sensitivity information has been returned. For patients with CF, aerosolised

aminoglycosides (such as tobramycin) have been shown to have efficacy when treating early acute infection [168]. Chronic infection in CF patients has proven impossible to eradicate however and critically ill patients with ventilator associated pneumonia must be treated promptly before culture isolates can be tested for sensitivity. Combined with a decreasing pool of antibiotics that remain efficacious and the frequency of nosocomial infections caused by *P. aeruginosa* there is a need for additional treatment options.

1.7 Pathogen Recognition, Inflammasome Formation and Initiation of Pyroptosis as an Anti-infective Mechanism of Epithelial Cells.

Airway epithelium is the first point of internal contact with the body for infectious bacterial microorganisms that utilise airborne and droplet transmission routes. Although inhalation of organisms and particles occurs all the time not all inhaled materials are pathogenic. Many species of bacteria may live commensally in the back of the nose and throat [227], whilst others are opportunistic pathogens only causing disease when local immune mechanisms are compromised.

Epithelial linings have an armoury of recognition and response mechanisms with which they protect the upper and lower respiratory tract (reviewed in [228]). These mechanisms may include the release of danger signals to mount an inflammatory response to a particular organism, or the induction of immune tolerance towards the presence of non-harmful organisms or by removing microbes with minimal alarm. Initially thought to provide merely a physical barrier between the outside world and the internal environment, epithelial cells are now understood to play a much more varied and important role in innate immunity and inflammatory pathways.

Respiratory epithelium is made up of ciliated epithelial cells, mucus-producing goblet cells and basal cells [221]. More than one type of epithelial cell exists in the airways and this varies with location within the tract. The oropharynx is protected by stratified squamous epithelium whilst the upper airway epithelial cells form a ciliated pseudostratified columnar epithelium. In addition to providing a physical barrier to the rest of the body an important mechanism for removal of organisms is the mucociliary escalator. Mucin-containing mucus, the production of which is controlled by MUC genes and goblet cells in the respiratory tract [229, 230], traps organisms which are then moved upwards by the beating of thousands of cilia so that microbe-containing mucus may be either ingested and dealt with my stomach acid or spat out and removed from the body entirely.

Where this mechanism fails such as in the case of cystic fibrosis (CF) patients whose mucus is dehydrated and difficult to remove from the airways [162] or in patients who have malfunctioning cilia [263], removal of organisms by this method is impaired. A major characteristic of the immune system however is redundancy. Aside from physical removal of

foreign particles there is also an array of lipid, protein, chemical and peptide mediators that either interact directly with the organism to kill or inhibit growth or act indirectly by directing other cells and components of the immune system. When pathogens are not removed by the mucociliary escalator and are allowed to interact with the epithelial layer then cellular recognition and defence mechanisms must be initiated to respond to the threat.

1.7.1 Pathogen Recognition Receptors (PRRs) and Inflammasome formation

Innate immune mechanisms are described as non-specific or, at best, broadly specific, a notion easy to comprehend when compared to the precise interaction between lymphocytes and their reciprocal antigens during an adaptive response. Nevertheless, alongside a wider appreciation of the role that innate mechanisms play in setting the scene for the type of adaptive response that follows, there is an increasing amount of evidence that suggests this view of non-specificity is perhaps a result of incomplete understanding. The following introduction aims to present the literature, which dissects the molecular recognition mechanisms that suggest that innate responses aren't as ”non-specific” as they are classically described..

1.7.2 Pathogen Recognition Receptor Signalling

Whilst cells of the adaptive response recognise specific molecular sequences via a combination of endocytosis by antigen presentation cells, MHC-restricted antigen presentation and co-stimulation from other cells and cytokines [169], cells of the innate system recognise disturbances in the body using an array of pathogen recognition receptors (PRRs, *table 1.1*) which respond to a diverse array of exogenous pathogen-associated molecular patterns (PAMPs) and/or endogenous host cell derived danger-associated molecular patterns (DAMPs) [170].

These diverse molecular patterns independently, or more often in combinations, initiate signal transduction pathways, determining the cytokine environment produced initially by the cells local to the infection or damaged tissue and later by activation of recruited immune cells. PRRs are differentially expressed on different cell types and “sentinel cells” such as

resident macrophages and dendritic cells possess a wider variety of receptors than non-myeloid cells such as epithelial cells. Upon ligation of a particular set of PRRs signalling pathways are initiated and, via an array of cytosolic mediators and accessory proteins (e.g. MyD88, TRIF, TRAM, MAPK), transcription factors involved in regulating cytokine production or cell death pathways translocate to the nucleus and alter gene expression in that cell (e.g. NF-κB) [171].

PRR	Location	Domains	Functions	
TLR (Toll-like Receptors)	Cell Membrane and endosomes	LRR domain in ectoderm (Leucine rich repeats) Transmembrane domain Cytoplasmic Toll/IL-1 receptor (TIR) domain	TLR 1 Bacterial lipoproteins TLR 2 Bacterial Lipoproteins, peptidoglycan, host HSP70 + HMGB1 TLR 3 ds RNA in endosome (Viral) TLR 4 LPS, HSPs, nickel TLR 5 Bacterial Flagellin TLR 6 Mycoplasma	TLR 7 ss RNA in endosome (Viral) TLR 8 ss RNA in endosome TLR9 Bacterial DNA (CpG motifs) in endosome TLR 10 – Unknown TLR 11 Profilin TLR 12 Profilin TLR 13 Pathogen rRNA
CLR (C-lectin type receptors)	Membranes and Secreted	Carbohydrate binding domains Variable other domains	Large variety of proteins and functions	
RLR (Rig-like helicases)	Cytosolic	CARD (Caspase activation and recruitment domain) RNA Helicase Repressor Domain	Recognition of Viral DNA/RNA Induction of type 1 IFN in response to infection	
AIM 2 (Absent in melanoma 2)	Cytosolic	PYD (Pyrin domains) HIN200	Foreign DNA recognition	
NLR (Nucleotide binding domain, leucine rich repeat)	Cytosolic	NOD 1 NOD 2 PYD NACHT (NOD) LRR BIR	23 in humans 34 in mice Main role is to regulate production of IL-1 and IL-18 and activate caspase-1	

Table 1.1: Types of Pathogen Recognition Receptors (PRR). PRRs that may be expressed by host cells in order to recognize damage or infection and mount an innate immune response accordingly [172-176]

1.7.2.1 *Toll-Like Receptors*

Toll-like receptors (TLRs) are membrane-spanning proteins that function as PRRs [172]. A flurry of activity followed the discovery of the first TLRs until several were identified along with their ligands. We now realize that epithelial, endothelial and immune cells all use these receptors to differentiate between threats from viral particles (ds or ss DNA and RNA), bacterial cells (Gram positive or negative, flagellated, capsulated etc.) as well as identify damaged tissues and host cell debris and respond accordingly. As effectively as these recognition receptors function however, they still seem a far cry from the specificity of an *Escherichia. coli* O157 IgG antibody in binding to the surface of that particular organism.

Yet in conjunction with TLR recognition of "type" of threat, these same cells can utilize cytosolic and endosomal PRRs such as the C-lectin type receptors [173], RIG-1 like receptors (RLRs) [174], AIM2 [175] and the nucleotide binding domain, leucine-rich repeat containing receptors (NLRs) [176], to detect changes in their intracellular environment such that the distinction between extracellular and intracellular insult can be made.

1.7.2.2 *RLRs*

Rig-1 like receptors [174] or retinoic acid inducible gene -1 like receptors are intracellular PRRs that bind RNA and initiate intracellular signalling responses via a caspase activation and recruitment (CARD) domain. They are important particular in the recognition of viral infection.

1.7.2.3 *NLRs*

Nod-like receptors (NLR) or nucleotide-binding oligomerisation domain-like receptors [176] are a group of 23 intracellular PRRs that share a similar structure. That is an N-terminal caspase recruitment, pyrin or baculovirus inhibitor repeat domain, a conserved nucleotide-binding oligomerisation domain and C terminal leucine rich regions that recognise a diverse array of intracellular pathogens.

1.7.3 Inflammasomes - large cytosolic complexes that form following intracellular insult to promote an inflammatory response.

Caspases [177] are a family of cysteine dependent aspartate-specific proteases that form cascades of activation, cleaving other pro-caspases and intracellular substrates. As this cleavage can result in activation of other proteins or destruction of cell contents regulation exists to avoid ill effects. One of these regulatory strategies is that caspases are produced in a zymogen form (*table 1.2*).

Inflammasomes are large cytosolic complexes (>700KDa) that recruit and activate certain pro-caspases. The oligomerisation of an inflammasome is triggered by a wide variety of molecules in a series of events which remain to be fully elucidated however the composition of the inflammasome, the surrounding extracellular environment and the type of cell in which it forms determines the effect this complex has on the function and fate of that cell.

The integral components of an inflammasome complex (for which they are named e.g. NLRP3 inflammasome) are NLRs, additionally, pro-caspase 1, accessory proteins (CARDINAL, BIR, NAIP5 or ASC) and sometimes pro-caspase 5 (*Fig. 1.6* and *table 1.2*). Upon recognition of particular ligands inside the cell NLRs dimerise and via their CARD (caspase activation and recruitment) domains or via the utilisation of ASC, which contains a CARD domain, pro-caspase 1 is recruited to the complex and activated [178].

Activation of caspases can occur in many scenarios, with or without inflammasome involvement. Caspase activation may result in cytoplasmic and nuclear condensation, DNA cleavage and laddering as seen during the process of apoptosis (caspases 3, 8, 9) [179]. Alternatively, inflammasome-associated caspase activation may cause the activation and release of pro-inflammatory cytokines such as IL-1 beta and IL-18, with or without inducing an inflammatory form of programmed cell death called pyroptosis (caspases 1, 4, 5 11) [180] which appears to be a mechanism for removing infected cells whilst summoning further inflammatory cells to deal with microbes that escape intracellular destruction.

Caspase	Activation	Effect
1	Inflammasomes containing CARD domains Caspase 1	• Pro-inflammatory • Decreases the growth of intracellular bacteria • Delivers bacteria to lysosomes • Maturation and secretion of IL-1, IL-18 and IL-33 • Pyroptosis
2	Extrinsic pathway	• Initiator caspase
3	Caspase 1, 8, 9, 10	• Effector caspase • Apoptosis
4	Caspase 1, 8, 10	• Pro-inflammatory
5	Inflammasomes containing CARD	• Pro-inflammatory
6	Caspase 8, 10	• Effector caspase •
7	Caspase 1, 8, 9, 10	• Effector caspase • Apoptosis
8	Death receptor mediated (Extrinsic) Caspase 10	• Initiator caspase • Apoptosis
9	Mitochondria mediated pathway (Intrinsic) Caspase 8, 9, 10	• Initiator caspase • Apoptosis
10	Death receptor mediated (Extrinsic)	•
11	Following bacterial evasion of endosome	• Pro-inflammatory • Pyroptosis
12	Caspase 7	• Pro-inflammatory

Table 1.2: Caspases – cysteine-aspartic proteases. Caspases belong to three groups, inflammatory, initiator of cell death and effectors of cell death [9]. Non-death functions of caspases have not been included in this table.

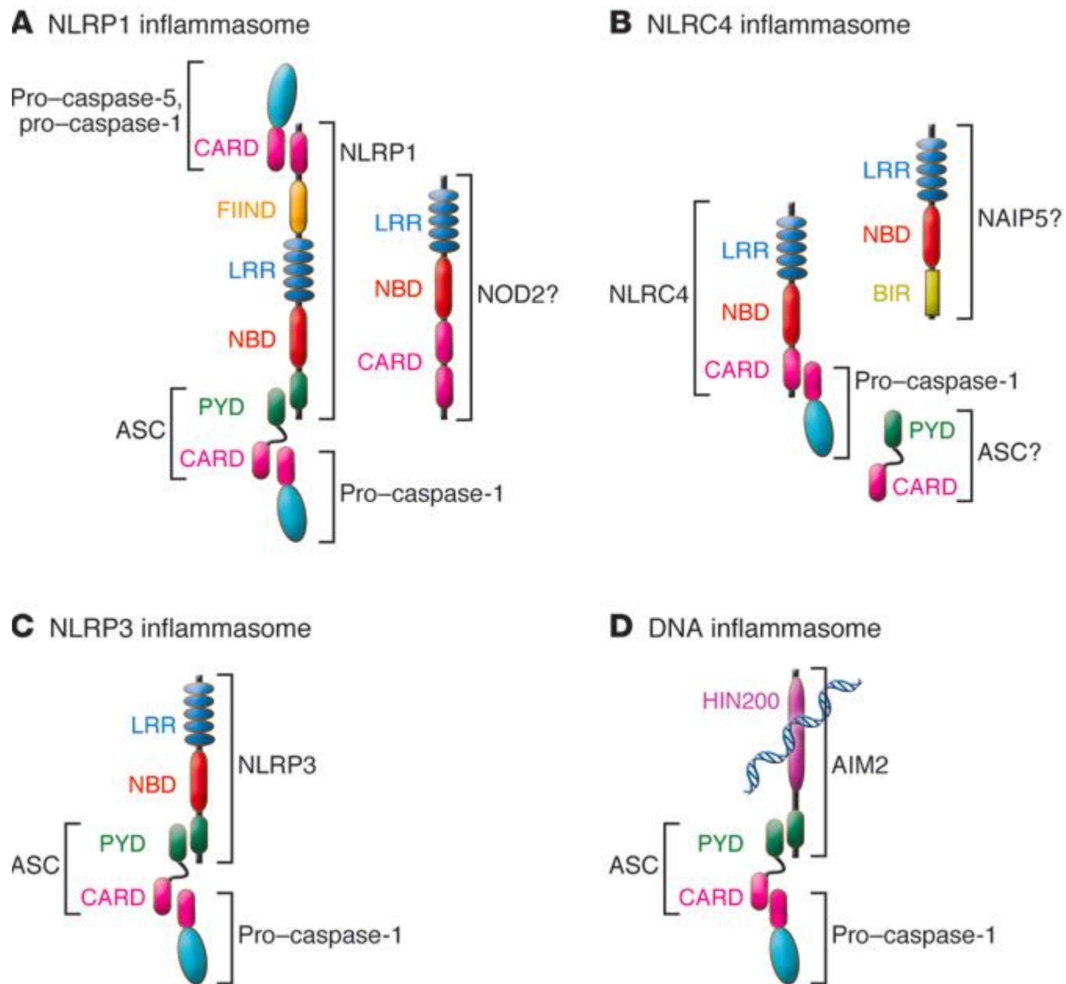

Figure 1.6: Types of Inflammasomes. The four best studied inflammasome formations. [182] CARD = Caspase activation and recruitment domain, FIIND = function to find domain, LRR = leucine rich repeat, NBD = nucleotide binding domain , PYD = pyrin domain , ASC = apoptosis-associated speck-like protein containing a CARD, NLRP1 = nod-like receptor protein 1 , NOD2 = nucleotide oligomerisation domain, BIR = baculoviral inhibition of apoptosis repeat domain, NAIP5 = NLR family apoptosis inhibitory protein, NLRP3 = nod-like receptor protein 3, HIN200 = , AIM2 = absent in melanoma 2

Inflammasome/NLR	Domains	Accessory/adapter molecules	Activated in response to
NLRP3	NACHT LRR PYD-domain containing protein	ASC (Apoptosis speck-like protein containing a CARD, PYD) CARDINAL Cryopyrin (NALP 3)	Pore forming toxins Extracellular ATP and PAMPS Uric acid vDNA RNA Asbestos UVB Lysosomal disruption
NLRC4 (IPAF)	NLR CARD-domain containing		Pseudomonas, Salmonella, Legionella, Listeria and Shigella
NLRP1	NLR		*B. anthracis* toxin
NAIP	NLR BIR (baculoviral inhibition of apoptosis repeat)		
AIM2 (Absent in melanoma 2)	PYD (engages HIN200 (binds ds DNA)		Inflammasome component that contains no NLRs, binds dsDNA IFN-inducible protein

Table 1.3: Nod-Like receptors (NLR). NLRs have nucleotide binding domains and leucine rich repeats and are intracellular PRRs known to be involved in inflammasome formation. Also shown is the NLR-independent AIM2 inflammasome [178, 199]

1.7.3.1 Inflammasomes are crucial for activation of the pro-inflammatory cytokines IL-1 and IL-18.

The IL-1 superfamily is a group of cytokines that include IL-1β, IL-18 and IL-33. IL-1β is an important cytokine and is known as the endogenous pyrogen for its fever inducing effects via the hypothalamus. It is not only involved in healthy inflammatory responses, enhancing leukocyte migration, cytokine and chemokine expression and host cell proliferation, differentiation and apoptosis but it is also strongly associated with pathology in various inflammatory diseases such as gout, rheumatoid arthritis and other autoimmune diseases [181]. IL-18 is also pro-inflammatory and can activate T cells and macrophages resulting in increased production of IFNγ. Expression of pro-forms of IL-1β and IL-18 are upregulated following TLR and NOD 1+2 receptor stimulation. However cleavage of the pro-forms by caspase-1 is essential for production of the active forms of both IL-1β and IL-18 [182-184], the relative expression of each appear to alter the course of infection in ways which are not yet well understood.

This exemplifies just how innate regulatory mechanisms can work in the sense of priming for a response and in perpetuating an inflammatory response. Extracellular signals tell the cell to prepare and store mediators and if the causative factors are not removed then intracellular signals prompt a course of attack using stored forces. IL-1β and IL-18 have no secretion signals and further to activation by caspase-1 may also be released from the cell by caspase-1 induced pores in the cell membrane [185]. Further to mediating cytokine release TIRAP, (Toll-like receptor domain containing adapter protein) a component of TLR pathways signals better in the presence of caspase-1 promoting an increase in TNF and IL-6 and macrophage activation in response to TLR2+4 ligands [186] A sequence of events for inflammasome formation and cytokine activation is shown in *fig. 1.7*

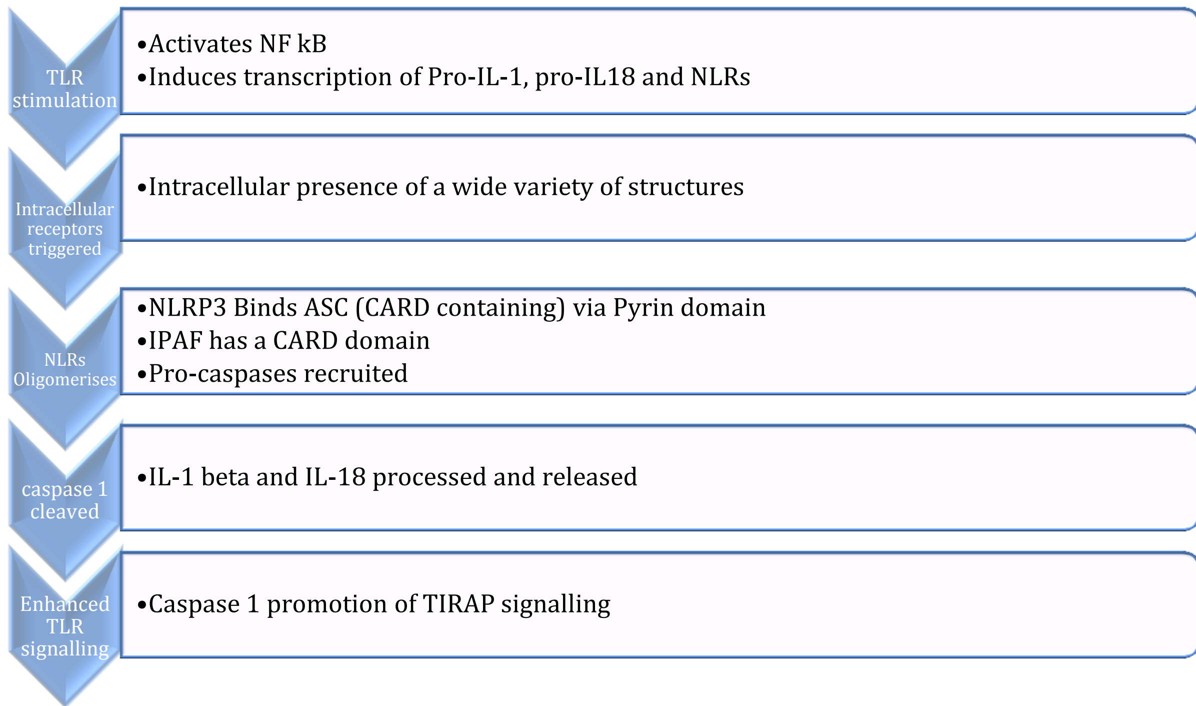

Figure 1.7: Coordinated responses of NLR and TLR signalling in response to PAMPS and/or DAMPS.

17.3.2 Intracellular sensing – The importance of K+ efflux, [ATP] and endosome integrity for activation of the NLRP3 inflammasome.

Interactions between cellular receptors and ligands rely on the structures of both parties fitting in a way similar to the lock and key concept of enzyme activation. NLRC4 inflammasomes are repeatedly shown to be activated by flagellin [210] and AIM2 inflammasomes by dsDNA. How then is it possible that inflammasomes formed by NLRP3 appear to be initiated by structurally diverse ligands?

Various proposals have been put forward to explain this. One is that it is alterations in intracellular K^+ that may be the trigger for NLRP3 dimerization and that all of the proposed ligands results in K^+ efflux via a variety of mechanisms [187-190]. For example, increases in extracellular ATP as well as the pleiotropic cathelicidin LL-37 interact with P_2X_7, [89, 107, 109, 118, 135] an ATP-gated receptor, which causes an efflux of K^+ and subsequent activation of inflammasome proteins and caspase-1 release. Ionic disturbances and inflammasome activation have also been shown during multiple viral and bacterial infections of cells [191]. In the case of bacterial infection it is commonly due to toxin-mediated damage of plasma membranes causing efflux of K^+. Another model for NLRP3 activation involves Cathepsin B, a lysosomal protease. Loss of lysosome membrane integrity causes a release of lysosome contents that activates NLRP3 [192]. Finally, events that increase the production of reactive oxygen species (ROS) can also induce NLRP3 inflammasome formation [193, 194].

1.7.3.4 NLRC4 and Pseudomonas

NLRC4 (previously named IPAF) acts as an intracellular PRR for *P. aeruginosa* infected macrophages, recognising flagellin and components of the type III and IV secretion systems [201, 211] both of which are virulence factors possessed by many Gram negative bacteria. Following direct or indirect binding of NLRC4, pro-caspase 1 is recruited and activated which in turn processes pro IL-1 and IL-18 to their active pro-inflammatory forms. ExoU expressing strains of *P. aeruginosa* are however, able to inhibit the inflammatory responses normally induced by caspase 1 [210].

1.7.4 Pyroptosis: Caspase-1 mediated inflammatory cell death as an anti-infective effector mechanism.

From initial distinctions between necrotic cell death as pathological, inflammatory and lytic and apoptosis being a tightly regulated “silent” cell death there are now descriptions of multiple forms of cell death. One such form has been coined pyroptosis; a caspase-1 mediated, inflammatory, anti-infective cell death (*Fig 1.8*), which is orchestrated by inflammasome formation and activated caspase-1 or caspase 11[195-197, 222]. Recently caspase-11 has also been shown to induce pyroptosis in cells following infection and evasion of the endo-lysosomal pathway by bacteria in the absence of IL-1 release [198].

In certain cell types, likely determined by the particular inflammasome components expressed by that cell as well as the virulence factors possessed by the invading microorganism, infectious stimuli especially associated with phagolysosome evasion, can induce an inflammatory lytic cell death following caspase-1 activation. Morphological differences are apparent in pyroptosis vs. apoptosis and it can be reasonably assumed that this is a defence mechanism for destroying compromised cells when first line phagocytosis has failed.

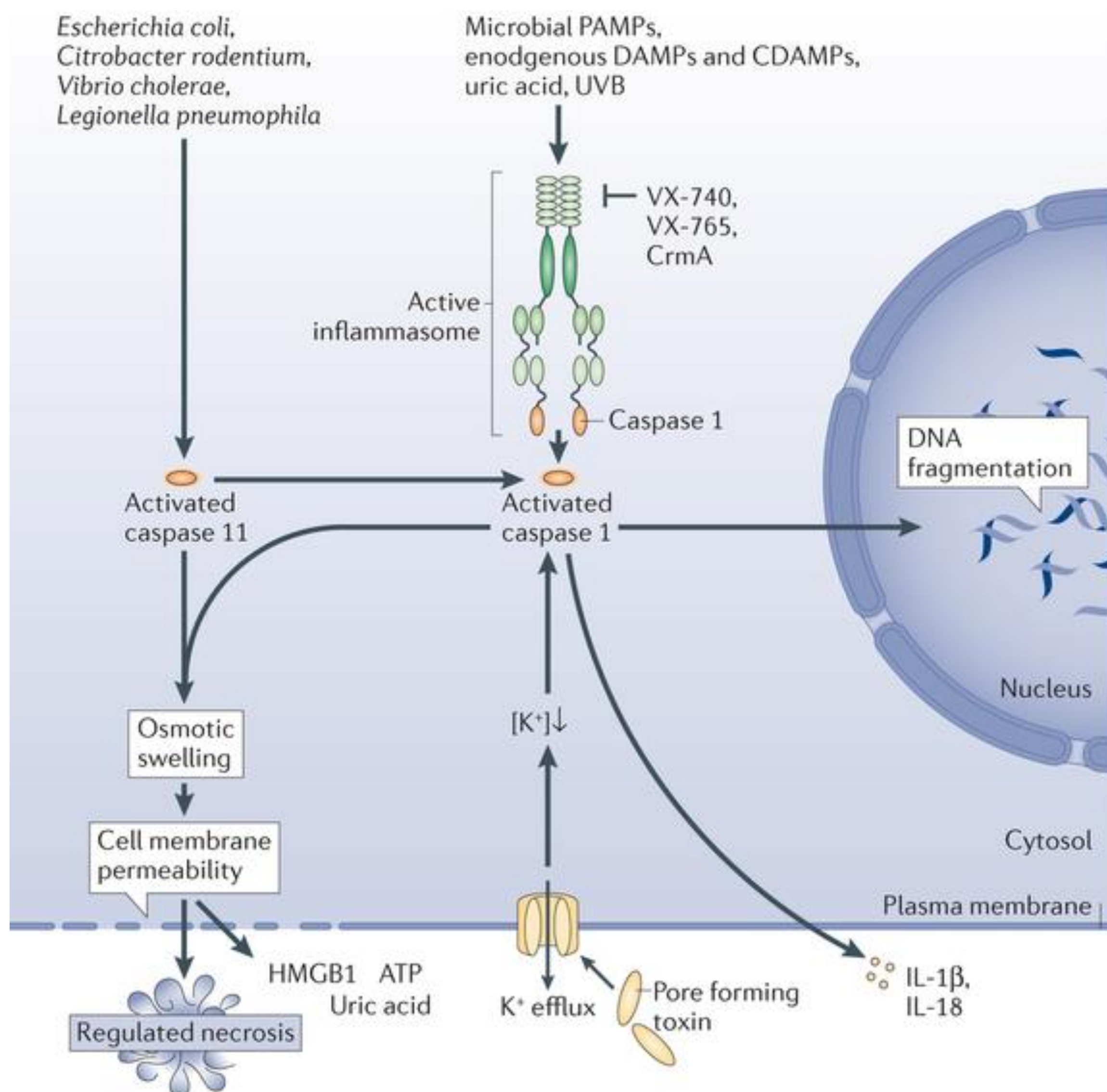

Figure 1.8 Pyroptosis; a caspase-1 mediated, inflammatory, anti-infective cell death [223]

1.8 Cell Death

The study of cell death pathways and the molecules that influence the form of death that is initiated is an ongoing area of research.

1.8.1 Apoptosis

Apoptosis is a form of "programmed" cell death that is considered a silent method of removing damaged or infected cells with no or minimal initiation of immune responses. It may be initiated by external death receptors signalling via caspase 8 or internal events such as the release of mitochondrial matrix proteins that activate caspase 9. Both methods of initiation culminate in the activation of caspase 3 and a common death pathway that results in cell shrinkage, DNA laddering, membrane blebbing [224-226] and the formation of a cell with "eat me" signals that may be recognised by macrophages.

1.8.2 Necrosis, necroptosis and secondary necrosis

Necrosis previously thought of as an uncontrolled process is an inflammatory cell death where damaged cells release intracellular contents that act as danger signals and initiate inflammatory responses. In contrast to apoptosis necrosis is morphologically characterized by rounding of the cell, cytoplasmic swelling with dilated organelles and an absence of chromatin condensation. Necroptosis is a form of necrosis dependant on the activity of serine–threonine kinase receptor-interacting protein 1 (RIP1) [212]. Secondary necrosis describes apoptotic cells that have not been cleared which after some time or following interaction with extracellular molecules such as LL-37 [93] lose their membrane integrity. It has been proposed by some that these secondary necrotic cells remain non-inflammatory.

1.8.3 Pyroptosis

Pyroptosis is a caspase-1 or caspase-11 mediated cell death thought to be an anti-infective mechanism to activate the cytokines IL-1 and IL-18 initiating an inflammatory response to remove compromised cells [195, 222].

1.8.4 NETosis

Netosis is a form of cell death of neutrophils where in the process of dying they spit out “nets” (trapping nearby pathogens) composed of nuclear chromatin and cytosolic proteins, many of which are antimicrobial, to destroy extracellular bacteria [213, 257].

1.8.5 Antimicrobial peptides and cell death

Based on the proposed mechanism of action of CHDPs (membrane insertion and disruption) it could reasonably be predicted that these peptides may also be cytotoxic though differences in membrane composition between microorganism and eukaryotes appear to offer protection against these effects. Nevertheless, cytotoxicity as well as initiation of cell death pathways, has been documented in response to CHDP and the effect these peptides have on host cell death is complex and dependent upon the particular peptide, presence of microbes, affected cell type and surrounding chemical milieu.

Early work proposing that cathelicidins from various species may be exploited as direct anti-microbials documented that these peptides are in fact often haemolytic [200]. In addition, according to recent work exposure to high levels of hCAP18/LL-37 causes a calpain and BAX-mediated apoptosis of epithelial cells [110]. Yuk [96] reported that hCAP18/LL-37 mediates autophagy initiation in mycobacterial-infected cells and has also been shown to induce a caspase-3 dependant cell death in epithelial cells [108] that is inhibited by human but not bovine serum.

Shown to have a role in resolution of inflammation a study undertaken in our lab confirms observations that hCAP18/LL-37 can convert apoptotic neutrophils into secondary necrotic cells with no resultant inflammatory response from resident phagocytes [93, 119, 120].

Keratinocytes are a large producer of hCAP18/LL-37 and as such are surrounded by high concentrations of the peptide. Proposing that there must be a mechanism for keratinocytes resistance to cytotoxicity Chamorro [112] looked at why these cells do not die and found that hCAP18/LL-37 induces the production of COX2 and consequently PGE2 in exposed keratinocytes with a resultant production of IAP2 and inhibition of cell death.

At the time of starting this thesis the role of host cell defence peptides in cell death remained unclear, however given that LL-37 induced cell death in both infected and uninfected cells in a dose dependant manner [108-109] yet was clearly protective to the host, this was to be a focus for me when studying lung infection with *Pseudomonas aeruginosa*.

Hypothesis

Cathelicidin-mediated immunomodulation, including preferential induction of cell death in infected epithelium, is a component of defence against bacterial infection at mucosal surfaces.

Project 1: Cathelicidin is differentially expressed in the female reproductive tract at times in the menstrual cycle and during pregnancy

Aims

1. To determine the cellular locations of LL-37 in the female upper reproductive tract by histology and take relative measurements of LL-37 mRNA expression in *ex vivo* endometrium and fallopian tube samples from throughout the menstrual cycle by quantitative PCR.

2. To assess progesterone regulation of LL-37 expression in endometrial epithelial cells *in vitro*.

Project 2: Human cathelicidin selectively induces death in infected epithelial cells and has a beneficial role during acute Pseudomonal lung infection.

Aims

1. To identify intracellular factors responsible for LL-37-mediated killing of *P. aeruginosa* infected airway epithelial cells *in vitro*.

2. To determine the contribution of common bacterial virulence factors in LL-37 mediated killing of infected airway epithelial cells *in vitro*.

3. To evaluate, *in vivo*, the role of cathelicidin in clearance of *P. aeruginosa* from the murine lung.

Chapter 2: Materials and Methods

Materials and Methods

Equipment

ABI Prism 7900	Applied Biosystems
WPA UV 1101, Biotech Photometer;	Biochrom Ltd., Cambridge, UK
96 well optical reaction plates	Applied Biosystems
NucleoCounter YC-100	(ChemoMetec, Allerød, Denmark)
Nanodrop	
Menzel-Glazer microscope slides	
Shandon cytospin 2 centrifuge	
Sequenza	Shandon
Shandon casettes	Thermo
Biometra cycler	
Peltier thermal cycler	Bio rad
PCR tubes	Axygen Scientific
Filter tips (DNAse free)	
Culture flasks	Corning Costar
Stripetttes	
6 well plates	Corning Costar

Reagents

2x Taqman universal master mix	PE Biosystems, UK
Taqman reverse transcription reagent kit	Applied Biosystems
Nuclease free water	Severn Biotech Ltd
Quick diff fix	Reastain(Recigina)
Acetone	
Methanol	
Xylene	
Alcohol	

Antigen retrieval solution	Vector
PBS Oxoid	
Avidin and Biotin	Vector
Protein block	Dako
R.T.U ABC reagent	Vector
Diamnobenzidine (DAB)	Vector
Haematoxalin	
Antibody diluent	Dako
anti-hCAP18/LL-37 IgG	Hycult Biotech
Biotinylated horse anti-mouse IgG	Vector,
Mouse IgG	Vector
RNAeasy minikit	Qiagen
β-mercaptoethanol	
Proteinase K solution	Qiagen
HAMS (+L-Glut)	Cambrex
F-10 HAM	Sigma
Insulin, transferrin, Selenium (ITS)	Cambrex
FCS	
Estradiol (1mM in ethanol)	
Medroxyprogesterone acetate (MPA) 50mM in DMF	
LL-37 synthetic peptide	
RU486 1mM in ethanol	

Bacterial Strains and Culture

These studies used the following strains of *P. aeruginosa*: PA01 (Standard lab strain) clinical isolate J1386 (a clonal isolate of J1385, originally from an individual with cystic fibrosis), ΔmexAB-oprM mutant (a gift from Keith Poole), PAO1exsA::Ω mutant (a gift from Dara Frank), *P. aeruginosa* pilA mutant (a gift from Eva Lorenz), and the isogenic PAO1 control strains for these mutants (Table 2.5). Studies involving genetically modified bacteria were performed according to Scientific Advisory Committee on Genetic Modification Health and Safety Executive Certificate GM207/07.2.

Strain	Origin/Description	Gifted by	Reference
PA01	Standard Laboratory strain	John Govan	[205]
J1386	Clinical isolate from a CF patient	John Govan	[206]
ΔmexAB-oprM	Efflux pump mutant	Keith Poole	[207]
PA01exsA	T3SS mutant	Dara Frank	[208]
pilA	Pili mutant	Eva Lorenz	[209]

Table 2.1: Strains of *P. aeruginosa* used in this study

All *P. aeruginosa* strains were grown in Luria Bertani (LB) broth at 37°C in an orbital shaker (250 rpm) overnight, to achieve a stationary-phase suspension. Before use, bacterial suspensions diluted 1:10 in fresh LB broth were incubated at 37°C for 90 minutes to reach logarithmic phase. Bacterial suspensions were standardized via dilution with LB broth to an optical density of 0.1 at 595 nm, using spectrophotometry (WPA UV 1101, Biotech Photometer; Biochrom Ltd., Cambridge, UK), centrifuged at 1,500 × g for 15 minutes (keeping supernatant where required for use in place of live bacteria), and resuspended in PBS.

To determine the direct microbicidal activity of LL-37, *P. aeruginosa* were resuspended in Ultroser G serum–substitute supplemented media before the immediate addition of LL-37 at the concentrations stated. After incubation for 1 hour at 37°C, serial dilutions were performed in PBS, and 100-μl aliquots of these (and the original bacterial suspension) were spread onto LB agar plates in triplicate, and incubated overnight at 37°C before counting the number of colony-forming units (CFUs).

For studies to determine if the function of the ΔmexAB-oprM mutant could be rescued by soluble factors released by PA01, PA01 was added to 16HBE14o− cells at a multiplicity of infection (MOI) of 10:1 and incubated for 18 hours at 37°C with 5% CO2. After incubation, the supernatant was collected and filtered through a 0.22-μm filter unit. The sterility of filtered supernatant was confirmed by culturing 50 μl on LB agar plates for 24 hours. Filtered supernatant (1:4 dilution in treatment medium) was simultaneously added together with LL-37 and ΔmexAB-oprM to 16HBE14o− cells, and the cells were incubated for 1 hour and analyzed using the mitochondrial depolarization technique.

Cell Culture

hTERT Cell culture. Human telomerase reverse transcriptase immortalised endometrial epithelial cells (hTERT EEpC) were used with kind permission of Prof Ian Mason. Cells were maintained in Hams F10 (Sigma, etc) with 10% foetal bovine serum (FBS) and Insulin, transferrin, selenium (ITS) (Cambrex, 1ml per 500 media) at 37°C + 5% $CO_{2.}$ cDNA previously prepared from these cells was obtained from Anne King for the purpose of confirming receptor expression. The cells in this case had been treated with increasing concentrations of IL-1.

hTERT EEpC were used to determine the effect of exogenous hormone application on LL-37 expression *in vitro*. 6 well plates were seeded with $3x10^5$ cells/well in FBS-containing media. After 24 hours, cells (for those experiments without serum) were serum starved using media containing ITS but no FBS for a further 24 hours at 37°C. Experiments containing serum were treated immediately after the first 24 hour incubation. Cells were treated in duplicate with Oestradiol (10^{-6}, 10^{-7}, 10^{-8}M), Progesterone (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}M), RU486 (10^{-6}M), Progesterone + Oestradiol (10^{-7}M + 10^{-6}M) progesterone + RU486 (10^{-7}M + 10^{-6}M). To determine if there was any feedback loop with LL-37 itself, synthetic LL-37 peptide at 3µg/ml was also added as a treatment for investigation. .Supernatants from treated cells were stored at -20°C for later analysis by ELISA to detect secreted LL-37 protein levels and cell lysates were harvested using RNA Later buffer (RLT, Qiagen) and immediately used with RNA extraction kit (RNeasy, Qiagen) or stored at -20°C overnight for RNA extraction the following day.

Decidualised endometrial stromal cells - (cells maintained and decidualised and RNA extracted by Anne King) stromal cells which had been separated from endometrial biopsies and then grown in the presence of Medroxyprogesterone acetate (MPA) (10^{-6}M), oestradiol (10^{-7}M) and 8-bromo-cAMP (0.1mg/ml) for 120 hours were used. RNA was collected from cells every 24 hours. At around 72 hours the cells started to show morphological changes. Decidualisation was confirmed by PCR for the presence of IGFBP1 (a marker of decidualisation).

Human uterus microvascular endothelial cells (hUMEC) (Cambrex, Lonza) – RNA from these cells was provided by Anne King.

The 16HBE14o− transformed human bronchial epithelial cells - were a kind gift from Dieter Gruenert at the University of California, San Francisco. Cells were grown in standard submerged cultured and maintained in DMEM supplemented with 1% L-glutamine, 1% NEAA , and 10% FBS. Culture flasks were coated with a basement layer of collagen IV (5 μg/ml), fibronectin (10 μg/ml), and BSA (100 μg/ml) before cell culture at 37°C, 5% CO2. Culture media was substituted with serum free Treatment media (phenol red-free DMEM supplemented with Ultroser G, L-glutamine and NEAA) for all experiments.

Normal human bronchial epithelial cells from a single donor were purchased from Clonetics, Lonza. Cells were grown in standard submerged culture and maintained in bronchial epithelial growth media (Lonza), supplemented with bronchial epithelial cell SingleQuots growth factors and supplements (Lonza) as a serum substitute, in strict accordance with the manufacturer's instructions. All assays were conducted in Ultroser G serum–substitute supplemented media, consisting of phenol red–free DMEM supplemented with 1% Ultroser G (vol/vol), 1% L-glutamine (vol/vol), and 1% NEAA (vol/vol). Cells were maintained at 37oC, 5% CO2

Cytospins and counts

BALF from *in vivo* murine experiments was centrifuged at 200×g for 5 minutes, and supernatant was removed for cytokine measurement. Pelleted cells were resuspended and counted by NucleoCounter YC-100 (ChemoMetec, Allerød, Denmark) automated cell number counting. 100 µl of cell suspension was then loaded onto a glass slide using a disposable sample funnel and cytocentrifuged at 10×g for 3 minutes in a Shandon Cytospin 2 centrifuge. Slides were air dried for 20 minutes, fixed in methanol for 20 minutes, stained with Diff Quik (Fisher Scientific, Loughborough, UK), and mounted in DPX Mountant (Fluka BioChemika/Sigma Aldrich, UK). Differential counts for neutrophils and monocytes were then performed by light microscopy at 20× magnification using an EVOS FL microscope (Peqlab, Sarisbury Green, UK).

ELISA

Enzyme Linked Immunosorbent Assay (ELISA) was used to assay cell supernatants for quantification of secreted LL-37 in response to treatments in cell assays. hCAP18/LL-37 sandwich ELISA (Hycult Biotechnology Ltd, Netherlands) was used as per the kit instructions.

Cytochrome c concentrations in each fraction (to confirm that cytochrome C was translocating from the mitochondria to the cytoplasm following exposure of 16HBE14o- cells to LL-37) was assessed using a Cytochrome c ELISA Kit (Merck Chemicals, Ltd.), according to the manufacturer's protocol.

Murine cytokines in BALF were measured using an ELISA (R&D Systems, UK) according to the manufacturer's instructions, for KC, MIP-2 alpha or by using a cytometric bead assay mouse inflammation kit (BD Biosciences, UK) for TNF, IL-6, MCP-1, IL-10, IFN-gamma, IL-12p70.

Gentamicin Exclusion Assay

The capacity of *P. aeruginosa* isolates and mutants to invade epithelial cells was assessed using a gentamicin exclusion assay. Briefly, 16HBE14o− cells were exposed to strains of log-phase *P. aeruginosa* (MOI 10:1) for 60 minutes in Ultroser G–serum-substitute supplemented media. The media were removed from all wells, and cells were incubated with fresh media for 60 minutes with or without gentamicin (50 μg/ml) to kill extracellular bacteria. The media was then aspirated from gentamicin-treated cells, and these cells were vigorously washed with PBS and lysed with PBS containing 0.1% Triton X-100, and then plated on LB agar to determine internalized bacterial numbers. Media and/or epithelial cell lysates from wells without gentamicin were also plated on LB agar, to determine the number of associated bacteria and total infectious load. The CFUs were quantified by culturing overnight on LB agar plates at 37°C.

Immunohistochemistry

A Diaminobenzidine (DAB) based detection system was employed to localise the hCAP18/LL-37 protein present in tissue sections. Sections (3μm) were de-waxed in xylene and antigen retrieval carried out using antigen unmasking solution (Vector) for 15 minutes at high power in the microwave. Sections were blocked with 3% hydrogen peroxide, avidin and biotin block (Vector) and with Dako protein block. Primary antibody for anti-hCAP18/LL-37 was mouse monoclonal IgG (Hycult Biotech, clone 3D11, 100μg/ml) used at 1/200. Secondary antibody was biotinylated horse anti-mouse IgG, (BA-2000, Vector, 1.5mg) used at 1/300. Synthetic LL-37 peptide (5mg/ml) was used at 10x antibody concentration and absorbed to primary antibody for 1 hour prior to use as a negative control. Mouse IgG (i-200, Vector) was used as a primary antibody isotype control. Sections were counterstained with Haematoxylin and Scott's tap water and dehydrated through alcohol back to xylene for mounting with pertex.

Mitochondrial Depolarization Assay (Mitocapture)

The 16HBE14o− cells were seeded at 2.5×10^4 cells per well in a 96-well plate and cultured at 37°C, 5% CO2. Cells were exposed to LL-37 (or scrambled LL-37 control peptide) at the concentrations described in the presence and absence of (1) log-phase *P. aeruginosa* at an MOI of 10:1; (2) heat-killed or UV-killed bacteria (MOI 10:1), *P. aeruginosa* PAO1 LPS (1 µg/ml) or *P. aeruginosa* supernatant, all prepared as described above; or (3) log-phase *P. aeruginosa* PAO1 (MOI 10:1), separated from the epithelial cells by a Transwell semipermeable polyester membrane with 0.4-µm pore size (Corning Life Sciences, Amsterdam, Netherlands), and incubated for 1 hour at 37°C, 5% CO2. All treatments were conducted in Ultroser G serum–substitute supplemented media. After treatments, cells were washed once with PBS prewarmed to 37°C, the supernatant was aspirated, and 50 µl of Mitocapture solution (Cambridge Bioscience, Cambridge, UK) at 37°C were added to each well, before incubation at 37°C for 30 minutes in the dark. Cells were then washed twice with PBS at 37°C, resuspended in 50 µl of Mitocapture buffer at 37°C, and imaged immediately using an Axiovert S100 inverted fluorescent microscope (Zeiss UK, Welwyn Garden City, UK). For each membrane, at least four random fields of view were counted with a minimum of 300 cells in total, and the number of apoptotic cells (displaying diffuse, green fluorescence) was expressed as a percentage of the number of healthy cells (displaying punctate red mitochondrial fluorescence). Data were corrected for a background level of approximately 10% positive cells observed in control untreated samples. For inhibition studies, the culture medium in each well was replaced with treatment medium containing 100 µM BIP-V5 for 1 hour before treatment.

Murine infection model

Mouse experiments were performed in accordance with Home Office UK project licence 60/4216, under the Animal (Scientific Procedures) Act 1986. Wild type control mice were all C57Bl/6 strain, supplied by Charles River Laboratories, UK, and housed at the University of Edinburgh for at least two weeks before use, or were animals bred from those mice. *Camp* $^{-/-}$

mice [16] were bred to congenicity on a C57Bl/6 strain background and were the offspring of homozygous mutant matings raised in the same facility at the University of Edinburgh. Both male and female mice were used, between 8–12 weeks old, housed in individually ventilated cages and randomly assigned to treatment groups (no significant difference were found in end points between male and female mice). Mice were weighed, given a general anaesthetic (isofluorane) in a category 2 biosafety hood, then held vertically by scruffing over the front of the thorax and inoculated by an intranasal delivery up to a total of 50 µl volume. Mice were inoculated with 3×10^7 colony forming units (cfu) of PAO1 or the same volume of PBS, and 10 µg LL-37 peptide in PBS or PBS only control. PBS alone (carrier for both bacteria and peptide) was used as a control (rather than scrambled peptide, which previous pulmonary infection studies indicated had no effects ([23] and unpublished data), in order that the wild type control infected animals were appropriate controls both for the LL-37-treated infected wild types and for the infected *Camp*$^{-/-}$ animals (in which no peptide was delivered). Mice were then returned to cages, placed on a heat mat to maintain body temperature, and monitored and scored for signs of infection every 2 hours, with peak of illness occurring at 6–8 hours post infection, followed by recovery with diminishing severity score. Mice were re-weighed and culled at selected timepoints (0, 6 or 24 hours), culled by pentobarbital injection and lungs and trachea exposed by dissection. Lungs were lavaged in 1 ml sterile PBS via intramedic polyethylene tubing (Sigma Aldrich, UK) inserted into the trachea, and bronchoalveolar lavage fluid (BALF) stored on ice. Following lavage, lungs were either homogenised in 2 ml sterile PBS for cfu counts or were perfused by PBS injection into the heart, then removed and frozen for RNA preparation.

For CFU counts, BALF or homogenised lungs were serially diluted in PBS, plated on Pseudomonas Isolation agar (Becton Dickinson Difco, Oxford, UK), incubated overnight at 37°C and bacterial colonies counted using a Stuart SC6 colony counter. Total colonies on the lowest dilution plate countable were multiplied by the appropriate dilution factors to determine the total CFU count of the lung tissue or BALF sample.

Peptide

LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES; MW 4493.33) was either synthesised by N-(9-fluorenyl) methoxycarbonyl chemistry at the Nucleic Acid/Protein Service unit at the University of British Columbia (UBC; Vancouver, Canada), or custom synthesised by Almac (East Lothian, Scotland) using Fmoc solid phase synthesis and reversed phase HPLC purification. Peptide identity was confirmed by electrospray mass spectrometry, purity (>95% area) by RP-HPLC and net peptide content determined by amino acid analysis. Lyophilised peptides were reconstituted in endotoxin free water at 5 mg/ml stock concentration and determined to be endotoxin-free using a Limulus Amebocyte Lysate Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, UK). Peptide functionality was confirmed by assessing anti-endotoxic activity.

Protein extraction

Total protein was extracted from cells by lysing adherent cells with M-PER mammalian protein extraction reagent (Thermo scientific) containing anti proteases and phosphatases (Halt Protease Inhibitor Cocktail Kit, cat 78410).

Quantitative (Real Time) Polymerase Chain Reaction (qPCR)

qPCR was used to detect transcription of LL-37 in endometrial and fallopian tube tissue as well as expression in these tissues of progesterone receptor forms PRA and PRB. mRNA levels of LL-37 in both cell lysates and in preserved tissue stored in RNA Later buffer (Qiagen) were carried out.

Briefly RNA was extracted from cells or tissues with the RNEasy extraction kit (Qiagen) RNA concentrations were measured using the nanodrop system and all samples prepared to a stock concentration of 100ng/µl RNA. Total cDNA was generated in a thermocycler (25°c for 20 mins, 42°C for 60 mins, 95°C for 5 mins) from each sample using random primers in a mix containing RT buffer, $MgCl_2$, dNTPs, RNase inhibitors and multiscribe reverse

transcriptase (Applied Biosystems reagent kit). Primers and probes are listed in Table 2.2. All samples were prepared in Taqman universal mastermix (Applied Biosystems) with 50nM of 18s for/rev primers and vic labelled probe, 300nM LL-37 for/rev primers and 200nM FAM labelled probe. 3µl cDNA per sample was added to mastermix. 2 negative controls were used in each assay one containing template RNA but no reverse transcriptase (RT negative) and the other containing reverse transcriptase with water in place of template RNA (RT H2O). Ribosomal 18s cDNA was used to correct for interassay variation and liver cDNA was used as a positive control. The $2^{-\Delta\Delta Ct}$ method ($\Delta \Delta$ Ct = ΔCt1 (treated) – ΔCt2 (control) was used to analyse expression of the gene of interest. PCR conditions were as follows, 50°C for 2 mins, 95°C for 10 mins then 40 cycles of 95°C for 15 secs and 60°C for 60 secs.and were run on the prism 7900 (Applied Biosystems).

Primer/probe	Sequence	Source	Conc.
LL-37 forward	TCA-CCA-GAG-GAT-TGT-GAC-TTC-AA	Eurogentec	300nm
LL-37 reverse	TGA-GGG-TCA-CTG-TCC-CCA-TAC	Eurogentec	300nm
LL-37 probe	AAG-GAC-GGG-CTG-GTG-AAG-CGG	Eurogentec	200nm
PRA+B forward	CAG-TGG-GCG-TTC-CAA-ATG-A	Eurogentec	300nm
PRA+B reverse	TGG-TGG-AAT-CAA-CTG-TAT-GTC-TTG-A	Eurogentec	300nm
PRA+B probe	AGC-CAA-GCC-CTA-AGC-CAG-AGA-TTC-ACT-TT	Eurogentec	200nm
PRB only forward	CGG-ACA-CCT-TGC-CTG-AAT-T	Eurogentec	300nm
PRB only reverse	CAG-GGC-CGA-GGG-AAG-AGT-AG	Eurogentec	300nm
PRB probe	CGG-CCA-TAC-CTA-TCT-CCC-TGG-ACG-G	Eurogentec	200nm
Ribosomal 18S Primers and probes		Applied Biosystems	All 50nm

Table 2.2 Primers and probes used in rtPCR experiments on reproductive cells and tissues

Polymerase Chain Reaction (PCR)

PCR was used to detect the presence of transcripts of inflammasome components in extracts of cultured cells. Briefly RNA was extracted from cells with the RNEasy extraction kit, RNA concentration was measured using the nanodrop system and all samples prepared to a stock concentration of 100ng/µl RNA. Total cDNA was generated in a thermocycler from each sample using random primers in a mix containing 10 x RT buffer, 25mM $MgCl_2$, dNTPs, RNase inhibitors and multiscribe reverse transcriptase. Primers and probes used are listed in

table 2.3. All samples were prepared in Taqman universal mastermix (Applied Biosystems) and were run on the prism 7900 (Applied Biosystems).

Primers	Sequence	Source	Product size
HPRT	GCT-CGA-GAT-GTG-ATG-AAG-GAG Tm59.97 CCT-GAC-CAA-GGA-AAG-CAA-AG Tm 59.85		306
CARD8 (cardinal)	GGG-ACA-TTC-CCA-GTG-TAT-CAG Tm 59.27 TGT-GAC-CTC-ATC-CCT-TAC-CAG Tm 59.27	Eurogentec	281
PYCARD (asc)	GAG-GAG-CTC-AAG-AAG-TTC-AAG-C Tm 59.78 GGC-TGG-TGT-GAA-ACT-GAA-GAG Tm 59.9	Eurogentec	396
AIM2	AAC-GTC-TTC-AGG-AGG-AGA-AGG Tm 59.86 TCT-TGG-GTC-TCA-AAC-GTG-GAA-G Tm 60.28	Eurogentec	277
NLRP3	AGG-AGT-GGA-TGG-GTT-TAC-TGG Tm 60.23 TCC-ATC-TTA-ATG-GGA-CTC-ACG Tm 59.94	Eurogentec	274
NLRC4	TTT-CAT-TTG-ACC-CAC-TTG-TCT-G Tm 60.01 GCT-GTT-CTA-GCA-CGT-TCA-TCC Tm 59.9	Eurogentec	256
NLRC2	AGG-AGG-AAA-GAG-CAA-GTG-TCC Tm 59.87 GCC-CAA-GTA-CCC-TTA-TTC-CAG Tm 59.84	Eurogentec	262
NAIP	CTG-GAA-ACC-ACA-AGT-GAA-AGC Tm 59.77 AGA-GGT-TCT-TGC-ACA-GGT-TTG Tm 59.39	Eurogentec	257
NLRC1	ACT-CAG-AGC-AAA-GTC-GTG-GTC Tm 59.51 CTT-TCC-TTG-AAG-CAG-CTG-AAC Tm 59.25	Eurogentec	392

Table 2.3 Human Primers for PCR reactions on 16HBE/A549 cells

Tissue sections

Formalin fixed tissues preserved in paraffin blocks which were previously collected were used throughout the study. Written informed consent was obtained from all patients involved and ethical approval was granted by the Lothian Research Ethics Committee. Normal across-cycle endometrium samples were collected either as papelles during routine gynaecological procedures or as full wedge tissues during hysterectomy for benign conditions. The cycle stage of these tissue samples was determined both by histological examination as well as serum levels of ovarian hormones. Decidua was obtained during either elective surgical termination of pregnancy (STOP) or from women undergoing treatment due to miscarriage of pregnancy. Mirena endometrium biopsies were taken from women complaining of bleeding problems whilst using the Mirena intra uterine system (IUS) contraceptive device. Fallopian tubes were obtained either at the time of hysterectomy for benign conditions or during management of ectopic pregnancy.

In Situ Cell Death Detection by Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay

Transwell polyester-permeable supports (pore size, 0.4 μm; diameter, 6.5 mm; Corning Life Sciences) were equilibrated for 45 minutes in culture media (DMEM supplemented with 10% FBS [vol/vol], 1% L-glutamine [vol/vol], and 1% NEAA [vol/vol]) before the addition of 100 μl of medium containing 2.5 × 105 16HBE14o− cells/ml into the apical compartment, with 600 μl culture medium in the basal compartment, and cultured at 37°C, 5% CO2. For primary bronchial epithelial cell experiments, Transwell supports were equilibrated with NHBE culture media for 45 minutes before the addition of 100 μl of NHBE media containing 2.5 × 10^5 NHBE cells/ml into the apical compartment, with 600 μL of NHBE culture medium in the basal compartment, and cultured at 37°C, 5% CO2. Before treatments, culture media in both the apical and basal compartments were replaced with Ultroser G serum–substitute supplemented media. Cells were exposed to LL-37 at the concentrations described in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1), and incubated for 6 hours at 37°C, 5% CO2. Cells were fixed in 10% neutral-buffered formalin (3.7% formaldehyde) for 10 minutes, washed once in PBS, permeabilized in ice-cold 0.1% Triton X-100/0.1% sodium citrate for 3 minutes, and washed twice with PBS. An in situ cell death detection kit (Roche Applied Science, West Sussex, UK) was used according to the manufacturer's instructions. The membranes with cells were mounted in 50 μL Vectashield Hardset (containing DAPI), and at least four random fields of view were counted (each containing more than 100 cells), using an Axiovert S100 fluorescent microscope, and analyzed using OpenLAB 3.0 software (Improvision/Perkin Elmer, Waltham, MA). The number of terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL)–positive cells was expressed as a percentage of the number of DAPI-positive nuclei. The total number of DAPI-positive nuclei counted for each condition was determined, to evaluate total cell number. For inhibition studies, cells were prepared as described, and culture medium in each well was replaced with treatment medium containing either 100 μM Bax inhibiting peptide V5 (Merck Chemicals, Ltd.) or 50 μM of the broad-spectrum caspase inhibitor, Z-VAD-FMK (Merck Chemicals, Ltd.), or the caspase 1 inhibitor for 1 hour before treatment.

Western Immunoblotting

The 16HBE14o− cells were seeded at 1 × 10^6 cells per well in six-well plates and cultured at 37°C, 5% CO2. Cells were exposed to LL-37 at the concentrations described, in Ultroser-G serum–substitute supplemented media, in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1) and incubated for 3 hours at 37°C, 5% CO2. Cells were washed once with ice-cold PBS and lysed with 300 µL M-PER protein extraction reagent (Thermo Scientific) containing a cocktail of protease, phosphatase, and metalloprotease inhibitors. Protein concentrations were determined by bicinchoninic acid assay (Thermo Scientific). Equivalent total protein concentration lysates (15–40 µg) were resolved on either 10% or 12% precast Precise Protein polyacrylamide gels (Thermo Scientific), transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Ltd., Hemel Hempstead, UK), blocked for 1 hour with Tris-buffered saline and 0.1% Tween-20 (TBST) containing 5% skimmed milk powder (TBST/milk), and then incubated with antibodies specific for cleaved caspase-3 (1 in 5,000 dilution), cleaved caspase-9 (1 in 1,000 dilution), XIAP (1 in 1,000 dilution), or pan-actin (1 in 2,000 dilution) in TBST/milk overnight at 4°C. Membranes were washed for 15 minutes in TBST and then incubated with a 1 in 5,000 dilution of HRP-conjugated goat anti-rabbit antibody (in TBST/milk) or a 1 in 5,000 dilution of HRP-conjugated goat anti-mouse antibody (in TBST/milk) for 1 hour at room temperature. Membranes were washed for 30 minutes and developed with chemiluminescence peroxidase substrate (Sigma-Aldrich) according to the manufacturer's instructions, and imaged on CL-Xposure film (Thermo Scientific). Equal loading of protein was confirmed by examining the expression of actin as a loading control.

Blots were stripped with RestorePlus Western Blot Stripping Buffer (Thermo Scientific, cat 46430) and then washed in Tris-buffered saline containing 0.05% Tween-20 prior to re-blocking and probing.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism version 5 for Windows (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed either using one-way ANOVA with Tukey's post hoc test, or two-way ANOVA with Bonferroni's post hoc test where appropriate. Kruskal-Wallis and Wilcoxon signed rrank 1-tailed test and Pearson correlation tests were also used where appropriate. $P \leq 0.05$ was considered significant.

Bacterial counts were normalised by logarithmic transformation before analysis by 2 way ANOVA with Bonferroni's post hoc tests where appropriate. Cell counts and cytokine concentrations were analysed by Mann Whitney test.

Chapter 3: Expression of hCAP18/LL-37 in the female reproductive tract

3.1 Introduction

Multiple antimicrobial peptides, including hCAP18/LL-37, are found in abundance in the lower urogenital tract, where it is likely they are acting in defence of an area constantly in contact with micro-organisms [203]. Infection in the upper reproductive tract is less common but has serious sequelae such as infertility, increased risk of ectopic pregnancy and may also pose a threat to the developing foetus [204]. There is however, relatively little literature on the actions of cathelicidin in the upper reproductive tract. Although it is certainly possible that the LL-37 in the upper reproductive tract has a role in destroying invading organisms it is also possible that it has immunomodulatory or physiological functions, as has been shown in other organs such as the lung. This is likely to be the case in the uterus as this is an area that for the most part is considered sterile. In addition, there is a requirement of the endometrium to replace a large number of cells after each menstruation and to replenish the rich blood supply and LL-37 is known to increase the rate of proliferation of epithelial cells and to have a role in promoting angiogenesis [35].

I aimed to identify the expression pattern of hCAP18/LL-37 in the female upper reproductive tract and establish *CAMP* mRNA expression in endometrium and fallopian tubes from different stages of the menstrual cycle. I wanted to investigate whether cathelicidin was expressed uniformly throughout the menstrual cycle, or cyclical, which may suggest functions over and above any antimicrobial action. In addition to healthy or “normal” tissue, tissues from patients who had been treated for ectopic pregnancy, elective surgical termination of pregnancy (STOP) and users of the Mirena (levonorgestrel-releasing) intrauterine contraceptive devices were also analysed. Finally, potential progesterone regulation of LL-37 expression was assessed *in vitro*.

3.2 hCAP18/LL-37 show a cyclical pattern of expression in tissue samples from the female reproductive tract.

In order to identify the cellular location of cathelicidin in tissues from female reproductive tract, immunohistochemistry was performed on a range of tissue sections. Using a DAB based detection system and monoclonal antibodies for hCAP-18/LL-37 we demonstrated that the pattern of LL-37 expression in the endometrium varied across the menstrual cycle (representative sections are shown in *Fig 3.1*). Tissues taken from endometrium at different stages of the menstrual cycle were obtained from women as either papelles during routine procedures or as wedges during hysterectomy (*Table 3.1*). The cycle stage was confirmed both morphologically on tissue architecture and also using patient data of last menstrual period and measured hormone levels at the time of tissue collection. In the proliferative stages (*Fig 3.1a*), hCAP-18/LL-37 was mainly concentrated in the epithelial cells surrounding the glands and intense staining can be seen on the apical surface of these cells indicating a protein secretion into the glands. At this stage some stromal staining can be seen as well as some endothelial staining (*Fig 3.1b*). In the secretory stage the pattern is much the same with less dark apical staining (*Fig 3.1c*). In the menstrual stage (*Fig 3.1d*) heavier staining can be seen across the whole tissue with individual cells staining intensely throughout the tissue. These cells appear to be leukocytes and most likely neutrophils, which are known to be present in large numbers during menstruation; however co-localisation of specific markers was not performed so this cannot be confirmed. Following implantation and decidualisation of the endometrium in pregnancy the pattern of staining changes and though glandular epithelial staining can still be detected (data not shown) there is predominantly a large amount of stromal staining (*Fig 3.1e*). To confirm that the positive staining was due to the presence of hCAP-18/LL-37 I included some control slides in each experiment. I used a non-specific pooled mouse IgG as an isotype control for the primary antibody as well as some slides where synthetic peptide had been incubated with the primary antibody prior to addition to the slides in order to inhibit further binding to the hCAP-18/LL-37 in the tissue. Both controls resulted in sections with no positive staining (*Fig 3.1f*).

Tissue Sample	Stage of menstrual cycle	Number of donors	Reason for surgery
Endometrium	Menstrual	2	Papelles taken during routine procedures or wedges taken following hysterectomy
	Proliferative	6	
	Early secretory	2	
	Mid Secretory	2	
	Late secretory	2	
Decidua	First Trimester	2	Surgical termination of pregnancy with misoprostol prostaglandin
Decidua	Mirena user	7	Users of Mirena system undergoing investigation for abnormal bleeding

Table 3.1 **Endometrial and decidua samples from patients**

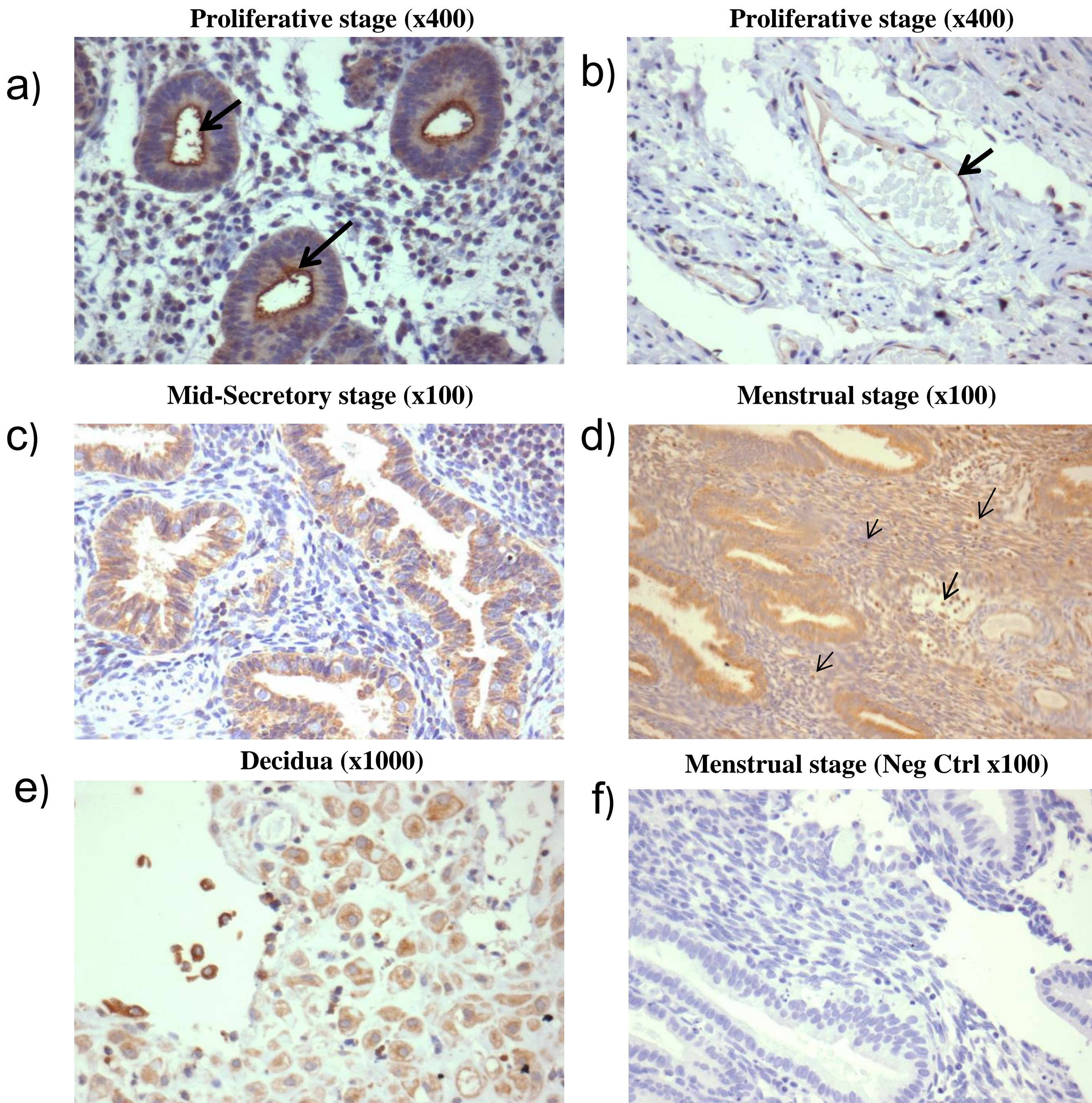

Figure 3.1 hCAP18/LL-37 expression in tissues from the human female endometrium. Sections were de-waxed in xylene and antigen retrieval carried out using antigen unmasking solution for 15 minutes at high power in the microwave. Sections were blocked with 3% hydrogen peroxide, avidin and biotin block and with protein block. Primary antibody for anti-hCAP18/LL-37 was mouse monoclonal IgG used at 1/200. Secondary antibody was biotinylated horse anti-mouse IgG, used at 1/300. Positive signal is shown as brown.

a) Proliferative endometrium representative of n=6 **b)** positive staining in endometrial cells in the proliferative endometrium **c)** mid secretory endometrium representative of n=2 **d)** menstrual endometrium representative of n=2 **e)** stromal staining predominates in decidua representative of n=2 **f)** Negative (Murine IgG Isotype) control.

Expression patterns determined for fallopian tube specimens differed from endometrium in that the expression pattern of cathelicidin we observed remained constant throughout the cycle (*Fig 3.2a*). Staining was observed to be intense in epithelial cells with very little, if any, stromal staining. Clear endothelial staining could be seen in vessels within the vascular smooth muscle surrounding the fallopian tubes (data not shown).

Interestingly, the sections taken from women using the Mirena IUS, were full wedge tissues and it could be seen that the basal layer of glandular epithelium did not stain for LL-37, unlike in the functional layer, however diffuse staining could be seen over the decidualised stromal cells (data not shown). Many intensely stained leukocytes could be seen scattered throughout the functional layers of these tissues (*Fig 3.2b*).

Thus, these data show that cathelicidin protein expression varied in amount and cellular location at different stages of the menstrual cycle and was not only produced by glandular epithelium but also observed in stroma and endothelial cells, which was an unexpected finding. In order to confirm these findings of cyclical, inducible expression of cathelicidin, an analysis of gene transcription was undertaken.

Tissue Sample	Stage of menstrual cycle	Number of donors	Reason for surgery
Fallopian tube	Menstrual	1	Total hysterectomy
	Proliferative	3	
	Mid secretory	3	
	Late secretory	3	
Fallopian tube	Ectopic	4	Ectopic Pregnancy

Table 3.2 **Fallopian tube tissue samples.**

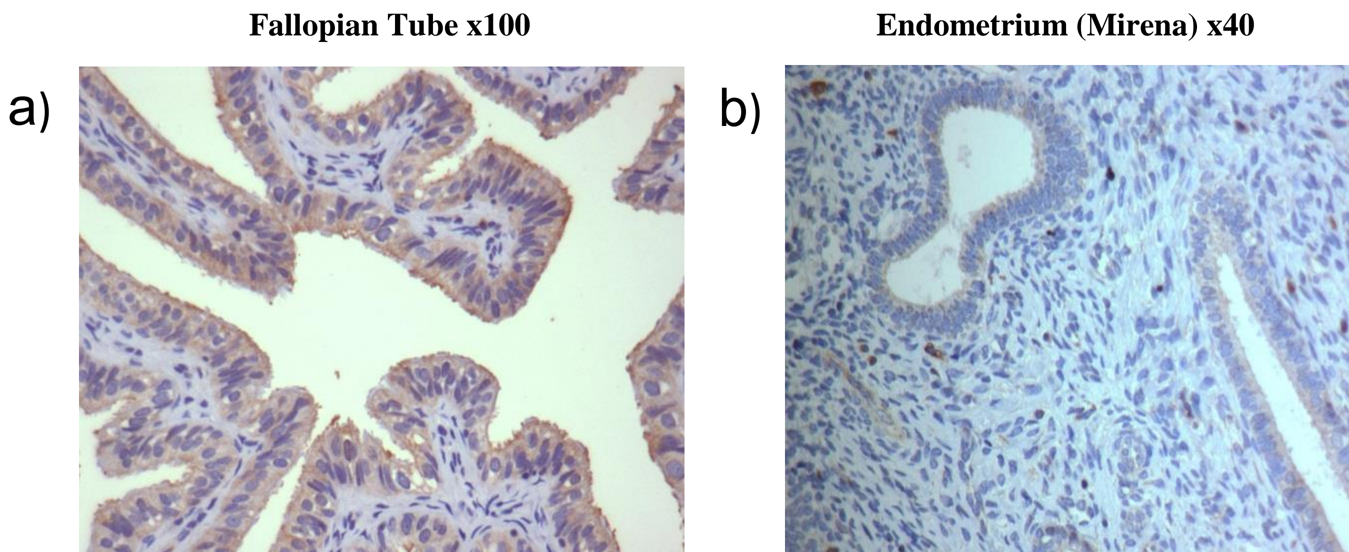

Figure 3.2 hCAP18/LL-37 expression in tissues from human female fallopian tube and endometrium. Sections were de-waxed in xylene and antigen retrieval carried out using antigen unmasking solution for 15 minutes at high power in the microwave. Sections were blocked with 3% hydrogen peroxide, avidin and biotin block and with protein block. Primary antibody for anti-hCAP18/LL-37 was mouse monoclonal IgG used at 1/200. Secondary antibody was biotinylated horse anti-mouse IgG, used at 1/300. **a)** fallopian tube from non-pregnant donor with hCAP18/LL-37 staining in epithelial cells concentrating on apical surface representative of n=10 **b)** endometrium from a Mirena IUS user. Representative of n=7

3.3 *CAMP* transcription in tissues from the female reproductive tract varies throughout the menstrual cycle and is maintained at high level in decidua.

In order to confirm whether the level of transcription of cathelicidin in endometria and fallopian tubes from women during different stages of the menstrual cycle was cyclical, reverse transcription and quantitative (real time) polymerase chain reaction (q-PCR) was utilised. *CAMP* mRNA was detected in normal endometrium from across the cycle. However there was a low level of *CAMP* transcription in the proliferative stage, which was significantly less (p=0.044) than at other times in the cycle (*Fig 3.3a*). Transcription of *CAMP* was otherwise stable over the rest of the menstrual cycle. Expression of *CAMP* in decidua was found to be higher than at any stage of the menstrual cycle although this trend did not reach significance. Levels of *CAMP transcription* in Mirena decidua, that is endometrium that has been artificially decidualised by high local dose progesterone from the Mirena intrauterine system (IUS), also remain high but is still approximately half of the fold change seen in decidua from pregnant women and comparable to mid-secretory levels. Taken together this variance in expression across the cycle and during pregnancy suggested a possible regulation by progesterone, the expression of which follows a similar pattern (*Fig 1.1*) and is sustained at high level following the implantation of the trophoblast.

Little difference was found between *CAMP* mRNA levels in the decidua of a terminated uterine pregnancy versus ectopic pregnancy (*Fig 3.3b*), however, interestingly, significantly lower levels of *CAMP* transcription was found in endometrial samples from the miscarriage group (p=0.018). The ectopic group was further subdivided into 2 groups. The group designated G came from donors with higher serum levels of the hormones hCG and progesterone. When divided into these 2 groups differences could be seen between those with higher levels and those with lower expression of hCAP18/LL-37, this was not however a significant difference. A subtle increase in transcription may be present in the fallopian tubes as the cycle progresses but the only striking difference seen is in the considerably higher levels in fallopian tubes of ectopic pregnancy (p-0.0023) which is to be expected due to local inflammation (Fig 3.3c).

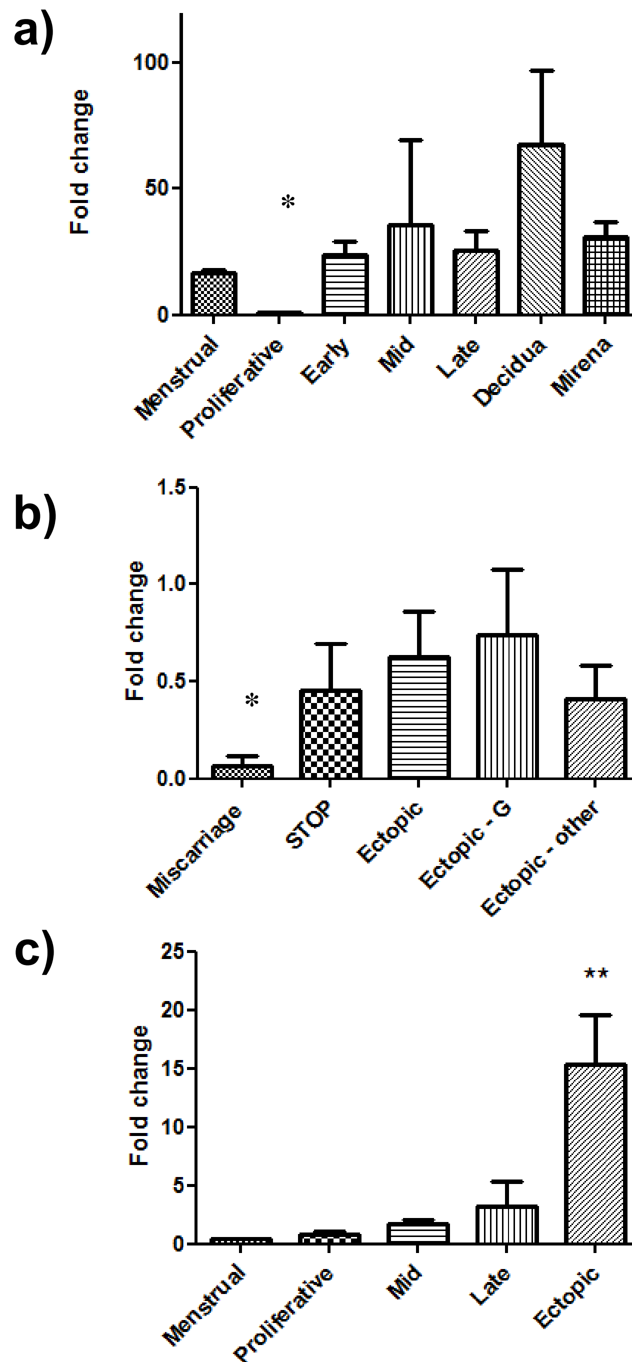

Figure 3.3 ***CAMP*** **mRNA in reproductive tract tissue samples:** real time PCR to detect *CAMP* expression was performed on cDNA made from a) normal endometrium across the cycle (n=12), endometrium from users of the Mirena coil (n=5), and decidua from patients undergoing surgical termination of pregnancy (STOP) (n=4). b) Decidua from patients undergoing STOP (n=8), miscarriage (n=4) or ectopic pregnancy (n=9) c) fallopian tubes taken from times across the cycle (n=13) and from individuals surgically treated for an ectopic pregnancy (n=4). Data was corrected to 18s housekeeping gene and show fold change in expression compared to liver DNA (*camp* +ve control). Data shown as mean + SEM for each sample. Data was analysed using non parametric Kruskal-wallis test * $p<0.05$ ** $p<0.01$

3.4 hTERT immortalised human endometrial epithelial cells express progesterone receptor A (PRA) and hCAP18/LL-37 *in vitro* but do not express progesterone receptor B (PRB)

In order to further investigate the hypothesis that the transcription of *CAMP* and expression of hCAP18/LL-37 can be regulated by steroid hormones in the female reproductive tract, an immortalised cell line was used. hTERT human endometrial epithelial cells (EEpCs) are primary endometrial cells that have been immortalised by the enzyme human telomerase reverse transcriptase (hTERT) [249]. Before addressing the hypothesis, it was first necessary to characterise this EEpC cell line for its capability to produce hCAP18/LL-37 and ability to respond to the hormone progesterone.

The two forms of progesterone receptor (A and B) are known to activate discrete genes [214] and are variably expressed on different cells, with the ligand progesterone capable of exerting a negative feedback regulation on the expression of the receptors. The activity of PRA may also inhibit the effects of PRB ligation.

To confirm that the cells to be used for investigation into the role of progesterone in hCAP18/LL-37 expression were capable of transcribing *CAMP* and responding to progesterone, qPCR using cDNA from endometrial epithelial cells (EEpC) was performed. I found that hTERT EEpC were positive for CAMP transcription confirming that these cells do have the ability to express hCAP-18/LL-37 (*Fig 3.4a*). The receptor for progesterone acts as a ligand activated transcription factor. As PRA is a truncated form of PRB (shortened by 164 N terminal amino acids) there is no primer that is specific for *PRA* only. Using primers which amplify both *PRA* and *PRB*, expression was detected in EEpC (*Fig 3.4b*). However, when primers specific for *PRB* only were used, no transcription was detected, indicating that all of the progesterone receptor expression in the hTERT epithelial cells is of the PRA form (*Fig 3.4b*). Interestingly, the addition of the cytokine IL-1β to these cells down-regulated the expression of progesterone receptor (*Fig 3b.*)

In order to further examine the immunohistochemical observations that endothelial cells also expressed hCAP18/LL-37 we performed the same q-PCR analyses with human endothelial cell (HUMEC) cDNA (*Fig 3.4*). The HUMEC cell line did not however express *CAMP*, *PRA* or *PRB* under cell culture conditions.

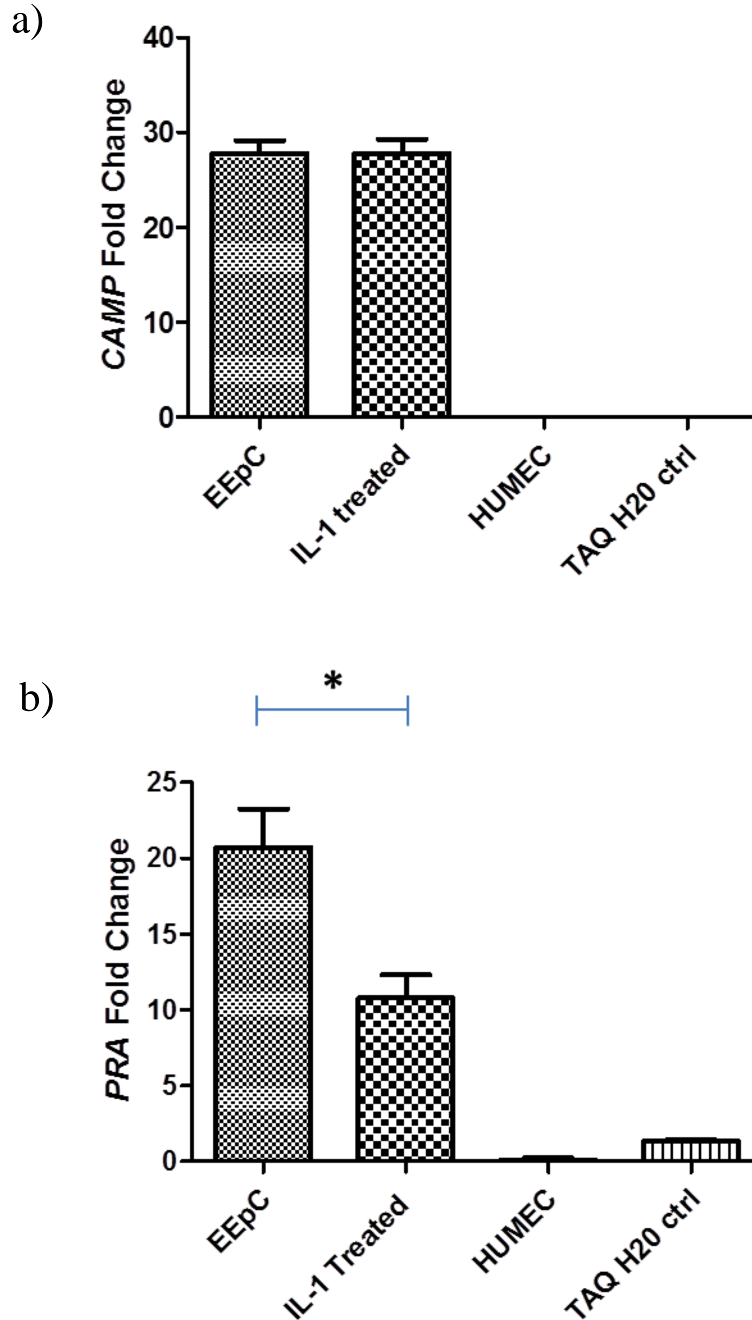

Figure 3.4 RT PCR on cDNA from hTERT endometrial epithelial cells (EEpC) or human endothelial cells (HUMEC) to detect transcription of CAMP, PRA and PRB : a) *CAMP* transcription was detected in EEpC (n=4) and was not affected by treatment of the cells with IL-1 (n=2). No CAMP transcription was seen in the cDNA from HUMEC (n=1) b) *PRA* transcription was seen in EEpC (n=4) and was significantly reduced in cells that had been treated with IL-1 (n=2). No PRA transcription was seen in the HUMEC (n=1). Data shows fold change in expression of genes compared to liver DNA (+ve control).

3.5 Progesterone has no direct effect on the level of *CAMP* mRNA transcription in endometrial epithelial cells *in vitro*

In order to determine the capacity of steroid hormones to regulate the transcription of *CAMP*, hTERT immortalized EEpC were cultured in 6 well plates for 24 hrs. Cells were given fresh media with or without serum for 12 hours prior to incubation for a further 24 hours with either LL-37 peptide, oestradiol (the predominant hormone during the proliferative phase), medroxyprogesterone acetate (MPA, the hormone secreted by the corpus luteum post ovulation that promotes the changes that occur in the secretory phase endometrium and maintains decidua), RU486 (a progesterone receptor antagonist used as an abortifacient) or a combination of oestradiol and MPA (as both hormones are present across the cycle at varying levels *in vivo*).

In the presence of serum no significant difference was observed in *CAMP* expression between treatments (*Fig 3.5a*). Cells temporarily starved of serum, which results in a synchronized culture of cells in the same stage of the proliferative cycle, responded to oestradiol with an increase in *CAMP* production (*Fig 3.5b*) however the experiment would need to be repeated to confirm this was not merely an artifact.

In the presence of serum there was a modest increase observed in *PRA* expression in cells treated with oestradiol greater than that of the DMF control alone (*Fig 3.5c*) In the absence of serum there was an increase in *PRA* transcription following treatment with exogenous LL-37 and with oestradiol as well as with DMF (a carrier for MPA) alone suggesting that the increase in expression observed in cells treated with MPA is due to the carrier (*Fig 3.5d*) *PRB* was not expressed in any cells analysed.

Although I found that EEpC transcribe the PRA form of progesterone receptor (but not PRB) there was no evidence in this pilot study to suggest that progesterone directly regulates cathelicidin in these cells. Oestradiol upregulated *PRA* expression in the presence of serum and Oestrodiol and LL-37 upregulated *PRA* expression in the absence of serum.

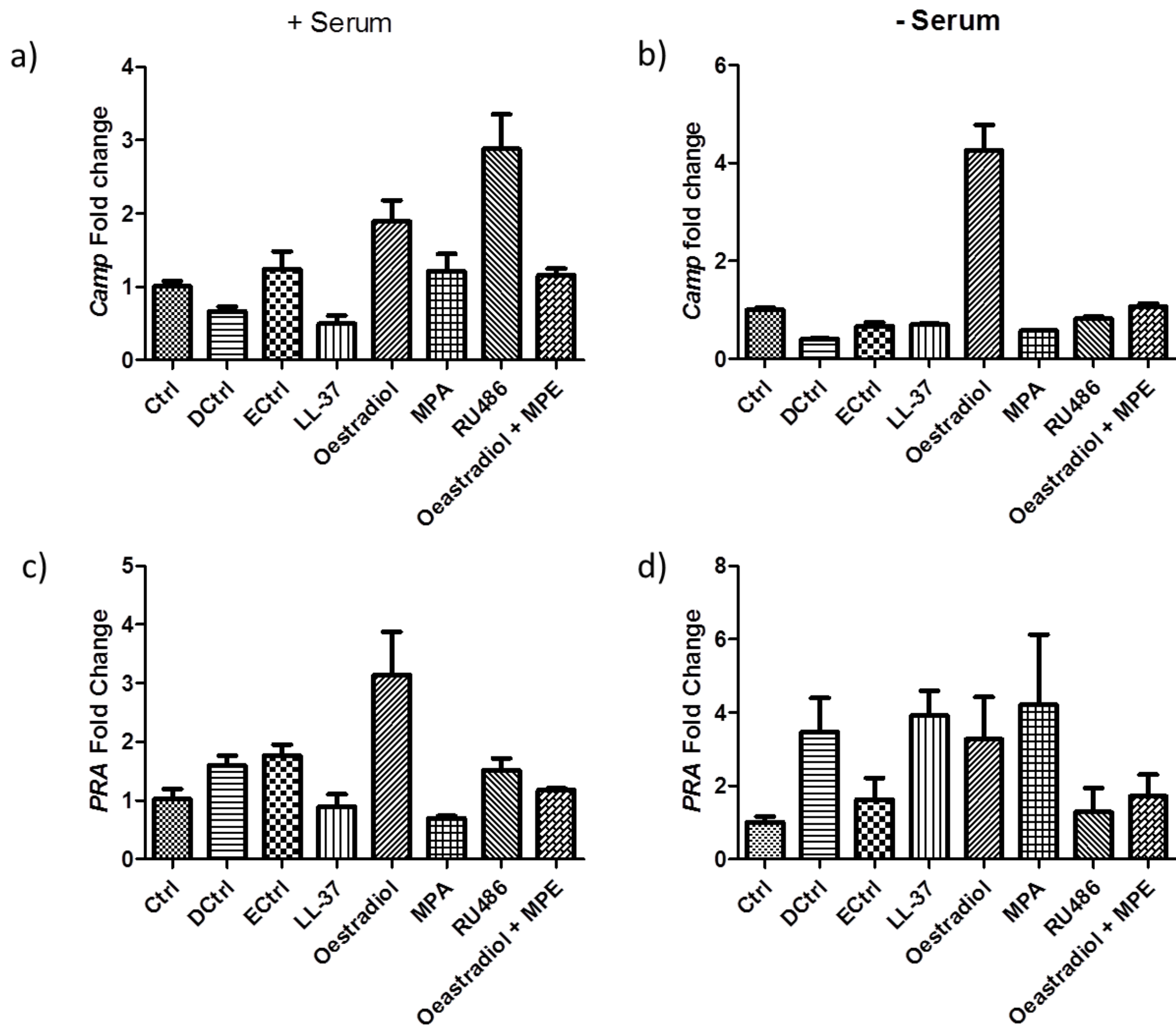

Figure 3.5 Transcription of *CAMP* and progesterone receptor following treatment with progesterone, oestradiol and RU486 EEpC were cultured in 6 well plates before treatment with LL-37 (3μg/ml), oestradiol (10^{-7}M) progesterone (MPA, 10^{-6}M), RU486 (progesterone receptor antagonist, 10^{-6}M), or progesterone and oestradiol together in the presence or absence of serum. DMF and ethanol were included as controls as they are carriers for progesterone and oestradiol respectively. a) *CAMP* transcription in EEpC in the presence of serum. n=2 b) *CAMP* expression in EEpC in the absence of serum. n=1 c) *PRA* expression in EEpC in the presence of serum. n=1 d) *PRA* expression in the absence of serum n=1. Error bars in b, c and d represent variation in technical replicates (n=3)

3.6 Cathelicidin is localised to stromal cells in decidua tissue samples but *CAMP* is not expressed in decidualised stromal cells *in vitro*

Immunohistochemistry on decidua tissue samples demonstrated hCAP-18/LL-37 colocalising with stromal cells (*Fig 3.1e*). To further investigate whether stromal cells upregulate CAMP following decidualisation I used q-PCR to investigate whether CAMP was being transcribed in stromal cells decidualised *in vitro*. Stromal cells were separated from endometrial biopsies by Dr Ann King who then cultured the cells in the presence of MPA (10^{-6}M), oestradiol (10^{-7}M) and 8-bromo-cAMP (0.1mg/ml) for 120 hours. RNA was collected from the cells every 24 hours. The cells start to show morphological changes around 72 hours and decidualisation was confirmed by IGFBP1 PCR, a marker of decidualisation. Dr King kindly gave me RNA from these cells to look for the presence of *CAMP* transcription. Controls confirmed that the q-PCR assay was working correctly but there was no *CAMP* transcription detected in these cells (data not shown).

3.7 Discussion

Antimicrobial peptides and cationic host defence peptides have been shown to be present in the female reproductive tract with particular peptides upregulated at different times during the menstrual cycle [139, 140, 143]. I wanted to identify if cathelicidin was produced in the upper female reproductive tissues and if so, identify the cellular location in endometrium and fallopian tissue. I also wanted to investigate whether *CAMP* expression also varied throughout the menstrual cycle and if so was this regulated by steroid hormones.

Immunohistochemistry for hCAP-18/LL-37 indicated expression of cathelicidin in endometrium which varied in amount across the menstrual cycle and increased upon successful implantation of the trophoblast and at the time of menstruation which is possibly due to the influx of neutrophils known to occur at this time [250]. mRNA levels detected by qPCR also indicated that expression varies during the cycle with low levels of *CAMP* expression in the proliferative phase and a trend for higher expression in decidua however

this was not a statistically significant increase. In contrast immunohistochemistry showed little change in hCAP-18/LL-37 staining from fallopian tubes taken at different stages of the menstrual cycle and *CAMP* transcription was low in cDNA from fallopian tubes with the exception of fallopian tubes from ectopic pregnancy showed 15-fold higher expression of *CAMP* than at any point in the menstrual cycle. However, in the absence of the relevant control fallopian tube samples from uterine pregnancies (ethically impossible to obtain) it is impossible to say whether this high level would be normal in pregnancy, or be a consequence of, or a causal factor in the ectopic event.

Despite our observations by immunohistochemistry that stromal expression of LL-37 occurs following decidualisation we did not see any mRNA production in stromal cells decidualised *in vitro*. This may be an anomaly due to the limitations of *in vitro* systems or alternatively further signals may be needed in addition to a rise in progesterone. For example, particular cytokines such as IL-1 or other stimuli such as human chorionic gonadotropin (hCG) in the case of decidualisation would be targets to investigate. The fact that tissues from Mirena users, which are not exposed to hCG, still show diffuse stromal staining would tend to cast doubt on the latter playing a role *in vivo*.

As Mirena tissues were taken from women undergoing hysterectomy for abnormal bleeding, the tissues samples available were all full thickness wedges. In these sections it was apparent that there were a large number of leukocytes present throughout the decidualised layer of the endometrium that stained positive for hCAP18/LL-37. This may indicate a pathological role for cathelicidin or, as they are major sources of stored hCAP-18, for neutrophils in the abnormal bleeding in these women. It could also be seen from these sections that the staining was restricted to the functional layer and that the basal glands did not stain for LL-37 protein. This was also seen in normal endometrium across the cycle wedges. With the Mirena system a high local dose of progesterone is delivered to the uterus. The decidua stained diffusely across the cells, as was seen in other decidualised stromal cells where progesterone levels were high.

A possible explanation for varying *CAMP* expression through the cycle and in decidua is that cathelicidin in the reproductive tract is regulated by steroid hormones. No effect of progesterone was seen on the production of *CAMP* mRNA in hTERT immortalised EEpCs *in vitro*. This may however exemplify the limitations of cell culture systems to reflect the

workings of *in vivo* systems. The hTERT endometrial epithelial cells that were used in these assays expressed PRA only. The two forms of progesterone receptor (A and B) are known to activate discrete genes [214]. PRA may also inhibit the effects of PRB ligation and as it is not known which form would be important in transcription of *CAMP* the outcome may have been different had PRB also been expressed. In addition, as only one time point was analysed for mRNA expression it is possible that these data are not representative. It would also be important to measure any variation in the levels of hCAP18/LL-37 peptide in the supernatant of these cells. Progesterone receptor activation of *CAMP* gene expression would likely be an indirect result of PR ligation. If PRA was having a suppressing effect then downregulation of the expression of this receptor by addition of progesterone could possibly account for the upregulation of LL-37 later in the cycle.

The passage number of the hTERT cells was high and although the original cell bank would be characterised to begin with it is difficult to say if any changes have been selected for since. T47d are breast carcinoma cells and are known to express both forms of PR. These cells would make an interesting comparison for assays investigating hormone effect. Additionally, there is known to be a great deal of crosstalk between the endometrial epithelial cells and the stromal cells which are of course absent in this cell culture system. Progesterone cannot be ruled out as a regulator as it may have an indirect effect by acting on stromal cells. Further studies could look at co-culturing these cells or use supernatant from stromal cells treated with progesterone on the EEpC instead, or in addition to progesterone.

The influence of progesterone on angiogenesis and endothelial cell proliferation is controversial [215, 216]. Two areas in which angiogenesis and cell proliferation are important are pregnancy and menstruation. In our study *CAMP* mRNA was shown to be particularly up-regulated in decidua and hCAP18/LL-37 was widespread in menstrual tissue. LL-37 has previously been shown to induce angiogenesis [35] and stimulate expansion of epithelial cells [125] which is speculated to be the reason for this peptide being over-expressed in ovarian tumours [129]. Clear evidence via immunohistochemistry in our study has shown that hCAP-18/LL-37 co-localises in endothelial cells both in the endometrium and in fallopian tube. The female reproductive tract is one of the few places in the body where new vessels are formed on a monthly cycle. Tissue remodelling occurs every cycle and with this is a need for new blood vessel formation. hCAP-18/LL-37 may have a role in promoting or regulating this

angiogenesis. Further studies could compare the hCAP-18/LL-37 protein levels in various bleeding disorders such as endometriosis.

Another explanation for the presence of cathelicidin in endothelial cells is that it is chemotactic for various leukocytes [81-83]. As the expression in endothelial cells could not be correlated to any cycle stage where large numbers of leukocytes are seen it is unlikely that this is the role. No mRNA could be detected in the RNA from HUMEC however this may be an inducible event and without knowing how first to treat these endothelial cells it is conceivable that gene transcription was not at this time in effect. An alternate explanation for detecting cathelicidin localising with endothelial cells by immunohistochemistry without detectable *CAMP* transcription is that cathelicidin has been shown to be released from neutrophils and transported across endothelium where it acts to promote adhesion of rolling leukocytes [248]. The peptide may then be detected with the endothelial cells without these cells being the source of the peptide.

A limitation of this study is that no significance could be shown in much of the cell data as experiments were only performed once for each set of conditions. To confirm and corroborate all findings, assays should be repeated at least three times (n=3). The biological component of the assay where cells are treated and the RNA extracted and used to produce complimentary DNA for qPCR measurement can vary and should be repeated on multiple occasions to minimise spurious results. Further, observations would benefit from clarification as to whether detectable protein is of the hCAP18 form or of the cleaved, functional LL-37 form as the protocols used for histological staining as well as protein detection by ELISA does not distinguish between the two.

This study was observational and aimed to detect the presence and cellular location of cathelicidin in the female upper reproductive tract. Although the presence of a protein in a tissue does not give information about whether the protein is causal to a particular physiological or pathological role, a differential expression in the amount or location of protein synthesised identifies targets on which to base further investigation. This study shows that *CAMP* expression in the female reproductive tract may be cyclical and may be relevant in menstruation and in reproductive tract pathologies. Investigation of hCAP18/LL-37 presence and function in the human reproductive tract is both an interesting and potentially important area that deserves attention and clarification.

Chapter 4: Expression of hCAP18/LL-37 in the lung in health and disease

4.1 Introduction

In healthy airways the amount of cathelicidin that can be measured is barely detectable. During inflammation or infection however, epithelial cells, alveolar macrophages and infiltrating neutrophils, all contribute to the production of local hCAP18/LL-37. The amount that can be quantified from bronchoalveolar lavage fluid (BALF), BAL cells or sputum (spontaneous or induced) is hugely variable in different lung pathologies despite serum levels of LL-37 generally remaining constant [32, 33, 153, 217-219].

In many inflammatory diseases in the lung, especially those that involve bacterial colonisation, hCAP18/LL-37 is raised. Bronchiolitis Obliterans syndrome (BOS) is an inflammatory state that occurs in the airways following lung transplantation. Although the pathologies necessitating a lung transplant vary, the levels of hCAP18/LL-37 in BALF is significantly higher in BOS patients irrespective of their level of neutrophil influx or the presence of pathogens when compared to patients who were stable following transplantation (10ng/ml vs. 0.4 ng/ml median values in the acellular portion of BALF) [33].

The concentration of hCAP18/LL-37 is notably increased in patients with cystic fibrosis and can be as high as 15 μg/ml BALF in patients where levels are found to correlate with neutrophil numbers as well as the extent of deterioration of lung function irrespective of the presence of bacteria [32]. Other studies measured induced sputum in CF patients with mild disease at closer to 80 ng/ml and 190 ng/ml in BALF [153] which was comparable to the levels they found in Chronic Obstructive Pulmonary Disease (COPD) patients both of which were significantly higher than that of healthy volunteers. Some of the discrepancies may lie in the methods used to measure the peptide and extent to which values reflect the concentration in the BALF itself or are extrapolated back to the estimated concentration in the airway surface liquid.

Further studiescompared induced sputum levels of hCAP18/LL-37 between non-smokers and smokers (with and without COPD) finding the same pattern of increased hCAP18/LL-37 with smoking and significantly greater increases with worsened lung disease. The authors report sputum levels in the range of 0.74ng/ml for non-smokers and 7.99ng/ml for severe COPD [217].

Other inflammatory conditions in the lung have surprisingly been shown to correlate with deficient cathelicidin production. Sarcoidosis is a poorly understood granulomatous condition, which affects the lung as well as other organs. Cathelicidin is decreased in severe sarcoidosis patients both at the transcriptional and protein level where it was undetectable in BAL [218], as well as in asthma patients who had a reduction in the level of cathelicidin in induced sputum as compared to healthy controls [153, 219]. Allergen exposure does however increase hCAP18/LL-37 in the lung of those with allergic asthma and allergic rhinitis. Those challenged with allergen increased to as much as 106 ng/ml BAL compared to a matched group who were given a saline control who did not produce more than 4.1ng/ml [219].

Given the variation in expression of the peptide in several documented lung diseases as well as the variable methods employed to estimate cathelicidin levels in the airways, I investigated, by ELISA, the expression of cathelicidin in patients with bronchiectasis, a chronic condition characterised by build-up of mucus in the airways with a propensity to infection and also in lung cancer patients before and after surgery.

4.2 Expression of hCAP18 / LL-37 in bronchiectasis

Sputum samples from Dr James Chalmers (University of Edinburgh) bronchiectasis clinic were supplied along with data he had collected on other parameters of clinical disease and inflammatory markers as measured according to appropriate ethical approval and consents (*Table 4.1*). hCAP18/LL-37 was quantified in each sample using a commercially available ELISA kit (*Fig 4.1*).

Wide variation in measurements of clinical severity and other inflammatory markers and infectious agents was seen in these patients. The amount of measureable cathelicidin also showed wide variation which ranged from undetectable levels up to 6045 ng/ml (median value 180ng/ml, IQR = 1067ng/ml).

There was no correlation found between concentrations of hCAP18/ LL-37 and the neutrophil chemokine IL-8 in the sputum samples (Pearson $r = 0.15$, $p = 0.41$), nor was there a correlation between LL-37 and bacterial load (Pearson $r = 0.09$, $p = 0.6$). There was a weak but significant correlation between LL-37 and the pro-inflammatory TNF (Pearson $r = 0.50$, $p = 0.04$) and between LL-37 and myeloperoxidase (MPO), an enzyme produced by neutrophils often measured as a proxy for neutrophil numbers (Pearson $r = 0.40$, $p = 0.001$). There was also a correlation between LL-37 and chronic colonisation with *Pseudomonas aeruginosa* (Pearson $r = 0.34$, $p = 0.04$).

A difficulty in drawing conclusions or correlations between the amounts of cathelicidin in our study of lung disease was that at the time of this work the only commercially available ELISA did not differentiate between the pro-peptide hCAP18 and the active fragment LL-37. Additionally the source of cathelicidin could not be ascertained (epithelial or neutrophilic) and concentrations are subject to sample variations in sputum production. In concordance with other observations we found that there are greater levels of cathelicidin when other markers of inflammatory lung disease or infection are also high.

Patient characteristic	Proportion % (total n=38)
Age	
21-54	18
55-69	45
>70	37
Female	53
Pseudomonas colonised	42
FEV1/FVC ratio	
<40 % (severe)	11
40-59 % (Moderate)	21
60-80 % (Mild)	52
>80 %	16
Number of hospitalisations in 1 yr.	
0	61
1-3	32
>3	7

Table 2.1 Characteristics of patients with bronchiectasis

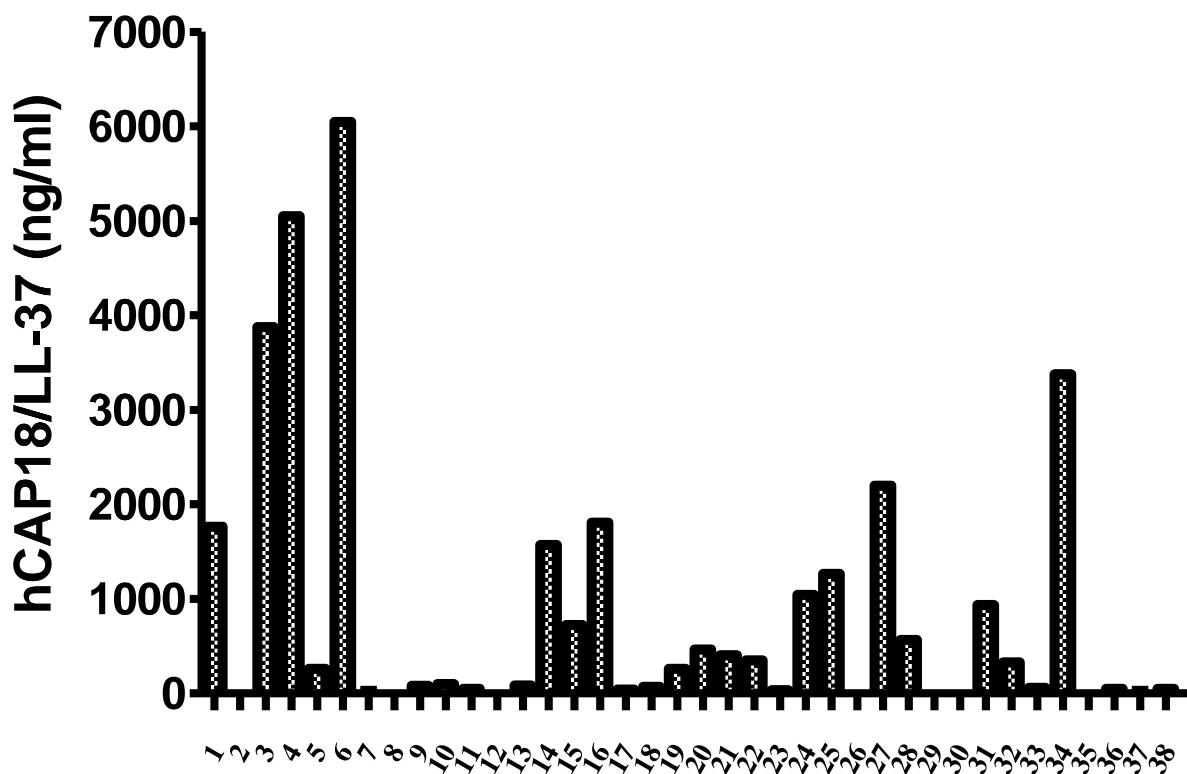

Figure 4.1 Cathelicidin in sputum varies widely in patients with bronchiectasis. hCAP18/LL-37 was measured in sputum samples from bronchiectasis patients using a commercially available ELISA kit. X axis shows individual patients (n=38). hCAP18/LL-37 values ranged from 0-6045ng/ml, median value of 180ng/ml

4.3 Expression of hCAP18 / LL-37 in individuals with lung cancer

Paired BALF samples taken from patients with lung cancer prior to and following surgery were supplied by Mr Richard Jones (University of Edinburgh) as well as serum samples from these same patients taken prior to surgery. hCAP18/LL-37 was quantified in each sample using a commercially available ELISA kit.

Considerably lower levels of cathelicidin were measured within the airways of lung cancer patients than in the bronchiectasis sputum (0-2.4ng with a median of 0.13ng/ml compared to 0-6045ng/ml with a median of 180ng). Results for a Wilcoxon signed rank 1-tailed test indicates that there is a significant difference pre and post-surgery with the amount of LL-37 being greater after surgery (P = 0.041), however as many of these values are close or below the limit of detection of the assay no conclusions can be drawn from this difference (*Fig 4.2a*).

Studies investigating cathelicidin expression during lung disease often note no difference in the serum levels of hCAP18/LL-37 between patients and healthy controls, even where differences are observed in the lung. The data in these samples indicate variation in systemic expression of hCAP18/LL-37 that may be detected in the serum of the lung cancer patients prior to surgery (*Fig 4.2b*). Levels ranged from 0 ng/ml - 31.98 ng/ml.

At the time of this work, commercially available ELISA kits did not distinguish between the pro-form of cathelicidin and the active, cleaved shorter peptide LL-37. In order to examine whether total hCAP18 levels in the serum may be misleading and may not reflect the levels of cleaved active LL-37, a selection of 8 of the BALF samples from patients who measured higher levels by ELISA were also investigated by western blotting to identify whether the samples contained both pro-form and mature peptide (*fig 4.2c*). Generally the more hCAP18 there was in a sample then the more LL-37 could be detected however the relationship is not this straightforward, cathelicidin is stored as hCAP18 and the proteases required for cleavage of mature peptide must also be present in the surrounding milieu for active peptide to be released. In this example of non-infectious, non-allergic lung disease the level of local cathelicidin is much lower than can be found in other lung disease and do not correlate with serum concentrations of cathelicidin.

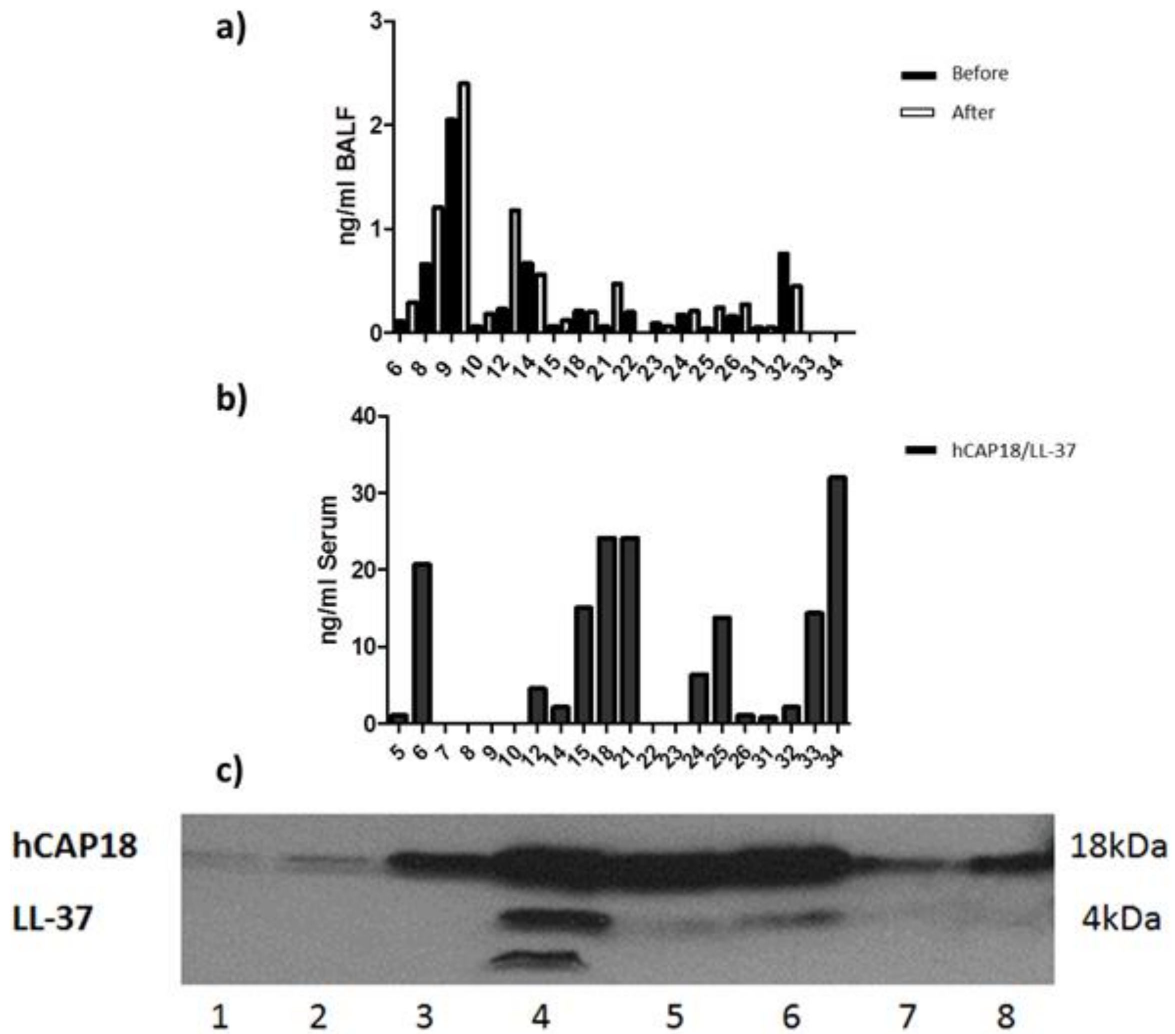

Figure 4.2 Cathelicidin in BALF and Serum from Lung Cancer Patients: hCAP18/LL-37 was measured by ELISA in a) BALF samples from lung cancer patients before and after surgery and b) serum samples from lung cancer patients prior to undergoing surgery were evaluated using a) & b) a commercially available ELISA kit. hCAP18/LL-37 values in BALF ranged from 0-2.4ng/ml. hCAP18/LL-37 values in serum ranged from 0-31.98ng/ml and c) by western blot using 8 BALF samples which were indicated to contain higher levels of cathelicidin by ELISA measurement. Proteins in BALF were separated by gel electrophoresis and probed with an anti-LL-37 antibody to identify the ratio of pro-peptide (hCAP18) and active, cleaved form (LL-37).

4.4 Discussion

Several groups have tried to identify the relative levels of cathelicidin expression in particular lung diseases [32, 33, 153, 217-219]. I was interested in looking at local production of cathelicidin in patients visiting a clinic for bronchiectasis (lung disease where abnormal widening of the bronchi and excess mucus production leave those affected prone to infection-related exacerbations) and lung cancer patients (a non-infectious, non-allergic condition) before and after undergoing surgical treatment. I was kindly permitted to measure, by ELISA and western blot, the amount of hCAP-18/ LL-37 in sputum samples from these two groups of patients.

Bronchiectasis is a lung disease that may be caused by a variety of factors and thus results in a range of severity of clinical symptoms depending on the causative agent [251]. I found that the amount of cathelicidin present in sputum from these patients also varied widely but correlated with markers of inflammation such as TNF alpha and neutrophil influx. Chronic colonisation with Pseudomonas also correlated with higher levels of cathelicidin. Studies of other inflammatory disease have reported levels of cathelicidin in the ng range [33,153] here I have found levels in bronchiectasis patients to range from undetectable to approximately 6μg/ml which is closer to the levels found in CF patients [32].Although hCAP-18/LL-37 is raised in response to infection where it has a beneficial effect in fighting infection, it would not be unreasonable to propose that chronic inflammation and dysregulated production of LL-37 may have a role in the pathogenesis of lung disease as well.

Some malignant cells have been shown to over express cathelicidin. I also measured the amount of cathelicidin in serum and sputum from patients undergoing surgery for lung cancer and found that the levels of cathelicidin were low, albeit similar to those reported in other non-infectious lung disease [33].

It is extremely difficult to compare studies investigating cathelicidin expression in the airways during lung disease, as there are many confounding factors when obtaining peptide concentrations in BALF and sputum. Determining the dilution factor that is to be used to accurately represent concentrations found in the airways, at cell surfaces and within mucous membranes is highly challenging and controversial and no attempt to correct was made in my study. In addition, the amount of saline recovered by lavage may be affected by the extent of

lung consolidation and fibrosis as well as the skill of the clinician performing the procedure and the extent to which charged peptides are effectively sampled from the airways is debated. Further, there are considerations when processing and storing samples to inhibit the work of proteases from degrading proteins of interest *ex vivo*.

That said, it is clear that the amount of cathelicidin expressed in airways is rapidly increased following infection such as in the case of CF and bronchiectasis, inflammation or injury to the lungs other than in conditions where immune modulation is a component such as allergy, asthma and sarcoidosis. What is less clear is what proportions of the amounts found are hCAP18 and what is the active LL-37 form, as commercial ELISAs do not distinguish between the two. As shown in my western blot data from the lung cancer patients, the relationship between the amount of propeptide and mature peptide is not straight forward and some patients whose sputum contained large amounts of hCAP18 did not also contain large amount of LL-37.

Interestingly, although many studies show comparable levels of serum cathelicidin between healthy volunteers and diseased states the data here suggest that there is a degree of variation in the serum from lung cancer patients which may warrant further investigation as LL-37 has been proposed to be a growth factor for malignant cells [220].

Physiological levels of cathelicidin in the lung are therefore difficult to define, though BAL levels are often within the ng range even in diseased states. Minimum inhibitory concentrations (MIC) for LL-37 against microbes *in vitro* are often greatly in excess of 10 μg/ml [5, 45, 46], which is much higher than the levels detected in *in vivo* studies. Additionally, the activity of cathelicidin is tightly regulated in the body with a host of factors that inhibit the activity of the peptide (see chapter 1). The question therefore arises as to how cathelicidins function as an antimicrobial agent *in vivo*. While antimicrobial effects might be mediated through direct microbicidal properties at sites of localised high peptide concentrations, or through synergy with other antimicrobial agents, perhaps the most important functions are indirect immunomodulatory effects.

Chapter 5: LL-37-induced cell death in infected airway epithelium

5.1 Introduction

Epithelium provides a physical barrier to microorganisms entering the body via the respiratory tract and, as previously discussed, in addition to the barrier function there are a host of other defence mechanisms employed by the epithelium to remove foreign particles and potential pathogens from this part of the body. In individuals in whom these mechanisms are impaired or absent, such as those with CF (in whom the mucocilliary escalator does not function correctly to remove bacteria), chronic infections may become established.

Pseudomonas aeruginosa is one such organism that causes opportunistic infections in those with immunocompromised airways. With increasing resistance to antibiotics demonstrated by this bacterium, and others, it is important to understand alternative means by which the epithelia can destroy these pathogens in order to augment these natural mechanisms with new therapeutics.

Work performed by Dr Peter Barlow, with my assistance, in the Davidson lab demonstrated that primary and transformed cultured epithelial cells underwent rapid Bax-dependent, but caspase-independent cell death, when incubated with high concentrations of synthetic LL-37 peptide alone (with an associated translocation of cytochrome C), but that at more physiologically relevant levels of LL-37 (at which peptide alone did not induce substantial cell death), this peptide induced epithelial cell death associated with activation of caspase-3 and -9 exclusively in cells infected with a lab strain of *P. aeruginosa* (PA01) (*Fig 5.1).*

In addition to the initial observation that LL-37 synergistically initiated cell death in the presence of PA01, Peter had also determined that this phenomenon was dependent upon whole, live bacteria (and could not be replicated using LPS, killed bacteria or bacterially-conditioned media). As part of these studies, I investigated whether other strains of *P. aeruginosa* would result in the same effect, if the expression of common bacterial virulence factors were important for cell death to occur, and examined a possible role for the inflammasome.

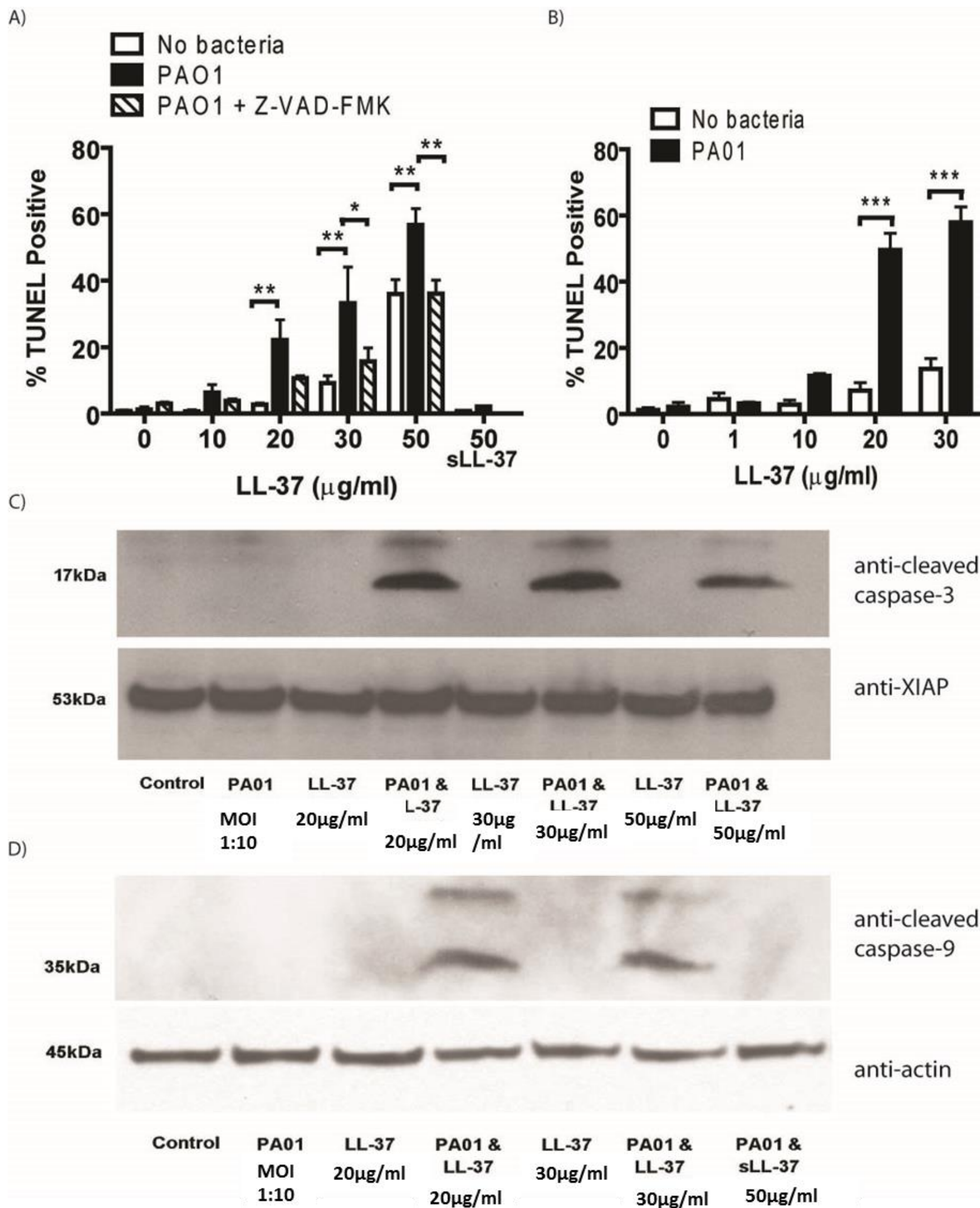

Figure 5.1 LL-37 and *P. aeruginosa* synergistically induce DNA fragmentation and caspase activation in airway epithelial cells. Human bronchial epithelial cell line 16HBE14o− (*A*, *C*, *D*) or primary human bronchial epithelial cells (*B*) were incubated for 6 hours (*A*, *B*) or 5 hours (*C*, *D*) over a range of LL-37 concentrations (or scrambled LL-37 [*sLL-37*] at 50 μg/ml) in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1) added concurrently. (*A*, *B*) Cells were treated as described, with or without preincubation for 1 hour with the polycaspase inhibitor Z-VAD-FMK (50 μM), and were then fixed. Apoptosis was assessed by TUNEL assay. Data represent mean values ± SEM, for $n \geq 3$ independent experiments for each condition. Two-way ANOVA with Bonferroni *post hoc* test was used to compare samples. $*P \leq 0.05$, $**P \leq 0.01$. (*C*, *D*) Whole-cell protein lysates were prepared and analyzed by SDS-PAGE and Western immunoblotting. Immunoblots were performed using antibodies specific for cleaved caspase-3, XIAP, cleaved caspase-9, or actin. Images shown are representative of $n \geq 3$ independent experiments.

5.2 Characterising the bacterial factors that affect synergism between LL-37 exposure and infection.

5.2.1 Synergistic induction of epithelial cell death by LL-37 and *P. aeruginosa* is independent of a type III secretion system or pilus expression.

Type IV pili may be found in varying numbers on the outer surface of *P. aeruginosa*. They are hair-like structures composed of pilin proteins and are important adhesins that the bacterium uses to interact with host cells. Type III secretion systems (T3SS) are needle like structures produced by some bacteria in order to transport bacterial proteins from the bacterial cytoplasm directly into host cells. Both pili and T3SS are important virulence factors in some Pseudomonal infections. To determine whether the synergistic induction of cell death observed was influenced by pili or T3SS, I utilised mutant strains of *P. aeruginosa* (*Table 5.1*) lacking these particular virulence factors and their isogenic parent strains as controls, using the mitocapture dye assay (which measures mitochondrial membrane depolarisation; an early event in the initiation of apoptosis) and the TUNEL assay (which labels the DNA nicks that occur during DNA fragmentation; a later event in some forms of cell death) to further characterise the bacterial factors involved in this phenomenon.

Using the mitocapture assay it was evident that the absence of either of these virulence factors did not prevent rapid LL-37 induced cell death when cells were cultured concurrently with LL-37 peptide and either the T3SS mutant (*Fig 5.2a*) or with the pilin mutant (*Fig 5.2b*) as compared to the isogenic controls.

Strain	Origin/Description	Gifted by	Reference
PA01	Standard Laboratory strain	John Govan	[205]
J1386	Clinical isolate from a CF patient	John Govan	[206]
Δ*mexAB-oprM*	Efflux pump mutant	Keith Poole	[207]
PA01exsA	T3SS mutant	Dara Frank	[208]
PA01pil	Pilus mutant	Bob Hancock	[209]

Table 5.1 Strains of *P. aeruginosa* used to investigate bacterial factors involved in LL-37 induced death of infected epithelial cells. Experiments were all conducted using the isogenic parent strain as a control. Bacterial cultures were received with thanks and stored at -20°C until required for culture. Working stocks were kept on agar on the bench and monitored for conversion to mucoidy.

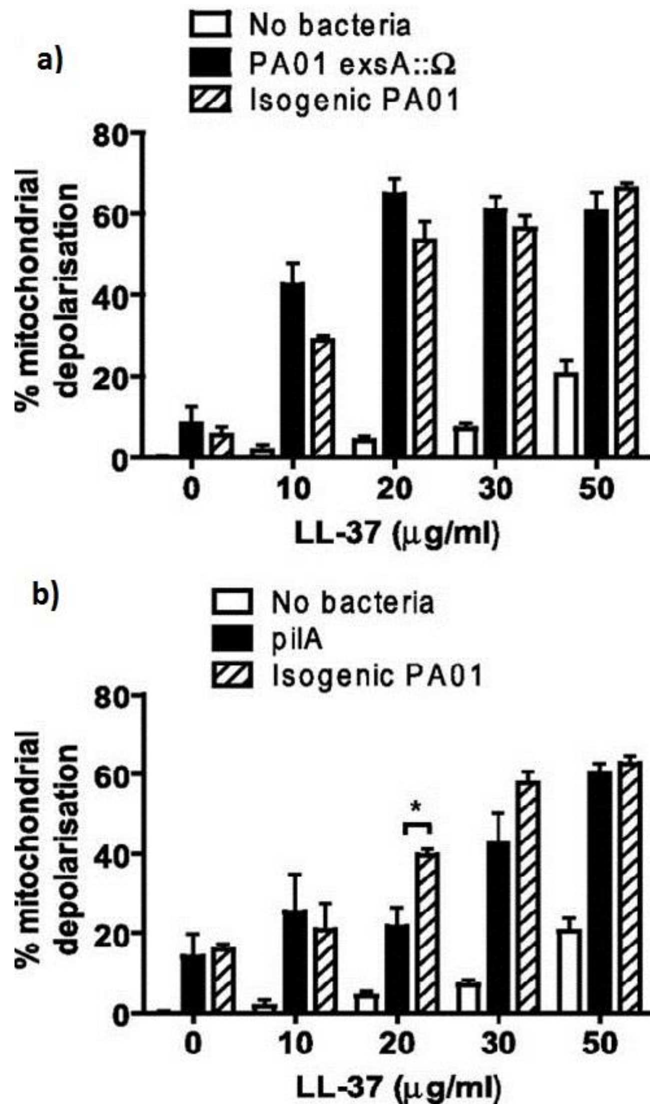

Figure 5.2: Synergistic induction of cell death by LL-37 and *P. aeruginosa* is isolate-specific and independent of type III secretion system and pilus expression. Human bronchial epithelial cells (16HBE14o−) were assessed for mitochondrial membrane depolarization using Mitocapture dye after incubation for 1 hour with a range of concentrations of LL-37, in the presence and absence of (*A*) log-phase *P. aeruginosa* PA01exsA::Ω or isogenic PAO1 control strain (MOI 10:1), and (*B*) log-phase *pilA P. aeruginosa* mutant or isogenic PAO1 control strain (MOI 10:1). Data represent mean values ± SEM, for n = 3 independent experiments for each condition. Two-way ANOVAs were performed to evaluate significance, with Bonferroni *post hoc* tests comparing (*A*) LL-37/*P. aeruginosa* to LL-37 alone, and (*B*) LL-37/*P. aeruginosa* mutant to LL-37/isogenic controls. *$P \leq 0.05$,***$P \leq 0.001$.

Studies conducted together with Peter Barlow.

5.2.2 Synergistic Induction of Cell Death by LL-37 and *P. aeruginosa* requires Epithelial Cell Internalization of Bacteria

Epithelial cells infected with *P. aeruginosa* PA01 in the presence of LL-37 results in an increase in cell death at concentrations of LL-37 where neither bacteria alone or LL-37 alone had any effect. The efflux mutant ΔMexAB-OprM [207], is reported to display delayed invasion into epithelial cells. This was confirmed using a gentamicin exclusion assay to measure internalized bacteria (*Fig 5.3a*).

When airway epithelial cells were infected with this mutant ΔMexAB-OprM and incubated with LL-37 there was a significant failure to induce the mitochondrial depolarisation in 16HBE14o$^-$ cells (*Fig 5.3b*) as compared to the parent strain. A TUNEL assay was used to evaluate the impact upon DNA fragmentation and also demonstrated enhanced significant difference between the mutant and the parent strain, with a loss of the peptide-induced cell death in 16HBE14o$^-$ cells under these conditions (*Fig 5.4*). A previous report [207] demonstrated that an unidentified secreted factor released by wild type PAO1 upon interaction with epithelial cells was not secreted by ΔMexAB-OprM and that supernatant from wild type PAO1 infected epithelial cell cultures could restore the ability of the mutant to invade /internalise into epithelial cells. Interestingly, replicating this approach in my studies, the addition of filtered supernatant from PA01-infected epithelial cultures with the ΔMexAB-OprM mutant strain and LL-37 restored the synergistic induction of cell death (*Fig 5.3c*).

The observation that of all the PA01 mutants investigated only ΔMexAB-OprM failed to induce cell death suggests invasion of the epithelial cell by the bacterium is required to promote LL-37-induced cell death of PAO1 infected cells.

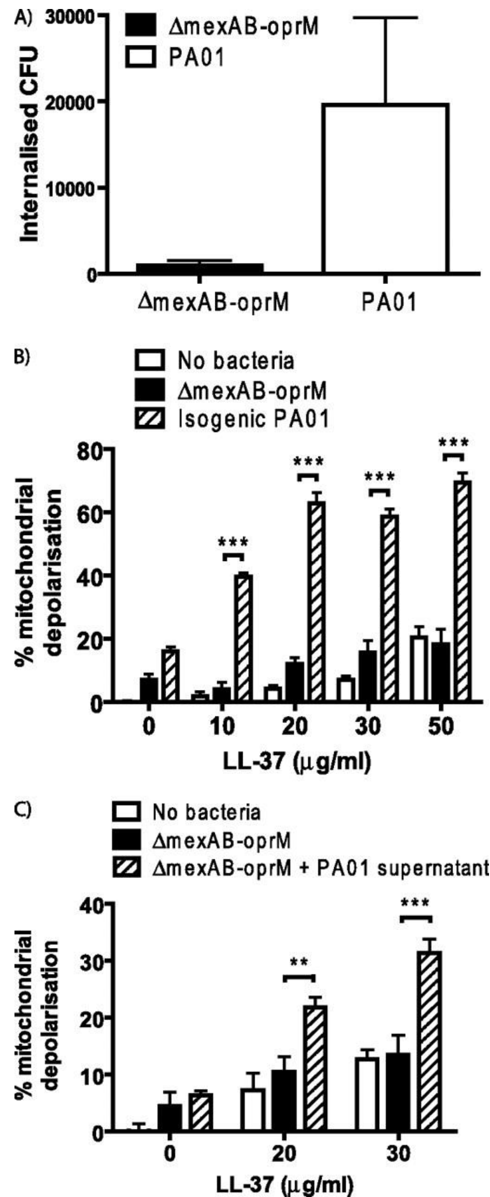

Figure 5.3. Synergistic induction of cell death by LL-37 and *P. aeruginosa* requires epithelial cell internalization of bacteria. Human bronchial epithelial cells (16HBE14o−) were incubated for 60 minutes in the presence and absence of (MOI 10:1) log-phase *P. aeruginosa* strains PA01, Δ*mexAB-oprM* mutant (*A*–*C*), isogenic PAO1 control strain (*B*), or Δ*mexAB-oprM* mutant added concurrently with sterile conditioned supernatant collected from 16HBE14o− cells infected with PA01 (*C*). (*A*) Invasion of epithelial cells by bacteria was determined by gentamicin exclusion, quantifying the number of viable CFUs surviving extracellular gentamicin treatment (50μg/ml). Data are plotted as mean values ± SEM, for n = 3 independent experiments plated in duplicate for each condition. (*B*, *C*) Infected epithelial cells were concurrently incubated with a range of concentrations of LL-37, and mitochondrial membrane depolarization was determined. Data represent mean values ± SEM, for n = 3 independent experiments for each condition. Two-way ANOVAs were performed to evaluate significance, with Bonferroni *post hoc* tests $^{**}P \le 0.01$, $^{***}P \le 0.001$. Studies conducted together with Peter Barlow.

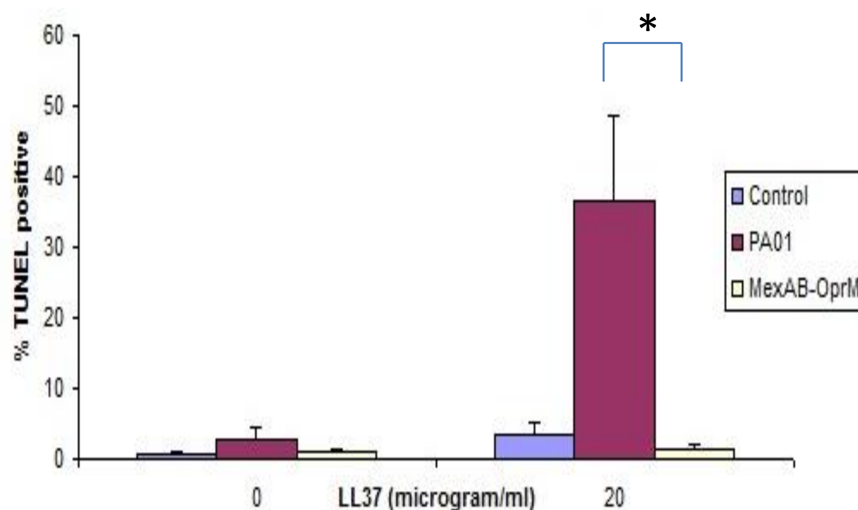

Figure 5.4 ΔMexAB-OprM fails to induce cell death in 16HBE14o⁻ cells with or without LL-37 after 6 hours as measured by TUNEL. Human bronchial epithelial cells (16HBE14o−) were incubated for 60 minutes in the presence and absence of (MOI 10:1) log-phase *P. aeruginosa* strains PA01, Δ*mexAB-oprM* mutant or the isogenic PAO1 control strain. Infected epithelial cells were concurrently incubated with culture media only or 20µg/ml of LL-37 in culture media before being assessed for cell death using the TUNEL assay. Results are means of n=3 independent experiments. Data is plotted as mean values +/- SEM * indicates P value of <0.05

5.2.3 LL-37 does not kill *P. aeruginosa* in Physiologically Relevant Media nor is the ΔMexAB-OprM Mutant more Susceptible to Direct Killing by this Peptide.

LL-37 has been reported to have direct antimicrobial killing activity [67] However, many studies were in non-physiological ionic environments. In order to study immunomodulation by LL-37 it was necessary to examine whether direct microbicidal activity of LL-37 could explain the results we were observing.

The strains of *P. aeruginosa* used to characterise LL-37 induced death of infected epithelial cells (*Table 5.1*) were all incubated with increasing concentrations of synthetic LL-37 peptide to determine the sensitivity of each strain to direct killing by the peptide. Strains tested include; PA01 (lab strain) ΔMexAB-OprM (Efflux mutant), ExsA (T3SS mutant) and J1386 (CF clinical isolate).

When incubated with LL-37 in PBS without Ca2+ and Mg2+ there is evidence of some direct antimicrobial activity against the clinical strain of *P. aeruginosa* J1386 (*Fig 5.5a*). To determine the direct microbicidal capability of LL-37 against *P. aeruginosa* in a media with a physiologically relevant ionic composition we tested whether co-incubation with the peptide in our treatment media would reduce the number of viable bacteria. When cell culture media was used there was no direct killing of bacteria as detected by viable counts (*Fig 5.5b*). The population doubling time for PA01 is more than 60 minutes and when cultures were incubated for 2 hours in the presence of peptide there was evidence of a possible bacteriostatic effect (data not shown).

These data show that there was no bactericidal effect of LL-37 in the media used for the cell death studies. Furthermore, the inability of ΔMexAB-OprM to promote LL-37 mediated cell death was not due to an increase in sensitivity of this strain to killing by LL37.

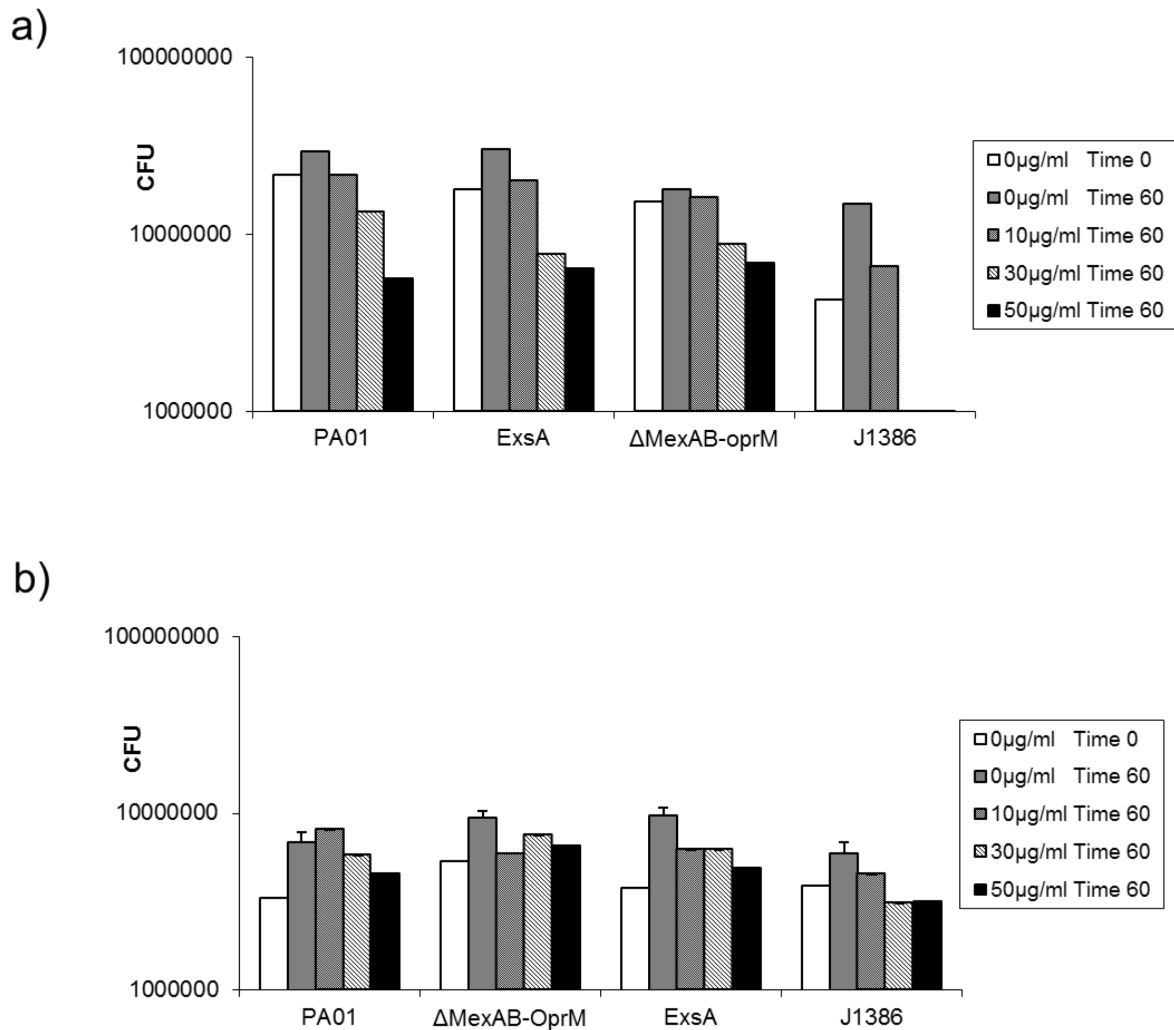

Figure 5.5 Susceptibility of *P. aeruginosa* strains to increasing concentrations of LL-37 after 1hr. Strains tested include; PA01 (lab strain) ΔMexAB-OprM (Efflux mutant), ExsA (T3SS mutant) and J1386 (CF clinical isolate) in either (a) PBS w/o Ca2+ and Mg2+. Figure shows means of 2 independent experiments or (b) physiologically relevant culture media (DMEM with 1% L-Glut 1% NEAA, ultroser G). Figure shows mean +/- SEM from 3 independent experiments per condition.

5.2.4 The Extent of Internalisation/Invasion of *P. aeruginosa* into Human Bronchial Epithelial Cells is Pseudomonas Strain Dependent.

The results of infection studies with the efflux pump mutant suggested that *P. aeruginosa* need to gain entry to the intracellular environment of the cell in order to make the cell susceptible to LL37-mediated cell death [52]. In order to determine the extent of internalisation/invasion of different strains of *P. aeruginosa*, the gentamicin exclusion assay was utilised. As this assay relies on the killing of extracellular bacteria for accurate quantification of intracellular bacteria it was first necessary to determine at what concentrations this would occur for these strains. Following incubation of bacteria with a range of concentrations of the antibiotic it was determined that 50 µg/ml gentamicin was sufficient to kill all strains of *P. aeruginosa* tested (data not shown). Controls for this assay were performed to exclude the possibility that any residual gentamicin-mediated effects occurred following washing and lysis steps (data not shown). PA01 was clearly shown to be invasive to the 16HBE14o^{-} cells at one hour (*Fig 5.3*) and PA01 and J1386 at two hours (*Fig 5.6*) after infection. ΔMexAB-OprM was shown to have negligible ability to invade the epithelial cells (*Fig 5.3 & 5.6*).

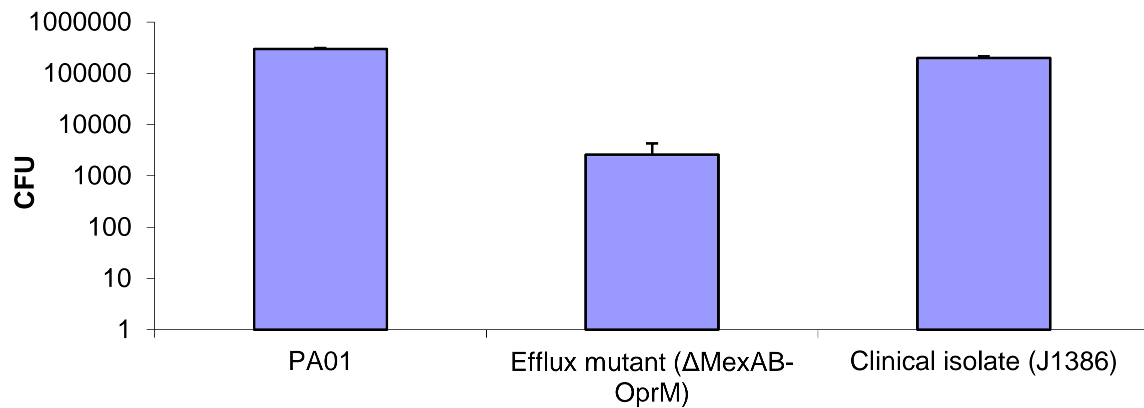

Figure 5.6: The efflux mutant of *P. aeruginosa* does not become intracellular Gentamicin exclusion assay results quantifying the number of colony forming units inside epithelial cells after 2 hours of infection with *P. aeruginosa* (PA01, efflux mutant ΔMexAB-OprM or the clinical strain J1386) and 1 hour of gentamicin exposure.

5.2.5 *P. aeruginosa* Clinical isolate (J1386) Induced Cell Death with or without LL37.

In order to determine the extent to which the observations made with PAO1 were representative of other strains of *P. aeruginosa*, a number of clinical isolates were examined in the same assay. The clinical strain J1386, an extremely motile, early isolate recovered from a child with cystic fibrosis showed a high level of cell death irrespective of the presence of LL-37 (*Fig 5.7 a & b*).

Intracellular numbers of J1386 were also investigated using the gentamicin exclusion assay. This isolate was shown to have a lower number of CFUs inside cells than the laboratory strain PA01 (*Fig 5.7c*). However, an apparent loss of confluency was observed visually in wells infected with J1386 and thus a loss of epithelial cells was presumed in wells containing J1386 following wash steps. It is possible that if this strain is directly cytotoxic then internal numbers were underestimated due to cell loss prior to counting. The possible loss of cell and the extent of the loss would need to be confirmed formally via nucleocounter and/or a haemocytometer cell count.

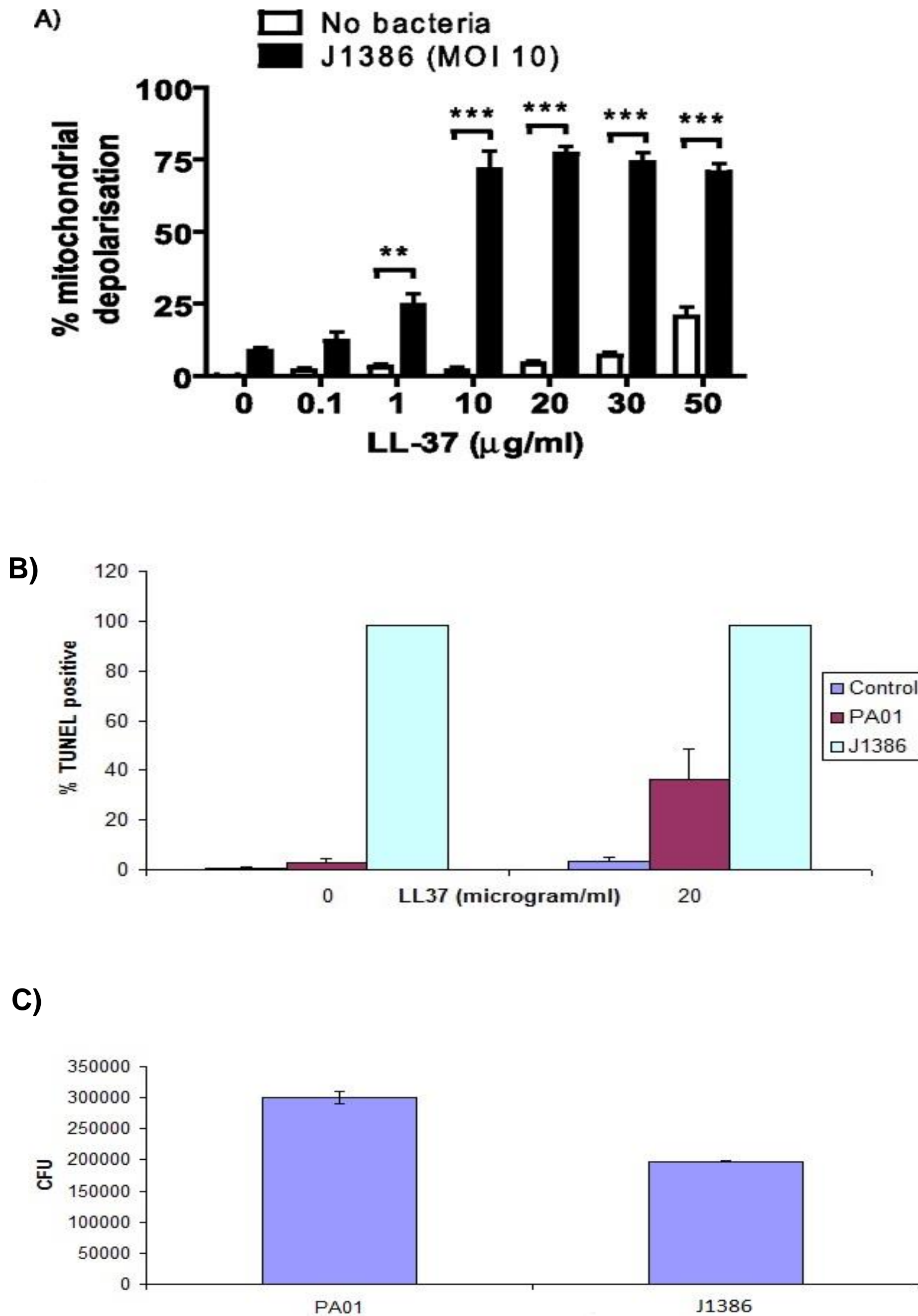

Figure 5.7 ***P. aeruginosa*** **Clinical strain J1386 induces a high proportion of cell death with or without the presence of LL-37.** 16HBE cells were incubated with *P. aeruginosa* clinical strain J1386 with or without increasing doses of LL-37 peptide. Cell death was assessed by a) Mitocapture assay and b) TUNEL staining C) Gentamicin exclusion assay results quantifying the number of colony forming units inside epithelial cells after 2 hrs of infection with *P. aeruginosa* (PA01 or J1386) and 1 hr gentamicin exposure.

5.3 The role of Caspases and Bcl2 Proteins

5.3.1 Combined use of the pan-caspase inhibitor (Z-VAD-FMK) with BAX peptide inhibitor (BipV5) reduces the proportion of TUNEL positive cells

As demonstrated in *Fig 5.1*, LL-37-induced cell death in *P. aeruginosa* infected epithelial cells is at least partially caspase dependent and may be inhibited by the poly-caspase inhibitor Z-VAD-FMK. Additional studies conducted by Peter Barlow also demonstrated that the cell death induced by higher concentrations of LL-37 in the absence of infection was mediated by the pro-apoptotic Bcl2 family member Bax [109]. Additional studies were conducted in order to evaluate the extent to which blockade of both pathways might abrogate LL-37-induced cell death.

These studies utilised BipV5 and Z-VAD-FMK (*Fig 5.8*). BipV5 is a peptide based on the Ku70 BAX inhibiting domain which prevents the translocation of Bax from the cytosol to the mitochondria, inhibiting mitochondrial induced apoptosis [252, 253]. Z-VAD-FMK is a non-specific caspase inhibitor that irreversibly binds to the active site of caspase proteases.

Although a striking reduction of TUNEL positivity was observed, this was not a complete inhibition relative to control levels. It is possible that these inhibitors may not enable total inhibition or that additional pathways may be triggered by LL-37 and *P. aeruginosa*.

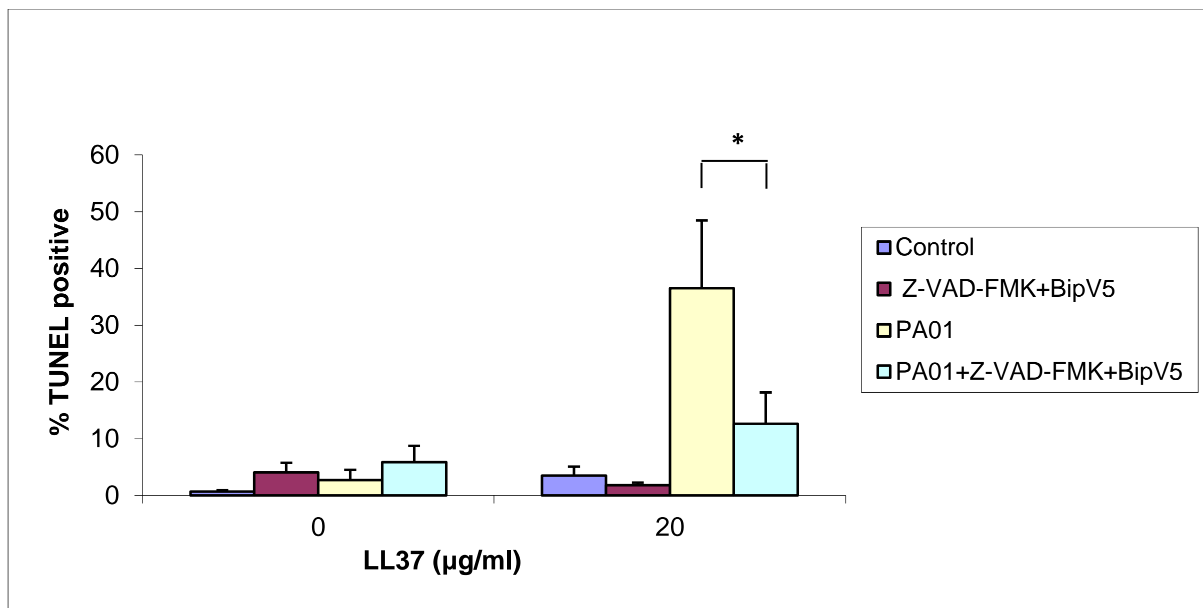

Figure 5.8: Synergistic inhibition of cell death by LL-37 is not completely abrogated by caspase and BAX inhibition. Cells were incubated with both BipV5 and Z-VAD-FMK for an hour then washed prior to treatment. Data shown are from n= 3 independent experiments. * indicates P value of <0.05

5.4 Role of Intracellular Pathogen Recognition Receptors and Inflammasome Activation by LL37

5.4.1 Synergistic induction of cell death in infected epithelia is caspase-1 dependent.

Based on the hypothesis that internalisation/invasion of epithelial cells is essential for the synergistic effect of LL-37 on cell death in infected cells, the role of intracellular pattern recognition pattern sensing was considered. Alternative types of cell death to apoptosis were considered, including the caspase-1 dependent cell death termed pyroptosis. Pyroptosis is an inflammatory form of cell death that is characterized by the activation of caspase-1 and processing and release of IL-1β and IL-18 and has been reported to be induced by *P. aeruginosa* in macrophages [248] following intracellular sensing and activation of the inflammasome.

Although our data demonstrated activation of caspase 9 and caspase 3 during LL-37-induced death of infected cells, and pyroptosis is not a caspase 3 dependent process, Cookson and colleagues also report activation of caspase 3 at later time-points in this form of cell death [180]. In addition, TUNEL positivity is observed in pyroptosis and poly-caspase inhibition (with Z-VAD-FMK) would be equally capable of inhibiting caspase-1 as the effector caspases of aopotosis. Additionally an unknown nuclease is thought to be responsible for death of cells by pyroptosis [192]. To investigate whether caspase 1 is being activated in my studies, a cell permeable caspase-1 inhibitor was used prior to treatment and cells analysed by TUNEL staining as before (*Fig 5.9*). These data demonstrate that the significant LL-37 mediated induction of cell death in PAO1 infected epithelial cells was abrogated in the presence of caspase 1 inhibition (*Fig 5.9*). This suggests that the cell death observed may be pyroptosis, induced by LL-37 and previously undescribed in epithelial cells.

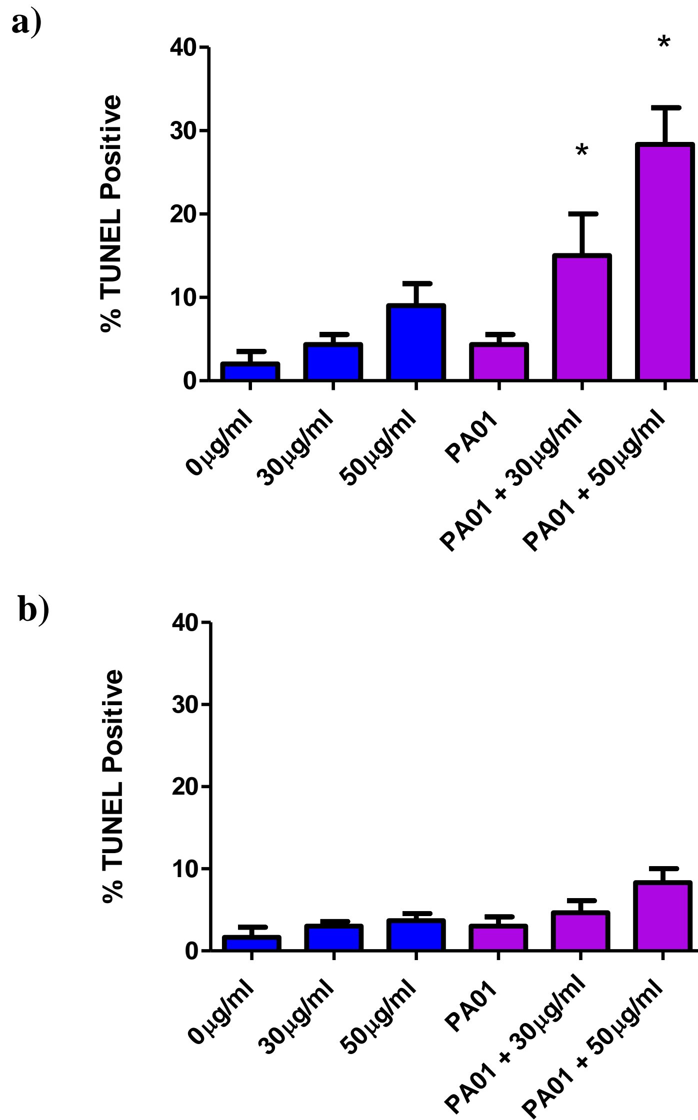

Figure 5.9 LL-37 induced death of infected epithelial cells is negated by caspase 1 inhibition. 16HBE cells were incubated with culture media only, media and LL-37 (30 or 50µ/ml), PA01 only or PA01 and LL-37 (30 or 50µg/ml). b) As for a except cells were pre-incubated with a cell permeable caspase 1 inhibitor prior to being incubated with the conditions above.

5.5 Inflammasome Activation and pyroptosis in Epithelial Cells

5.5.1 The Human Bronchial Epithelial Cell Line 16HBE as well as the cell line A549 Express Components of Multiple Inflammasome Pathways

The majority of published scientific literature describes the pathways and inducers of inflammasome formation and pyroptosis in cells of the myeloid lineage. There are however recent preliminary descriptions of cell death characteristic of pyroptosis in epithelial cells [259-260]. One study report seeing significant increases in caspase-1, caspase-11 and IL-1β following renal ischaemia-reperfusion injury with cell death, presumed to be pyroptosis in renal tubule epithelial cells [259] whilst another group have published their study showing NLRP3 dependent pyroptosis in HBE cells in response to carbon nanotubes which was partially inhibited by silencing NLRPs in the cells or by the use of a caspase 1 or cathepsin B inhibitor [260].

Studying LL-37-induced cell death of infected epithelial cells raised the question of whether the cells in our *in vitro* model were capable of forming inflammasomes. Qualitatively we could see from PCR, western and qPCR data that mRNA coding for various inflammasome components including ASC, NLRP3, CARD8 and NLR4 were all expressed by epithelial cells (*Fig 5.10*). This was true for 16HBE cells as well as the A549 epithelial cell line and also primary NHBE cells.

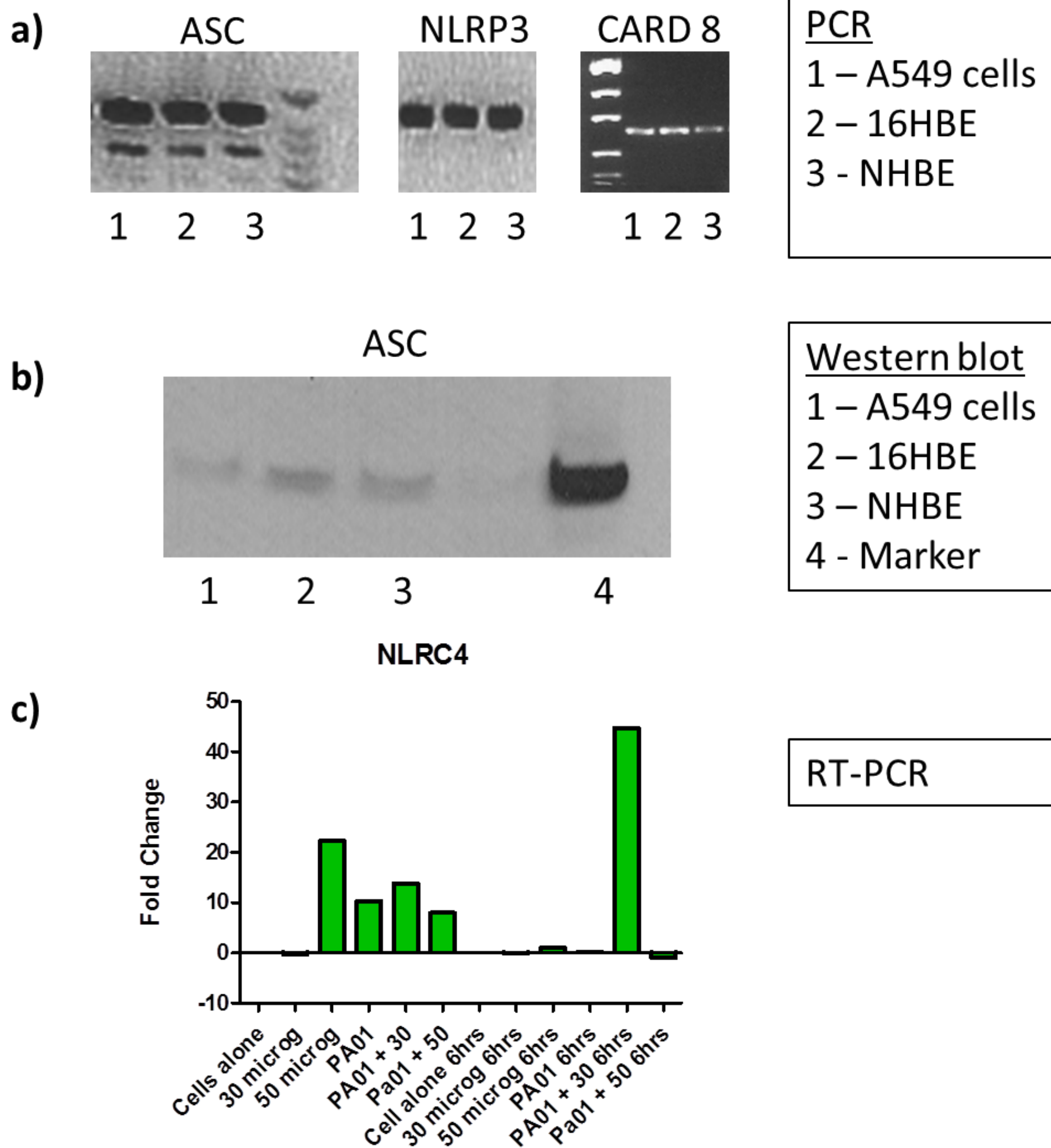

Figure 5.10 Inflammasome components are expressed in airway epithelial cells. A) PCR results indicate that airway epithelial cells contain genes for the inflammasome components ASC (apoptosis-associated speck like protein containing a CARD), NLRP3 (Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing family) and CARD8 (Caspase activation and recruitment domain 8 b) Western blot to detect the protein ASC in airway epithelial cells c) Change in expression of the gene for NLRC4 shown as fold change in 16HBE cells after 1hr or 6hrs compared to the untreated control at time 0.

5.5.2 Caspase 1 dependent cytokine IL-1β may be released by infected cells exposed to LL-37.

In order to further evaluate whether the LL-37-induced cell death observed in infected epithelial cells was caspase-1 dependent pyroptosis, cells were assessed for IL-1β, cleavage and release, which is caspase-1 dependent [255]. Initial investigation to quantify IL-1β and its precursor molecule pro-IL-1β from treated cell lysates by western immunoblot clearly showed that the proform of the cytokine was detected only when cells were infected with *P. aeruginosa* and that the cleaved form was only present if those infected cells were also exposed to LL-37 (*Fig 5.11*). This result proved difficult to replicate in subsequent experiments.

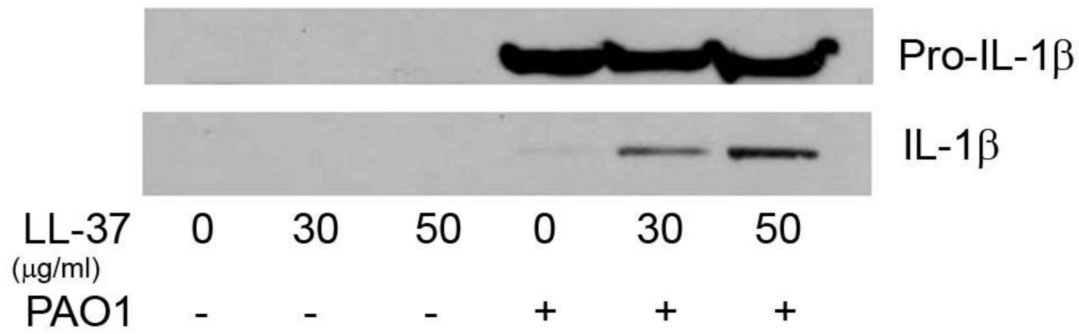

Figure 5.11 IL-1β expression in PA01-infected 16HBEs. The pro-form of IL-1β can be seen by western blot only when cells are infected with PA01. Cleaved IL-1β can be detected in increasing quantities in infected cells that were treated with increasing doses of LL-37

5.6 Discussion

Cathelicidins have clearly been shown to have protective capacity *in vivo* in the context of infection [27, 28], and yet are capable of inducing host cell death during infection at epithelial surfaces [109, 110, 145]. This may at first seem counter intuitive, unless the death of an infected epithelial cell removes a compromised cell and helps to alert the host to danger. CHDPs such as cathelicidin have been proposed for use as novel antimicrobial therapeutics due to their microbicidal properties at high concentrations. Thus, understanding the capability of cathelicidins to induce cell death and the regulation of such properties is relevant to both the understanding of host defence against infection as well the development of novel therapeutics based on these peptide structures. My studies, and those of Peter Barlow in the Davidson lab have demonstrated that low levels of LL-37, which do not cause cell death on their own, are capable of inducing a rapid death of *Pseudomonas aeruginosa* infected epithelial cells [52]. I propose this to be a novel component of the normal defence mechanisms of the lung to avoid colonisation in the airways early in infection when bacterial numbers are low.

The airway epithelial cell line 16HBE14o^{-}, infected with *P. aeruginosa* (PA01) and treated with LL-37, underwent rapid cell death in response to concentrations of peptide that did not induce death in healthy cells [52]. This suggests a synergistic induction of cell death pathways between the peptide and the bacterium. The intrinsic pathway of apoptoss is characterised by permeabilistion of mitochondria with subsequent release of cytochrome C and caspase activation. The synergistic cell death I observed with LL-37 and *P. aeruginosa* was characterised by mitochondrial depolarisation, release of cytochrome C from the mitochondria to the cytoplasm and an increase in terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick-end labelling (TUNEL) positive cells. Cleaved caspase-3 and -9 was observed in these cells by western blotting, only in conditions where cells are treated with bacteria and LL-37 and not with either treatment alone. Further investigation revealed that this synergy was dependent on direct contact between the cell and intact, live bacteria and, although independent of a functional type 3 secretion system (T3SS) or flagella production, and did not occur using the ΔmexAB-oprM mutant of *P. aeruginosa*, proposed to be defective in epithelial cell internalisation [231]. The internalisation of *P. aeruginosa* by epithelial cells, and the induction of apoptosis in infected pulmonary cells *in vivo* have been proposed to be important in innate host defence against this organism [114, 115].

Cell death pathways are complex and sometimes interlinked. We had shown that LL-37 induced host cell death at high concentrations was characteristic of apoptosis with the involvement of mitochondrial factors BAX and caspase -9 and -3 activation but that the inhibition of these factors did not fully negate the LL-37 induced death of infected epithelial cells. I produced further work following the publication of our initial data that showed that the cathelicidin-mediated death of *Pseudomonas aeruginosa* infected airway epithelium could be significantly abrogated when caspase-1 was inhibited.

Inflammasomes are large cytosolic complexes (>700KDa) containing NLRs that recruit and activate pro-caspases. Caspase-1 is one such inflammasome-activated caspase and is required for cleavage and activation of the pro-inflammatory cytokines IL-1β and IL-18. Pyroptosis is a caspase-1 dependent form of inflammatory cell death that has been best studied in infected macrophages and is characterised by the presence of caspase-1 and pro-inflammatory cytokine release. Investigation of airway epithelial cells did in fact show a capability to express inflammasome components and in conjunction with the results I had produced showing abrogation of cell death when caspase-1 was inhibited I hypothesised that during intracellular infections cathelicidins act as danger signals to instruct inflammasome formation and destruction of compromised airway epithelial cells as an anti-infective mechanism. This hypothesis was supported by the detection of pro-IL-1β from infected cells and pro and active IL-1β from infected cells treated with LL-37 by western blotting however it proved to be a difficult result to replicate. Although IL-1β has been reported to be expressed following contact with *P. aeruginosa* other groups have found little detectable levels of this pro-inflammatory cytokine being produced by epithelial cells therefore this is not a conclusive finding as to whether the death is occurring by pyroptosis or not. ELISA could however be used to investigate whether epithelial cells infected with PA01 in the presence of LL-37 produce IL-1β.

Regulation of cell death pathways may be used by the host as a means of resolving infection and removing destructive cells that are no longer needed such as neutrophils from sites of inflammation [201]. A common theme in removal of pathogens from the epithelial lining is prompt desquamation of affected or infected epithelial cells. This has been described in bladder epithelial cells following bacterial attachment of uropathogenic *Eschericia coli* [202] within 2 hours in a murine model of urinary tract infection as indicated by TUNEL positive cells. In bladders where areas of cell death were evident there was a reduction in bacterial

numbers that was not seen when exfoliation of host cells was inhibited with a pan-caspase inhibitor. A comparable clearance mechanism is demonstrated in airway epithelial cells during *P. aeruginosa* pneumonia whereby efficient epithelial cell apoptosis is needed in order to clear Pseudomonal lung infections. Thisprocess is reliant on the Fas/FasL stimulated induction of cell death, the inhibition of which abrogates any protective effects of clearance by this mechanism [115].

Several possible explanations could be proposed to explain the induction of infected epithelial cell death with low concentrations of LL-37. Antigens inducing cell death may only be exposed following endosomal processing. Alternatively the organism may exit the endosome and trigger cytosolic signaling following membrane disruption by LL-37 interacting with the endosomal membrane. Future work could use confocal microscopy to visualise what happens to both TAMRA labeled LL-37 and fluorescent bacteria once inside the cells. There are multiple fluorescent stains and molecules that may be used to analyse the location, acidity and integrity of endo-lysosomes in live cells. DQ albumin and acridine orange may be used to determine the location of endosomes and whether there is any leakage of contents and/or exit of *P. aeruginosa* into the cytosol following LL-37 exposure. A lysosomal protease, Cathepsin B has been shown to activate Nalp3-containing inflammasome formation following release of endosomal contents [192]. Multiple nod-like receptors (NLRs) can sense intracellular pathogens and be involved in inflammasome formation and caspase 1 activation leading to cell death and further work is warranted to investigate whether there is any role for the NLRs/inflammasome in LL-37-mediated induction of cell death of infected cells.

In acute infections, *P. aeruginosa* at the time of colonisation usually express T3SS toxins; however, later in chronic infections these genes are switched off. We have already seen that infection with a T3SS mutant unable to express the translocon proteins still promoted LL-37-induced cell death (data not shown). However we have not looked at the effect of growing the bacteria in conditions that will promote T3SS expression (e.g. calcium depleted medium) or characterised the effector protein expression within each of our working isolates. Strains producing the T3SS proteins ExoS and ExoT have a more invasive phenotype whereas ExoU is rapidly cytotoxic to many cells which may account for the difference seen between the lab strain and the clinical isolate. 20-28% of *P. aeruginosa* strains are known to be invasive but also directly cytotoxic to cells due to the production of the protein ExoU. Although PA01 is

known to express Exo S, ExoT and ExoY it does not express ExoU. It is possible that the results we saw using the clinical strain J1386 is due to the expression by this strain of ExoU which would make it directly cytotoxic however this would need to be formally validated by determining the exoU status of this strain. Analysis of *P. aeruginosa* mutants which do or do not cause cell death could lead to the purification of bacterial components to use as tools to investigate the host factors involved. These data suggest that the effects of LL-37 on the death of infected cells may only be relevant in non-cytotoxic bacterial strains, or isolates at certain stages in infection/colonisation of the host but a larger panel of strains would need to be tested to determine this.

Further investigation to understand the mechanisms behind LL-37 induction of cell death could utilise FLICA® assays (fluorescent caspase probe based assays) to look at caspase activation in cells using real-time cell imaging, western blots to look at caspase activation, P2x7 receptor inhibition (to prevent certain types of inflammasome formation) and the use of siRNA to silence components of inflammasomes such as ASC (apoptosis-associated speck like protein containing a CARD domain) and NALP3 (a nod-like receptor) in cells prior to incubation with PA01 and peptide. BipV5 may inhibit the pathway initiated by LL-37 alone, however in an infected cell there may also be other initiators and effectors of cell death activated. Future investigations could determine the involvement in this system, if any, of apoptosis inducing factor (AIF), Inhibitors of apoptosis (IAP) and the inhibitor of these IAP's Smac/Diablo. In addition the effect of overexpression of the protective anti-apoptotic protein Bcl2 could be examined.

Chapter 6 : Cathelicidin mediated modulation of pulmonary infection *in vivo*

6.1 Developing a mouse model of acute lung infection: Does intranasal infection of C57/Bl6J mice with PA01 result in observable pneumonia?

To study the capacity of cathelicidin to modulate pulmonary inflammatory responses to *P. aeruginosa* infection *in vivo* it was necessary to establish a non-lethal model of acute lung infection that would induce a measurable inflammatory response to this bacterium in the airways. Results from studies in the literature vary widely when C57/Bl6J mice are infected with *P. aeruginosa* depending on the strain of bacteria used [40, 246, 247], The doses used in other labs when intra-nasally administering *P. aeruginosa* to C57/Bl6J was between 10^4-10^9 colony forming units (cfu). As mentioned previously, PA01 is a lab strain lacking cytotoxicity and other virulence factors and thus a higher dose was predicted to be necessary to establish a clear inflammatory response to infection.

In order to establish a suitable model, dose finding pilot studies were performed. I lightly anaesthetised male and female C57/Bl6J (wild type) mice between the ages of 6-10 weeks with isofluorane and oxygen before innoculating intranasally with doses of $3x10^5$, $3x10^6$ or $3x10^7$ cfu *P. aeruginosa* PA01 or with saline only controls and monitored the progress of infection every 2 hours until the mice were culled at 6 or 24hrs p.i. I determined that all three concentrations of *P. aeruginosa* PA01 given to mice intra-nasally resulted in a self-limiting pneumonia whereby (when assisted by the use of heat mats to prevent a drop in body temperature of convalescing mice), all infected mice recovered spontaneously within 48 hours of infection resuming normal activities (frequency of movement and grooming/feeding) with cfu numbers returning to or approaching zero (data not shown). $3x10^7$ cfu was chosen as an appropriate dose to use in further studies as the dose which produced a measurable pneumonia and cellular response in the mice airways but was also not cleared too rapidly without intervention. This was important as mice clear PA01 very effectively without major inflammatory responses and so in order to establish a model where conditions can be adjusted to study the capacity of LL-37 to enhance the clearance we must first have a response to measure and be able to enhance. $3x10^7$ cfu was the lowest dose at which there was a strong inflammatory response and delayed clearance in wild type animals that resolved without unnecessary suffering to the animal.

Having established this model it was then used to test three main questions:

1. Does endogenous murine cathelicidin (mCRAMP) play a role in murine pulmonary clearance of *P. aeruginosa* , and, if so, by what mechanisms.
2. Can therapeutically delivered synthetic human cathelicidin (LL-37) enhance murine pulmonary clearance of *P. aeruginosa* , and, if so, by what mechanisms.
3. In the absence of endogenous murine cathelicidin (mCRAMP) can therapeutically delivered synthetic human cathelicidin (LL-37) enhance murine pulmonary clearance of *P. aeruginosa*, and, if so, by what mechanisms.

The importance of maintaining body temperature in infected animals was highlighted during an early pilot experiment in which a socket that was used to power a heat mat was turned off by a member of technical staff in error prior to leaving for the evening. The absence of temperature regulation resulted in the death of 2 out of 3 wild type mice and 3 out of 3 *Camp-/-* mice treated with PBS as a control and infected with *P. aeruginosa*, but all the of the LL-37-treated wild type and *Camp-/-* mice infected with *P. aeruginosa* survived (*Fig 6.1*). These compelling data helped to inform the future studies.

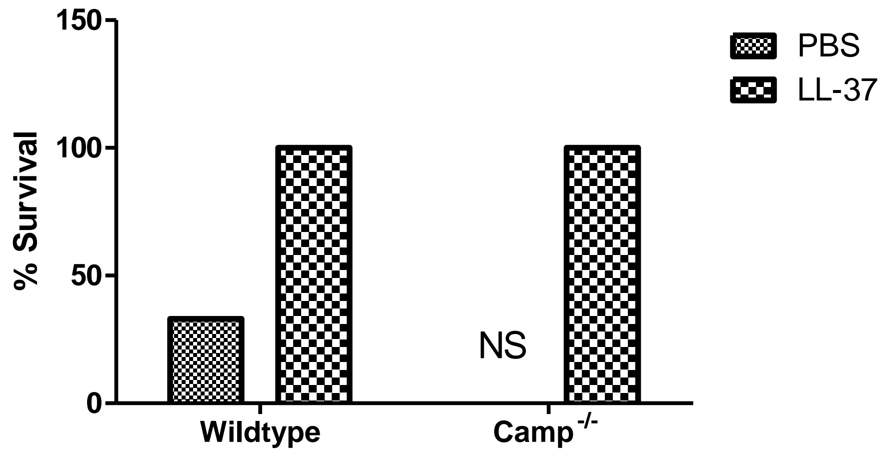

Figure 6.1 Survival of wild type and *camp* $^{-/-}$ mice at 24 hours. C57Bl/6J or *camp* $^{-/-}$ mice were intranasaly infected with $3x10^7$ PA01 n=3 per group

6.2 Does the absence of endogenous cathelicidin alter the course of infection?

In order to study whether the absence of endogenous mCRAMP (the murine homologue of LL-37) alters the course of infection, a series of experiments were performed comparing wild type mice and knock-out mice lacking the *Camp* gene. C57Bl/6J and *Camp*$^{-/-}$ mice (congenic on a C57Bl/6J strain background) were infected via the intranasal route, as before, with $3x10^7$ cfu PA01 and culled 6 or 24 hours later. The parameters of % weight loss (*Fig 6.2*), recoverable viable bacteria from BAL and lung homogenates (*Fig 6.3*) and differential inflammatory cell counts from lung lavages (*Fig 6.4*), were used to begin to study the course of disease and cellular response in each strain of mouse.

Infected animals lost no weight for 6 hrs after infection (*Fig 6.2a*) however a significant ($P<0.0001$) amount of weight (approx. 10% loss of initial weight) was lost by 24 hours following infection compared to animals having received PBS only (*Fig 6.2b*). This is most likely due to a reduced intake of food and fluid and an increase in shivering. Genotype had no significant effect on the amount of weight lost in each group ($P>0.05$).

In order to assess whether there was any difference in clearance of PA01 from the airways of mice deficient in mCRAMP compared to wild type control mice, animals from both strains were intranasally infected with $3x10^7$ cfu PA01 and culled 6 or 24 hours later. Immediately following being culled the airways were lavaged with PBS and the lungs homogenised. Viable bacteria were cultured from BALF and lung homogenate overnight. At 6 hours (*Fig 6.3a*) a significant ($P<0.05$) clearance defect was observed in BALF from *Camp*$^{-\backslash-}$ mice, but not in lung homogenate although there was a trend for higher bacterial loads in lungs of *Camp*$^{-\backslash-}$ compared to wild type. By 24 hours both genotypes had successfully cleared the majority of bacteria compared to the bacterial loads seen 6 hours post infection. My lab work demonstrated that there is a clear trend for delayed clearance in both the airways and the lung tissue in mice deficient in mCRAMP compared to wild type animals (*Fig 6.3b, c, d*). Further work performed by others in the Davidson laboratory after the completion of my PhD lab work to increase the numbers of mice in the study demonstrated that this trend is statistically significant at 24 hours p. i.

In order to evaluate the cellular inflammatory response to infection, mice were culled at 6 and 24 hours following inoculation and the airways lavaged once with 1 ml of ice cold PBS. The total number of cells retrieved in the BALF was determined by the use of a nucleocounter and the proportion of these cells which were monocytes or PMN was determined by counting the percentage of 100 cells in a 100 μl sample on a cytospin slide that were morphologically monocytes or PMN under a light microscope. This proportion was multiplied by the total cell numbers to calculate monocyte cell or PMN cell numbers retrieved from the airways at these times following infection (*Fig 6.4*).

There was no difference in monocyte response to infection at 6hrs (*Fig 6.4a*) and only a trend towards an increased number of monocytes by 24hrs (*Fig 6.4b*) in the BALF of infected animals compared to those inoculated with PBS only. No significant difference was seen in the monocyte response to PBS or any difference in monocyte number in response to PA01 between genotypes.

There was an initial low level PMN response to infection at 6 hrs. which was not significantly different between genotypes (*Fig 6.4c*), this PMN influx was much greater by 24 hrs in wildtype mice but had not further increased in *Camp*$^{-\backslash-}$ mice (*Fig 6.4d*) compared to the response at 6 hrs. This difference observed between the two genotypes was significant ($p<0.05$). These data demonstrate a CRAMP-dependency of the later increased influx of PMNs but not the initial PMN response to PA01 in the airways. No PMNs were present in the BALF from PBS controls.

To establish whether the induction of local cytokines was altered in mice deficient in cathelicidin in response to infection I measured the concentration of several cytokines in BALF from mice of both genotypes which were culled 6 or 24 hours after intranasal delivery of PA01 or PBS. BALF was centrifuged to remove cellular content prior to measuring the cytokines with a commercially available kit. There was no difference in the cytokine profile at 6 or 24hrs between the genotypes (*Fig 6.5 & 6.6*). The cytokines IL-6 and TNF alpha as well as the chemokines KC and MIP-1α were induced in animals infected with PA01 by 6 hrs after intranasal delivery of PA01 (*Fig 6.5*) and had mainly returned to base levels by 24 hrs (*Fig 6.6*) as compared with PBS treated controls ($p<0.01$). The chemokine MCP-1 however, was induced later in the infection and was significantly increased in mice of both genotype by 24 hrs but not at 6 hrs post infection. ($P<0.01$).

No substantial production of IL-10, IFN gamma or IL-12 was detected in any mice and the similar pattern of cytokines studied offers no explanation as to why there is delayed clearance of PA01 or impaired later neutrophil influx in *Camp* $^{-/-}$ mice.

6.2.1 Mice infected with *P. aeruginosa* lose approximately 10% of their bodyweight in the first 24 hours following intranasal inoculation.

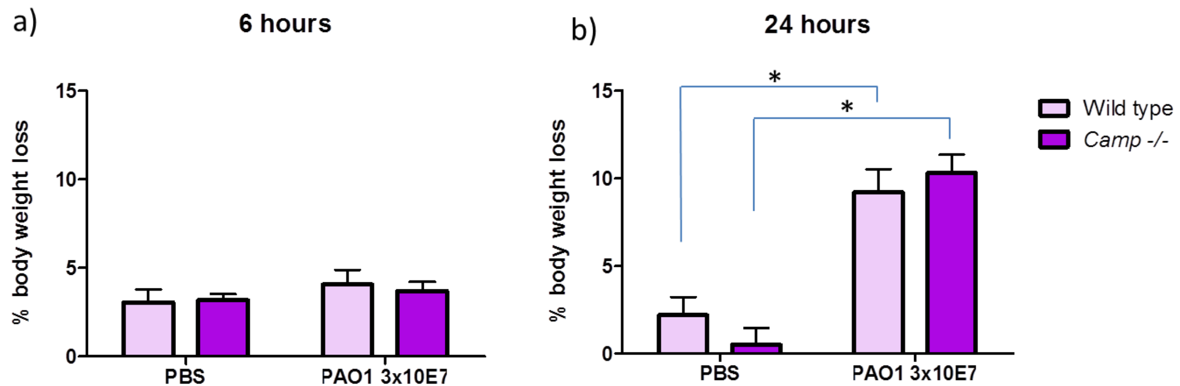

Figure 6.2 Wild type and *Camp*$^{-/-}$ mice intra-nasally infected with 3x10^7 *P. aeruginosa* lose an equivalent proportion of body weight by 24hrs post infection. Wild type C57Bl/6J mice and *Camp*$^{-\backslash-}$ mice were weighed, then inoculated with 3x10^7 cfu of *P. aeruginosa* PAO1 or PBS by intranasal delivery. a) 6 hours after inoculation of all mice, a subset were re-weighed prior to being culled (C57Bl/6J n=10, *Camp*$^{-\backslash-}$ n=8) or b) 24 hours after inoculation mice were re-weighed and culled (C57Bl/6J n=13, *Camp*$^{-\backslash-}$ n=6). Data shows mean percentage weight loss +/- SEM. Statistical analyses were conducted using 2 way ANOVA with Bonferroni's post tests; * $p < 0.01$.

6.2.2 Mice deficient in endogenous cathelicidin demonstrate delayed clearance of PAO1 from the airways.

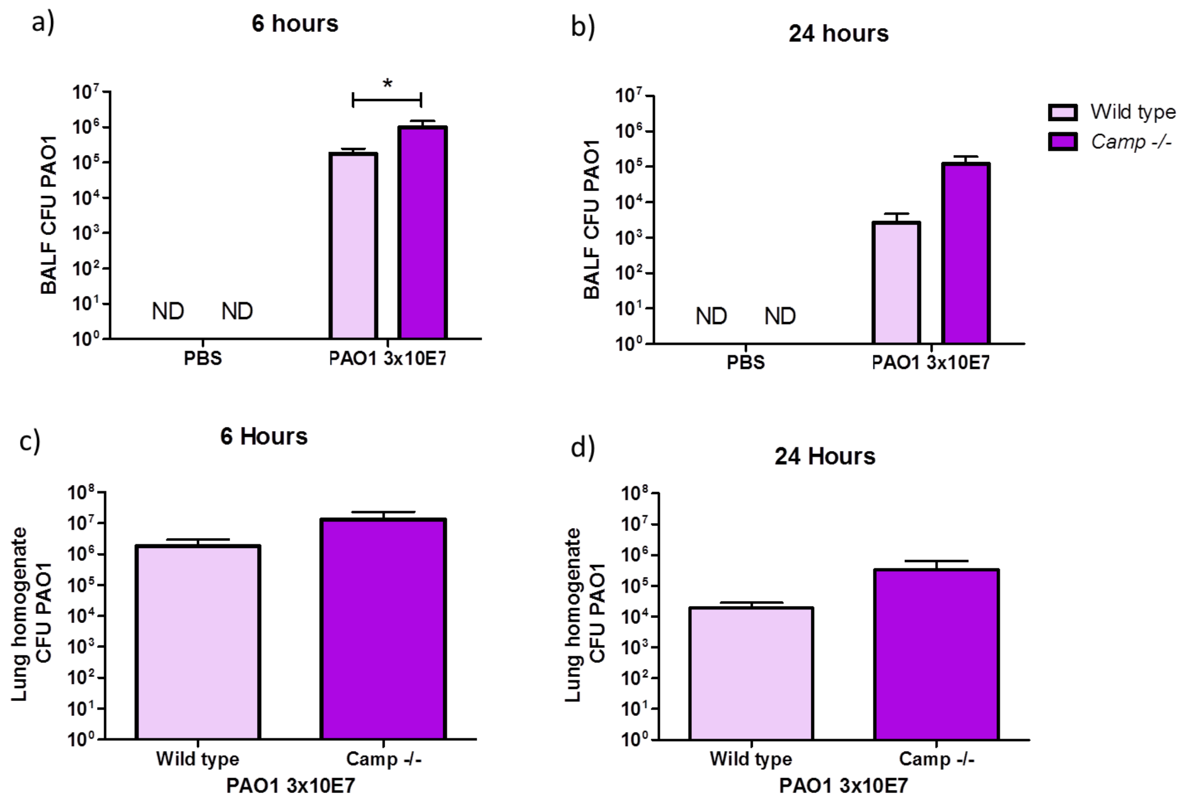

Figure 6.3 Mice deficient in mCRAMP show delayed clearance of PA01 from the airways compared to wild type animals. C57Bl/6J wild type controls and *Camp*$^{-/-}$ mice were inoculated with $3x10^7$ cfu of *P. aeruginosa* PAO1 or PBS by intranasal delivery. At 6 or 24 hours after inoculation mice were culled and their lungs were lavaged before homogenisation. BALF and lung homogenates were serially diluted, plated and incubated overnight at 37˚C before bacterial colonies were counted and corrected for volume. Mean PAO1 cfu +/- SEM in the BALF (a & b) or lung homogenate (c & d) from infected animals (n≥6 per condition) are displayed. No bacteria were detected in samples from uninfected mice (ND). For statistical analyses bacterial counts were normalised by logarithmic transformation. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests for BALF data and two-tailed t-tests for Lung data; * $p < 0.05$.

6.2.3 Infection with PA01 induces an acute influx of neutrophils to the lungs which is increased and sustained in the later phase of infection by mice expressing endogenous cathelicidin.

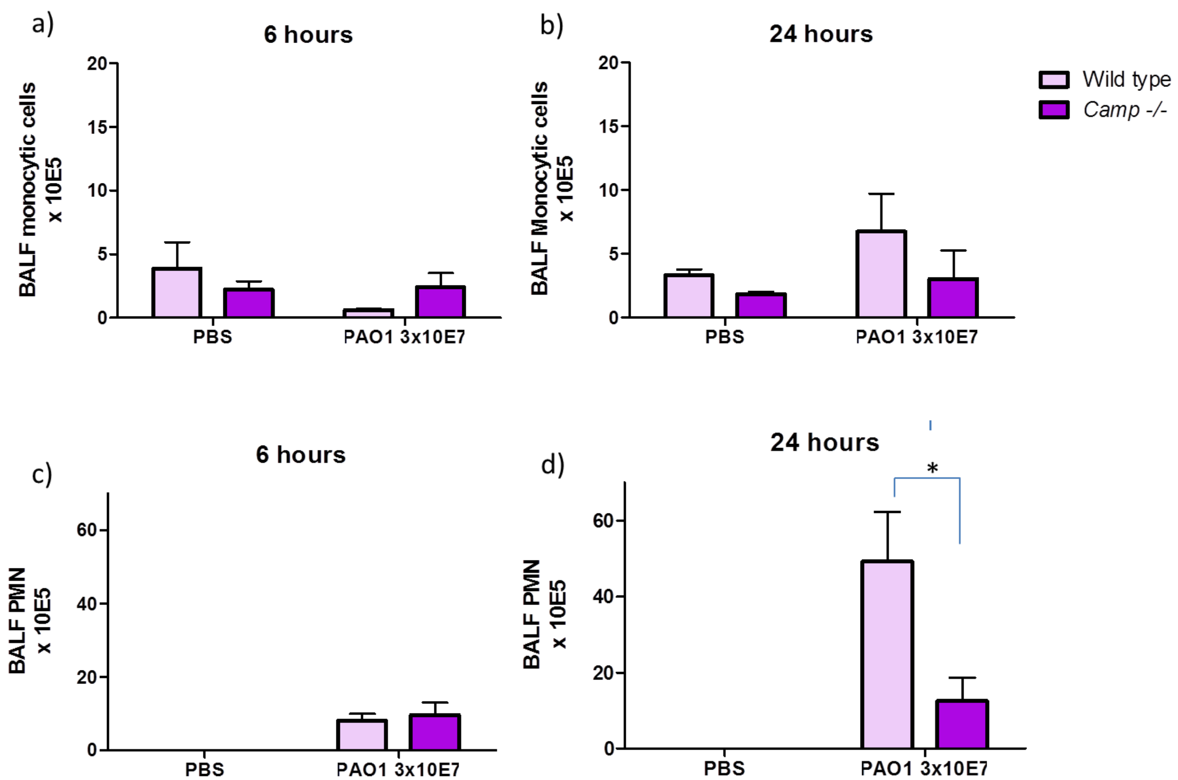

Figure 6.4 Infection of C57Bl/J6 wild type mice results in significantly higher PMN influx to the airways than during infection of *Camp* $^{-/-}$ mice by 24hrs p.i. *Camp*$^{-/-}$ mice and C57Bl/J6 wild type controls were inoculated with PBS or 3x10^{7} cfu of *P. aeruginosa* PAO1 by intranasal delivery (n≥8 for each condition). At 6 hours (a & c) or 24 hours (b & d) after inoculation mice were culled and their lungs were lavaged. BALF was cytocentrifuged and differential counts were conducted for monocytes (a & b) and neutrophils (c & d). Data shows mean cell counts +/- SEM. Statistical analyses were conducted using 2 way ANOVA with Bonferroni's post tests; * $p < 0.05$.

6.2.4 Delayed clearance of PA01 in Camp $^{-/-}$ mice is not explained by differences in local cytokine profiles.

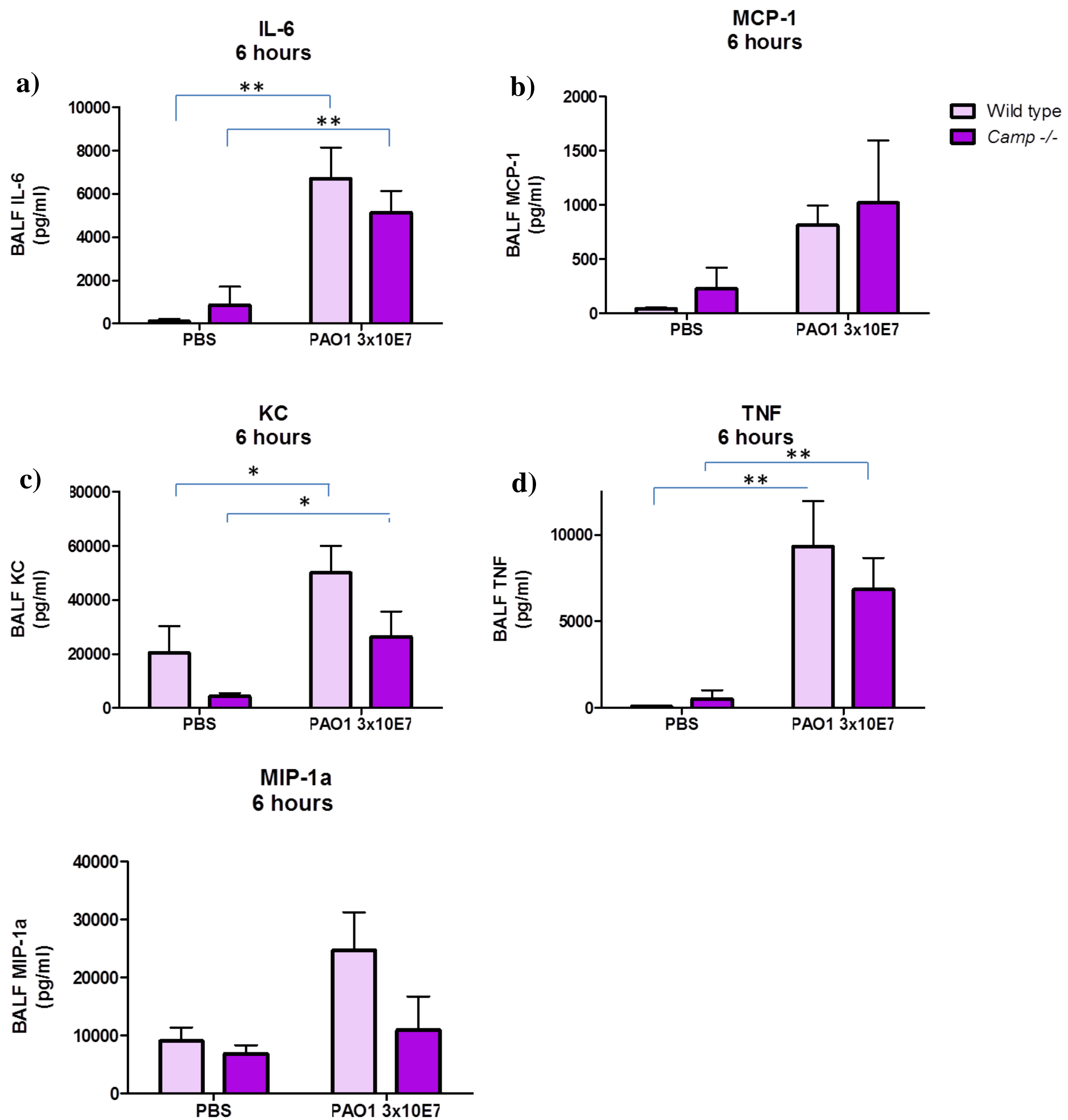

Figure 6.5 Delayed clearance of PA01 by mice deficient in mCRAMP is not explained by differences in local cytokines recovered from BALF at 6hrs post infection. Wild type C57Bl/6 mice and *Camp*$^{-/-}$ mice were inoculated with $3x10^7$ cfu of *P. aeruginosa* PAO1 or PBS by intranasal delivery. At 6 hours after inoculation, mice were culled and their lungs were lavaged. BALF was centrifuged to remove cells and levels of IL-6 (a), MCP-1 (b), KC (c), TNF (d) and MIP-1α (e) were determined using cytometric bead arrays (a, b, d) or ELISA (c & e). Data show bars for n ≥ 9 animals per condition. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests. *p<0.05, **p<0.01

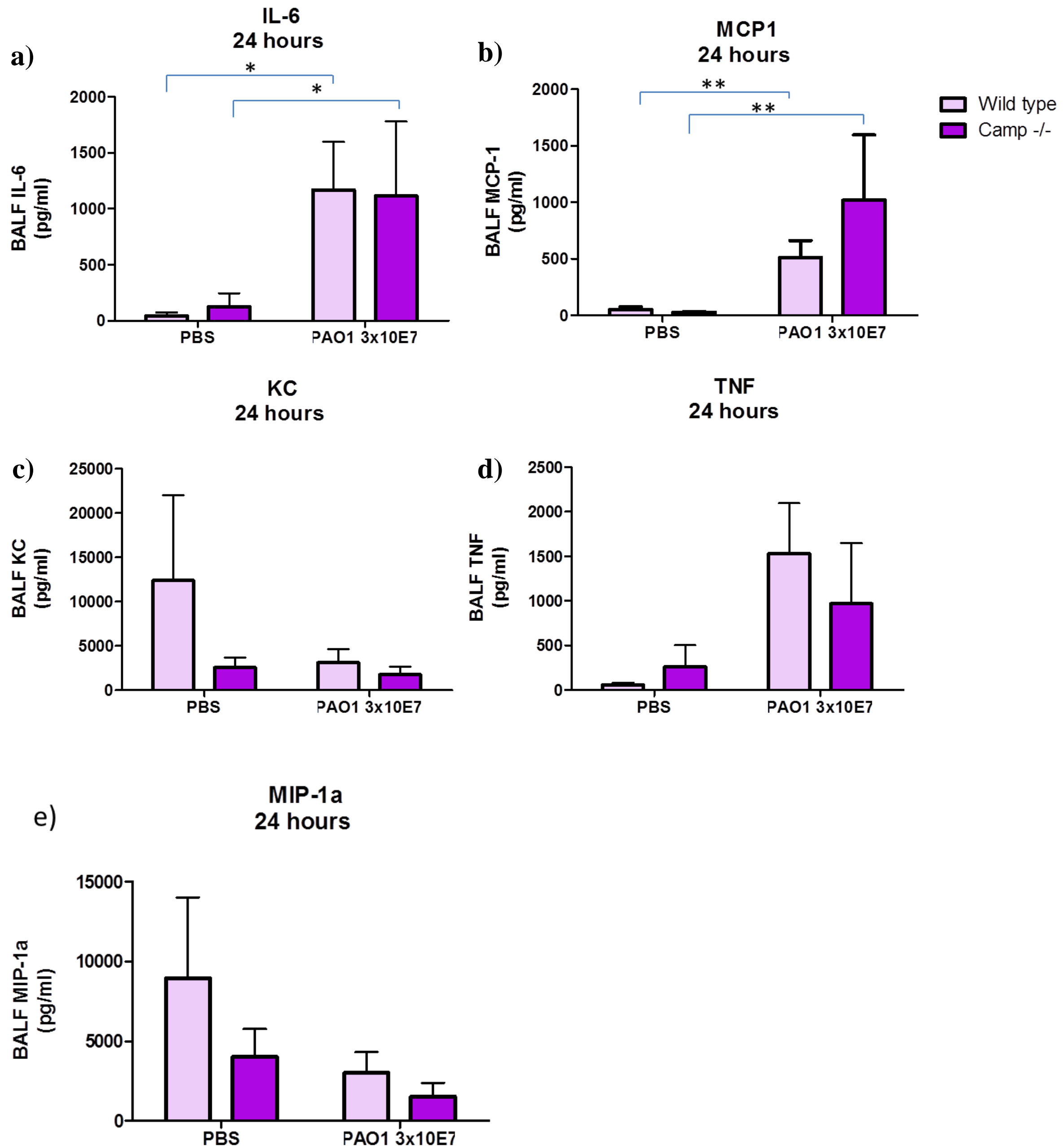

Figure 6.6 Delayed clearance of PA01 by mice deficient in mCRAMP is not explained by differences in local cytokines recovered in BALF at 24hrs post infection. Wild type C57Bl/6 mice and *Camp-/-* mice were inoculated with $3x10^7$ cfu of *P. aeruginosa* PAO1 or PBS by intranasal delivery. At 24 hours (a - e) after inoculation, mice were culled and their lungs were lavaged. BALF was centrifuged to remove cells and levels of IL-6 (a), MCP-1 (b), KC (c), TNF (d) and MIP--1α (e) were determined using cytometric bead arrays (a, b, d) or ELISA (c & e). Data show bars for ≥ 5 animals per condition. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests. *p<0.01

6.3 Does exogenous cathelicidin enhance clearance of pulmonary bacteria in wild type mice?

Having shown a clear role for endogenous cathelicidin in the clearance of bacteria from the airways and considering that host defence peptides pose likely candidates for therapeutic use I next wanted to know what effect early exogenous peptide would have on infection in wild type mice. As therapeutic cathelicidin in humans would be based on exogenous LL-37 to complement the role of endogenous peptide, synthetic LL-37 was used for these sets of experiments.

To determine whether early delivery of exogenous LL-37 in an acute *P. aeruginosa* challenge could alter clearance of infection from the murine airways, wild type mice were inoculated as before with PBS or PA01 concomitantly with either 10 µg of synthetic LL-37 (a concentration that I had previously shown was not bactericidal for PA01 in a physiological relevant milieu) or the same volume of PBS as a control (*Fig 6.7*). Mice were culled immediately (0 hrs.), 6 hrs. or 24 hrs. following intranasal inoculation and the airways lavaged and lungs homogenised as described previously.

Results from culturing samples from the airways of mice designated the 0 hr. time point demonstrated no significant difference in the bacterial load of mice with or without LL-37. This was despite the fact that the LL-37 and PAO1 delivered to the lung had ~ 60 minutes after infection before lung homogenisation (given time for completion of delivery, dissection, transfer back to laboratory for processing). These data indicate that the addition of LL-37 did not have a directly bactericidal effect (data not shown).

After 6 hrs. (*Fig 6.7*), there was a significantly enhanced ($p<0.05$) clearance of bacteria from BALF, but not lung homogenate, in animals that were inoculated with PA01 and LL-37 compared to PA01 and PBS controls (a & c). After 24 hrs. this enhancement was significant for both BALF and lung tissue (b & d). ($p<0.05$ & $p<0.01$). These data clearly show that early therapeutic administration of exogenous LL-37 augments clearance of pulmonary infection with *P. aeruginosa*, even in the absence of any early direct microbicidal properties.

To investigate the cellular response to inoculating mice with LL-37 +/- PA01 the total cells in the BALF from the experiment above were, as before, counted by nucleocounter and then

differential counts made using cytospins and a light microscope (*Fig 6.8*). Inoculating wild type animals with LL-37 in the absence of infection did not induce a greater number of monocytes at 6 or 24 hours than PBS alone (a & b). In the absence of infection LL-37 did not induce any influx of neutrophils by 6 hrs. and only a low number of PMNs by 24 hours following intranasal delivery (c & d). Infected animals showed no significant increase in monocytes to the lungs at either timepoint, however displayed an increase in the number or neutrophils in the lungs at 6 hrs. which was sustained and increased by 24 hrs. In animals concomitantly infected with PA01 and LL-37 there was a significant early increase in the number of neutrophils in the lungs at 6 hrs. compared to animals receiving PA01 with PBS as control (Fig 6.8 c; $p<0.05$). These data suggest that LL-37 did not act as a chemoattractant in the absence of infection, however in the presence of PA01, LL-37 enhanced early neutrophil influx to the lungs.

To determine whether there were any differences in the cytokines in the lungs in response to LL-37 treatment the BALF was spun as before to remove cells before using a flow cytometry based assay to measure the level of several mouse cytokines (*Fig 6.9 & 6.10*). As shown previously, inflammatory cytokines and chemokines rise in response to infection and decline by 24 hours post infection. Administration of LL-37 did not alter the expression of local cytokines compared to PBS controls in infected or uninfected animals.

6.3.1 The administration of exogenous LL-37 at the time of infection enhances clearance of pulmonary *P. aeruginosa* in wild type mice.

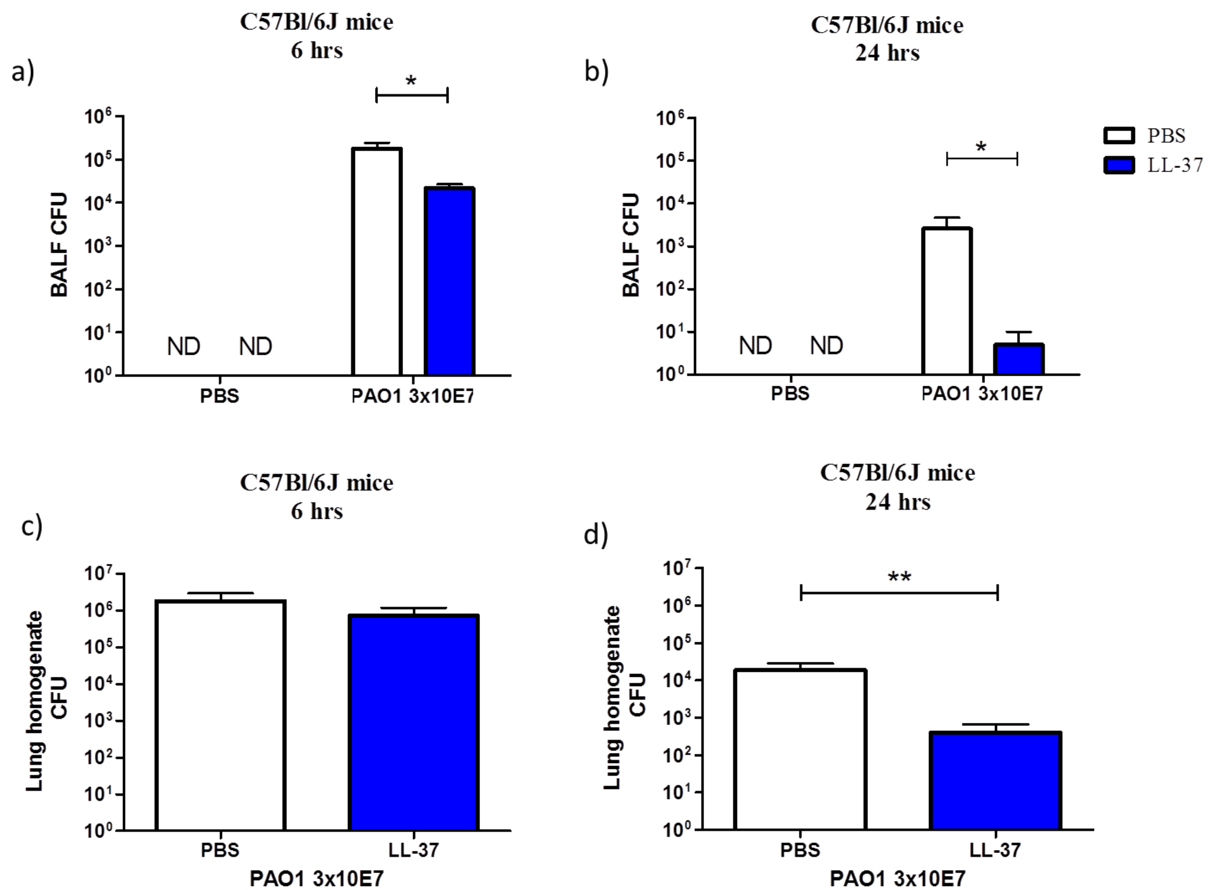

Figure 6.7 Exogenous LL-37 enhances pulmonary clearance of Pseudomonas from wild type mice. Wild type C57Bl/6J mice were inoculated with 10 µg LL-37 peptide alongside either $3x10^7$ cfu of *P.aeruginosa* PAO1 or PBS by intranasal delivery. 6 or 24 hours after inoculation mice were culled, and their lungs were lavaged once with 1ml PBS before homogenisation. BALF and lung homogenates were serially diluted, plated and incubated overnight at 37˚C before bacterial colonies were counted and corrected for volume. Mean PAO1 cfu +/- SEM in the BALF (a & b) or lung homogenate (c & d) for infected animals (n≥6 per condition) are displayed. No bacteria were detected in samples from uninfected mice. For statistical analyses bacterial counts were normalised by logarithmic transformation. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests; * $p < 0.05$, ** $p < 0.01$.

6.3.2 In the presence of pulmonary infection with *P. aeruginosa*, administration of exogenous LL-37 results in an enhanced, early influx of neutrophils.

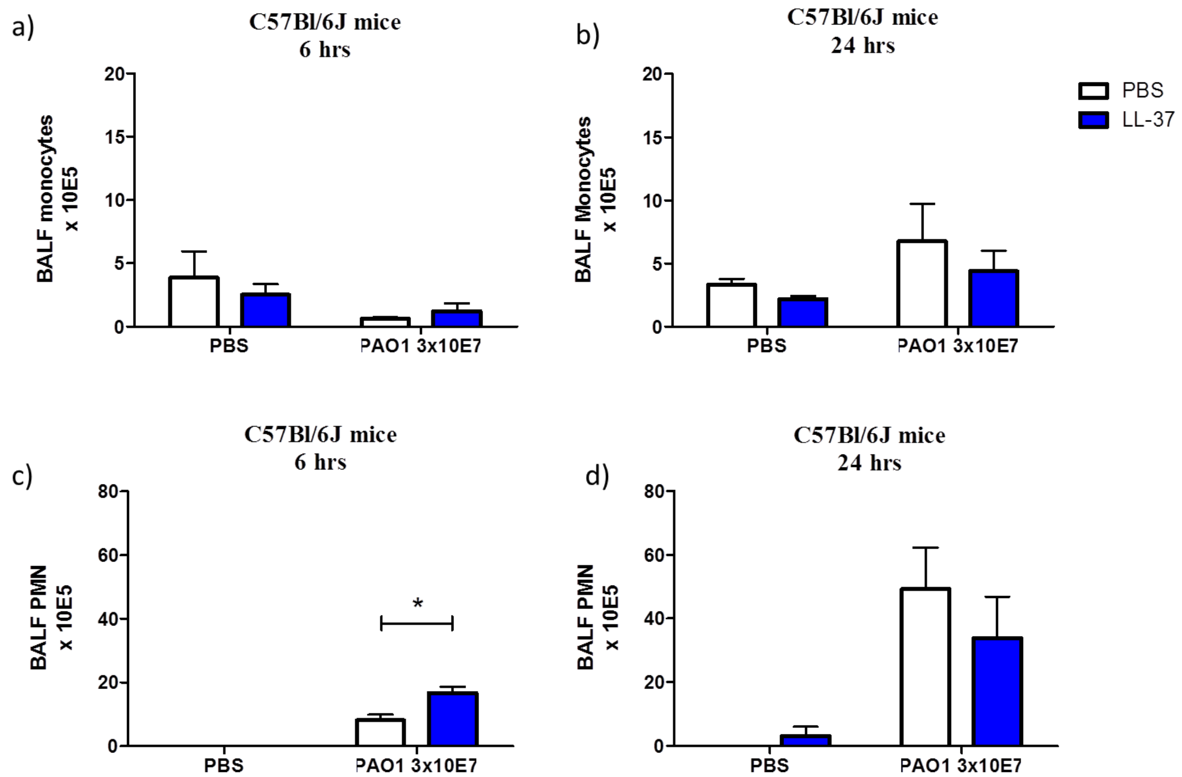

Figure 6.8 Administration of LL-37 at the time of infection results in an enhanced influx of neutrophils early in infection. Wild type C57Bl/6J mice were inoculated with 10 µg LL-37 peptide alongside either 3x10^7 cfu of *P.aeruginosa* PAO1 or PBS by intranasal delivery. At 6 hours (a & c) or 24 hours (b & d) after inoculation mice were culled and their lungs were lavaged. BALF was cytocentrifuged and differential counts were conducted for monocytes (a & b) and neutrophils (c & d). Bars show data from mice n ≥5 per condition. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests * $p < 0.05$.

6.3.3 Intranasal adminstration of exogenous LL-37 does not alter the cytokine response to pulmonary infection.

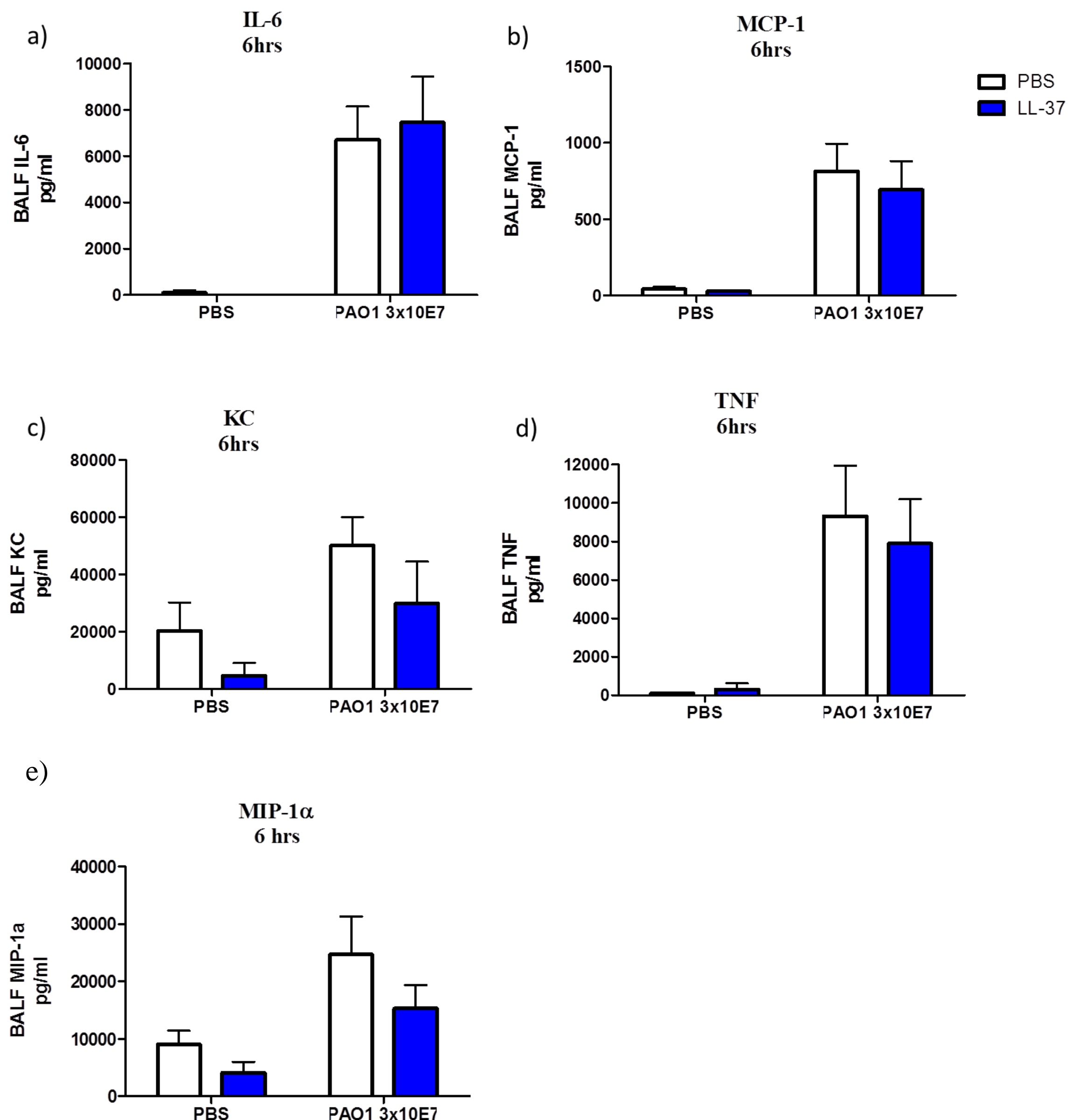

Figure 6.9 Administration of exogenous LL-37 does not alter the cytokine profile in infected or uninfected wild type animals at 6hrs. Wild type C57Bl/6 mice were inoculated with $3x10^7$ cfu of *P. aeruginosa* PAO1 and LL-37 or PBS by intranasal delivery. At 6 hours after inoculation, mice were culled and their lungs were lavaged. BALF was centrifuged to remove cells and levels of IL-6 (a), MCP-1 (b), KC (c), TNF (d) and MIP-1α (e) were determined using cytometric bead arrays (a, b, d) or ELISA (c & e). Data show bars for n ≥ 9 animals per condition. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests.

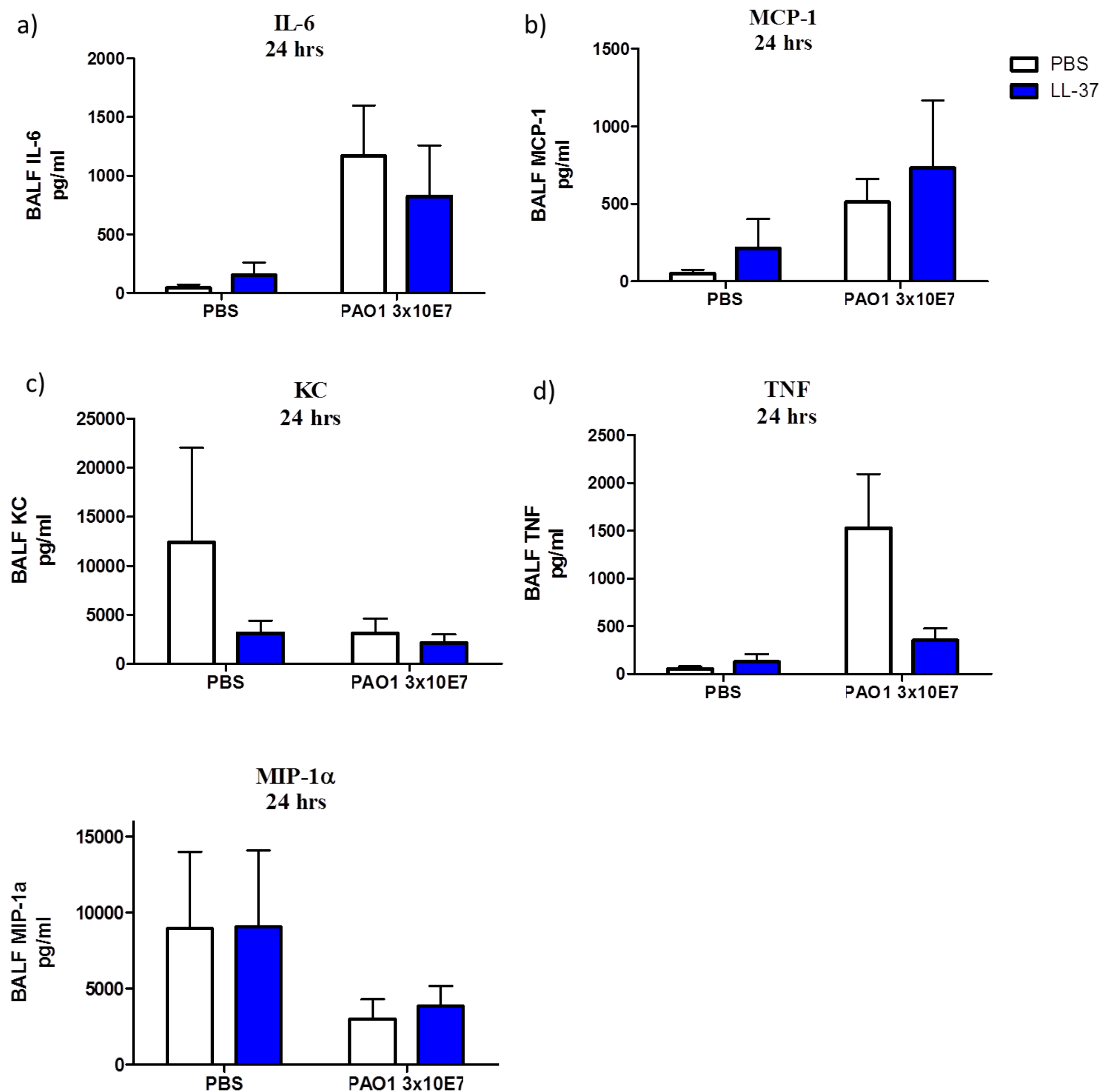

Figure 6.10 Administration of exogenous LL-37 does not alter the cytokine profile in infected or uninfected wild type animals at 24hrs. Wild type C57Bl/6 mice were inoculated with $3x10^7$ cfu of *P. aeruginosa* PAO1 and LL-37 or PBS by intranasal delivery. 24 hours after inoculation, mice were culled and their lungs were lavaged. BALF was centrifuged to remove cells and levels of IL-6 (a), MCP-1 (b), KC (c), TNF (d) and MIP-1α (e) were determined using cytometric bead arrays (a, b, d) or ELISA (c & e). Data show bars for n ≥ 9 animals per condition. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests.

6.4 Does exogenous cathelicidin rescue the defect in clearance of bacteria in CRAMP deficient mice?

Having shown that LL-37 is not only tolerated in wild type mice but enhances clearance of bacteria from the airways with an increase in the early influx of neutrophils to the lungs observed, the pertinent question was whether LL-37 could correct the defect in clearance seen in animals lacking endogenous cathelicidin.

Camp$^{-/-}$ mice given LL-37 at the time of infection showed a significantly enhanced clearance of bacteria (*Fig 6.11*) compared to animals given PA01 and PBS, $p<0.05$. There was also a trend to suggest early enhanced neutrophil response but this did not reach significance (*Fig 6.12*).

The augmented clearance of bacteria in *Camp*$^{-/-}$ mice given LL-37 and the corresponding cellular responses reflect the results seen in wild type animals demonstrating that it is possible to compensate to some extent for the lack of mCRAMP production using the human cathelicidin. It also demonstrates that endogenous mCRAMP production is not required for the protective effects of exogenous LL37.

6.4.1 Administration of exogenous cathelicidin at the time of infection restores the ability of mice deficient in mCRAMP to clear PA01.

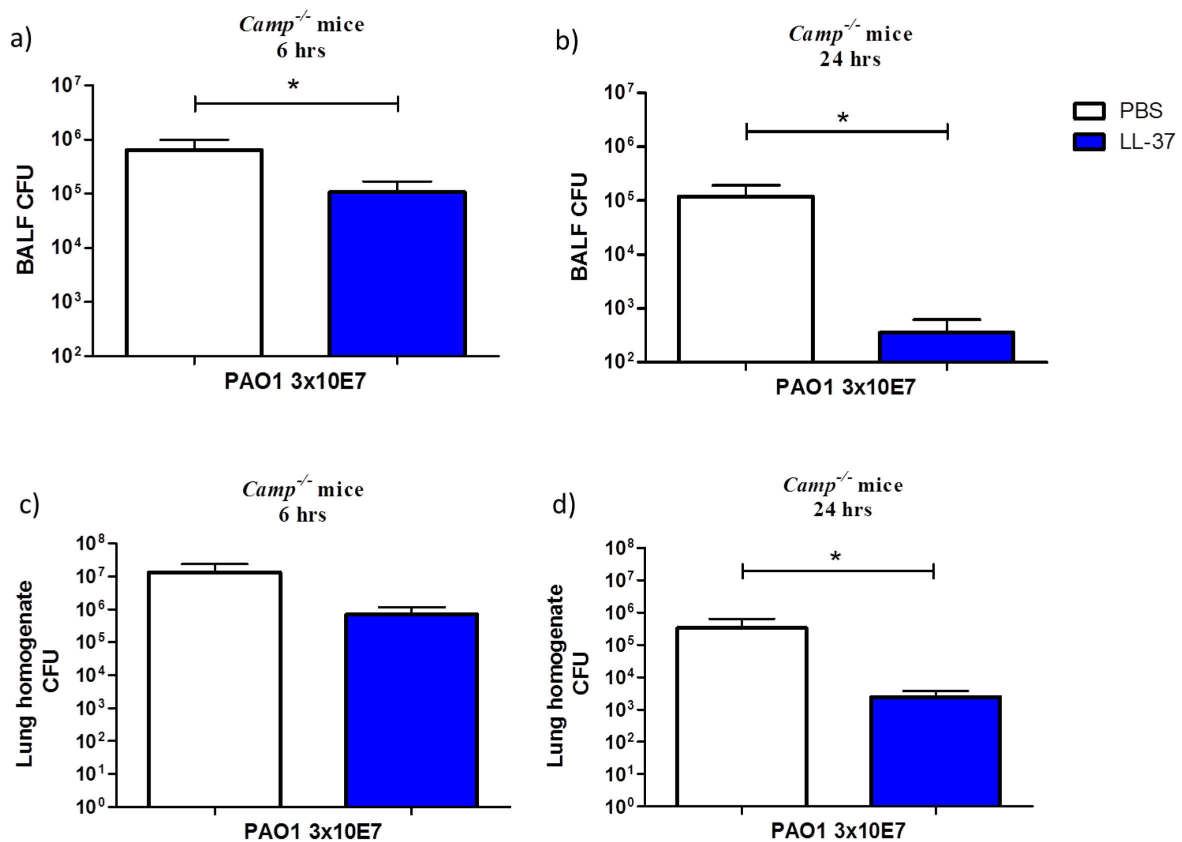

Figure 6.11 Exogenous LL-37 enhances pulmonary clearance of Pseudomonas from *camp*$^{-\backslash-}$ mice *Camp*$^{-/-}$ mice deficient in endogenous cathelicidin were inoculated with 10 µg LL-37 peptide alongside either $3x10^7$ cfu of *P.aeruginosa* PAO1 or PBS by intranasal delivery. 6 or 24 hours after inoculation mice were culled, and their lungs were lavaged once with 1ml PBS before homogenisation. BALF and lung homogenates were serially diluted, plated and incubated overnight at 37˚C before bacterial colonies were counted and corrected for volume. Mean PAO1 cfu +/- SEM in the BALF (a & b) or lung homogenate (c & d) for infected animals (n≥6 per condition) are displayed. No bacteria were detected in samples from uninfected mice. For statistical analyses bacterial counts were normalised by logarithmic transformation. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests; * $p < 0.05$

6.4.2 Administration of exogenous cathelicidin at the time of infection induces an early, increased influx of neutrophils in mice deficient in mCRAMP.

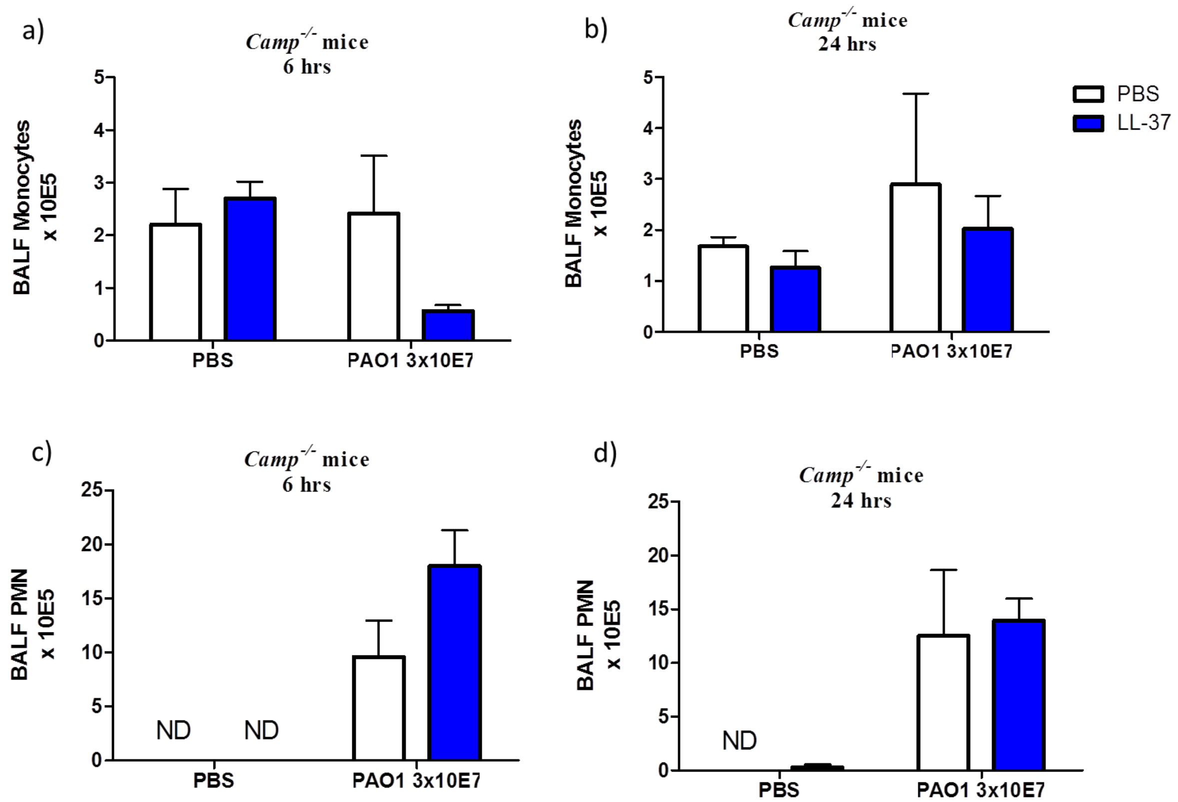

Figure 6.12 Administration of LL-37 at the time of infection results in an enhanced influx of neutrophils early in infection. *Camp*$^{-/-}$ mice were inoculated with 10 µg LL-37 peptide alongside either $3x10^7$ cfu of *P.aeruginosa* PAO1 or PBS by intranasal delivery. At 6 hours (a & c) or 24 hours (b & d) after inoculation mice were culled and their lungs were lavaged. BALF was cytocentrifuged and differential counts were conducted for monocytes (a & b) and neutrophils (c & d). Bars show data from mice n ≥5 per condition. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests

6.5 Discussion

Cathelicidins have been shown to possess an enormous array of microbicidal and innate immunomodulatory properties *in vitro*. This has unsurprisingly generated interest in these peptides with a view to adapt the peptide structure itself or manipulate the endogenous expression thereof for therapeutic benefit. Although studies existed that had started to address the function of this peptide in various body systems there was, at the time of this PhD, little description *in vivo* of LL-37 having a protective role during infection of the lung. I therefore set out to develop a cohesive murine infection model to assess the role of this peptide in response to lung pathogens *in vivo*.

Camp $^{-/-}$ knock out animals (from a C57Bl/6J background) deficient in endogenous mCRAMP production (the murine orthologue of LL-37) were compared to wild type C57Bl/6J mice that express functional mCRAMP to assess any differences in clearance and response to acute bacterial lung infection. As LL-37 is not species specific and maintains function in murine systems as in human we were also able to investigate the effect of exogenous LL-37 peptide on infection with intranasal *P. aeruginosa.*

Following intranasal inoculation of *P. aeruginosa* there were two phases of neutrophil influx to the murine lungs in response to infection. Results after 6 hours of infection indicated that the first phase of neutrophil influx was of a similar low level in animals independent of endogenous cathelicidin production. However a second phase of continued neutrophil influx was observed only in animals expressing endogenous mCRAMP. In addition to differences in the cellular influx between wildtype and mCRAMP deficient mice there was a delayed clearance of *P. aeruginosa* from the airways of mice deficient in endogenous cathelicidin at 24hrs post infection. LL-37 has been shown to be chemotactic for neutrophils but there was no difference in cytokines (IL-1, MCP-1, KC, TNF, and MIP-1α) measured in the lungs of wild type or *Camp* $^{-/-}$ mice.

Intranasal administration of exogenous LL-37 peptide was not directly microbicidal to *P. aeruginosa* however after 6hrs resulted in enhanced clearance of bacteria accessible by BAL (though not in whole lung) and after 24hrs resulted in increased clearance from the airways of mice independent of endogenous cathelicidin production which was accompanied by an early increase in neutrophil influx that did not occur in the absence of infection.

Cathelicidin in the lung is elevated in response to infection. LL-37 secreted in the airways of healthy, uninfected individuals is estimated to be less than 5µg/ml of bronchoalveolar lavage fluid (BALF) however when this peptide is measured in BALF from children with lung infections concentrations are closer to 30 µg/ml [256]. Levels of LL-37 in human lung disease such as sarcoidosis are also associated with increased LL-37 production alongside upregulation of other CHDPs, which is thought to explain why these patients do not succumb to respiratory infections [31]. LL-37 is also however found at higher than normal levels in the lungs of cystic fibrosis patients suffering chronic lung infections (around 15µg/ml BALF) and increasing amounts correlate with disease severity rather than protection [32] this is likely as a correlate of neutrophil infiltration to the lungs and the damage caused by chronic inflammation in the absence of successful clearance of the pathogen. Cathelicidin may be immunopathological at high concentrations, especially in chronic infections where the stimuli for upregulation of hCAP18 is not being effectively removed, however I saw no evidence of ill effects when treating mice with LL-37 during infection at the doses of peptide used.

As evidence to the important role that the cathelicidin LL-37 plays in the defence against bacterial infections it is worth noting that those with Morbus Kostmann, a disease which results in neutropenia and absence of cathelicidin do not have normal saliva concentrations of LL-37 and consequently they present with chronic periodontal infections [27]. Additionally, the murine orthologue of hCAP18, cathelicidin related antimicrobial peptide (mCRAMP) has been shown in a knockout model to be important in the clearance of gastrointestinal, skin, corneal and urinary tract infections [36-39].The susceptibility to lung infection in this model had not yet been characterised, but overexpression of LL-37 in wild type mouse lung had been shown to increase the clearance of *Pseudomonas aeruginosa* via an undefined mechanism [44].

At the beginning of my work on the *in vivo* role of cathelicidin during infections in the lung little was known in the literature about opportunistic infections in *Camp* $^{-/-}$ mice respiratory tracts. An interesting study performed recently to demonstrate the protective role that prior immunisation with flagellin can have on subsequent lung infections with *P. aeruginosa* [40] also found that when infecting mice that are unable to express mCRAMP (*camp*$^{-/-}$) the protective effects of flagellin immunisation were significantly lost with only a third of mice deficient in mCRAMP surviving as compared to 100% survival in WT animals. Further, despite the fact that neutrophils provide the most abundant source of cathelicidin, depleting

PMNs did not decrease the partially mCRAMP-dependant protection following immunisation. Perhaps indicating a role for the epithelial sources of cathelicidin produced in response to the infection by respiratory epithelial cells and in fact this is what the investigators discovered in airway epithelial cells within 8hrs of flagellin exposure.

Receptor specific actions of LL-37 may be investigated independently by selective use of knockout mice e.g. fprl-$1^{-/-}$ mice to remove the neutrophils chemotactic property attributed to this peptide. The expertise exists in our department to allow us to look at live animal imaging to track the movement of both labelled peptide and bacteria.

The sole human cathelicidin hCAP18/LL-37 is a multifunctional CHDP with direct microbicidal potential and the capacity to modulate inflammation and immune responses through a broad range of mechanisms. It has been implicated in host defence and disease pathogenesis in multiple systems and conditions, and represents both a fascinating target for clinical intervention and promising template for the development of novel antimicrobial, immunomodulatory therapeutics. Early clinical trials using synthetic analogues of CHDP were designed to maximise microbicidal activity, but achieved only moderate efficacy, perhaps due to failure to recognise the importance of their immunomodulatory functions. A recent approach, using non-microbicidal analogues that retained other bioactive functions, has demonstrated effective host defence augmentation in mice. These studies suggest that realising the full therapeutic potential requires further research to more clearly understand the precise mechanisms of action underpinning the inflammomodulatory and immunomodulatory properties and the *in vivo* effects of these peptides' pleiotropic functions in specific clinical conditions.

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Appendix 1

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Cathelicidin Host Defence Peptide Augments Clearance of Pulmonary *Pseudomonas aeruginosa* Infection by Its Influence on Neutrophil Function *In Vivo*

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Abstract

Cathelicidins are multifunctional cationic host-defence peptides (CHDP; also known as antimicrobial peptides) and an important component of innate host defence against infection. In addition to microbicidal potential, these peptides have properties with the capacity to modulate inflammation and immunity. However, the extent to which such properties play a significant role during infection *in vivo* has remained unclear. A murine model of acute *P. aeruginosa* lung infection was utilised, demonstrating cathelicidin-mediated enhancement of bacterial clearance *in vivo*. The delivery of exogenous synthetic human cathelicidin LL-37 was found to enhance a protective pro-inflammatory response to infection, effectively promoting bacterial clearance from the lung in the absence of direct microbicidal activity, with an enhanced early neutrophil response that required both infection and peptide exposure and was independent of native cathelicidin production. Furthermore, although cathelicidin-deficient mice had an intact early cellular inflammatory response, later phase neutrophil response to infection was absent in these animals, with significantly impaired clearance of *P. aeruginosa*. These findings demonstrate the importance of the modulatory properties of cathelicidins in pulmonary infection *in vivo* and highlight a key role for cathelicidins in the induction of protective pulmonary neutrophil responses, specific to the infectious milieu. In additional to their physiological roles, CHDP have been proposed as future antimicrobial therapeutics. Elucidating and utilising the modulatory properties of cathelicidins has the potential to inform the development of synthetic peptide analogues and novel therapeutic approaches based on enhancing innate host defence against infection with or without direct microbicidal targeting of pathogens.

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Introduction

Cationic host-defence peptides (CHDP; also known as antimicrobial peptides or AMPs) are important components of early innate host defences. In addition to their physiological roles, these peptides and their derivatives have been proposed as future antimicrobial therapeutics, relatively unaffected by the development of sustained microbial resistance [1]. Although initially characterised as directly microbicidal agents, it is now clear that many CHDP also have multiple functions as modulators of inflammation and immunity [2,3,4], with emerging roles in diseases affecting multiple organs including the lung, skin and gastrointestinal tract. Human clinical trials using analogues of CHDP modified to maximise direct microbicidal function have achieved only moderate efficacy [5], perhaps due to failure to recognise the importance of the immunomodulatory functions of the native peptides. Interestingly, studies using non-microbicidal analogues of naturally-occurring CHDP that retained other bioactive functions, have demonstrated effective host defence augmentation in mice [6,7]. These studies raise questions about the relative roles of microbicidal and immunomodulatory properties of naturally-occurring CHDP in infections.

Cathelicidins are multipotent immunomodulatory CHDP [8]. The sole human cathelicidin Human Cationic Antimicrobial Peptide of 18 kDa (hCAP-18; encoded by *CAMP*) is expressed by multiple cell types including neutrophils, where it is stored in specific granules and proteolytically cleaved following release, to produce a 37 amino acid mature peptide fragment named LL-37 [9,10]. hCAP-18/LL-37 is upregulated in pulmonary infections [11] and, in children with RSV bronchiolitis, low serum cathelicidin is correlated with more severe disease [12]. Mice, like humans, express only a single cathelicidin gene; *Camp* (encoding the mCRAMP peptide) [13], with similar patterns of expression, which is cleaved to produce an active 34 amino acid peptide [14]. Mice deficient in mCRAMP (*Camp*−/−) have increased suscep-

tibility to bacterial infections of the skin [15], intestinal tract [16], cornea [17] and urinary tract [18]. These *Camp*−/− mice also have impaired host defence against lung infection [19,20], while therapeutic use of LL-37 and/or mCRAMP in wild type mice is protective in models of pulmonary infection with *P. aeruginosa* [21] or influenza virus [22]. These studies demonstrate a critical, non-redundant role for endogenous cathelicidin in host defence against lung infection and the therapeutic potential of the unmodified peptides, but the mechanisms by which pulmonary host defence is enhanced *in vivo* remains unclear. Although generally presented as being primarily a consequence of direct microbicidal activity, this is not fully consistent with *in vivo* concentrations and microbicidal properties in a physiological environment. However, the extent to which any of the plethora of immunomodulatory properties ascribed to cathelicidins play a significant role during infection has never been demonstrated *in vivo*. Understanding the critical modulatory roles of native CHDP and how these contribute to innate host defence against infection, may prove to be vital in development of specific pathogen-targeted analogues of these peptides for therapeutic use.

Respiratory diseases are among the most common causes of morbidity and account for 1 in 5 deaths in the UK [23]. A third of mortalities are due to acute respiratory infections, influenza or pneumonia and pathogens resistant to conventional therapeutics represent an increasing clinical challenge. *Pseudomonas aeruginosa* is the primary cause of nosocomial pulmonary infections and pulmonary colonisation with this pathogen is considered to be responsible for the fatal deterioration of lung function in patients with cystic fibrosis (CF) (reviewed in [24]). This opportunistic pathogen is difficult to treat because of its widespread resistance to multiple antibiotics [25], with the limited number of effective antimicrobial treatments reduced further by the emergence of carbapenem-and polymyxin-B resistant isolates [26]. A greater understanding of the natural host defence mechanisms involved in pulmonary defence against this organism is required in order to develop novel therapeutic approaches. Cathelicidins can alter susceptibility to pulmonary infection with *P. aeruginosa* in murine models [19,20,21], despite this pathogen being resistant to the directly microbicidal effects of these peptides in the presence of physiologically relevant levels of cations *in vitro* [27,28,29]. Thus, the *in vivo* roles of endogenous cathelicidin in host defence against *P. aeruginosa*, the relative effects of microbicidal and modulatory properties, and the consequences of therapeutic targeting of cathelicidin expression or exogenous delivery of peptide remain unknown.

We demonstrate that therapeutically administered synthetic LL-37 peptide can enhance the clearance of *P. aeruginosa* from the murine lung, in the absence of demonstrable direct microbicidal effects, and can induce an upregulation of the early neutrophil response to pathogen in the lungs that is dependent both upon the presence of the peptide and the pathogen. We show that despite a normal early neutrophil response, second phase pulmonary neutrophil influx was deficient in *Camp*−/− mice, with impaired clearance of pulmonary *P. aeruginosa*. Delivery of LL-37 to these cathelicidin-deficient mice enhanced the neutrophil response and restore bacterial clearance, demonstrating proof of principle for therapeutic use of LL-37 in cathelicidin deficiency. These studies indicate that the protective effects of cathelicidins in *P. aeruginosa* infection *in vivo* can result from modulatory effects in innate immune responses, synergising with infectious stimuli to enhance a protective neutrophil response.

Materials and Methods

Peptide

LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVP-RTES; MW 4493.33) was either synthesised by N-(9-fluorenyl) methoxycarbonyl chemistry at the Nucleic Acid/Protein Service unit at the University of British Columbia (UBC; Vancouver, Canada), or custom synthesised by Almac (East Lothian, Scotland) using Fmoc solid phase synthesis and reversed phase HPLC purification. Peptide identity was confirmed by electrospray mass spectrometry, purity (>95% area) by RP-HPLC and net peptide content determined by amino acid analysis. Lyophilised peptides were reconstituted in endotoxin free water at 5 mg/ml stock concentration and determined to be endotoxin-free using a Limulus Amebocyte Lysate Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, UK). Peptide functionality was confirmed by assessing anti-endotoxic activity [30].

Bacteria

Pseudomonas aeruginosa strain PAO1 was grown in Luria Bertani (LB) broth at 37°C in an orbital shaker (250 rpm) overnight to achieve a stationary-phase suspension. Bacterial cultures were then diluted 1:10 in fresh LB broth and incubated at 37°C for 90 min to reach logarithmic phase. Standardisation was performed by dilution with LB broth to an optical density of 0.1 at 595 nm using spectrophotometry (WPA UV 1101, Biotech Photometer), then bacteria were centrifuged at 1,500×g for 15 min and resuspended in PBS for use.

Murine infection model

Mouse experiments were performed in accordance with Home Office UK project licence 60/4216, under the Animal (Scientific Procedures) Act 1986. Wild type control mice were all C57Bl/6 strain, supplied by Charles River Laboratories, UK, and housed at the University of Edinburgh for at least two weeks before use, or were animals bred from those mice. *Camp* −/− mice [15] were bred to congenicity on a C57Bl/6 strain background and were the offspring of homozygous mutant matings raised in the same facility at the University of Edinburgh. Both male and female mice were used, between 8–12 weeks old, housed in individually ventilated cages and randomly assigned to treatment groups (no significant difference were found in end points between male and female mice). Mice were weighed, given a general anaesthetic (isofluorane) in a category 2 biosafety hood, then held vertically by scruffing over the front of the thorax and inoculated by an intranasal delivery up to a total of 50 µl volume. Mice were inoculated with 3×10^7 colony forming units (cfu) of PAO1 or the same volume of PBS, and 10 µg LL-37 peptide in PBS or PBS only control. PBS alone (carrier for both bacteria and peptide) was used as a control (rather than scrambled peptide, which previous pulmonary infection studies indicated had no effects ([22] and unpublished data), in order that the wild type control infected animals were appropriate controls both for the LL-37-treated infected wild types and for the infected *Camp*−/− animals (in which no peptide was delivered). Mice were then returned to cages, placed on a heat mat to maintain body temperature, and monitored and scored for signs of infection every 2 hours, with peak of illness occurring at 6–8 hours post infection, followed by recovery with diminishing severity score. Mice were re-weighed and culled at selected timepoints (0, 6 or 24 hours), culled by pentobarbital injection and lungs and trachea exposed by dissection. Lungs were lavaged in 1 ml sterile PBS via intramedic polyethylene tubing (Sigma Aldrich, UK) inserted into the trachea, and bronchoalveolar lavage fluid (BALF) stored on ice. Following

lavage, lungs were either homogenised in 2 ml sterile PBS for cfu counts or were perfused by PBS injection into the heart, then removed and frozen for RNA preparation.

CFU counts

BALF or homogenised lungs were serially diluted in PBS, plated on *Pseudomonas* Isolation agar (Becton Dickinson Difco, Oxford, UK), incubated overnight at 37°C and bacterial colonies counted using a Stuart SC6 colony counter. Total colonies on the lowest dilution plate countable were multiplied by the appropriate dilution factors to determine the total CFU count of the lung tissue or BALF sample.

Cytospins and counts

BALF was centrifuged at 200×g for 5 minutes, and supernatant was removed for cytokine measurement. Pelleted cells were resuspended and counted by NucleoCounter YC-100 (ChemoMetec, Allerød, Denmark) automated cell number counting. 100 µl of cell suspension was then loaded onto a glass slide using a disposable sample funnel and cytocentrifuged at 10×g for 3 minutes in a Shandon Cytospin 2 centrifuge. Slides were air dried for 20 minutes, fixed in methanol for 20 minutes, stained with Diff Quik (Fisher Scientific, Loughborough, UK), and mounted in DPX Mountant (Fluka BioChemika/Sigma Aldrich, UK). Differential counts for neutrophils and monocytes were then performed by light microscopy at 20× magnification using an EVOS FL microscope (Peqlab, Sarisbury Green, UK).

ELISAs

BALF was used to measure cytokine levels by ELISA according to manufacturer's instructions, for KC, MIP-2 alpha (R&D Systems, UK) or using a cytometric bead assay mouse inflammation kit (BD Biosciences, UK) for TNF, IL-6, MCP-1, IL-10, IFN-gamma, IL-12p70.

qRT-PCR

Mouse lung tissue was homogenized in Qiagen RLT buffer (Qiagen, Manchester, UK) using a Precellys 24 homogeniser with Precellys-Keramik-kit ceramic beads (PeqLab). RNA was then prepared from homogenised mouse lung tissue using RNeasy mini kits (Qiagen), according to the manufacturer's instructions. After DNase treatment with RQ1 DNase (Promega, Southampton, UK), cDNA was prepared from RNA using TaqMan reverse transcriptase reagents and random hexamer primers (Life Technologies Ltd, Paisley, UK), according to the manufacturer's instructions. Quantitative Real Time PCR was performed on a StepOne Real Time PCR machine (Life Technologies), using Gene Expression Mastermix and TaqMan gene expression assays for *Camp* (assay I.D. Mm00438285_m1) and 18S (assay I.D. Mm03928990_g1). Relative quantitation of *Camp* was calculated using the ΔCT method.

Analysis of mCRAMP protein expression

Harvested lung tissue was placed in 600 µl M-PER Mammalian Protein Extraction Reagent with Complete Protease Inhibitor Cocktail (Roche Applied Science, Burgess Hill, UK) added, and homogenised using a Precellys 24 homogeniser with Precellys-Keramik-kit ceramic beads (PeqLab). Homogenised tissue was shaken on an IKA-Vibramax-VXR (Sigma Aldrich, UK)) for 20 minutes at 4°C and lysates were subsequently centrifuged at 15,000×g for 10 minutes at 4°C to pellet insoluble material. Protein concentration in lysates was measured by Pierce BCA assay (Thermo Scientific), according to manufacturer's instructions. Lysate concentrations were equalised with lysis buffer, and subsequently boiled at 96°C for 5 minutes in the presence of loading buffer and reducing agent (Life Technologies), then run on Novex NuPAGE 4–12% Bis-Tris pre-cast gels, in MOPS buffer (Life Technologies), and subsequently transferred to Novex 0.2 µm pore Nitrocellulose Membrane. mCRAMP was detected with rabbit anti-mouse mCRAMP antibody (R-170, Santa Cruz Biotechnology, Heidelberg, Germany), followed by staining with IRDye 800CW anti-rabbit secondary antibody, with subsequent detection using a LI-COR Odyssey Infrared Imaging System. Rabbit pan-actin antibody (Cell Signalling Technology, Danvers, MA, USA) staining was used as a loading control, detected with anti-rabbit secondary antibody and infrared imaging as above. Mouse CRAMP staining was then quantitated using LI-COR Odyssey software.

Statistics

Statistical analyses were performed using Graphpad Prism version 5.04 for Windows. Bacterial counts were normalised by logarithmic transformation before analysis by 2 way ANOVA with Bonferroni's post tests where appropriate. Cell counts and cytokine concentrations were analysed by Mann Whitney test. Differences were considered statistically significant at $P<0.05$.

Results

LL-37-mediated microbicidal activity against *P. aeruginosa* PAO1 is not observed *in vivo*

LL-37 and other CHDP were initially described as having rapid direct microbicidal properties based on *in vitro* studies conducted under favourable ionic environments (reviewed in [27]). However, the activity of LL-37 can be inhibited by physiological levels of divalent cations [27,28], serum apolipoprotein, f-actin and DNA [31,32,33]. Thus, the relative contributions of microbicidal versus modulatory properties in conferring the protective effects of this peptide *in vivo* remain uncertain. We and others have shown that LL-37 has negligible microbicidal activity against *P. aeruginosa in vitro* [27,28,29]. In order to evaluate the contribution of any early direct microbicidal properties of exogenously delivered LL-37 peptide in a murine pulmonary *P. aeruginosa* infection model, mice were culled immediately after the intranasal delivery of bacteria with peptide or carrier-only control to the cohort. Lungs were removed, homogenised and plated to evaluate the number of viable bacteria in the lungs. These samples are referred to as t = 0, however homogenisation did not occur until 60 minutes after inoculation, during which time interaction between peptide and bacteria could occur. No significant difference was observed between infected mice receiving LL-37 and control infected animals (Fig 1a) demonstrating that LL-37, under these conditions, had no discernable early microbicidal affects.

Therapeutic delivery of LL-37 is protective against *P. aeruginosa* infection *in vivo*

In order to evaluate the protective antimicrobial properties of LL-37 against *P. aeruginosa* PAO1 *in vivo*, mice were infected with or without concomitant delivery of LL-37. All mice lost ~4% body weight in the first 6 hours post procedure (data not shown), with infected animals continuing to lose weight over 24 hours, but no significant effect of LL-37 treatment was observed (Fig 1b). Mice were culled 6 and 24 hours post-infection and the total number of viable bacteria in the BALF (bronchoalveolar lavage fluid) and lung homogenate was assessed (Fig 1 c–f). At 6 hours post-infection, treatment with LL-37 showed no statistically significant effect on the total number of bacteria in the lung homogenates, but

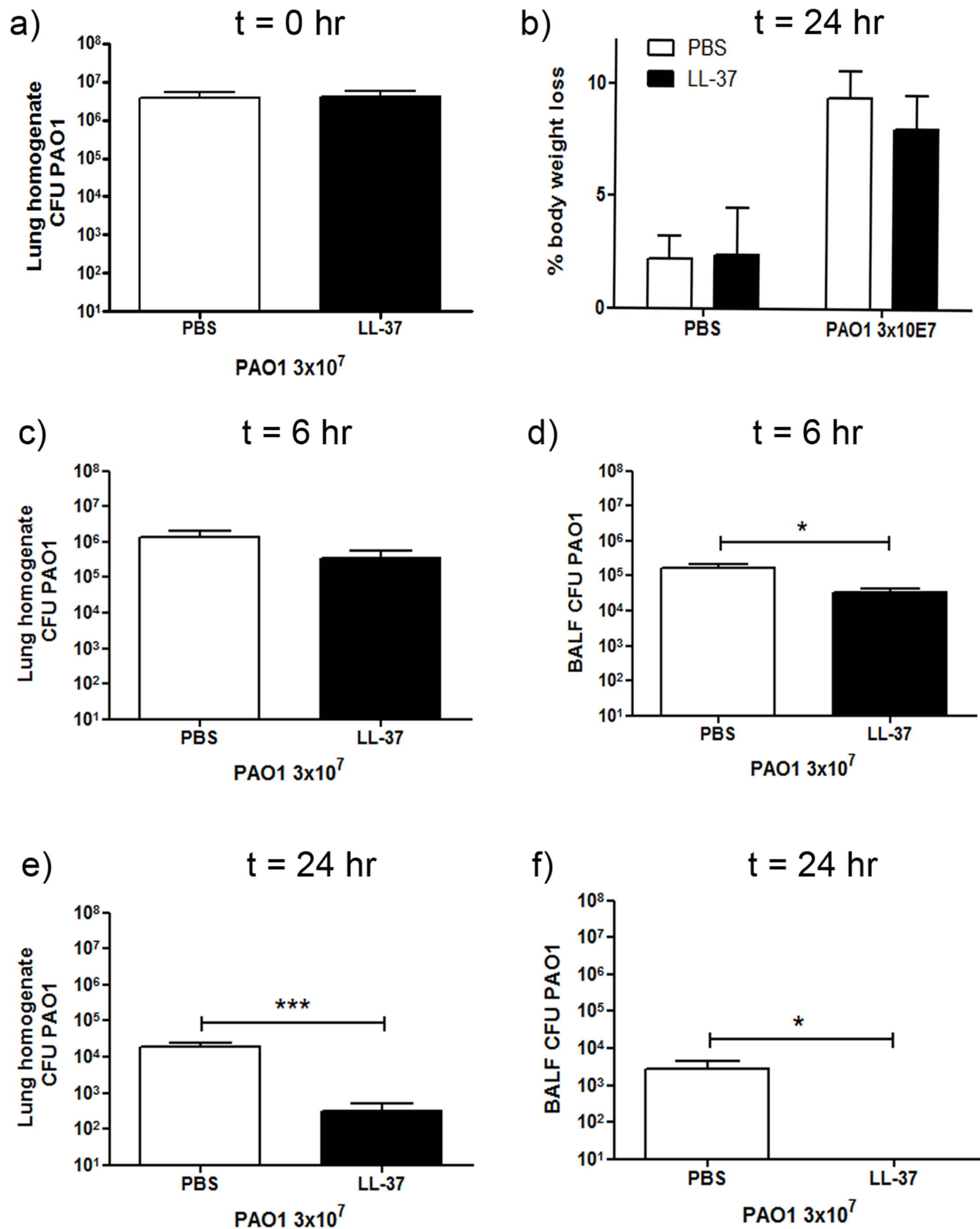

Figure 1. Exogenous LL-37 enhances pulmonary clearance of *P. aeruginosa*. Wild type C57Bl/6 mice were weighed, then inoculated with 3×10^7 cfu of *P. aeruginosa* PAO1 or PBS and 10 µg LL-37 peptide or PBS by intranasal delivery. a) Immediately after inoculation of all mice, a subset (called 0 hr; n = 3 per group) were culled and their lungs homogenised (60 minutes after initial inoculation), or b–f) 6 or 24 hours after inoculation mice were re-weighed and culled, and their lungs were lavaged before homogenisation. BALF and lung homogenates were serially diluted, plated and incubated overnight at 37°C before bacterial colonies were counted and corrected for volume. Mean PAO1 cfu +/− SEM in the lung homogenate (a, c & e) or BALF (d & f) for infected animals (n≥9 per condition) are displayed. No bacteria were detected in samples from uninfected mice. b) Data show mean percentage weight loss +/− SEM. For statistical analyses bacterial counts were normalised by logarithmic transformation. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests; * $p<0.05$, ** $p<0.01$.
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did result in significantly lower levels of bacteria in the BALF (Fig 1c/d). By 24 hours post-infection, LL-37 treatment had significantly enhanced pathogen clearance from the lungs, compared to controls, leaving only a residual infection, inaccessible to BAL (Fig 1e/f). These data demonstrate the capacity of LL-37 to enhance pulmonary bacterial clearance in the absence of early microbicidal properties.

Therapeutic delivery of LL-37 enhances neutrophil responses in infected animals

Cathelicidins have been proposed to have multiple inflammomodulatory properties that could modulate the clearance of infection *in vivo* [2], including direct chemotactic activity of LL-37 for neutrophils and monocytes [34,35,36,37,38]. Differential cytospin cell counts were performed on the BALF from LL-37-treated and control-treated infected and uninfected mice at 6 and 24 hours after infection. LL-37 treatment resulted in a significantly upregulated neutrophil response to infection (over 2 fold increase in median cell number) compared to control infected animals at 6 hours post-infection (Fig 2a). No neutrophils were detected in the BALF from uninfected mice, regardless of LL-37 treatment, at this timepoint (Fig 2b). Whereas a larger second phase neutrophil response was observed by 24 hours in control-treated infected mice, the mean neutrophil number in the LL-37-treated infected animals was actually significantly lower than the controls at this timepoint, in line with the enhanced earlier clearance of the infection (Fig 2c). A degree of neutrophil influx was observed in response to LL-37 alone at 24 hours in uninfected mice (Fig 2d). In contrast, no significant LL-37-mediated effects on pulmonary monocyte numbers were observed at 6 or 24 hours in infected (Fig 2 e/f) or uninfected (data not shown) mice.

Therapeutic delivery of LL-37 does not affect pulmonary cytokine responses in infected animals

In order to determine whether LL-37-mediated enhanced neutrophil responses were secondary to modulation of pulmonary cytokine and chemokine responses, CBA (Cytometric Bead Array) and ELISA assays were performed on the BALF from LL-37-treated and control-treated infected mice at 6 and 24 hours after infection, to determine the concentrations of TNF, IL-6, MIP-2, KC, MCP-1, IL-10, IFNγ and IL-12. Although TNF, IL-6, MIP-2, KC and MCP-1 were all highly expressed in response to infection at 6 hours (Fig 3a–e) compared to baseline levels in uninfected mice (data not shown), and resolving by 24 hours (Fig 3f–j), treatment with LL-37 had no significant effect on any of the cytokines measured. In contrast, IL-10, IFNγ and IL-12 were not detected in significant quantities.

Pulmonary infection with *P. aeruginosa* induces *Camp* expression in the murine lung

In this model, therapeutic administration of LL-37 was in addition to any effects of endogenous murine cathelicidin mCRAMP produced in the murine lungs in response to infection. mCRAMP has also been shown to have neutrophil chemotactic properties *in vivo*, in an air pouch model [36]. Thus, in order to establish the temporal expression pattern of *Camp* in *P. aeruginosa* infected mice, qRT-PCR and western immunoblot analyses were performed on lung homogenates at 0, 2, 6 and 24 hours post-infection. *Camp* transcription was not detected at 0 hour, but was detectable at very low levels by 2 hours after infection. Transcription was dramatically increased at 6 and 24 hours after infection (upregulated 1886-fold, +/−137, and 1124-fold, +/−66 respectively, relative to the 2 hour timepoint), with mCRAMP protein clearly detectable at these timepoints (data not shown) in keeping with previously published data [20]. Thus, the inflammatory responses were potentially modified by cathelicidin from around 6 hours post-infection in all mice, but additionally modified by cathelicidin within the first few hours in mice receiving an intranasal bolus of LL-37.

Endogenous mCRAMP is protective against *P. aeruginosa* infection *in vivo*

In order to evaluate the protective antimicrobial properties of induced endogenous mCRAMP against *P. aeruginosa* PAO1 in this model, *Camp* −/− mice were infected and compared to wild type control animals. The profile of weight loss in infected *Camp* −/− mice was not significantly different from the wild type control (data not shown). Mice were culled 6 and 24 hours post-infection and the total number of viable bacteria in the BALF and lung homogenate was assessed (Fig 4 a–d). At 6 hours post-infection *Camp* −/− mice showed no statistically significant difference in the total number of bacteria in the lung homogenates or BALF when compared to infected wild type controls (Fig 4 a/b). However, by 24 hours post-infection, wild type mice had more effectively cleared the bacteria, with significantly higher pathogen loads found in both the lung homogenates and BALF from the *Camp* −/− mice (Fig 4c/d). These data demonstrate the capacity of endogenous mCRAMP to enhance pulmonary bacterial clearance, occurring after inducible *Camp* expression is detectable in the lungs of wild-type mice.

Endogenous mCRAMP enhances neutrophil responses in infected animals

The extent to which endogenous mCRAMP might play a role in the pulmonary neutrophil response to *P. aeruginosa* infection was examined by comparing BALF cytospin differential cell counts from infected *Camp* −/− mice and wild type mice at 6 and 24 hours after infection. No significant differences were observed in neutrophil or monocyte counts at 6 hours (Fig 5a/b), with an initial neutrophil influx occurring similarly in both genotypes (Fig 5a). However, *Camp* −/− mice failed to further upregulate this response, demonstrating a significantly less elevated neutrophil count than wild type controls at 24 hours (Fig 5c). A trend towards fewer monocytes was also observed, but did not reach significance (Fig 5d). These data indicate that endogenous mCRAMP is not involved in the first phase of neutrophil influx, but is required, following induction, for the second phase neutrophil response to *P. aeruginosa* infection. TNF, IL-6, MIP-2, KC and MCP-1 were all highly expressed in response to infection in *Camp* −/− mice at 6 hours (Fig 6a–e), and resolving by 24 hours (Fig 6f–j), but were not significantly different from the responses quantified in infected wild type mice.

Therapeutic delivery of LL-37 can restore protection against *P. aeruginosa* infection in cathelicidin deficient mice

Cathelicidin insufficiency has been associated with increased susceptibility to infection in humans [12,39,40]. Thus, in order to determine whether therapeutic use of synthetic human LL-37 could restore cathelicidin-mediated protective antimicrobial function in cathelicidin deficiency, *Camp* −/− mice were infected with *P. aeruginosa* PAO1 *in vivo*, with or without concomitant delivery of LL-37. The profile of weight loss in infected *Camp* −/− mice was not significantly altered by delivery of LL-37 (data not shown). Mice were culled 6 and 24 hours post-infection and the total number of viable bacteria in the BALF (bronchoalveolar lavage fluid) and lung homogenate was assessed (Fig 7 a–d). At 6 hours post-infection, treatment with LL-37 showed no significant effect on the total number of bacteria in the lung homogenates (despite a trend towards enhanced clearance), but led to significantly lower levels of bacteria in the BALF (Fig 7a/b). By 24 hours post-infection, LL-37 treatment had very significantly enhanced pathogen clearance from the lungs, compared to control-treated

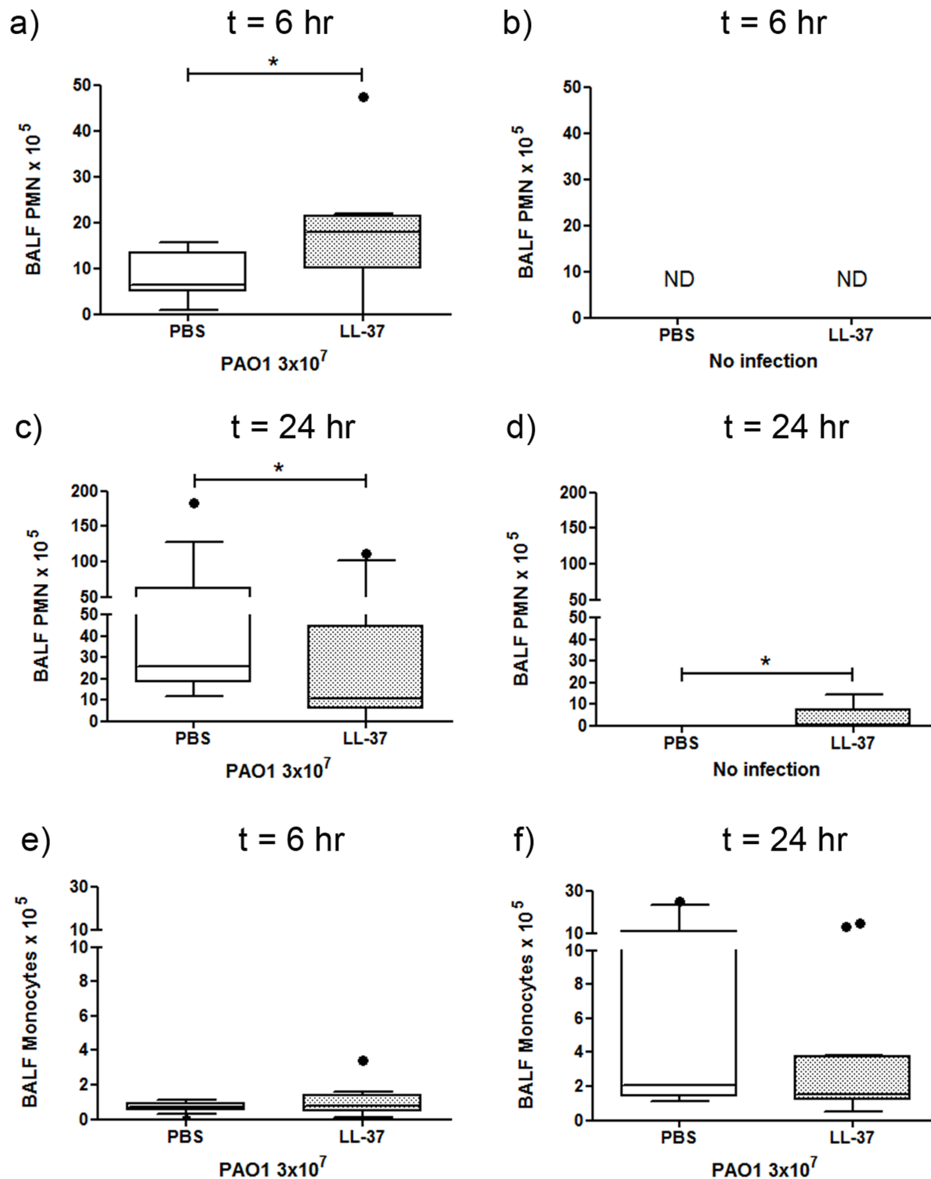

Figure 2. Exogenous LL-37 promotes an early neutrophil response to *P. aeruginosa*. Wild type C57Bl/6 mice were inoculated with 3×10^7 cfu of *P. aeruginosa* PAO1 or PBS and 10 µg LL-37 peptide or PBS by intranasal delivery. At 6 hours (a, b & e) or 24 hours (c, d & f) after inoculation mice were culled and their lungs were lavaged. BALF was cytocentrifuged and differential counts were conducted for neutrophils (a–d) and monocytes (e & f). Data show Tukey box and whiskers plots for infected (a, c, e & f) (n≥9 per condition) and uninfected (b & d) animals (n≥5 per condition). Analyses were conducted using the Mann Whitney test; * $p<0.05$. ND denotes "not detected".
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Camp −/− (Fig 7c/d). These data demonstrate that delivery of exogenous synthetic LL-37 can enhance host defence against infection by mechanisms that do not require endogenous host cathelicidin production, and show cross-species functionality of these peptides.

Therapeutic delivery of LL-37 promotes an early neutrophil response to *P. aeruginosa* infection, associated with enhanced clearance

Cathelicidin-mediated enhancement of bacterial clearance was associated with upregulated neutrophil influx in LL-37-treated infected wild type mice (compared to untreated infected controls). However, in addition, endogenous cathelicidin clearly also had a critical role in the induction of a maximal neutrophil responses to infection. Thus, in order to determine whether LL-37-mediated enhanced neutrophil responses were independent of endogenous cathelicidin production, BALF cytospin differential cell counts were also evaluated from LL-37-treated and control infected *Camp* −/− mice at 6 and 24 hours after infection. As also observed in LL-37-treated wild type mice (Fig 2a), early, infection-induced neutrophil influx (at 6 hours) was significantly greater in LL-37-treated mice (Fig 8a), but this early effect of the therapeutic bolus was lost by 24 hours (Fig. 8b). However, whereas wild type mice showed a robust later neutrophil response to infection regardless of peptide treatment (Fig 2c), this second phase neutrophil response failed to occur in infected *Camp* −/− mice, irrespective of peptide treatment (Fig 8b), demonstrating the dependence of this later response upon pathogen-induced *Camp* expression. No significant

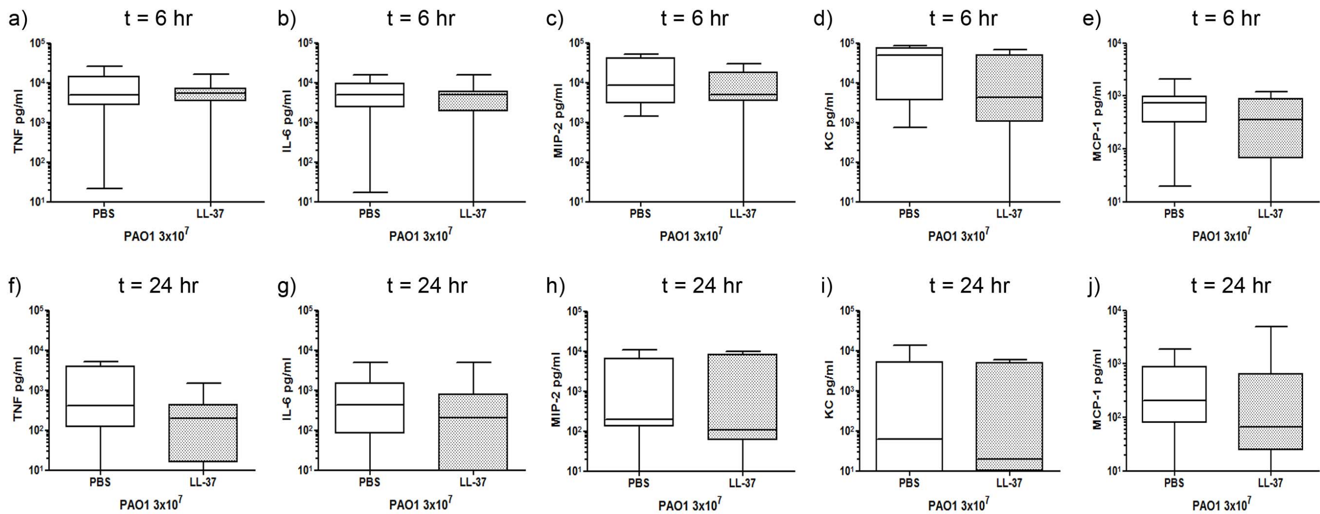

Figure 3. *P. aeruginosa*, but not exogenous LL-37, induces pulmonary cytokine responses. Wild type C57Bl/6 mice were inoculated with 3×10^7 cfu of *P. aeruginosa* PAO1 and 10 µg LL-37 peptide or PBS by intranasal delivery. At 6 hours (a–e) or 24 hours (b–j) after inoculation, mice were culled and their lungs were lavaged. BALF was centrifuged to remove cells and levels of TNF (a, f), IL-6 (b, g), MIP-2 (c, h), KC (d, i) and MCP-1 (e, j) were determined. Data show Tukey box and whiskers plots for n≥9 animals per condition. Analyses were conducted using the Mann Whitney test.
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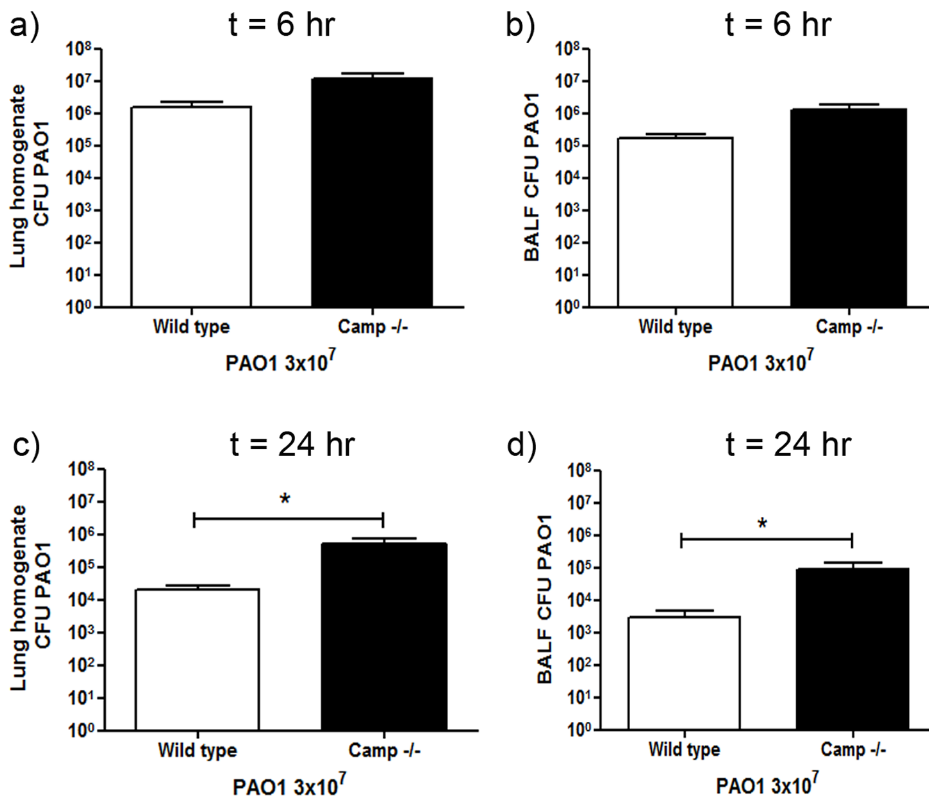

Figure 4. Cathelicidin-deficient mice display impaired pulmonary clearance of *P. aeruginosa*. *Camp−/−* mice and wild type controls were inoculated with 3×10^7 cfu of *P. aeruginosa* PAO1 or PBS by intranasal delivery. At 6 or 24 hours after inoculation mice were culled and their lungs were lavaged before homogenisation. BALF and lung homogenates were serially diluted, plated and incubated overnight at 37°C before bacterial colonies were counted and corrected for volume. Mean PAO1 cfu +/− SEM in the lung homogenate (a & c) or BALF (b & d) for infected animals (n≥10 per condition) are displayed. No bacteria were detected in samples from uninfected mice. For statistical analyses bacterial counts were normalised by logarithmic transformation. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests; * $p<0.05$.
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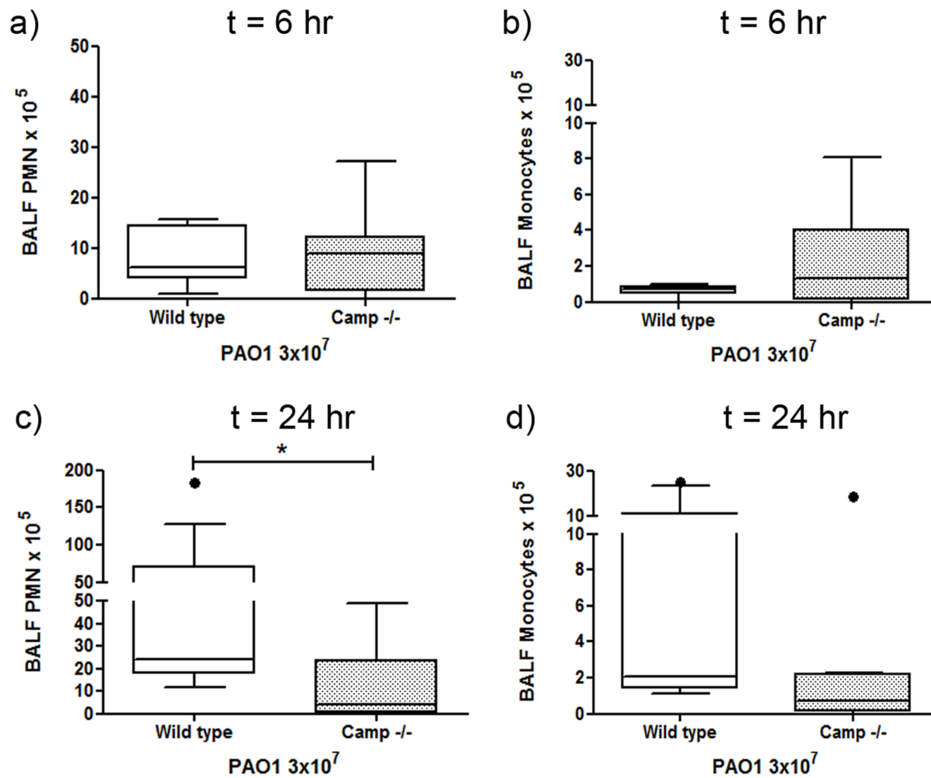

Figure 5. Cathelicidin-deficient mice display impaired late neutrophil responses to *P. aeruginosa*. *Camp−/−* mice and wild type controls were inoculated with 3×10^7 cfu of *P. aeruginosa* PAO1 by intranasal delivery. At 6 hours (a & b) or 24 hours (c & d) after inoculation mice were culled and their lungs were lavaged. BALF was cytocentrifuged and differential counts were conducted for neutrophils (a & c) and monocytes (b & d). Data show Tukey box and whiskers plots for n≥8 animals per condition. Analyses were conducted using the Mann Whitney test; * $p<0.05$.
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effects upon monocyte counts were observed (data not shown). These data demonstrate that the early infection-mediated neutrophil response, enhanced by the bolus of LL-37, was independent of endogenous cathelicidin expression and associated with enhanced clearance of pulmonary *P. aeruginosa*.

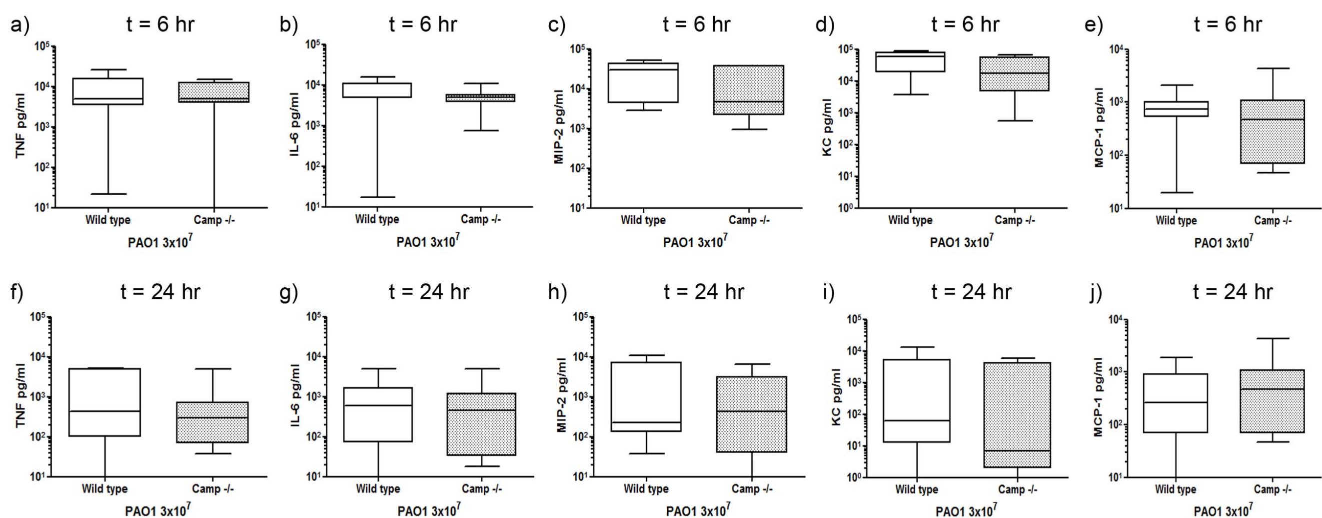

Figure 6. *P. aeruginosa*, but not cathelicidin sufficiency, induces pulmonary cytokine responses. *Camp−/−* mice and wild type controls were inoculated with 3×10^7 cfu of *P. aeruginosa* PAO1 by intranasal delivery. At 6 hours (a–e) or 24 hours (b–j) after inoculation, mice were culled and their lungs were lavaged. BALF was centrifuged to remove cells and levels of TNF (a, f), IL-6 (b, g), MIP-2 (c, h), KC (d, i) and MCP-1 (e, j) were determined. Data show Tukey box and whiskers plots for n≥8 animals per condition. Analyses were conducted using the Mann Whitney test.
doi:10.1371/journal.pone.0099029.g006

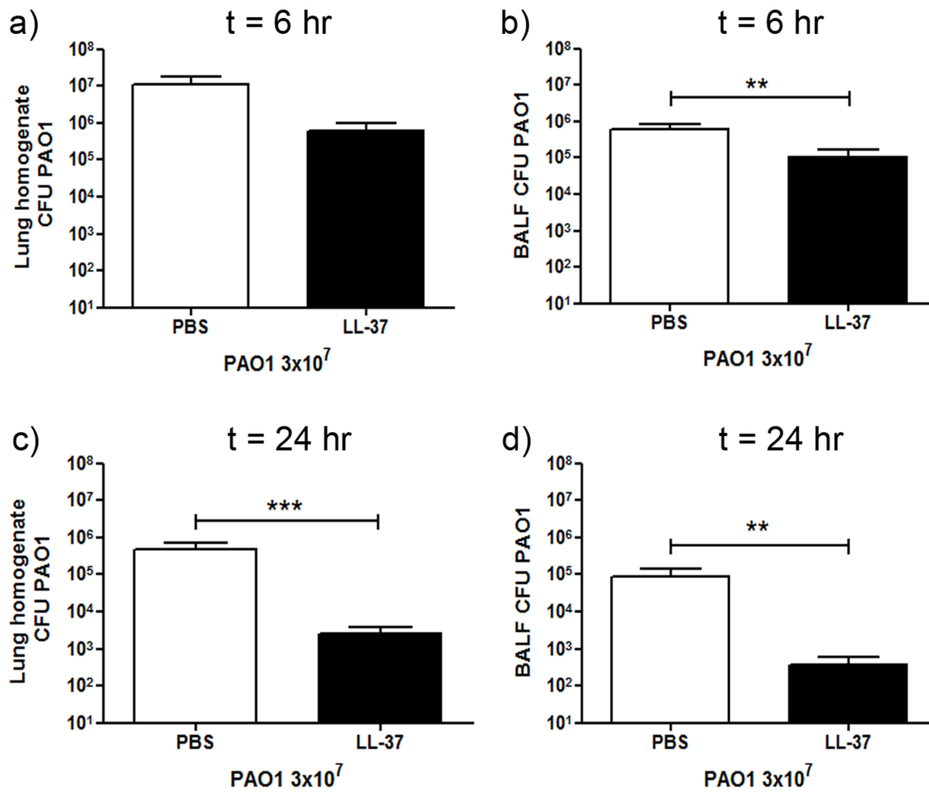

Figure 7. Exogenous LL-37 enhances pulmonary clearance of *P. aeruginosa* in cathelicidin-deficient mice. *Camp*−/− mice were inoculated with 3×10^7 cfu of *P. aeruginosa* PAO1 or PBS and 10 μg LL-37 peptide or PBS by intranasal delivery. At 6 or 24 hours after inoculation mice were re-weighed and culled, and their lungs were lavaged before homogenisation. BALF and lung homogenates were serially diluted, plated and incubated overnight at 37°C before bacterial colonies were counted and corrected for volume. Mean PAO1 cfu +/− SEM in the lung homogenate (a & c) or BALF (b & d) for infected animals (n≥6 per condition at 6 hours and n≥10 per condition at 24 hours) are displayed. No bacteria were detected in samples from uninfected mice. For statistical analyses bacterial counts were normalised by logarithmic transformation. Analyses were conducted using 2 way ANOVA with Bonferroni's post tests; ** $p<0.01$, *** $p<0.001$.
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Discussion

Cathelicidins are recognised as key multifunctional modulators of innate immunity and host defence against infection, and offer possible novel therapeutic templates. In addition to directly microbicidal potential, these peptides have been described as having a broad range of inflammomodulatory and immunomodulatory properties [2]. However, no clear evidence exists for these functions being involved in cathelicidin-mediated enhanced host defence against pulmonary infection *in vivo*, with the relative significance of microbicidal potential and modulatory functions remaining unclear. Using a murine model of acute *P. aeruginosa* lung infection, we demonstrate cathelicidin-mediated enhancement of bacterial clearance *in vivo* in the absence of direct early

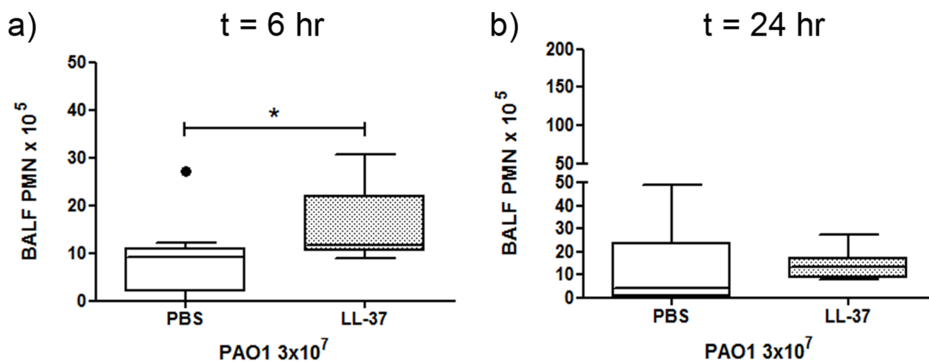

Figure 8. Exogenous LL-37 promotes an early neutrophil response to *P. aeruginosa* in cathelicidin-deficient mice. *Camp*−/− mice were inoculated with 3×10^7 cfu of *P. aeruginosa* PAO1 and 10 μg LL-37 peptide or PBS by intranasal delivery. At 6 hours (a) or 24 hours (b) after inoculation mice were culled and their lungs were lavaged. BALF was cytocentrifuged and differential counts were conducted for neutrophils. Data show Tukey box and whiskers plots for n≥8 animals per condition. Analyses were conducted using the Mann Whitney test; * $p<0.05$.
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microbicidal activity. Administration of synthetic LL-37 promoted an upregulation of the early neutrophil response that was dependent upon both infection and peptide, but was independent of native cathelicidin production, and enhanced bacterial clearance from the lung. Mice deficient in endogenous mCRAMP had a normal initial neutrophil response to infection, during a period in which *Camp* was not initially expressed in the wild type lung. However, these *Camp*−/− mice were deficient in the larger second phase neutrophil response observed in *Camp*-expressing infected wild type mice and had an impaired capacity to clear the infection. To our knowledge, this is the first demonstration of cathelicidins enhancing host defence against infection through primarily modulatory mechanisms *in vivo* and suggests induction of a cathelicidin-mediated protective proinflammatory response.

The associations between hCAP-18/LL-37 expression and susceptibility to infection in humans [39][40][11][12] suggest an important, but as yet undefined role for hCAP-18/LL-37 in innate host defence against infection in humans. Although this peptide has microbicidal potential, its activity is poor against many microorganisms in physiologically relevant environments at the low concentrations found *in vivo* in most systems [27,28,29]. However, cathelicidins have additionally been shown to have multiple modulatory activities, including chemotactic function [34,35,36,37,38], the ability to modulate chemokine, cytokine and cellular responses [30,41,42,43], the capacity to alter leukocyte differentiation and function [44,45,46] and cell death modulating properties [28,47,48]. Critical to understanding these peptides and utilising their properties therapeutically is the need to clarify their modes of action *in vivo* in infectious contexts.

In this study, an acute murine pulmonary infection model with *P. aeruginosa* was utilised in order to evaluate the capacity of cathelicidins to enhance host defence against infection with a microbe which is largely resistant to these peptides under physiological conditions *in vitro* [27,28,29]. Under favourable *in vitro* conditions in which microbicidal properties are evident for LL-37, this peptide has been shown to permeabilise bacterial membranes within minutes [49]. However, we found no evidence for direct microbicidal activity against *P. aeruginosa* after co-incubation with LL-37 *in vivo*, yet exogenously delivered LL-37 was found to significantly enhance pathogen clearance over 24 hours. Although we cannot exclude some alternative form of late direct microbicidal activity of LL-37, even by 6 hours after infection no significant impact on bacterial load of the whole lung could be demonstrated in response to LL-37 treatment, although interestingly a therapeutic bolus of peptide was found to diminish the number of live bacteria accessible to bronchoalveolar lavage at this time point. The reason for this is unclear, but may relate to early removal of the most accessible bacteria by the enhanced neutrophil influx observed. A previous study using adenoviral vectors carrying the cDNA for hCAP18/LL-37, to overexpress the human cathelicidin in the murine lung over the 5 days prior to infection, resulted in the promotion of a small, but significant enhancement of *P. aeruginosa* clearance from the murine lung over a 24 hour period [21]. This was observed to be accompanied by decreased pulmonary TNF levels, but the mechanism underpinning this therapeutic effect was not evaluated and was assumed to be microbicidal. In contrast, we found no evidence to support a microbicidal effect, but demonstrate a peptide-mediated enhanced early neutrophil influx *in vivo*.

Prior research has demonstrated the capacity for cathelicidins to have direct chemotactic activity for human neutrophils and monocytes and murine leukocytes *in vitro* [34,36] and for murine leukocytes in an experimentally-formed murine air pouch model [36]. In that *in vivo* model, injection of 2 μM LL-37 or mCRAMP into the air pouch significantly enhanced the influx of neutrophils and monocytes within a 4 hour period. This is in contrast to the complete absence of neutrophils observed in our studies in the murine lung 6 hours after instillation of LL-37 alone (Figure 2b). In addition, LL-37 was not found to mediate any significant effects on the number of monocytes in the BALF, in contrast to the previously published findings in other systems [35,36]. A small, but significant neutrophil response was observed in the lungs of LL-37-treated uninfected mice at 24 hours after instillation (Figure 2d), demonstrating some LL-37-mediated neutrophil influx. However, indirect effects cannot be excluded and LL-37 has also been shown to enhance the production of neutrophil chemokines by other cells [30,50]. Nevertheless, despite this absence of any substantial response to LL-37 alone, a significantly enhanced pulmonary neutrophilia was observed in response to LL-37 upon concomitant infection (Figure 2a). With regard to this apparent contradiction to previous findings, it is worth noting that even the control air pouches in the previous report yielded a substantial number of neutrophils [36], indicating that this was already an inflamed environment and may in fact be analogous to infected lungs in our study. The requirement for concomitant infection in order to establish the early enhanced LL-37-mediated neutrophil response suggests a synergy with inflammatory mediators that remain to be identified, with no peptide-mediated modulation having been observed in the levels of the chemokines studied (including KC, MIP2, MCP-1, TNF and IL-6). Nevertheless, these data provide clear evidence for the *in vivo* capacity of exogenous LL-37 to modulate the innate cellular immune response in the context of pulmonary infection, enhancing pathogen clearance in the absence of microbicidal activity and having potential therapeutic implications.

In addition to the potential therapeutic roles of exogenous cathelicidins, the primary roles of endogenous peptides in pulmonary infection remain unclear. Studies using *Camp*−/− mice have demonstrated a deficiency in the clearance of both pulmonary *Klebsiella pneumoniae* and *P. aeruginosa* infections [19,20]. *K. pneumoniae* promoted a later induction of pulmonary *Camp* expression than *P. aeruginosa* and mCRAMP appeared to have a more potent effect on *K. pneumoniae*, with *Camp*−/− mice having a significant and severe clearance defect at 24 hours, resulting in more florid inflammation by 48 hours in the absence of endogenous cathelicidin and increased mortality [20]. Given that, in the case of *K. pneumoniae*, mCRAMP was reported to have microbicidal effects at a relatively modest 1 μM, it is possible that the phenotype in this particular infection was influenced by loss of a relevant microbicidal agent in the face of a lethal infectious dose. Indeed, the late induction of *Camp* in wild type mice infected with *K. pneumoniae* may suggest less relevance for the peptide in the inflammatory response to this particular infection. In contrast, we demonstrate that *P. aeruginosa* infection can ultimately be controlled and cleared even in the absence of mCRAMP, and the earlier expression in wild type mice may indicate a more important role in the inflammatory response. The previous study reported that *Camp*−/− mice infected with *P. aeruginosa* (at a lower infectious dose than in our study) has a significantly impaired bacterial clearance at 48 hours, with a decreased neutrophil response at 24 hrs [20]. These observations are compatible with our study, but were attributed to a loss of a direct chemotactic response to endogenous cathelicidin. Our new data, examining earlier timepoints, indicate that the initial murine pulmonary neutrophil response to *P. aeruginosa* infection precedes induction of and is independent of mCRAMP, and thus proceeded normally in *Camp*−/− mice. However, second phase neutrophil influx was dependent upon mCRAMP expression, which may synergise with

infection-induced factors as yet unidentified, and was therefore defective in *Camp*−/− mice, in whom impaired pathogen clearance then occurred. These data suggest that in individuals with impaired endogenous cathelicidin production, an effective, protective pulmonary inflammatory response will be suboptimal.

hCAP-18/LL-37 in humans is pre-formed in neutrophil granules, but can also be induced in a vitamin-D dependent manner in epithelial cells and macrophages [51,52,53,54]. In addition, strategies to induce LL-37 expression are under development, including the use of compounds such as 4-phenylbutyrate (reviewed in [55]), which can effectively upregulate hCAP-18/LL-37 expression *in vitro*, including in airway epithelial cells [56], and *in vivo* in a model of Shigella infection [57]. Such approaches may be of value in enhancing protective cathelicidin expression in humans, particularly in vitamin-D insufficient seasonal conditions. However, it was also important to consider whether therapeutic application of cathelicidin could provide rapid short term improvement of host defence in the absence of effective endogenous cathelicidin expression. In this regard, our studies demonstrate that the delivery of LL-37 to *P. aeruginosa* infected mice could promote an early neutrophil response and enhanced pathogen clearance in *Camp*−/− mice as effectively as in wild type mice. These data indicate that this protective effect was in response to the exogenous LL-37 delivered, independent of endogenous mCRAMP expression and of native cathelicidin release from incoming PMN and supports the potential for the use of exogenous peptides in infection.

Thus, using a murine model of acute *P. aeruginosa* lung infection, we demonstrate cathelicidin-mediated enhancement of bacterial clearance *in vivo* in the absence of direct microbicidal activity. The delivery of exogenous cathelicidin functioned to enhance a protective pro-inflammatory response to infection, promoting bacterial clearance from the lung, with an infection- and peptide-dependent early neutrophil response that was independent of native cathelicidin production. Furthermore, although *Camp*−/− mice had an intact early cellular inflammatory response (which was comparable to cathelicidin-sufficient animals in the period preceding the induction of mCRAMP expression), they had significantly impaired bacterial clearance and absence of a second phase neutrophil response to infection. These finding demonstrate the importance of the inflammomodulatory properties of cathelicidins in pulmonary infection *in vivo* and highlight the significance of understanding and utilising these properties in the development of novel therapeutic approaches.

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Author Contributions

Conceived and designed the experiments: PEB BM JRWG AJS DJD. Performed the experiments: PEB BM EGF AM KJM. Analyzed the data: PEB BM DJD. Contributed reagents/materials/analysis tools: RLG JRWG. Wrote the paper: BM DJD. Expert revision of the manuscript: RLG AJS.

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The Human Cathelicidin LL-37 Preferentially Promotes Apoptosis of Infected Airway Epithelium

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Cationic host defense peptides are key, evolutionarily conserved components of the innate immune system. The human cathelicidin LL-37 is an important cationic host defense peptide up-regulated in infection and inflammation, specifically in the human lung, and was shown to enhance the pulmonary clearance of the opportunistic pathogen *Pseudomonas aeruginosa in vivo* by as yet undefined mechanisms. In addition to its direct microbicidal potential, LL-37 can modulate inflammation and immune mechanisms in host defense against infection, including the capacity to modulate cell death pathways. We demonstrate that at physiologically relevant concentrations of LL-37, this peptide preferentially promoted the apoptosis of infected airway epithelium, via enhanced LL-37–induced mitochondrial membrane depolarization and release of cytochrome *c*, with activation of caspase-9 and caspase-3 and induction of apoptosis, which only occurred in the presence of both peptide and bacteria, but not with either stimulus alone. This synergistic induction of apoptosis in infected cells was caspase-dependent, contrasting with the caspase-independent cell death induced by supraphysiologic levels of peptide alone. We demonstrate that the synergistic induction of apoptosis by LL-37 and *Pseudomonas aeruginosa* required specific bacteria–epithelial cell interactions with whole, live bacteria, and bacterial invasion of the epithelial cell. We propose that the LL-37–mediated apoptosis of infected, compromised airway epithelial cells may represent a novel inflammomodulatory role for this peptide in innate host defense, promoting the clearance of respiratory pathogens.

Keywords: cationic host defense peptide; antimicrobial peptide; innate immunity; *Pseudomonas*; apoptosis

Cationic host defense peptides (CHDPs; also known as antimicrobial peptides) are key, conserved components of innate host defenses. The broad-spectrum, direct microbicidal potential of CHDPs has made these peptides attractive therapeutic agents. However, many CHDPs were further demonstrated to exert multiple potential immunomodulatory functions, including the modulation of cell death, raising questions about the nature of their primary physiologic roles and the possibility of developing novel therapeutics with both microbicidal and immunomodulatory activities.

One of the CHDPs demonstrating the most significant immunomodulatory potential to date is LL-37. This cationic, amphipathic peptide is the predominant cleavage product of human cationic antimicrobial peptide (hCAP)-18, the sole human cathelicidin (reviewed by Zanetti) (1). LL-37 is stored at high concentrations in the specific granules of neutrophils, and is produced by epithelial cells and some leukocytes. It can be detected in airway surface liquid, plasma, sweat, and other body fluids, and is upregulated in response to infection and inflammation (1, 2). In addition to direct microbicidal capability, the modulatory potential of LL-37 is considerable, with *in vitro* and *in vivo* studies suggesting a broad range of activities that could modify innate inflammatory processes and adaptive immune responses (2). The physiological significance of LL-37 to human disease is demonstrated by the increased susceptibility to infection of individuals with morbus Kostmann (in which defective neutrophils are cathelicidin-deficient) (3), and is also suggested by the association between hCAP-18 expression and susceptibility to skin infections in psoriasis and atopic dermatitis (4). In addition, studies using a mouse model deficient in cathelin-related antimicrobial peptide (mCRAMP), the murine ortholog of LL-37, demonstrated increased susceptibility to infections of the skin, gastrointestinal system, urinary tract, and cornea (5–8). Despite this clear evidence of a critical role for cathelicidin expression in innate defense against infection, the relative roles of the microbicidal and immunomodulatory activities of this peptide remain unclear.

Gene therapy augmentation demonstrated that the expression of LL-37 in the murine lung can enhance the clearance of pulmonary *Pseudomonas aeruginosa* (9), an important opportunistic pulmonary pathogen of immunocompromised individuals and those with cystic fibrosis (10). However, the mechanisms underlying enhanced defense against infection in this model remain unclear, with the concentrations of LL-37 detected unlikely to be directly microbicidal under physiological conditions (9, 11). Multiple mechanisms are likely involved in the host defense against lung infection with *P. aeruginosa*, ranging from simple mucociliary clearance and innate microbicidal components of airway surface liquid, to the activity of professional phagocytes. In addition, the apoptosis and subsequent removal of infected epithelial cells were described as innate defense mechanisms at diverse epithelial surfaces (12–14), required for the clearance of invasive *P. aeruginosa* from the murine lung (12). Such a mechanism may be an important component of host defenses, removing bacteria that have evaded other defenses and invaded epithelial cells.

LL-37 was previously demonstrated to modulate cell death pathways (15–21). We previously demonstrated that high concentrations of LL-37 can induce apoptosis in airway epithelial cell lines and primary cells *in vitro*, and in murine airways *in vivo* (15, 17). Moreover, LL-37 was shown to induce mitochondrial depolarization in alveolar epithelial cells (18).

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However, the roles of the Bcl2-family proteins, which can regulate mitochondrial membrane potential, and of the key apoptosis-inducing caspase proteins in LL-37, which can induce apoptosis of airway epithelial cells, remain uncertain. Further, it is unclear whether LL-37–induced apoptosis might be primarily detrimental, with overexpression of LL-37 damaging normal epithelial integrity, or whether at much lower, more physiological concentrations, LL-37 expression could enhance innate defenses by promoting targeted apoptosis to facilitate the clearance of pathogens. To address these issues, we studied the ability of LL-37 to induce apoptosis in airway epithelial cells infected with the invasive lung pathogen *P. aeruginosa*.

We demonstrate that LL-37 can induce Bax-dependent mitochondrial membrane depolarization in airway epithelial cells in a dose-dependent manner, with the release of cytochrome *c*, and that this is synergistically enhanced by infection with *P. aeruginosa*. However, at physiologically relevant concentrations of LL-37, the activation of caspase-9 and caspase-3, and DNA fragmentation, only occurred in the presence of both peptide and bacteria, but not with either stimulus alone. This synergistic induction of apoptosis was caspase-dependent and partly Bax-dependent, and required specific bacteria–epithelial cell interaction with whole, live bacteria capable of epithelial-cell invasion.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, non-essential amino acids (NEAAs), PBS, trypsin/EDTA, and FBS were all purchased from PAA Laboratories (Somerset, UK). Primary normal human bronchial epithelial (NHBE) media and growth supplements were purchased from Lonza (Wokingham, UK). Fibronectin, BSA, Tween-20, Luria Bertani broth, formalin, chemiluminescence peroxidase substrate, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate were all supplied by Sigma-Aldrich (Dorset, UK). Mouse collagen type IV (Cultrex) was purchased from Stratech Scientific, Ltd. (Suffolk, UK). Ultroser G was obtained from Pall Pharmaceuticals (Hampshire, UK). Precise protein polyacrylamide gels, M-PER protein extraction reagent, and protease/phosphatase inhibitor cocktails were supplied by Thermo Scientific (Loughborough, UK). Vectashield Hardset mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) was supplied by Vector Laboratories (Peterborough, UK). The Bax-inhibiting peptide V5 (BIP-V5) and Z-VAD-FMK were supplied by Merck Chemicals, Ltd. (Nottingham, UK). Rabbit anti-human cleaved caspase-3, caspase-8, and caspase-9 antibodies (catalogue numbers 9661, 9496, and 9505, respectively), anti-human X-linked inhibitor of apoptosis protein (XIAP) (catalogue number 2045), anti-human pan actin (catalogue number 4968), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies were purchased from Cell Signaling Technologies (Danvers, MA). We extracted *Pseudomonas aeruginosa* PAO1 lipopolysaccharide (LPS) using a 90% aqueous phenol solution at 65°C and ultracentrifugation, followed by quantification using a limulus amebocyte lysate assay (Cambrex, Wokingham, UK). LL-37 (sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES; molecular weight 4,493.33) was synthesized by *N*-(9-fluorenyl) methoxycarbonyl chemistry at the Nucleic Acid/Protein Service Unit at the University of British Columbia (Vancouver, Canada), as described previously (22). Scrambled LL-37 control peptide (sequence RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL) was purchased from CSS-Albachem, Ltd. (East Lothian, UK). Peptides were purified by reverse-phase high-performance liquid chromatography, and were at least 98% pure. LL-37, and scrambled LL-37 control peptide, were dissolved in endotoxin-free water (Sigma-Aldrich) and stored at −20°C until further use. The concentration of peptides in solution was determined by amino-acid analysis, and tested to ensure that they were free of endotoxin.

Primary and Transformed Epithelial-Cell Culture

The 16HBE14o$^-$ transformed human bronchial epithelial cells were a kind gift from Dieter Gruenert at the University of California, San Francisco. Cells were grown in standard submerged cultured and maintained in DMEM supplemented with 1% L-glutamine (vol/vol), 1% NEAA (vol/vol), and 10% FBS (vol/vol). Culture flasks were coated with a basement layer of collagen IV (5 μg/ml), fibronectin (10 μg/ml), and BSA (100 μg/ml) before cell culture at 37°C, 5% CO_2. Normal human bronchial epithelial cells from a single donor were purchased from Lonza. Cells were grown in standard submerged culture and maintained in bronchial epithelial growth media (Lonza), supplemented with bronchial epithelial cell SingleQuots growth factors and supplements (Lonza) as a serum substitute, in strict accordance with the manufacturer's instructions.

All assays were conducted in Ultroser G serum–substitute supplemented media, consisting of phenol red–free DMEM supplemented with 1% Ultroser G (vol/vol), 1% L-glutamine (vol/vol), and 1% NEAA (vol/vol).

Bacterial Strains and Culture

In addition to *P. aeruginosa* PAO1, these studies used the following strains of *P. aeruginosa*: clinical isolate J1386 (a clonal isolate of J1385, originally from an individual with cystic fibrosis) (23), Δ*mexAB-oprM* mutant (a gift from Keith Poole) (24), PAO1*exsA*::Ω mutant (a gift from Dara Frank) (25), *P. aeruginosa pilA* mutant (a gift from Eva Lorenz) (26), and the isogenic PAO1 control strains for these mutants. Studies involving genetically modified bacteria were performed according to Scientific Advisory Committee on Genetic Modification Health and Safety Executive Certificate GM207/07.2.

All *P. aeruginosa* strains were grown in Luria Bertani (LB) broth at 37°C in an orbital shaker (250 rpm) overnight, to achieve a stationary-phase suspension. Before use, bacterial suspensions diluted 1:20 in fresh LB broth were incubated at 37°C for 90 minutes to reach log phase. Bacterial suspensions were standardized via dilution to an optical density of 0.1 at 595 nm, using spectrophotometry (WPA UV 1101, Biotech Photometer; Biochrom Ltd., Cambridge, UK), centrifuged at 1,500 × *g* for 15 minutes (keeping supernatant where required for use in place of live bacteria), and resuspended in PBS before immediate addition to epithelial cells. Where required, bacteria were heat-killed (60°C for 60 minutes in an orbital shaker) or ultraviolet light (UV)-killed (exposed to a constant UV source for 2 hours in a sealed glass Petri dish), with killing confirmed by overnight culture. To determine the direct microbicidal activity of LL-37, *P. aeruginosa* were resuspended in Ultroser G serum–substitute supplemented media before the immediate addition of LL-37 at the concentrations stated. After incubation for 1 hour at 37°C, serial dilutions were performed, and 100-μl aliquots of these (and the original bacterial suspension) were spread onto LB agar plates in triplicate, and incubated overnight at 37°C before counting the number of colony-forming units (CFUs). For studies to determine if the function of the Δ*mexAB-oprM* mutant could be rescued by soluble factors released by PA01, PA01 was added to 16HBE14o$^-$ cells at a multiplicity of infection (MOI) of 10:1 and incubated for 18 hours at 37°C with 5% CO_2. After incubation, the supernatant was collected and filtered through a 0.22-μm filter unit. The sterility of filtered supernatant was confirmed by culturing 50 μl on LB agar plates for 24 hours. Filtered supernatant (1:4 dilution in treatment medium) was simultaneously added together with LL-37 and Δ*mexAB-oprM* to 16HBE14o$^-$ cells, and the cells were incubated for 1 hour and analyzed using the mitochondrial depolarization technique.

Mitochondrial Depolarization Assay

The 16HBE14o$^-$ cells were seeded at 2.5×10^4 cells per well in a 96-well plate and cultured at 37°C, 5% CO_2. Cells were exposed to LL-37 (or scrambled LL-37 control peptide) at the concentrations described in the presence and absence of (1) log-phase *P. aeruginosa* at an MOI of 10:1; (2) heat-killed or UV-killed bacteria (MOI 10:1), *P. aeruginosa* PAO1 LPS (1 μg/ml) or *P. aeruginosa* supernatant, all prepared as described above; or (3) log-phase *P. aeruginosa* PAO1 (MOI 10:1), separated from the epithelial cells by a Transwell semipermeable polyester membrane with 0.4-μm pore size (Corning Life Sciences,

Amsterdam, Netherlands), and incubated for 1 hour at 37°C, 5% CO_2. All treatments were conducted in Ultroser G serum–substitute supplemented media. After treatments, cells were washed once with PBS prewarmed to 37°C, the supernatant was aspirated, and 50 μl of Mitocapture solution (Cambridge Bioscience, Cambridge, UK) at 37°C were added to each well, before incubation at 37°C for 30 minutes in the dark. Cells were then washed twice with PBS at 37°C, resuspended in 50 μl of Mitocapture buffer at 37°C, and imaged immediately using an Axiovert S100 inverted fluorescent microscope (Zeiss UK, Welwyn Garden City, UK). For each membrane, at least four random fields of view were counted with a minimum of 300 cells in total, and the number of apoptotic cells (displaying diffuse, green fluorescence) was expressed as a percentage of the number of healthy cells (displaying punctate red mitochondrial fluorescence). Data were corrected for a background level of approximately 10% positive cells observed in control untreated samples. For inhibition studies, the culture medium in each well was replaced with treatment medium containing 100 μM BIP-V5 for 1 hour before treatment.

Cytochrome *c* Assay

The 16HBE14o$^-$ cells were seeded at 1×10^6 cells per well in a six-well plate and cultured at 37°C, 5% CO_2. Cells were exposed to LL-37 at the concentrations described in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1), and incubated for 90 minutes at 37°C, 5% CO_2. Cells were washed once with PBS, and 0.5 ml of trypsin/EDTA was added to each well to detach cells. Ultroser G serum–substitute supplemented media (0.5 ml) was added to each well and suspensions were centrifuged in microtubes at $850 \times g$ for 2 minutes. Mitochondrial and cytosolic fractions were then prepared using a Mitochondrial Isolation Kit (Thermo Scientific, Loughborough, UK) according to the manufacturer's instructions. Cytochrome *c* concentrations in each fraction were assessed using a Cytochrome *c* ELISA Kit (Merck Chemicals, Ltd.), according to the manufacturer's protocol.

Western Immunoblotting

The 16HBE14o$^-$ cells were seeded at 1×10^6 cells per well in six-well plates and cultured at 37°C, 5% CO_2. Cells were exposed to LL-37 at the concentrations described, in Ultroser-G serum–substitute supplemented media, in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1) and incubated for 3 hours at 37°C, 5% CO_2. Cells were washed once with ice-cold PBS and lysed with 300 μL M-PER protein extraction reagent (Thermo Scientific) containing a cocktail of protease, phosphatase, and metalloprotease inhibitors. Protein concentrations were determined by bicinchoninic acid assay (Thermo Scientific). Equivalent total protein concentration lysates (15–40 μg) were resolved on either 10% or 12% precast Precise Protein polyacrylamide gels (Thermo Scientific), transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Ltd., Hemel Hempstead, UK), blocked for 1 hour with Tris-buffered saline and 0.1% Tween-20 (TBST) containing 5% skimmed milk powder (TBST/milk), and then incubated with antibodies specific for cleaved caspase-3 (1 in 5,000 dilution), cleaved caspase-9 (1 in 1,000 dilution), XIAP (1 in 1,000 dilution), or pan-actin (1 in 2,000 dilution) in TBST/milk overnight at 4°C. Membranes were washed for 15 minutes in TBST and then incubated with a 1 in 5,000 dilution of HRP-conjugated goat anti-rabbit antibody (in TBST/milk) or a 1 in 5,000 dilution of HRP-conjugated goat anti-mouse antibody (in TBST/milk) for 1 hour at room temperature. Membranes were washed for 30 minutes and developed with chemiluminescence peroxidase substrate (Sigma-Aldrich) according to the manufacturer's instructions, and imaged on CL-Xposure film (Thermo Scientific). Equal loading of protein was confirmed by examining the expression of actin as a loading control.

In Situ Cell Death Detection by Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay

Transwell polyester-permeable supports (pore size, 0.4 μm; diameter, 6.5 mm; Corning Life Sciences) were equilibrated for 45 minutes in culture media (DMEM supplemented with 10% FBS [vol/vol], 1% L-glutamine [vol/vol], and 1% NEAA [vol/vol]) before the addition of 100 μl of medium containing 2.5×10^5 16HBE14o$^-$ cells/ml into the apical compartment, with 600 μl culture medium in the basal compartment, and cultured at 37°C, 5% CO_2. For primary bronchial epithelial cell experiments, Transwell supports were equilibrated with NHBE culture media for 45 minutes before the addition of 100 μl of NHBE media containing 2.5×10^5 NHBE cells/ml into the apical compartment, with 600 μL of NHBE culture medium in the basal compartment, and cultured at 37°C, 5% CO_2. Before treatments, culture media in both the apical and basal compartments were replaced with Ultroser G serum–substitute supplemented media. Cells were exposed to LL-37 at the concentrations described in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1), and incubated for 6 hours at 37°C, 5% CO_2. Cells were fixed in 10% neutral-buffered formalin (3.7% formaldehyde) for 10 minutes, washed once in PBS, permeabilized in ice-cold 0.1% Triton X-100/0.1% sodium citrate for 3 minutes, and washed twice with PBS. An *in situ* cell death detection kit (Roche Applied Science, West Sussex, UK) was used according to the manufacturer's instructions. The membranes with cells were mounted in 50 μL Vectashield Hardset (containing DAPI), and at least four random fields of view were counted (each containing more than 100 cells), using an Axiovert S100 fluorescent microscope, and analyzed using OpenLAB 3.0 software (Improvision/Perkin Elmer, Waltham, MA). The number of terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL)–positive cells was expressed as a percentage of the number of DAPI-positive nuclei. The total number of DAPI-positive nuclei counted for each condition was determined, to evaluate total cell number. For inhibition studies, cells were prepared as described, and culture medium in each well was replaced with treatment medium containing either 100 μM Bax inhibiting peptide V5 (Merck Chemicals, Ltd.) or 50 μM of the broad-spectrum caspase inhibitor, Z-VAD-FMK (Merck Chemicals, Ltd.), for 1 hour before treatment.

Gentamicin Exclusion Assay

The capacity of *P. aeruginosa* isolates and mutants to invade epithelial cells was assessed using a gentamicin exclusion assay. Briefly, 16HBE14o$^-$ cells were exposed to strains of log-phase *P. aeruginosa* (MOI 10:1) for 60 minutes in Ultroser G–serum-substitute supplemented media. The media were removed from all wells, and cells were incubated with fresh media for 60 minutes with or without gentamicin (50 μg/ml) to kill extracellular bacteria. The media were then aspirated from gentamicin-treated cells, and these cells were vigorously washed with PBS and lysed with PBS containing 0.1% Triton X-100, and then plated on LB agar to determine internalized bacterial numbers. Media and/or epithelial cell lysates from wells without gentamicin were also plated on LB agar, to determine the number of associated bacteria and total infectious load. The CFUs were quantified by culturing overnight on LB agar plates at 37°C.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism version 5 for Windows (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed either using one-way ANOVA with Tukey's *post hoc* test, or two-way ANOVA with Bonferroni's *post hoc* test where appropriate. $P \leqslant 0.05$ was considered significant.

RESULTS

LL-37 and *P. aeruginosa* Synergistically Induce Epithelial Cell Death

To determine the capacity of LL-37 to induce cell death in infected airway epithelial cells, the human bronchial epithelial cell line 16HBE14o$^-$ was infected with *P. aeruginosa* PAO1, with or without concurrent exposure to LL-37. These cells were examined for nuclear DNA fragmentation by TUNEL assay at 6 hours (Figure 1A). *Pseudomonas aeruginosa* alone did not induce cell death, and LL-37 alone induced cell death only at higher concentrations. However, concurrent exposure to both stimuli synergistically induced significant levels of cell

death at greater than or equal to 20 μg/ml of LL-37 ($P \leqslant 0.01$), and even at an LL-37 concentration of 20 μg/ml that had no effect alone. A control scrambled LL-37 peptide had no effect. Total cell counts demonstrated no loss of cells during analysis (data not shown). To confirm these observation in nontransformed cells, primary human airway epithelial cells were used, and demonstrated the same response, with significant cell death induced in the presence of physiologically inflammatory levels of LL-37 only when infected with *P. aeruginosa* PAO1 (Figure 1B).

To determine whether the cell death observed was apoptosis, cleavage of the key executioner caspase, caspase-3, was determined by Western immunoblot in 16HBE14o^{-} cells at 3 to 6 hours after infection with *P. aeruginosa* PAO1, with or without concurrent exposure to LL-37. No activation was detected in response to LL-37 alone, or *P. aeruginosa* alone. However, concurrent exposure to both stimuli resulted in caspase-3 activation at 4 hours and thereafter (Figure 1C and data not shown). These data indicate that the cell death induced synergistically by LL-37 and *P. aeruginosa*, but not by high concentrations of LL-37 alone, is caspase-dependent apoptosis. This finding is supported by the observation that preincubation with the polycaspase inhibitor Z-VAD-FMK significantly ($P \leqslant 0.001$) inhibited the synergistic induction of cell death by *P. aeruginosa* and LL-37 (Figure 1A), reducing it to approximately the level induced by LL-37 alone at that concentration.

In addition, cleavage of caspase-9 (a key cytochrome *c*–activated initiator caspase) was also observed in response to infection with *P. aeruginosa* PAO1, only in the presence of LL-37 (Figure 1D). Caspase-9 activation was not detected in response to LL-37 alone, or *P. aeruginosa* alone. In contrast, the activation of caspase-8 (a key death receptor–activated initiator caspase) was not evident (data not shown). These data demonstrate a synergistic induction of intrinsic apoptosis-inducing pathways.

Given the absence of caspase-3 activation in response to concentrations of LL-37 at which peptide alone induced cell

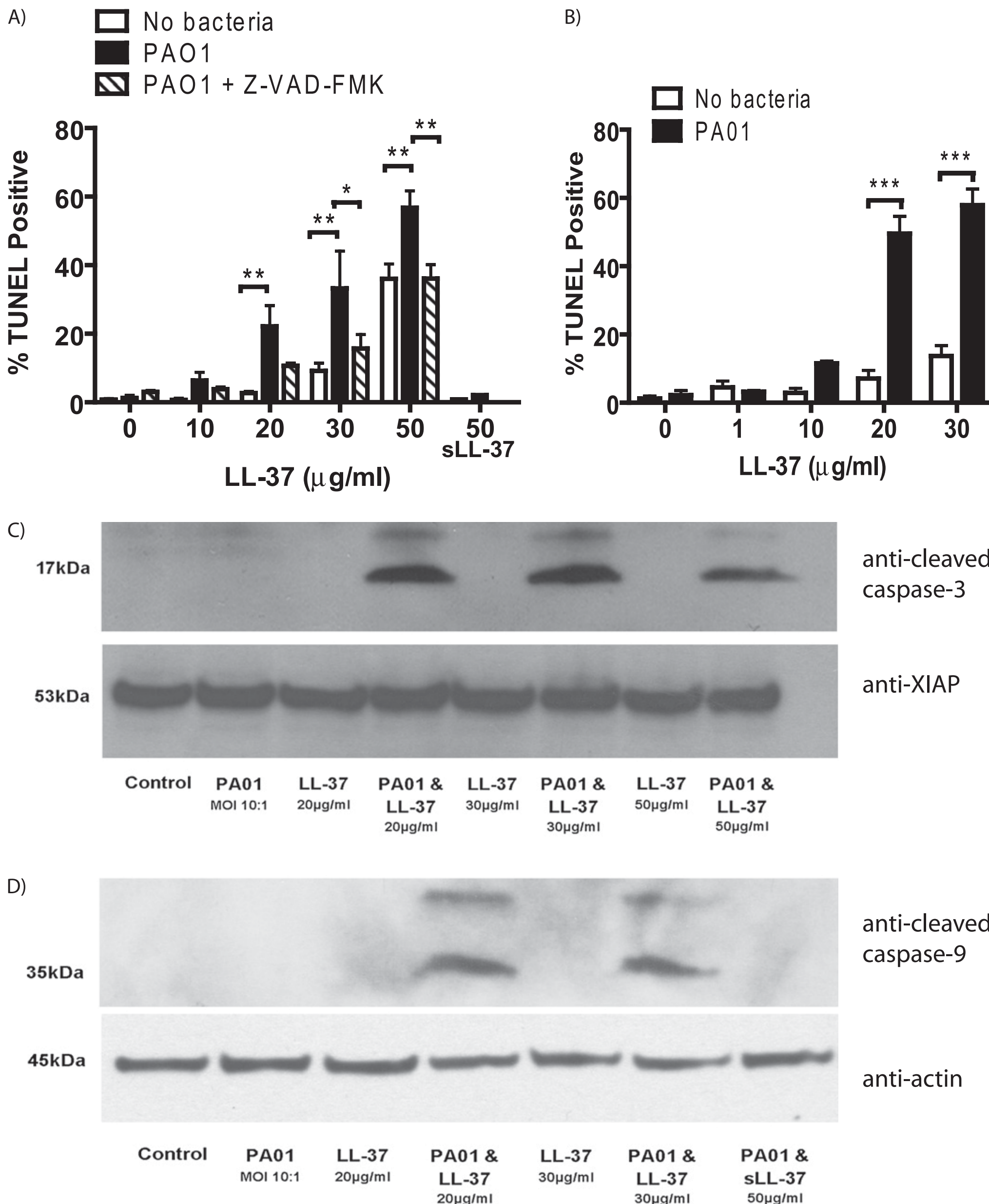

Figure 1. LL-37 and *P. aeruginosa* synergistically induce DNA fragmentation and caspase activation in airway epithelial cells. Human bronchial epithelial cell line 16HBE14o^{-} (*A*, *C*, *D*) or primary human bronchial epithelial cells (*B*) were incubated for 6 hours (*A*, *B*) or 5 hours (*C*, *D*) over a range of LL-37 concentrations (or scrambled LL-37 [*sLL-37*] at 50 μg/ml) in Ultroser G serum–substitute supplemented media, in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1) added concurrently. (*A*, *B*) Cells were treated as described, with or without preincubation for 1 hour with the polycaspase inhibitor Z-VAD-FMK (50 μM), and were then fixed. Apoptosis was assessed by TUNEL assay. Four random fields of view, each containing more than 100 cells, were counted for each sample. and the number of TUNEL-positive cells was expressed as a percentage of the number of DAPI-positive nuclei. Data represent mean values ± SEM, for $n \geqslant 3$ independent experiments for each condition. Two-way ANOVA with Bonferroni *post hoc* test was used to compare LL-37/*P. aeruginosa*–treated samples with LL-37 only–treated samples at corresponding concentrations, or LL-37/*P. aeruginosa*/Z-VAD-FMK–treated samples with LL-37/*P. aeruginosa*–treated samples at corresponding concentrations. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$. (*C*, *D*) Whole-cell protein lysates were prepared and analyzed by SDS-PAGE and Western immunoblotting. Immunoblots were performed using antibodies specific for cleaved caspase-3, XIAP, cleaved caspase-9, or actin. Images shown are representative of $n \geqslant 3$ independent experiments.

death, the expression levels of XIAP (a potent caspase inhibitor) were examined, but no effect on expression levels was evident (Figure 1C).

Pseudomonas aeruginosa Infection of Airway Epithelial Cells Synergistically Enhances LL-37–Mediated Mitochondrial Depolarization and Cytochrome *c* Release

To determine the role of mitochondria in LL-37–induced cell death, 16HBE14o$^-$ cells were infected with *P. aeruginosa* PAO1, with or without concurrent exposure to LL-37. After 1 hour, the mitochondrial membrane potential was determined as an early indicator of apoptosis (Figure 2A). The LL-37 alone induced a dose-dependent increase in mitochondrial depolarization at greater than or equal to 20 μg/ml. *Pseudomonas aeruginosa* alone had no effect, but synergized with LL-37 to induce significantly greater mitochondrial depolarization than LL-37 alone, even inducing increased depolarization at low (10 μg/ml) LL-37 concentrations that had no effect alone ($P \leqslant 0.05$). Scrambled LL-37 peptide had no effect (Figure 2A). To determine whether this synergistic effect required an initial interaction between LL-37 and either the epithelial cell or the bacteria, which could subsequently alter bacteria–epithelial cell interactions, 16HBE14o$^-$ cells were infected with *P. aeruginosa* PAO1 for 1 hour, and washed before incubation with LL-37 for 1 hour. Under these conditions, the synergistic induction of mitochondrial depolarization was still evident, and even amplified at lower LL-37 concentrations (Figure 2B). This result indicates that infection with *P. aeruginosa* promotes airway epithelial cell susceptibility to LL-37-induced apoptosis.

To evaluate the consequences of mitochondrial depolarization, the intracellular localization of cytochrome *c* was examined 90 minutes after 16HBE14o$^-$ cells were infected with *P. aeruginosa* PAO1, with or without concurrent exposure to LL-37 (Figure 2C). The LL-37 alone induced a dose-dependent relocalization of cytochrome *c* from the mitochondria to the cytoplasm, reflecting the mitochondrial depolarization and TUNEL positivity observed, and reaching significance at 50 μg/ml LL-37 ($P \leqslant 0.01$). *Pseudomonas aeruginosa* alone had no effect, but synergized with LL-37 to induce a highly significant translocation of cytochrome *c* at all concentrations of LL-37 tested ($P \leqslant 0.001$). This latter effect was surprisingly pronounced, with very significant translocation observed even at 10 μg/ml of LL-37, a concentration at which significant cell death was not evident. Effects as yet unexplained on the mitochondria under these conditions (but not in response to peptide alone or bacteria alone) may have led to further translocation of cytochrome *c* from the mitochondria during sample preparation, with a resultant amplification of the effect observed. Cytoplasmic cytochrome *c* was detected by Western immunoblot in response to 10–30 μg/ml LL-37 only in infected cells (data not shown). Thus, the cytoplasmic translocation of cytochrome *c* was clearly evident under these conditions.

To determine whether the LL-37–mediated induction of apoptosis was dependent on the key proapoptotic Bcl-2 family protein Bax, the effects of exposure to LL-37 and *P. aeruginosa* on mitochondrial depolarization (Figure 3A) and DNA fragmentation (Figure 3B) were evaluated after preincubation with the Bax-inhibiting peptide V5 (BIP-V5). At high concentrations of LL-37, at which LL-37 alone induced substantial mitochondrial depolarization and apoptosis, the inhibition of Bax significantly ($P \leqslant 0.01$) and almost completely blocked these effects. In contrast, Bax inhibition only partly inhibited the combined effect of LL-37 and *P. aeruginosa*. These data demonstrate that caspase-independent induction of cell death by LL-37 alone is Bax-dependent. However, additional, and as yet unidentified, components are required for the synergistic enhancement of mitochondrial depolarization and induction of caspase-dependent apoptosis by LL-37 in *P. aeruginosa*–infected cells.

Synergistic Induction of Apoptosis by LL-37 and *P. aeruginosa* Requires Specific Bacteria–Epithelial Cell Interactions with Live Bacteria

To exclude the possibility that LL-37 exerted directly microbicidal effects on *P. aeruginosa* PAO1, bacterial viability was determined after exposure to LL-37 over the range of concentrations and in the culture media used for these studies (Figure 4A). No significant, direct microbicidal activity was evident.

To examine whether the synergistic induction of apoptosis by LL-37 and *P. aeruginosa* required infection with live bacteria, and/or could result from secreted products, 16HBE14o$^-$ cells were exposed to a range of bacterial stimuli in the presence or absence of concurrent exposure to 30 μg/ml LL-37, and assessed for mitochondrial depolarization (Figure 4B). The highly significant ($P \leqslant 0.001$), synergistic induction of mitochondrial depolarization observed after exposure to live *P. aeruginosa* and LL-37 was completely lost if the bacteria used were dead (heat-killed or UV-killed), or substituted with bacterial culture supernatant, or LPS prepared from PAO1 (1 μg/ml). Furthermore, physical separation of the epithelial cells from the bacteria by a semipermeable membrane also completely prevented this effect. These data indicate that the synergistic induction of apoptosis by LL-37 and *P. aeruginosa* requires a physical interaction between the epithelial cells and viable bacteria, and is not simply the result of pathogen-sensing by extracellular pattern recognition receptors.

Synergistic Induction of Apoptosis by LL-37 and *P. aeruginosa* Is Isolate-Specific, and Independent of Type III Secretion System and Pilus Expression

To exclude the possibility that the synergistic induction of apoptosis by LL-37 and *P. aeruginosa* was specific to PAO1, a clinical *P. aeruginosa* isolate J1386 was examined (Figure 5A). A synergistic induction of mitochondrial depolarization was also observed in response to this clinical isolate in the presence of LL-37. This finding was substantially enhanced in comparison to that observed using the laboratory strain PAO1, with significant effects observed in infected cells after incubation with concentrations of LL-37 greater than or equal to 1 μg/ml ($P \leqslant 0.01$). No direct microbicidal effect of LL-37 was observed on *P. aeruginosa* isolate J1386 (data not shown).

To examine whether common virulence factors differentially expressed by divergent *P. aeruginosa* isolates were necessary for this effect, mutant strains of *P. aeruginosa* PAO1 were used (Figures 5B and 5C). No substantial difference was evident when comparing an ExsA mutant with a defective type III secretion system (PAO1*exsA*::Ω; Figure 5B) or a pilus mutant (*pilA* mutant; Figure 5C) with their corresponding isogenic strains. No direct microbicidal effect of LL-37 was evident in either mutant strain (data not shown). These data demonstrate that common determinants of virulence associated with epithelial-cell interactions (pilus) and bacterially induced epithelial cell death (type III secretion system) are not required for the synergistic induction of apoptosis in LL-37–treated infected epithelial cells.

Synergistic Induction of Apoptosis by LL-37 and *P. aeruginosa* Requires Epithelial-Cell Internalization of Bacteria

The internalization of *P. aeruginosa* by airway epithelial cells was proposed as a key component of the innate pulmonary host defense that is defective in cystic fibrosis (27). To determine the significance of bacterial internalization, a MexAB-OprM de-

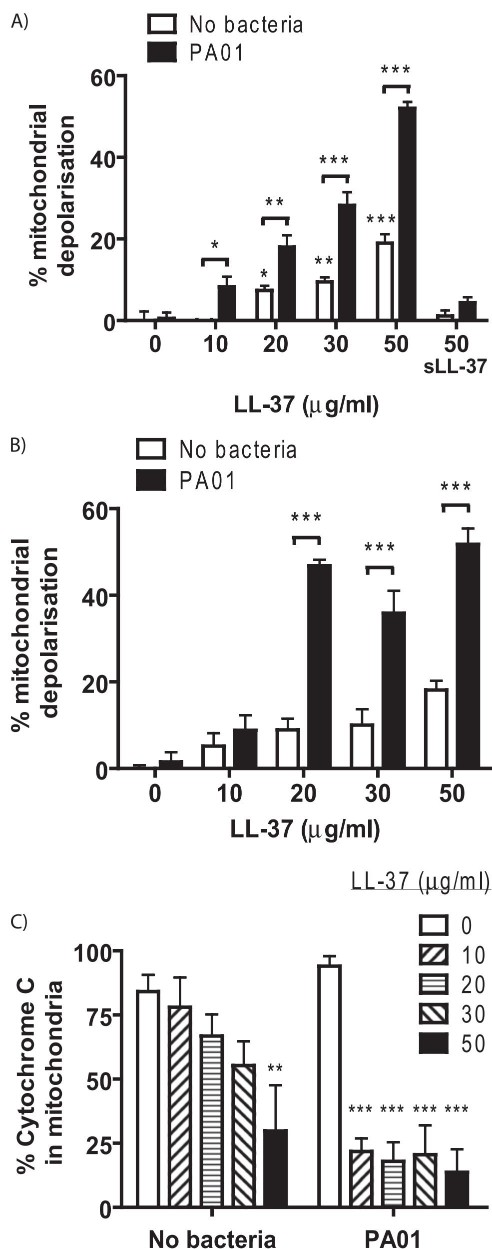

Figure 2. *Pseudomonas aeruginosa* infection of airway epithelial cells synergistically enhances LL-37–mediated mitochondrial depolarization and cytochrome *c* release. Human bronchial epithelial cells (16HBE14o^{-}) were incubated with a range of LL-37 concentrations (or scrambled LL-37 [*sLL-37*] at 50 μg/ml) in Ultroser G serum–substitute supplemented media, in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1). Bacteria and LL-37 were added concurrently and incubated for 60 minutes (*A*) or 90 minutes (*C*), or epithelial cells were preinfected with bacteria for 60 minutes, washed, and exposed to LL-37 for 60 minutes (*B*). (*A*, *B*) Mitochondrial membrane depolarization was determined using Mitocapture dye, quantifying the percentage of apoptotic cells displaying diffuse *green* fluorescence (cells with depolarized mitochondria), compared with healthy cells displaying punctuate *red* fluorescence (cells with polarized mitochondrial membranes). Four random fields of view were counted for each sample (minimum of 300 cells per sample), and number of apoptotic cells was expressed as a percentage of the total number of cells. Data were corrected for a background level of approximately 10% positive cells in control untreated samples, and plotted as mean values ± SEM, for $n = 6$ (*A*) or $n = 3$ (*B*) independent experiments for each condition. Two-way ANOVA with Bonferroni *post hoc* test was performed to determine significance. *$P \leqslant 0.05$, **$P \leqslant 0.01$, ***$P \leqslant 0.001$. (*C*) Cellular localization of cytochrome *c* was assessed by ELISA analysis of mitochondrial fractions after subcellular fractionation. Data represent the mean percentage of cytochrome *c* present in this fraction as a proportion of total cytochrome *c* detected in each sample ± SEM for $n = 3$ independent experiments, measured in duplicate for each condition. Two-way ANOVA was performed with Bonferroni *post hoc* test to compare each treatment to appropriate LL-37–free negative control sample. **$P \leqslant 0.01$, ***$P \leqslant 0.001$.

letion mutant (Δ*mexAB*-oprM), described as containing a defect in its ability to invade epithelial cells (28), was used. The 16HBE14o^{-} cells were infected with PAO1Δ*mexAB*-oprM, or the isogenic control, and a gentamicin-exclusion assay was performed to determine the extent of internalization into the epithelial cells (Figure 6A). Whereas internalization of the isogenic strain could be clearly demonstrated, negligible internalization of the PAO1Δ*mexAB*-oprM bacteria occurred. Furthermore, the LL-37–mediated synergistic enhancement of apoptosis was significantly ($P \leqslant 0.001$) and completely lost when using PAO1Δ*mexAB*-oprM (Figure 6B). The invasion defect of PAO1Δ*mexAB*-oprM was previously shown to result from the absence of a bacterial secreted factor, and can be restored by the addition of supernatants from isogenic control bacteria exposed to epithelial cells (28). The LL-37–mediated synergistic enhancement of apoptosis was significantly restored ($P \leqslant 0.01$) when 16HBE14o^{-} cells were infected with PAO1Δ*mexAB*-oprM in the presence of both LL-37 and supernatants from isogenic control bacteria exposed to epithelial cells (Figure 6C). These data demonstrate that the epithelial-cell internalization of *P. aeruginosa* is required to facilitate the LL-37–mediated induction of apoptosis at physiologically inflammatory concentrations of peptide.

DISCUSSION

Cationic host-defense peptides, including LL-37, have been demonstrated to have multiple properties capable of modulating inflammation and immunity. The full extent of these properties remains to be determined, but understanding the physiological roles of CHDPs in health and disease, and their development as antimicrobial therapeutics, is clearly significant. Our results suggest a novel innate inflammomodulatory role for LL-37, preferentially inducing apoptosis in infected epithelial cells, with the potential to exert protective or detrimental effects.

The most critical mechanisms by which cathelicidins contribute to host defense against infections remain uncertain. In various models, LL-37 and mCRAMP exert antimicrobial effects *in vivo* (5–9), despite high minimum inhibitory concentration values that often exceed detectable physiologic levels. Recent studies implicated the vitamin D–dependent up-regulation of LL-37 in the intracellular killing of mycobacteria in mononuclear leukocytes (29, 30), perhaps in synergy with β-defensin 4 (31), and mCRAMP impairs the intracellular replication of *Salmonella* (32). Therefore, these peptides likely have direct antimicrobial roles where peptides are concentrated in favorable, controlled ionic conditions, and perhaps function synergistically with other agents. However, the function of LL-37 at epithelial surfaces, at the peptide concentrations reported, is less clear. In lungs, hCAP-18 was detected in bronchoalveolar lavage fluid from healthy infants at approximately 5 μg/ml, and was found at up to approximately 25 μg/ml and at approximately 15 μg/ml in infants with pulmonary infections and individuals with cystic fibrosis lung

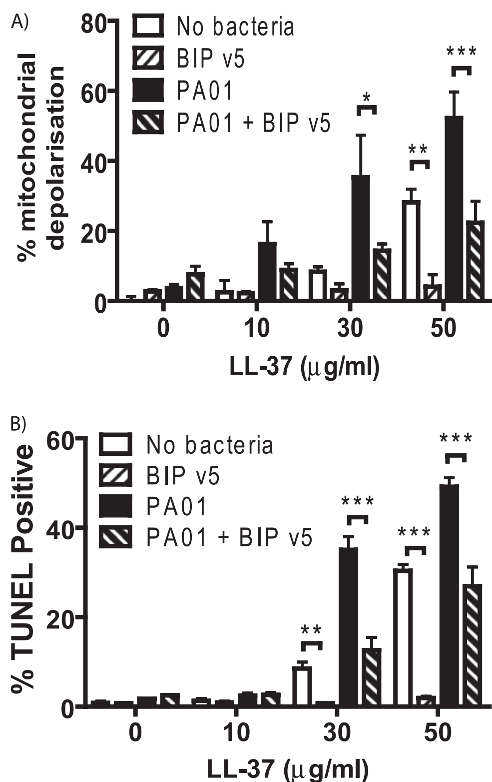

Figure 3. LL-37–induced mitochondrial depolarization and DNA fragmentation involve Bax-dependent mechanisms. Human bronchial epithelial cells (16HBE14o⁻) were incubated for 1 hour (*A*) or 6 hours (*B*) over a range of LL-37 concentrations in Ultroser G serum–substitute supplemented media, in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1) added concurrently, with or without preincubation for 1 hour with Bax-inhibiting peptide V5 (BIP-V5; 100 μM). (*A*) Mitochondrial membrane depolarization was determined using Mitocapture dye, quantifying the percentage of apoptotic cells displaying diffuse *green* fluorescence (cells with depolarized mitochondria), compared with healthy cells displaying punctuate *red* fluorescence (cells with polarized mitochondrial membranes). Four random fields of view were counted for each sample (minimum of 300 cells per sample), and the number of apoptotic cells was expressed as a percentage of total number of cells. Data were corrected for a background level of approximately 10% positive cells in control untreated samples, and plotted as mean values ± SEM, for $n = 3$ independent experiments for each condition. A two-way ANOVA with Bonferroni *post hoc* test was used to compare LL-37–only treated samples with LL-37/BIP-V5–treated samples, or LL-37/*P. aeruginosa*–treated samples with LL-37/*P. aeruginosa*/BIP-V5–treated samples at corresponding concentrations. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. (*B*) Cells were fixed and apoptosis was assessed by TUNEL assay. Four random fields of view, each containing more than 100 cells, were counted for each sample, and the number of TUNEL-positive cells was expressed as a percentage of the number of DAPI-positive nuclei. Data represent mean values ± SEM, for $n = 3$ independent experiments for each condition. Two-way ANOVA with Bonferroni *post hoc* test was used to compare LL-37 only–treated samples with LL-37/BIP-V5–treated samples, or LL-37/*P. aeruginosa*–treated samples with LL-37/*P. aeruginosa*/BIP-V5–treated samples at corresponding concentrations $**P \leq 0.01$, $***P \leq 0.001$.

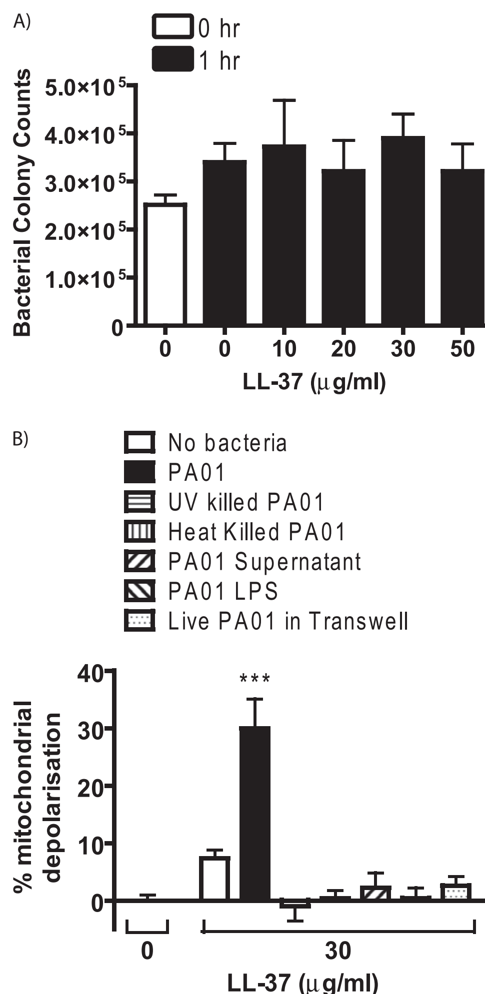

Figure 4. Synergistic induction of apoptosis by LL-37 and *P. aeruginosa* requires specific bacteria–epithelial cell interactions with whole, live bacteria. (*A*) *P. aeruginosa* PA01 was cultured to log-phase, then exposed to LL-37 over a range of concentrations for 1 hour at 37°C in Ultroser G serum–substitute supplemented media. Serial dilutions were performed, incubated on LB agar plates in triplicate, and cultured for 16 hours before colony-forming units were counted. Data represent mean values ± SEM, for $n = 3$ independent experiments for each condition. (*B*) Human bronchial epithelial cells (16HBE14o⁻) were assessed for mitochondrial membrane depolarization using Mitocapture dye, as described in MATERIALS AND METHODS, after incubation for 1 hour with a range of concentrations of LL-37, in serum-substitute supplemented media, in the presence and absence of live log-phase *P. aeruginosa* PA01 (MOI 10:1), heat-killed or UV-killed PA01 (MOI 10:1), *P. aeruginosa* PA01 LPS (1 μg/ml), *P. aeruginosa* PA01 conditioned medium, or live *P. aeruginosa* PA01 (MOI 10:1) separated from the cells via a semipermeable polyester membrane with 0.4-μm pore size. Data represent mean values ± SEM, for $n = 3$ independent experiments for each condition. Two-way ANOVAs were performed to evaluate significance, with Bonferroni *post hoc* tests comparing LL-37 alone to LL-37/stimuli. $***P \leq 0.001$.

disease (in steady state), respectively (33, 34). At these sites, immunomodulatory roles may be of primary significance. Indeed, the potential significance of such activities was recently demonstrated in terms of the *in vivo* protection against infection in animal models using a synthetic CHDP derivative with no direct antimicrobial activity *in vitro* (35).

A variety of CHDPs, including bovine cathelicidins and human α-defensins, were shown to affect eukaryotic cell death (18, 36). We previously showed that high (potentially supraphysiologic) concentrations of LL-37 induced apoptosis in pulmonary epithelial cells *in vitro* and *in vivo* (15, 17). However, the mechanisms involved remain undetermined. We demonstrate here that at these higher concentrations, LL-37 can induce mitochondrial depolarization and cytochrome *c* release

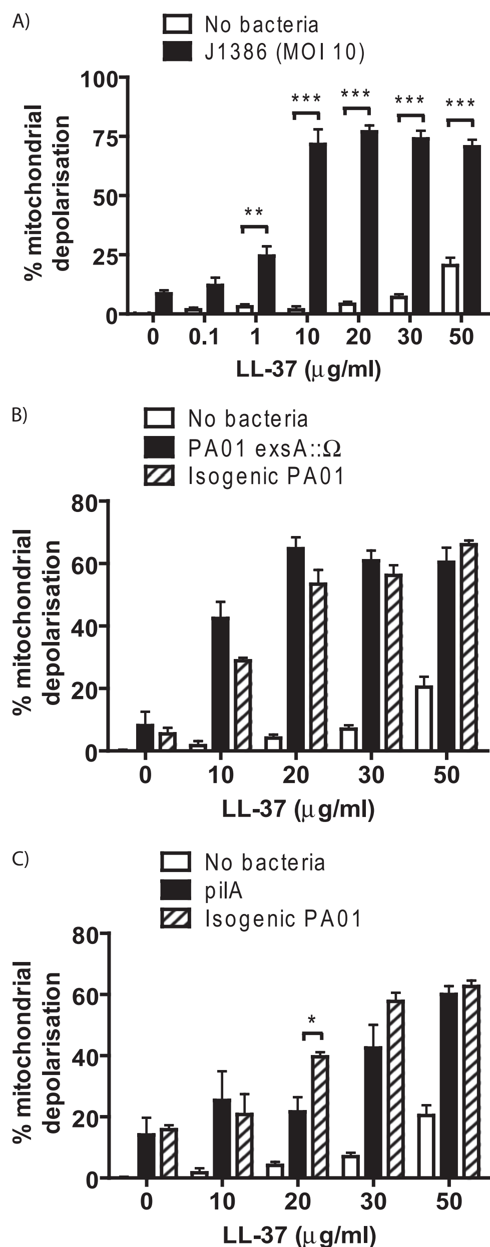

Figure 5. Synergistic induction of apoptosis by LL-37 and *P. aeruginosa* is isolate-specific and independent of type III secretion system and pilus expression. Human bronchial epithelial cells (16HBE14o$^-$) were assessed for mitochondrial membrane depolarization using Mitocapture dye, as described in MATERIALS AND METHODS, after incubation for 1 hour with a range of concentrations of LL-37, in Ultroser G serum–substitute supplemented media, in the presence and absence of (*A*) log-phase clinical *P. aeruginosa* isolate J1386 (MOI 10:1), (*B*) log-phase *P. aeruginosa* PA01exsA::Ω or isogenic PAO1 control strain (MOI 10:1), and (*C*) log-phase *pilA P. aeruginosa* mutant or isogenic PAO1 control strain (MOI 10:1). Data represent mean values ± SEM, for $n = 3$ independent experiments for each condition. Two-way ANOVAs were performed to evaluate significance, with Bonferroni *post hoc* tests comparing (*A*) LL-37/*P. aeruginosa* to LL-37 alone, and (*B*) LL-37/*P. aeruginosa* mutant to LL-37/isogenic controls. *$P \leq 0.05$,***$P \leq 0.001$.

in airway epithelial cells, confirming previous findings in alveolar epithelial cells (18). In addition, the LL-37–mediated induction of mitochondrial depolarization and the subsequent apoptosis of these cells can be completely blocked using the BIP-V5 peptide inhibitor of the proapoptotic Bcl-2 family protein Bax. The BIP-V5 peptide mimics the Bax-binding domain of Ku70, preventing Bax translocation from cytosol to the mitochondria (37). This translocation is a central event in mitochondria-dependent apoptosis, with the subsequent activation and oligomerization of Bax and Bak resulting in either the nonspecific rupture of, or the formation of specific channels in, the outer mitochondrial membrane and release of cytochrome *c* (38). Interestingly, we demonstrate that the Bax-dependent LL-37–mediated release of cytochrome *c* did not cause an activation of caspase-3 or caspase-9 after exposure to LL-37 alone, yet resulted in a Bax-dependent DNA fragmentation. In addition, polycaspase inhibition resulted in only a partial inhibition of the apoptosis induced by high levels of LL-37 (15). These data suggest that the induction of apoptosis by high concentrations of LL-37 alone appears to be a Bax-dependent and predominantly caspase-independent process, and may implicate the liberation and activation of mitochondrial apoptosis–inducing factor (AIF) and/or endonuclease G. The mechanism by which LL-37 can interact with or activate Bax in airway epithelial cells is unclear. LL-37 could induce an opening of the mitochondrial permeability transition pore, as proposed for bovine myeloid antimicrobial peptide-28 (BMAP-28) (36). However, a study published during preparation of our manuscript described a calpain-dependent mechanism of LL-37–mediated Bax translocation to the mitochondria, responsible for the AIF-mediated apoptosis induced by very high concentrations (50–200 μg/ml) of LL-37 in Jurkat T leukemia cells. These findings are compatible with our data (21). Irrespective of this, we demonstrate that concentrations of LL-37 considered to be physiologically relevant during lung inflammation (10–30 μg/ml) induce minimal apoptosis in human airway epithelial cell lines and primary cells, in the absence of infection. This result suggests that under normal physiological conditions, LL-37 on epithelial surfaces would not be damaging.

In contrast to the effects of LL-37 alone, cells infected with *P. aeruginosa* demonstrated an enhanced susceptibility to the induction of apoptosis upon exposure to concentrations of LL-37 that had no effect alone, but not to control scrambled LL-37 peptide. This effect comprised a pronounced synergistic increase in mitochondrial depolarization, cytochrome *c* release, and DNA fragmentation, and was at least partly Bax-independent. In addition, the LL-37–mediated activation of caspase-3 and caspase-9 was evident only in infected cells, demonstrating activation of the intrinsic pathway of apoptosis. Although *P. aeruginosa* infection alone has been shown to induce extrinsic pathways of apoptosis via CD95/CD95L (12), we saw no activation of capase-8 and no significant cell death in response to bacteria alone in our system. This finding may relate to the fairly low MOI used, and the timeframe examined in our studies, suggesting that the LL-37–mediated induction of apoptosis in infected epithelial cells is a much earlier (and mechanistically distinct) form of cell death compared with previously described, bacterially induced death receptor–mediated apoptosis. The intrinsic pathway of apoptosis is a mitochondrial-dependent mechanism of caspase activation involving cytochrome *c*–induced oligomerization of the cytosolic apoptotic protease activating factor-1 (Apaf-1), which recruits and activates procaspase-9, an upstream activator of effector caspases, such as caspase-3 (39). In addition, the mitochondrial release of Smac/DIABLO (second mitochondrial activator of capases/direct IAP binding protein with low PI) (40) and Omi (also known as high temperature requirement factor A2 [HtrA2]) (41) leads to an inactivation of the inhibitor-of-apoptosis proteins (IAPs) that normally inhibit caspase activity. The increased apoptosis observed via TUNEL assay in infected cells exposed to LL-37 could be inhibited by the polycaspase in-

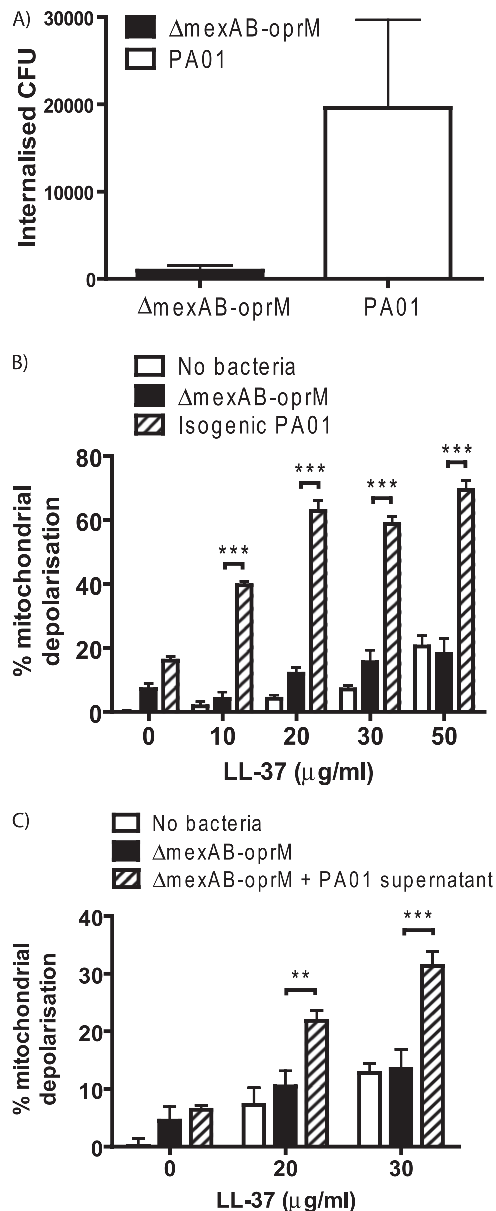

Figure 6. Synergistic induction of apoptosis by LL-37 and *P. aeruginosa* requires epithelial-cell internalization of bacteria. Human bronchial epithelial cells (16HBE14o$^-$) were incubated for 60 minutes in Ultroser G serum–substitute supplemented media, in the presence and absence of (MOI 10:1) log-phase *P. aeruginosa* strains PA01, Δ*mexAB-oprM* mutant (*A*–*C*), isogenic PAO1 control strain (*B*), or Δ*mexAB-oprM* mutant added concurrently with sterile conditioned supernatant collected from 16HBE14o$^-$ cells infected with PA01 (*C*). (*A*) Invasion of epithelial cells by bacteria was determined by gentamicin exclusion, quantifying the number of viable CFUs surviving extracellular gentamicin treatment (50 μg/ml). Data are plotted as mean values ± SEM, for $n = 3$ independent experiments plated in duplicate for each condition. (*B*, *C*) Infected epithelial cells were concurrently incubated with a range of concentrations of LL-37, and mitochondrial membrane depolarization was determined using Mitocapture dye, as described in MATERIALS AND METHODS. Data represent mean values ± SEM, for $n = 3$ independent experiments for each condition. Two-way ANOVAs were performed to evaluate significance, with Bonferroni *post hoc* tests comparing (*B*) LL-37/Δ*mexAB-oprM* mutant to LL-37/isogenic controls, and (*C*) LL-37/Δ*mexAB-oprM* mutant to LL-37/Δ*mexAB-oprM* mutant in PAO1-conditioned supernatant. $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$.

hibitor Z-VAD-FMK, reducing it to levels similar to those induced by LL-37 alone. Thus the synergistic effects are caspase-dependent, and occur in addition to predominantly caspase-independent pathways induced by higher concentrations of LL-37 alone. The caspase inhibition by IAPs may be reduced in infected cells, and although XIAP levels were unaffected, the roles of Smac/DIABLO and Omi HtrA2 in this system remain unknown. Therefore, a caspase-dependent pathway downstream of mitochondrial depolarization, induced by an alternate mechanism from that used by LL-37 alone, is responsible for the capacity of LL-37 to promote the apoptosis of cells infected with *P. aeruginosa*.

The nature of the interaction between epithelial cells and bacteria required to make these cells susceptible to the apoptosis-inducing effects of LL-37 was investigated under a number of conditions and using mutants of *P. aeruginosa*. Neither dead bacteria nor soluble products produced by untreated or LL-37–treated bacteria could promote these synergistic effects. In the absence of physical contact between the epithelial cells and live bacteria, no effects were observed. In contrast, the effect of LL-37 was even more profound when a clinical strain of *P. aeruginosa* J1386 (isolated from an individual with cystic fibrosis) (23) was used, suggesting that this effect might be modified by isolate variation in virulence factors. PAO1 is classified as an "invasive" rather than "cytotoxic" strain of *P. aeruginosa* (although both can invade eukaryotic cells), and this invasiveness is proposed to require contact between bacteria and epithelial cells to stimulate the efflux of bacterial "invasive factors" (28). The Δ*mexAB-oprM* deletion mutant of *P. aeruginosa* PAO1 (24) is defective in terms of epithelial-cell invasion (despite normal adherence), and has diminished virulence *in vivo* as a consequence of the loss of the MexAB-OprM efflux system, proposed to be responsible for the efflux of these putative "invasive factors" (28). A synergistic induction of apoptosis was not evident in LL-37–treated epithelial cells infected with this mutant strain, but could be replicated by the addition of these unknown "invasive factors" from the isogenic wild-type PAO1 strain, demonstrating a requirement for invasiveness. In contrast, the PAO1*exsA*::Ω mutant (25), in which the ExsA mutation impairs the ExsA-regulated type III secretion system, behaved identically to its isogenic wild-type PAO1 strain. Although a functional ExsA allele is required for *P. aeruginosa*–induced cytotoxicity, epithelial-cell invasiveness is independent of ExsA expression (42). Similarly, a *P. aeruginosa pilA* mutant (26) was largely able to synergize with LL-37 to induce apoptosis as effectively as its isogenic PAO1 wild-type strain. In this strain, *pilA* mutation results in an absence of pilus, proposed to be an important adhesin involved early in epithelial-cell interactions with *P. aeruginosa* (43). Interestingly, differences were observed in the sensitivity to the LL-37–induced mitochondrial depolarization of cells infected with our original PAO1 isolate, compared with isogenic controls for some of the mutants used. Additional investigations using these isolates may help in further defining the key events involved in this interaction. Nevertheless, the data suggest that the bacterial invasion of airway epithelial cells, but not ExsA-regulated type III secretion or pili expression, is critical in inducing enhanced susceptibility to LL-37–mediated apoptosis.

Our results describe a novel innate inflammomodulatory role for LL-37, preferentially inducing the apoptosis of infected epithelial cells. However, the extent to which this might contribute to innate epithelial defenses, or be manifest in pathologic damage to epithelial-barrier integrity, is unknown, and a fine balance could exist. Although LL-37 clearly has important roles in innate host defense against infection, chronically increased hCAP-18/LL-37 concentrations in cystic fibrosis

lung disease are correlated with increased lung damage (34), and elevated hCAP-18/LL-37 concentrations are associated with bronchiolitis obliterans syndrome (44) and the pathogenesis of psoriasis (45). Pulmonary epithelial-cell apoptosis plays a significant role in *P. aeruginosa* clearance from the murine lung (12). In addition, bladder epithelial-cell exfoliation after bacterial attachment plays a role in innate defense against invasive *Escherichia coli* (14), preventing the establishment of a safe niche and intracellular biofilm-like growth (46). Furthermore, the susceptibility of individuals with cystic fibrosis to pulmonary *P. aeruginosa* infection is proposed to relate, in part, to the failure of airway epithelial cells to internalize this bacterium, and thus an inability to clear *P. aeruginosa* by desquamation of infected cells (27). Thus, we propose that in the healthy host, LL-37, up-regulated during infection and inflammation, may promote the apoptosis and consequent clearance of *P. aeruginosa*–infected airway epithelial cells, as a component of the innate host defense against this pathogen. However, under pathologic conditions of excessive, chronic LL-37 exposure, or a failure of epithelial-cell internalization of *P. aeruginosa* (such as in cystic fibrosis), the epithelial-cell death induced by high concentrations of LL-37 alone may be detrimental to the host and contribute to chronic lung damage. The extent to which this effect might be common to other invasive bacteria, or else specific to *P. aeruginosa*, remains to be determined, but has clear significance for the possible use of LL-37 and related CHDPs as antimicrobial therapeutics.

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Dying and necrotic neutrophils are anti-inflammatory secondary to the release of α-defensins

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Abstract

Neutrophils are recruited to sites of injury but their timely removal is thought to be vital to prevent exacerbating inflammation. In addition, the recognition of apoptotic cells by cells of the innate immune system provides potent anti-inflammatory and anti-immunogenic signals. In this paper we describe how human neutrophils dying by apoptosis or necrosis release anti-inflammatory peptides, the alpha defensins. This family of small cationic peptides, effectively inhibits the secretion of multiple pro-inflammatory cytokines and nitric oxide from macrophages, the main innate immune cell found at sites of chronic inflammation. In addition, the systemic administration of necrotic neutrophil supernatants and alpha defensins protects mice from a murine model of peritonitis. Hence their effects may be far reaching and serve to kill microbes whilst regulating a potentially tissue destructive inflammatory response.

Keywords

Macrophage; Apoptosis; Neutrophil; Inflammation

Introduction

Polymorphonuclear cells (PMNs) are the most abundant type of leukocyte, rapidly recruited to sites of inflammation by pathogen-derived stimuli or host derived danger signals (1). Subsequent activation of PMN triggers the release of reactive oxygen species and an arsenal

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of non-specific cytotoxic compounds. This has led researchers to consider that the safe disposal of neutrophils as early as possible is essential to the maintenance of immunological homeostasis and the resolution of inflammation (2). However the data that exposes the pathogenic role of late apoptotic and necrotic neutrophils is conflicting. Elastase, which is released by necrotic neutrophils, has been reported to induce resting macrophages to secrete pro-inflammatory cytokines (3). In contrast, other studies indicate that necrotic neutrophils are phagocytosed by macrophages in a non-phlogistic manner and even down regulate CD80, CD86 and CD40 on immature DC, rendering them unable to induce T cell proliferation in an MLR (4, 5). In addition whilst necrotic cell lines are able to induce DC maturation, necrotic primary cells are not (6-8), suggesting that necrotic cells cannot by themselves be considered dangerous, without reference to the cell type and the way in which they are exposed to the immune system.

Defensins are widely distributed in nature, being expressed by leukocytes and epithelial cells lining the environmental interface. They are divided into alpha and beta defensins based on their tertiary structure, which has a characteristic six cysteine motif; pairing to form three intramolecular disulphide bonds. α-Defensins are small cationic and amphipathic peptides with a molecular weight of 3–5 kDa (9). Of the six α-defensins, four (HNP1-4) are major constituents of human neutrophils, where they are found stored in the azurophilic (primary) granules. The other two (HD5-6) are expressed in the Paneth cells, which are secretory epithelial cells located in the small intestinal crypts (10). Whilst rats and rabbits express neutrophil α-defensins, mice do not; but they do express homologues of human HD5-6 in the Paneth cells, known as cryptidins (11). The secretion of α-defensins by epithelial cells is an important component of innate immunity. This is highlighted by mice that lack matrilysin-7 and cannot secrete active cryptidins, due to an inability to process Paneth cell α-defensin precursors. Despite the fact that they secrete a number of other antimicrobial molecules they are more susceptible to an oral challenge with a virulent strain of *S. typhimurium* and mount a more severe inflammatory response (11). In contrast mice transgenic for the human crypt α-defensin, HD-5, are protected from a normally lethal dose of *Salmonella* (12). Recently α-defensins have been reported to block the release of IL-1β from monocytes whilst having no effect on the release of TNF-α (13). Monocytes, which are found circulating in the blood, mature into macrophages upon egress from the circulation and entry into tissues. Here they interact with activated neutrophils in the absence of serum proteins that are known to inhibit α-defensin function (14, 15). In this paper we describe how α-defensins, released by dying and necrotic neutrophils exert a powerful anti-inflammatory effect on human macrophages whilst still maintaining significant anti-microbial activity.

Materials and Methods

Reagents

Purified HNP1-3 was supplied by Hycult biotechnology. Synthetic HNP1, linearized HNP1 and the D enantiomer of HNP was kindly provided by Prof Wuyuan Lu. Linear (or linearized) HNP1 is an unstructured form of the α-defensin, in which the six Cys residues have been replaced by Ala. LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES; MW 4493.33) was synthesized by N-(9-fluorenyl) methoxycarbonyl chemistry at the Nucleic Acid/Protein Service unit at the University of British Columbia (UBC; Vancouver, Canada), as described previously (Barlow et al, J. Leuk. Biol, 1996, 80:509-520). *R*-roscovitine, (*R*)-2-[[9-(1-methylethyl)-6-[(phenylmethyl)amino]-9*H*-purin-2-yl]amino]-1-butanol (A.G. Scientific) was kindly provided by Prof A. Rossi and used at 20uM. HMDMs were stimulated with CD40 ligand (Peprotech, UK) at 3ug/ml and IFN-γ (Peprotech, UK) at 5ng/ml. LPS (Sigma) was used at 1ng/ml.

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Mice

6-8 week old female C57BL/6, mice (Harlan-UK), were used at 8-9 weeks of age and were sex and age-matched within experiments. All experiments were covered by a Project License granted by the Home Office under the Animal (Scientific Procedures) Act 1986. Locally, this license was approved by the University of Edinburgh Ethical Review Committee.

Generation of apoptotic cells

Human neutrophils were extracted from peripheral blood of healthy volunteers, as described previously (16). Blood was separated using dextran sedimentation and a Percoll gradient. This yielded highly pure human neutrophils (>95%). Neutrophils were cultured in serum free IMDM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin for various periods of time and the cell free medium collected and ultracentrifuged at 100,000g for 1 hour prior to using immediately or storing at −70°C. Necrotic neutrophils were generated from freshly isolated neutrophils by freeze thawing them 5 times after which no complete cells remained. Membranes were removed by ultracentrifuging them at 100,000g for 1 hour. In all in vitro experiments the number of neutrophils used was 12×10^6/ml. An equivalent number of necrotic neutrophils were generated by freeze thawing per ml of culture medium and the membrane free supernatant was used at this concentration. Necrotic thymocytes were generated from thymi removed from 6 week old syngeneic mice, teased into single cell suspensions and freeze thawed 5 times as described for necrotic neutrophils. Murine neutrophils were isolated from the bone marrow of syngeneic mice by percoll gradient and then treated in the same way as human neutrophils to obtain necrotic membrane free cell fractions at the same concentration.

Macrophage culture

Human monocytes were extracted from peripheral blood of healthy volunteers according to Lothian Research Ethics Committee approval (LREC/2001/4/56), using dextran sedimentation and a Percoll gradient as previously described (16). They were cultured in IMDM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin, and 10% autologous platelet-rich plasma-derived serum. Mature macrophages were used on day 7. Murine bone marrow derived macrophages were cultured, as described previously (4) and used between day 7 and 10 of culture. All assays were done in serum free medium.

Depletion of α-defensins using R2 or dynabeads beads

R2 beads (Applied Biosystems), which bind hydrophobic proteins, were incubated with membrane free necrotic neutrophil supernatants for 2 hours. Beads were then removed following centrifugation and proteins in the supernatant or on the beads was analysed by NuPage 10% Bis-Tris gel with MES running buffer, Invitrogen. Proteins were visualized by silver stain. The band of peptides at 3-5 kd was cut out, reduced, alkylated and digested with trypsin. Chromatographic separation of tryptic digests was conducted by an Ultimate 3000 nanoLC system (Dionex, Mountain View, CA) and peptides were analyzed by an HCT Ultra PTM ion trap instrument (Bruker Daltonics) equipped with a nano-ESI source. Acquired spectra were analyzed using the MASCOT search engine (Matrix Science) (D. Compopiano and D. Clarke-University of Edinburgh). To specifically deplete NN of α-defensin, Dynabeads M280 coated with sheep anti mouse IgG (Invitrogen) were bound to mouse anti-human HNP 1-3 (Hycult) as per the manufacturers instructions. These anti-HNP 1-3 coated beads were then used to deplete NN of α-defensin and beads with bound α-defensin were removed with a magnet (BD Pharmingen). Complete and specific depletion of α-defensin was checked both by HNP 1-3 ELISA (Hycult) and by protein gel.

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Tests of cell viability

LDH released from the cytoplasm of dying macrophages into the assay medium was used as a measure of membrane integrity and viability. A cell cytotoxicity colorimetric kit was used according to the manufacturers instructions (Sigma Aldrich) The assay utilizes NAD, reduced by the released lactate, which induces a colour change in a tetrazolium dye that can be detected using a spectrophotometric method. As a positive control the protein synthesis inhibitor cyclohexamide (10ug/ml) was used to reduce cell viability after 24 hours. The Alamar Blue assay is a sensitive non-radioactive means of measuring cell viability based on the addition of a fluorogenic redox indicator to cells in culture. When taken into cells, Alamar Blue becomes reduced and turns red. This reduced form of Alamar Blue is highly fluorescent. The extent of this conversion, which is a reflection of cell viability, was quantified by its optical density. Alamar Blue was used at a 1:10 dilution and added to the assay medium for the duration of the culture.

Eating assay

Cells were pretreated with α-defensin or a positive control puromycin known to reduce cell viability (Sigma /Aldrich at 50ug/ml). After 24 hours the HMDMs were washed and fresh medium containing 1.25×10^6 flourscent beads (Fluoresbrite Plain YG 3.0 micron microspheres; PolySciences, Inc. warrington, PA) was added per 0.5×10^6 HMDMs. After 1h unbound cells were removed and cells washed ×3 with PBS containing magnesium and calcium. Cells were removed from the wells using Trypsin/EDTA, washed again and resuspended in FACs buffer (PBS + 2% FCS) prior to analysis on a FACs machine.

Sterile Peritonitis

Peritonitis was induced by i.p. injection of 0.5 ml of 10% thioglychollate. Mice underwent peritoneal lavage at various time points following thioglychollate injection.

Bacterial in vitro and in vivo infections of macrophages

Murine BMDM were cultured as described above. Salmonella *enterica* serovar Typhimurium strain SL3261 (17), which was live or had been heat killed was added to murine BMDM at an MOI (multiple of infection) of 10:1 bacteria to macrophages. After 1 hour excess bacteria were washed away and Gentamicin at 100ug/ml was added for 1 hour to kill any residual extracellular bacteria. Cells were washed again and HNP1 or medium alone was added for various time-points after which supernatants were collected for cytokine estimation prior to lysing the macrophages with 1% Triton X for 15 minutes. Lysed cells containing live bacteria were collected and plated onto agar and incubated for 18 hours after which colonies were counted. In a similar way Pseudomonas Aeruginosa PA01 was added to HMDM at an MOI of 10. After 4 hours supernatants were collected prior to lysing the cells with 0.1% Triton X and the number of live colonies counted after a further 18 hours of culture.

ELISA

Supernatants collected after specified culture periods were analyzed for production of cytokines by a sandwich ELISA according to the manufacturers instruction (R&D systems, UK). HNP 1-3 was measured using an HNP1-3 ELISA according to the manufacturers instructions (Hycult Biotechnology). All experiments were performed in triplicate.

Statistics

Data are expressed, when appropriate, as mean and SEM. Significance was assessed using unpaired t tests, and p-values <0.05 were considered significant.

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Results

Apoptotic neutrophils do not require contact to inhibit inflammatory macrophages

Our interest in a soluble factor released by dying human neutrophils was initiated by the observation that co-culture of apoptotic neutrophils separated from activated human monocyte derived macrophages (HMDMs) by transwells led to the inhibition of pro-inflammatory cytokine secretion (Fig 1 a/b). TGF-β is thought to play a pivotal role in the inhibition of HMDM TNF-α secretion by apoptotic neutrophils (18). However the addition of blocking anti-TGF-β to apoptotic neutrophils, in contact with LPS-stimulated HMDM had only a moderate inhibitory effect but no effect on CD40L/IFN-γ stimulated HMDM. Apoptotic cells generate apoptotic bodies (19), which may be able to pass through the pores of a transwell. To control for this we ultra-centrifuged supernatants derived from neutrophil cultures to remove apoptotic bodies and all membrane constituents. The active inhibitory factor contained within this neutrophil-conditioned medium (NCM) was released by dying neutrophils in a time-dependent manner. It was able significantly to inhibit the secretion of TNF-α from macrophages stimulated by both LPS and CD40L/IFN-γ by 4 hours after culture, when neutrophils are beginning to undergo apoptosis (Fig 1c/d). TGF-β measured in supernatants from CD40L/IFN-γ- and LPS-stimulated HMDM was not significantly raised whilst levels of IL-1β, IL-6, IL-8 and IL-10 and nitric oxide were all decreased (data not shown).

To ask if neutrophil apoptosis augmented the release of the soluble factor we cultured neutrophils in the presence of R-roscovitine, which is known to induce neutrophil apoptosis (20). Following 6 hours of culture the percentage of neutrophils positive for annexin-V increased from 14% to 56.4% (Fig 2a). R-roscovitine did not itself inhibit TNF-α secretion from LPS-stimulated HMDMs (Fig 2b). However culture supernatants from R-roscovitine-treated neutrophils inhibited pro-inflammatory cytokine secretion significantly more than untreated NCM (Fig 2b-c). In contrast, if apoptosis was inhibited, (by culturing neutrophils at 4°C overnight) (Fig 2d) the ability of the NCM was lacking in anti-inflammatory activity. If the same neutrophils were then allowed to undergo apoptosis for 6 hours by culturing at room temperature the inhibitory factor was released into the NCM (Fig 2e).

Necrotic neutrophils are also anti-inflammatory

We wondered whether primary or secondary necrotic neutrophils would also release the active immunosuppressive factor. To generate necrotic neutrophils we freeze thawed fresh neutrophils 5 times after which more than 90% of the neutrophils had lysed (data not shown). These lysed cells were then ultracentrifuged to remove membranous material and the remaining necrotic neutrophil conditioned medium (NN) added to LPS stimulated macrophages. Titration of the NN revealed a dose dependent inhibition of TNF-α secretion by the activated macrophages, which was even more effective than using NCM at the same dilution (Fig 3a). The TNF-α ELISA was able to detect both mature and precursor TNF-α. In addition, TNF–α converting enzyme (TACE) levels were measured and found to be unchanged (data not shown). Multicytokine analysis confirmed that NN was also able to inhibit the production of cytokines including IL-6, IL-1β, IL-8, and IL-1 (Fig 3b). In addition NN also inhibited the generation of nitric oxide (Fig 4c). The concentration of TGF-β was either decreased or similar to stimulated cells (data not shown). Identical results were obtained using NN prepared from secondarily necrotic neutrophils that had previously undergone 24 hours of culture (data not shown).

Necrotic neutrophils but not other necrotic cells are anti-inflammatory

Necrotic cells are generally considered to pose a danger to the immune system resulting in auto-antibody generation and a breakdown in tolerance to self with subsequent

autoimmunity (21-23). We were interested to know if necrotic neutrophils were unique in their ability to release a soluble anti-inflammatory factor or if this could be generalised to other primary cells or tumour cell lines. The anti-inflammatory activity of necrotic neutrophils was compared to supernatants from necrotic murine thymocytes (NT) and from the necrotic human tumour cell line, Mutu (NM). While necrotic thymocyte supernatants had a limited ability to suppress TNF-α secretion from LPS-stimulated HMDM, necrotic tumor cells had none (Fig 4a) and both necrotic thymocytes and tumour cells were pro-inflammatory to CD40L/IFN-γ-stimulated HMDMs (Fig 4b). In contrast NN was markedly anti-inflammatory inhibiting both TNF-α and nitric oxide (NO) generation (Fig 4c/d). This indicates that compared to the cells tested, the release of a soluble anti-inflammatory factor is specific to neutrophils.

Alpha defensins are the active anti-inflammatory factor released by apoptotic/necrotic neutrophils

To delineate further the active immunosuppressive factor we tested the NN that had been depleted of hydrophobic proteins using R2 beads and found that depleted NN now lacked the ability to inhibit LPS (Fig 5a) or CD40L/IFN-γ (Fig 5b) stimulated HMDM release of TNF-α. The R2 beads had partially removed a range of proteins from the NN, but completely removed a band of proteins between 3-5kDa in size (Fig 5c). This band was digested and sequenced by MS/MS and found to be the anti-microbial peptide α-defensins (data not shown). When purified alpha defensins (AD) were added back to the R2 depleted NN the immunosuppressive activity of the NN was restored, indicating that one of the active inhibitory factors released by and contained within the neutrophils was α-defensins. However R2 beads removed a range of proteins from the NN and to ensure specificity, α-defensins were depleted from NN using anti-human HNP 1-3 bound to dynabeads. The complete removal of α-defensins was confirmed with an HNP1-3 ELISA whilst the specificity of the antibody bound beads was confirmed by protein gel analysis (data not shown). When α-defensins were specifically depleted from the NN the ability of NN to inhibit TNF-α production by CD40L/IFN-γ stimulated HMDMs was completely lost (Fig 5di), but was regained upon addition of HNP-1. However NN was still able to significantly inhibit TNF-α production by HMDMs stimulated with LPS (Fig 5dii) because the NN retained LL37, which is known to bind to LPS and inhibit its pro-inflammatory potential (24). When HNP-1 was added back though the full inhibitory capacity of the NN was restored.

α-Defensins exist as 4 types in human neutrophils; human neutrophil peptides 1-4 (HNP1-4). HNP1-3 constitute more than 5% of the total cellular protein in human neutrophils and 99% of the total defensin content of neutrophils with traces of HNP4. We measured HNP1-3 released by neutrophils undergoing apoptosis in culture and found that the concentration of HNP1-3 increased progressively with time reaching a peak by 9 hours suggesting that the release of α-defensins is associated with ongoing neutrophil apoptosis (Fig 5e). The level of the α-defensins in necrotic neutrophil supernatants was consistently higher at between 8-15 +/− 0.45 ug/ml, depending on the human donor. We also assessed the concentration of HNP 1-3 in the synovial fluid of 12 patients suffering with a flare of rheumatoid arthritis undergoing arthrocentesis for an acutely swollen knee, which was found to range between 3-25ug/ml with an average of 12.4ug/ml indicating that the concentration reached in tissues is not dissimilar to that tested in our assays.

α-Defensins do not kill macrophages

A number of reports have described how α-defensins are able to kill eukaryotic cells reviewed in (25). In contrast, L929 cells a murine fibroblast cell line, is resistant to killing by α-defensins (26). We asked if α-defensins decreased the cytokine production of

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macrophages through a delayed effect on cell viability. We found that α-defensin (25ug/ml) pre-treatment for 1 hour prior to stimulating HMDMs with LPS inhibited the ability of macrophages to generate TNF-α, but 20 hours following the removal of α-defensins they were able to secrete equivalent amounts of TNF-α when compared to untreated control macrophages (Fig 6ai). In addition HMDMs cultured in the presence of α-defensin for 24 hours were more refractory to stimulation with LPS and only fully recovered their ability to secrete TNF-α after 72 hours (Fig 6aii). However the fact that they do completely recover indicates that α-defensin treated macrophages, (which do not proliferate in culture) are still viable and able to respond to LPS as well as control cells after a period of time. In addition we performed LDH assays to assess the viability of macrophages after α-defensin treatment. Lactate dehydrogenase (LDH), which is released as cells die, was not significantly elevated when compared to both resting and CD40L/IFN-γ stimulated HMDMs (Fig 6b) after 24 hours of culture with α-defensins. We utilised an additional test of cell viability, the Alamar Blue assay, which relies on detecting the reduced form of Alamar Blue generated by reductase enzymes present in viable cells. When cells were cultured in the presence of the cytotoxic agent, puromycin for 24 hours and then stimulated with LPS a definite decrease in the reduction of Alamar blue is seen secondary to a reduction in cell viability. In contrast no change in reductive capacity is seen in HMDMs pretreated with α-defensin (25ug/ml) for the same length of time indicating that viability was maintained (Fig 6c). Finally we assessed the other main function of HMDMs, their ability to phagocytose (beads) following pre-treatment for 24 hours with either α-defensin or puromycin (Fig 6d). In comparison to control untreated HMDMs (**6ci**) puromycin treated HMDMs showed a reduction in the ability to phagocytose fluorescent beads (**6cii**); but HMDMs pre-treated with α-defensins had a significantly augmented phagocytic capacity when compared to untreated macrophages (**6ciii**), suggesting that α-defensins had functionally altered the macrophage to a pro-resolution, pro phagocytic phenotype.

Alpha defensins inhibit the pro-inflammatory cytokine production by macrophages in the presence of both live and dead whole bacteria

We went on to ask if α-defensins were able to inhibit macrophage pro-inflammatory function and still inhibit the growth of bacteria. We first looked at the response of HMDMs to infection with the human opportunistic pathogen Pseudomonas Aeruginosa PA01. HMDMs infected with live bacteria (Fig 7ai) at a MOI of 10 and treated with α-defensins or with an equivalent number of dead whole bacteria (Fig 7aii) also showed an inhibited secretion of TNF-α, IL-8, IL-6 and IL-1β. In spite of the reduced pro-inflammatory cytokine secretion bacterial counts were not increased when compared to control infected HMDMs (Fig 7b). Hence α-defensin treatment inhibits an excessive pro-inflammatory cytokine response from the HMDM despite the presence of both live and dead Pseudomonas Aeruginosa PA01, but this does not subsequently allow for excessive pathogen replication. We went on to ask if α-defensins could affect a murine model of infection. We used the murine pathogenic Salmonella *enterica* serovar Typhimurium strain SL3261 to infect mice and sacrificed them on day 7 at the height of infection (Fig 7c). We found that the administration of NN had a significant effect on reducing bacterial counts in the spleen (**7ci)** and also reduced TNF-α in the serum (**7cii**).

Alpha defensins but not LL37 inhibits both T cell mediated and LPS mediated activation of macrophages

Neutrophils contain within the secondary granules cathelicidin, an anti microbial peptide of comparable electrophorectic mobility to α-defensins. LL37, the active fragment of the only human cathelicidin hCAP-18 is known to bind LPS and inhibit LPS mediated activation of macrophages (24, 27). To test the possibility that one of the inhibitory factors contained within the NN was LL37 we titrated LL37 into both LPS (Fig 8a) and CD40L/IFN-γ (Fig

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8b) stimulated HMDMs and compared this with the ability of apoptotic neutrophils (N) or NN to inhibit TNF-α secretion. Whereas LL37 was able to inhibit TNF-α secretion from LPS activated HMDMs, it behaved as a pro-inflammatory peptide to CD40L/IFN-γ stimulated HMDMs. This indicates that LL37 is not the active factor that inhibits both CD40L/IFN-γ and LPS stimulated macrophages. We titrated purified HNP 1-3 into LPS (Fig 8c) or CD40L/IFN-γ (Fig 8d) stimulated HMDMs and found that this peptide preparation was able to significantly inhibit pro-inflammatory cytokine secretion by activated HMDMs. As HNP1 constitutes the major alpha defensin in the primary granules of neutrophils (25) we used synthetically-derived HNP-1, finding similar levels of immunosuppressive activity (Fig 8e). HNP2 and HNP3 were also able to significantly inhibit TNF-α secretion by LPS or CD40L/IFN-γ stimulated HMDMs (data not shown). The requirement for structural integrity of HNP1 was examined by comparing the ability of linearized α-defensin, to inhibit TNF-α secretion from CD40L/IFN-γ. HMDMs; this confirmed that the three dimensional structure of HNP-1 was essential for anti-inflammatory activity, which was completely lost when the peptide was linearized (Fig 8f).

Alpha defensins do not affect the release of pro-inflammatory cytokines from macrophages

We asked if α-defensins elicited their anti-inflammatory properties *via* a direct effect on cell membranes preventing the release of cytokines contained within secretory vesicles of HMDMs. To address this we stimulated mature HMDMs with CD40L/IFN-γ plus or minus HNP1-3. At specified time-points culture supernatants were collected and analysed for TNF-α protein by ELISA. TNF–α levels climbed steadily after stimulation in control wells reaching a peak after 8 hours. However in stimulated and HNP1-3 treated wells TNF-α appeared to plateau soon after 3 hours and remained low for the duration of the experiment (Fig 9a). To ask if the TNF-α may be prevented from leaving the cells, macrophages were lysed at 4 hours after stimulation. The concentration of cytokines contained within the macrophage (Fig 9bi) and secreted into the culture medium was then compared by ELISA (Fig 9bii). No significant differences were seen in the ratio of secreted to retained TNF-α in either LPS or CD40L/IFN-γ stimulated HMDMs treated with α-defensins suggesting that TNF-α was not being sequestered within the macrophage. The low levels of NO found after α-defensin treatment would also be in keeping with our data as this is not stored in secretory vesicles (Fig 4c/d).

Necrotic neutrophils and HNP-1 protect mice from experimental inflammation

To assess the local effect of α-defensins on an established inflammatory response *in vivo* we used the thioglychollate model of peritonitis and found HNP1 and NN reduced the cellular infiltrate of neutrophils and macrophages (Fig 10a). We did not find a significant reduction in the inflammatory cell influx using necrotic mouse neutrophils (prepared in an identical way to human NN and at the same concentration), which lack α-defensins nor did the injection of whole AC or LL37 at 5 ug/ml affect the accumulation of inflammatory cells. In separate experiments to test the possibility that the reduced influx of inflammatory cells was secondary to the inhibition of resident peritoneal macrophages, these cells were isolated from the peritoneum of untreated mice, adhered to plastic overnight and stimulated with CD40L/IFN-γ along with added α-defensins (Fig 10b). Resident peritoneal macrophages treated with α-defensin were completely unable to respond to the stimulus and secrete TNF-α. Identical results were obtained following LPS stimulation (data not shown).

Discussion

It is currently widely believed that macrophages must engulf apoptotic neutrophils before they become necrotic to prevent the release into the tissues of potentially toxic and

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immunogenic intracellular substances (28). We have now discovered that both apoptotic and necrotic neutrophils elicit a profound anti-inflammatory response in macrophages that does not require cell contact. We have identified the anti-inflammatory mediator they release as α-defensins. The α-defensins inhibit macrophage pro-inflammatory function driven both by the microbial cell wall constituent LPS and a T cell surrogate stimulus CD40L/IFN-γ. When HMDMs are infected with Pseudomonas α-defensins effectively prevented the macrophages from inducing an exaggerated pro-inflammatory cytokine response, whilst not compromising the ability of macrophages to keep bacterial viability in check. This was mirrored in an in vivo model of infection with the pathogenic Salmonella Typhimurium using NN where both bacterial cell counts and serum TNF-α measured at the height of the infection were reduced.

Alpha-defensins are released by neutrophils as early as 4 hours after *in vitro* culture and continue to be released reaching a peak when neutrophil apoptosis is established. Importantly, the α-defensins are also released from necrotic cells when they disintegrate, explaining the protective effect of necrotic neutrophils when injected *in vivo* in a murine model of inflammation and infection. The finding that human NN, (which contains α-defensins) were able to reduce the influx of neutrophils and inflammatory macrophages in a murine model of peritonitis was surprising given that they have been shown to be chemotactic for immature dendritic cells and lymphocytes, though interestingly do not activate them (29). In addition necrotic human neutrophil supernatants were devoid of membranous products (following ultracentrifugation) but were otherwise replete with preformed enzymes that would be expected to be pro-inflammatory in their own right (1, 3). In contrast murine necrotic neutrophils, which do not contain α-defensins but are otherwise similar to human neutrophils did not affect the influx of inflammatory cells into the peritoneum suggesting that this effect was specific for the presence of the peptide (30). The effect of α-defensins on macrophages may be specific as α-defensin treatment did not inhibit the activation of human neutrophils by TNF-α, as measured by the loss of surface CD62L (L-selectin) and CD11b upregulation. Myeloperoxidase release from these activated neutrophils and the degranulation of murine peritoneal mast cells was also unaffected (data not shown). One may speculate that the reduced influx of inflammatory cells in the peritoneum may relate to an initial dampening of the inflammatory response of resident macrophages normally seen when the irritant and innate immune stimulus, thioglychollate is administered. This in turn would lead to a reduction in cellular influx of neutrophils and inflammatory macrophages. In support of this, *in vitro* experiments on resting resident peritoneal macrophages that have been stimulated with α-defensins show that they are completely inhibited from responding to concomitant stimulation with CD40/IFN-γ and this inhibition may override any chemotactic effect of α-defensin alone. α-Defensins have recently been shown to inhibit specifically the secretion of IL-1β by monocytes attesting to their anti-inflammatory role (13). Interestingly recent reports have linked the absence of intestinal Paneth cell α-defensins to chronic colitis seen both in animal models and in humans with Crohn's disease (11, 31). As Crohn's disease is likely due to an aberrant response to commensal bacteria which normally pose no risk to healthy adults (32-34), one could speculate that the lack of these α-defensins may deprive these patients not only of an antimicrobial peptide but also of an important anti-inflammatory and immuno-regulatory signal in the distal small intestine (13). Indeed the effect of α-defensins on macrophages, reducing the secretion of multiple pro-inflammatory cytokines whilst checking the growth of bacteria attests to its ability to prevent an excessively pro-inflammatory macrophage response whilst not sacrificing its ability to function as an antimicrobial peptide. The mechanism by which α-defensins inhibit such a broad swathe of pro-inflammatory cytokines and NO is unknown. As an anti-microbial peptide, they induce pores in bacterial membranes but the exact means by which they kill microbes remains a mystery (35). Analysis of treated macrophages used in our assays showed no evidence of macrophage

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apoptosis following culture with α-defensins. Prolonged treatment for up to 24 hours with α-defensins did not result in a delayed decrease in viability as measured by Alamar blue and LDH assays. In addition macrophages regained the ability to respond to pro-inflammatory stimuli producing equivalent amounts of TNF-α, compared to control untreated macrophages following a delay that was proportional to the time that they had been initially exposed to α-defensins. Pre-treatment with α-defensins led to an increase in phagocytic capacity, which suggests that they do not simply inhibit macrophage function but alter it to a pro-phagocytic pro-resolution phenotype. Time-course studies of secreted TNF-α indicate that released cytokine fails to ever reach control levels following α-defensin treatment but lysates of cells did not contain TNF-α, suggesting that it was not prevented from leaving the cells as Shi et al have found specifically for IL-1β in monocytes. We would speculate that α-defensins may affect the translation of pro-inflammatory cytokines through an effect on mRNA stability or alternatively through the inhibition of the pro-inflammatory transcription factor, nuclear factor-*k*B. Future work clearly needs to elucidate the molecular mechanism by which they inhibit the inflammatory phenotype of macrophages and to ask if this could be useful as a therapeutic option in autoimmune diseases such as rheumatoid arthritis in which the inflammatory macrophage mediates the final assault on normal healthy tissue.

Currently, the prevailing view is that necrotic cells present danger signals to the immune system; for instance necrotic fibroblasts are found to be immunostimulatory to DC (36, 37). Thus, it is generally accepted that the presence of necrotic cells, especially neutrophils, is pro-inflammatory (3, 28). This is despite reports of the inhibitory effect of necrotic neutrophils on dendritic cell (DC) maturation and the ability of macrophages to respond to necrotic neutrophils in a non-phlogistic way, (4, 38). It seems likely that not all necrotic cells pose a danger. Our data clearly shows that necrotic human neutrophils are, in fact, anti-inflammatory and if a macrophage encounters such a cell its ability to secrete pro-inflammatory cytokines and NO is inhibited, whilst its ability to phagocytose material is increased. Thus, neutrophil necrosis at sites of inflammation far from driving the process, initiates its resolution.

Tissue resident macrophages are among the first cells to detect microorganisms that have crossed an epithelial barrier. They then recruit large numbers of neutrophils, followed by blood monocytes that differentiate into macrophages upon entry into the affected tissue. Both cell types become activated, phagocytose microorganisms and in the case of neutrophils then undergo apoptosis. The presence of these apoptotic cells then alters the macrophage response, switching it from an inflammatory to a pro-resolution phenotype (39). If necrotic neutrophils were pro-inflammatory and if the ability of macrophages to phagocytose them was overwhelmed even temporarily; then the inevitable result would be further inflammation. In this scenario the immune system would be permanently poised on a knife-edge, dependent entirely upon the rate at which apoptotic neutrophils were removed. In our model, the finding that necrotic human neutrophils are uniquely anti-inflammatory attests to the importance of avoiding this catastrophic possibility. In fact during an inflammatory response the apoptosis of neutrophils (and subsequent interaction with macrophages), is correlated temporally with the resolution of inflammation (40, 41). Physiologically, α-defensins released by dying neutrophils may then exert potent anti-inflammatory effects on macrophages, providing the perfect counterbalance to the arsenal of cytotoxic compounds contained within them. The release of alpha defensins means that the pro-resolution effect of apoptotic/necrotic neutrophils on inflammatory macrophages is not limited to those cells the neutrophil specifically contacts. In conclusion, neutrophils secrete both an antimicrobial and an anti-inflammatory peptide as they die and undergo necrosis, so that even in death they continue to exert an immunomodulatory and anti-microbial phenotype fighting pathogens whilst preventing an excessive inflammatory response that would place healthy tissue at risk of further damage.

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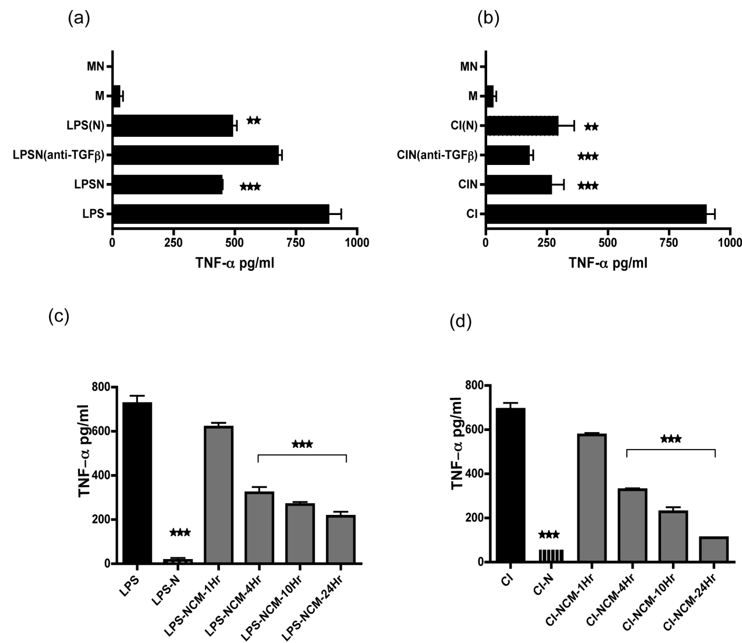

Figure 1. Neutrophils secrete a soluble anti-inflammatory factor

(a+b) HMDM were stimulated with either LPS or CD40L/IFN-γ (CI) along with added apoptotic neutrophils (LPSN or CIN) for 18 hours, prior to harvesting culture supernatants for assay of TNF-α by ELISA. In triplicate wells anti-TGFβ was added to assess the role of TGFβ in mediating the immunosuppressive effect of apoptotic neutrophils [LPSN(anti-TGFβ) or CIN(anti-TGFβ)]. In addition apoptotic neutrophils were separated from activated HMDM by a transwell [LPS(N) or CI(N)] for the duration of the culture period. Macrophages alone (M) or unstimulated macrophages cultured with apoptotic neutrophils (MN) did not secrete TNF-α.

(c+d) Neutrophils were cultured for up to 24 hours, harvested at set timepoints and ultracentrifuged to remove cell membranes and apoptotic bodies. This neutrophil conditioned medium (NCM) was added (as 25% final vol) to LPS or CD40L/IFN-γ (CI) stimulated macrophages. After 18 hours of culture HMDM culture supernatants were harvested and assayed for TNF-α by ELISA.

Representative of 10 experiments performed with different human donors. Error bars represent SEM and significance of *** p 0.0002, ** p 0.002, * p 0.02.

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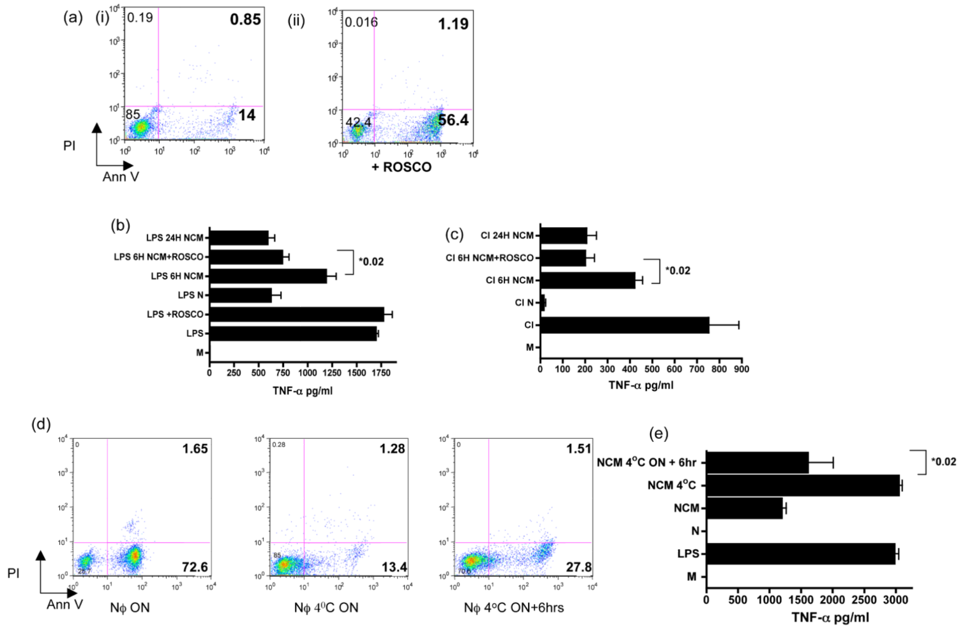

Figure 2. Neutrophils release an active anti-inflammatory factor as they become apoptotic
(a-i-ii) Facs analysis of neutrophils cultured in 20uM Roscovitine (Rosco) for 6 hours and stained with Annexin V/PI to detect apoptotic and necrotic cells respectively. NCM from neutrophils cultured with or without Roscovitine for 6 hours was co-cultured with either CI **(b)** or LPS **(c)** stimulated macrophages for 18 hours after which macrophage culture supernatants were collected and tested for TNF-α content by ELISA. (**d**) FACS analysis of neutrophils stained with Annexin V/PI in which apoptosis was inhibited by culturing them at 4°C overnight (NCM 4°C) and then allowed to undergo apoptosis for 6 hrs by culturing at 37°C (NCM 4°C+6hr). Analysis of the ability of NCM from these 3 neutrophil populations to inhibit LPS activated HMDM cytokine secretion was assessed by ELISA following an 18 hour incubation with LPs stimulated macrophages (**e**). Data representative of 3 separate experiments with different donors. Error bars=SEM and **=p<0.02.

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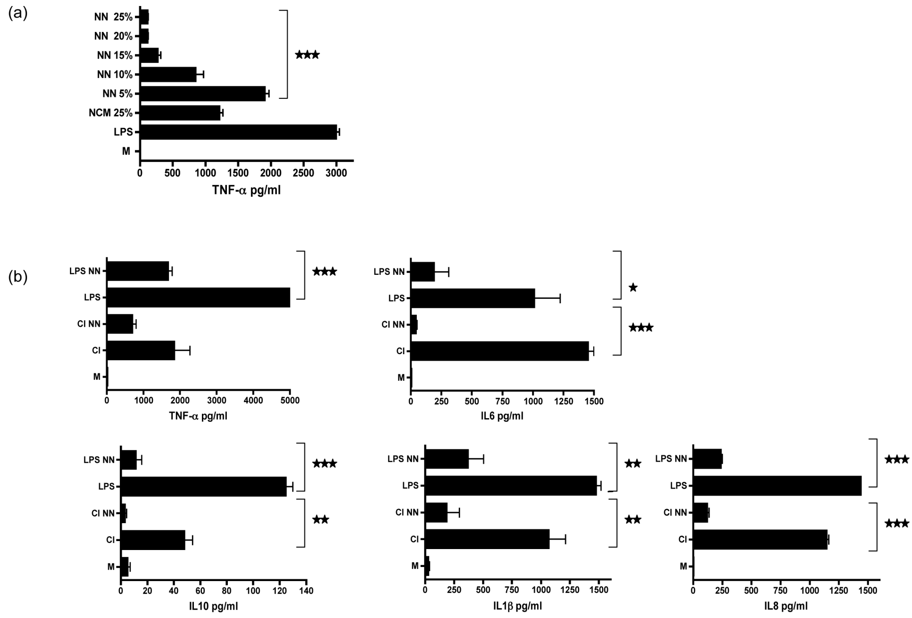

Figure 3. Necrotic neutrophils are anti-inflammatory

(**a**) Fresh neutrophils were freeze thawed and then ultracentrifuged to generate membrane-free necrotic neutrophil supernatants (NN). NN was titrated into cultures containing LPS activated HMDMs. This was compared with the ability of NCM at a final vol:vol of 25% released from apoptotic neutrophils to inhibit TNF-α secretion. TNF-α in the supernatants collected from these stimulated macrophages (after 18 hrs of culture) was quantified by ELISA.

(**b**) Multicytokine analysis of these supernatants to show that NN inhibited the secretion of a wide range of pro-inflammatory cytokines as well as IL-10 by activated macrophages stimulated with LPS or CD40L/IFN-γ (CI). *** p 0.0002, ** p 0.003, * p 0.03.error bars =SEM. Experiments representative of 1 from 5 using separate donors.

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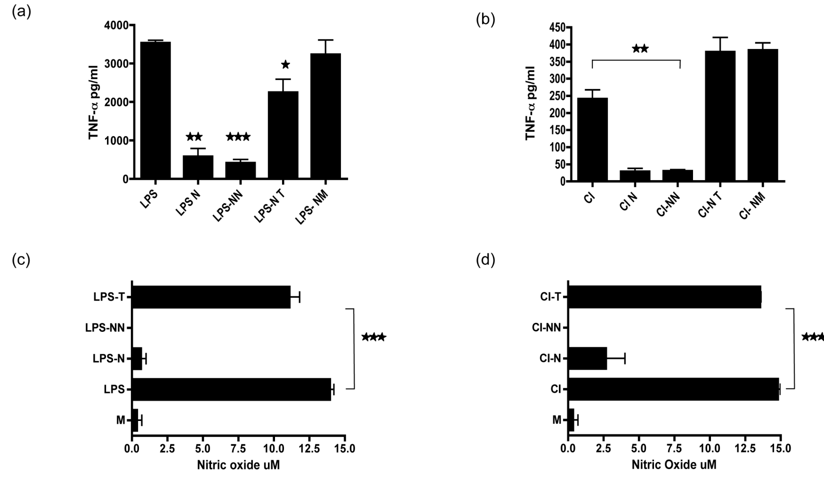

Figure 4. Necrotic neutrophils but not other necrotic cells are anti-inflammatory

Apoptotic neutrophils (N), necrotic neutrophil supernatants (NN), necrotic thymocyte supernatants (NT) and necrotic supernatants from the tumour cell line Mutu (NM) were added to either LPS (a) or CD40L/IFN-γ (CI) (b) stimulated macrophages and culture supernatants collected 18 hours later were analysed by ELISA for TNF-α. Murine bone marrow derived macrophages were also stimulated with either LPS (c) or CD40L/IFN-γ [CI] (d) with added NN or NT and culture supernatants tested for NO by the Griess reaction. *** p 0.0001, ** p 0.002, * p 0.02.error bars =SEM.

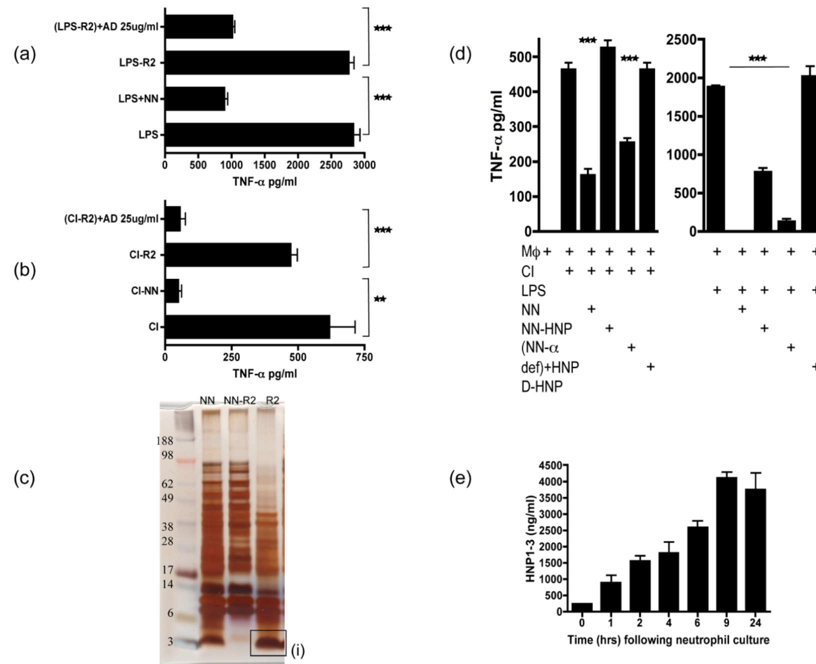

Figure 5. Alpha defensins are one of the the active anti-inflammatory factors released by apoptotic/necrotic neutrophils

LPS (a) or CI (b) stimulated HMDM were cultured with NN, NN depleted of hydrophobic molecules by R2 beads (NN-R2) and NN-R2 where α-defensins were added back at 25ug/ml [(NN-R2)+AD]. Culture supernatants were collected after 18 hours of culture and tested for TNF-α secretion by ELISA. (c) A protein gel of NN indicated the large number of proteins released by necrotic neutrophils (NN). NN were depleted of hydrophobic proteins by R2 beads (NN-R2) and the proteins bound to the R2 beads (R2) were identified. R2 beads completely depleted a large band of small proteins between 3-5kD. This band was digested and sequenced by HPLC and identified as the anti-microbial peptides, α-defensins. (d) The actual release of α-defensins over 24 hours by cultured neutrophils undergoing apoptosis was quantified by HNP 1-3 ELISA. (e) To ensure that the R2 beads had not depleted other anti-inflammatory factors, α-defensins in NN were specifically depleted using anti-HNP antibodies bound to dynabeads. HMDMs were then stimulated with CI or LPS along with added NN depleted of α-defensins (NN-α def) or depleted NN where HNP 1-3 has been added back at 25ug/ml (NN-α def)+HNP. As an additional control HMDMs were stimulated with CI or LPS in the presence of the D-enantiomer of HNP1-3, which lacks anti-inflammatiory activity and is protease resistant.. *** p 0.0001, ** p 0.04. error bars =SEM.

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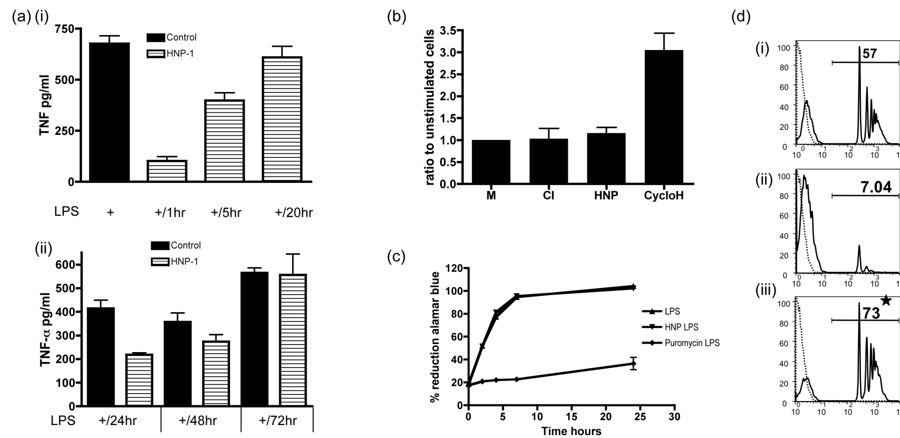

Fig 6. Alpha defensins do not kill macrophages and actually enhance their phagocytic capacity

(a) HMDMs were pre-treated with HNP1-3 for 1 hour (i) and then allowed to rest for 1 (+/1hr), 5 (+/5hr) or 20 hours (+/20hr) prior to stimulating them with LPS for a further 18 hours, after which supernatants were collected and tested for TNF-α by ELISA. (ii) The same as (i) but HMDMs were pre-treated with HNP 1-3 for 24 hours and then rested for 24 (+/24hr), 48 (+/48hr) or 72 hours (+/72hr) prior to stimulating with LPS. Control HMDMs were pre treated with vehicle alone for the same time period. (b)LDH levels were measured from supernatants taken from HMDMs stimulated with CD40/IFN-γ (CI), HNP1-3 (HNP) or cyclohexamide for 18 hours. (c) HMDMs were pre treated with HNP 1-3 or Puromycin for 24 hours prior to stimulating with LPS for a further 18 hours. Alamar blue was used to determine the the presence of reductive enzymes seen in viable cells. (d) Histograms of HMDMs that were treated with vehicle (i), puromycin (ii) or HNP1-3 (iii) for 24 hours, prior to adding fluorescent beads for 1 hour. Cells were washed, lifted from the cell culture plates and the degree of eating quantified by FACs (black line). Dashed line represents control HMDMs without added fluorescent beads. * p 0.01. error bars =SEM.

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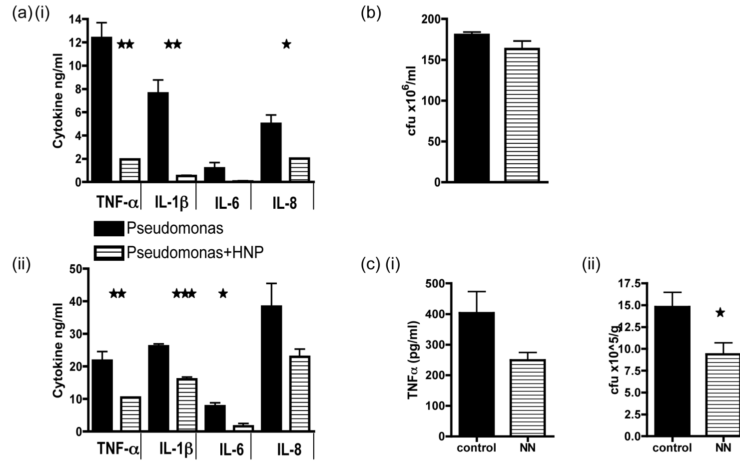

Fig 7. Alpha defensins can still inhibit pro-inflammatory cytokine secretion by HMDMs despite infection with whole bacteria

(a) HMDMs were co-cultured with dead Pseudomonas Aeruginosa PA01 for 24 hours (i) or live bacteria (at an MOI of 10) for 4 hours (ii) after which culture supernatants were collected and cytokine secretion quantitated by ELISA. (b) For the live bacterial experiment HMDMs were lysed after 4 hours and bacteria cultured for a further 18 hours on agar prior to counting the number of live colonies. (c) Mice were injected with $10^{\times 6}$ of live Salmonella *enterica* serovar Typhimurium and PBS or NN was administered on days 0,1,2,4 and 6. Mice were sacrificed on day 7 and TNF-α was measured in the serum (i). In addition the number of live bacteria retrieved from lysed splenocytes after an overnight culture was calculated following a further 18 hours of culture on agar (ii). *** p 0.0004, ** p 0.003, * p 0.02. error bars =SEM

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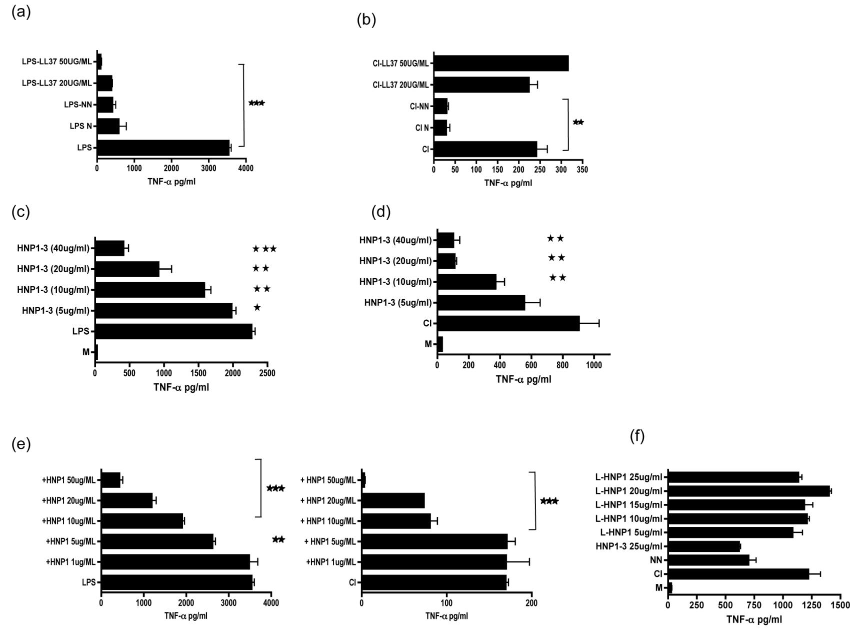

Fig 8. Alpha defensins but not cathelicidins inhibit both T cell mediated and LPS mediated activation of macrophages

HMDM were stimulated with either LPS (a) or CD40L/IFN-γ (CI) [b] and apoptotic neutrophils (N), necrotic neutrophil supernatants (NN) or LL37 at the indicated doses. In separate experiments HNP1-3 (c-d) or purified HNP-1 was titrated into CI or LPS activated HMDMs. The anti-inflammatory potential of synthetically derived HNP-1 that had been linearized was compared to α-defensins using CD40L/IFN-γ (CI) stimulated HMDMs. Culture supernatants were harvested after 18 hours and tested for TNF-α secretion by ELISA. *** p   0.0001, ** p   0.001, * p   0.01.error bars =SEM when compared to HMDMs treated with stimulus alone .

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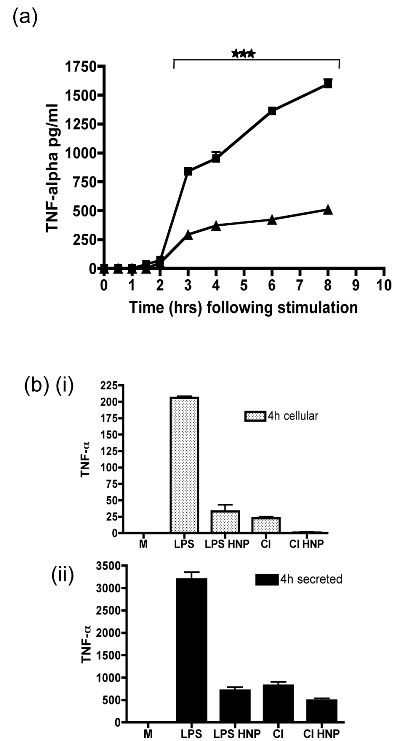

Figure 9. Alpha defensins do not inhibit the exocytosis of TNF-α

(a) HMDM were stimulated with CD40L/IFNγ (CI) either alone or in the presence of HNP1-3 (25ug/ml) for the indicated times. TNF-α protein released by HMDMs was measured by ELISA. (b) HMDMs were stimulated with LPS or CD40L/IFN-γ (CI) and treated with HNP1-3 (25ug/ml) or vehicle alone. At 4 hours post stimulation culture supernatants (i) were harvested prior to lysis of the HMDMs to reveal TNF-α retained within the cells (ii). *** p   0.001, error bars =SEM.

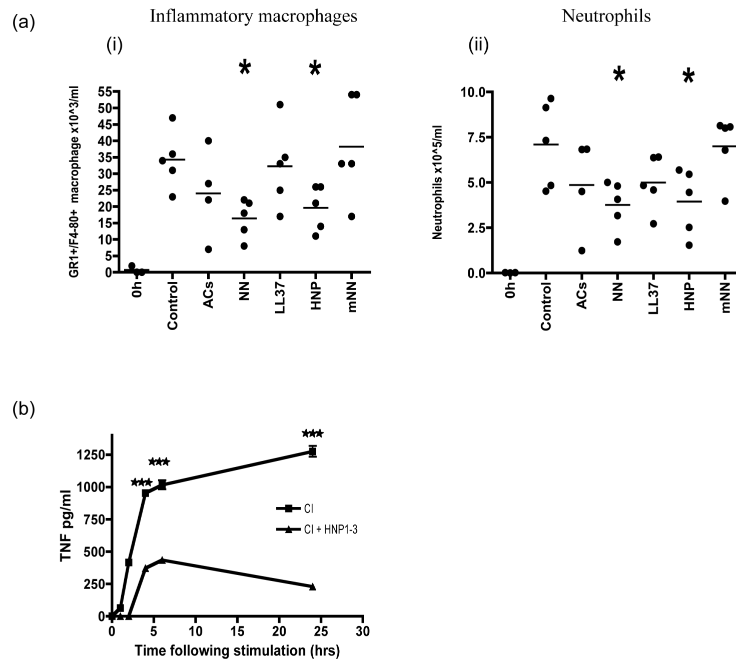

Figure 10. Necrotic neutrophils and α-defensins protect mice from experimental inflammation
Sterile peritonitis was induced by injecting thioglychollate, along with either PBS, apoptotic cells (AC), necrotic human neutrophils (NN), LL37, HNP1-3 or mouse necrotic neutrophils (mNN). After 4 hours peritoneal lavages were used to isolate inflammatory GR1+F480+ macrophages (a) and neutrophils (b), which were characterised by FACS and compared to cell numbers in control mice with peritonitis given PBS. Experiment is representative of 2 separate expts with 5 mice per group. (c) In separate experiments resting murine peritoneal macrophages were isolated and stimulated *in vitro* with CD40/IFN-γ along with α-defensins and supernatants were collected at various timepoints and tested for TNF-α. *** p 0.001, * p 0.01, error bars =SEM.

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