The impact of cationic host defence peptide LL-37 on human neutrophil death and inflammatory responses

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Declaration

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with reference to the literature, and acknowledgement of collaborative research and discussions. The data in this thesis have not been submitted anywhere for any degree or award.

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List of Abbreviations

AIM2	The protein absent in melanoma 2
AIF	Apoptosis-inducing factor
Apaf-1	Apoptosis protease activating factor-1
APC	Antigen-presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BAI1	Brain-specific angiogenesis inhibitor 1
Bcl-2	B-cell lymphoma-2
Bid	BH3-interacting domain death agonist
BIR	Baculovirus IAP repeat
BPI	Bactericidal /permeability-increasing protein
BTHS	Barth's syndrome
CAD	Caspase-activated DNAse
CARD	Caspase activation and recruitment domain
Caspase	Cysteinyl aspartate-specific proteases
CCL4	Chemokine (C-C motif) ligand 4
CCR2	Chemokine (C-C motif) receptor 2
CCR6	Chemokine (C-C motif) receptor 6
CDK	Cyclin-dependent kinase
CDK	Cyclin-dependent kinase
CF	Cystic fibrosis
CHDP	Cationic host defence peptides
c-IAP	Cellular inhibitor of apoptosis protein
cIAP2	Cellular inhibitor of apoptotic protein 2
CLP	Cecal ligation and puncture
CLR	C-type lectin receptor
CR3	Complement receptor (CD11b/CD18)
CR4	Complement receptor (CD11c/CD18)
CRAMP	Cathelin-related antimicrobial peptide
CTL	Cytotoxic T cell
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL10/ IP-10	Chemokine ligand 10/ interferon-γ-inducible protein-10
CXCR2	CXC chemokine Receptor 2
CXCR4	CXC chemokine Receptor 4
Cyt c	Cytochrome c
DAMP	Danger-associated molecular pattern
dATP	Deoxyadenosine triphosphate
DC	Dendritic cell
DD	Death domain

DED	Death effector domain
DFF	DNA fragmentation factor
Diablo	Direct IAP binding protein with low pI
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
Endo G	Endonuclease G
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FADD	Fas-Associated protein with Death Domain
FasL	Fas ligand or CD95L
FCS	Foetal calf serum
fMLF	Formyl-methionyl-leucyl-phenylalanine
FPRL-1	Formyl peptide receptor-like 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gas6	Growth arrest-specific 6
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HBD	Human β-defensin
HBSS	Hank's balanced salt solution
HMGB1	High-mobility group box
HNP	Human neutrophil peptide
HOCl	Hypochlorous acid
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein
ICAM	Intracellular adhesion molecule
IDR	Innate defence-regulator peptide
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-18	Interleukin-18
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IMDM	Iscove’s Dulbecco’s modified Eagles medium (+ L-Glutamine,

	+25mM HEPES)
INF-α	Interferon-alpha
INF-β	Interferon-beta
INF-γ	Interferon-gamma
IPAF	ICE protease-activating factor
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
IRG-1	Immunoresponsive gene 1
LDH	Lactate dehydrogenase
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LRP	LDL-receptor-related protein
LRR	Leucine-rich repeats
LTA	Lipoteichoic acids
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein-1
mCRAMP	Mouse cathelin-related antimicrobial peptide
MDM	Monocyte-derived macrophage
MFG-E8	Milk fat globule protein
MIC	Minimum inhibitory concentration
Mincle	Macrophage-inducible C-type lectin
MIP-1α	Macrophage inflammatory protein-1α
MIP-3α	Macrophage inflammatory protein-3α
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeability
MPO	Myeloperoxidase
mPT	Mitochondrial permeability transition
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NALP	NACHT domain-leucine-rich repeat-, and PYD-containing protein
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-kappaB
NGAL	Neutrophil gelatinase-associated lipocalin
NK cell	Nature killer cell
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
OVA	Ovalbumin

PAF	Platelet-activating factor
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen-associated molecular pattern
PARP-1	Poly (ADP-ribose) polymerase 1
PBMC	Human peripheral blood monocytes
PBS	Phosphate buffer saline
PCD	Programmed cell death
PGN	Peptidoglycan
PI	Propidium iodide
PMN	Polymorphonuclear leukocyte
PPAR-δ	Perxisome proliferator-activated receptor-δ
PRR	Pattern recognition receptor
PS	Phosphotidylserine
PSR	Phosphotidylserine receptor
PTX	Pertussis toxin Bordetella pertussis
RAGE	The receptor for advanced glycation end products
RANTES	Regulated upon activation, normal T cell expressed and secreted
RIP	Receptor interacting protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SIPRα	Signal regulatory protein alpha
SLE	Systemic lupus erythematosus
Smac	Second mitochondria-derived activator of caspase
SR	Scavenger receptor
TAK1	Transforming-growth-factor-β-activated kinase 1
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-beta
Tim4	T-cell immunoglobulin- and mucin-domain-containing molecule 4
TIR	Toll/IL-1 receptor
TIRAP	Toll/interleukin-1 receptor domain-containing adapter protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAF6	Tumour necrosis-factor-receptor-associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
VnR/CD36	Vitronectin receptor/CD36 complex
XIAP	X-linked inhibitor of apoptosis protein

Abstract

Cathelicidins are cationic host defence peptides (CHDP) with essential roles in the innate defence system. These peptides have antimicrobial potential and the capacity to modulate innate immunity and inflammatory processes. Neutrophils (PMN) are the main reservoir of cathelicidins and play key roles in first line defence against infection. The appropriate regulation of PMN function, death, and clearance is critical to innate immunity, and the efferocytosis of apoptotic PMN, in contrast to necrotic cells, is proposed to promote the resolution of inflammation. In this thesis I demonstrate that the human cathelicidin LL-37 rapidly induced secondary necrosis of apoptotic human PMN and identify the essential C-terminal region of LL-37 required for this activity. In addition to the induction of secondary necrosis, higher concentrations of LL-37 also promoted PMN granule contents release. LL-37-induced secondary necrosis did not affect PMN ingestion by human monocyte-derived macrophages and, in contrast to expectation, was not pro-inflammatory. Interestingly, the anti-inflammatory effects of apoptotic PMN on activated macrophages were retained and even potentiated where LL-37-mediated secondary necrosis induced anti-inflammatory granule content release. Consistent with the results of *in vitro* studies, *in vivo* murine sterile peritonitis model revealed the same phenomenon: LL-37-induced secondary necrosis diminished inflammatory responses with decreased PMN influx. I also present data on LL-37-mediated modulation of innate immune effector cell cytokines responses to inflammatory signals. I propose that during acute inflammation LL-37 can modulate innate immune responses through its activity on cytokine production, and that LL-37-mediated secondary necrosis of apoptotic PMN has anti-inflammatory

effects, but may also mediate host damage by promoting the release of potentially harmful intracellular contents under chronic or dysregulated conditions.

Chapter 1

Introduction

1. Introduction

1.1. General introduction

All living organisms are exposed constantly to microorganisms that are present in the environment and need to cope with invasion of these organisms into the body. The host response to infection and injury depends on both innate and adaptive immune systems. The adaptive immune system primarily relies on the actions of B and T lymphocytes that activate and develop in response to threats. In contrast, the innate immune system is a germ-line encoded scheme to recognise conserved patterns. The response of innate immunity is immediate and depends on the activity of cells located at primary barriers between host tissue and environment such as macrophages, neutrophils (PMN), epithelial cells, mast cells, and natural killer cells (NK). These cells express a wide variety of pattern recognition receptors (PRR), such as toll-like receptors (TLRs) and scavenger receptors that recognise structurally conserved molecules derived from microorganisms. The detection of microbes results in the release and activation of numerous effector molecules and mediators of host defence, including the complement cascade, cytokines, chemokines, and cationic host defence peptides (CHDP).

Cationic host defence peptides, existing in organisms from plants to insects to invertebrates and vertebrates, are involved in many aspects of innate host defences

associated with inflammation. Not only do they demonstrate direct antimicrobial activity against pathogens, but they also have multiple modulatory properties in immune responses [1, 2]. These peptides are mainly stored in the granules of innate immune effector cells, such as PMN, monocytes, NK cells, and mast cells, or expressed and produced de novo by epithelial cells [3, 4]. Although pleiotropic functions of CHDP have been studied, the key mechanisms involved in many of the immunomodulatory properties have not been completely understood.

Emerging evidence demonstrates the impacts of CHDP on the functions and fates of immune effector cells [2, 5], with perhaps the most promising target being PMN. PMN are one of the most prominent effector cells of the innate immune system [6]. They are armed with a variety of weapons allowing them to combat a broad range of microbes, including generation of reactive oxygen species (ROS) and discharge of granule contents to kill pathogens [7]. PMN have a short lifespan, and undergo spontaneous apoptosis to ensure their histotoxic constituents remain packaged inside the intact cell membrane, followed by the clearance by phagocytes. Efficient removal of dead PMN is a prerequisite for the resolution of inflammation. Proper regulation of PMN recruitment, activation, death, and removal is thus critical to maintain homeostasis; dysregulation of these processes has been implicated in the pathogenesis of many inflammatory diseases [8].

Under physiological conditions, apoptotic PMN are phagocytosed by macrophages to promote the resolution of inflammatory responses rapidly with limited tissue damage [9]. Many receptors, adaptors, and chemotactic molecules

are reportedly involved in the process of removing apoptotic cells in various tissues [10, 11]. Growing evidence indicates that the phagocytic clearance of apoptotic cells and bodies is not only important for the disposal of effete cells, but can also result in powerful anti-inflammatory and immunosuppressive effects [12, 13]. In contrast to apoptosis, recognition of necrotic cells by phagocytes is less understood and cell death by necrosis is typically regarded as being associated with inflammation [14]. However, an increasing number of recent studies have shown that necrotic cells were not necessarily always pro-inflammatory, and that apoptotic cell clearance could also have inflammatory consequences [15]. Irrespectively, these reports suggest that the modes of cell death can have substantial influences on inflammatory responses and are not as simple as what was proposed.

At inflamed sites, increased PMN influx to affected areas and release of their stored CHDP can help to contain harmful pathogens. Released CHDP also possess multiple effects on PMN. For example, the human cathelicidin LL-37 (a well-characterised CHDP) induce the generation of ROS from human PMN and enhances the engulfment of bacteria [16, 17]. LL-37 also modulates the response of PMN to LPS [16, 18] and promotes immune responses by attracting PMN and other immune effector cells [19]. In addition, CHDP have a complex influence on PMN death [20, 21]. However, the mechanisms by which CHDP modulate PMN death remain elusive, and whether CHDP modulate inflammatory responses by altering innate immune cell death is still unknown. My studies therefore focus on the effects of CHDP on human PMN death, trying to characterise the mechanism

involved. In addition, I address the impacts of CHDP on inflammatory responses, by modulating cell death and by altering cytokine responses in response to pro-inflammatory stimuli *in vitro* and *in vivo*.

CHDP have been regarded as potential candidates to develop novel therapeutic drugs due to their multiple functions in modulating immune responses. Studying the definitive pathways by which they interact with immune cells provides chances to exploit their potential to fight harmful pathogens and avoid side effects of over-reacted inflammatory responses. It is hoped that better understanding of the mechanisms involved in modulation of PMN death by CHDP, the effects of CHDP on phagocyte clearance and the subsequent inflammatory response, and the physiological significance of these observations, can provide advanced knowledge for which to develop new therapeutics for inflammatory diseases [22-24].

1.2. Cationic host defence peptides

1.2.1. Introduction

Cationic host defence peptides (CHDP) play critical roles in innate immune responses. They are found in all classes of life and are evolutionarily conserved. These peptides share many key features such as positive charges, ranging in size from 12 to 80 amino acid residues, and an amphipathic structure [24]. Initially it was believed that their sole role in innate immunity was to kill invading microorganisms thus they were described as “antimicrobial peptides”. However, *in vitro* and *in vivo* evidence now suggests that these peptides play diverse and complex roles in the immune system, such as anti-endotoxic capacity, stimulation of cell cytokine production, chemotaxis of immune effector cells, alteration of transcriptional responses of antigen presenting cells, modulation of dendritic cell differentiation and function, triggering of mast cell degranulation, promotion of angiogenesis and wound healing, and modulation of cell death [2, 25] via unknown or known receptors (Table 1.1) and pathways.

Peptide	Receptor	Function	Target cells
Human alpha defensins	Unknown (chemokine receptor)	Chemotaxis	naïve CD45RA/CD4 T cells, CD8 T cells, immature dendritic cells [26]
Human beta defensins	CCR6	Chemotaxis	immature dendritic cells, memory CD45RO/ CD4 T cells [27]
LL-37	FPRL-1	Chemotaxis	human PMN, monocytes, and T cells [19]
LL-37	two unknown receptors	Chemotaxis, degranulation	mast cells [28]
LL-37	$P2X_7$	Promote processing of IL-1beta	human monocyte [29]
LL-37	EGFR	Activate MAPK/ERK, release IL-8	epithelial cells [30]
LL-37	Atypical endocytic processes	MAPK signalling, chemokine, cytokine production	epithelial cells [31]
LL-37	CXCR2	Migration, function	PMN [32]
LL-37	EGFR	Migration	keratinocyte [33]
LL-37	FPRL-1	angiogenic activation, proliferation	endothelial cells [34]
CRAMP	FPRL-1/ mouse FPR-2	Chemotaxis	human monocytes, PMN, macrophages, mouse peripheral blood leukocytes [35]
LL-37, CRAMP, IDR	GAPDH	MAPK signalling, chemokine, cytokine production	Monocytes [36]

Table 1.1 Receptors of CHDP and the multiple functions of CHDP to target cells.

1.2.2. Major families of CHDP

Two major families of host defence peptides in mammals are widely studied. The first family, the defensins, containing six highly conserved cysteine residues which form three pairs of intramolecular disulfide bonds, are classified into α-, β-, and θ-defensins based on alignment of these disulfide bonds (α-defensins are linked in a 1-6, 2-4, and 3-5 pattern, whereas the disulfide bonds in β-defensins are in a 1-5, 2-4, and 3-6 pattern. θ-defensins are circular without a free N- or C-terminus) [37]. Alpha-defensins are stored in the primary granules of granulocytes [38] and are made in Paneth cells in crypts of small intestine [39, 40] while beta-defensins are generated by monocytes, macrophages, keratinocytes, or mucosal epithelial cells of the respiratory, digestive, urinary, and reproductive systems [41, 42]. θ-defensins can be isolated from PMN and bone marrow of non-human primates [43]. The expression of these peptides is greatly increased following injury or infection.

Cathelicidins are another family of cationic host defence peptides and named based on the highly conserved N-terminal region known as the cathelin (cathepsin L inhibitor) domain. They fold into amphipathic structure, retaining both cationic and hydrophobic faces to facilitate the interaction with negatively charged membrane [44]. In humans, only one cathelicidin gene (CAMP) has been found to date, on chromosome 3p21.3. It encodes an inactive precursor protein with an approximate mass of 18 kDa and is thus named hCAP18. hCAP18 is mainly stored in PMN specific granules at a concentration as high as 630 $\mu g/10^9$ cells [45, 46]

and is mainly processed by a PMN proteinase (protease-3) to release its mature form, LL-37 [47]. Other granulocytes (such as macrophages, NK cells and mast cells) [48], epithelial cells from a variety of tissues (such as urinary and respiratory tracts), and colon enterocytes also release or secrete this peptide during infection, injury, or inflammation [49-56]. LL-37 is composed of 37 amino acids rich in lysine and arginine which begins with two leucine residues with +6 net charge and forms an α-helix structure [44]. In addition to LL-37, the prostate-derived protease gastricsin can also process hCAP18, generating the alternative cleavage product ALL38 [57]. At the human skin surface, LL-37 is further processed by serine proteases or other proteases produced by the skin microflora into different derivatives (e.g. RK-31, KS-30 and K20) that have alternate activities [58, 59]. Thus, although there is only one cathelicidin gene in humans, the peptides generated can be varied by post-translational modification.

In addition to PMN granules, human cathelicidin can be found in a number of bodily fluids, including gastric juices, saliva, seminal plasma (42-143 μg/ml), sweat (5 μg/ml), blood, airway surface liquid, and breast milk [60, 61]. LL-37 detected in the bronchoalveolar lavage of healthy individuals is at concentrations around 2-5 μg/ml, and is increased by 2-3 folds during infections [4]. In addition, plasma has been reported to contain hCAP18 bound to lipoproteins at a concentration of 1.2 μg/ml [62].

The induction of CHDP often involves signalling mediated by PRR or responses to pro-inflammatory cytokines. For example, although keratinocytes express

human beta-defensin hBD1 mRNA constitutively, hBD2, hBD3, and hBD4 are induced by stimulation with TLR ligands, TNF-α, IL-1β, INF-γ, and 1,25-dihydroxyvitamin D_3 [63-73]. Cathelicidin expression is moderately inducible upon stimulation with pro-inflammatory cytokines such as IL-1α [74] or bacterial components [75]. Recently, it was found that active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) induced gene expression and protein production of cathelicidins in isolated human epithelial cells, keratinocytes, macrophages, monocytes, and PMN [53, 72]. A recent study demonstrated that LL-37, induced by active form of vitamin D, triggered autophagy in affected cells and promoted the eradication of intracellular pathogens [76, 77].

1.2.3. Physiological significance of CHDP

The importance of CHDP to human immunity is indicated by the increased susceptibility to infection of individuals with a specific granule deficiency and patients with morbus Kostmann [78, 79] (conditions in which PMN deficiency in defensins and cathelicidin, respectively, constitute key components of the disease). Additionally, smaller amounts of Paneth cell α-defensins are found in patients with Crohn's disease compared with the amount in normal controls [80]. Moreover, in the case of chronic cystic fibrosis lung disease or psoriatic skin lesion, LL-37 expression has been detected at higher concentrations (~15 μg/ml and ~1.5 mg/ml respectively) than that observed in unaffected individuals (~5 μg/ml and ~0 μg/ml) [3]. Conversely, expression of hBD2 and the cathelicidin LL-37 is diminished substantially in atopic dermatitis, a skin condition often accompanied by bacterial,

fungal, or viral infection [3]. These clinical findings suggest that CHDP play an important role in first-line defence, but uncontrolled upregulation of these peptides can result in chronic inflammatory disorders.

1.2.4. Antimicrobial capability of CHDP

In vitro, most CHDP have antimicrobial activity against a range of microbes including gram-positive and gram-negative bacteria, protozoa, fungi and some viruses [2]. Studies have shown their capacity to directly disrupt the microbial cell membrane and thereby result in killing [81, 82]. In general, positively charged peptides are attracted by electrostatic forces to the negative phospholipid head groups on the membrane surface capsular polysaccharides, including lipopolysaccharide (LPS) in Gram-negative bacterial cell wall and teichoic acids and lipoteichoic acids (LTA) in Gram-positive bacteria. Then the nonpolar face of peptides will allow insertion into the membrane through hydrophobic interactions. Once CHDP gain access to the cytoplasmic membrane they interact with the lipid bilayer, alter membrane structure and create a physical hole causing cellular contents to leak out [81, 82].

However, several mechanisms have been described for microbial resistance to CHDP. For example, the expression of LL-37 and hBD1 was reduced or turned off by *Shigella* infection [83]. In addition, microbes such as *S. aureus, Bacillus anthracis,* and *Burkholderia cenocepacia* produce metalloproteinases which can effectively degrade LL-37, contributing to the resistance of these pathogens to LL-

37 [84-86]. Another effective strategy used by *S. aureus* is to change the composition of its cell membrane. The alterations decrease the susceptibility of pathogens to the peptides and contribute to their pathogenicity [87]. Furthermore, LPS of *P. aeruginosa* found in cystic fibrosis airway has altered lipid A structure associated with resistance to CHDP [88], suggesting another mechanism underlying the sustained infection and inflammation.

It should be emphasised that most of antimicrobial experiments were conducted in conditions with low ionic strength and neutral pH *in vitro* which may not reflect real physiological situations. The antimicrobial activities of many CHDP are inhibited by the presence of serum and divalent cations (1-2 mM) [1]. This observation casts doubt on the idea that these peptides have a purely antibiotic function *in vivo* [89, 90]. For instance, the concentration of CHDP is normally around than 2-5 μg/ml at mucosal sites, whereas the MIC of LL-37 for a range of common bacteria is between 15-30 μg/ml under optimal ionic conditions. Although it is possible that at certain settings CHDP can reach high levels to exert their microbe-killing capability (eg. leukocyte granules, or extracellular traps composed of DNA, histone, and CHDP [91, 92], inflamed sites), the additional or alternative immunomodulatory activities of CHDP are proposed to be more critical.

1.2.5. Properties of modulating cell death

1.2.5.1. Cytotoxicity

Because of the differences of plasma membrane contents in prokaryotic and eukaryotic cells (cholesterol content and asymmetric distribution of phospholipids in the cytoplasmic membrane in eukaryotic cells), it is proposed that CHDP are not toxic to host cells. In addition, the cytotoxic effects of peptides to eukaryotic hosts *in vivo* may also be substantially attenuated by serum components by binding to the peptides [62, 89, 90]. However, several studies have also reported that these peptides at high concentrations also destroy eukaryotic cells *in vitro*. For example, higher concentrations (exceeding 13 μM) of LL-37 is cytotoxic to eukaryotic cells such as peripheral blood leukocytes and T cell line [25], which may be associated with cationic peptide-induced membrane permeabilisation [93, 94]. In addition, two bovine cathelicidins, BMAP-27 and BMAP-28, were found to induce cell death in cell lines and fresh haematopoietic tumour cells [94-96], leading to the hypothesis that CHDP might also have a role in tumour surveillance [93, 97, 98].

Tumour cells differ in membrane composition from nontransformed cells and such differences can result in higher susceptibility of tumour cells to membrane-permeabilising peptides [99]. CHDP inhibit cancer progression either by triggering necrosis via the cell membrane lytic effect or inducing apoptosis via mitochondrial membrane disruption. For example, treatment with or intracellular expression of α-defensins induces tumour cell apoptosis or necrosis, inhibiting tumour growth [100,

101]. A murine model showed that intratumoural expression of these peptides decreased neovascularisation and increased dendritic cell recruitment and maturation, suggesting a potential in cancer therapy [102]. However, contrast findings addressed that ovarian and breast cancers had overexpressed hCAP18/LL-37, suggesting that the peptide may contribute to tumourigenesis through direct stimulation of tumour cells, initiation of angiogenesis, recruitment of immune cells, and promotion of cancer metastasis [103, 104]. Taken together, the cytotoxic activity of CHDP on eukaryotic cells may depend on their unique structure and would be affected by the properties of target cells and the working conditions (eg. pH, ions, serum components).

1.2.5.2. Modulation of cell death

CHDP have the capability to modulate inflammatory responses by modifying immune cell death. For instance, β-defensins prolong PMN lifespan by downregulation of pro-apoptotic proteins, upregulation of anti-apoptotic proteins, and inhibition of mitochondrial membrane potential change and caspase-3 activity [105]. Murine β-defensin 2 (mDF2β) induced DC maturation, but also promoted atypical death of antigen-presenting cells (APC) [106].

In addition to the lytic activity on cells, high concentrations of LL-37 induced apoptosis in airway epithelial cells *in vivo* [107], and LL-37 at physiologically relevant concentrations induced apoptosis in infected airway epithelial cells via mitochondrial depolarisation and caspase-dependent pathways [20, 107, 108].

Moreover, in carcinoma and Jurkat lymphoma cell line, LL-37 contributed to caspase-independent apoptosis [109, 110]. In certain circumstances, the membrane perturbation induced by LL-37 could be antagonised by the presence of serum components in human serum and is not dependent upon signals via known receptors (FPRL-1, EGFR) [107].

By contrast, LL-37 has also been described as a potent inhibitor of human PMN apoptosis [20, 21]. The alternate consequences of LL-37-mediated modulation of apoptotic pathways in different human primary cells suggest an immunomodulatory role for this peptide in innate host defence. In addition, LL-37 protects primary human keratinocytes from apoptosis through up-regulation of cyclooxygenase-2 (COX-2) expression, suggesting a role of this peptide in reduced keratinocyte apoptosis in psoriasis [111]. Moreover, human cathelicidin is required for vitamin D_3-induced autophagy in human monocytes and for suppressing intracellular *Mycobacterium tuberculosis* survival [76]. Nevertheless, it remains unclear how this peptide interacts with eukaryotic cells and how it affects immune consequences by altering cell death. Given that the mode of cell death can direct subsequent inflammatory response, peptide-modified cell death might be expected to have significant physiological effects.

1.2.6. Anti-endotoxic and anti-inflammatory properties

1.2.6.1. TLR superfamily

TLRs are evolutionarily conserved receptors expressed by various immune cells and non-immune cells such as macrophages, DC, PMN, epithelial cells, keratinocytes, and fibroblasts [112-115]. TLRs play a vital role in immune system, belonging to a major family of pattern-recognition receptors (PRRs) to detect signature molecules of pathogens or danger signals from damaged self. TLRs are composed of N-terminal extracellular leucine-rich repeats (LRRs) which are responsible for recognition of pathogen components or danger molecules, a membrane-spanning domain, and a conserved C-terminal cytoplasmic domain Toll/IL-1 receptor (TIR) domain, which is required for downstream signalling [116-118]. In addition to the physiological surveillance functions, TLRs are upregulated in the affected tissue in inflammatory disorders, implying that their signalling is involved in the pathogenesis of chronic inflammation [119].

To date, 10 TLR members have been identified in humans and each of them recognise distinct molecules [113]. For instance, lipoproteins or lipopeptides, such as peptidoglycan (PGN) and lipoteichoic acid (LTA), are recognised by TLR2 in complex with TLR1 or TLR6, viral double-stranded RNA is recognised by TLR3, lipopolysaccharide (LPS) is recognised by TLR4, flagellin is recognised by TLR5, single-stranded RNA is recognised by TLR7 or TLR8, and microbial DNA is

recognised by TLR9. TLRs are expressed at different cellular compartments. TLR1, TLR2, TLR4, TLR5, and TLR6 are found on the cell surface, while TLR3, TLR7, TLR8, and TLR9 are located at cytosolic organelles like endosome and ER [118]. TLRs sense ligands and induce conformational dimerisation to initiate downstream signalling pathways. The signalling pathways of TLRs can be roughly divided into two routes: MyD88-dependent pathway and MyD88-independent pathway (Fig. 1.1) [116, 118, 120, 121].

MyD88-dependent pathway

Upon stimulation, all TLRs except TLR3 use MyD88 as the adaptor molecule to activate downstream signalling pathway while TLR2 and TLR4 require an additional adaptor to bridge MyD88 and TIR, namely TIRAP [122, 123]. MyD88 immediately recruits IL-1R-associated kinase (IRAK) members and tumor necrosis-factor-receptor-associated factor 6 (TRAF6). TRAF6 subsequently recruits transforming-growth-factor-β-activated kinase 1 (TAK1) and other enzymes (UEV1A, UBC13), resulting in the activation of NF-κB and MAPK pathways [116, 118]. The activation of NF-κB induces pro-inflammatory cytokine and chemokine production, such as TNF and IL-6. In TLR7, TLR8, and TLR9 signalling, MyD88 is required for the induction of type I IFNs (INF-α) through IRAK-TRAF6-IRF7 pathway [116, 118, 120].

MyD88-independent pathway (TRIF-dependent pathway)

TLR3 and TLR4 initiate a TRIF-dependent pathway (MyD88-independent pathway). TRIF directly engages with TLR3, whereas TRAM bridges the TLR4 interaction [123]. TRIF-dependent pathway mediates the activation of NF-κB (through TRAF6 or RIP1) and IRF3, which induces INF gene transcription and INF production (INF-α and INF-β) [124, 125]. INF-β subsequently induces the expression of a number of genes, such as immunoresponsive gene 1 (IRG1) and the gene encoding CXC-chemokine ligand 10 (CXCL10; the product of which is also known as INF-γ-inducible 10 kDa protein, IP-10) [118, 126, 127]. In addition to inducing the expression of INF-inducible genes, the MyD88-independent pathway leads to the upregulation of costimulatory molecules of APCs through the induction of INF-β [128]. Taken together, TLR recognition involves complicated molecular interactions and signalling pathways which provide a powerful mechanism to respond to a variety of foreign pathogens or endogenous danger signals.

1.2.6.2. Anti-inflammatory effect of CHDP on TLR signalling

Uncontrolled upregulation of TLR-mediated signalling may lead to excessive or persistent inflammation and severe immune pathology to the host, including septic shock, autoimmunity, metabolic syndrome, and chronic inflammatory diseases [114, 115, 129, 130]. CHDP are known to have selective anti-inflammatory effect

on TLR stimulation in different cell types [89, 131-134]. For example, in human monocytes, cathelicidin LL-37 neutralises the activation of macrophage by LPS and LTA at very low concentrations (≤ 1 μg/ml) in physiological salt conditions [89, 90, 131, 135]. The peptide has a selective anti-inflammatory effect on TLR stimulation [89, 131], selectively inhibiting pro-inflammatory cytokine release whereas retaining the TLR-induced signals for cell recruitment [131]. In human monocyte-derived dendritic cells, LL-37 not only suppresses pro-inflammatory cytokine responses, but also inhibits the surface expression of co-stimulatory molecules and decreases the activation of T cells by TLR ligands [133]. In addition, LL-37 attenuates pro-inflammatory responses of non-immune cells such as human gingival fibroblasts to TLR2/1, TLR3, and TLR4 ligands [132]. Moreover, the murine homologue of LL-37, mCRAMP, is capable to blunt activation of MAPK and decrease TNF-α release in murine macrophages in response to the agonists of TLR4 (LPS), TLR2 (LTA), and TLR5 (flagellin) [134]. Recent study using LL-37 fragments and physico-chemical parameters revealed that the capability of cathelicidins to modulate TLR responses was dependent on their cationicity and hydrophobicity [136]. The mechanisms by which CHDP suppress TLR inflammatory responses remain elusive and require further studies.

In contrast to the inhibition of TLR-mediated pro-inflammatory cytokine responses, cathelicidins can enhance inflammatory responses accompanied with certain TLR stimulation in specific cell types [132]. Cathelicidins can activate TLR9 by forming a complex with inert extracellular self-DNA fragments, triggering robust IFN responses by pDC in chronic skin inflammation [137]. It is

likely that CHDP play a dual role in modulating TLR signalling, either mediating anti-inflammatory effects or promoting immune responses, dependent on stimuli and different cell types. Better understanding of the underlying mechanisms involved in the interaction of TLR signaling pathways and CHDP may offer a potential for developing anti-inflammatory therapeutics.

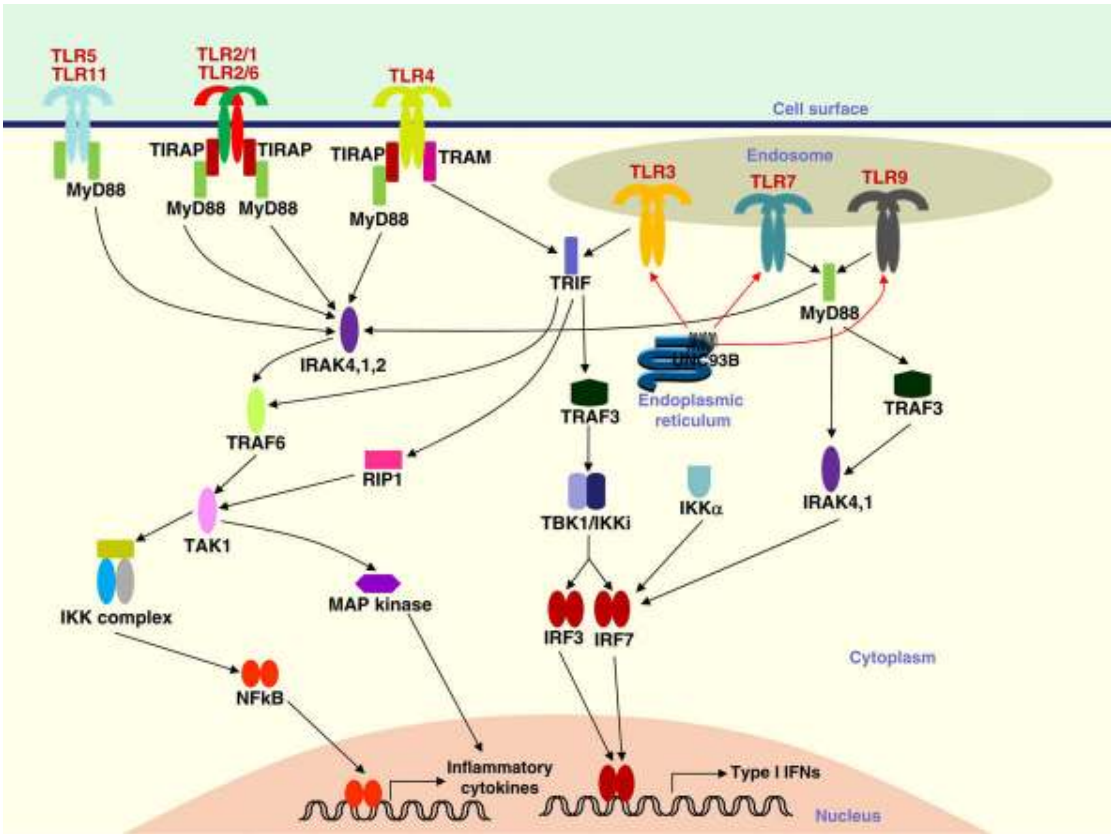


Fig. 1.1 TLR signalling in immune cells. TLR1, TLR2, TLR4, TLR5, TLR6 are located on the cell surface while TLR3, TLR7, TLR8, and TLR9 are located in intracellular organelles. All TLRs, except TLR3 recruit MyD88 to activate downstream signalling pathways. TLR2 and TLR4 require an additional adaptor molecule, TIRAP, to link their TIR domain and MyD88. On the other hand, TLR3 and TLR4 initiate MyD88-independent pathway through recruiting another adaptor molecule TRIF (TLR4 needs TRAM for bridging). In the MyD88-dependent pathway, MyD88 recruits the IRAK family and TRAF6. TRAF in turn activates TAK1, which induces the activation of downstream MAP kinase and NF-κB, resulting in transcription of inflammatory cytokine genes. In the TRIF-dependent pathway, TRIF interacts with RIP1 and TRAF6, leading to the activation of NF-κB and MAP kinase. In addition, TRIF also interacts with TRAF3, causing the activation of downstream IRF3 and IRF7, subsequently initiating the transcription of type I interferons. (The figure is taken from Kumar *et al.* Biochemical and Biophysical Research Communications 388 (2009) p.623[121])

1.2.7. Stimulation of cytokine and adhesion molecule expression

CHDP can also stimulate cytokine responses. HNP have been reported to stimulate IL-8 production by human bronchial and lung epithelial cells [138, 139] and increase TNF-α and IL-1β production by human monocytes [140]. However, an *in vivo* study revealed that α-defensins blocked the release of the pro-inflammatory cytokine IL-1β from LPS-stimulated monocytes, indicating the possible role of the peptides in inflammatory diseases [141]. In addition, hBD-2, -3, -4, and cathelicidin LL-37 have been found to individually and synergistically increase human keratinocyte gene expression and protein production of IL-6, IL-10, IP-10, MCP-1, MIP-3α and RANTES through GPCR, providing a mechanism for the pathogenesis of skin diseases such as psoriasis [142].

LL-37 stimulates the expression of a wide variety of genes involved in the innate immune response, including those encoding chemokines. For example, LL-37 up-regulates the expression of MCP-1 in macrophages, and induces the secretion of IL-8 from human epithelial cell line, keratinocytes, and PMN [30, 89, 143, 144]. LL-37 also induces the release of IL-4, IL-5, and IL-1β from mast cells [145]. A study showed that at physiological concentrations (2-5 μg/ml), LL-37 significantly increased the production of IL-6 and IL-8 by epithelial cells and keratincytes in response to pro-inflammatory stimuli such as IL-1β and TLR agonists, suggesting it may play an important role in the initiation and regulation of inflammation in

epithelia [146]. In addition, LL-37 up-regulates the chemokine receptors CXCR4, CCR2, and IL-8RB in macrophages, indicating the multifunctional role of the peptide in immune responses [25]. Furthermore, LL-37 induces processing and release of the pro-inflammatory cytokine IL-1β by LPS-primed monocytes via $P2X_7$ receptor [29]. Along with its individual cytokine modulating effects, LL-37 can synergise with other mediators to enhance cytokine production, for example, the IL-1β and GM-CSF-induced production of cytokines (IL-6, IL-10) in human peripheral blood monocytes (PBMC) [147]. Above observations reveal that CHDP have massive impacts on many aspects of host defence system by modulating numerous cytokine responses.

1.2.8. Other immunomodulatory functions

1.2.8.1. Chemotactic activity

Human α-defensins (HNP-1 and HNP-2, but not HNP-3) have been shown to have chemotactic activity mediating the recruitment of monocytes to inflammed sites [148]. In addition, the β-defensins hBD3 and hBD4 have been reported to attract monocytes and macrophages [149], and hBD2 is chemotactic for mast cells [150]. Interestingly, hBD1 and hBD3 are chemotactic for immature dendritic cells and memory T-cells through human CC chemokine receptor 6, whereas human α-defensins selectively induce the migration of human naïve $CD4^+CD45^+$ and $CD8^+$cells [26, 27]. In addition to defensins, LL-37 uses FPRL-1 and CXCR2 as receptors to chemoattract human peripheral blood PMNs, monocytes, eosinophils,

and T cells [19, 32, 151]. LL-37 has been also reported to be chemotactic for human mast cells using two different receptors, a high-affinity that is not a GPCR and a low-affinity GPCR [28]. These findings indicate that these peptides can attract immune cells to affected sites and further promote immune responses or inflammatory resolution.

1.2.8.2. Leukocyte granule content release

Another mechanism exploited by CHDP to modulate inflammatory reactions is to promote leukocyte granule content release. Studies investigating cellular content release revealed that hBD2 and LL-37 stimulate mast cell degranulation by mobilising intracellular Ca^{2+} in a GPCR-dependent manner [152]. In addition, LL-37 increases the gene expression and extracellular release of human α-defensins [17]. These data suggest that CHDP can enhance immune responses by augmenting other immune mediator or peptide release.

1.2.8.3. Promotion of wound healing

It has been documented that CHDP are mitogenic for epithelial cells and fibroblasts and may play a central role in the wound healing process [153, 154]. For example, hBD2 was described as being a potent promoter of human endothelial cell migration, proliferation and, in the presence of angiogenic factors, tube formation, which accelerated wound closure [87, 155]. Additionally, the reduction of the cathelicidin LL-37 in chronic wounds impairs re-epithelialisation,

suggesting that this peptide may play a part in wound closure [156]. LL-37 also induces keratinocyte migration through transactivation of EGFR [33, 157] and induces angiogenesis through FPRL-1 [34]. Furthermore, an observation revealed that fibrosis in human dermal keloids was inversely related to the expression of cathelicidins, suggesting their antifibrotic activity during the wound repair process [158]. CHDP-mediated promotion of wound repair serves as another mechanism by which these peptides strengthen host defences against invasions.

1.2.9. Impacts on adaptive immunity

The chemotactic capacities of both defensins and cathelicidins predict that they might play a role in recruiting T cells, immature DCs and monocytes to sites of infection, thereby facilitating the initiation of adaptive antimicrobial immune responses [26, 27]. Studies using a mixture of HNP1-3, when simultaneously administered with ovalbumin (OVA) intranasally into C57BL/6 mice, enhanced the production of OVA-specific serum IgG antibody and the generation of IFN-γ, IL-5, IL-6 and IL-10 by OVA-specific $CD4^+$ T cells [154]. Thus, defensins enhance the specific immune response by inducing the production of cytokines, which promote the adaptive immune response. Also, LL-37 was demonstrated as a potent modifier of DC differentiation, up-regulating endocytic capacity and surface expression of costimulatory molecules, enhancing secretion of Th-1 inducing cytokines, and promoting Th1 responses *in vitro* [159]. Taken together, CHDP are able to bridge innate and adaptive immunity to amplify immune responses.

1.2.10. *In vivo* protection against infection

Evidence supports that hypothesis that CHDP play critical roles in immune defence against a broad variety of pathogens in physiological conditions, probably dependent on their multiple immunomodulatory functions. This proposal has been supported by an innate defence-regulator peptide, IDR-1 a synthetic peptide derived from LL-37. Although devoid of direct antimicrobial activity, IDR-1 is protective in mouse models of infection with Gram-positive and Gram-negative pathogens, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, and *Salmonella enterica serovar Typhimurium* [160].

In addition, mouse models deficient in cathelin-related antimicrobial peptide (mCRAMP), the murine homologue of LL-37, have demonstrated increased susceptibility to infection in the skin, urinary tract, gut, and lung [161-164]. Transgenic mouse studies have provided more evidence for the physiological function of CHDP in antimicrobial host defence. One study showed that matrix metalloproteinase-7 [(MMP-7) (an enzyme required for conversion of inactive pro-α-defensins to active cryptdin peptides)] –null ($Mm7^{-/-}$) mice had decreased clearance of noninvasive *E.coli* in the small bowel. These mice had higher susceptibility to oral challenge with *S. typhimurium*, indicating the importance of defensin expression in intestinal immunity to enteric pathogens [165]. In addition, targeted deletion of the mouse β-defensin-1 gene resulted in mice deficient in the clearance of *Haemophilus influenzae* from the lung [166].

On the other hand, mice expressing a human α-defensin gene (*DEFA5*) had different enteric microbiota composition and fewer IL-17-producing lamina propria T cells [167]. Studies demonstrated that HD-5 transgenic mice, which expressed human HD-5 small intestinal Paneth cells, were markedly resistant to oral challenge with virulent *Salmonella typhimurium* [168]. Moreover, overexpression of LL-37 in the mouse airway by adenovirus-mediated gene transfer resulted in the increased ability to reduce bacterial load from *Pseudomonas aeruginosa* challenge and improved survival after administration of lethal doses of LPS [169]. These *in vivo* studies demonstrate the pivotal roles of CHDP in protecting against infection.

1.2.11. Conclusion

It is therefore clear that CHDP are crucial components of the body's defence against infections and are able to modify the immune responses. Understanding the mechanisms by which CHDP act, has the potential to inform the development of effective therapeutics with the capacity to modulate the host defence.

1.3. Polymorphonuclear leukocytes (PMN)

1.3.1. Introduction

Polymorphonuclear leukocytes (PMN), also called granulocytes, are the most abundant white blood cells in humans and an essential component of the innate immune system. There are three types of granulocytes: neutrophil, eosinophil, and basophil, distinguished by their granules when stained.

The bone marrow of a healthy adult produces up to 10^{11} PMN a day [170]. PMN are terminally differentiated cells. They originate from a precursor pool of stem cells and develop in bone marrow. PMN differentiation has classically been divided into six stages (myeloblast, promyelocyte, myelocyte, metamyelocyte, band, and mature PMN) on the basis of cell size, nuclear morphology, and granule contents [171]. After release from bone marrow, PMN enter the circulation where they represent more than 50% of circulating leukocytes.

1.3.2. PMN functions

PMN are the first line defence cells against bacteria and fungi infections. They ingest microbes by a process known as phagocytosis, and destroy ingested microorganisms by the combination of production of reactive oxygen species (ROS) and disgorgement of granule contents (degranulation) [172-174]. ROS are derived

from superoxide which is produced by a multisubunit enzyme complex called nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) at the phagosomal membrane. NADPH oxidase catalyses the production of superoxide (O_2^-), which dismutates rapidly to H_2O_2. Both O_2^- and H_2O_2 are effectively antimicrobial. In addition, granule components such as myeloperoxidase (MPO) catalyse a reaction with H_2O_2 and chloride to form hypochlorous acid (HOCl) and singlet oxygen. NADPH oxidase and the MPO-halide system serve as redundant mechanisms to kill ingested microbes. This explains why MPO deficiency in humans does not necessarily correlate with morbidity from infection [172, 175]. In the phagosome, microorganisms are exposed to high concentrations of ROS and antimicrobial peptides, both of which are responsible for microbial killing. In addition, a novel form of PMN killing technique has been described, named neutrophil extracellular traps (NETs). Stimulated PMN release granule proteins and chromatin that together form extracellular fibres to bind microorganisms. These NETs degrade virulence factors and kill pathogens. NETs appear to be a form of innate response that binds microorganisms, prevents them from spreading, and ensures a high concentration of antimicrobial peptides at the structure to kill microbes [92].

In addition to the direct antimicrobial activity, PMN also orchestrate the functions of other immune effector cells, integrating the innate and adaptive limbs of the immune response [176-179]. For example, PMN have the ability to produce numerous cytokines and chemokines following phagocytosis such as TNF-α, IL-1β, IL-8, and macrophage inflammatory protein-1α (MIP-1α) [180]. These

cytokines contribute to the inflammatory response and the attraction of other immune effector cells [181, 182]. PMN can also release granule proteins, enhancing the activation of macrophages, promotion of bacterial phagocytosis [183], and attraction of monocytes, dendritic cells, and lymphocytes [28, 179, 184]. Moreover, PMN also generate CXCL10/ IP-10, which acts as an important chemoattractant for activate NK and T-helper type 1 cells [185]. Furthermore, by releasing their granule contents, PMN are capable of mobilising and activating dendritic cells, thus augmenting adaptive immune responses [179]. Taken together, PMN are multifunctional immune cells, destroying microorganisms by phagocytosis, superoxide production, degranulation, and being able to orchestrate inflammatory and immune processes.

1.3.3. PMN granules

Morphologically, PMN are characterised by a lobulated chromatin-dense nucleus and their granules. The different types of granules—primary or azurophil granules, secondary or specific granules, gelatinase or tertiary granules and secretory vesicles—are not merely containers of proteolytic and bactericidal substances, they also serve as reservoirs of membrane receptors [186] (Table 1.2). According to the targeting-by-timing hypothesis, PMN granules are formed sequentially during myelopoiesis. Primary granules are produced at the stage of the early promyelocyte, secondary granules are created in myelocytes, and gelatinase granules are formed at the metamyelocyte and band cell stages. Secretory vesicles are formed by endocytosis at late stages.

1.3.3.1. Primary granules

Primary granules (azurophil granules), which are also described as peroxidase-positive granules, can be further divided into two major subsets dependent on the appearance of defensins: early-appearing defensin-poor azurophil granules and late-appearing defensin-rich azurophil granules [186, 187]. A number of proteins has been identified in azurophil granules described as follows.

Myeloperoxidase

MPO is a microbicidal hemoprotein, released into the phagosome or to the extracellular space upon PMN activation. MPO reacts with H_2O_2, formed by NADPH oxidase, to induce formation of toxic oxidants and radicals which can attack the surface membranes of microorganisms [173].

Alpha-defensins

PMN contain four closely-related α-defensins (HNP1-4), which are small cationic peptides with antimicrobial and immunomodulatory properties (details are found in Chapter 1.2 CHDP).

Bactericidal /permeability-increasing protein (BPI)

BPI is a highly cationic peptide binding to negatively charged LPS in the outer membrane of Gram-negative bacteria, promoting the killing of pathogens [188].

Serprocidins (serine proteases with microbicidal activity)

Azurophil granules contain three structurally related serprocidins: proteinase-3, cathepsin G, and elastase [189]. Serprocidins are cationic polypeptides with proteolytic activity of degrading a variety of extracellular matrix components, such as elastin, fibronectin, laminin, collagens, and proteoglycans [189]. They can also target and degrade virulence factors and outer membrane components of pathogens, contributing to PMN-mediated killing of microbes [190-192]. Serprocidins can induce activation of defence cells [193] or process CHDP (eg. proteinase-3 processes human cathelicidin hCAP-18 to generate active form of peptide, LL-37 [47]).

1.3.3.2. Specific granules (secondary granules) and gelatinase granules (tertiary granules)

In contrast to primary granules, specific and gelatinase granules are peroxidase-negative. These granules possess three metalloproteases, namely collagenase (MMP-8), gelatinase (MMP-9), and leukolycin (MMP-25). MMPs are capable of degrading major structural components of the extracellular matrix and are important in degrading vascular basement membranes and interstitial structures during PMN extravasation and migration [187, 193]. In addition to metalloproteases, specific granules contain other proteins distinct from that of gelatinase granules described as follows.

Lactoferrin

Lactoferrin is glycoprotein with a broad spectrum of antimicrobial activity based on its iron-binding property which impairs bacterial growth by sequestration of iron [194]. In addition to its well-established anti-microbial properties, lactoferrin can mediate immunomodulatory functions through its direct activity on innate immune cells and molecules [195].

hCAP-18/ LL-37

hCAP18 is mainly stored in PMN specific granules and is mainly processed by a PMN proteinase (protease-3) to release its mature form, LL-37 [47]. Like α-defensins, it is a small cationic, antimicrobial peptide with multiple immunomodulatory peptides (details are found in Chapter 1.2 CHDP).

Neutrophil gelatinase-associated lipocalin (NGAL)

NGAL exerts its bacteriostatic activity by binding bacterial catecholate-type ferric siderophores and sequestrating ferric-siderophore complexes [196].

Lysozyme

Lysozyme is a cationic antimicrobial peptide present in all granule subsets, with peak concentrations in specific granules [197]. Lysozyme is able to cleave peptidoglycan polymers of bacterial cell walls and bind LPS to exert its bactericidal activity [198, 199].

1.3.3.3. Secretory vesicles

Secretory vesicles appear in segmented PMN. The membranes of secretory vesicles are rich in the β_2-integrin (CD11b/CD18), receptors for formylated bacterial peptides, the LPS/ lipoteichoic acid receptor CD14, and FcγIII receptor CD16, all of which are incorporated in the plasma membrane after degranulation [174]. Upon stimulation with inflammatory mediators, secretory vesicles mobilise and enrich the PMN surface with β_2-integrin CD11b/CD18, enhancing the adhesion of endothelial cells (by binding ICAM-1) and promoting PMN transmigration [200].

	Azurophil granules	**Specific granules**	**Gelatinase granules**	**Secretory vesicles**
Membrane proteins		CD11b/CD18 CD66, CD67 TNFR	CD11b/CD18 CD67 TNFR	CD11b/CD18 CD67 TNFR, CXCR FcR, TLR CD14 MyD88, MD-2
Matrix Proteins	Elastase Cathepsin G Proteinase 3 Defensins MPO, BPI Lysozyme	Collagenase Gelatinase hCAP18 Lactoferrin NGAL Lysozyme	Gelatinase Leucolysin Lysozyme	

Table 1.2 PMN granule proteins [modified from Table 1, *TRENDS in Immunology*, (2007) **Vol.** 28, p.341]

1.3.3.4. Degranulation

Degranulation is defined by the translocation of granules to the phagosomal or plasma membrane, where granules dock and fuse with the membrane to release their contents. Degranulation occurs when receptors (GPCR, such as the formyl peptide receptors FPR, FPRL1 [201], chemokine receptors CXCR1, CXCR2 [202], and complement receptor C5a receptor [203]) are stimulated with wide variety of stimulants such as formyl-methionyl-leucyl-phenylalanine (fMLF) [7], C5a [204], platelet-activating factor (PAF) [205], TNF [206], LPS [207], and proteins derived from pathogens [208]. The excessive PMN granule content release would cause many inflammatory disorders, such as acute lung injury, severe asthma, rheumatoid arthritis, and septic shock [204]. Therefore, degranulation process of PMN should be tightly regulated to avoid uncontrolled release of tissue-destructive constituents.

It is postulated that degranulation involves four distinct steps: granule recruitment, granule docking, granule priming, and granule fusion to target membrane [209]. The translocation and exocytosis of granule contents of PMN require increases of intracellular Ca^{2+} and the reorganisation of the actin cytoskeleton [204]. Using Ca^{2+} ionophores such as A23187 or ionomycin induces a hierarchy of granule secretion [210]. The order of release in responses to elevating concentrations of Ca^{2+} is secretory vesicles > tertiary granules (gelatinase granules) > secondary granules > primary granules, which also reflects their responses in physiological conditions [211]. Upon initiation of inflammation, PMN interact

with the endothelium which results in discharge of secretory vesicles. While PMN pass through the endothelium and basement membrane, high concentration of gelatinase, a collagenolytic protein, is being released. Once PMN reach the extravascular tissue, primary and secondary granules undergo exocytosis, acting as defences against insults [186].

1.3.4. PMN death

1.3.4.1. Apoptosis

PMN are inherently short-living cells with a half life of 6-10 hours in the circulation before undergoing spontaneous apoptosis. Constitutive death of PMN is a critical process for modulating PMN number and function and plays an essential role in PMN homeostasis. The removal of apoptotic PMN by macrophages is a mechanism to clear effete PMN and to facilitate the resolution of inflammation [9].

Apoptotic PMN display morphological characteristics such as cell shrinkage, condensation of chromatin, and loss of the multilobed shape of their nucleus. They are nonfunctional, with impaired chemotaxis, respiratory burst, and degranulation [212]. Molecular alterations on their cell surface have also been reported [213, 214]. For example, a marked downregulation of FCγRIII (CD16) [213], leukosialin (CD43), and $_{L}$-selectin (CD62L) [215] have been found in PMN undergoing apoptosis. The apoptotic pathway in PMN is executed by caspases and involving the balance of pro-apoptotic and anti-apoptotic proteins (Fig. 1.2).

Caspases

Caspases are cysteine proteinases that initiate or execute cellular programs. The caspase family is composed of two major subfamilies that are related to either inflammation (e.g. caspase-1, 4, 5, 13) or apoptosis (e.g. caspase 2, 3, 6, 7, 8, 9, 10). Caspases are expressed as proenzymes (zymogens) that contain three domains: an N-terminal prodomain, the p20, and a small C-terminal domain p10 subunit. PMN have been reported to express a variety of caspases, including caspase-1, -3, -4, -8, and -9 and their activation during PMN death is well documented [216-218].

Two main pathways lead to the activation of caspase cascades. The first of these depends on the participation of mitochondria (receptor-independent; intrinsic pathway) and the second involves the interaction of a death receptor with its ligands (extrinsic pathway). The intrinsic apoptosis pathway is characterised by the loss of mitochondrial transmembrane potential and the release of pro-apoptotic factors such as cytochrome c, apoptosis-inducing factor (AIF), and endonuclease G (EndoG) into the cytosol or nuclei. Binding of cytochrome c and dATP causes the adaptor molecule Apaf-1 to form a large multimeric complex called the apoptosome. Apaf-1 in the apoptosome recruits procaspase-9 which in turn is processed by autocatalysis to activate the downstream execution phase of apoptosis [219].

Although PMN contain a paucity of mitochondria and low levels of cytochrome c, they possess a lower threshold requirement for cytochrome c to activate caspases and their low content of cytochrome c is compensated by the elevated expression

of Apaf-1 [220]. In addition to cytochrome c, the loss of mitochondrial membrane potential results in the release of AIF and Endo G. Both AIF and EndoG can translocate into nuclei and cause nucleosomal DNA fragmentation, one of the hallmarks of apoptosis, leading to cell death independent of caspases [221-223].

Different from the intrinsic pathway, the extrinsic apoptosis pathway is initiated by the activation of "death receptors" of the tumour necrosis factor receptor (TNFR) family instead of involving mitochondria factors. Members of this family are characterised by two to five copies of cysteine-rich extracellular repeats. Death receptors also have an intracellular amino acid stretch within the carboxy terminus of the receptor called the "death domain" (DD). Ligation of death receptors causes the rapid formation of a death-inducing signalling complex through the receptors DD, which is responsible for coupling the death receptor either to the activation of the NF-κB signalling pathway or to a cascade of caspases, resulting in apoptotic cell death [224].

In activating the caspase cascade, DD recruits procaspase-8. Via aggregation of two or more procaspase-8 molecules, procaspase-8 becomes activated and then goes on to trigger the apoptotic cascade [225-227]. Recent studies also revealed that caspase-8 could be activated by cathepsin D, which was released from azurophilic granules in a ROS-dependent manner, suggesting another mechanism of induction of PMN death mediated by granule content [228]. In addition, evidence indicated that inflammatory caspase members such as caspase-1 may also have the capacity to mediate PMN apoptosis. PMN from caspase-1-deficient mice

had delayed constitutive apoptosis, and LPS could not inhibit apoptosis in these cells, implying a combination caspases functions related to inflammation and apoptosis [229]. Little is known to date about the role of inhibitor of apoptosis proteins (IAPs), which inhibit activated caspases, in PMN apoptosis. Studies demonstrated that G-CSF can up-regulate cIAP2 (cellular IAP2) to promote PMN survival [230]. Overexpression of XIAP protects cell survival [231] while this factor is negatively regulated by a pro-apoptotic factor, Smac/DIABLO, which is released from mitochondria along with cytochrome c [232].

Bcl-2 family

PMN also express a number of proteins from the Bcl-2 family. Bcl-2 members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1-BH4) and can be subdivided into three categories according to their function and structure. The first class is anti-apoptotic members, such as Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1, which exert anti-cell death activity and most contain all four BH domains. The second class is pro-apoptotic members, such as Bax, Bak, Bok, which share sequence homology in BH1, BH2, and BH3, but not in BH4. The third class is "BH3-only" pro-apoptotic members, which include Bid, Bad, Bim, Bik, and possess only the central short BH3 domain. Subcellular localisation studies have shown that Bcl-2 and Bcl-X_L reside on the mitochondrial outer membrane, while the pro-apoptotic family members may be either cytosolic or present on the mitochondrial membrane. During apoptosis, the pro-apoptotic Bcl-2 family members are activated and undergo a conformational change to form channels or to induce the rupture of outer mitochondrial membrane. The translocation of pro-

apoptotic Bcl-2 family members to mitochondria facilitates cytochrome c release and leads to downstream caspase activation. In contrast, the anti-apoptotic proteins Bcl-2 and Bcl-X_L prevent cytochrome c release from mitochondria and thereby preserve cell survival [233, 234].

It is now agreed that human PMN do not express the anti-apoptotic protein Bcl-2 but do express Mcl-1 and A1. Mcl-1 was initially isolated from a human myeloblastic leukemia cell line and it is increasingly recognised as a key regulator of immune cell proliferation and longevity. While PMN are undergoing apoptosis, Mcl-1 levels decline rapidly, suggesting that PMN survival is regulated by Mcl-1 expression [235]. A1 is another protein important for PMN survival and is upregulated by G-CSF or LPS, mediators known to promote PMN survival [236]. PMN also express the pro-apoptotic proteins Bax, Bid, Bak, and Bad, which have a relatively longer half life than anti-apoptotic proteins and hence their constitutive expression explains why bloodstream PMN have such a short survival time [235]. Bax, which inserts to mitochondrial membrane and causes cytochrome c release, has been demonstrated as an important molecule in regulation of constitutive PMN apoptosis [237]. In addition, deletion of the BH3-only protein Bim results in PMN accumulation at inflammatory sites and delayed apoptosis [238]. Moreover, one of the BH3-only proteins, Bid, is cleaved by caspase-8 and translocates to the mitochondria, where it triggers cytochrome c release, demonstrating a crosstalk between intrinsic and extrinsic pathways [239].

Calpain

A role of calpain, a non-caspase cystein protease, in the regulation of PMN apoptosis has also been suggested. PMN apoptosis is blocked by inhibitors of calpains [240] and accelerated by reducing the levels of endogenous calpain inhibitor, calpastatin [241]. In support of that PMN from patients with cystic fibrosis that exhibited delayed apoptosis have increased calpastatin and decreased calpain protein levels compared with PMN from control individuals. Inhibiting calpain prevents the mitochondrial release of cytochrome c and blocks caspase-3 processing and activation, leading to prolonged PMN lifespan [242]. These findings indicate another mechanism involving calpastatin-calpain system in the regulation of PMN death.

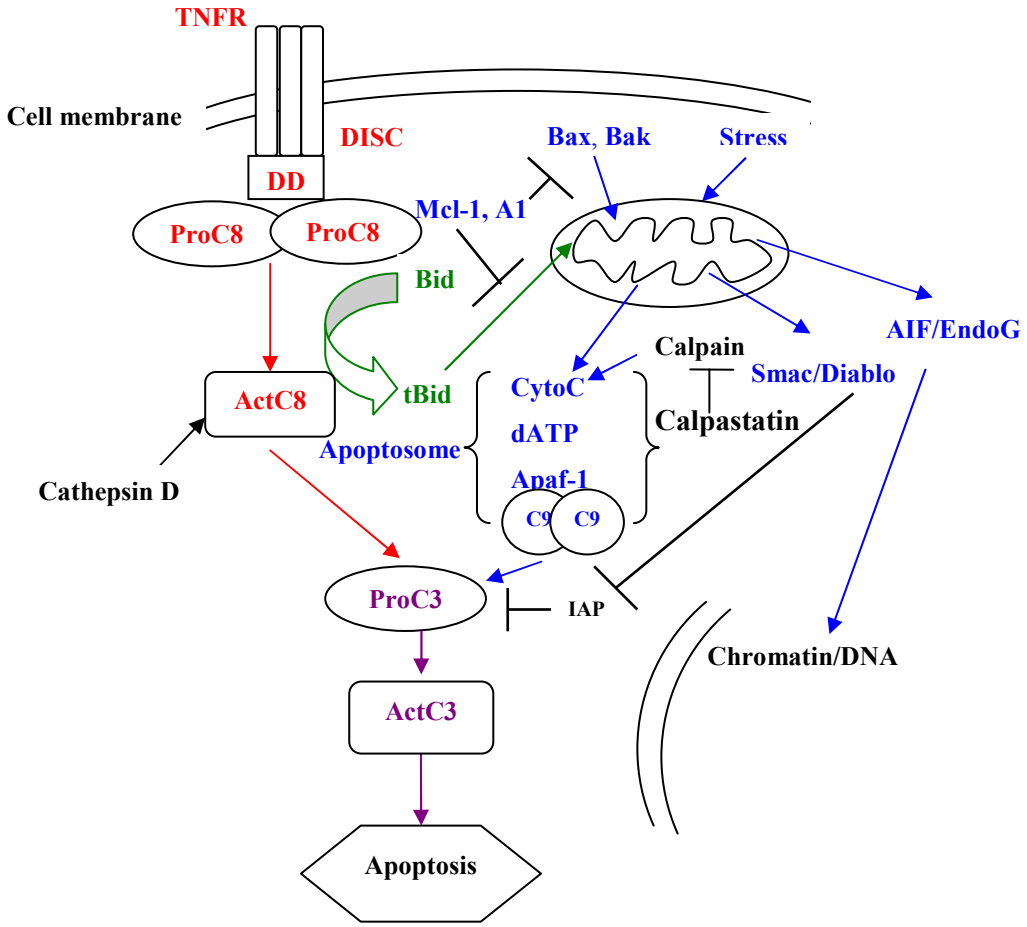


Fig. 1.2 Molecules involved in PMN apoptosis. Two main pathways are involved in PMN apoptosis: intrinsic pathway (main molecules involved are presented as blue color) and extrinsic pathway (red color). Stress stimuli or pro-apoptotic Bcl-2 members Bax and Bak cause the release of cytochrome c from mitochondria. Cytochrome c accompanies with Apaf-1, dATP, and recruited pro-caspase-9 (C9) to form apoptosome, which can catalyse and activate downstream executioner caspase-3 (ProC3), leading to apoptosis. The effects of pro-apoptotic Bcl-2 members can be antagonised by the increased level of anti-apoptotic Bcl-2 proteins, such as Mcl-1 and A1. Extrinsic pathway, on the other hand, is mediated by the interaction of death receptors and their ligands. Pro-caspase-8 (proC8) is recruited by death domain and autocatalysed, followed by activating caspase-3. In addition, Bid, a pro-apoptotic Bcl-2 member, is cleaved by caspase-8, and triggers cytochrome c release, representing a crosstalk between these two pathways (green color). Other mechanisms may also involve in PMN apoptosis, such as cathepsin D and calpain induce apoptosis by activating caspase-8 and inducing cytochrome c release, respectively. AIF and EndoG are released with cytochrome c from mithochondria to nuclei, inducing PMN death in caspase-independent manner. By contrast, IAP and calpastatin inhibit PMN apoptosis by inhibiting caspases and calpain, and the inhibitory effects of IAP can be in turn inhibited by Smac/ Diablo released from mitochondria.

Molecules altering PMN apoptosis

PMN death is altered in the presence of stimuli such as pro-inflammatory cytokines, pathogen components, phagocytosis [243, 244], and other blood cells [245, 246], either accelerate or suppress PMN apoptosis [247]. Inflammatory cytokines such as IL-1β, INF-γ, G-CSF, GM-CSF, and TLR agonists have been demonstrated to prolong PMN survival [248-252]. The reported effects of IL-6 and TNF-α on PMN apoptosis are variable [253-255]. While prolonged incubation (>12 h) of human PMN with TNF-α can cause a decrease of apoptosis, TNF-α can also induce apoptosis in a sub-population of cells at earlier times of incubation (< 8 h). TNF-α may activate a death pathway such as the mitogen-activated protein kinases (MAPK) pathway or caspase-8 pathway in susceptible cells, but stimulate an anti-apoptotic pathway mediated by NF-κB in surviving cells [180, 253, 256]. In addition to cytokines, hypoxic conditions at inflammatory sites delay apoptosis in human PMN [257, 258]. Ligation of PMN adhesion receptors can also extend PMN lifespan [259, 260]. Moreover, some antioxidants such as catalase delay apoptosis when PMN are incubated under normoxic conditions [258, 261]. Furthermore, dexamethasone, despite being a potent anti-inflammatory agent, has the paradoxical effect of inhibiting PMN apoptosis [262, 263]. Suppression of PMN apoptosis has been implicated in the enhancement of inflammatory responses and pathogen clearance.

PMN apoptosis can also be actively promoted. PMN are susceptible to Fas-induced apoptosis and an interaction between Fas and Fas ligand (FasL) was originally suggested as a mechanism to explain constitutive PMN apoptosis [264].

However, although PMN express both Fas receptor and Fas ligand, PMN from Fas receptor or Fas ligand deficient mice showed normal apoptotic rate as control mice, arguing against the Fas/FasL system as the mechanism underlying spontaneous apoptosis induction in PMN [265]. In addition, PMN apoptosis is accelerated following bacterial ingestion as part of effective immunity, but is also accelerated by bacterial exotoxins as a mechanism of immune evasion. Pyocyanin, a toxic metabolite generated by *Pseudomonas aeruginosa*, has been shown to dramatically increase PMN apoptosis both *in vitro* and *in vivo* [266, 267].

Chemicals such as cycloheximide and actinomycin D (inhibitors of translation and transcription, respectively) accelerate PMN apoptosis *in vitro*. An elegant study also showed that cyclin-dependent kinase (CDK) inhibitors, which were used as an anti-tumour drug, induced caspase-dependent PMN apoptosis *in vitro* by reducing anti-apoptotic protein Mcl-1. *In vivo,* CDK inhibitors also enhanced the resolution of established inflammation by promoting apoptosis of inflammatory cells, demonstrating a therapeutic potential of CDK inhibitors for inflammatory disorders [268].

1.3.4.2. Necrosis

Necrosis is a form of cell death characterised by cell swelling and lysis. External factors such as infection, toxins, or trauma induces necrosis and results in the release of intracellular contents. As PMN are very rich in peptides, proteases, and oxidants, it is expected that the leakage of their highly cytotoxic contents would

potentiate inflammation and cause tissue injury [269]. Indeed, excessive releases of proteases such as elastase, a variety of inflammatory cytokines, and reactive oxygen species are observed in chronic inflammatory lung disorders where accumulated PMN lose their plasma membrane integrity [270-276]. It is also suggested that there is a deficiency in the capacity to engulf apoptotic PMN in chronic lung inflammatory diseases such as COPD and cystic fibrosis, resulting in pathogenic PMN apoptotic secondary necrosis [277]. Secondary necrosis of PMN, like primarily necrotic PMN, is believed to be pro-inflammatory by discharging their histotoxic mediators to the surroundings, causing tissue injury. It is also implicated in lung infections by Gram-negative bacteria which induce PMN death and impair the uptake of scavenger phagocytes [267, 278], leading to detrimental inflammatory conditions. In addition to inflammation, secondarily necrotic PMN have been found in autoimmune disorders where cell clearance is impaired and cells release their autoantigens and cytotoxic molecules [279-282]. These findings support the concept that prompt removal of dead PMN is critical to promote the resolution of inflammation whereas dysregulation of the process can result in persistent inflammatory conditions.

1.3.4.3. Autophagy

Human PMN can undergo autophagy-like cell death following Sialic acid binding immunoglobulin-like lectin-9 (Siglec-9) ligation and concurrent stimulation with certain PMN survival cytokines [283]. However, a widely used inhibitor of autophagic cell death, 3-MA, failed to delay PMN spontaneous death,

suggesting that autophagy may not be a predominant mechanism for PMN spontaneous death [284].

1.3.4.4. NETosis

A novel form of PMN death named NETosis has been described. This death process involves extrusions of plasma membrane and nuclear material composed of granule components and histone in an NADPH oxidase dependent manner [92]. Distinct from other modes of death, NET-induced death shows neither classical apoptotic morphology, nor necrotic features [285]. It is a form of active cell death occurring in activated PMN and allows them to bind and kill microbes effectively, even beyond their lifespan.

1.3.5. Conclusion

PMN are a key component of immunity and play a major role in inflammatory events. Appropriate control of granulocyte function, death, and removal is critical to maintain tissue homeostasis. Therefore, it is necessary to gain a better understanding of the physiology of these versatile cells.

1.4. Cell death

1.4.1. Introduction

Cells can die in a genetically controlled process (programmed cell death) or in response to noxious external stimuli, including heat shock, infection, oxidative stress, toxic challenge, or radiation, resulting in accidental cell death. Programmed cell death (PCD) is a well-orchestrated mechanism to eliminate damaged, used-up, or misplaced cells during development, homeostasis, and immune regulation of multicellular organisms [286, 287]. Dysregulation of cell death is associated with numerous pathologies, such as degeneration, autoimmunity, immune deficiency, and cancers. Distinct cell death pathways results in different morphological and physiological outcomes. Three major types of cell death, namely apoptosis, necrosis, and pyroptosis, will be discussed in this introduction.

1.4.2. Apoptosis

The term “apoptosis” was first coined by Currie and colleagues in 1972 to describe a type of programmed cell death that the authors repeatedly observed in various tissues and cell types. ‘Apoptosis’ in Greek is used to describe the ‘falling off’ of leaves from a tree and is to indicate the dying cells releasing contacts with neighboring cells and being detached from the surrounding tissue [288]. The authors defined apoptotic cells as sharing many morphological features including

cell shrinkage, chromatin condensation, and internucleosomal cleavage of genomic DNA [225]. The plasma membrane also underwent blebbing and divided cells into small apoptotic bodies which contained condensed organelles. Importantly, throughout the apoptotic process the plasma membrane on the dying cells remained intact without release of intracellular contents to surroundings, suggesting that apoptosis was a well-organised process of cell demise.

1.4.2.1. Mechanism of apoptosis

Caspase-dependent cell death

The knowledge of the regulation of apoptosis is mainly based on studies of programmed cell death in *C. elegans* [289]. In this nematode, cell death protease (CED-3) is associated with regulated cell demise. Homologous proteins have been found in organisms throughout the animal kingdom, and these proteins are related to cell death with an apoptotic morphology [290]. To date, 13 caspases have been identified in mice and humans, belonging to the apoptotic and the inflammatory subfamilies. Apoptotic caspases can be further subdivided into initiator and executioner caspases. The subset of caspases that cleave selected substrates to produce the changes associated with apoptosis are known as executioner caspases, which in mammals are caspase-3, -6, and -7. In most instances, these executioner caspases are activated by the activated initiator caspases, such as caspase-8, caspase-10, caspase-2, and caspase-9 [291]. Caspase activation involves finely-controlled cascades and directs regulated cell death.

All caspases are expressed as inactive proenzymes consisting of an N-terminal prodomain, followed by a large subunit (p20) and a small C-terminal subunit (p10). The long prodomain of the initiator caspases contains death domain (DD), including protein-protein interaction motifs belonging to death effector domains (DEDs) and caspase activation and recruitment domains (CARDs). Initiator caspases are recruited to platform molecules via these protein-protein interaction domains and are activated by oligomerisation and proximity-induced autoproteolysis. Executioner caspases, as mentioned above, are activated by proteolytic cleavage of upstream caspases [292, 293].

Two major caspase-dependent signalling pathways are known in mammals: extrinsic pathway and intrinsic pathway. In the extrinsic apoptotic pathway, extracellular ligands such as TNF-α, FasL or TRAIL bind to the extracellular domains of TNF-receptor superfamily and trigger the receptor multimerisation and a conformational change on the cytoplasmic face of the receptors [294]. Recruitment of the adaptor FADD ensues, followed by recruitment and activation of caspase-8 [224]. Active caspase-8 then cleaves and activates the downstream executioner caspases-3, which is pivotal for the execution of apoptotic cell death.

Intrinsic pathway, by contrast, is not activated through a transmembrane signalling receptor, but through the release of mitochondrial factors. Bcl-2 family proteins, which control mitochondrial integrity [295, 296], consists of both pro- and anti-apoptotic members that elicit opposing effects on mitochondria. Pro-apoptotic members can induce mitochondrial outer membrane permeability

(MOMP) resulting in the release of pro-apoptotic factors. In contrast, anti-apoptotic members such as Bcl-2 preserve mitochondrial integrity, blocking the discharge of intermembrane pro-apoptotic proteins [297]. During cell stress, pro-apoptotic proteins are activated and do antagonise the anti-apoptotic Bcl-2 family members. Pro-apoptotic proteins oligomerise and form a channel on mitochondria membrane through which cytochrome c is released into cytosol. After that, cytochrome c associated with Apaf-1, and dATP, form a platform which is called apoptosome, to recruit and activate procaspase-9. Like caspase-8, activated caspase-9 subsequently cleaves and activates the downstream executioner caspase-3.

Both extrinsic and intrinsic pathways converge on activating downstream executioner caspases, which then cleave structure components, such as nuclear lamins and cytoskeletal proteins, and inhibitor of DFF (DNA fragmentation factor)/CAD (caspase-activated DNAse) [298-300], contributing to the classical morphology of apoptosis (cell shrinkage, chromatin condensation, and DNA fragmentation).

There are other pathways mediate the activation of caspases, namely the endoplasmic reticulum (ER)-mediated pathway and the granzyme B-mediated pathway. Stress induces the translocation of caspase-7 from the cytosol to ER surface, which then activates procaspase-12 on the cytoplasmic side of the ER by cleaving its prodomain [301]. The activated caspase-12 subsequently activates procaspase-9, and the activation further activates caspse-3, -6, and -7. Since

mitochondrion is not involved in this pathway, there is no cytochrome c release from mitochondria [291]. Moreover, granzyme B, which is a serine protease that is present in cytotoxic granules of CTLs and NK cells, along with other pro-apoptotic mediators, can induce the activation of caspase-3 [302, 303]. Granzyme B also cleaves Bid, which translocates to mitochondria and recruits Bax, inducing apoptosis in a caspase-independent manner [304]. These examples demonstrate that apoptosis is not only mediated by either simple extrinsic or intrinsic pathway, but that numerous molecules and pathways may be involved in this programmed cell death modality.

Unsurprisingly, the expression, processing, activation, and inactivation of caspases are precisely controlled in normal cells. The most well-known regulators of caspases are inhibitor of apoptosis proteins (IAPs) [305]. IAPs are a family of cellular proteins, which are characterised by N-terminal baculovirus IAP repeat (BIR) domains and a conservative C-terminal RING domain. The BIR domain can bind to caspases and inhibit the activity of caspases. Although IAPs do not directly bind and block caspase-8, they inhibit its substrate procaspase-3, arresting the cascade of extrinsic apoptotic pathway. In intrinsic pathway, IAPs (mainly XIAP, c-IAP1, and c-IAP2) directly bind to the principal caspase, procaspase-9, preventing the apoptosis induced by cytochrome c release [306, 307]. Like caspases, the activity of IAPs is also regulated by other proteins. Smac/Diablo and Omi/ HtrA2, which are simultaneously released from the mitochondria with cytochrome c, antagonise the action of inhibitor of apoptotic proteins (IAPs), relieving IAPs' inhibitory interactions with executioner caspases [308-310]. From

these reports, it is concluded that caspase cascades are tightly controlled, ensuring the proper regulation of cell death.

Caspase-independent cell death

Increasing evidence supports the claim that apoptosis is not exclusively controlled by caspase activation. As mentioned above, cytochrome c is not the only protein that is released from mitochondria following MOMP, and that other proteins originating from mitochondria may mediate cell death via different mechanisms. For instance, apoptosis-inducing factor (AIF) and endonuclease G (EndoG) are other proteins shown to be released from mitochondria during apoptosis. These proteins translocate to nuclei and mediate caspase-independent nuclear features of apoptosis such as chromatin condensation and large-scale (~50 kb) DNA degradation [222, 311, 312]. However, in-depth studies suggest that AIF-associated cell death appears to require specific upstream components and pathways. One important player is poly (ADP-ribose) polymerase 1 (PARP-1), a nuclear enzyme involved in DNA repair [313]. In response to excessive DNA damage, PARP-1-dependent death program is activated to induce the translocation of AIF from mitochondria to the nucleus, nuclear condensation, and phosphatidylserine exposure. The collapse of the mitochondrial membrane and translocation of AIF can not be blocked by broad-spectrum caspase inhibitors, suggesting this pathway is caspase-independent [314].

In addition, endogenous proteases such as caplains and cathepsins can induce cell apoptosis. A moderate increase in cytosolic calcium is sufficient for calpain

activation, which cleaves the anti-apoptotic Bcl-X_L and Bax (the truncated form of which is a more potent inducer of apoptosis than the full-length form [315]), promoting cell apoptosis. Moreover, these proteases cleave mitochondrial Na^+/Ca^{2+} exchanger, inducing Ca^{2+} overload in the mitochondria and leading to sustained ROS production by these organelles [316], resulting in cell death. Besides calpains, cathepsins which are released from lysosomes to cytosol, can target mitochondria and Bcl-2 family members Bid, Bax, and Bak, inducing cell apoptosis [317]. Studies also show that endogenous calpain and cathepsins can promote AIF release, leading to caspase-independent cell apoptosis [318, 319]. These data indicate that other proteases and molecules rather than caspases can initiate the cell death programme.

1.4.2.2. Biological significance of apoptosis

Apoptotic cell death has an important role in organ and tissue remodeling during development. In adult organisms, apoptosis is also crucial in maintaining homeostasis. Several studies indicate that the role of clearance of apoptosing cells is not only to eliminate cell corpses, but also to degrade these cells into harmless and reusable molecules [320]. Apoptotic cells can be engulfed very early which may explain why under normal physiological conditions cells with typical apoptotic morphology are rarely seen in tissues [321, 322]. Dysregulation of apoptosis can result in the development of various pathologies, such as cancer, autoimmune diseases, or sepsis. Importantly, since apoptotic cells remain their plasma membrane integrity before being recognised and ingested by phagocytes, it

is generally believed that apoptotic cell death is immunologically silent and does not provoke inflammation [286].

1.4.3. Necrosis

In contrast to apoptosis, necrosis was considered as a disorderly mode of cell death. Necrotic cells take up water and swell, instead of condensing and shrinking as apoptotic cells do. This swelling causes the plasma membrane to burst and release the cytoplasmic contents to the surroundings. Although it was proposed that apoptosis and necrosis were two different modes of cell death involving distinct dying mechanisms, increasing evidence now suggests that these two dying types represent only the extreme ends of a continuum of cell death with shared events. The extent of oxidative stress, the concentration of cellular ATP content, the level of Bcl-2, and the mechanisms underlying the opening of mitochondria pores can determine the switch of these two types of cell death [323-325].

Secondary necrosis, also called late apoptosis, is the disintegration of the cells that have undergone apoptosis, supporting the hypothesis that apoptosis and necrosis are part of continuum, rather than fundamentally different types of cell death. Secondary necrosis is presented as a mixture of genetically controlled apoptotic processes and a necrotic outcome with rupture of the cytoplasmic membrane and autolytic cell integration into cell debris [321]. Two assays which detect cytoplasmic permeability, namely propidium iodide (PI) and intracellular enzyme lactate dehydrogenase (LDH) are frequently used to evaluate necrosis, but

cannot distinguish between primary necrosis and secondary necrosis. In fact, secondary necrosis has a unique morphology characterised by the co-existence of apoptotic features (like nuclear fragmentation and chromatin condensation) and necrotic alterations (like cytoplasmic membrane permeabilisation). These cells may exhibit specific molecular features (eg. caspase activation, mitochondrial protein releases) as they have gone through the apoptotic pre-necrotic phase.

1.4.3.1. Molecular mechanisms of necrosis

The mechanisms involved in necrotic cell death were less understood than those in apoptotic cell death. Accidental necrosis results from physicochemical damage without involvement of underlying signalling events leading to plasma membrane rupture. However, over the past decade it has become evident that under certain circumstances, necrosis is the result of strictly regulated dying process [326]. Signalling pathways involved in apoptosis such as death receptor activation, the caspase cascade, the activation of proteases, and the mitochondrial pathway also participate in programmed necrosis [327-329].

It was observed that some cells stimulated with death ligands along with the inhibitors of apoptotic caspase cascade did not survive but died by necrosis [326, 330, 331], suggesting the dynamic switch between necrosis and apoptosis. Studies in RIP1$^{-/-}$ Jurkat cells demonstrated that propagation of necrosis induced by triggering of death receptors (eg. Fas, TNF-R, and TRAIL-R) depended on the presence of modulator RIP1 (receptor interacting protein 1), a serine/threonine

kinase [332]. The term necroptosis was introduced to designate programmed necrosis induced by triggering of death receptors under conditions when apoptotic cell death execution is prevented (eg. by caspase inhibitors) [332, 333]. Although it occurs under regulated conditions, necroptotic cell death is characterised by the same morphological features as unregulated necrotic death [333].

In addition to RIP1, recent studies identified RIP3 as a crucial upstream activating kinase that regulates RIP1-dependent necroptosis [334-336]. TNF treatment or triggering pathogen recognition receptors (PRRs) induce the formation of a RIP1-RIP3 pro-necrotic complex and subsequently induce cell necrosis by increasing ROS production [336, 337]. Under normal physiological conditions, the ligation of cells with TNF receptor stimulates either cell survival via NF-κB activation or apoptosis mediated through a cascade of caspase activation [327, 338]. However, when caspase activation is blocked, cells are redirected to programmed necrosis, apparently acting as a kind of back-up cell death pathway [286, 327, 337].

Mitochondrial pathways are also involved in programmed necrosis. The BH3-only protein Bcl-2 modifying factor (Bmf) binds to Bcl-2 and Bcl-X_L, leading to mitochondrial outer membrane permeabilisation (MOMP) and apoptosis. Bmf is also identified as a core component of programmed necrosis by interfering with Bcl-2-mediated blockade of mitochondrial permeability transition (mPT), indicating roles of this factor and mitochondria at the crossroad of apoptosis and necrosis [328, 339]. Additionally, it has been demonstrated that sequential

activation of PARP-1, calpains and Bax following excessive DNA damage is required for AIF-induced necrosis [329], further suggesting the importance of mitochondria in cell necrosis.

In contrast to the increasing clarification of the mechanisms underlying primary necrosis, the factors involved in secondary necrosis are still scarce. It is agreed that secondary necrosis is a separate process occurring after completion of apoptosis, followed by a transition to a necrotic cell. In the first process, molecular alterations induce the classic apoptotic morphology with intact plasma membrane enclosing apoptotic cells or apoptotic bodies. The typical apoptotic process is subsequently followed by development of necrotic morphology, with the alterations involving activation of self-hydrolytic enzymes, swelling of the cells or apoptotic bodies, and rupture of the cytoplasmic membrane [277]. Similar to primary necrosis, the involvement of ROS production is suggested in the transition to secondary necrosis [277]. In addition, calpains and lysosomal enzymes have been found to participate in the cytoplasmic membrane damage in secondary necrosis [340-342]; inhibition of lysosomal enzymes results in inhibition of secondary necrosis in epithelial cell lines [343]. These findings indicate that molecules directly involved in the induction of primary necrosis may also be significant in the induction of secondary necrosis.

1.4.3.2. Physiological significance of necrosis

It has been proposed that under some conditions necrosis is involved in physiologically relevant processes and can substitute for apoptosis to eliminate unwanted cells. For example, during the development, removal of interdigital cells in the presence of the caspase inhibitor or in Apaf-$1^{-/-}$ mice occurs by a caspase-independent necrosis-like process [344]. Additionally, necrosis can function as a backup mechanism to eliminate damaged cells infected by some viruses and intracellular bacteria which suppress caspases or apoptotic signals [334, 337, 345]. Induction of necrosis under these conditions will also activate immune responses, promoting recovery of hosts. However, inefficient clearance of necrotic cells may result in the spread of pathogens.

It is generally believed that necrotic cell death is often associated with pathological conditions. In necrosis, the cellular content leakage and cytokine secretion into the extracellular environment [15], where they may act a “danger signal” and are associated inflammation. Several mediators have been described as endogenous danger signals, including defensins, cathelicidins, uric acid, proteases, nucleosomes, high-mobility group box (HMGB1) protein, and heat shock proteins (HSP) [346-350]. As an example, high mobility group 1 (HMGB1) protein, which acts as an architectural chromatin-binding factor, is secreted passively by necrotic or damaged cells. Outside the cells, it binds with high affinity to RAGE (the receptor for advanced glycation end products) and TLR, mediating inflammatory signals [351, 352]. In apoptotic cells, HMGB1 is bound firmly to chromatin

because of generalised underacetylation of histone, or is oxidised by activated caspases during apoptosis, thus withholds the signal to activate inflammatory and immune responses [346, 353, 354]. Recently, Yamasaki *et al.* demonstrated that a nuclear protein (SAP130) is released by late apoptotic or necrotic cells and can be recognised by Mincle, a transmembrane C-type lectin receptor (CLR) mainly expressed in macrophages. Macrophages are then activated to produce inflammatory cytokines and chemokines, confirming that "danger signals" from necrotic cell death can promote inflammation [355].

The biological significance of secondary necrosis is less understood [356] as initially it was regarded as an *in vitro* condition deprived of proper scavenger system that does not occur *in vivo*. However, more evidence suggests that secondary necrosis does exist in physiolgical conditions, such as in lactating breast [277]. When secondarily necrotic cells are shed into ducts (like breast alveolar lumen, guts, and airway lumen), they could be expelled and the release of their cellular contents may not be critical [277]. However, when the load of dying cells exceeds the local capacity for phagocyte-mediated clearance in solid organs, secondary necrosis could be pathological through the uncontrolled release of cellular components. The leakage of cell contents may cause tissue injury, and the secondarily necrotic cells or their debris may be taken by antigen presenting cells, inducing inflammation and autoimmunity. For example, secondary necrosis of hepatocytes resulting in severe hepatitis has been described in animal models with extensive hepatocyte apoptosis induced by stimuli (eg. death receptor ligands, toxic substances) [357-359]. Secondary necrosis is also found in brain subjected to focal

ischemia [360], acute myocardia infarction [361], and cystic fibrosis (CF), and systemic lupus erythematosus (SLE) [12]. Thus, extensive and persistent secondary necrosis may play major roles in various pathological conditions.

1.4.4. Pyroptosis

Pyroptosis is a more recently recognised form of regulated cell death with morphological and biochemical properties distinct from necrosis and apoptosis [362]. Pyroptosis occurs most frequently in monocytes, macrophages, and dendritic cells upon infection with intracellular pathogens (for example, *Salmonella*, *Francisella* and *Legionella*) and is likely to form part of the antimicrobial response. This death modality is caspase-1-dependent and characterised as cell death with plasma membrane rupture, cellular swelling, osmotic lysis, and activation and release of IL-1 family cytokine and other cellular contents. Features characteristic to apoptotic pathways such as loss of mitochondrial integrity and release of cytochrome c do not occur during pyroptosis [363]. Although pyroptosis is regarded to be a form of programmed cell death, it is clearly different from the immunologically silent cell death represented by apoptosis. Thus, the term pyroptosis (from the Greek ‘pyro’, relating to fire or fever, and ‘ptosis’, meaning a falling) is used to describe the inherently inflammatory process of this caspase-1-dependent programmed cell death [364].

1.4.4.1. Mechanism of pyroptosis

Pyroptosis retains morphological features from both apoptosis and necrosis. In pyroptotic cell death, caspase-1 mediates pore formation on the plasma membrane of host cells, resulting in dissipation of the cellular ionic gradient. The drop of ionic gradients produces a net increased osmotic pressure, water influx, and cell swelling, eventually resulting in osmotic lysis and release of inflammatory intracellular contents [365]. Pyroptotic cells also undergo DNA fragmentation and nuclear condensation like apoptotic cells but the mechanisms underlying this are distinct and still unclear [366].

Instead of the apoptotic caspases, caspase-1 is the enzyme that mediates this process of cell death. Like all caspases, caspase-1 is present in the cytosol as an inactive zymogen. After stimulation by microbial and endogenous signals, the dormant procaspase-1 zymogen is self-activated by proteolytic cleavage into the enzymatically active heterodimer composed of two 10- and 20- kilodalton subunits [367]. In analogy to activation of caspase-9 in the apoptosome, caspase-1 is activated in a complex called the inflammasome. The inflammasome, a molecular platform for recruitment and activation of procaspase-1, is composed of Nod-like receptor (NLR) family members and the adaptor ASC [368]. NLRs detect conserved microbial structures called ‘pathogen-associated molecular patterns’ (PAMP) or danger-associated molecular patterns (DAMP) (i.e. uric acid crystals, asbestos, or silica) in host cytosol and drive the formation of inflammasomes [369, 370]. To date, four inflammasomes have been characterised, namely NLRP1,

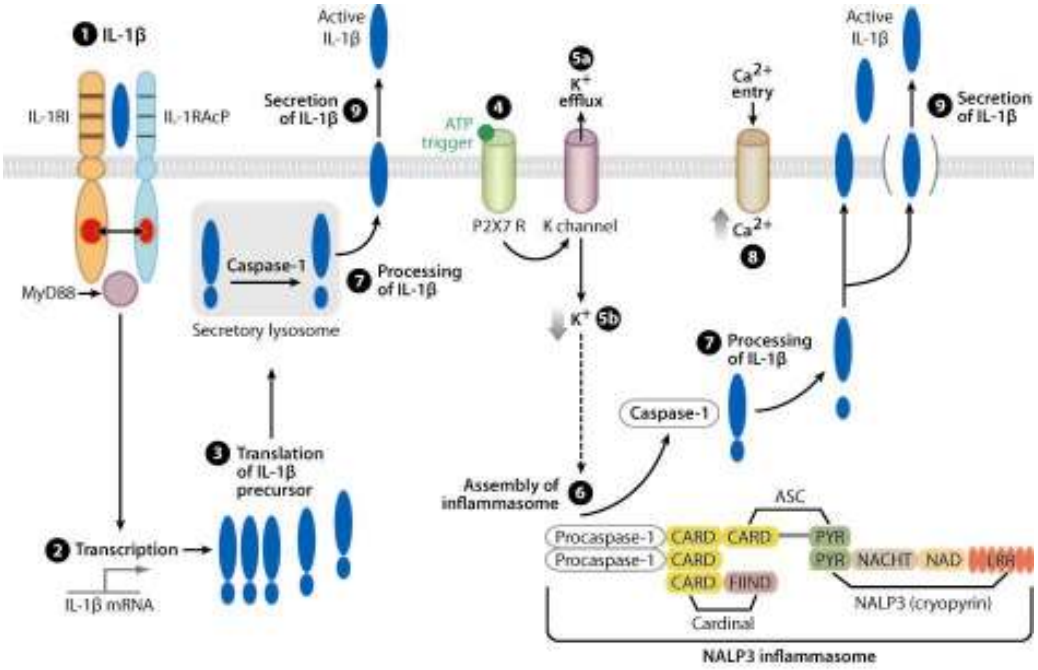
NLRP3, NLRC4/IPAF and AIM2 inflammasomes according to the NLR involved [366, 371]. However, the precise mechanism by which the activation of various inflammasomes by diverse stimuli remains elusive.

Active caspase-1, however, does not only contribute to cellular demise, but also to the cleavage of pro-inflammatory cytokines into their mature, biologically active form. The inflammatory cytokines IL-1β and IL-18 undergo caspase-1-dependent activation and secretion during pyroptosis [366]. IL-1β is a pro-inflammatory cytokine produced mainly by immune effector cells. It functions in many aspects of immune and inflammatory responses by generating fever, activating immune cells, and promoting the recruitment of leukocytes to the affected sites. IL-1β is the primary cause of inflammation and has been implicated in a variety of inflammatory disorders [372-374]. IL-18 induces INF-γ production and is important for the activation of T cells, macrophages and other cell types [375, 376]. Through these mechanisms, caspase-1-mediated processing of IL-1β and IL-18 is a crucial component of the host defence against infections.

Two signals are required for the production and release of IL-1β and IL-18 (Fig. 1.3). The first signal is pro-inflammatory cytokines such as TNF-α, IL-1, and TLR ligands which induce the transcription of IL-1β mRNA in immune effector cells [377, 378]. The second signal, such as ATP, triggers the $P2X_7$ receptor, causing potassium efflux [379, 380]. The drop of intracellular potassium results in assembly of the inflammasome components with inactive procaspase-1. Procaspase-1 is then processed into active form, followed by process of the 31-kDa

inactive IL-1β precursor form into the bioactive 17-kDa IL-1β. An influx of calcium into the cell with an increase in intracellular calcium levels provides a mechanism by which mature IL-1β is released from the cell [373]. Other second signals can come from the cathelicidin-derived peptide LL-37 [29] or bacterial toxin nigericin ionphore [381], leading to pore formation and intracellular potassium efflux. This two-step process ensures the well-regulated release of pro-inflammatory IL-1β and IL-18.

Interestingly, although formation of caspase-1-dependent pores in the plasma membrane is correlated with cytokine release [365], cell lysis or cell death is not always required for the release of activated IL-1β and IL-18. Pharmacological inhibition of cell lysis does not prevent caspase-1-dependent pore formation or cytokine secretion [365], suggesting that cytokine secretion and cell death are both downstream consequences of caspase-1-dependent pore formation.



Dinarello CA. 2009.
Annu. Rev. Immunol. 27:519–50

Fig. 1.3 Steps in the synthesis and secretion of IL-1β induced by IL-1β (or can be substituted by other pro-inflammatory cytokines and TLR ligands). Primary blood monocytes or tissue macrophages are activated by pro-inflammatory stimuli (step 1) to increase the transcription (step 2) and translation (step 3) of precursor IL-1β. Secondary stimulus such as ATP, nigericin, or bacterial toxins then signals through $P2X_7$ receptor (step 4), leading to a rapid efflux of potassium from the cell (step 5a). The drop of intracellular levels of potassium (step 5b) triggers the assembly of inflammasome (step 6). The assembled components of the inflammasome initiate the processing of procaspase-1, resulting in the formation of active caspase-1. Active caspase-1 processes the IL-1β precursor (step 7) to generate mature IL-1β, followed by secretion of active mature form of IL-1β to extracellular environment (step 8 & 9). The figure is taken from Dinarello CA. Annu. Rev. Immunol. 27 (2009) p.529 [373].

1.4.4.2. Biological significance of pyroptosis

Pyroptosis is part of the host defence system against pathogens and a potent inducer of inflammation. Mice deficient in caspase-1 (which lack the capacity of pyroptosis and activation of the pro-inflammatory cytokines IL-1β and IL-18) are more susceptible to infection than wild type mice and IL-1β and IL-18 double knockout mice. These observations suggest that pyroptosis or other caspase-1-dependent events, instead of cytokines alone, are involved in the control of infection. However, inappropriate or overwhelming caspase-1 activation can be detrimental and results in pathological inflammatory diseases [366].

1.4.5. Conclusion

Cells can die by distinct mechanisms with differing impacts on inflammation and immune response. Physiological cell death such as apoptosis is thought to be anti-inflammatory and intrinsically immune tolerogenic, whereas pathological cell death (necrosis or pyroptosis) is inherently immunogenic and elicits inflammatory reaction [382]. Apoptotic cells remain intact membrane integrity, preventing intracellular content from being discharged, and also possess modified immunostimulatory molecules to promote tolerance [383]. However, this concept is oversimplified and controversial as cells dying by apoptosis can be highly immunogenic [384-386], whereas necrotic cells can be anti-inflammatory in certain circumstances [387, 388].

Different forms of cell death may occur concomitantly under physiological or pathophysiological conditions, and their impacts on inflammation and immune responses merit further investigation. It seems that cells are equipped with a range of machinery to undergo different forms of cell death, and that different stimuli in various environments at varied time points can result in diverse outcomes. Thus, the identification of the mechanisms that regulate the different modalities of cell death may provide potential therapeutic strategies for diseases [389].

1.5. Phagocytosis of dead cells

1.5.1. Introduction

Phagocytosis (from Greek, phago- 'eating', -cyte 'vessel', -osis 'process') was described in the late 19th century by the Russian biologist Ilya Metchnikoff, who observed that amoeboid-like cells in transparent sea star larvae contained ingested cells. In lower organisms this process is used to internalise nutrients from their surrounding milieu, whereas in multicellular organisms, phagocytosis has adopted an important role in host defence and tissue homeostasis [390].

Phagocytosis is defined as a process of uptake of large particles (> 0.5 μm) into cells dependent on receptor recognition. The engulfing processes form phagosomes and subsequently fuse with lysosomes to become phagolysosomes where the ingested material is digested or degraded [391]. Monocytes/ macrophages, immature DC, and PMN are very efficient at internalising particles thus have been referred to as professional phagocytes. On the other hand, most of cells (except lymphocyte and red blood cells) have some phagocytic capacity and are termed as nonprofessional phagocytes such as renal mesangial cells and epithelial cells [392].

1.5.2. Main purposes of phagocytosis

Two main purposes of phagocytosis have been described: “sensing the invaders” and “burying the corpses” [390]. Containment of infection can be achieved by ingestion and degradation of pathogens. A wide range of receptors act in surveillance and recognise the conserved molecular pattern of microorganisms, followed by degradation of microbes. The other goal of phagocytosis is to ensure the safe disposal of unwanted cells from self, which is a fundamental element to regulate normal tissue turnover and homeostasis. [393]. In this chapter, I will focus on phagocytosis of dead cells and the ensuing biological responses.

1.5.3. Factors affect the rates of phagocytosis

The capacity and efficiency of phagocytosis can be modulated by the presence of cytokines, pathogen metabolites, and pharmacological agents [266, 394-397]. Studies reveal that pro-inflammatory mediators such as LPS, HMGB1, and TNF-α inhibit apoptotic PMN being taken up [396, 398]. Additionally, bacteria metabolites and proteases during infection may also interfere with the efferocytosis of dead cells [399, 400]. Glucocorticoid, by contrast, reverses the pro-inflammatory stimuli-mediated inhibition of phagocytosis and plays an important role in therapeutics of inflammatory diseases [394, 396]. These findings suggest that several factors present at affected sites can modulate the efficiency of cell clearance by phagocytes.

1.5.4. Molecules involved in recognition and clearance of apoptotic cells

The most favourable fate for dying cells, to avoid inflammation and host damage, is removed by phagocytes, avoiding dead cells from releasing cytotoxic substances that can damage neighboring cells [15, 390, 401]. This process has been named 'efferocytosis' (from effero-to carry to the grave, to bury in Greek). Clearance of apoptotic cells involves an elaborate combination of mechanisms of "tethering" and "tickling" [402]. This involves a large variety of eat-me signals which ensure the first tethering of the apoptotic cell to the phagocyte, followed by the interaction of molecules and receptors on target cells and phagocytes, triggering the process of internalisation [401, 402].

1.5.4.1. Come-get-me signal

The first step in the process is recruitment of professional phagocytes to the sites of the apoptotic cells. To attract phagocytes, apoptotic cells can secret lipid factors such as lysophosphatidylcholine (LPC) in a caspase-3-dependent fashion [403]. A recent study revealed that nucleotides released by apoptotic cells can also act as a find-me signal to promote recruitment of phagocytes, facilitating dead cell clearance [404]. Another interesting report showed that apoptotic cells can also release lactoferrin, which inhibits migration of granulocytes but attracts mononuclear phagocytes. These data suggest a well-regulated system controlling recruitment of phagocytes to remove unwanted dead cells [405, 406].

1.5.4.2. "Eat-me" signal

Once recruited phagocytes arrive in the sites, they have to recognise their targets first. Molecules that newly appear on the cell surface and existing molecules altered by oxidation and modification during apoptosis serve as "eat me" signals to facilitate recognition and ingestion by phagocytes [407-409]. The best characterised is the loss of phospholipid asymmetry and the translocation of phosphotidylserine (PS) from the inner to the outer leaflet of the plasma membrane, which occurs very early during apoptosis [407, 409]. Studies showed that apoptotic targets that failed to express PS or that had masked PS were not recognised and engulfed by phagocytes [410, 411], suggesting the importance of PS exposure for phagocytosis. However, a study showed that PS externalisation on apoptotic cells alone is not sufficient to trigger cell clearance. Instead, the presence of oxidised PS in conjunction with non-oxidised PS on cell surface is required for removal of apoptotic cells [412]. In addition, a study using nematode *Caenorhabditis elegans*, demonstrated that annexin I, which co-localises with PS and is also externalised during apoptosis, is required for efficient clearance of apoptotic cells [413]. Interestingly, living cells also expose PS, such as activated B cells and T lymphocytes although the change of PS distribution serves different functions [414, 415], suggesting that PS externalisation is not the only factor to serve as recognition ligand. Indeed, calreticulin is another general ligand of apoptotic cells, binding and activating LDL-receptor-related protein (LRP) on the engulfing cells [416]. Since both surface PS and calreticulin can also be found on viable cells, it

was proposed that there must also be molecules expressed on living cells which protect them from efferocytosis [416-418].

1.5.4.3. "Don't eat-me" signal

Living cells protect themselves from engulfment by expressing "don't eat-me" signals. Brown *et al.* demonstrated that an interaction between CD31 molecules on living leukocytes and macrophages mediated the detachment of leukocytes from macrophages [419]. During apoptosis, this detachment signal is disabled, therefore enabling the phagocytosis of dying cells. Thus, CD31, a member of the immunoglobulin superfamily functions as a "don't eat-me" signal [419]. In addition, CD47 on red blood cells serves as a don't-eat-me signal, acting through the SIPRα receptor [416-418]. The knowledge of "don't eat-me" signal had been limited on CD31 and CD47 until recently, a novel "don't eat-me" signal, plasminogen activator inhibitor-1 (PAI-1), was found [420]. PAI-1, an inhibitor of fibrinolysis, has been found having elevated levels in inflammatory conditions, in which activated PMN accumulate in tissues and contribute to organ dysfunction. Study showed that phagocytosis of viable PAI-1 deficient PMN and PMN treated with anti-PAI-1 antibodies was enhanced associated with the increased expression of calreticulin and LRP [420]. These data suggest that both "eat-me" and "don't eat-me" signals participate in the process of detecting dead cells.

1.5.4.4. Receptors

Several macrophage receptors have been implicated in efferocytosis, including phosphatidylserine (PS) receptor [421], complement receptors [422], scavenger receptors [423], the $\alpha_v\beta_3$/CD36/thrombospondin recognition receptors [424], CD14 [425], and CD44 [426]. Blocking each of these receptors alone causes partial inhibition of apoptotic cell engulfment, suggesting a redundancy in the engulfment pathways [427].

Phosphatidylserine receptor (PSR)

As mentioned above, transposition of PS from the inner leaflet of the plasma membrane to the outer leaflet is a hallmark of cells undergoing apoptosis. Several groups had claimed that PSR was required for clearance of apoptotic cells *in vitro* and *in vivo* [413, 428, 429]. It was believed that human and mouse macrophages express the *Psr* gene, and mice genetically deficient in this gene had accumulation of dead cells in the lung and brain during embryogenesis, suggesting its role in dead cell removal [429]. However, later studies challenged this original hypothesis, demonstrating the nuclear localisation of Psr protein, rather than a transmembrane receptor for apoptotic cell recognition [430]. In addition, a comprehensive investigation of apoptotic cell clearance *in vivo* and *in vitro* demonstrated that engulfment of apoptotic cells was normal in *Psr* knockout mice, suggesting that other receptors of PS may be involved in efferocytosis.

Recently, researchers discovered three novel PSR through which apoptotic cells are removed by macrophages. Park *et al.* showed that stabilin-2, which is a scavenger receptor, functioned as a PSR and downregulation of this receptor on macrophages resulted in decreases of apoptotic cell removal [431]. In addition, BAI1, belonging to G-protein-coupled receptor family, served as an engulfing receptor in both the recognition and subsequent internalisation of apoptotic cells through binding to PS. Knockout of BAI1 in phagocytes resulted in a decreased uptake of apoptotic cells [432]. Moreover, Miyanishi *et al.* identified Tim4 as a PSR, showing that inhibition of Tim4 *in vivo* leaded to an accumulation of apoptotic corpse in the thymus and the development of autoantibodies [433]. These reports demonstrate that several receptors are involved in the recognition of PS expressed on apoptotic cells.

Other receptors than PSR

In addition to the extensively-studied PS receptors, other receptors also mediate the recognition and ingestion of dead cells. For example, complement receptors of macrophages (CR3 and CR4) can recognise apoptotic cells through detecting the complement proteins (iC3b) which bind to exposed PS [422, 434]. Moreover, CD36, a class B scavenger receptor, associated with vitronecin ($\alpha_v\beta_3$ integrin) and bridging molecule thrombospondin, promotes binding and uptake of apoptotic cells by macrophages [424, 435, 436]. Macrophages derived from CD36 and $\alpha_v\beta_3$ integrin knockout mice show significantly reduced apoptotic cell phagocytosis [437]. Scavenger receptors other than CD36 have also been implicated in the clearance of dying cells [423]. For instance, class A macrophage scavenger

receptor (SR-A) has been shown to play a role in the engulfment of apoptotic thymocytes. Blockage of the receptor by antibody or disruption of receptor genes reduces the dead cell ingestion [423]. Furthermore, CD14 and CD44 on the surface of macrophages play important roles in efferocytosis [425]. CD14 mainly functions as a co-receptor for LPS/ LPS-binding protein, and can interact with apoptotic cells with a region identical to, or closely associated with, a region known to bind LPS [425]. CD44, a receptor with broad ligand specificity, has been proposed to trigger the rearrangement of cytoskeletal proteins upon stimulation [438]. Ligation of human macrophage surface CD44 by divalent monoclonal antibodies augments the capacity of ingesting apoptotic PMN *in vitro*, suggesting that receptor cross-linking is a prerequisite for this process [439]. *In vivo* study showed that CD44-deficient mice had reduced capacity to clear apoptotic PMN, demonstrating a critical role for this receptor in clearance of dying cells [440]. In summary, there is a redundancy of receptors involved in the engulfment pathways to ensure efficient removal of apoptotic cells.

1.5.4.5. Bridging molecules

Most of receptors mentioned above do not directly bind to apoptotic cells, but engage dying cells via a broad range of bridging molecules. For example, β2 glycoprotein [441], milk fat globule protein (MFG-E8) [442], protein S [443], growth arrest-specific 6 (Gas6) [444], and thrombospondin [424] facilitate interactions between macrophages and exposed PS on apoptotic cells. Bridging molecules preferentially interact with the oxidised form of PS and promote the

recognition of surface-modified dying cells. Thus, the process of recognition and clearance of apoptotic cells involves numerous molecules, dependent on the divergent receptors expressed on phagocytes.

1.5.5. Molecules involved in recognition and clearance of necrotic cells

It is still elusive how phagocytes recognise necrotic cells although it has been shown that necrotic cells also externalise PS [445] and can be recognised through a PS-dependent mechanism [446, 447]. The studies also reveal that phagocytosing necrotic cells is less effective than phagocytosing apoptotic cells [446, 447], suggesting that the response to phagocytosis of dead cells is more complex than simply a response to externalised PS. In addition, studies have been suggested overlap in other receptors involved, with the $\alpha_v\beta_3$/CD36 system and CD14 implicated in uptake of primary necrotic lymphocytes by macrophages as well as in the clearance of apoptotic cells [388]. However, others have demonstrated that recognition of apoptotic and necrotic cells occurs via distinct non-competing mechanisms [446, 448]. These observations indicate that the existence of common pathways for recognising dead cells, with additional mechanisms specific to clearance of dead cells of different modalities.

1.5.6. Macrophages ingest apoptotic PMN

Clearance of dead PMN by phagocytes is a critical process to modulate inflammtory responses. Under physiological conditions, the speed of phagocytosing demised PMN is very quick to prevent toxic intracellular content release. Removal of dying PMN has been studied mainly using human monocyte-derived macrophages. The recognition of apoptotic PMN by macrophages involves a change of membrane polarity and a reduction in the expression of surface molecules, such as CD16 and CD32. The reduction of CD16 occurs simultaneously with PS externalisation while the decrease of CD32 increases the binding of opsonins, both of which enhance phagocytosis [449, 450]. Scavenger receptors, lectin-like receptors, and CD44 have also been implicated in ingestion of apoptotic PMN, but CD14 has only minor significance [435].

Contrary to the previously dominant paradigm, the recognition of PS as an ‘eat me’ signal may not be critical for the removal of apoptotic PMN by resting macrophages. Growing evidence suggests other molecules may be involved in signalling the clearance. For example, Barth’s syndrome (BTHS), where nearly 80% of circulating PMN have externalised PS, does not increase the engulfment by macrophages [451]. In addition, pretreatment of apoptotic PMN with bacterial proteinase resulted in a significant increase in cell uptake by macrophages without any change in PS distribution [400]. However, although data showed that unstimulated macrophages phagocytose apoptotic PMN mainly using the $\alpha_v\beta_3$/CD36/TSP mechanism, glucan-stimulated macrophages exploit PSR to

recognise apoptotic PMN [435]. Thus, the system used for phagocytosing apoptotic PMN, like uptake of other types of dead cells, involves a variety of receptors and molecules, providing efficient ways of clearance.

1.5.7. Modes used by macrophages to clear apoptotic and necrotic cells

Two modes of phagocytosing dead cells have been observed dependent on morphological differences: zipper-like phagocytosis and spacious macropinocytosis. Zipper-like interaction is found when targets are surrounded by pseudopods from phagocytes and surfaces of targets and phagocyctes are tightly apposed with no solute taken up during internalisation [452]. In contrast, macropinocytosis is characterised with cell surface ruffling and formation of membrane invaginations wherein large amounts of external solute are internalised together with targets [453].

The mechanisms responsible for the clearance of dying cells are still controversial. Some studies by microscopy show that apoptotic cells are taken up by complete engulfment of membrane-bound apoptotic bodies which form tight fitting phagosomes without ingestion of fluorescent fluid phase tracer. In contrast, primary and secondary necrotic cells are internalised by formation of spacious macropinosomes accompanied by uptake of fluid [446, 454, 455]. However, other groups have the opposing findings, demonstrating ingestion of apoptotic cells by macropinocytosis rather than zipper-like phagocytosis [456]. Irrespective of these

findings, these data suggest that different modes for internalisation of apoptotic and necrotic cells do exist and may well exist and may be significant in explaning the different biological consequences [15, 446].

1.5.8. Consequences for inflammatory and immune responses

Increasing number of studies indicate the importance of the nature of cell death and phagocytosis in determining following inflammatory consequences. It has been widely accepted that the removal of apoptotic cells by phagocytes is non-phlogistic whereas the clearance of necrotic cells promotes inflammation [457].

1.5.8.1. Inflammatory impacts of apoptotic cell phagocytosis

Fadok *et al.* first revealed that phagocytosis of apoptotic PMN was not only a non-inflammatory process but also actively inhibited the production of cytokines such as IL-1β, IL-8, IL-10, GM-CSF, and TNF-α by activated human monocyte-derived macrophages *in vitro* [13]. In contrast, the production of anti-inflammatory cytokines like TGF-β1 and prostaglandin E2 was increased, suggesting that the interaction with apoptotic cells induced anti-inflammatory or suppressive properties in human macrophages [13]. Further studies have supported these observations, claiming that efferocytosis of apoptotic cells by phagocytes is anti-

inflammatory and immunosuppressive [14, 458-460]. Molecules involved in orchestrating clearance of apoptosis and promotion of tolerance are widely studied. Recently, PPAR-δ, a sensor of fatty acids, was also identified as a critical receptor to sense lipid flux of engulfing apoptosis, leading to an inflammation-dampening result [461].

Although efferocytosis of apoptotic cells may be primarily anti-inflammatory, different receptors involved in apoptotic cells uptake may alter inflammatory outcomes. In contrast to the anti-inflammatory effects of apoptotic PMN uptake through VnR/CD36 mechanism, phagocytosis of immunoglobulin G-opsonised apoptotic cells, mediated by the FcR, increased pro-inflammatory cytokine production [13]. In contrast, complement-dependent apoptotic cell clearance by MDM has been shown to be anti-inflammatory by blocking NF-κB activation albeit that the anti-inflammatory effects are independent of IL-10 and TGF-β [462]. However, other reports suggest that apoptotic cell interaction or contact with the phagocyte is sufficient to inhibit pro-inflammatory cytokine production, prior to internalisation or phagocytosis [462, 463]. The complexity of molecules and mechanisms involved in dead cell phagocytosis may explain these results in different experimental systems.

In addition to *in vitro* studies, an *in vivo* mouse model has been used to demonstrate that administration of apoptotic cells can protect mice against LPS-induced shock. The study suggested that LPS can quickly bind to apoptotic cells and these LPS-coated apoptotic cells can be recognised and cleared by

macrophages in a CD14/thrombospondin/vitronectin receptor-dependent manner, accompanied with suppression of proinflammatory cytokine production [464]. Anti-inflammatory effects of apoptotic cells are therefore not only seen *in vitro* conditions, but also mediate protective function against inflammation *in vivo*.

However, other studies suggest that apoptosis is not anti-inflammatory nor beneficial to the host under certain conditions. For instance, it has been shown that Fas/ FasL-mediated lung epithelial apoptosis induced release of pro-inflammatory cytokines such as TNF-α, leading to inflammation and progression to fibrosis [465]. In addition, another *in vivo* study demonstrated that transfer of apoptotic cells exacerbated CLP-induced sepsis [466]. Furthermore, the inflammomodulatory effects of apoptosis can be exploited by intracellular pathogens such as *Trypansoma cruzi* to subvert macrophage response, with the induction of anti-inflammatory mediators enhancing the growth and survival of the parasites [467].

Taken together, all of these findings indicate a diversity of inflammatory consequence of phagocytosing apoptotic cells. This may be attributable to the complexity of interplay between phagocytes and target cells, and indicates that the initial paradigm may be overly simplistic.

1.5.8.2. Inflammatory impacts of necrotic cell phagocytosis

When it comes to the inflammatory consequences of removal of necrotic or lysed cells, the opinions are controversial. However, the prevailing paradigm has been claimed that necrotic cells are pro-inflammatory to phagocytosis. Several studies suggest that necrotic cells act as a 'danger signal' to initiate inflammatory responses and mediate activation and maturation of professional phagocytes, such as macrophages and dendritic cells [14, 355, 468-470]. Necrotic cells release their intracellular proteases which can cleave phagocytic receptors or molecules, hindering the clearance of dying cells and resulting in persistent inflammation [269, 275, 471, 472]. In addition, necrosis leads to release of histotoxic contents like proteases and danger signals, leading to tissue damage and further promoting inflammation. However, contrasting findings indicate that not all necrotic cells behave in the same way. Under certain conditions, necrosis exerts anti-inflammatory effects on macrophages [446, 447], which may be dependent on PS exposure [473]. However, it is unclear whether such anti-inflammatory signals may be overridden by pro-inflammatory factors released during necrosis. Because necrosis is such a heterogenous cell death mode, it is perhaps not surprising that necrotic cells have divergent impacts on inflammatory responses when they are removed.

1.5.8.3. Inflammatory impacts of disposal of secondary necrosis

The effect of phagocytosis of late apoptotic cells (apoptotic cells that have lost membrane integrity), also referred to as post-apoptotic or secondarily necrotic cells, is more uncertain [356]. According to many models, late apoptotic cells should behave like necrotic cells, due to the loss of cell membrane integrity and release of potentially inflammatory intracellular contents [474, 475]. Delayed or altered clearance of apoptotic cells leads to sustained inflammation and a variety of autoimmune diseases [279, 280, 476]. Cystic fibrosis (CF) lung, for example, is a chronic inflammatory condition characterised by chronic bacterial infection and massive PMN accumulation. CF patients have significantly higher levels of secondarily necrotic PMN in lung, which may be associated with the persistent chronic inflammation and clinical impairment [278, 472]. Furthermore, autoantibodies from SLE patients opsonise secondarily necrotic cells, and can enhance the secretion of inflammatory cytokines upon phagocytosis [477]. However, inconsistent with the idea that secondary necrosis is pro-inflammatory, some groups have shown that irrespective of membrane integrity, apoptotic cells do not become pro-inflammatory through the induction of secondary necrosis [478, 479]. Late apoptotic cells have equivalent anti-inflammatory effects [448] and delayed clearance alone cannot lead to autoimmunity [479-481]. Thus, the impact of secondary necrosis upon inflammation remains unclear.

1.5.8.4. Immune responses of disposal of dead cells

Apoptosis was proposed to be immunologically silent, whereas necrosis was proposed to stimulate an immune response [288]. The uptake of apoptotic cells by DCs fails to stimulate activation and antigen presentation [482, 483], whereas exposure to necrotic cells, particularly those derived from tumours and their lysates, does [14]. However, this idea has been challenged by various observations showing that certain subtypes of apoptosis can be immunogenic. For example, the apoptosis of tumor cells induced by certain chemotherapy was shown to prime an immune response [384]. In addition, antigens from apoptotic cells have been shown to be effectively cross-presented to cytotoxic T cells (CTLs) and prime an immune response [385, 386, 484]. Recently study further demonstrated that a C-type lectin receptor CLEC9A (also known as DNGR-1) on dendritic cells can recognise signals exposed on necrotic cells, resulting in immunogenicity [470]. Taken together, whether a cell undergoes necrosis or apoptosis does not predict whether the outcome will be an immunogenic or immunosuppressive.

1.5.9. Conclusion

The biological significance of phagocytosis of dying cells is not only to remove dead cells but also to serve a mechanism to modulate consequent inflammatory responses. The mechanisms of phagocytosis of dying cells and consequent responses are dependent upon the cell type, the means of induction of cell death, the stage of apoptosis or necrosis, the type and state of differentiation of the

phagocyte, and the surrounding microenvironment [10]. The complexity of factors involved may provide an explanation to the various inflammatory and immune outcomes after cell clearance.

However, the inflammatory and immune consequences of phagocyte uptake of dead cells appear to be more complex than initially proposed. At inflamed sites, mixtures of apoptotic and necrotic cells may be presented to phagocytes in the presence of other inflammatory factors. Despite data demonstrating anti-inflammatory effects of both early and late apoptotic cells are completely dominant over the pro-inflammatory effects of the necrosis [448, 462, 479], it is unclear whether this applies in all scenarios. More studies are required to investigate the inflammatory impacts of dead cells on physiological or pathological circumstances.

1.6. Hypothesis and aims of work

CHDP possess multiple immunomodulatory functions on immune effector cells, particularly on the first-line defence cell, PMN. Since PMN death and removal play pivotal roles in determining the following inflammatory consequences, I hypothesised that LL-37 could modulate cell death in human PMN, *in vitro* and *in vivo*, to influence inflammatory responses. My studies therefore focus on the effects of CHDP on human PMN death, and the impacts of CHDP on inflammatory responses, by modulating cell death and by altering cytokine production in response to pro-inflammatory stimuli.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Reagents

Arg-Gly-Glu-Ser (RGES), Arg-Gly-Asp-Ser (RGDS), oATP, KN-62, and nigericin were bought from Sigma-Aldrich (Poole, UK). WRW4, pertussis toxin Bordetella pertussis (PTX), and caspase-1 inhibitor (cell-permeable, YVAD-CHO) were purchased from Calbiochem/ Merck Biosciences Ltd. (Nottingham, UK). AZ-167 was synthesized by Keith Finlayson at Centre for Translational Chemistry and Biology, the University of Edinburgh (UK). rhGM-CSF was purchased from Research Diagnostics Inc. (Flanders, NJ, USA). *R*-Roscovitine was supplied by A.G. Scientific (San Diego, CA, USA). Ultra-pure Lipopolysaccharide (LPS) from the *Escherichia coli* 0111:B4 strain was purchased from InvivoGen (San Diego, CA, USA). Dexamethasone was purchased from Organon Laboratories Ltd. (Cambridge, UK). rhCD40L and INF-γ were purchased from PeproTech ED Ltd. (London, UK). Human TLR agonist kit was supplied by Autogen Bioclear (Nottingham, UK), Brewer thioglycollate medium was supplied from Sigma-Aldrich (Poole, UK). Rabbit polyclonal antibodies against caspase-1, IL-1β, caspase-3, cleaved caspase-3, pan-actin, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G antibodies were purchased from Cell Signaling Technology (Beverly, MA). LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVRPTES) and mCRAMP (GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ) were synthesised by N-(9-fluorenyl) methoxycarbonyl chemistry at the Nucleic Acid/ Protein Service Unit at

the University of British Columbia (Canada). Peptides were purified by reverse-phase HPLC and were at least 98% pure. The concentrations of the peptides in solution were determined by amino acid analysis. Scrambled LL-37 (RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL) was purchased from CSS-Albachem Ltd. (Gladsmuir, Scotland, UK), and the panel of 16 overlapping 22-mer partial peptides (from N-terminal peptide LLGDFFRKSKEKIGKEFKRIVQ through to C-terminal peptide EFKRIVQRIKDFLRNLVRPTES) was synthesized by Jen W. Drijfhout at Leiden University Medical Centre (The Netherlands). Human β-defensin-3 was purchased from Scientific Marketing (Kent, UK). HNP1-4 was a kind gift from Ole Sørensen (Lund Univeristy). Peptides were dissolved in endotoxin-free water (Sigma-Aldrich, Poole, UK) and stored at -20°C until further use. All reagents were tested using a kinetic-quantitative chromogenic LAL assay (Cambrex, Walkersville, MD, USA) to ensure that they were free of endotoxin and reconstituted in endotoxin-free water.

2.2. Methods

2.2.1. Effects of CHDP on PMN death

2.2.1.1. Isolation of human blood PMN

Fresh human venous blood was collected according to Lothian Research Ethics Committee approval #08/S1103/38 or #1702/95/4/72 using sodium citrate anticoagulant (Phoenix Pharma Ltd., Gloucester, UK). Collected blood was centrifuged at 300 xg for 20 min at room temperature, platelet-rich plasma was removed and added 220 μl $CaCl_2$ (Sigma-Aldrich)/ 10 ml plasma added to generate autologous serum. Cells were then gently suspended in 6% Dextran 500 (Amersham Pharmacia Biotech, UK) in 0.9% saline (Baxter) and sedimented for 25 min at room temperature. The leukocyte-rich upper layer was then fractionated by using three-step discontinuous, isotonic Percoll gradients [213]. Briefly, leukocytes were centrifuged at 350 xg for 6 min, resuspended in 55% isotonic Percoll (GE Healthcare, Buckinghamshire, UK), layered on top of 70% and 81% isotonic Percoll layers, and centrifuged at 720 xg for 20 min at room temperature. PMN were then collected from the interface between 70% and 81% Percoll layers, washed in phosphate buffer saline (PBS) without calcium or magnesium (PAA Labortories, Somerset, UK) and resuspended in IMDM (PAA Labortories) with 10% fetal bovine serum (FCS, Biosera, East Sussex, UK). Purity of PMN was assessed by morphological criteria using cytocentrifuge preparations and FACS

analyse. Antibodies against CD14, CD3, and CD19 (Caltag-Medsystems, Ltd.) were used to distinguish PMN from PBMC. Granulocyte purity of >98% was yielded by this method, and granulocytes were typically 95-98% neutrophils by cytocentrifuge identification. Total cell number was assessed by hemacytometer counts and by NucleoCounter YC-100 (Chemometec, Allerød, Denmark) automated cell number counting.

Alternatively, another method to isolate blood leukocytes was tried [485]. In this method, PMN were purified by Ficoll-Paque (Amersham Pharmacia Biotech, Buckinghamshire, UK) gradient centrifugation at 4°C to avoid activation. Erythrocytes were lysed hypotonically with distilled water and PMN were washed with Krebs-Ringer phosphate buffer (Sigma-Aldrich, Poole, UK) containing glucose and Mg^{2+}. Collected PMN were resuspended in IMDM with 10% fetal bovine serum.

2.2.1.2. PMN death assessment

Freshly isolated human PMN were incubated at 37°C, 5% CO_2, at 5×10^6/ml in IMDM with or without 10% (v/v) FCS in flexible, 96-well culture plates in the presence of LL-37, GM-CSF, *R*-Roscovitine, LPS, oxidised ATP, WRW4, PTX, AZ-167, mCRAMP, scrambled LL-37, human β-defensin-3, HNP, or partial peptides at the stated concentrations or in control media in triplicate over the time periods detailed. To induce primary necrosis, freshly isolated cells were heated at 65°C for 30 min. Cell death was assessed by light microscopic evaluation of

apoptotic morphology such as cell shrinkage, chromatin condensation, and cytoplasmic vacuole formation. Samples (100 μl) of cells were cytocentrifuged, fixed in methanol, stained with Reastain Quick-Diff (Reagena, UK), and examined at least 100 cells under microscopy. Cells were also examined by stained with FITC-labeled AV (Roche Applied Sciences, West Sussex, UK), diluted 1:2000 in HBSS (Sigma-Aldrich, Poole, UK) with 5 mM $CaCl_2$, and 3 μg/ml propidium iodide (Invitrogen Ltd., Paisley, UK) for flow cytometric evaluation at 4°C. FACS Calibur was used, counting ≥ 10, 000 cells, and the data were analysed using FlowJo software (TreeStar Inc., Ashland, OR, USA). Total cell counts were determined using a NucleoCounter YC-100.

2.2.1.3. TEM

Fresh isolated human PMN were incubated with or without 5 μg/ml LL-37 for 20 hr, as described above. Overnight treated or untreated PMN were sorted by FACS into Annexin V^- PI^-, Annexin V^+ PI^- (apoptotics), and Annexin V^+ PI^+ (necrotics) populations. Unsorted and sorted cells were collected and centrifuged at 250 xg for 5 min at room temperature, and resuspended gently in 1 ml 2.5% glutaraldehyde (Sigma-Aldrich) in 0.1 M sodium cacodylate buffer, pH 7.3 (Sigma-Aldrich), for 1 h. Cells were washed in three 10-min changes of 0.1 M sodium cacodylate and postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 45 min before three 10-min changes of 0.1 M sodium cacodylate. Samples were then dehydrated in 50%, 70%, 90%, and 100% normal grade acetones before two 10-min changes in analar acetone and embedded in Araldite

resin. Toluidine blue-stained sections (1 μm) were previewed before 60 nm ultrathin sections were cut from selected areas, stained in uranyl acetate and lead citrate, and then viewed in a Philips CM120 transmission electron microscope (FEI UK Ltd., Cambridge, UK). Images were taken on a Gatan Orius charged-coupled device camera (Gatan UK, Oxon, UK). FACS sorting was conducted with assistance from Mrs Fiona Rossi and TEM was performed by Mr Stephen Mitchell.

2.2.1.4. Western immunoblotting

Fresh human PMN were exposed to LL-37 at different concentrations for 20 hours. Cells were washed with PBS without Ca^{2+} and Mg^{2+}, and proteins were extracted using M-PER Mammalian Protein Extraction Reagent (PIERCE/ Perbio Science UK, Cheshire, UK) containing 30 μl/ml Halt^{TM} protease inhibitor cocktail, 30 μl/ml Halt^{TM} phosphatase inhibitor cocktail, 10 μl/ml EDTA solution (all PIERCE/ Perbio Science UK, Cheshire, UK), 30 μM pepstatin (Sigma-Aldrich), and 10 μM lactacystin (Calbiochem/ Merck Biosciences Ltd., Nottingham, UK). Total protein concentrations were determined using a bicinchoninic acid assay (PIERCE/ Perbio Science UK, Cheshire, UK). Equivalent total protein (20-50 μg) was resolved in Pierce Precise gels (PIERCE/ Perbio Science UK, Cheshire, UK), transferred to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA) which were then blocked for 1 hour with 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 (TBST) containing 5% skimmed milk powder (TBST/ milk). Subsequently, the nitrocellulose membranes were incubated with anti-human cleaved caspase-3 rabbit polyclonal antibody and

reprobed with anti-pan-actin (both Cell signaling Technology, Beverly, MA, USA) for protein loading correction via densitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.2.1.5. Burkitt's lymphoma death assessment

Mutu I Burkitt lymphoma (BL) line was cultured in RPMI 1640 medium with 10% FCS. Apoptosis was induced by exposure of cells to ultraviolet B (UVB) radiation (100 mJ/cm^2). Four hours after irradiation, cells were left untreated or exposed to 5 μg/ml LL-37 10 min before being assessed by FACS Calibur using Annexin V FITC and PI.

2.2.2. Phagocytic studies

2.2.2.1. Culture human monocyte-derived macrophages

Human peripheral blood mononuclear cells (PBMC) were isolated by isotonic Percoll gradient centrifugation as described above and incubated at 4×10^6/ml in pre-warmed IMDM followed by plating cells out at 0.5 ml per well in 48-well plates incubating at 37°C, 5% CO_2, for 1 hr to allow monocytes to adhere. Nonadherent cells were then washed out with pre-warmed IMDM; adherent monocytes were cultured for 5-7 days with IMDM and 10% autologous serum to generate MDM.

2.2.2.2. Microscopy-based phagocytosis assay

PMN were incubated previously for 20 hr, with or without LL-37 (or scrambled LL-37) at the stated concentrations, and the level of apoptosis was assessed as described. PMN (2.5×10^6; ~2.5:1 ration of PMN: MDM) were overlaid onto HBSS-washed MDM monolayer for 1 hr at 37°C. MDM were gently washed four times with IMDM to remove noningested PMN and fixed with 2.5% paraformaldehyde (Sigma-Aldrich) for 10 min. PMN MPO was stained with 0.1 mg/ml dimethoxybenzidine (Sigma-Aldrich) and 0.03% (v/v) hydrogen peroxide (Sigma-Aldrich) before analysis by light microscopy, counting at least 200 MDM in five randomly selected fields of view to evaluate the proportion of MDM-containing peroxidase-positive cells. Only MDM that had engulfed PMN clearly were scored as positive.

2.2.2.3. Flow cytometry-based phagocytosis assay

Isolated fresh human PMN were then stained with Cell Tracker Green (Molecular Probes, CMFDA retained in cell cytoplasm) at 37°C for 20 minutes. Labelled PMN were washed twice with PBS and cultured for 20 hr at 37°C in the presence of 10% FCS. Prior to the phagocytotic assay the level of apoptosis were assessed by cytospin. 4×10^6/ml apoptotic PMN in 0.5 ml suspension were then overlayed on HBSS (PAA Labortories)-washed macrophage monolayers. After 1 hr incubation at 37°C, macrophages were detached using trypsin/ EDTA solution (Cambrex). Samples were analysed by FACS Calibur. Apoptotic cells and

macrophage populations were identified by their distinct laser scatter properties. The number of FL-1-positive events in the macrophage gate was divided by the total number of macrophages to obtain the proportion of macrophages that have internalised apoptotic cells.

2.2.2.4. Cytokine production studies

Isolated human blood PMN were incubated at 5×10^6/ml in IMDM and 10% FCS for 20 hr in the appearance or absence of LL-37, scrambled LL-37, or partial peptides p1 (LLGDFFRKSKEKIGKEFKRIVQ), p2 (KIGKEFKRIVQRIKDFLRNLVP), or p3 (EFKRIVQRIKDFLRNLVPRTES) at the concentrations indicated. Samples (1 ml) were collected and each split in two, and half of each sample was centrifuged at 230 xg before collection of supernatant and resuspension of the cells in 0.5 ml X-vivo 10 media (Lonza Biologics, Slough, UK), with or without 10% FCS. Washed MDM, with or without concomitant exposure to 10 ng/ml *E.coli* 0111:B4 LPS or 3 μg/ml rhCD40L/ 5 ng/ml rhIFN-γ, were then incubated at 37°C, 5% CO_2, for 18 hr with 0.5 ml PMN in fresh X-vivo 10, PMN in the original IMDM with 10% FCS, IMDM with 10% FCS supernatant collected from overnight-incubated PMN, or media alone. MDM alone and PMN alone in the presence/ absence of LPS and /or LL-37 were also studied as controls. Supernatants were collected, centrifuged at 230 xg for 6 min to remove cells and particulate debris, and then stored in aliquots at -70°C. The concentrations of TNF-α, IL-6, IL-10, IL-12p70, and IL-1β in the supernatants were measured using BD Cytometric Bead Array human inflammation kits with a BD FACSArray (BD

Biosciences) or Human TNF-α DuoSet ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.2.2.5. MTT cell viability assay

After supernatants were collected for cytokine evaluation, 100 μl X-vivo 10 media and 40 μl MTT working solution (CellTiter 96® Non-Radioactive Cell Proliferation Assay, Promega UK Ltd, Hampshire, UK) were added to the macrophages attached on plates. Macrophages were incubated for another 1 hr at 37°C, 5% CO_2 followed by measurement of optical density by microplate reader at 490 nm.

2.2.2.6. Myeloperoxidase (MPO) release

Isolated PMN were suspended at 5×10^6/ml in IMDM with 10% FCS and incubated with different concentrations of LL-37 or scrambled peptide for 20 or 38 hr, centrifuged at 230 xg for 6 min, and placed immediately on ice, and the supernatants were collected . To detect MPO, 100 μl supernatant samples (used neat or diluted in PBS) were incubated in the dark for 20 min at room temperature with 100 μl substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine; R&D Systems), followed by addition of 50 μl 2N H_2SO_4 stop solution (R&D Systems) and colorimetric analyses at 450 nm using a microplate reader. The concentration of MPO in the supernatants was evaluated as a percentage of MPO

release after complete lysis of fresh control PMN using 0.1% Triton X-100 (Sigma-Aldrich).

2.2.2.7. Anti-endotoxic effects of HNP and HBD3

MDM were concomitantly incubated with 10 ng/ml LPS and 5 μg/ml HBD3 or 5 μg/ml HNP for 18 hr in IMDM in the presence or absence of 10% FCS. Untreated MDM or MDM treated with LPS or peptides alone were also studied as controls. Supernatants were collected and centrifuged at 230 xg for 6 min to remove cells and particulate debris, and then stored in aliquots at -70°C. The concentrations of TNF-α in the supernatants were measured using human TNF-α DuoSet ELISA, according to the manufacturer's instructions.

2.2.3. In vivo studies

2.2.3.1. Mice

Six to eight week old female Balb/c mice (Harlan, UK) were used. All experiments were covered by a Project Licence granted by the Home Office under the Animal (Scientific Procedures) Act 1986. Locally, this licence was approved by the University of Edinburgh Ethical Review Committee.

2.2.3.2. Preparation of 10% (w/v) thioglycollate

Ten gram thioglycollate powder was dissolved in 100 ml PBS. The greenish or brown solution was then autoclaved and left in fridge for at least one week before use.

2.2.3.3. Sterile peritonitis

Mice were intraperitoneally injected with 0.5 ml of 10% thioglycollate to induce sterile peritonitis or 0.5 ml of PBS as controls. Three hours after injection, mice were sacrificed by cervical dislocation and peritoneal lavage was performed. Briefly, peritoneal wall was explored and 5 ml cold PBS was injected into peritoneum with 19 G needles. Peritoneal fluids were then slowly withdrawn and collected in 15 ml tubes on ice. Studies were performed with assistance of Ms Annie Mackellar and Mr Spike Clay.

2.2.3.4. Effects of LL-37 on thioglycollate-induced sterile peritonitis

To evaluate the effects of LL-37 on inflammation *in vivo*, sterile peritonitis was induce in mice followed by the administration of LL-37 peptide or LL-37-mediated secondary necrosis of human PMN. Isolated human blood PMN were incubated at 5×10^6/ml in IMDM and 10% FCS for 20 hr in the appearance or absence of 25

μg/ml LL-37. Samples were then centrifuged at 230 xg before resuspension of the cells at 60×10^6/ml in 100 μl X-vivo 10 media. Mice were injected with 0.5 ml of PBS or 0.5 ml of 10% thioglycollate along with 100 μl X-vivo 10 media, 100 μl human overnight untreated (apoptotic) PMN, 100 μl LL-37-exposed (secondarily necrotic) PMN, or 25 μg/ml LL-37 peptide alone. Three hours after injection, peritoneal lavage was performed. Lavage fluids were assayed by Nucleocounter to determine cell numbers. To determine the PMN influx, cells were immunolabelled with FITC-conjugated anti-mouse Ly6G and assessed by FACS Calibur. The concentration of cytokines in lavage fluids were evaluated by BD Cytometric Bead Array mouse inflammation kits with a BD FACSArray (BD Biosciences). Studies were performed with the assistance of Ms Annie Mackellar and Mr Spike Clay.

2.2.4. Effects of LL-37 on IL-1β release

2.2.4.1. Isolation of CD14 positive monocytes

Freshly isolated PBMC were added with CD14 magnetic microbeads (Miltenyi Biotec, Surrey, UK) with the concentrations according to manufacturer instruction and placed at 4°C in dark for 20 min. Cells then passed through MACS separator in separation LS columns (Miltenyi Biotec, Surrey, UK). CD14-labelled cells retaining in columns were isolated and washed with FACS buffer [PBS with 2% FCS and 5 mM EDTA (Invitrogen Ltd., Paisley, UK)] the purity reaches 96% by FACS check with antibody against CD14.

2.2.4.2. IL-1β release studies

Isolated CD14 positive monocytes or human MDM were incubated at 10^6/ml in IMDM and 10% FCS for 3 hr in the appearance or absence of 10 ng/ml LPS. Then 900 μM oATP, 1 μM KN-62, 0.1 μM AZ-167, or 10 μM caspase-1 inhibitor were added before LL-37 (50 μg/ml), ATP (5 mM), or nigericin (0.5 μM) stimuli. Monocytes were treated with LPS, LL-37, ATP, or nigericin alone, or left untreated as cpntrols. One hr after the second stimulus, supernatants were collected, centrifuged at 230 xg for 6 min to remove cells and particulate debris, and then stored in aliquots at -70°C. The concentrations of IL-1β, pro-IL-1β, TNF-α, LDH in the supernatants were measured using human IL-1β DuoSet ELISA, human Pro-IL-1 beta/IL-1F2 Quantikine ELISA Kit, TNF-α DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) or CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega UK Ltd, Hampshire, UK) according to the manufacturer's instructions.

2.2.4.3. MTT cell viability assay

After supernatants were removed for cytokine measurement, 100 μl X-vivo 10 media and 40 μl MTT working solution were added to monocytes in the plates and incubated for 1 hr at 37°C, 5% CO_2. Optical density was then determined by microplate reader at 490 nm.

2.2.4.4. Western immunoblotting

Isolated CD14 positive monocytes or human MDM were treated with 10 ng/ml LPS for 3 hr and second stimuli for 1 hr or left untreated. Supernatants were collected followed by the addition of M-PER Mammalian Protein Extraction Reagent to extract cell proteins. Total protein concentrations of supernatants and cells were determined using a bicinchoninic acid assay. Equivalent total protein (20-50 μg) was resolved in Invitrogen Bis Tris gels (Invitrogen Ltd, Paisley, UK), transferred to iBlot nitrocellulose membranes (Invitrogen Ltd, Paisley, UK) which were then blocked for 1 hour with 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 (TBST) containing 5% skimmed milk powder (TBST/ milk). Subsequently, the nitrocellulose membranes were incubated with anti-human IL-1β rabbit polyclonal antibody and reprobed with anti-human caspase-1 rabbit polyclonal antibody (both Cell signaling Technology, Beverly, MA, USA).

2.2.5. LL-37 effects on TLR-stimulated hMDM

MDM were treated with TLR1/2 (Pam3CSK4), TLR2 (HKLM), TLR3 (poly I:C LMW), TLR4 (*E. coli K12* LPS), TLR5 (*S. typhimurium* Flagellin), TLR6/2 (FSL1), TLR7 (Imiquimod), TLR8 (ssRNA40) or TLR9 (ODN2006) at the concentrations suggested by the manufacture in the presence or absence of 5 μg/ml LL-37 for 18 hr in IMDM with 10% FCS. Supernatants were collected and evaluated centrifuged at 230 xg for 6 min to remove cells and particulate debris, and then stored in aliquots at -70°C. The concentrations of TNF-α and IP-10 in the

supernatants were measured using human TNF-α DuoSet ELISA and human CXCL10/ IP-10 DuoSet ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.2.6. Statistical Analysis

GraphPad Prism 5 statistical software was used to determine statistical significance. One-way ANOVA, two-way ANOVA, and student's t-tests were performed depending on data, and $p \leq 0.05$ was considered significant. Values shown are expressed as mean + SEM except particular mention.

Chapter 3

Effects of CHDP on PMN death

3. Effects of CHDP on PMN Death

3.1. Introduction

CHDP have been described as potent modulator of cell death pathways in different human primary innate-immune effector cells. The modulation of survival and death of innate-immune effector cells by CHDP could be critical to enhancing the clearance of infection and resolution of inflammation. My studies thus focus on the effects of CHDP, in particular human cathelicidin LL-37, on the cell death of the key first-line defence cell, PMN. I initiated these studies with the hypothesis that LL-37 can modulate the cell death in human PMN, *in vitro* and *in vivo*, to promote inflammation and enhance clearance of pathogens.

3.2. Results

3.2.1. The Percoll gradient protocol is an efficient method to obtain more purified PMN compared to Ficoll isolation

To compare two methods of PMN isolation, separation of fresh human peripheral blood from the same donor was performed simultaneously using two protocols as described in the Materials and Methods. Harvested cells were characterised by microscopic identification and FACS analyses of immuofluorescently labelled cells to determine cell number, purity, and activation status (Table. 3.1). Antibodies against CD66B and CD16b were used to detect granulocytes [486, 487], against CD3 to detect T lymphocytes [488], against CD14

to detect monocytes [489], and against CD19 to identify B lymphocytes [490]. Activation status was determined by the fluorescent intensity of CD18, a component of the leukocyte adhesion molecules (CD11/CD18), and using the expression level of adhesion receptor CD62L (L-selectin), which is shed upon PMN activation [491, 492]. PMN isolated using Ficoll-Hipaque based (method 2) had a higher contamination rate with other cell types, and the total number of cells acquired per ml of blood was less than that prepared using a Percoll-based method (method 1). Isolating PMN by Percoll gradient, which was routinely utilised in our lab, was therefore considered better practice to obtain purified granulocytes. In contrast, method 2 was more technically demanding and resulted in greater PBMC contamination. Monocytes and lymphocytes were visualised on cytospin slides and using FACS antibody analyses, with increased contamination with CD3 (~9%), CD14 (~8%), and CD19 (~8%)-positive cells observed when using Method 2. Neither method significantly activated the PMN as assessed by expression of CD62L and CD18. PMN obtained by the two methodologies were also later examined for their responses to LL-37 (cells were gated in the appropriate region excluding lymphocytes and monocytes), and showed broadly similar patterns in response to LL-37 (data not shown). Thus, the Percoll gradient protocol was chosen to isolate PMN for all future experiments.

Protocol	Cell number/ ml blood	Purity					Activation status	
		CD66B (+)	CD16b (+)	CD3 (+)	CD14 (+)	CD19 (+)	Median CD18	Median CD62L
Method 1	$1.5\times10^{6}\pm7\times10^{5}$	97.62%±0.28%	94.58%±0.44%	1.23%±1.52%	0.05%±0.07%	1.43%±1.94%	46.64	116.28
Method 2	$8.6\times10^{5}\pm8\times10^{4}$	78.05%±2.15%	76.66%±4.61%	8.99%±2.00%	8.11%±0.86%	8.27%±1.22%	41	125.74

Table 3.1 Comparisons of PMN obtained by Method 1 and Method 2. Separation of fresh human peripheral blood from the same donor was performed simultaneously using two protocols as described. Harvested cells were counted by haemocytometer and FACS analyses of immuofluorescently labeled cells to determine cell number, purity, and activation status. Data indicate mean values ± SEM for using 3 different donors.

3.2.2. Human cathelicidin LL-37 decreases PMN apoptosis and increases PMN necrosis after 20 hr incubation

Our lab and others [20, 21] have demonstrated that the proportion of apoptotic (AV^+PI^-) PMN after 20 hr incubation *in vitro* was decreased significantly when cells were cultured with LL-37, in a dose-dependent manner. The most prominent consequence of this was an increased proportion of necrotic (AV^+PI^+) PMN. To clarify the effect of LL-37 on PMN death, a large donor pool (n = 28) was evaluated. Fresh human peripheral blood PMN were isolated and incubated in culture over a range of LL-37 concentrations in the presence of 10% FCS. The level of apoptosis was assessed by FACS Calibur quantification of FITC-labelled annexin V and propidium iodide staining (Fig. 3.1). Confirmation of apoptosis was obtained by morphological evaluation of cytospins (Fig. 3.2) and gave comparable results. Cell exposed to LL-37 were demonstrated to have lower apoptotic rates at 20 hr (Fig. 3.3A). The decrease was dose dependent, with significant decreases at concentrations of 1 μg/ml or greater ($p \leq 0.05$). In parallel to the reduction of PMN apoptosis, exposing PMN to LL-37 also produced a significant increase in the percentage of cells becoming necrotic, especially at 5 μg/ml, up to 40% (Fig. 3.3B). Viable cell numbers, unexpectedly, did not change significantly over a range of LL-37 (Fig. 3.3C). This contrasted with previous observations in the laboratory [20]. This discrepancy likely relates to artifacts from loss of cells to detection at the higher concentrations of LL-37 used in the earlier study, the size of donor pool, and the statistical methodology employed (ANOVA vs. t-test). LL-37-induced

necrotic PMN were observed to be similar to “late apoptotic” cells observed in control samples at 20 hr, with less severe loss of membrane integrity than primary necrosis induced by 65°C heat treatment for 30 min (Fig. 3.1D). Transmission Electron Microscopy was used to confirm the capacity of LL-37 to promote PMN necrosis after 20 hr incubation when compared with control cells (Fig. 3.4). Untreated control PMN showed typical apoptotic morphology with condensed chromatin and intact cell membrane (Fig. 3.4B), while PMN exposed to LL-37 for 20 hr revealed broken membrane and leakage of intracellular contents (Fig. 3.4C). Taken together, these data suggested that human cathelicidin LL-37 could modulate PMN death, decreasing apoptosis and increasing necrosis.

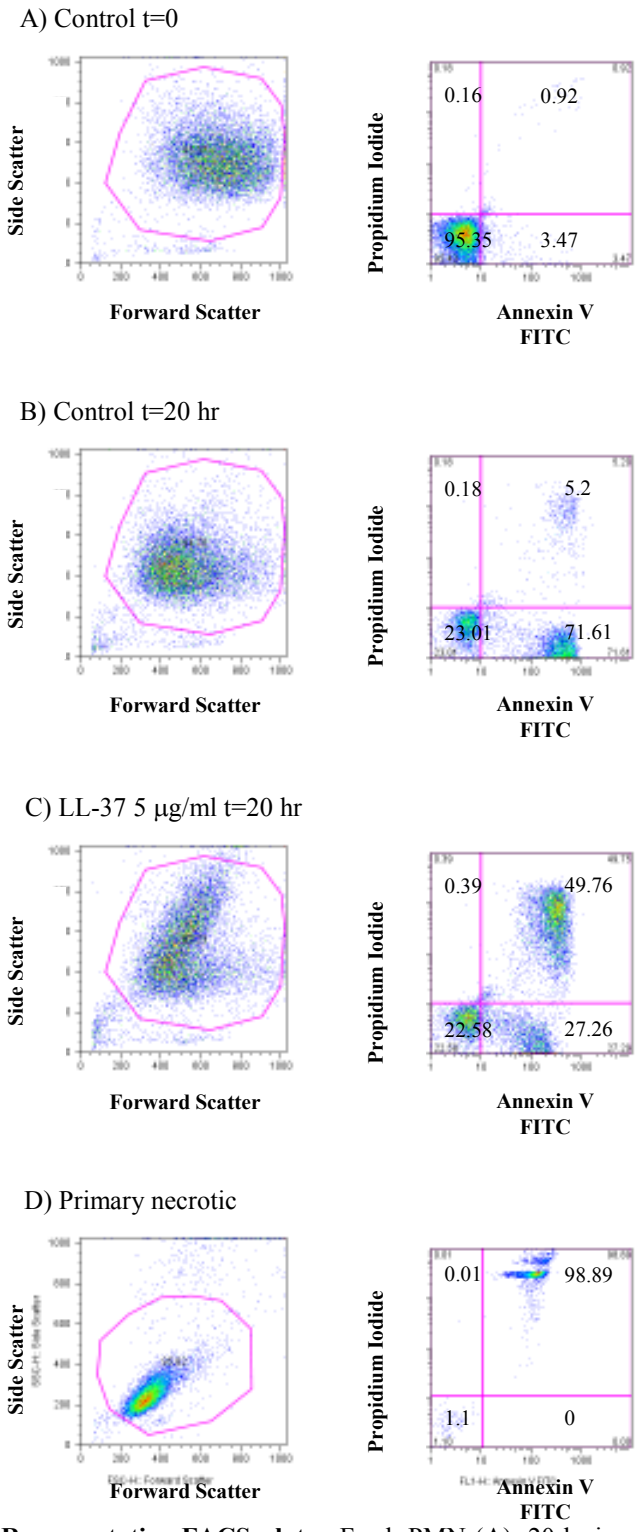
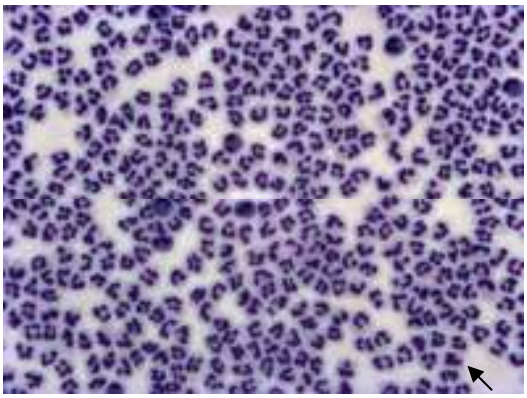
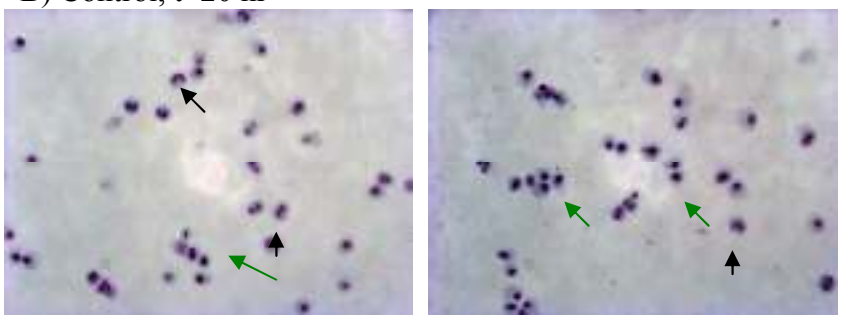


Figure 3.1 Representative FACS plots. Fresh PMN (A), 20 hr incubated PMN without or with LL-37 exposure (B & C), and heat-treated PMN (D) were analysed by FACS quantification of annexin V FITC and propidium iodide staining. Apoptotic (Bottom-right; AV-FITC-positive, PI-negative), necrotic (Top-right; AV-FITC-positive, PI-positive), and live (Bottom-left; AV-FITC-negative, PI-negative).

A) Control t=0



B) Control, t=20 hr



C) LL-37 5 µg/ml, t=20 hr

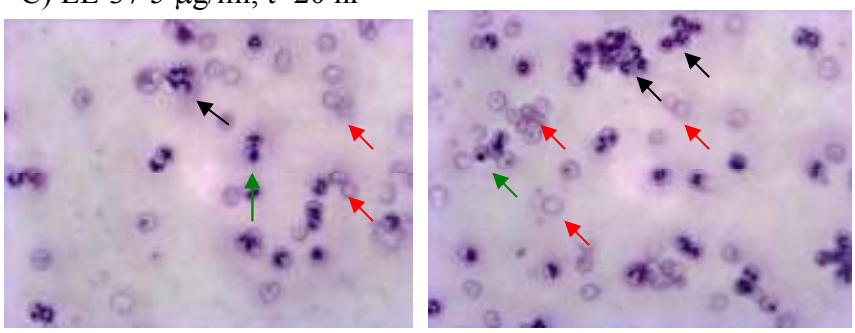


Figure 3.2 Representative cytospin images. (A) fresh PMN, (B) untreated overnight PMN, and (C) PMN exposed to 5 µg/ml LL-37 for 20 hr. Representative live PMN are indicated with black arrows; apoptosis is determined by morphological characteristics such as cell shrinkage, condensation of chromatin, cytoplasmic vacuole formation, and loss of the multilobed shape of their nucleus and is indicated with green arrows; necrotic PMN are indicated with red arrows.

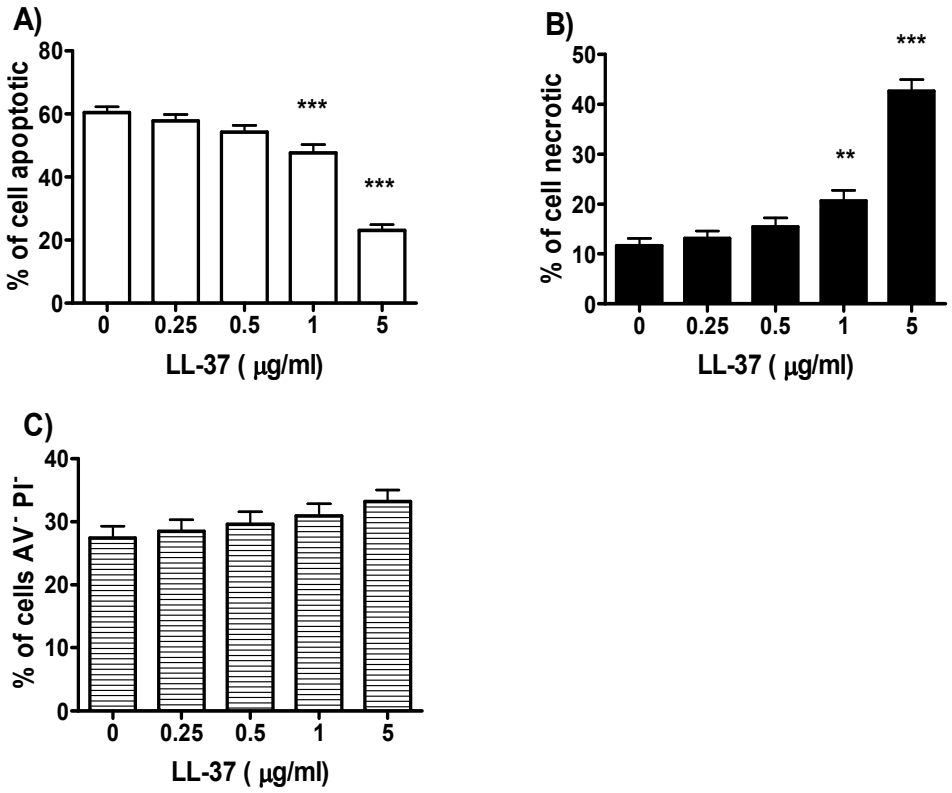
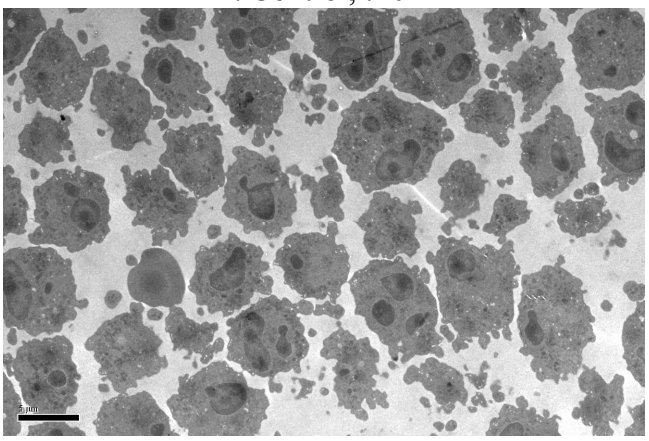
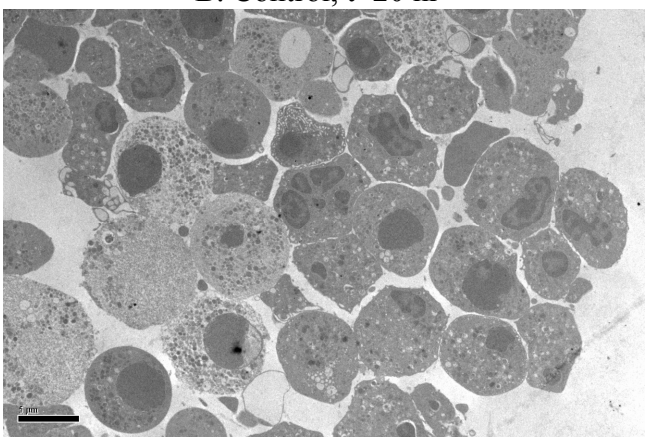


Figure 3.3 LL-37 decreases PMN apoptosis and increases PMN necrosis after 20 hr incubation. PMN were incubated for 20 hr over a range of LL-37 concentrations in the presence of 10% FBS. Modulation of apoptosis and necrosis were examined by FACS analysis in triplicate. FACS analysis was used to determine the percentage of PMN, which were (A) apoptotic, (B) necrotic, and (C) viable. Figures indicate mean values + SEM for n = 28 for each condition using 28 different donors. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test to compare controls with samples exposed to LL-37 of different concentrations. **, $p \leq 0.01$; ***, $p \leq 0.001$.

A. Control, t=0



B. Control, t=20 hr



C. LL-37 5 μg/ml, t=20 hr

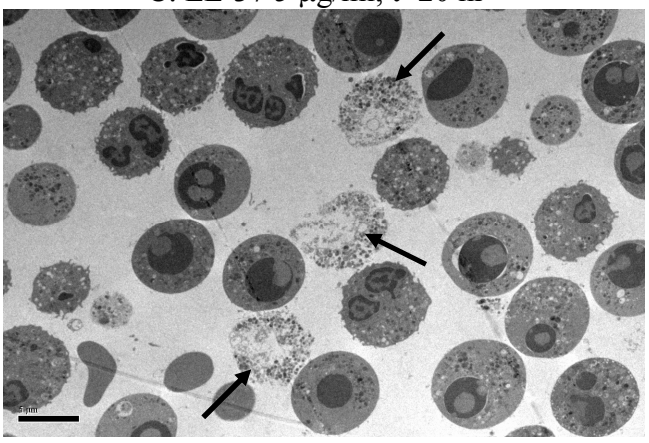


Figure 3.4 Representative TEM images of (A) fresh PMN, (B) untreated, apoptotic PMN, and (C) PMN exposed to 5 μg/ml LL-37 for 20 hr. Arrows indicate examples of necrotic PMN.

3.2.3. Higher concentrations of LL-37 do not significantly promote PMN viability

Previous data showed that after 20 hr exposure to higher concentrations of LL-37, slightly higher proportions of viable PMN were observed. Isolated fresh human peripheral blood PMN were incubated in culture with LL-37 up to 25 μg/ml in the presence of 10% FCS. The level of apoptosis was assessed by FACS Calibur quantification of FITC-labeled annexin V and propidium iodide staining (Fig. 3.5). A decrease in apoptosis and increase in necrosis were observed as expected. Although a trend towards an increased proportion of viable cells was observed at higher LL-37 concentrations, this was not statistically significant. We hypothesised that increased necrosis at high concentrations of LL-37 may result in a complete loss of intact cells from analysis, thus skewing results. To evaluate this accurately, total cell numbers were determined using a NucleoCounter YC-100 (ChemoMetec) after 20 hr culture over a range of LL-37 concentrations (≤ 25 μg/ml). LL-37 ≥ 10 μg/ml (~2 μM) was found to induce a significant and concentration-dependent loss in total detectable cell number ($p<0.01$) (Fig. 3.6), suggesting that the apparent increase in viable cells at higher concentrations of LL-37 may be an artifact. In contrast, incubation with ≤ 5 μg/ml LL-37 did not result in a significant decrease, although a degree of cell loss was observed sometimes. LL-37 was therefore used at ≤ 5 μg/ml for further studies examining LL-37-mediated modulation of PMN cell death pathways.

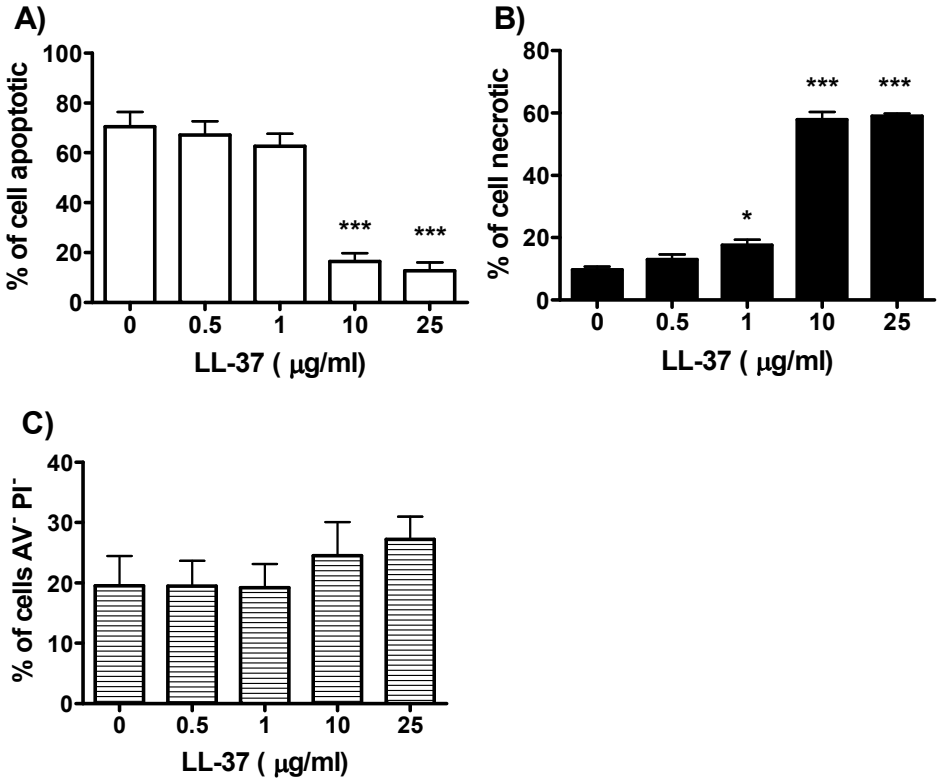


Figure 3.5 LL-37 at higher concentrations (> 10 μg/ml) does not significantly increase the proportion of viable PMN detected. PMN were incubated for 20 hr over a range of LL-37 concentrations in the presence of 10% FBS. The proportions of apoptosis, necrosis, and viable cells were examined by FACS analysis in triplicate. (A-C) FACS analysis was used to determine the percentage of PMN, which were (A) apoptotic, (B) necrotic, and (C) viable. Figures indicate mean values + SEM for n = 3 for each condition using 3 different donors. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing controls with samples exposed to LL-37 of different concentrations. *, $p \leq 0.05$; ***, $p \leq 0.001$.

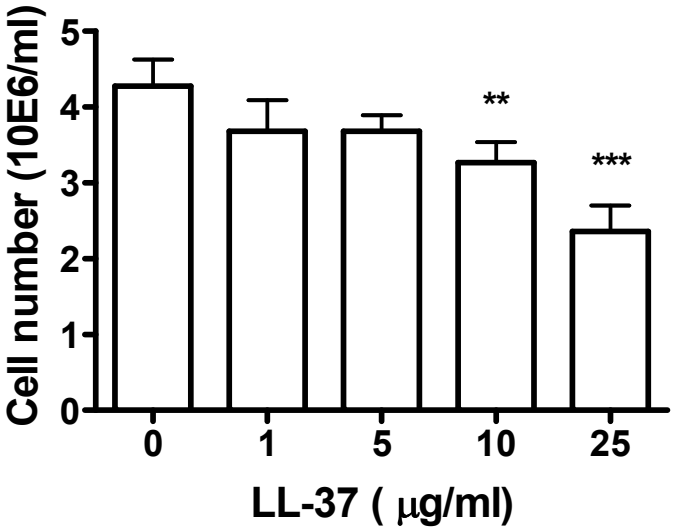


Figure 3.6 Higher concentrations of LL-37 cause loss of detectable PMN after 20 hr incubation. PMN were incubated for 20 hr over a range of LL-37 concentrations in the presence of 10% FBS. Total cell numbers were assessed by nucleocounter. Figures indicate mean values + SEM for $n \geq 11$. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test to compare controls with samples exposed to LL-37 of different concentrations. **, $p \leq 0.01$; ***, $p \leq 0.001$.

3.2.4. The proportion of eosinophils in the blood preparation does not affect the interpretation of LL-37 effects on granulocyte death

The method I used to isolate neutrophils has great efficiency to separate PMN and PBMC, but cannot deplete eosinophils from the PMN population. Eosinophils have longer half life than that of neutrophils. They also have different apoptotic pathways from neutrophils [493, 494], and anti-inflammatory agents such as glucocorticoids can have opposing effects on the rate of apoptosis in neutrophilic and eosinophilic granulocytes [495]. I therefore evaluated whether the proportion of eosinophils in blood preparations affected the ability of cathelicidins to modulate death of the purified granulocytes. The proportion of eosinophils was assessed by cytospins to allocate donors into three groups, namely, eosinophil <5%, eosinophil 5-10%, and eosinophil ≥10% (Fig. 3.7). Twenty-eight different donors were recruited in this study (11 for eosinophil <5%, 11 for 5%-10%, and 6 for eosinophil ≥ 10%). The effect of LL-37 upon cell death was consistent in all groups. Statistical analyses revealed that the number of eosinophils in the granulocyte preparation did not significantly alter the numbers of apoptotic and necrotic cells after LL-37 exposure. Thus, although a variable number of eosinophils is present in the blood granulocyte preparations, these do not seem to alter the effects of LL-37, and this granulocyte preparation will hereafter be referred to as PMN.

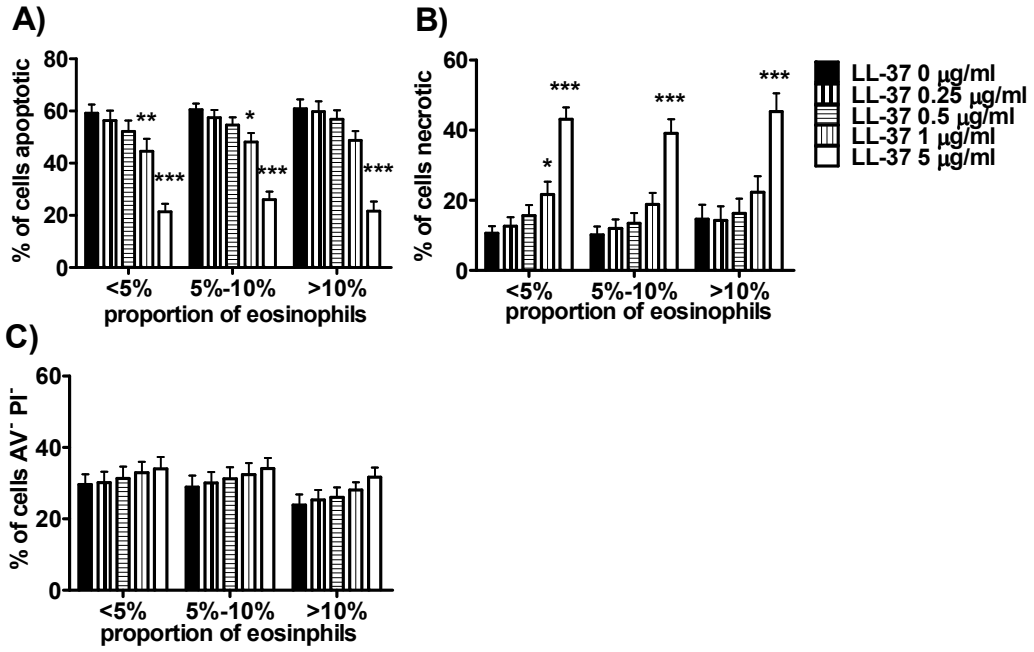


Figure 3.7 The proportion of eosinophils in the blood preparation does not affect the interpretation of the effects of LL-37 on PMN death pathways. Fresh human peripheral blood granulocytes were isolated and 200 cells were counted on cytospins to determine the proportion of eosinophils. Donors were allocated into three groups, eosinophil < 5%, eosinophil 5% - 10%, and eosinophil ≥ 10%. PMNs were incubated in culture over a range of LL-37 concentrations (0 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, and 5 µg/ml) in the presence of 10% FCS for 20 hr. Apoptotic, necrotic, and viable percentages were examined by FACS analysis in triplicate. (A-C) FACS analysis was used to determine the percentage of PMN, which were (A) apoptotic, (B) necrotic, and (C) viable cells. Figures indicate mean values + SEM for n = 11 in eosinophil < 5% group, n= 11 in eosinophil 5-10% group, and n = 6 in eosinophil ≥ 10% group using 28 different donors. Significance was assessed by two-way ANOVA with Bonferroni's multiple comparison test to compare samples exposed to LL-37 over a range of concentrations and the effects of proportion of eosinophils on PMN responses. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

3.2.5. Time course studies show that LL-37 is not lytic to PMN and does not induce death of PMN at early time points

Studies showed that LL-37 is cytotoxic to peripheral blood leukocytes at higher concentrations (> 50 μg/ml) [25]. Although the concentrations of LL-37 used in my experiments were far lower than that having lytic activity, experiments were still conducted to determine any direct cell lysis in this system. Fresh human peripheral blood PMN were isolated and incubated in culture over a range of LL-37 concentrations for 0 hr, 1 hr, 4 hr, 6 hr, 12 hr and 20 hr (Fig. 3.8). The proportions of apoptotic, necrotic, and viable cells were assessed by microscopic observations and FACS Calibur analyses as previously described. Flow analyses and light microscopic observation demonstrated that at early time points, (0 hr, 1 hr, 4 hr, and 6 hr), no substantial cell death was detected irrespective of LL-37 concentrations, indicating that LL-37 was not lytic nor inducing primary necrosis at these time points and concentrations. Apoptotic and necrotic percentages remained steady even after 6 hr at 5 μg/ml. The LL-37-mediated decrease in apoptosis and increase in necrosis were observed to occur simultaneously after 12 hr incubation, by which time a substantial proportion of PMN had undergone spontaneous apoptosis. This suggests that prior induction of apoptosis may be necessary and that LL-37 might induce secondary necrosis.

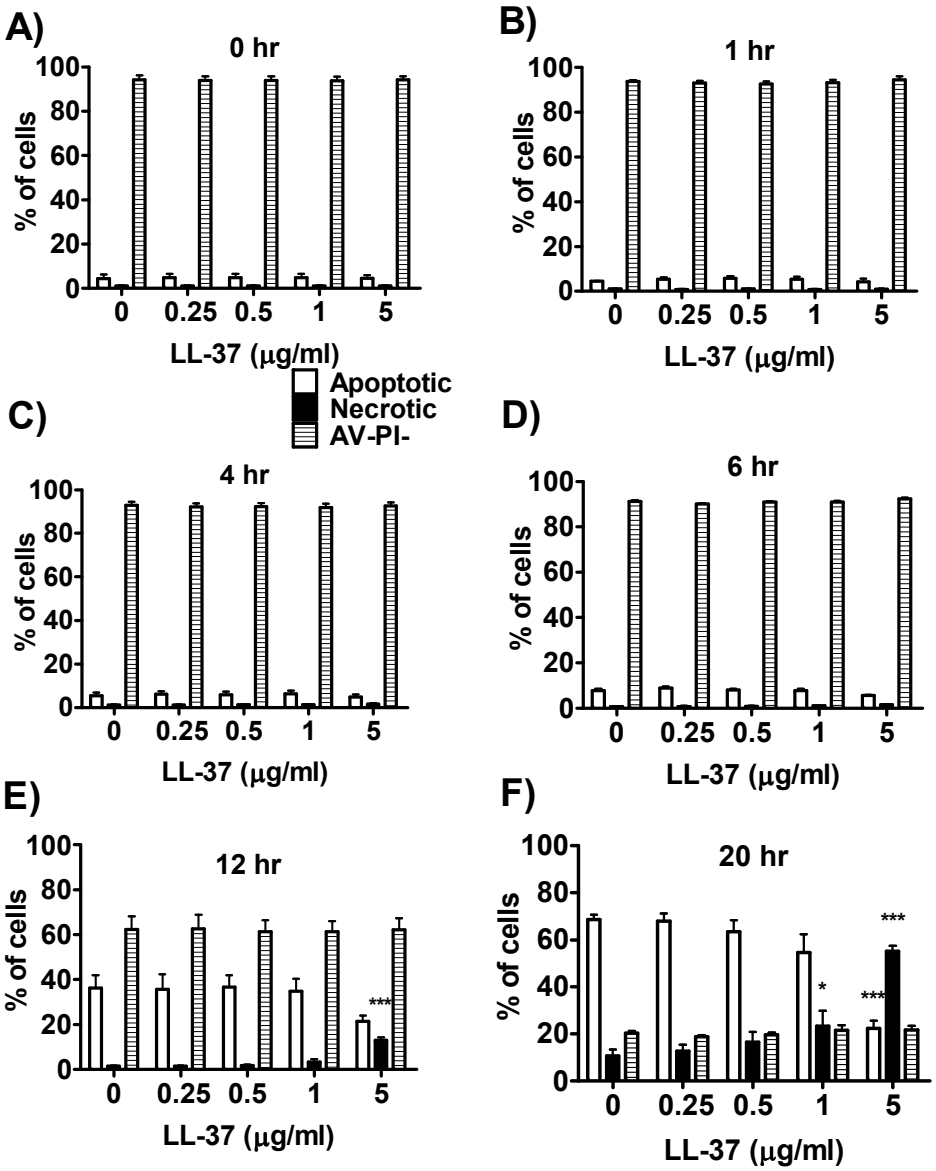


Figure 3.8 Time course studies show that LL-37 at physiological concentrations does not have cytotoxic properties at early time points. Fresh human peripheral blood PMN were isolated and incubated in culture over a range of LL-37 concentrations in the presence of 10% FBS for 0 hr, 1 hr, 4 hr, 6 hr, 12 hr, and 20 hr. (A-F) FACS analysis was used to determine the percentage of apoptotic, necrotic, and viable cells. Figures indicate mean values + SEM for n = 3 for each condition in triplicate. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test to compare control with samples exposed to LL-37 over a range of concentrations. *, $p \leq 0.05$; ***, $p \leq 0.001$.

3.2.6. Pulse treatments demonstrate that LL-37-mediated modulation of PMN death occurs rapidly at later time points

Time course studies indicated that the death-modulating effects of LL-37 on PMN were observed at later time points, once PMN apoptosis had occurred. Thus I hypothesised that LL-37 induced secondary necrosis of apoptotic cells. In order to evaluate this, untreated PMN cultured overnight (20 hr) were exposed to LL-37 over a range of concentrations for just 10 min before flow cytometric analyses. The LL-37-mediated increase in necrosis, and reduction in apoptosis, were found to be even more pronounced in these pulse-treated PMN compared to overnight LL-37-exposed cells, particularly in terms of necrotic rates ($p = 0.02$) (Fig. 3.9). In contrast, PMN exposed to a range concentrations of LL-37 for 4 hr, then washed before a 16 hr incubation period in control media, were not significantly different when compared to untreated controls (Fig. 3.10). Thus, it is concluded that LL-37 induces secondary necrosis at later time points once PMN apoptosis has occurred, but does not modulate cell death at earlier time points.

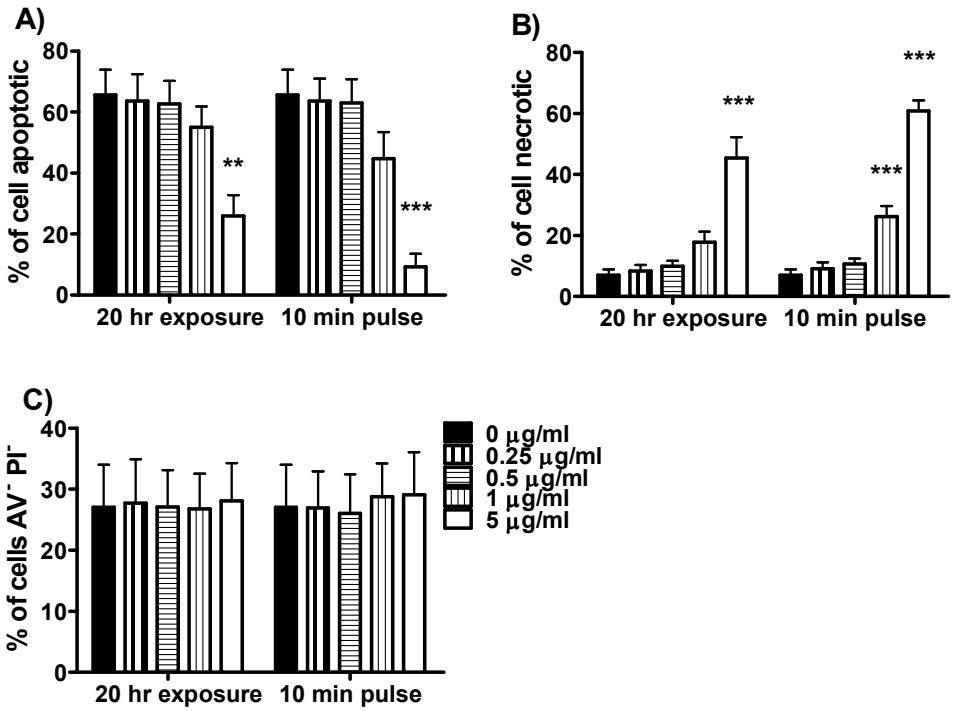


Figure 3.9 Pulse treatments reveal that LL-37-modified PMN death pathways occur swiftly at late time points. Fresh human peripheral blood PMN were isolated and incubated with LL-37 over a range of concentrations (20 hr exposure) or without LL-37 in the presence of 10% FCS for 20 hr. The overnight untreated PMN were then given LL-37 10 min before flow cytometry analyses (10 min pulse). (A-C) FACS analysis was used to determine the percentage of apoptotic, necrotic, and viable cells. Figures indicate mean values + SEM for n = 3 for each condition in triplicate. Significance was assessed by two-way ANOVA with Bonferroni's multiple comparison test to compare control with samples exposed to LL-37 over a range of concentrations and compare the effects of overnight LL-37 and those of pulse LL-37. **, $p \leq 0.01$, ***, $p \leq 0.001$.

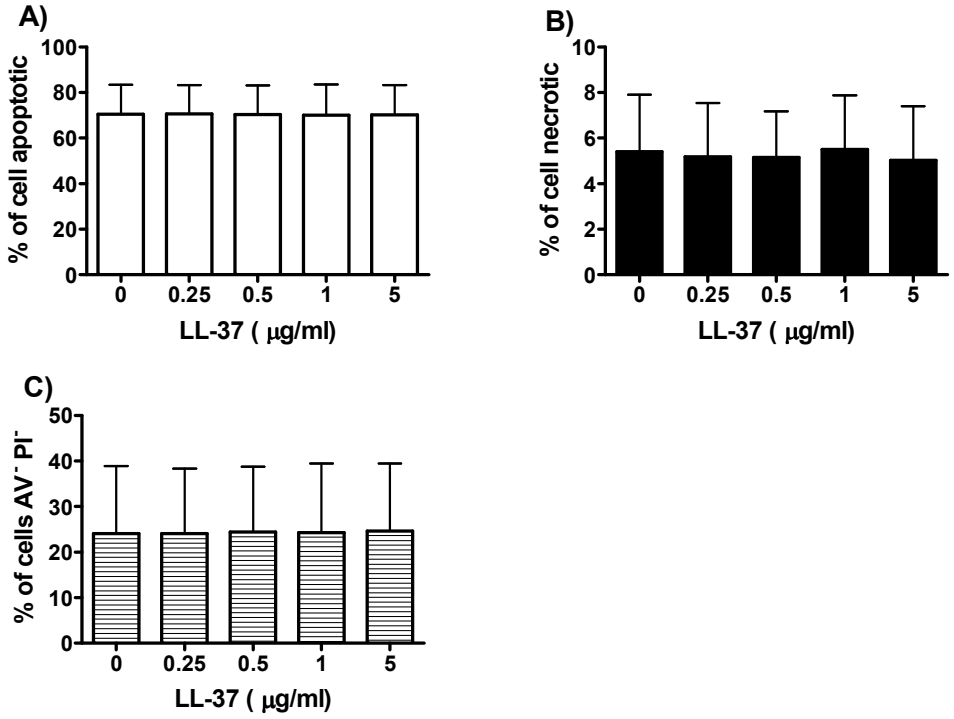


Figure 3.10 Early pulse treatments of LL-37 removed before induction of apoptosis have no significant effects on PMN death. Fresh human peripheral blood PMN were isolated and incubated with LL-37 over a range of concentrations or without LL-37 in the presence of 10% FCS for 4 hr, followed by washing and incubation in control media for 16 hr. (A-C) FACS analysis was used to determine the percentage of apoptotic, necrotic, and viable cells. Figures indicate mean values + SEM for n = 3 for each condition in triplicate. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test to compare control with samples exposed to LL-37 over a range of concentrations.

3.2.7. Critical proteins involved in the caspase cascade are unchanged in human PMN after LL-37 exposure, suggesting the induction of secondary necrosis, rather than decreased apoptosis with consequent primary necrosis

Time course and short time pulse studies suggested that LL-37-mediated alteration of PMN death occurred at late time points. The increase of necrosis was always observed when a proportion of PMN had undergone spontaneous apoptosis. This phenomenon was more pronounced at very late stages when more apoptotic cells were exposed to fresh LL-37, and exposure to LL-37 after the induction of apoptosis was required. To examine whether the decrease of apoptosis resulted from the anti-apoptotic activity of LL-37 on PMN as previously described [21], Western immunoblotting for critical proteins involved in the induction of apoptosis via the caspase cascade was performed. If LL-37 was anti-apoptotic in PMN, there would be expected to be fewer cells at 20 hr with cleaved caspase-3; if LL-37 induces secondary necrosis of apoptotic PMN, caspase-3 at late time should be unaffected. Isolated PMN were cultured with LL-37 for 20 hr. Proteins were extracted from incubated PMN using M-PER with proteinase inhibitor reagents. PMN proteins were subjected to Western immunoblotting to study the expression of key proteins involved in apoptotic caspase cascade. Results showed that 20 hr LL-37 exposure did not change the level of cleaved caspase-3, an executioner caspase which is a major convergence point of intrinsic and extrinsic apoptotic pathways (Fig. 3.11). This result further supported the hypothesis that rather than

mediating anti-apoptotic effects, LL-37 induced already apoptotic cells to become secondarily necrotic.

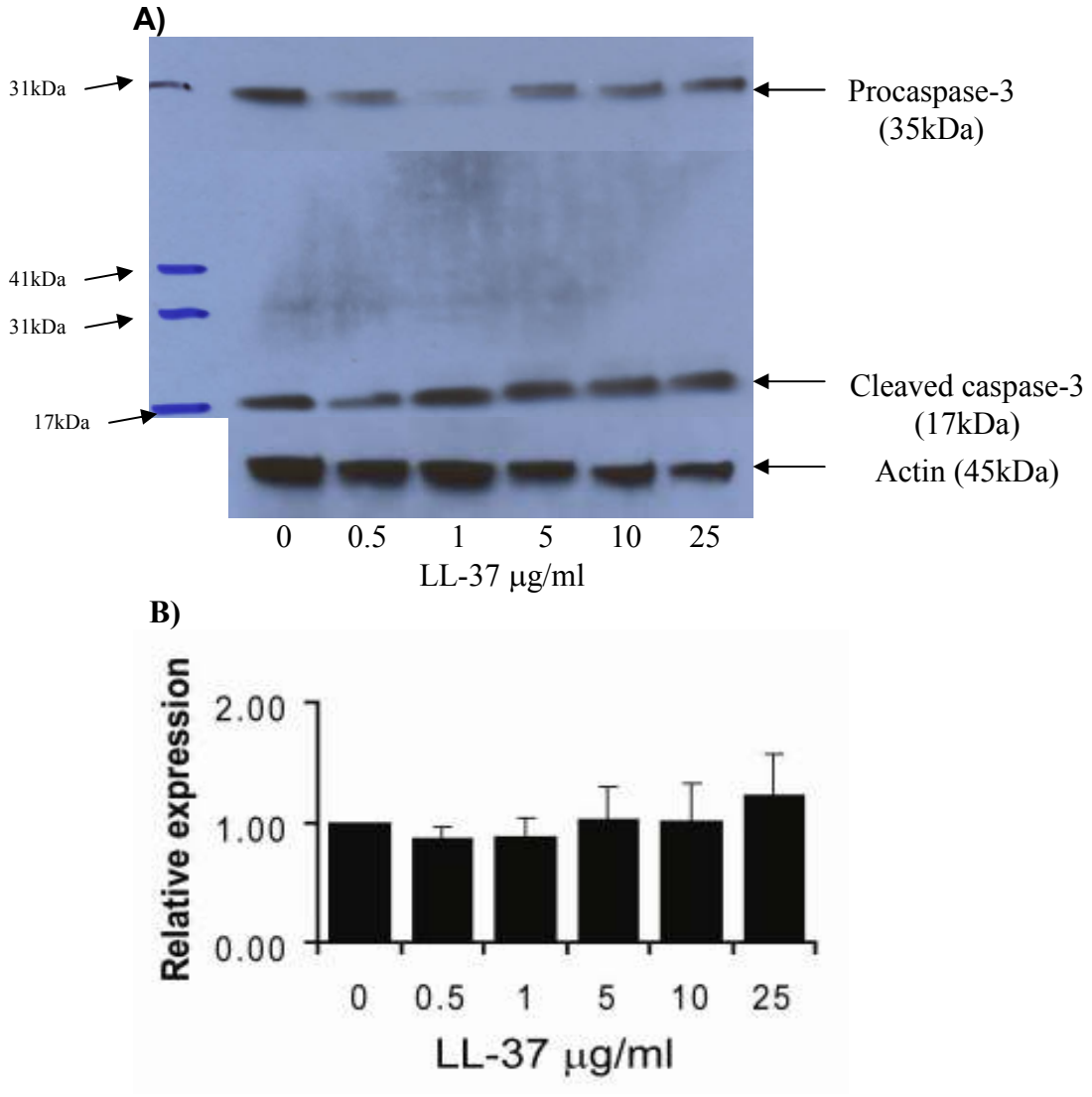


Figure 3.11 Western immunoblot for cleaved caspase-3. Whole cell lysates from freshly isolated human PMN incubated for 20 hr were examined by Western immunoblotting for procaspase-3 (antibody dilute 1:1000), cleaved caspase-3 (antibody dilute 1:1000), which are the critical proteins executing apoptosis, and pan-actin (antibody dilute 1:2000) for protein loading control. A) Representative image; B) relative quantity compared to control samples (after correction for protein loading), mean + SEM, n = 4.

3.2.8. In serum-free conditions, overnight LL-37-mediated secondary necrosis of PMN is attenuated, but the effects of pulse treatment are enhanced.

Serum proteins have been shown to play a pivotal role in inhibiting microbicidal and cytotoxic activities of LL-37 [93], and are required for LL-37-induced activation of the MAPKs, ERK1/2, and p38 [143]. To study whether varied serum conditions affected the function of LL-37 to modulate PMN death pathways, cells were incubated in serum-free medium in the presence or absence of a concentration range of LL-37 for 20 hr. Baseline apoptotic and necrotic rates were higher in the absence of serum than previously observed in the presence of 10% FCS, accompanied by less viable cells. LL-37-mediated induction of secondary necrosis was largely attenuated in overnight serum-free conditions (Fig. 3.12). In addition, to evaluate whether apoptotic PMN were differentially susceptible to LL-37-mediated induction of secondary necrosis, cells were cultured for 20 hr in LL-37-free, serum-free media before being exposed to a pulse of LL-37 for the final 10 min before analyses. Under these conditions LL-37-mediated secondary necrosis with a concomitant decrease in apoptosis, was observed in pulse LL-37-exposed PMN in the absence of serum (Fig. 3.12 A & B). These data suggest that LL-37-induced secondary necrosis is not a serum-dependent phenomenon, but that under serum-free conditions LL-37 added at the start of a 20 hr incubation period is not available to induce secondary necrosis by the time spontaneous apoptosis occurs.

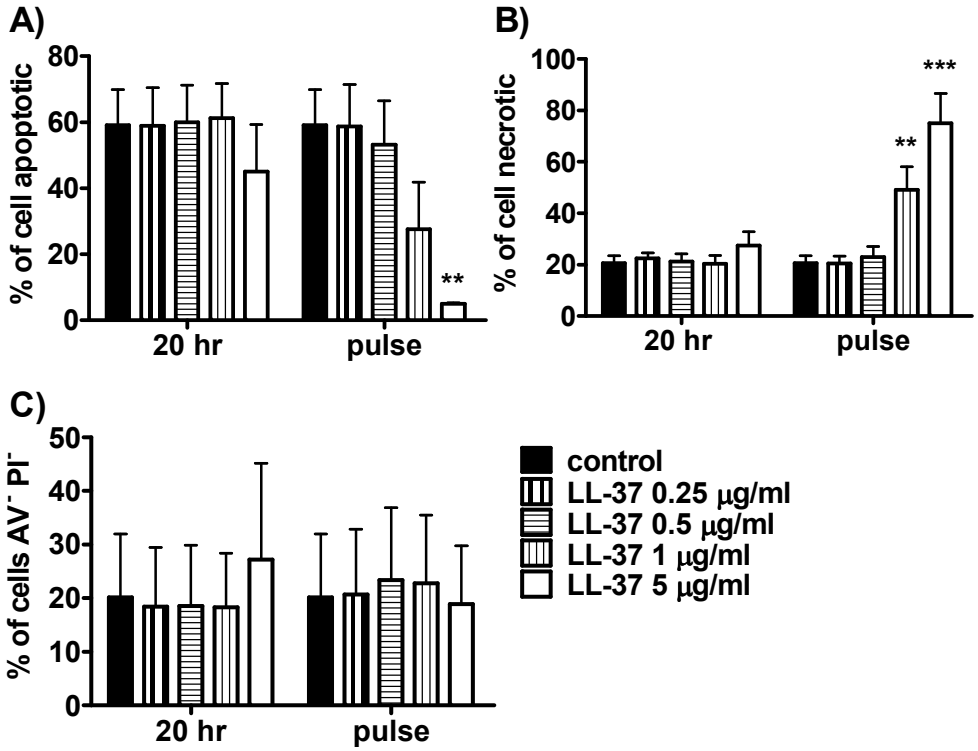


Figure 3.12 I**n serum-free conditions, overnight LL-37-mediated modulation of PMN death is attenuated, but the effects are enhanced following pulse treatment.** Fresh human peripheral blood PMN were isolated and incubated in the absence of 10% FCS in the absence and presence of a range concentration of LL-37 for 20 hr, or cells in LL-37-free conditions overnight were given a range concentrations of LL-37 10 min before analyses. (A-C) FACS analysis was performed in triplicate to determine the percentage of apoptotic, necrotic, and viable cells. Figures indicate mean values + SEM for n = 3 for each condition using three different donors. Significance was assessed by two-way ANOVA with Bonferroni's multiple comparison test to compare samples in 20 hr LL-37 treatment with those of LL-37 pulse exposure and their responses to a range concentration of LL-37. **, $p \leq 0.01$, ***, $p \leq 0.001$.

3.2.9. Interaction of LL-37 with known modulators of PMN apoptosis

To investigate whether LL-37 could interact with other modifiers of PMN apoptotic pathways, cells were incubated with LL-37 in the presence or absence of GM-CSF (a PMN survival factor [496]) or *R*-Roscovitine (a cyclin-dependent kinase inhibitor and inducer of PMN apoptosis [268]). GM-CSF reduced PMN apoptosis significantly at 20 hr, increasing the proportion of live cells without affecting necrosis. Concomitant treatment with 5 μg/ml LL-37 had no effect on the capacity of GM-CSF to promote cell survival but significantly ($p \leq 0.05$) increased the proportion of necrotic cells with a reciprocal loss of apoptotic cells when compared with GM-CSF alone (Fig. 3.13A). *R*-Roscovitine induced PMN apoptosis significantly at 6 hr with minimal necrosis. LL-37 alone (≤ 25 μg/ml) had no effect on cell death at this time point. Concomitant LL-37 treatment had no effect on the capacity of *R*-Roscovitine to induce cell death but significantly ($p \leq 0.01$) increased the proportion of necrotic cells in a dose-dependent manner with a reciprocal loss of apoptotic cells when compared with *R*-Roscovitine alone (Fig. 3.13B). These data demonstrated no interaction with these direct modifiers of PMN apoptosis, but LL-37 induced apoptotic PMN secondary necrosis, relating in magnitude to the extent of induction of apoptosis.

In addition, studies were performed to assess the impact of LL-37 (a well-characterised anti-endotoxic agent [89]) on the anti-apoptotic effects of LPS in PMN [497]. LPS promoted PMN survival significantly (Fig. 3.13D), inhibiting

apoptosis without increasing necrosis. Concomitant incubation with LL-37 inhibited this effect significantly in a dose-dependent manner (Fig. 3.13D). LL-37 (5 μg/ml) blocked the pro-survival effects of LPS completely and significantly increased the proportion of dead cells that were necrotic ($p \leq 0.01$) (Fig. 3.13C). However, the capacity of LL-37 to induce secondary necrosis was diminished significantly by LPS ($p \leq 0.001$) (Fig. 3.13C). These data demonstrate LL-37-mediated inhibition of the anti-apoptotic effects of LPS and suggest a mutually inhibitory interaction with LPS diminishing LL-37-induced secondary necrosis.

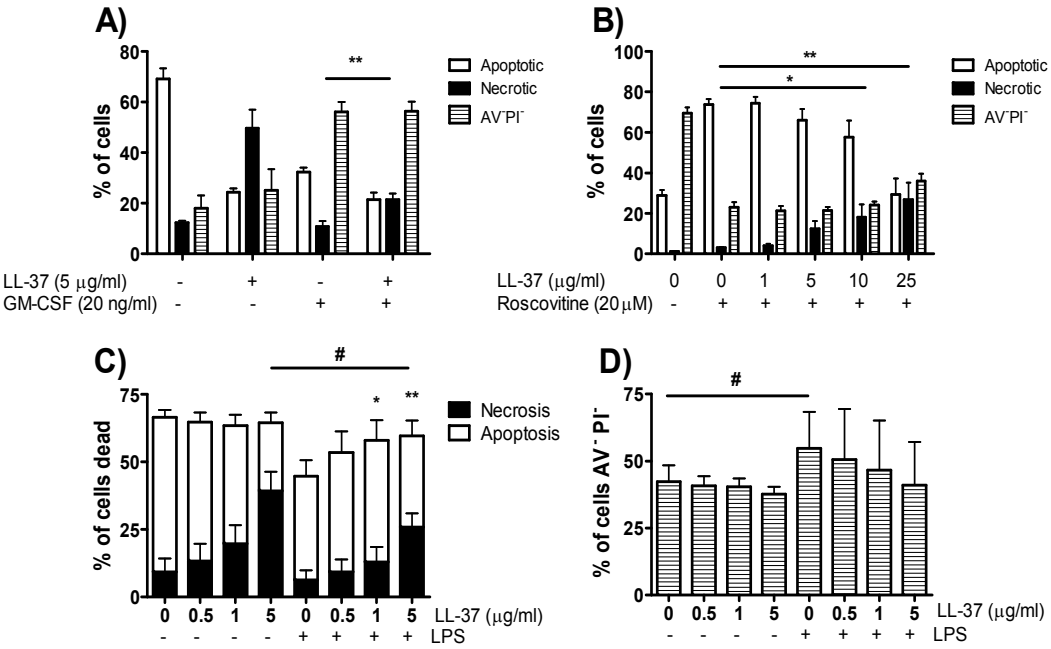


Figure 3.13 LL-37 exposure of PMN in the presence of modifiers of apoptotic pathways. Freshly isolated human PMN were incubated with LL-37 (at the concentrations indicated) for 20 hr in the presence or absence of 20 ng/ml GM-CSF (A), for 6 hr in the presence or absence of 20 μM *R*-Roscovitine (B), or for 20 hr in the presence or absence of 500 ng/ml *E.coli* LPS (C and D). Cell death was examined by FACS analyses. Figures represent mean values + SEM for $n \geq 3$ donors performed in triplicate. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control; *, $p \leq 0.05$; **, $p \leq 0.01$, for necrotic cells in LL-37-treated samples compared with controls without LL-37 in the presence of (A) GM-CSF, (B) *R*-Roscovitine, or (C) LPS; #, $p \leq 0.001$, for necrotic (C) or viable (D) cells in LPS-treated samples compared with controls without LPS in the comparable concentrations of LL-37.

3.2.10. The effects of LL-37 on apoptotic PMN are not inhibited by the presence of Annexin V

My data thus far suggested possible differences in apoptotic membranes, when compared to those of viable cells, enabled induction of membrane permeabilisation by LL-37. One clearly described change in apoptotic membrane is the externalisation of PS, which is usually resticted to the inner leaflet of cell membranes. In order to determine whether LL-37 may interact with PS, Annexin V, which has a high affinity to PS in the presence of physiological concentrations of calcium, was used to try to block such an interaction. Overnight untreated PMN were prepared to generate apoptotic PMN. These cells were then resuspended in HBSS, Annexin V buffer (which contained 5mM $CaCl_2$) or Annexin V buffer containing Annexin V (diluated 1:2000), before a 10 min exposure pulse treatment of LL-37. Despite a trend towards diminished secondary necrosis, neither buffer containing high divalent ions nor Annexin V stain significantly inhibited the capacity of LL-37 to induce the secondary necrosis of apoptotic PMN (Fig. 3.14). The minor effects on the proportion of apoptotic cells observed appeared to be dependent upon the presence of higher concentrations of Ca^{2+}, rather than inhibition by Annexin V. Although not conclusive, this experiment suggests that induction of secondary necrosis by LL-37 is not primarily due to a direct interaction between the peptide and PS.

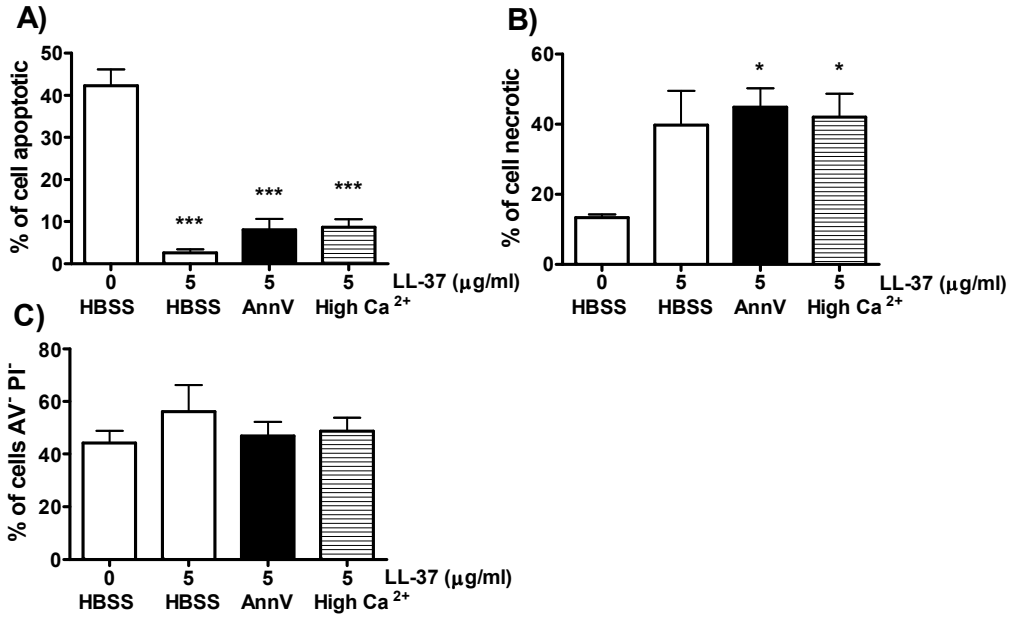


Figure 3.14 The effect of LL-37 on apoptotic PMN is not inhibited by the presence of Annexin V. Fresh human peripheral blood PMN were isolated and incubated without LL-37 in the presence of 10% FCS for 20 hr. The overnight untreated PMN were then resuspended in HBSS, in the absence or presence of 5 mM $CaCl_2$ (High Ca^{2+} FACS buffer) or FACS buffer containing Annexin V FITC (dilute 1:2000 as for FACS staining) just before a 10 min exposure to pulse LL-37 and flow cytometric analyses. (A-C) FACS analysis was used to determine the percentage of apoptotic, necrotic, and viable cells. Figures indicate mean values + SEM for n = 3 for each condition in triplicate. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test to compare control with samples exposed to LL-37 in different media conditions. *, $p \leq 0.05$; ***, $p \leq 0.001$.

3.2.11. G-protein-coupled receptor and $P2X_7$ receptor antagonists do not inhibit the capacity of LL-37 to induce secondary necrosis of PMN

The modulatory effects of LL-37 on cell death have been proposed to be dependent on signalling through G-protein-coupled receptor FPRL-1 and the purinergic receptor $P2X_7R$ [20, 21]. In order to clarify if LL-37-induced secondary necrosis was a receptor-dependent or a membrane-perturbing event, antagonists of these known receptors were used. Fresh PMN were incubated with PTX (pertussis toxin, to inhibit G-protein-coupled receptors), WRW4 (specific inhibitor of FPRL-1 [498]), oATP (oxidised ATP) or AZ-167 (specific inhibitors of $P2X_7$ [499]) 30 min before exposure to a concentration range of LL-37. The levels of cell death were subsequently determined after 20 hr incubation (Fig. 3.15). The inhibitors alone had no effects on PMN death compared to untreated control cells (data are not shown). Although there was a trend towards inhibition with oATP in apoptosis, none of these antagonists at the optimal concentrations significantly impaired the activity of LL-37 in inducing secondary necrosis. These data indicate that the capacity of promoting secondary necrosis is not a receptor-dependent phenomenon, and our collaborator in Dr. Johan Bylund's laboratory further demonstrated a swift event of membrane permeabilisation [500].

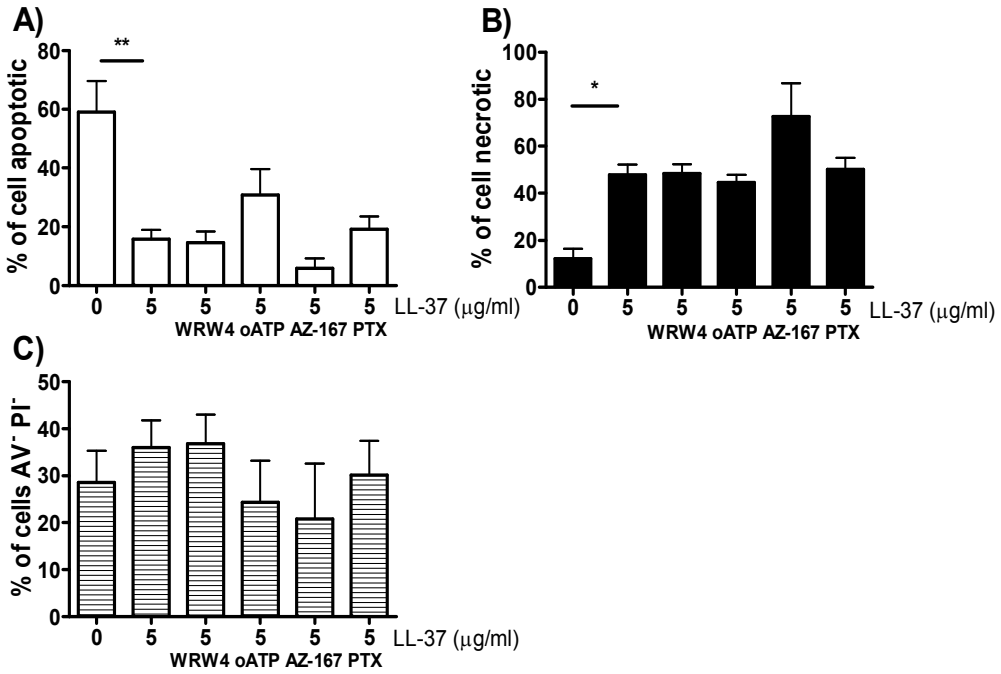


Figure 3.15. LL-37-induced secondary necrosis of apoptotic PMN is not dependent on known receptors of LL-37. Fresh human peripheral blood PMN were isolated and incubated with 30 min 10 μM WRW4, 100 μM oxidised ATP, 0.1 μM AZ-167, or 200 ng/ml PTX, followed by addition of 5 μg/ml LL-37 or without LL-37 in the presence of 10% FCS for 20 hr. (A-C) FACS analysis was used to determine the percentage of apoptotic, necrotic, and viable cells. Figures indicate mean values + SEM for n = 3 for each condition in triplicate. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test to compare samples exposed to LL-37 alone with samples exposed to receptor antagonists and LL-37. *, $p \leq 0.05$; **, $p \leq 0.01$.

3.2.12. LL-37 also selectively permeabilises apoptotic Burkitt lymphoma cell line

In order to test whether the induction of secondary necrosis of apoptotic cells was specific to PMN, or if it represented as a general mechanism acting on other cell types as well, a Burkitt lymphoma cell line Mutu I was used. Burkitt lymphoma cells display high baseline levels of apoptosis, a property that is retained constitutively in these tumor-derived cells, and can be further upregulated by irradiation. Irradiated Burkitt cells incubated for 4 hr displayed 60% apoptosis. When these highly apoptotic Burkitt cells were treated with LL-37 (5 μg/ml), they quickly became secondarily necrotic (Fig. 3.16). Thus the selective permeabilisation of apoptotic cells is not specific to PMN.

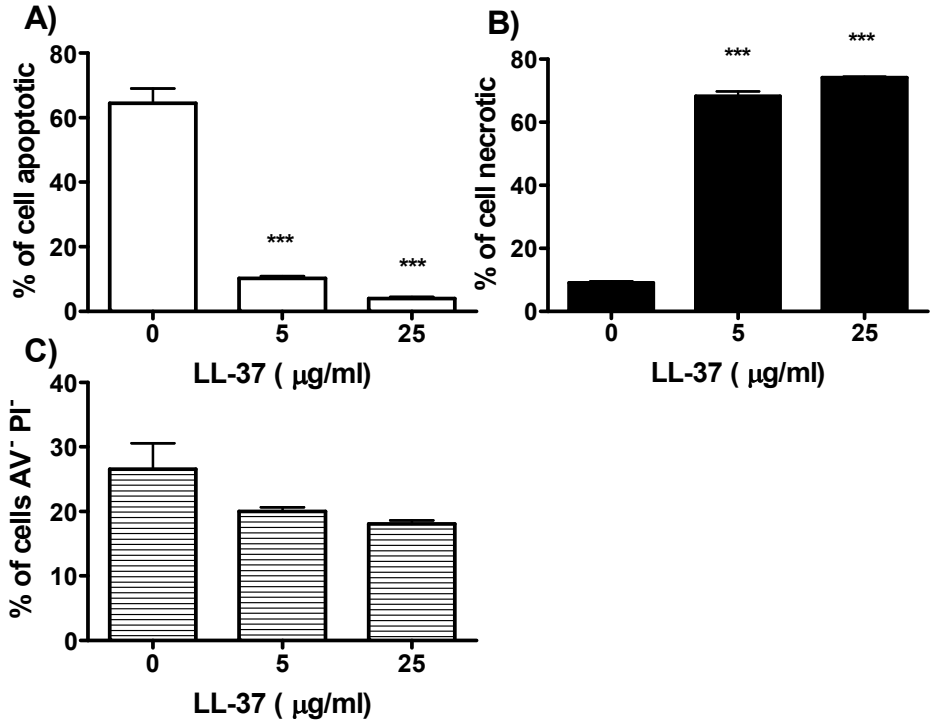


Figure 3.16 LL-37 permeabilised apoptotic Burkitt lymphoma cells. Burkitt lymphoma cell line was irradiated and incubated for 4 hr in the presence of 10% FCS. Burkitt cells were then exposed to LL-37 for 10 min before flow cytometric analyses. (A-C) FACS analysis was used to determine the percentage of apoptotic, necrotic, and viable cells. Figures indicate mean values + SEM for n = 3 for each condition in triplicate. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test to compare control with samples exposed to LL-37 over a range of concentrations. ***, $p \leq 0.001$.

3.2.13. LL-37-mediated induction of secondary necrosis is conserved in murine homologue mCRAMP and C-terminal partial peptides but not scrambled LL-37 peptide

To examine the important characteristics of LL-37 for the capacity to induce secondary necrosis of PMN, FACS-based studies were performed using a scrambled LL-37 peptide (with conserved charge but altered amino acid sequence), the murine homologue mCRAMP, and a panel of sixteen 22-mer partial peptides spanning the sequence of LL-37 (a kind gift from Prof. Pieter Hiemstra) [501] (Table 3.2). Incubation of PMN with scrambled LL-37 peptide had no effect, whether exposed over 20 hr or only as a final 10-min pulse (Fig. 3.17). In contrast, PMN exposed to mCRAMP closely replicated the effects of LL-37 with significant induction of secondary necrosis (Fig. 3.18). Exposure to N-terminal 22-mer partial LL-37 peptides (from the peptide incorporating amino acids (aa) 1-22 through to that spanning aa 10-31) had no significant effects (Fig. 3.19). In contrast, C-terminal partial peptides (from the peptide incorporating aa 11-32 through that spanning aa 16-37) induced significant secondary necrosis ($p \leq 0.01$) but to a lesser extent than full-length LL-37 (Fig. 3.19). This identified a putative minimal core functional region for induction of secondary necrosis spanning aa 16-32. The effects of this panel of peptides showed no correlation with charge, which ranged from +3 to +6. These data demonstrate that the capacity to induce secondary necrosis is evolutionarily conserved between mouse and human cathelicidin, is not

primarily dependent on peptide charge, and is retained by C-terminal but not N-terminal-truncated peptides of LL-37.

Peptides	Sequences N terminus----------C terminus	Charge
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	**+6**
mCRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ	**+6**
Scrambled LL-37	RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL	**+6**
Partial peptide	LLGDFFRKSKEKIGKEFKRIVQ	**+4**
p1	LGDFFRKSKEKIGKEFKRIVQR	**+5**
Partial peptide	GDFFRKSKEKIGKEFKRIVQRI	**+5**
Partial peptide	DFFRKSKEKIGKEFKRIVQRIK	**+6**
Partial peptide	FFRKSKEKIGKEFKRIVQRIKD	**+6**
Partial peptide	FRKSKEKIGKEFKRIVQRIKDF	**+6**
Partial peptide	RKSKEKIGKEFKRIVQRIKDFL	**+6**
Partial peptide	KSKEKIGKEFKRIVQRIKDFLR	**+6**
Partial peptide	SKEKIGKEFKRIVQRIKDFLRN	**+5**
Partial peptide	KEKIGKEFKRIVQRIKDFLRNL	**+5**
Partial peptide	EKIGKEFKRIVQRIKDFLRNLV	**+4**
p2	KIGKEFKRIVQRIKDFLRNLVP	**+5**
Partial peptide	IGKEFKRIVQRIKDFLRNLVPR	**+5**
Partial peptide	GKEFKRIVQRIKDFLRNLVPRT	**+5**
Partial peptide	KEFKRIVQRIKDFLRNLVPRTE	**+4**
p3	EFKRIVQRIKDFLRNLVPRTES	**+3**

Table 3.2 The sequences and charges of LL-37, mCRAMP, scrambled LL-37, and the panel of partial LL-37.

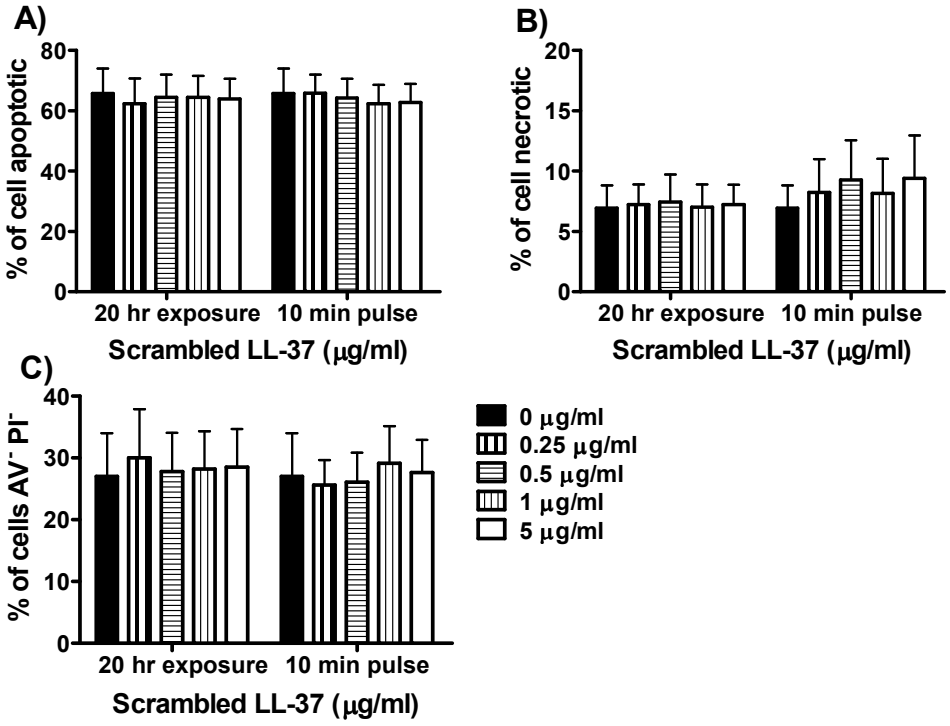


Figure 3.17 Scrambled LL-37 peptide has no secondary necrosis-inducing effect on apoptotic PMN. Fresh isolated human PMN were incubated in the presence of 10 % FCS with a range of concentrations of scrambled LL-37 peptide for 20 hr, or 10 min after 20 hr incubation in the absence of peptide (pulse); n = 3 donors performed in triplicate. Cell death was examined by FACS analyses as described. Figures represent mean values + SEM. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control.

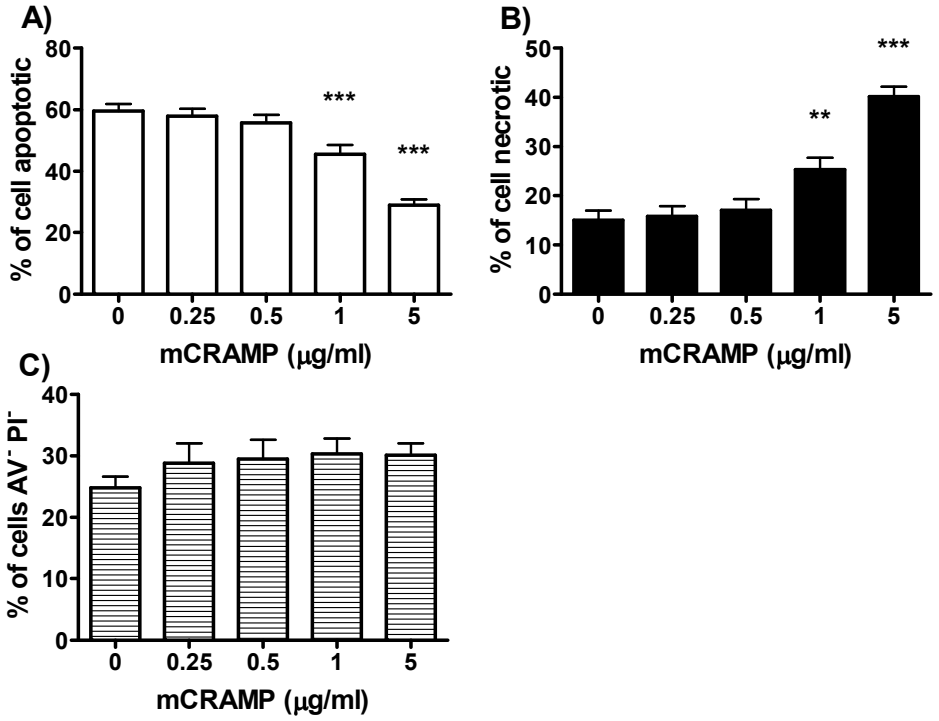


Figure 3.18 Murine homologue mCRAMP closely replicates the effects of LL-37 with significant induction of secondary necrosis. Fresh isolated human PMN were incubated with a range of concentrations of mCRAMP peptide for 20 hr in the presence of 10% FCS. Cell death was examined by FACS analyses as described; n = 16 donors performed in triplicate. Figures represent mean values + SEM. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control. **, $p \leq 0.01$; ***, $p \leq 0.001$.

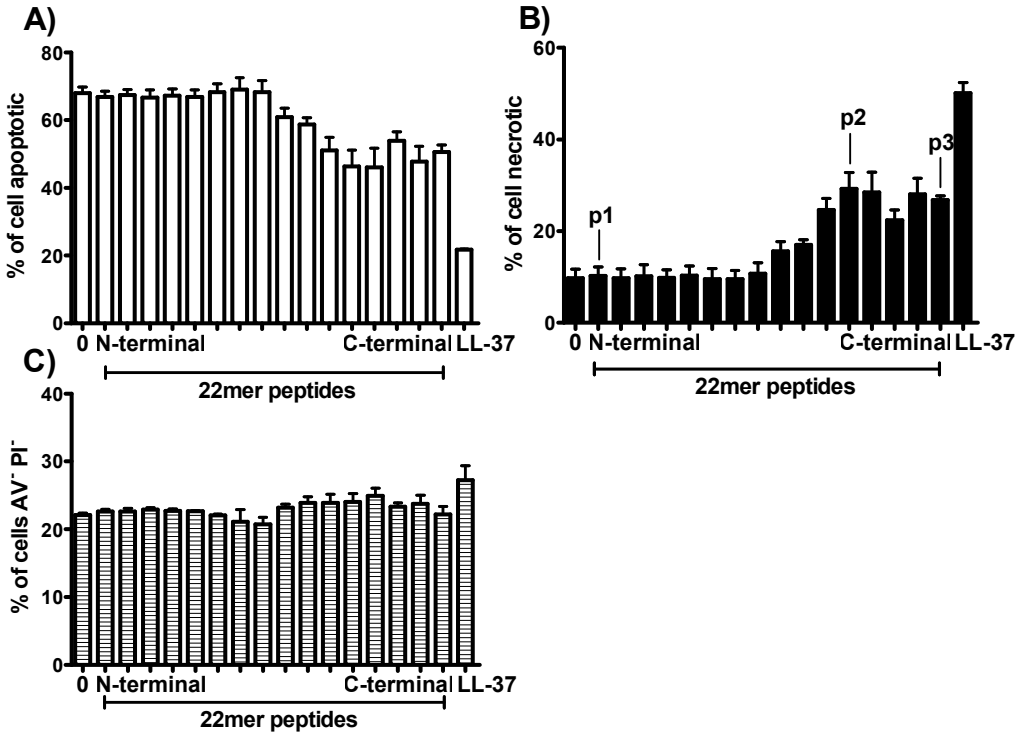


Figure 3.19 The capacity to induce PMN secondary necrosis is retained by C-terminal but not N-terminal-truncated peptides of LL-37. Fresh isolated human PMN were incubated with 5 μg/ml partial LL-37 peptides from a panel of 16 overlapping 22-mers using N-terminal peptide (aa 1-22), then peptide spanning aa 2-23, and so on through C-terminal peptide (aa 16-37) or full-length LL-37 for 20 hr in the presence of 10% FCS. Cell death was examined by FACS analyses as described; n = 3 donors performed in triplicate. Figures represent mean values + SEM. Peptides p1, p2, and p3 are identified, and referred to later in these thesis.

3.2.14. The property of induction of secondary necrosis is not a unique feature of cathelicidins

In order to test whether the capability to induce secondary necrosis was specific to cathelicidins, another family of CHDP was evaluated. PMN were incubated with α-defensins (HNP1-4) purified from human PMN (a kind gift from Dr Ole Sorensen) or synthetic β-defensin HBD3 in the presence of 10% FCS for 20 hr. Interestingly, despite the shared features of these two subgroups of defensins (e.g. positive charges, 28-42 amino acid length, and containing three intramolecular disulfide bonds), only HBD3 had the capacity to induce secondary necrosis (Fig. 3.20). As other effects of α-defensins can be inhibited by serum components [502, 503], I also conducted the experiment in serum-free media. Incubation of PMN with α-defensin peptide had no effect, no matter whether exposed over 20 hr in the presence of serum or as a final 10 min pulse in serum free media (Fig. 3.21). I then confirmed that HNP1-4 were biologically active by testing their anti-endotoxic activity which is referred to later in this thesis (Fig. 4.17) and by demonstrating detection of the peptides with antibody in Western immunoblotting (data are not shown). Taken together, these results demonstrated that the capacity to induce secondary necrosis is not restricted to cathelicidins, but nor is it a property of all CHDP.

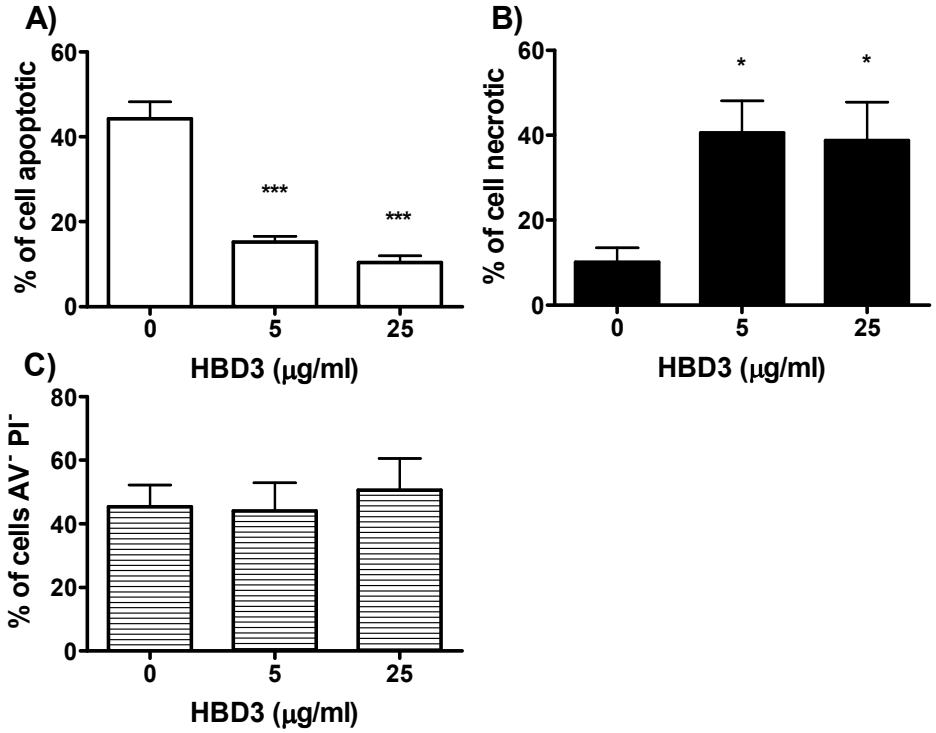


Figure 3.20 Human β-defensin HBD3 induces secondary necrosis of apoptotic PMN. Fresh isolated human PMN were incubated with a range of concentrations of HBD3 peptide for 20 hr in the presence of 10% FCS. Cell death was examined by FACS analyses as described; n = 3 donors performed in triplicate. Figures represent mean values + SEM. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control. *, $p \leq 0.05$; ***, $p \leq 0.001$.

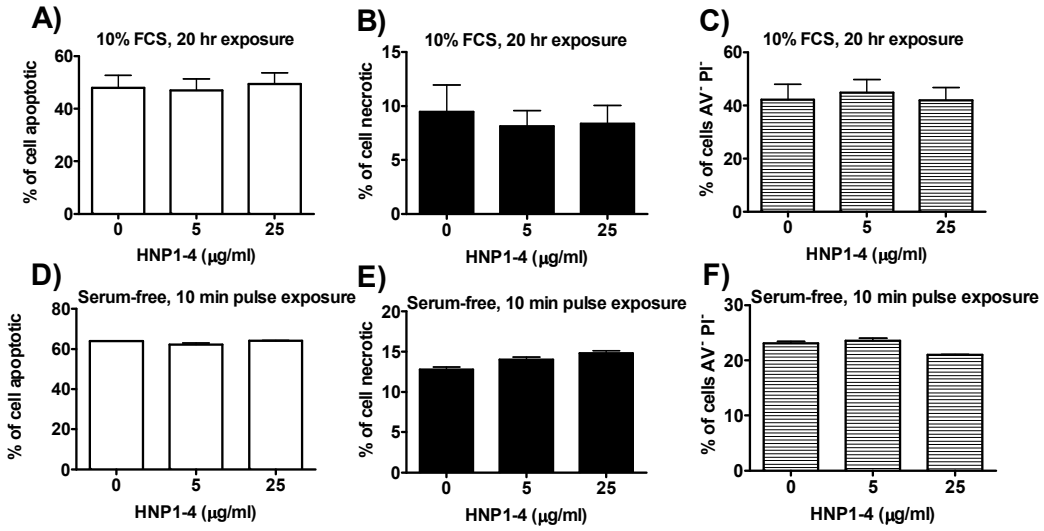


Figure 3.21 Human neutrophil peptides HNP1-4 did not induce secondary necrosis of apoptotic PMN. Fresh isolated human PMN were incubated with a range of concentrations of alpha-defensin HNP peptide for 20 hr in the presence of 10% FCS (A-C), or 10 min peptide exposure after 20 hr incubation in the absence of serum (pulse) (D-E); n = 3 donors performed in triplicate. Cell death was examined by FACS analyses as described. Figures represent mean values + SEM. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control.

3.2.15. LL-37-induced secondary necrosis of PMN can release granule contents.

As shown by my data in earlier sections, LL-37 rapidly induced secondary necrosis of apoptotic PMN, and our collaborator demonstrated that LL-37 selectively permeabilised plasma membrane of apoptotic PMN [500]. However, it was not known whether organelle membranes were similarly affected. To determine granule content release (or granule release) from LL-37-treated PMN, MPO (stored at the primary granules) levels in the cell supernatants was evaluated at 20 hr, under conditions in which secondary necrosis was significantly induced by LL-37 (Fig. 3.22). MPO release was not detected above background at 20 hr time point following exposure to ≤ 10 μg/ml LL-37 (concentrations at which substantial secondary necrosis occurred) or in response to scrambled LL-37. However, significant levels of MPO were observed in response to ≥ 25 μg/ml LL-37 ($p \leq 0.01$) at levels of around 40% of total MPO released by lysis with Triton X-100 (Fig. 3.22). These data suggest that LL-37 at higher concentrations is not only able to permeabilise cell membrane, but is also able to destabilise granules and lead to the release of their intracellular contents.

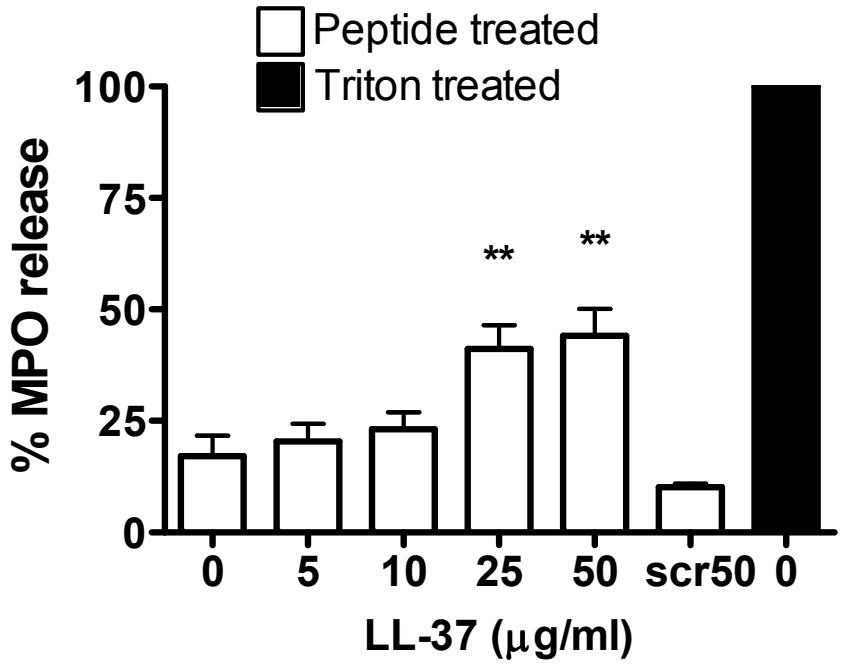


Figure 3.22 LL-37-induced secondary necrosis of PMN can release granule contents. Isolated human PMN were incubated for 20 hr with a range of concentrations of LL-37 or 50 μg/ml scrambled LL-37 (scr50) in the presence of 10% FCS. The concentration of MPO in the supernatants was evaluated as a percentage of total MPO release after lysis of fresh control PMN using 0.1% Triton X-100. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test; n=7 donors; $p \leq 0.01$, compared with untreated PMN.

3.3. Discussion

CHDP have been demonstrated to have multiple immunomodulatory capabilities. LL-37, a mature and functional CHDP, is a multifaceted modulator of innate immune responses, with properties including direct antimicrobial function, chemokine production, anti-endotoxin activity, and chemotaxis of inflammatory and immune cells [2, 5]. PMN are first-line innate immune cells and the regulation of their function, death, and removal is critical. Several studies have demonstrated that the direct effects of LL-37 on PMN, including enhancing their activity against pathogens [16, 17] and modulating their survival [20, 21]. At inflamed sites, increased concentrations of LL-37 and increased numbers of PMN are observed; thus, we hypothesised that LL-37 could modulate inflammation via effects on PMN cell death pathways.

LL-37 has been reported to induce apoptosis in airway epithelial cells and keratinocytes, indicating its capacity to modulate cell death in innate effector cells [108, 111]. The antimicrobial capability of this peptide has been ascribed to its membrane pore-forming activity [504, 505], but eukaryotic cells are less susceptible to membrane attacks from LL-37 [2]. This is proposed to relate to differences in membrane composition [2]. It is well known that cells change the composition of their membranes during apoptosis, a process that is important in order to be recognised by phagocytes and ensure safe disposal. My data demonstrate that LL-37, and certainly other CHDP, can induce secondary necrosis of apoptotic PMN, altering the state of cell death. This alteration in PMN death

might provide another mechanism by which CHDP modulate inflammatory and immune responses.

My data demonstrate that LL-37 rapidly induces secondary necrosis of apoptotic PMN. In order to confirm that the appearance of increased necrosis was not simply an artifact of loss of intact cells, total cell numbers were evaluated and showed there was not a substantial cell loss at the concentrations used in the experiments. In addition, in order to exclude the possibility of an interaction between Annexin V and the peptide which may skew results as decreasing apoptosis, our collaborators in Dr. Johan Bylund's laboratory (University of Gothenburg) employed CD16 as a marker for PMN apoptosis rather than Annexin V. CD16 is a surface protein present on viable PMN and is lost upon apoptosis. They showed that only PMN negative for CD16 became necrotic after addition of LL-37, confirming that LL-37 selectively targeted apoptotic cells [500].

At inflamed sites, a variety of mediators can affect the life span of PMN [216, 266]. GM-CSF or LPS has been demonstrated to prolong PMN survival whereas some pharmacological agents (eg. *R*-Roscovitine) effectively promote PMN apoptosis [216, 268]. Thus, these PMN survival factors or death signals were tested in the presence of LL-37 to estimate their net biological effects on human PMN death. The results support the hypothesis that LL-37 induces apoptotic PMN towards secondary necrosis, with the necrotic rates correlate to the extent of apoptosis. Combination of LPS and LL-37 (a well-characterised anti-endotoxic

agent [89]) demonstrate a mutually inhibitory effect on the modulation of PMN death, suggesting the antagonistic interaction between these two factors *in vivo*.

It was proposed that LL-37-induced secondary necrosis was a serum-dependent event. LL-37 binds to at least one serum protein, and that this binding modifies its antibacterial and cytotoxic activities [107, 506]. In addition, for primary blood derived monocytes the presence of serum seems to be required for this peptide to activate MAPK, ERK1/2, and p38 [143]. In contrast, my data showed that LL-37-induced secondary necrosis was not a serum-dependent event. Although the absence of serum in overnight incubation with LL-37 diminished the level of secondary necrosis of apoptotic cells, short, late stage pulse treatment studies demonstrated that serum presence was not necessary for LL-37-mediated secondary necrosis in PMN. Two possibilities may explain this phenomenon: 1) LL-37 could be more easily degraded in serum-free media; or 2) LL-37 could be internalised more rapidly in the absence of serum and localised intracellularly, as previously observed using biotinylated LL-37 [31]. In both cases lower levels of active LL-37 would be available in the media to induce secondary necrosis once apoptosis occurs. Although so far there are no data to prove or disprove my assumptions, experiments can be done by evaluating the concentrations and functions of residual LL-37 after overnight incubation in serum-free media, or using fluorescent LL-37 to localise the peptide while interacting with cells.

Interestingly, the ability of LL-37 to induce secondary necrosis was not specific to apoptotic PMN, but also observed with a Burkitt lymphoma cell line and, by our

collaborators, primary natural killer (NK) cells [500]. These observations suggest that LL-37 might target a general membrane composition change in certain apoptotic cells, or receptor/ membrane protein the expression of which is altered by apoptosis in many diverse cell types. The fact that LL-37 selectively permeabilises membranes of apoptotic cells, which are nonfunctional and unable to transmit signals, diminishes the likelihood of specific active receptor signalling involvement. Experiments conducted with antagonists of known receptors of LL-37 also support this hypothesis. Apoptotic cells flip the negatively charged PS from the inner leaflet of the lipid bilayer to the outer leaflet. PS expression on the cell surface might promote susceptibility of apoptotic cells to the cationic LL-37. However, my data and that of our collaborators fail to support this hypothesis. Blocking PS by Annexin V did not inhibit the capability of LL-37 in the induction of secondary necrosis, nor did liposomes incorporating with PS confer any permeabilising activity of LL-37 [500]. It is possible that apoptotic membrane has other surface molecules than PS altered which may be recognised and targeted by LL-37. It could also be hypothesised that LL-37 interacts with outer membranes of both viable and apoptotic cells and temporarily disrupts them. The peptide can be removed from the membranes by internalisation in healthy cells with limited membrane damage whereas apoptotic cells are non-functional and unable to repair the disturbed membrane [500]. Taken together, the permeabilisation of apoptotic cells is unlikely to depend on LL-37 interaction with specific functional surface receptors or PS expression.

LL-37 and mCRAMP are structurally similar [44] and have a conserved capacity to induce secondary necrosis in apoptotic human PMN. In contrast, scrambled LL-37 does not have secondary necrosis-inducing properties on apoptotic cells, suggesting that cationicity alone is not sufficient to confer this effect. Studies on the panel of partial peptides also show no correlation with positive charges and the capacity to induce secondary necrosis, but may correlate with peptide amphipathicity. However, defensins, although positively charged and amphipathic in structure, are not all capable of inducing secondary necrosis of apoptotic PMN; HBD3 had the capacity to induce secondary necrosis whereas HNP1-4 did not). Our collaborators evaluated the antibacterial/ immunomodulatory peptide Hp(2-20), a peptide that combines cationicity with an alpha-helical nature, and found that peptide was unable to permeabilise apoptotic leukocytes [500]. These findings show that the selective action described for LL-37 is not a general feature for cationic, alpha-helical, amphipathic antibacterial peptides [500]. Future research will hopefully unravel the structural determinants responsible for the permeabilisation of apoptotic cells.

3.4. Conclusion

Consistent with other recent reports, my data demonstrate that LL-37 specifically induces secondary necrosis of apoptotic cells and may destroy the integrity of the plasma membrane [144, 500]. I also demonstrated LL-37-induced release of granule contents occurs, although only at higher peptide concentrations. These data suggest that LL-37-mediated secondary necrosis of apoptotic cells can

be a potent means of transferring the intracellular substances out to an extracellular location. Interestingly, lower concentrations of LL-37 do not lead to significant increases of granule content release, despite the induction of secondary necrosis. This may be due to decreased concentrations of LL-37 available intracelluarly for a given extracellular concentration, but could also possibly relate to differential membrane susceptibility. These observations suggest that during inflammation when recruited PMN become apoptotic and LL-37 is upregulated there may be massive PMN intracellular content release at affected sites.

Apoptosis is viewed as a silent, physiological mechansim for removing PMN, without host damage or increasing inflammation, and contributing to the resolution of the inflammatory process. In contrast, necrosis is regarded as a pathological and inflammatory type of cell death. The membranes of necrotic cells are no longer intact and the cell contents are free to leak out to the extracellular milieu, causing tissue destruction and persistent inflammation [322]. Thus the impact of LL-37-mediated secondary necrosis of apoptotic PMN on inflammatory responses was the subject of further analysis for this thesis.

Chapter 4
The impact of LL-37-induced secondary necrosis on inflammatory responses

4. The impact of LL-37-induced secondary necrosis on inflammatory responses

4.1. Introduction

Cell death by apoptosis, followed by efficient efferocytosis by professional phagocytes, is proposed to ensure safe cell removal without the release of harmful intracellular contents and to promote anti-inflammatory responses to enhance resolution [12]. In contrast, cell death by necrosis is proposed to be pro-inflammatory. However, the effects of primary necrosis, as compared to secondary necrosis of apoptotic cells remain unclear. Although this axiom is widely accepted and applied to many experimental systems, there are still arguments against this oversimplified proposal [15]. It is clear that the mechanism of cell death do matter in determining ensuing biological responses, but more work is required to clearly clarify the inflammatory properties of different forms of cell death.

I have demonstrated (in chapter 3) that human cathelicidin LL-37 is a potent inducer of secondary necrosis of apoptotic PMN. This observation raises several intriguing questions: does this LL-37-mediated secondarily necrosis of PMN affect their clearance by phagocytic cells, does this influence the consequent inflammatory responses, do these secondarily necrotic PMN release their intracellular constituents to alter the process of inflammation, and do these cells have any biological importance in real physiological or pathological conditions? In order to address these questions, the ingestion of dying PMN by primary human

monocyte-derived macrophage, and the consequences for inflammation, was used as a study model.

4.2. Results

4.2.1. LL-37-induced secondary necrosis of apoptotic PMN does not affect efferocytosis by monocyte-derived macrophages

To determine whether MDM ingestion of dead PMN would be altered by LL-37-mediated secondary necrosis, MDM phagocytosis of LL-37-treated and untreated PMN was studied by light microscopic enumeration (Fig. 4.1 & 4.2) and by FACS analyses. FACS method was validated by Dransfield *et al*. using a fluorescent chloromethyl dye that diffuses freely through plasma membranes to label the cytoplasm of cells [507] and phagocytic rate was determined by measuring ingestion of fluorescence-labelled PMN by MDM (Fig. 4.3 & 4.4). Dexamethasone-primed MDM which have previously been shown to have an increased capacity for apoptotic PMN ingestion [394], were used as positive control, to demonstrate that differences in the magnitude of ingestion could be evaluated using these methods. The data show significantly increased efferocytosis ($p \leq 0.05$) by dexamethasone-primed MDM, but demonstrate that LL-37-induced secondary necrosis of PMN has no significant effects on the magnitude of PMN ingestion by MDM.

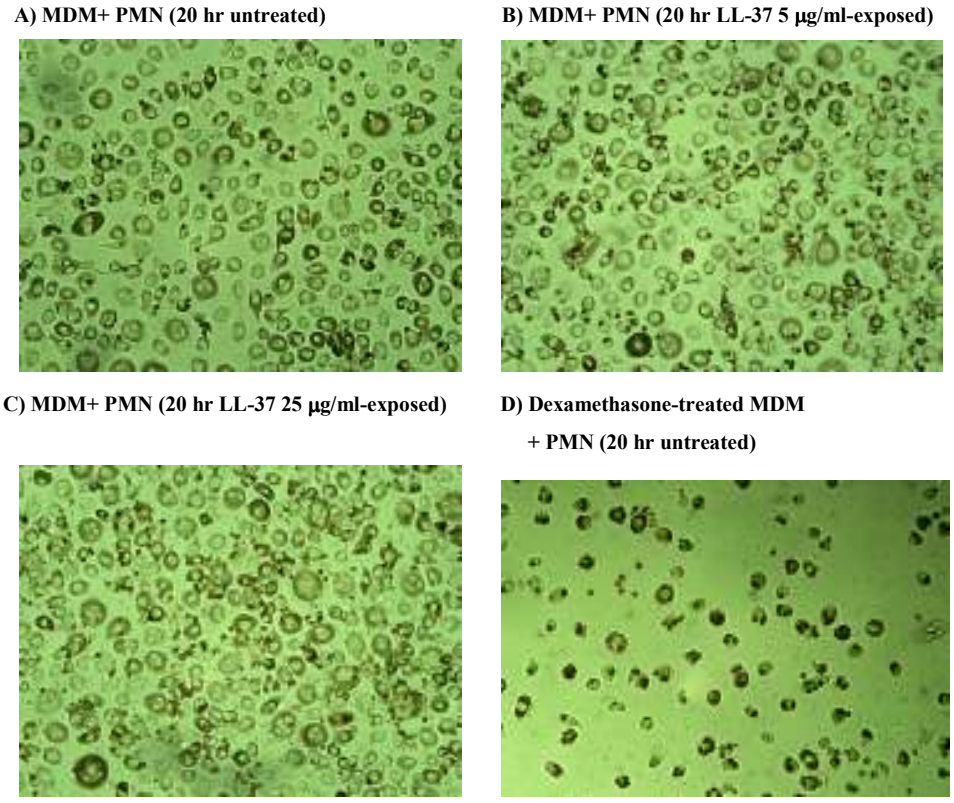
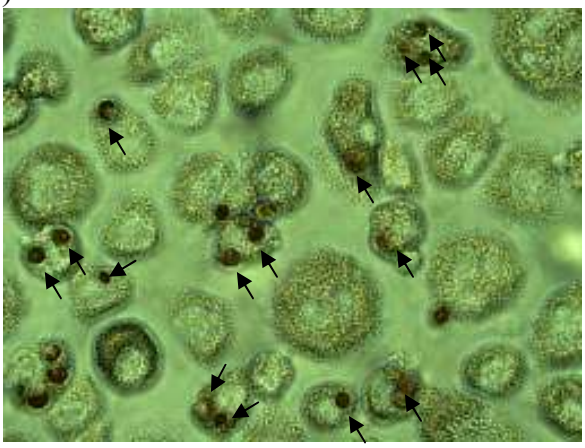


Figure 4.1 Representative images of phagocytosis (light microscopic enumeration). PMN were incubated for 20 hr, in the absence (A, D) or presence (B, C) of LL-37 at the stated concentrations and then incubated with MDM for 1 hr before four times of washing steps to remove noningested PMN. For positive control, hMDM were exposed to 1 µM Dexamethasone for 5 days before incubation with PMN (D). PMN MPO was stained with 0.1 mg/ml dimethoxybenzidine and 0.03% (v/v) H_2O_2.

A)



B)

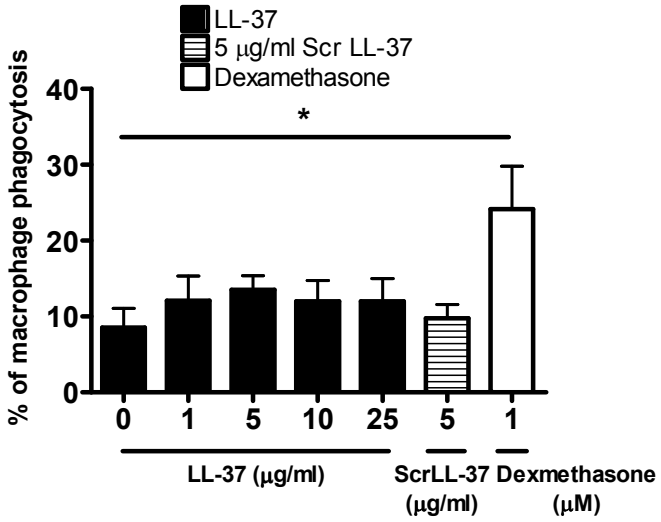


Figure 4.2 LL-37-induced secondary necrosis of PMN does not affect rates of MDM phagocytosis (light microscopic enumeration). PMN were incubated for 20 hr, with or without LL-37 (or scrambled LL-37) at the stated concentrations and then incubated with MDM for 1 hr before removal of noningested PMN. Phagocytosis was assessed by light microscopy, counting the proportion of MDM peroxidase-positive cells (A, indicated by arrows)**.** As a positive control, wells of adherent monocytes were also culture in the presence of 1 μM dexamethasone for 5 days to up-regulate MDM phagocytosis of dead PMN. (A) representative image (B) mean + SEM for n = 3 donors with each condition in duplicate. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison post-tests comparing each treatment with control; *, $p \leq 0.05$.

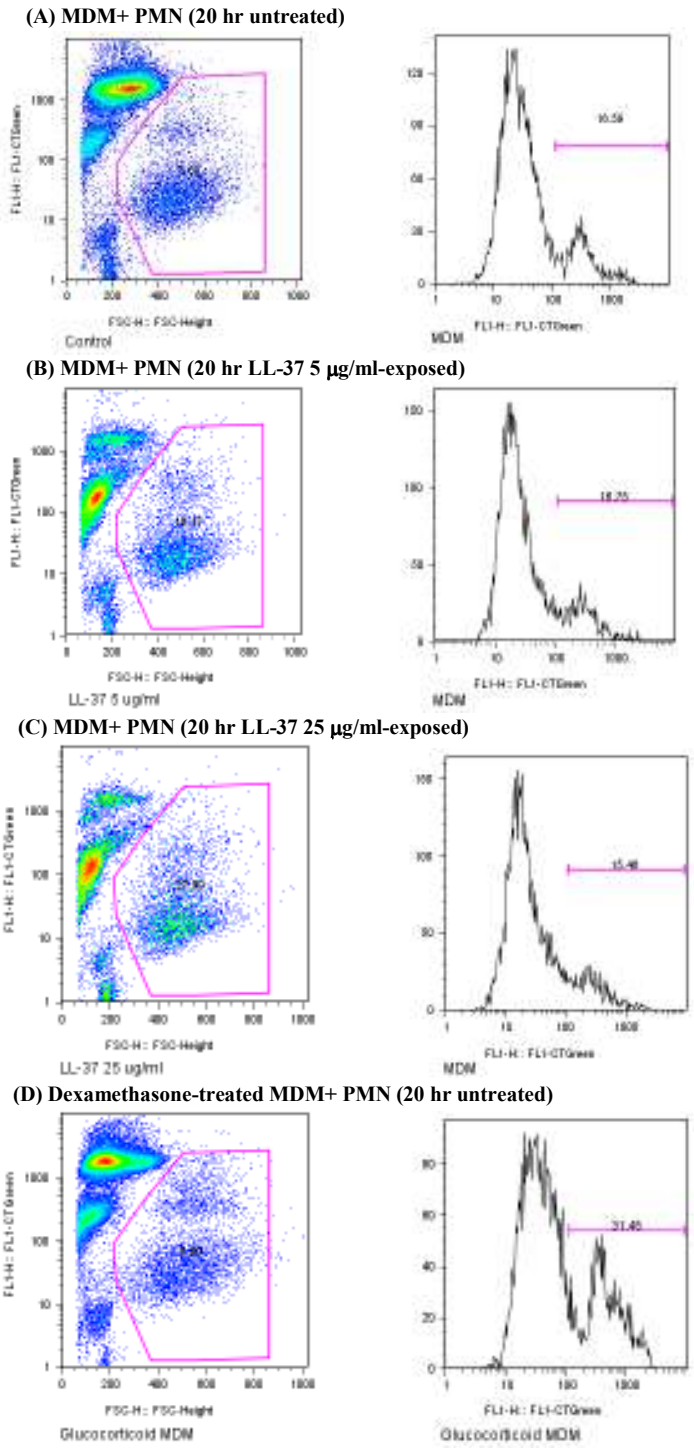


Figure 4.3 Representative figures of phagocytosis (FACS-based analyses). Dot plots represent MDM exposed to fluorescently-labelled PMN (left-sided panel). Histograms represent the percentage of MDM containing fluorescent PMN (right-sided panel). Details are found in **Figure 4.4**.

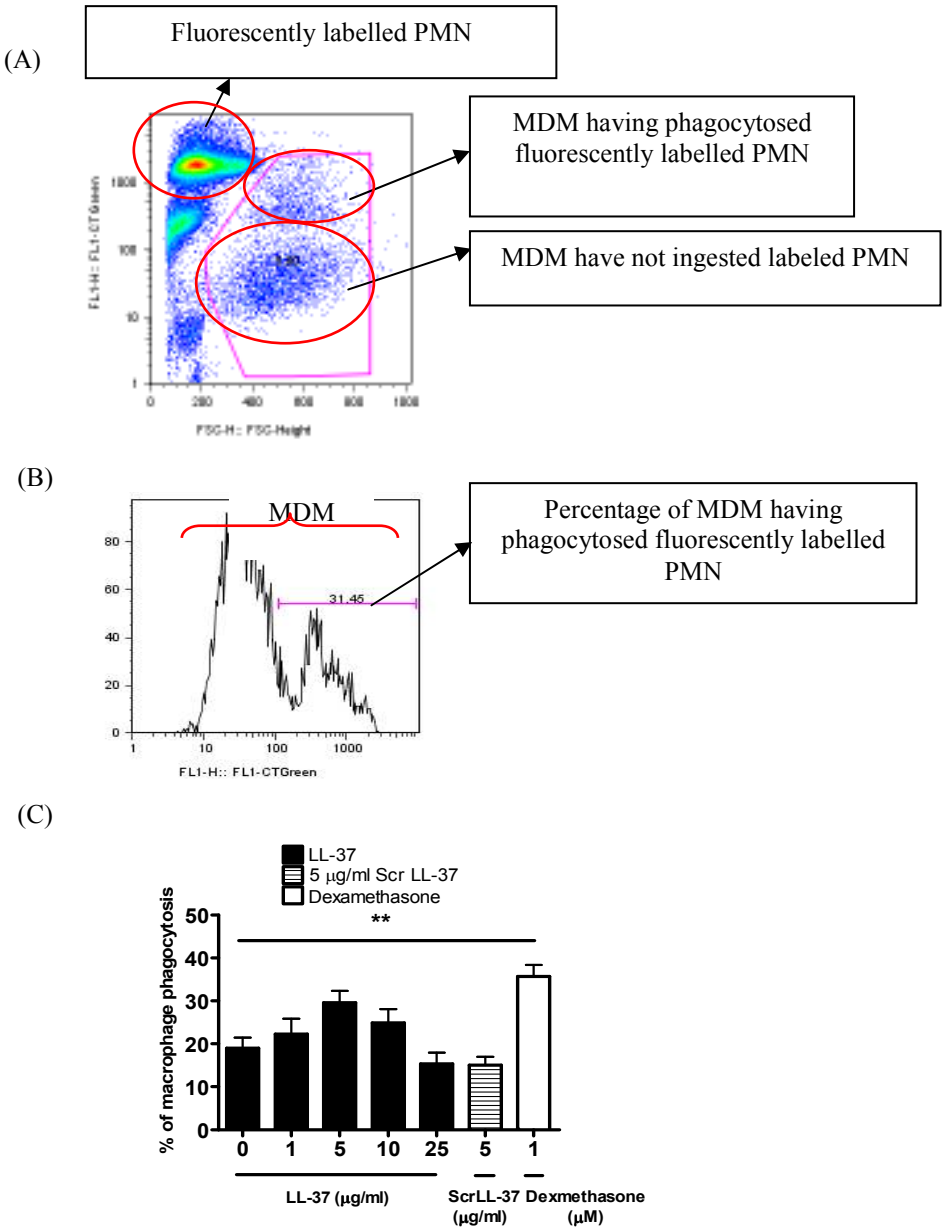


Figure 4.4 LL-37-induced secondary necrosis of PMN does not affect rates of MDM phagocytosis (FACS-based analyses). PMN were incubated for 20 hr, with or without LL-37 (or scrambled LL-37) at the stated concentrations and then incubated with MDM for 1 hr before removal of noningested PMN. Dexamethasone-treated MDM were used as positive controls. Phagocytosis was assessed by flow cytometric analyses, evaluating the proportion of MDM ingesting Cell Tracker Green-labelled PMN (A, B); mean + SEM for $n \geq 3$ donors for each condition in duplicate (C). Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control; **, $p \leq 0.01$.

4.2.2. Uptake of PMN is not inhibited by blocking $\alpha_v\beta_3$/CD36/TSP system

It has been well documented that several receptors are involved in phagocytosis of apoptotic PMN by MDM such as PSR, scavenger receptors, lectin-like receptors, CD44, and $\alpha_v\beta_3$/CD36/TSP complex [508]. The mechanisms of recognition of apoptosis are influenced by the activating status of macrophages, macrophages at different tissue, and the surrounding milieu. However, there are limited data about mechanisms by which late apoptotic or secondarily necrotic cells are phagocytosed. In order to determine whether LL-37-induced secondarily necrotic PMN and apoptotic PMN were phagocytosed by MDM using alternate pathways, inhibition of the $\alpha_v\beta_3$/CD36/TSP system was conducted chemically and using an inhibitory antibody as described in a previous report [435]. The tetrapeptide RGDS was used to block integrin ($\alpha_v\beta_3$) binding, and a mouse anti-human CD36 were used to block CD36, with RGES and mouse IgG isotype antibody as negative controls. However, no effective inhibition of MDM ingestion of apoptotic PMN was observed (Fig. 4.5), suggesting that there was a receptor redundancy in the process of clearance of dead cells. Thus, phagocytic mechanisms by which MDM ingest LL-37-mediated secondarily necrotic PMN were not further investigated by this method.

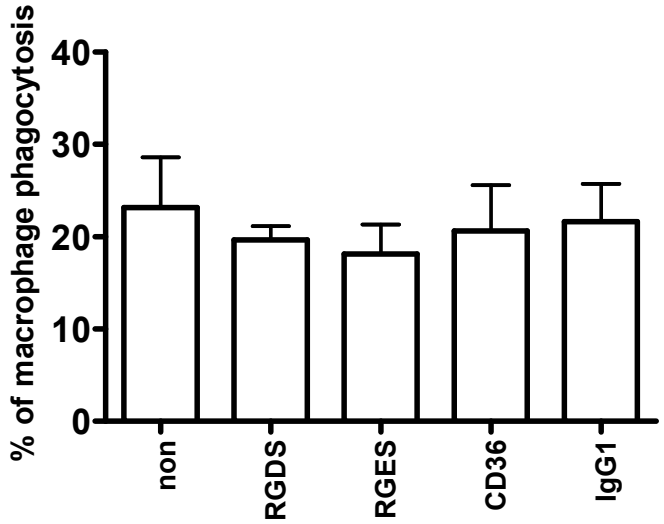


Figure 4.5 $\alpha_v\beta_3$/CD36 inhibition does not alter uptake of dead PMN by MDM. MDM were pretreated with 1 mM RGDS or mouse anti-human CD36 (1:500) for 30 min before the addition of overnight incubated apoptotic PMN. As negative controls, 1 mM RGES and mouse anti-human IgG antibody (1:500) were used respectively. Cells were allowed to interact for 1 hr before removal of noningested PMN. Phagocytosis was assessed by light microscopy counting; data are shown as mean + SEM for n = 3 donors for each condition in duplicate. Significance was assessed by one-wayANOVA with Bonferroni's multiple comparison post-test comparing each treatment with untreated control MDM.

4.2.3. LL-37-induced secondarily necrotic PMN are not pro-inflammatory to phagocytosing MDM

It is widely accepted that apoptotic cell death does not provoke inflammation, whereas necrotic cell death releases a variety of danger signals and stimulates a host inflammatory responses. To compare the effects of LL-37-induced secondarily necrotic human PMN on phagocytosing MDM, cytokine responses from macrophages were evaluated after 18 hr exposure to those PMN, which replicated the model used by Fadok *et al.* to characterise the inflammatory property of apoptotic PMN [13]. To separate any effect of residual, functional LL-37 in the media from the effects of the dead PMN and the effects of cell bodies from released soluble mediators and cellular contents, PMN were incubated for 20 hr at a range of concentrations of LL-37 (or scrambled LL-37) and then divided into three groups (Fig. 4.6): ① peptide-treated/ untreated PMN which were resuspended in fresh serum-free media, ② unwashed peptide-treated/ untreated PMN in their original media (containing 10% FCS), and ③supernatant from peptide-treated /untreated PMN (containing 10% FCS). The responses of MDM exposed to these three conditions were then evaluated and contrasted. MDM exposed to control apoptotic, LL-37-induced secondarily necrotic PMN, or the supernatants from these cells did not produce detectable levels of TNF-α, IL-6, IL-10, (Fig. 4.7), IL-12p70, or IL-1β (data of IL-12p70 and IL-1β are not shown). There were no significant differences between responses to LL-37-treated and control untreated PMN. These data suggest that LL-37-induced secondarily

necrotic PMN are not pro-inflammatory to phagocytosing MDM, nor releasing pro-inflammatory contents into the supernatant.

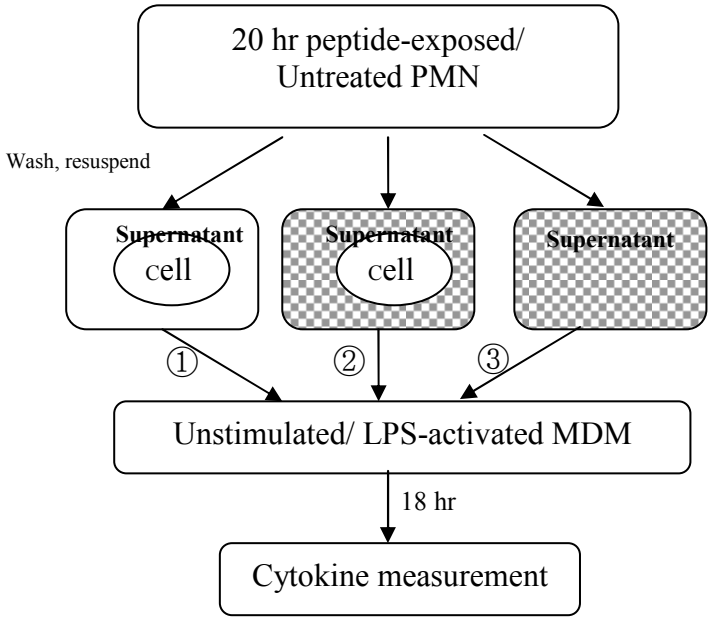


Figure 4.6 Methods of measuring cytokine responses to MDM phagocytosis of dead PMN. Human PMN were incubated for 20 hr in the presence or absence of LL-37 or scrambled LL-37, then were divided into three groups to be added to unstimualted/ LPS-activated MDM: ① Peptide-treated/ untreated PMN which were resuspended in fresh serum-free media, ② unwashed peptide-treated/ untreated PMN in their original media (containing 10% FCS), and ③ supernatant from peptide-treated /untreated PMN (containing 10% FCS). Blank pattern represents serum-free media, and spot pattern represents serum-present media.

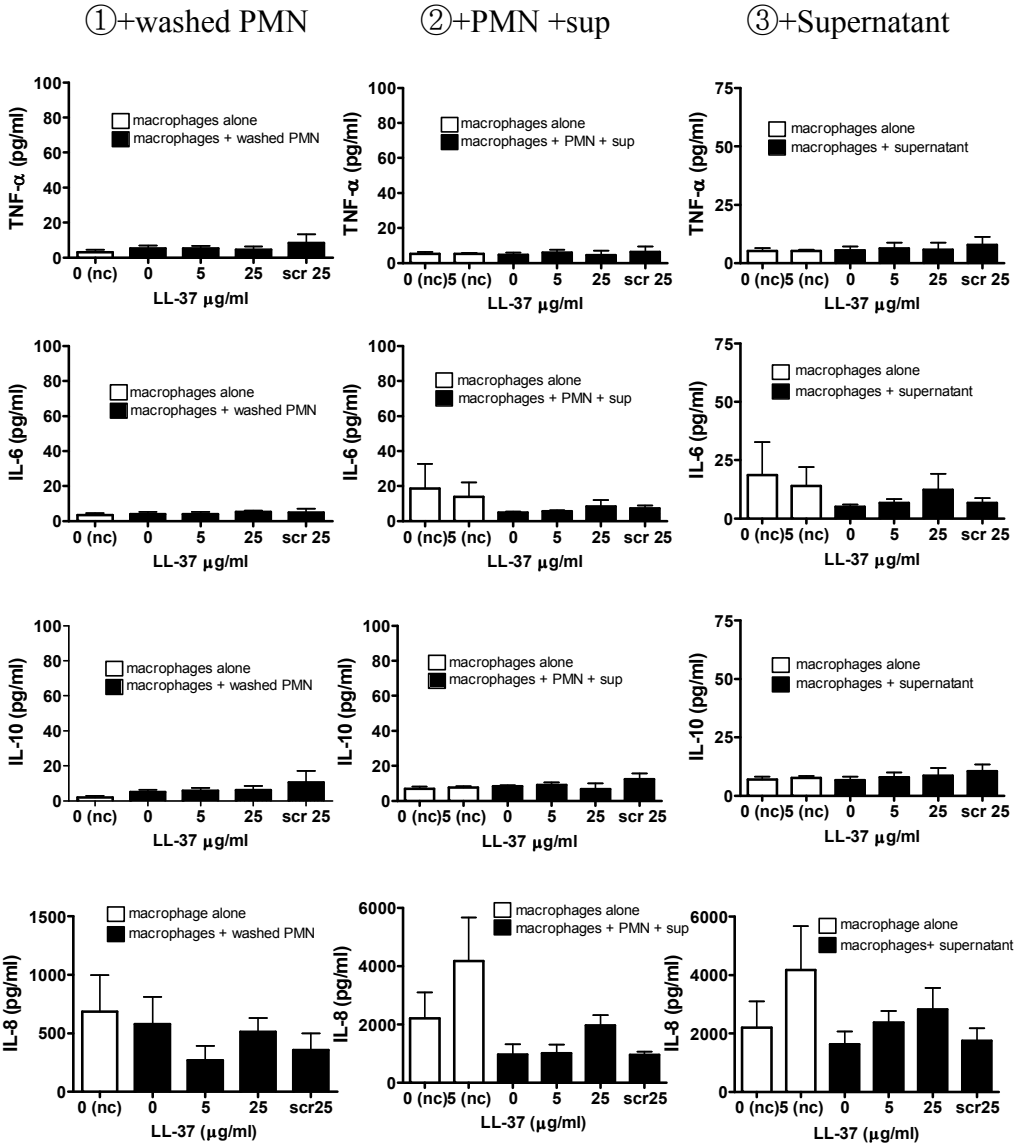


Figure 4.7 LL-37-induced secondarily necrotic PMN are not pro-inflammatory for MDM. Human PMN were incubated for 20 hr in the presence or absence of LL-37 or scrambled LL-37 at the concentrations indicated, from which three preparations were made: ① washed cells resuspended in serum-free media, ② unwashed in the IMDM + 10 % FCS media, or ③ supernatant from peptide-treated /untreated PMN. MDM were then incubated with these PMN. Alternatively, MDM were incubated for 18 hr without PMN (nc), in the presence or absence of fresh LL-37 as controls. Supernants were evaluated for cytokine responses. Column ① represents MDM incubated with washed cells resuspended in serum-free media, column ② shows MDM exposed to unwashed PMN in the IMDM + 10 % FCS original media, and column ③ reveals MDM exposed to supernatants from PMN (in original media containing 10% FCS). Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test compared with MDM in the absence of PMN; data are shown as mean + SEM for n = 5 donors.

4.2.4. LL-37-induced secondarily necrotic PMN retain anti-inflammatory activity against LPS-stimulated MDM in serum-free media.

Given the unexpected absence of pro-inflammatory activity of LL-37-induced secondarily necrotic PMN, the effects of these cells on activated MDM were then compared to that of anti-inflammatory apoptotic PMN. To determine the effect of secondarily necrotic PMN in the context of pro-inflammatory stimuli and MDM activation, MDM were exposed concomitantly to 10 ng/ml *E. coli* LPS (Fig. 4.8). MDM treated with LPS alone had significant TNF-α, IL-6, IL-10, and IL-8 production, compared to untreated cells. Using method ① to replicate the previous study [13], this LPS-induced pro-inflammatory response (TNF-α, IL-6) was significantly diminished by the addition of control apoptotic PMN in serum-free media ($p \leq 0.01$). In contrast to expectation, LL-37-induced secondarily necrotic PMN also significantly inhibited LPS-induced pro-inflammatory cytokine responses, and the effects were even potentiated under the conditions with the most induction of secondary necrosis (LL-37 25 μg/ml), with significantly greater inhibition of TNF-α ($p \leq 0.01$). This demonstrates that extensive secondary necrosis of PMN, unexpectedly, retains anti-inflammatory property as apoptotic cells, and the anti-inflammatory effects are even more pronounced. Interestingly, in contrast, exposure of MDM to control untreated PMN in their original supernatant (method ②) had no effect on the LPS-induced production of TNF-α, IL-6, IL-10, and IL-8 by MDM and was essentially the same as exposure to supernatant alone. This suggests that some factor in the media, or the FCS, was able to inhibit the

anti-inflammatory effects of these apoptotic cells. However, exposure to unwashed, LL-37-treated PMN in their original supernatant did inhibit the LPS-induced production of TNF-α, IL-6, IL-10, and IL-8 by MDM. This could indicate that a factor released during secondary necrosis had an anti-inflammatory effect that was not inhibited in the manner observed for the apoptotic cell bodies. However, fresh LL-37 alone, added directly to LPS-stimulated MDM in the absence of PMN, was able to replicate this inhibitory effect, suggesting that the anti-endotoxic effects of residual functional LL-37 in the original supernatant may be responsible for the results observed in these later studies. Taken together, these data show that: 1) LL-37-induced secondarily necrotic PMN are anti-inflammatory, 2) secondary necrosis induced by higher concentrations of LL-37 has more pronounced anti-inflammatory effects, and 3) the anti-inflammatory effects of original supernatants may be due to the anti-endotoxic activity of residual LL-37.

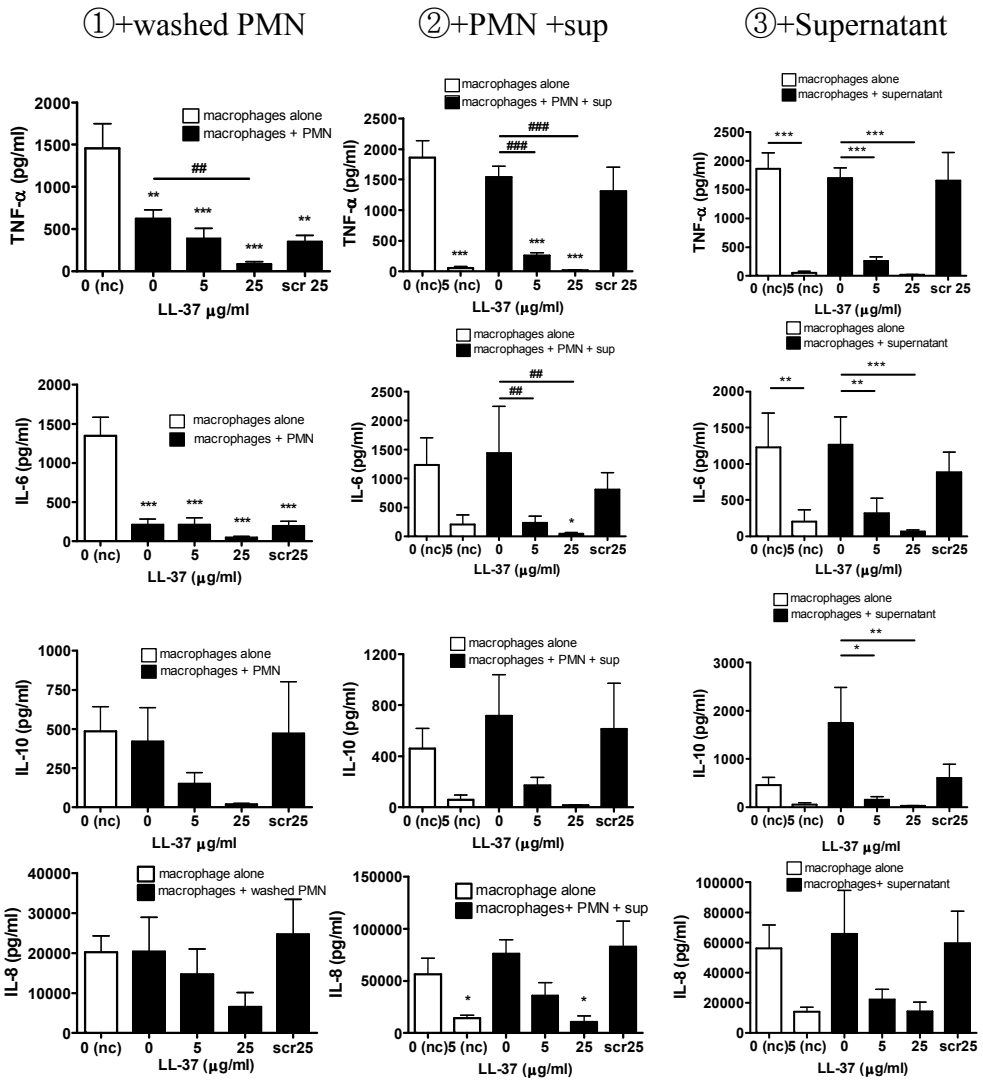


Figure 4.8 LL-37-induced secondarily necrotic PMN retain anti-inflammatory properties. Human PMN were incubated for 20 hr in the presence or absence of LL-37 or scrambled LL-37 at the concentrations indicated and then either washed and resuspended in serum-free media, or used unwashed in the original IMDM + 10% FCS original media. MDM were then incubated, with concomitant exposure to 10 ng/ml *E. coli* 0111:B4 LPS and PMN for 18 hr, or LPS without PMN (nc), in the presence or absence of fresh LL-37. Supernatants were evaluated for cytokine responses. Column ① represents MDM incubated with washed cells resuspended in serum-free media, column ② shows MDM exposed to unwashed PMN in the IMDM + 10 % FCS original media, and column ③ reveals MDM exposed to supernatants from PMN (in original media containing 10% FCS). Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test; data are shown as mean + SEM for n = 10 donors (TNF-α); n = 5 donors (IL-6/IL-10/IL-8); *, $p \leq 0.05$; **, $p \leq 0.01$, ***, $p \leq 0.001$, compared with MDM exposed to LPS in the absence of PMN; ##, $p \leq 0.01$; ###, $p \leq 0.001$, compared with MDM exposed to control apoptotic PMN untreated with LL-37.

4.2.5. MDM do not die during the incubation with dead PMN or LL-37 peptide.

Although it has been documented that LL-37 is not cytotoxic to MDM below the concentration of 250 μg/ml [25], the generalised reduction of cytokine responses in MDM exposed to LL-37-treated PMN (or LL-37) raised a question of whether MDM died during the process of phagocytosis or died during the incubation with LPS, apoptotic PMN, or secondarily necrotic PMN. MTT assay was conducted to assess cell viability. Although a trend was observed towards a small decrease in total viable cells when MDM were exposed to the PMN with the highest LL-37-induced secondary necrosis, this was not significant nor of sufficient magnitude to account for the pronounced effects on cytokine production. These data indicated that there was no significant change in functional cell numbers in response to peptide or dead PMN over the incubation period (Fig. 4.9). These findings suggest that the observed anti-inflammatory effects can be attributed to the effect of active suppressive effects of apoptotic or secondarily necrotic PMN, components release into the supernatant, or LL-37 peptide on phagocytosing MDM.

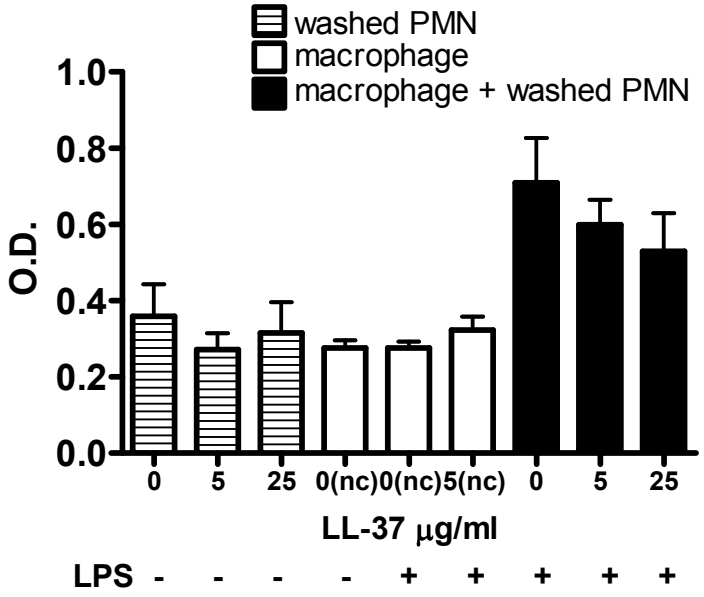


Figure 4.9 MDM do not die during the incubation with dead PMN or LL-37 peptide. Human PMN were incubated for 20 hr in the presence or absence of LL-37 at the concentrations indicated and used as washed cells resuspended in serum-free media (striped bars, PMN alone). MDM were then incubated with concomitant exposure to 10 ng/ml *E. coli* 0111:B4 LPS and PMN for 18 hr (black bars), or LPS without PMN (nc) in the absence/ presence of LL-37 (white bars). Supernatants were discarded followed by the addition of MTT reagent according to the manufacturer's instructions (volume of media: MTT solution = 5:1). Absorbance at 490nm wavelength was measured 1 hr after incubation; data are shown as mean + SEM for n = 3 donors.

4.2.6. Washing steps do not significantly alter the death modes of PMN.

In order to evaluate whether the washing and resuspending steps applied to some of the PMN could damage the integrity of PMN cell membrane and diminish the number of intact cells, FACS analysis (with Annexin V and PI staining) was used to study washed and unwashed PMN. Absolute numbers of apoptotic, necrotic, and live cells were determined by multiplying the percentage of each group of cells (determined by FACS) by total cell number (assessed by nucleocounter). Data showed that the procedures of washing and resuspending did not have substantial effects (Fig. 4.10), and a single washed step was continued for all study.

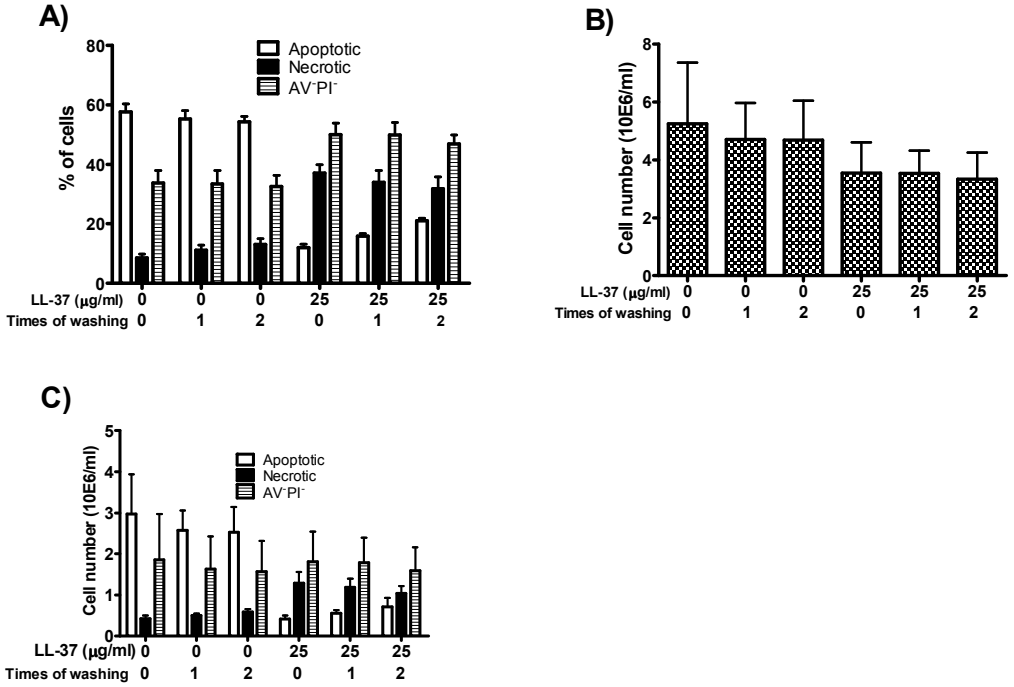


Figure 4.10 Washing steps do not have substantial effects on dead PMN. Human PMN were incubated for 20 hr in the presence or absence of LL-37 at the concentrations indicated and then were washed and resuspended once or twice in serum-free media. Cell death was assessed by FACS analyses of cells stained with Annexin V and PI (A). Total cell numbers were determined by nucleocounter (B). Absolute cell numbers of apoptotic, necrotic and AV^-PI^- cells were determined by multiplying percentages by total cell numbers (C); data are shown as mean + SEM for n=4 donors in triplicate.

4.2.7. Short exposures of LPS-stimulated MDM to LL-37-induced secondarily necrotic PMN retain anti-inflammatory effects.

One of the potential problems with allowing the apoptotic PMN to remain in contact with the MDM for 18 hr was the possibility of ongoing secondary necrosis throughout the period of incubation that could influence the results. Although Fadok *et al.* claimed that dead PMN were still anti-inflammatory to phagocytosing MDM even incubated for 18 hr when most of cells were supposed to be secondarily necrotic [13], experiments were conducted to confirm that. LPS-activated MDM were allowed to phagocytose apoptotic or LL-37-induced secondarily necrotic PMN, or were incubated with serum-free media in the absence of PMN for 1 hr, after which time residual cells were removed by washing, followed by the addition of fresh serum-free media and the second dose of 10 ng/ml LPS to ensure a continued activating pro-inflammatory stimulus. Supernatants were collected 17 hr after incubation to evaluate cytokine production. Although the total cytokine concentrations were significantly lower compared to 18 hr incubation without washing steps ($p \leq 0.001$), 1 hr of LPS-stimulated MDM to apoptotic or secondarily necrotic PMN decreased TNF-α production (Fig. 4.11B). However, in contrast to the 18 hr continuous exposure, the potentiating anti-inflammatory effects of LL-37-mediated secondary necrosis of PMN (at LL-37 25 μg/ml) were not observed in short exposure experiments (Fig. 4.11A vs. 4.11B or 4.11C vs. 4.11D). These data demonstrate that only a brief exposure to dead PMN is sufficient to have anti-inflammatory effects, but raise the possibility

that the more pronounced effects of PMN previously incubated with high concentrations of LL-37 may relate to the altered breakdown of these cells at later timepoints.

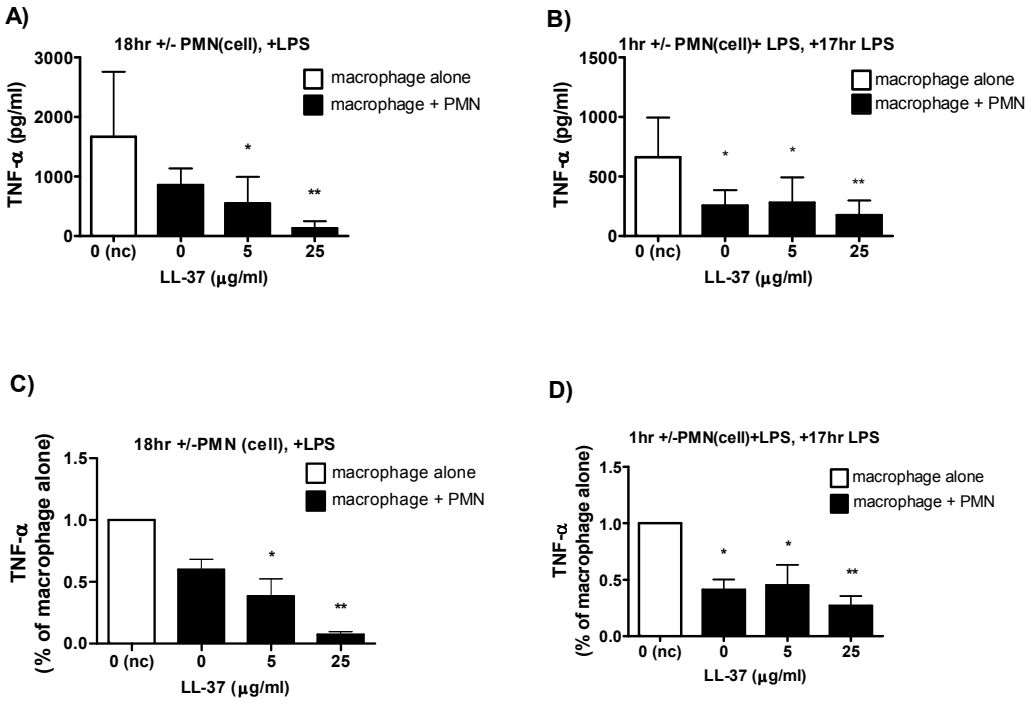


Figure 4.11 Short exposure of LPS-stimulated MDM to LL-37-induced secondarily necrotic PMN remains anti-inflammatory effect. Human PMN were incubated for 20 hr in the presence or absence of LL-37 at the concentrations indicated, washed and resuspended in serum-free media. MDM were then incubated with 10 ng/ml *E. coli* 0111:B4 LPS for 1 hr, in the presence or absence (nc) of PMN. Noningested PMN were then removed followed by the addition of fresh media and 10 ng/ml *E. coli* 0111:B4 LPS for another 17 hr (B, D), or PMN and LPS were left coincubated with MDM for a total of 18 hr (A, C). Supernatants were evaluated for cytokine responses. Significance was assessed by two-way ANOVA with Bonferroni's multiple comparison test; n=5 donors; *, $p \leq 0.05$; **, $p \leq 0.01$, compared with MDM exposed to LPS in the absence of PMN. Figure panel A & B demonstrated mean + SEM, whereas to correct for significant donor variation in absolute responses in these studies panels. C & D demonstrate the mean + SEM of the percentage of TNF-α production by MDM exposed to PMN, as a proportion of the total TNF-α production by activated MDM in the absence of PMN for each donor.

4.2.8. Serum inhibits the anti-inflammatory effects of apoptotic and LL-37-induced secondarily necrotic PMN.

To determine whether the loss of inhibitory activity of apoptotic PMN observed with they were used in their original supernatant (Fig. 4.8), related to factors secreted into the media by the dying cells, or to the presence of serum, 20 hr incubated PMN were washed and then resuspened in fresh media with or without 10% FCS. The anti-inflammatory properties of these cells were then evaluated against LPS-activated MDM. Apoptotic PMN completely lost their anti-inflammatory properties in the presence of 10% FCS (Fig. 4.12). In addition, the presence of 10% FCS had a very significant impact on the anti-inflammatory properties of LL-37-induced secondarily necrotic PMN (Fig. 4.12), with only the most necrotic cells (with secondary necrosis induced by 25 μg/ml LL-37) retaining any significant anti-inflammatory effects in the presence of serum. This data demonstrate an as yet unidentified inhibitory serum factor.

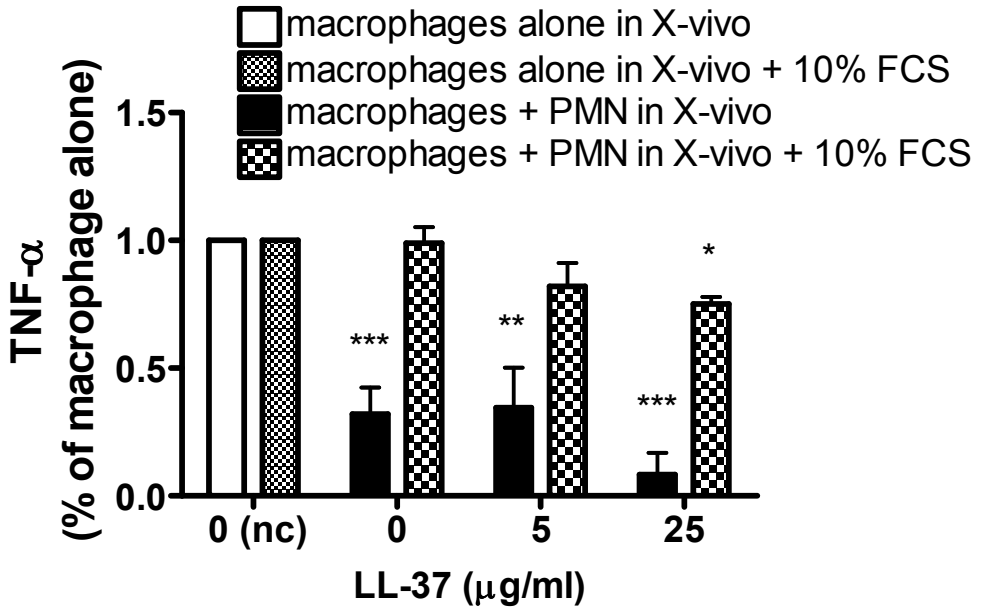


Figure 4.12 Serum inhibits the anti-inflammatory effects of apoptotic and LL-37-induced secondarily necrotic PMN. Human PMN were incubated for 20 hr in the absence or presence of LL-37 at the concentrations indicated. PMN were resuspened in X-vivo 10 media or X-vivo media with 10% FCS. MDM were exposed to these PMN with concomitant exposure to 10 ng/ml *E.coli* 0111:B4 LPS for 18 hr. Activated MDM alone without PMN, in the presence or absence of serum were also studied as controls. Supernatants were evaluated for cytokine responses. Significance was assessed by two-way ANOVA with Bonferroni's multiple comparison test; data are shown as mean + SEM as a proportion of the total TNF-α production by activated MDM in the absence of PMN for each donor; n = 3 donors; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared with activated MDM in the absence of PMN under the same conditions.

4.2.9. Partial LL-37 peptide-induced secondarily necrotic PMN are also anti-inflammatory to LPS-activated MDM, irrespective of anti-endotoxic activity.

In contrast to the original hypothesis, LL-37-induced secondarily necrotic PMN retain potent anti-inflammatory property. One possible confounding factor remaining in interpreting the data thus far could be that LL-37 has anti-endotoxic activity, and therefore any residual LL-37 remaining with the washed secondarily necrotic PMN could inhibit MDM activation and skew the result. Thus, three 22-mer partial peptides from the partial peptide library, for which I have evaluated the capability to inducing secondary necrosis, were selected (Fig. 3.19). The anti-endotoxic activity of these three peptides were characterised by TNF-α production by LPS-activated MDM exposed to these peptides (Fig. 4.13A white bars). N-terminal peptide p1, which induced no PMN secondary necrosis (Fig. 3.19), also had no direct anti-endotoxic effect (Fig. 4.13A). C-terminal peptides p2 and p3 induced significant PMN secondary necrosis (Fig. 3.19), but whereas p2 had direct anti-endotoxic effects, p3 did not have any significant anti-endotoxic properties (Fig. 4.13A). When 20 hr treated PMN in their original media were incubated with MDM concomitantly with LPS, their anti-inflammatory capacity clearly correlated with the anti-endotoxic properties of the peptide those PMN has been exposed to (Fig. 4.13A). Under these conditions, FCS in the original media inhibited the anti-inflammatory effects of the cells, and residual functional peptide in the media appears to be responsible for the anti-inflammatory effects of full-length LL-37

and partial peptide p2. However, in contrast, when these same apoptotic or secondarily necrotic PMN incubated previously for 20 hr with these peptides, were resuspended in fresh serum-free media, these cells were all capable of mediating a significant inhibition of LPS-induced MDM TNF-α production, identical to untreated control apoptotic cells (Fig. 4.13B). These data demonstrated a) that no negligible functional residual peptide is carried over after washing the dead PMN, b) that peptide-induced secondarily necrotic PMN (here induced by LL-37, p2, or p3) had anti-inflammatory activity against LPS-stimulated MDM comparable to apoptotic PMN (controls or p1 treated), and c) that this inflammation-dampening activity is independent of anti-endotoxic property of peptides.

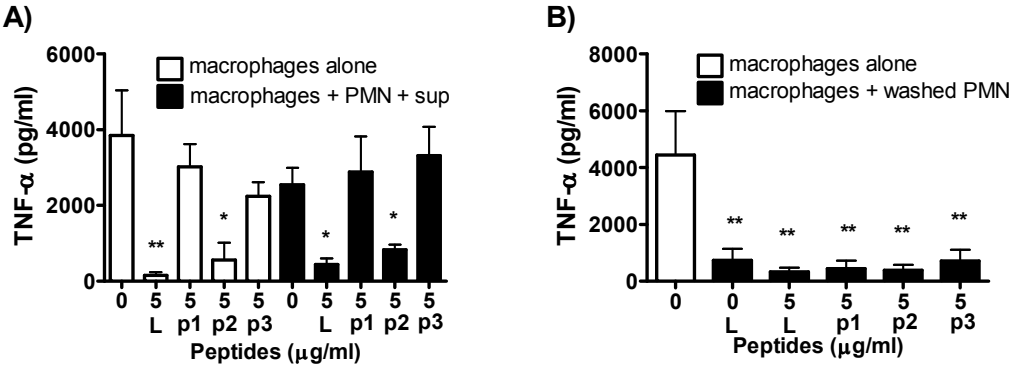


Figure 4.13 The anti-inflammatory properties of LL-37-induced secondarily necrotic PMN are independent of the anti-endotoxic activity of peptides. Human PMN were incubated for 20 hr in the presence or absence of LL-37 (L) or partial peptides (p1, p2, or p3) at the concentrations indicated. PMN were washed and resuspended in X-vivo 10 media (B), or used unwashed in the IMDM +10% FCS media supernatant, in which they had been incubated overnight (A). MDM were incubated alone or with these PMN with concomitant exposure to 10 ng/ml *E.coli* 0111:B4 LPS for 18 hr. LPS-activated MDM without PMN were also stuided, in the presence or absence of peptides, to evaluate direct anti-endotoxic activity of peptides (white bars in Fig A). Supernatants were evaluated for cytokine responses. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test; data are shown as mean + SEM for n = 4 donors; *, $p \leq 0.05$; **, $p \leq 0.01$, compared with activated MDM in the absence of PMN.

4.2.10. LL-37-induced secondarily necrotic PMN are also anti-inflammatory to rhCD40L/INF-γ-activated MDM.

To further confirm that the anti-inflammatory effects of LL-37-induced secondarily necrotic PMN were independent of the anti-endotoxic effects of the peptide, and had broader anti-inflammatory properties, rhCD40L and INF-γ treatment were used as an alternative activating signal for MDM (Fig. 4.14). LL-37 had no significant capacity to directly inhibit the MDM-activating properties of this stimulus (Fig. 4.14A), and indeed showed a non-significant trend towards potentiating the activation at higher concentrations of LL-37. However, both apoptotic PMN and LL-37-induced secondarily necrotic PMN were capable of significantly inhibiting MDM activation ($p \leq 0.001$), as assessed by pro-inflammatory TNF-α cytokine production as the results observed in LPS-activated MDM. These results demonstrate equivalence with the results observed in LPS-activated MDM, and further confirm that the anti-inflammatory effects of these LL-37-induced secondarily necrotic PMN are not dependent upon the anti-endotoxic effects of the peptide.

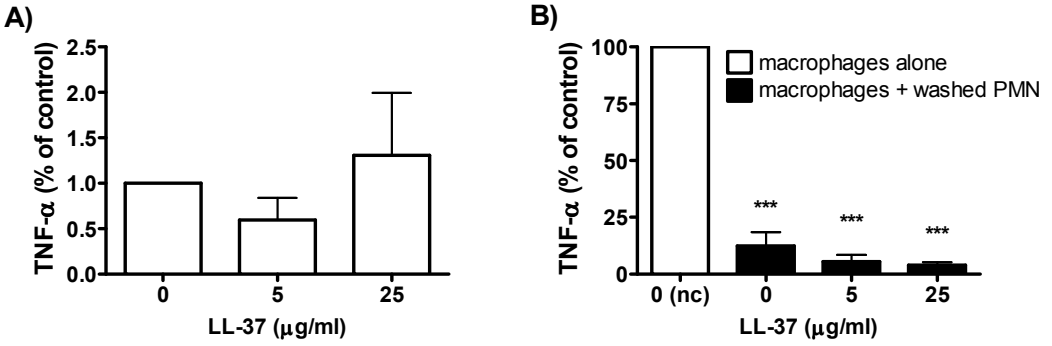


Figure 4.14 LL-37-induced secondarily necrotic PMN are anti-inflammatory to rhCD40L/INF-γ-activated MDM. A) MDM were activated with 3 μg/ml rhCD40L +5 ng/ml rhINF-γ for 18 hr in the presence or absence of LL-37 at the stated concentrations. B) Human PMN were incubated for 20 hr in the absence or presence of LL-37 at the concentrations indicated. PMN were washed and resuspened in serum-free X-vivo 10 media. MDM were incubated with these PMN, with concomitant exposure to 3 μg/ml rhCD40L + 5 ng/ml rhINF-γ for 18 hr. Supernatants were evaluated for cytokine responses. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test; data are shown as mean + SEM as a proportion of the total TNF-α production by activated MDM in the absence of PMN for each donor, n = 3 donors; ***, $p \leq 0.001$, compared with activated MDM in the absence of PMN under the same conditions.

4.2.11. LL-37-induced secondary necrosis of PMN can release cytoplasmic and granule contents.

As previously described (Fig. 3.22), LL-37 at higher concentrations (> 25 μg/ml) can induce the release of PMN granule contents. MPO release was not detected above background at 20 hr time point following exposure to ≤ 10 μg/ml LL-37 (concentrations at which substantial secondary necrosis occurred, Fig. 4.15D) or in responses to scrambled LL-37. However, LL-37 at higher concentrations (≥ 25 μg/ml) induced release of MPO from azurophilic granules. In contrast, in collaboration with Dr. Robert Gray, we demonstrated that 20 hr PMN exposed to 5 μg/ml LL-37, at which concentration substantial secondary necrosis was induced as evaluated by PI permeability, induced significant increase of a cytoplasmic protein calprotectin release ($p \leq 0.05$) (Fig. 4.15A). These data suggest that LL-37 at lower concentration (< 10 μg/ml) can permeabilise cell plasma membrane, leading to the leakage of cytoplasmic contents, whereas LL-37 at higher concentration (>25 μg/ml) not only breaks up cytoplasmic membrane but also permeabilises granules. In addition to evaluating granule content release at 20 hr (when secondary necrosis was significantly induced by LL-37, but negligible in control cells), I evaluated the effect after 38 hr incubation to include full period of incubation of PMN in the MDM stimulation assay (Fig. 4.15C). In contrast to 20 hr incubation, PMN cultured for 38 hr have increased MPO release, even under control conditions. This is to be expected given the extent of necrosis by this timepoint, even in the control cells. Indeed the level of secondary necrosis observed at 38 hr was equivalent in all the samples irrespective of peptide

treatment (Fig. 4.15E). Intriguingly, LL-37 at higher concentration (≥ 25 μg/ml) significantly induced significantly greater MPO release at 38 hr ($p \leq 0.01$) than control cells, even although the necrotic rate was equivalent, as assessed by FACS study (Fig. 4.15E). These data indicate that higher concentrations of LL-37 are able to destabilise granules and lead to the release of their intracellular contents. Whereas, untreated apoptotic cells, despite gradually undergoing secondary necrosis and release of their granules into culture media, remain intact for longer.

A)

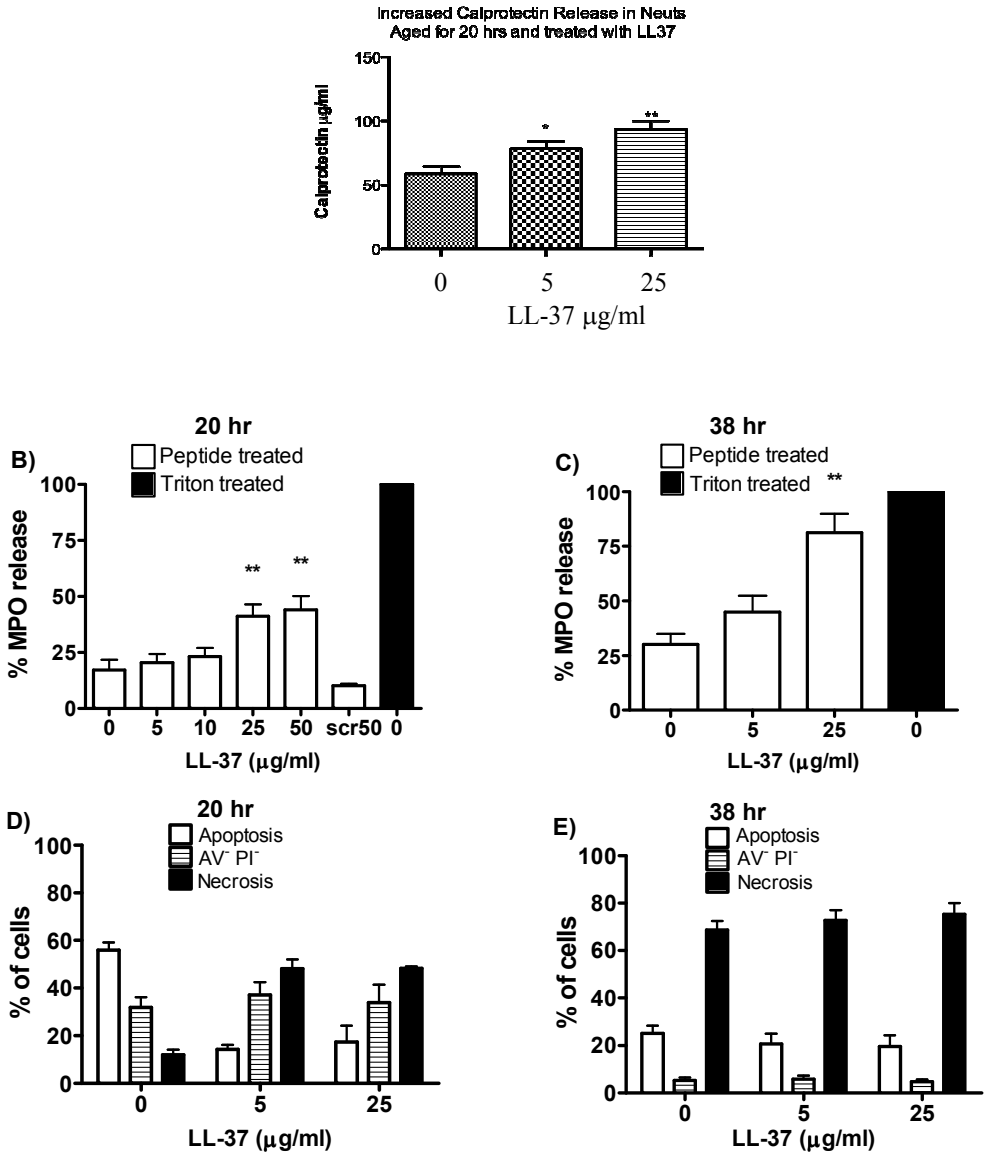


Figure 4.15 LL-37-induced secondary necrosis of PMN can release cytoplasmic and granule contents. A) Isolated human PMN were incubated for 20 hr without or with exposure to LL-37 at concentrations stated in the presence of 10% FCS. The concentration of calprotectin in the supernatant was evaluated by ELISA by Dr. R. Gray. B-E) Isolated human PMN were incubated for 20 hr or 38 hr over a range of concentrations of LL-37 or scrambled LL-37 (scr50). The concentrations of MPO in the supernatant were evaluated as a percentage of total MPO release after lysis of fresh control MPO using 0.1% Triton X-100 (B, C). PMN death was also assessed by FACS analysis (D, E). Significance of protein release was assessed by one-way ANOVA with Bonferroni's multiple comparison test; data are shown as mean + SEM for n = 4 donors (calprotectin), or n = 7 donors (MPO); *, $p \leq 0.05$ **, $p \leq 0.01$, compared with untreated control PMN.

4.2.12. "Freeze-and-thaw-induced" primary necrotic PMN are anti-inflammatory to phagocytosing MDM.

Necrotic cells are generally considered to pose a danger to the immune system, resulting in pro-inflammatory responses. Since secondary necrosis of PMN were anti-inflammatory in my system, the contrasting inflammatory potential of primary necrotic PMN compared to apoptotic PMN as previously proposed [457], was investigated. Freeze-and-thaw-induced primary necrotic PMN were incubated with LPS-stimulated MDM in the absence or in the presence of 10% FCS. In contrast to our original expectation, primary necrotic PMN were anti-inflammatory both in serum-free and in serum-present media (Fig. 4.16). The data are compatible to a study published recently in which dying and necrotic PMN were found to be anti-inflammatory secondary to the release of anti-inflammatory peptide α-defensins [387]. Apoptotic, LL-37-mediated secondarily necrotic, and freeze-thaw-induced primary necrotic PMN were therefore all anti-inflammatory to phagocytosing MDM in this *in vitro* system, suggesting that the inflammatory consequences of dead PMN are more complicated than were proposed before.

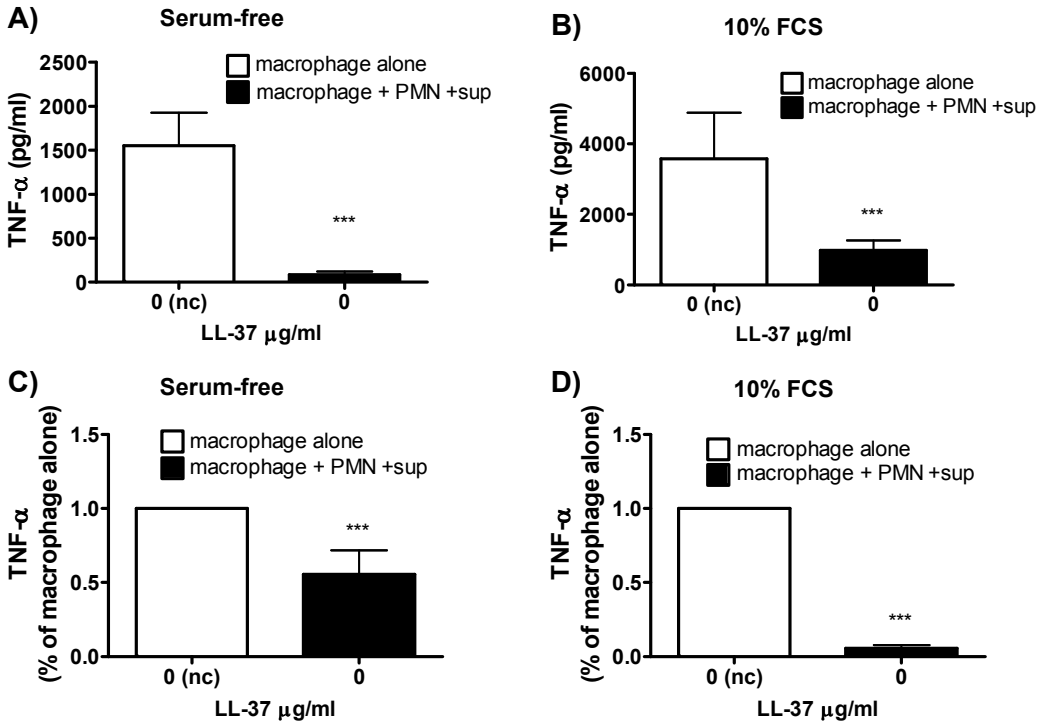


Figure 4.16 Primary necrotic PMN are anti-inflammatory to activated MDM both in the absence or presence of serum. Fresh human PMN were suspended in IMDM in the absence of serum (A, C) or in the presence of 10% FCS (B, D). PMN were frozen and thawed for 3 cycles to induce primary necrosis. MDM were then incubated with these primary necrotic PMN with concomitant exposure to 10 ng/ml *E.coli* 0111:B4 LPS for 18 hr. Activated MDM without PMN (nc), in the presence or absence of serum, were also studied as controls. Supernatants were evaluated cytokine responses. Data were presented as raw TNF-α concentrations (A, B) and as percentages of TNF-α production by activated MDM (C, D); data are shown as mean + SEM for n ≥ 12 donors. Significance was assessed by Student's t-test compared with activated MDM in the absence of PMN; ***, $p \leq 0.001$.

4.2.13. The activity of α-defensins is inhibited by serum components, whereas LL-37 retains its anti-endotoxic property in the presence of serum.

HNP, cathelicidins, and other PMN granule components are expected to be released during the process of granule membrane permeabilisation in necrosis. Whether these components, released from necrotic PMN, could be responsible for effects observed, was unknown. Thus the anti-inflammatory activity of HNP and LL-37 were evaluated in the presence or absence of serum. Both of peptides have potent anti-endotoxic activity in the absence of serum (Fig. 4.17A, C). However, unlike LL-37, for which anti-endotoxic function was retained in the presence of serum (Fig. 4.17D), the anti-endotoxic effects of HNP were inhibited by unknown serum factors (Fig. 4.17B). These findings suggest that both HNP and LL-37 possess anti-endotoxic capability while demonstrating that this activity is serum-sensitive in HNP.

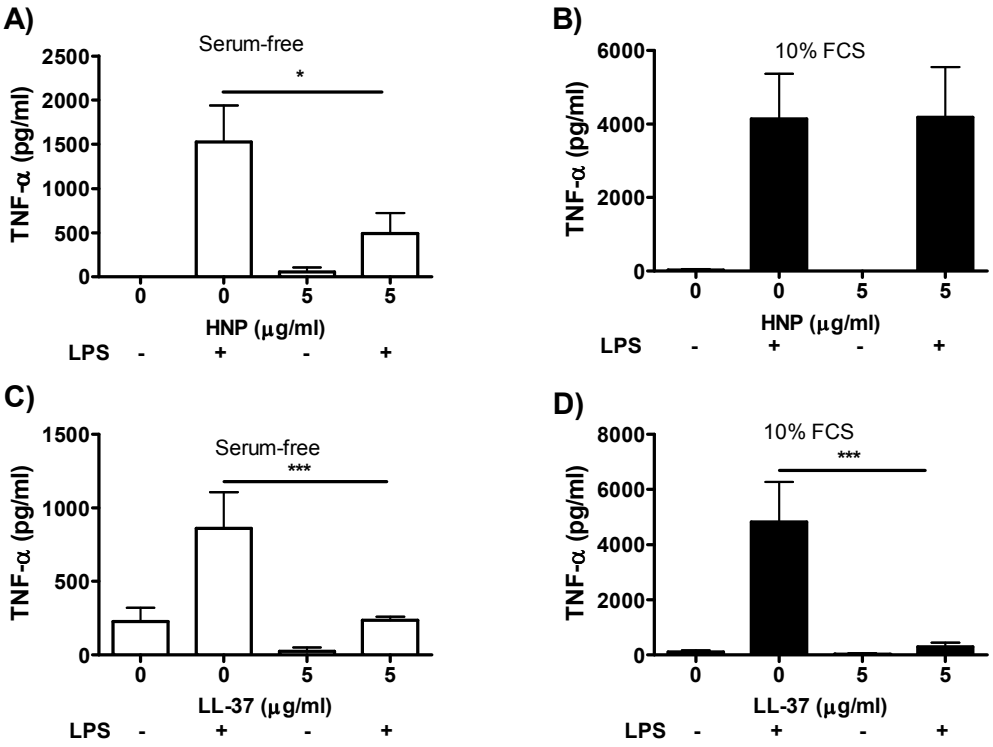
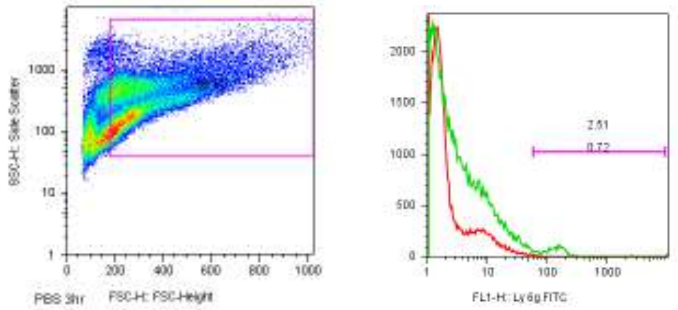


Figure 4.17 The activity of α-defensins is inhibited by serum components, whereas LL-37 retains its anti-endotoxic property in the presence of serum. MDM were untreated/ treated with 5 μg/ml HNP (A, B) or LL-37 (C, D) concomitantly exposure to 10 ng/ml *E.coli* 0111:B4 LPS for 18 hr in the absence (A, C) or presence of 10% FCS (B, D). Untreated MDM and peptide-exposed MDM were also studied. Supernatants were evaluated cytokine responses; n = 3 donors. Significance was assessed by two-way ANOVA; data are shown as mean + SEM; *, $p \leq 0.05$; ***, $p \leq 0.001$, compared with LPS-activated MDM.

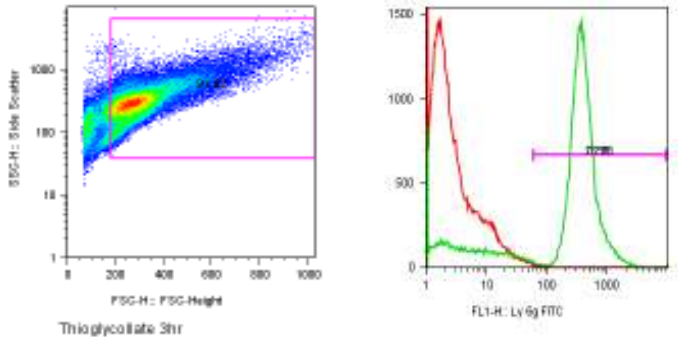
4.2.14. Thioglycollate-elicited sterile peritonitis induces PMN influx.

LL-37-induced secondary necrosis of apoptotic PMN is not pro-inflammatory in *in vitro* studies, thus the question arose of whether secondarily necrotic PMN are anti-inflammatory *in vivo*. The thioglycollate-induced sterile peritonitis mouse model was chosen as a well-characterised model, with significant PMN influx, in which the effects of LL-37 on these recruited PMN would be investigated. In order to establish this model, mice were intraperitoneally injected with 0.5 ml 10% thioglycollate or PBS as control, and peritoneal lavages were performed, either 3 hr or 24 hr after initial thioglycollate injection. PMN in peritoneal lavage were assessed by FACS to determine the percentage of cells expressed Ly6G, a specific protein expressed on mouse peripheral PMN. Data revealed that thioglycollate effectively induced PMN influx at 3 hr and 24 hr, providing an acute inflammatory model for further LL-37 studies (Fig. 4.18 & 4.19).

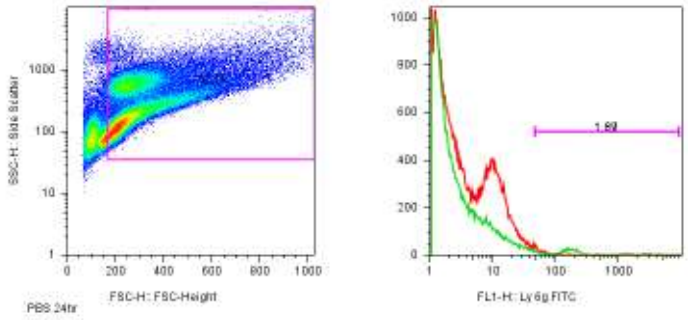
(A) PBS 3hr



(B) Thioglycollate 3hr



(C) PBS 24hr



(D) Thioglycollate 24hr

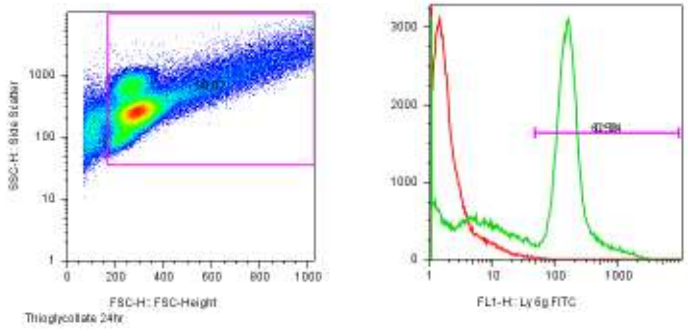


Figure 4.18 Representative FACS plots. Dot plots represent cell population selected for analysis (left-sided panel). Histograms represent the percentage of cell with positive Ly6G expression (Green graph) and antibody isotype control (red graph) (right-sided panel).

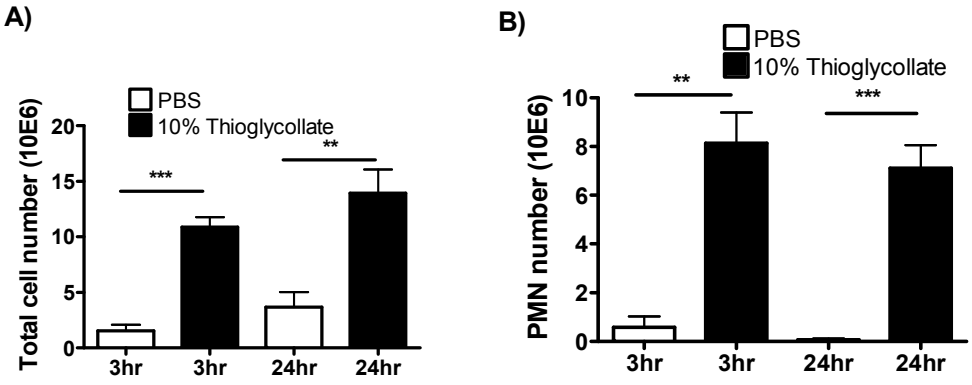


Figure 4.19 Thioglycollate induces PMN influx in sterile peritonitis model. Six to eight week old Balb/c mice were injected intraperitoneally with 0.5 ml 10% thioglycollate or PBS as control, and the peritoneal lavages were performed after 3 hr and 24 hr. Total cell numbers were counted by nucleocounter and the percentages of PMN in peritoneal fluids were assessed by FACS. PMN numbers were then determined by multiplying total cell numbers by percentages of PMN; data are shown as mean + SEM for $n \geq 4$. Significance was assessed with Student *t*-test to compare PBS and thioglycollate injection; **, $p \leq 0.01$; ***, $p \leq 0.001$.

4.2.15. LL-37 is anti-inflammatory during sterile thioglycollate-induced peritoneal inflammation

In order to evaluate the effects of LL-37 on the induction of PMN influx during sterile inflammation, 25 μg/ml LL-37 was injected simultaneously with 0.5 ml PBS or 0.5 ml 10% thioglycollate to mouse peritoneum. Three hours after injection, peritoneal lavages were performed and the number of PMN in peritoneal fluids was evaluated. As report in the literature [19], LL-37 had chemotactic property, demonstrating 2-3 fold greater peritoneal lavage total cell and PMN counts in LL-37 + PBS-injected mice, when compared to PBS-only mice (although this did not reach statistical significance). Interestingly, in contrast, LL-37 diminished PMN influx in thioglycollate-induced peritonitis (even though this did not reach statistical significance) (Fig. 4.20), demonstrating its anti-inflammatory properties. In addition, LL-37 significantly inhibited pro-inflammatory cytokine (IL-6 and MCP-1) production ($p \leq 0.05$), and diminished TNF-α production in thioglycollate-mediated inflammation (Fig. 4.21). These data demonstrate complex *in vivo* anti-inflammatory immunomodulatory functions of LL-37, with modulation of immune effector cells influx and inhibition of inflammatory cytokine responses during inflammation.

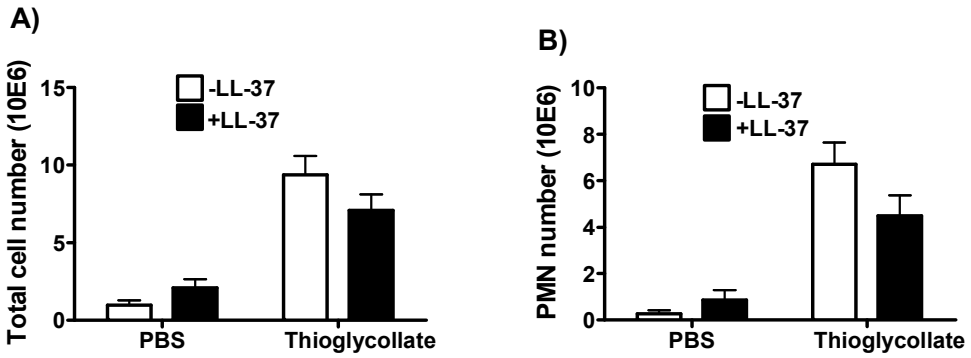


Figure 4.20 LL-37 modulates inflammatory cell numbers *in vivo* during thioglycollate-induced inflammation. Six to eight week old Balb/c mice were intraperitoneally injected with 0.5 ml 10% thioglycollate or 0.5 ml PBS in the presence or absence of 25 μg/ml LL-37 (12.5 μg peptide in 0.5 ml PBS or thioglycollate as mentioned). The peritoneal lavages were performed 3 hr after injection. Total cell numbers were counted by nucleocounter (A) and the number of PMN was determined by FACS and total cell number (B); data are shown as mean + SEM for n = 6 mice. Significance was assessed with two-way ANOVA with Bonferroni's multiple comparison tests, compare mice with or without LL-37 exposure under the same reagent injection (PBS/ Thioglycollate).

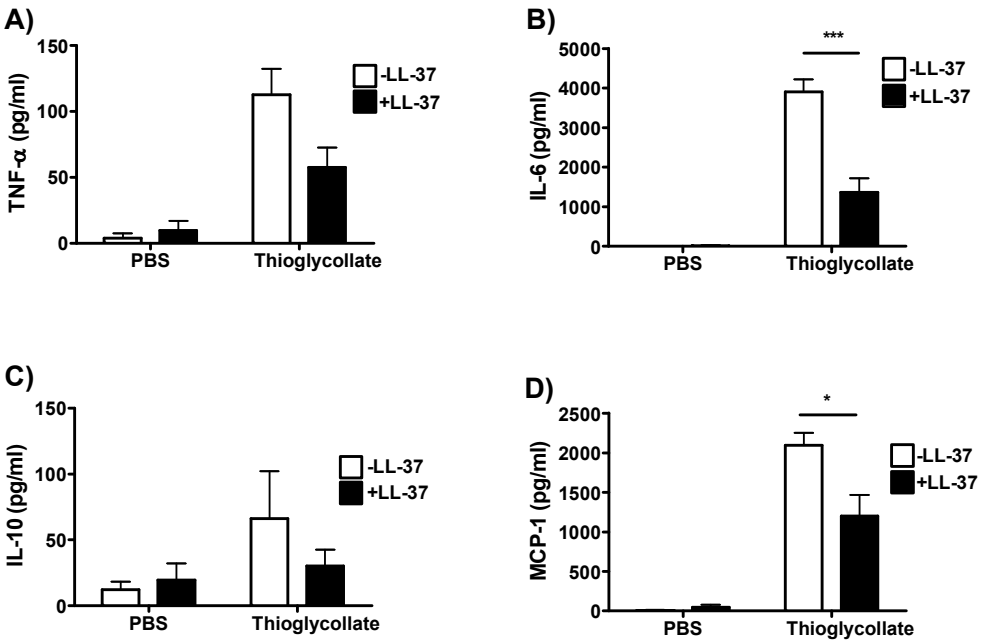


Figure 4.21 LL-37 inhibits pro-inflammatory cytokine production during thioglycollate-induced sterile inflammation. Six to eight week old Balb/c mice were injected intraperitoneally with 0.5 ml 10% thioglycollate or 0.5 ml PBS in the presence or absence of 25 μg/ml LL-37 (12.5 μg peptide in 0.5 ml volume). The peritoneal lavages were collected 3 hr after injection and cytokine production was evaluated by CBA analysis; data are shown as mean + SEM for n = 6 mice. Significance was assessed with two-way ANOVA with Bonferroni's multiple comparison tests, compared mice with or without LL-37 exposure under the same reagent injection (PBS/ thioglycollate); *, $p \leq 0.05$; ***, $p \leq 0.001$.

4.2.16. LL-37-induced secondarily necrotic PMN are anti-inflammatory *in vivo*.

LL-37-mediated secondary necrosis of apoptotic PMN is not pro-inflammatory *in vitro*, and PMN exposed to higher concentrations of LL-37 have even more pronounced anti-inflammatory effects. To determine the inflammatory impacts of the secondary necrosis of PMN in inflammation *in vivo*, overnight incubated apoptotic human PMN, LL-37-induced secondarily necrotic PMN, or control media were administered in thioglycollate-elicited sterile peritonitis or PBS-injected mice. Three hours after injection peritoneal cells were harvested and analysed by FACS. Consistent with *in vitro* findings, apoptotic and LL-37-induced secondarily necrotic cells were anti-inflammatory in thioglycollate-induced peritonitis, demonstrating a trend towards inhibition of PMN influx (Fig. 4.22) and significantly inhibiting TNF-α production (Fig. 4.23) ($p \leq 0.05$). However, in these studies the effects of apoptotic and LL-37-induced secondarily necrotic cells were largely comparable. These data indicate that in sterile inflammation, secondarily necrotic PMN retain the anti-inflammatory properties of apoptotic cells, rather than becoming proinflammatory.

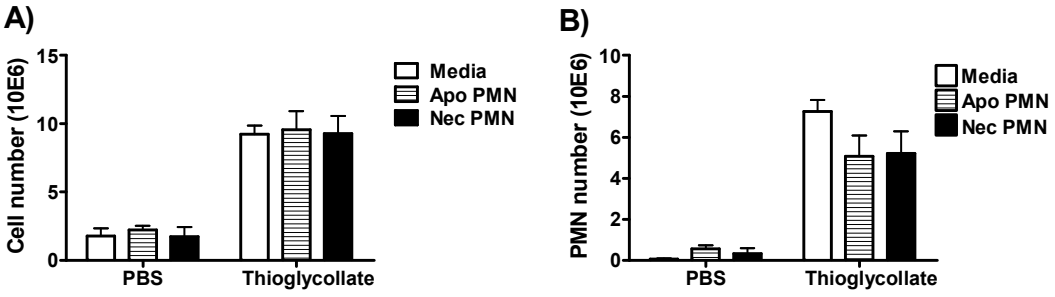


Figure 4.22 LL-37-induced secondarily necrotic PMN diminish PMN influx in thioglycollate-induced sterile peritonitis. Six to eight week old Balb/c mice were injected intraperitoneally with apoptotic human PMN, LL-37 (25 μg/ml)-induced secondarily necrotic PMN, or media concomitantly with 0.5 ml 10% thioglycollate or 0.5 ml PBS. The peritoneal lavages were collected 3 hr after injection. Total cell numbers were counted by nucleocounter (A) and PMN numbers were determined by FACS and total cell number (B); data are shown as mean + SEM for n = 4 mice. Significance was assessed with two-way ANOVA with Bonferroni's multiple comparison post-tests used to compare mice with or without PMN injection under the same conditions (PBS/ thioglycollate).

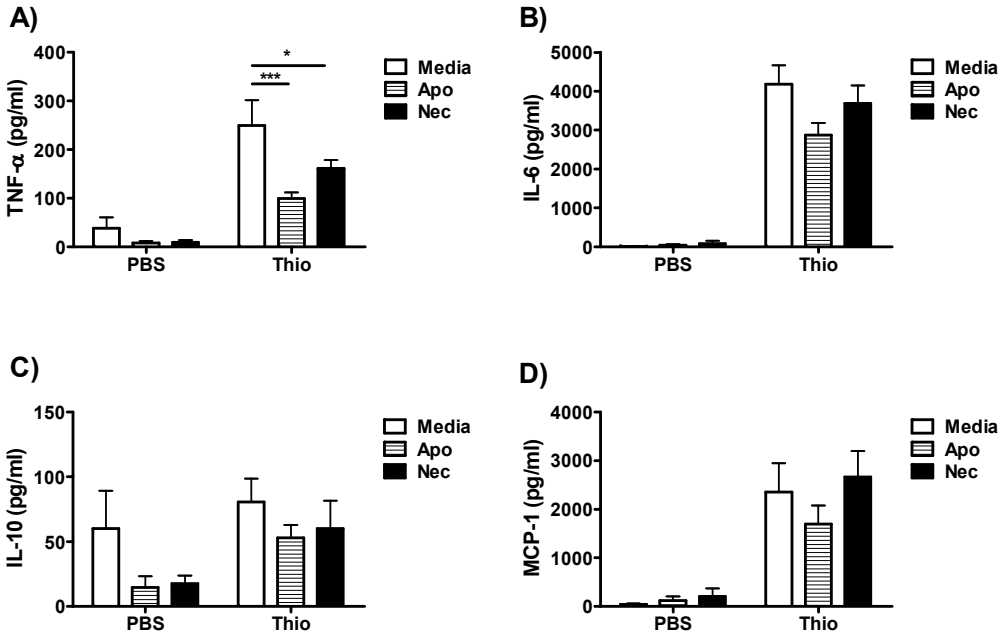


Figure 4.23 LL-37-induced secondarily necrotic PMN are anti-inflammatory in thioglycollate-induced sterile peritonitis. Six to eight week old Balb/c mice were injected intraperitoneally with apoptotic human PMN, LL-37 (25 μg/ml)-induced secondarily necrotic PMN, or media concomitantly with 0.5 ml 10% thioglycollate or 0.5 ml PBS, and mice without PMN injection were used as controls. The peritoneal fluids were collected 3 hr after injection and cytokines were evaluated by CBA analysis; data are shown as mean + SEM for n = 4 mice. Significance was assessed with two-way ANOVA with Bonferroni's multiple comparison post-tests used to compare mice with and without PMN injection under the same conditions (PBS/ thioglycollate); *, $p \leq 0.05$; ***, $p \leq 0.001$.

4.3. Discussion

In comparison to the well characterised effects of apoptosis, studies on death by secondary necrosis are still scarce. Although secondary necrosis has been implicated as a major pathogenic mechanism in several inflammatory disorders [509], the biological significance of this cell death remains unclear. I have demonstrated that LL-37, a multi-faceted modulator of inflammation and immune responses, is a potent inducer of PMN secondary necrosis. Given the conventional views, in which apoptosis is regarded as anti-inflammatory while necrosis is considered as a pro-inflammatory event, were true, LL-37-mediated secondary necrosis of PMN was expected to be pro-inflammatory in nature. However, my data contrast with these initial expectations, suggesting that the inflammatory consequences determined by the various modalities of cell death are more complicated than initially proposed.

It was proposed that different forms of cell death affected the efficiency of phagocytosis, with phagocytes may recognising and ingesting dead cells by different mechanisms, dependent upon their mode of death. My data fail to support this assumption, demonstrating similar phagocytic rates by MDM in uptake of apoptotic and secondarily necrotic PMN. Two methods were used to ensure the accuracy of assessment. Although it is noted that microscopy-based enumeration has generally lower phagocytic rates than FACS-based assessment (probably due to the extra washing steps in the former method removing the MDM having phagocytosed the most PMN, and the potential inclusion of adherent, but not

internalised PMN by FACS), the overall uptake is consistent, irrespective of the induction of secondary necrosis. The possibility that MDM may use different mechanisms to engulf apoptotic and secondarily necrotic PMN can not be excluded by this experimental approach, but these data suggest the possibility that apoptotic PMN induced to undergo secondary necrosis in response to LL-37 are recognised by the same mechanisms as apoptotic PMN.

In addition, it should be noted that the influences of LL-37 itself on the process of phagocytosis could not be ruled out. In phagocytic studies, MDM were incubated with LL-37-mediated secondarily necrotic PMN with concomitant exposure to the original cultured media containing residual peptide. As the presence of cationic amino acids can inhibit recognition mechanisms of macrophages [510], LL-37, which is a peptide with positive charges, may inhibit the cell clearance. On the other hand, Davidson *et al.* demonstrated that LL-37 could significantly enhanced phagocytosis by professional phagocytes [159]. However, in the absence of discernable differences in my studies, I would conclude that apoptotic PMN induced to secondary necrosis by LL-37 do not alter the efficiency of phagocytosis, possessing similar clearance rates compared with apoptotic cells.

In keeping with the pioneer study, phagocytosis of apoptotic PMN (in contrast to immunoglobulin G-opsonised apoptotic cells) actively inhibited the production of TNF-α, IL-10, and IL-8 by MDM. However, in contrast to initial expectations, ingestion of LL-37-induced secondarily necrotic PMN was not a pro-inflammatory

event, and did not promote the pro-inflammatory cytokine release. Furthermore, these secondarily necrotic PMN were actively anti-inflammatory, and the anti-inflammatory effects were enhanced in response PMN with the most extensive secondary necrosis. These data suggest functional equivalence, irrespective of membrane integrity, of apoptotic PMN and LL-37-induced secondarily necrotic PMN in this system, with an additional anti-inflammatory effect dependent upon secondary necrosis induced by high concentrations of LL-37.

The exact mechanism by which dead PMN inhibit the activation of MDM is still unknown. Previous reports suggested the possible role of anti-inflammatory cytokines TGF-β1 and IL-10 in suppression of inflammatory responses [13, 459]. However, other studies suggested that cell contact was the main determinant of anti-inflammatory consequence [462, 463]. Although my experiments do not specifically address the importance of these mediators and cell interaction in inflammation-dampening results, it is clear that this peptide can modulate inflammatory responses by altering the mode of PMN death.

Short exposure of MDM to apoptotic or LL-37-induced secondarily necrotic PMN was sufficient to dampen pro-inflammatory reaction. However, the potentiated anti-inflammatory responses of secondary necrosis in 18 hr continuous exposure were not observed in short exposure. In conjunction with my observations on LL-37-mediated granule content release, this suggest that secondary necrosis induced by higher concentrations of LL-37 results in steady releases of anti-inflammatory granule contents, whereas short exposure to the dead

cells does not allow this extra anti-inflammatory reinforcement. The data also suggest that from the earliest stages after engulfment MDM produce mediators to promote their later cytokine production, with removal of these mediators resulting in diminished total cytokine production.

Unknown serum factors were found to inhibit the anti-inflammatory effects of apoptotic PMN and secondarily necrotic PMN (to a variable extent, dependent upon the concentrations of LL-37 used to induce secondary necrosis), probably through blocking the undefined anti-inflammatory activity of cellular components (eg. cell membrane, cytoplasmic proteins, granule contents) or of mediators released from dead cells (eg. cytokines). As serum contains abundant proteins which function as opsonins [422, 462, 511-513], bridging molecules, binding proteins, and proteases, it can be expected that the presence of serum can alter cell engulfment, degrade the activity of soluble mediators, inhibit anti-inflammatory molecules, and modulate the eventual inflammatory outcomes. To further characterise the inhibitory factors of serum, fractionated serum could be used in the future to identify key proteins influencing the inflammatory results of dead PMN.

The anti-inflammatory effects of LL-37-induced secondary necrosis could have been the result of the anti-endotoxic effects of residual LL-37 in the media, or even residual peptide retained on PMN membranes. Partial peptide with poor anti-endotoxic capability and CD40L/INF-γ were used to exclude these possibilities. Although recent data revealed that LL-37 could also inhibit cellular responses to INF-γ in monocytic cells, showing suppression of cell activation and pro-

inflammatory cytokine production [514], my results did not support these observations. LL-37 peptide did not inhibit pro-inflammatory cytokine production, by CD40/INF-γ-stimulated MDM, but LL-37-induced secondary necrosis of apoptotic PMN did have anti-inflammatory effects. The data indicate that LL-37-altered PMN death possess modulatory activities of inflammation which is independent of the anti-endotoxic property of the peptide.

In contrast to original expectations, freeze-and-thaw-induced necrosis of PMN was not pro-inflammatory in my studies, an observation which was also reported by Fadok *et al.* although their results were inconsistent [13]. Although Fadok *et al.* later demonstrated that necrotic PMN could stimulate production of cytokines such as IL-8, TNF-α, and IL-10 by phagocytosing macrophages through releasing pro-inflammatory proteases [269], using separation of necrotic PMN into membrane and soluble fractions, they showed that the PMN membranes behaved like apoptotic cells. In addition, the authors demonstrated that late apoptotic PMN released very little elastase and the anti-inflammatory effects of the membrane could predominate over pro-inflammatory properties [269].

Another report indirectly supports the anti-inflammatory property of necrotic PMN, showing that necrotic PMN do not release pro-inflammatory mediator HMGB1, in contrast to apoptotic cells from as most tumour cell lines [515]. These disparities in studies of the inflammatory impacts of necrosis may be due to variations in the necrotic cell preparation and the unique properties of specific cell types used. As mentioned above, PMN can release anti-inflammatory peptides

during death [387], which may explain why this type of cell has an anti-inflammatory propensity, regardless of the mode of cell death. Also, it may be possible that apoptotic process degrades pro-inflammaotry PMN proteases such as elastase, and oxidises pro-inflammatory mediators, such as HMGB-1 [353, 354], but preserves the activity of anti-inflammatory peptides, resulting in the tendency to inhibiting inflammation of late apoptotic/ secondarily necrotic PMN.

During the process of inducing secondary necrosis by LL-37, PMN which were exposed to higher concentrations of LL-37 release greater amount of granule contents (eg. HNP, LL-37), which may enhance their anti-inflammatory effects. This proposal is supported by recent work from Dr. Mohini Gray's group, demonstrating the anti-inflammatory potential of necrotic PMN, and that α-defensins in the membrane-free fraction of necrotic PMN are potent anti-inflammatory mediators, in the absence of serum [387]. Interestingly, in keeping with my data, inhibition of the effects of α-defensins in these membrane-free fractions from necrotic PMN completely inhibited the anti-inflammatory properties against INFγ/CD40L (agsinst which stimulus α-defensins have potent activity), but only partially inhibited the anti-inflammatory effects against LPS. This suggests a primary role for α-defensins against the former stimulus, but roles for both LL-37 and α-defensins against the latter stimulus. However, my studies demonstrate that the activity of HNP is inhibited by the presence of serum, and only PMN releasing the greatest amount of granule contents (and therefore the most "serum resistant" LL-37) retained the anti-inflammatory capability in the presence of serum. The induction of granule content release from secondarily necrotic PMN and primary

necrotic PMN provide a reasonable hypothesis for their potent anti-inflammatory property.

However, studies increasingly demonstrate that the anti-inflammatory impact of apoptosis is dominant over the pro-inflammatory effect of necrosis [448, 462, 463]. It is technically challenging to separate dead cells into pure populations for studying, however, such mixtures should reflect real inflammatory conditions, in which we can expect the existence of mixed populations of dead cells. Nevertheless, according to this hypothesis, the anti-inflammatory properties of mixtures of apoptotic and LL-37-induced secondarily necrotic PMN (as found in my experimental system), might be predicted to be equivalent to the anti-inflammatory effects of even a minority of apoptotic cells. Although this could provide a possible explanation for the apparent equivalence of apoptotic and secondarily necrotic PMN, such a hypothesis would not explain the enhanced anti-inflammatory properties of the most secondarily necrotic PMN.

Irrespective, the anti-inflammatory nature of LL-37-induced secondarily necrotic PMN, which raises another interesting question: is LL-37-induced secondary necrosis immunogenic? In addition to macrophages, DC are another type of APC with specific response to dying cells. *In vivo* observations suggest that there is a balance between macrophage- and dendritic cell-mediated clearance of dead cells. Macrophages may maintain homeostasis by promote the silent disposal of dying cells, whereas clearance by dendritic cells favours an immune responses [458, 484]. LL-37-modulated PMN death may have significant impacts on immune responses

once phagocytosed by DC or non-professional phagocytes. Cancer research reveals that post-apoptotic tumors are more immunogenic than early apoptosis and DC have higher efficiency to cross-present them [516], while others state that only certain extent of necrosis can release DC maturation factor and post-apoptotic cells behave more like early-apoptotic cells [14]. The immunogenicity of LL-37-mediated secondary necrosis may acquire physiological significances but was beyond the scope of this thesis.

The sterile peritonitis model demonstrates that LL-37-induced secondary necrosis is anti-inflammatory *in vivo*, as predicted by *in vitro* data. This model system induces inflammatory responses with a swift PMN influx. Although the model is too simple to reflect complexity of specific disease states, in which many mediators are concomitantly present, it provides interesting initial confirmation of the *in vitro* observations. Although cell death was evaluated to determine whether injection of LL-37 could induce secondary necrosis of recruited apoptotic mouse PMN, the rates of apoptosis detectable were too low (data are not shown) to address this question. Thus, human PMN exposed to LL-37 were used as a secondarily necrotic PMN stimulus. My data showed that LL-37-induced secondarily necrotic PMN behaved in an anti-inflammatory manner comparable to that of apoptotic PMN in the mouse peritonitis model, the possibility that cells from different species will result in different inflammatory responses cannot be ruled out.

Under physiological conditions, PMN undergo spontaneous apoptosis and are swiftly removed by phagocytes, probably minimising the number of apoptotic cells available for LL-37-induced secondary necrosis. However, under pathological conditions, with excessive neutrophilia, apoptosis can be facilitated by the presence of pathogens or cytokines, with the additionally delayed clearance [509]. Under these conditions, LL-37 may induce secondary necrosis of PMN with anti-inflammatory, protective consequences. However, it is possible that the release of other PMN factors could have deleterious consequences in this scenario, with the balance of effects therefore unknown. More *in vivo* studies are therefore required in the future, promoting extensive neutrophilia, and inducing massive apoptosis (eg. Fas ligand, *R*-Roscovitine, bacterial metabolites) to overload the clearance capacity, and inducing secondary necrosis by LL-37 (or its homologue mCRAMP) to better characterise the biological significance of this mode of PMN death.

4.4. Conclusion

LL-37-induced secondary necrosis of human PMN is anti-inflammatory *in vitro* and *in vivo*, dampening the pro-inflammatory cytokine production. However, LL-37-induced secondary necrosis has the potential to induce the release of potentially harmful granule contents, which could have deleterious consequences for the host, particularly in chronic disease states. Further understanding of the biological significance of secondary necrosis in physiological or pathological conditions will assist the establishment of novel strategies to modulate inflammatory responses in diseases and promote the resolution of inflammation.

Chapter 5

Other immunomodulatory capabilities of CHDP

5. Other immunomodulatory capabilities of CHDP

5.1. Introduction

In addition to the capability of modulating cell death, CHDP possess other immunomodulatory functions including anti-endotoxic activity and modulation of cytokine responses in immune effector cells [5]. These key properties of CHDP were further investigated to better characterise their roles in host defence.

5.2. Results

5.2.1. The anti-endotoxic and anti-inflammatory properties of CHDP

5.2.1.1. LL-37 inhibits pro-inflammatory cytokine production by MDM in response to TLR3 or TLR4 agonist.

LL-37 has been shown to bind LPS (a TLR4 agonist) with high affinity and has potent anti-endotoxic activity [89]. To evaluate the influences of LL-37 on innate immunity through other TLRs, the cytokine responses of MDM exposed to a panel of TLR agonists were studied in the presence or absence of LL-37, added concomitantly. In order to evaluate the downstream pathways through which LL-37 affected TLR signalling, cytokines characteristic of responses from separate

pathways were evaluated. TNF-α, which can be upregulated through both MyD88 dependent signalling and via TRIF pathway (MyD88-independent pathway) was contrasted with IP-10 production, an interferon-regulated protein which mainly depends on the TRIF pathway (Fig. 1.1) [121].

Data showed that TLR1/2 (Pam3CSK4), TLR2 (HKLM), TLR3 (poly I:C LMW), TLR4 (*E. coli K12* LPS), TLR5 (*S. typhimurium* Flagellin), TLR6/2 (FSL1), TLR8 (ssRNA) and TLR9 (ODN2006) agonists all effectively activated MDM, resulting in significantly increased TNF-α production ($p \leq 0.05$) (Fig. 5.1). The addition of LL-37 significantly inhibited the pro-inflammatory cytokine production by MDM in response to TLR3 and TLR4 agonists only (Fig. 5.1C & 5.1D). Given that TLR3 signals exclusively through TRIF, TLR4 uses both TRIF and MyD88-dependent signaling, and other TLR primarily uses MyD88-dependent signaling [118, 124, 517], these data raise the possibility that the inhibitory effects may be primarily on the TRIF pathway.

Significant IP-10 responses were only induced by TLR3 and TLR4 agonists, as expected based on their use of TRIF signalling (Fig. 5.2C & 5.2D). However, LL-37 did not inhibit TLR3 or TLR4-induced IP-10 production (Fig. 5.2C & 5.2D), indicating that the inhibitory effects were not simple blockade of the TRIF pathway, and may not affect the interferon-regulated responses. However, we could not exclude the possibility that LL-37 had selective direct affinity/ interactions with, different agonists, accounting for some of these observations [131]. These data suggest that LL-37 selectively inhibits certain components of TLR signalling

pathways, with cytokine specific results. However, further studies are clearly required to clarify this complex interaction.

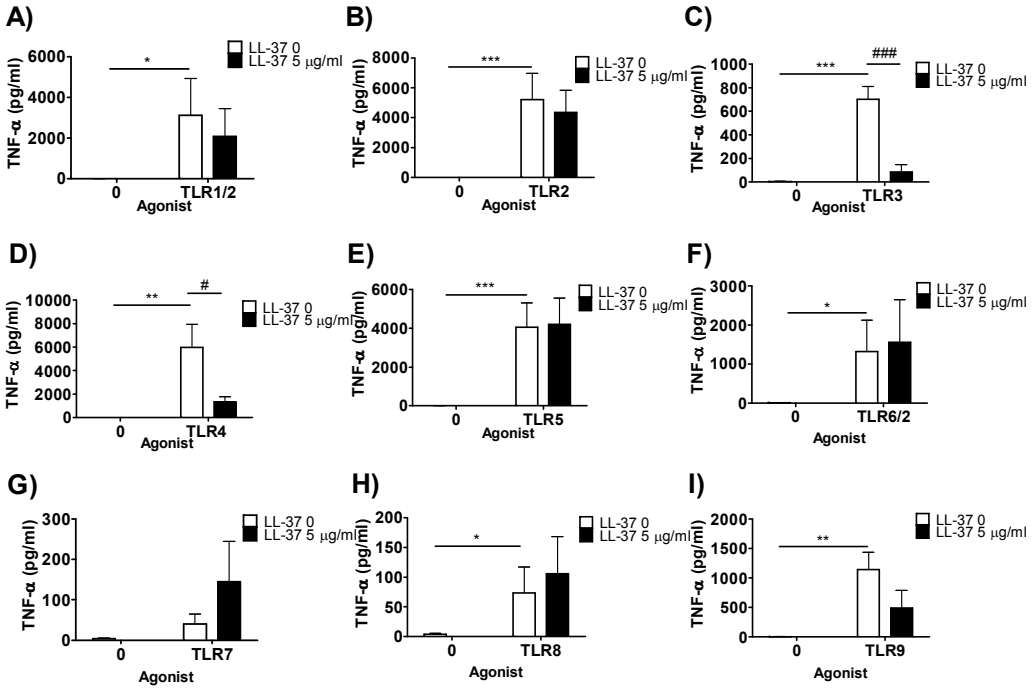


Figure 5.1 LL-37 inhibits pro-inflammatory cytokine production by MDM in response to TLR3 or TLR4 agonist. MDM were incubated with a panel of TLR agonists (TLR1/2: Pam3CSK4 0.1 μg/ml; TLR2: HKLM 10^8 cells/ml; TLR3: Poly (I:C) LMW 10 μg/ml; TLR4: LPS 10 ng/ml; TLR5 :Flagellin 10 ng/ml; TLR6/2: FSL1 1 ng/ml; TLR7: Imiquimod 0.25 μg/ml; TLR8: ssRNA40 0.25 μg/ml; TLR9: ODN2006 5μM) in the absence or presence of 5 μg/ml LL-37 in media with 10% FCS for 18 hr. Supernatants were evaluated for TNF-α responses by ELISA. Significance was assessed by two-way ANOVA with Bonferroni's multiple comparison test; data are shown as mean + SEM for n = 5 donors; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared with untreated MDM; #, $p \leq 0.05$; ###, $p \leq 0.001$, compared with MDM exposed to TLR agonists in the absence of LL-37.

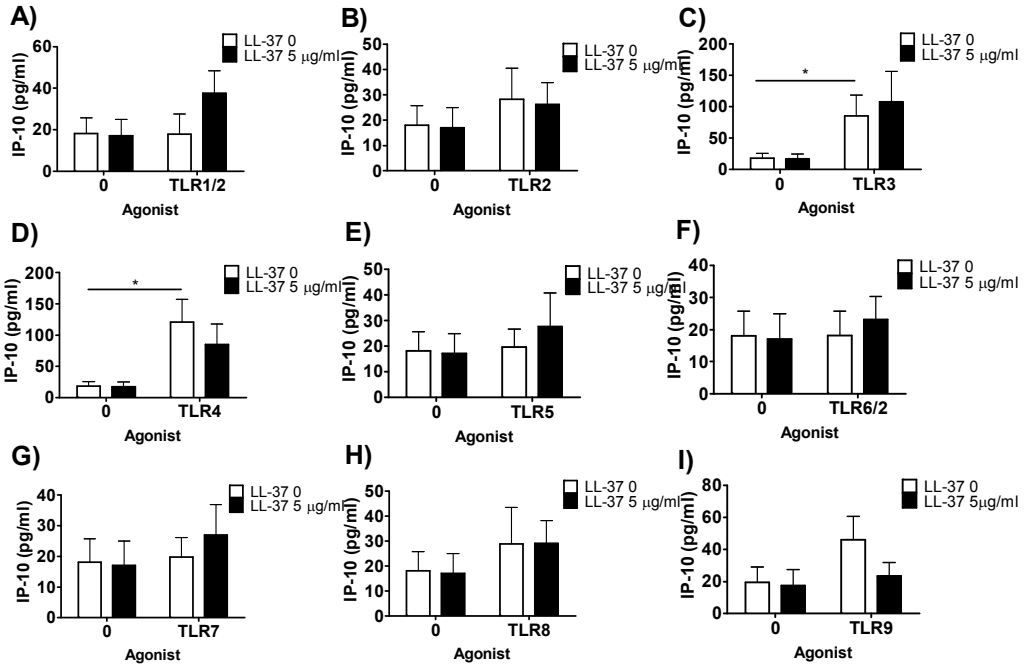


Figure 5.2 LL-37 does not directly inhibit TRIF pathway. MDM were incubated with a panel of TLR agonists (TLR1/2: Pam3CSK4 0.1 μg/ml; TLR2: HKLM 10^8 cells/ml; TLR3: Poly (I:C) LMW 10 μg/ml; TLR4: LPS 10 ng/ml; TLR5 :Flagellin 10 ng/ml; TLR6/2: FSL1 1 ng/ml; TLR7: Imiquimod 0.25 μg/ml; TLR8: ssRNA40 0.25 μg/ml; TLR9: ODN2006 5μM) in the absence or presence of 5 μg/ml LL-37 in media with 10% FCS for 18 hr. Supernatants were evaluated for IP-10 production by ELISA. Significance was assessed by two-way ANOVA with Bonferroni's multiple comparison test; data are shown as mean + SEM for n = 5 donors; *, $p \leq 0.05$, compared with untreated MDM.

5.2.1.2. HBD3 (human β-defensin 3) has anti-endotoxic activity.

The role of LL-37 as an anti-endotoxic agent is well described, but defensins have been less clearly characterised in this area. HBD3 is a β-defensin with a broad spectrum of microbicidal property, which also induces secondary necrosis of apoptotic PMN (Fig. 3.20). In order to evaluate whether HBD3 has anti-endotoxic activity similar to that observed for LL-37, LPS-induced pro-inflammatory cytokine responses were studied in MDM exposed concomitantly to HBD3 (or LL-37 as a positive control). Data showed that HBD3 and LL-37 had similar capacities to reduce pro-inflammatory cytokine responses in LPS-stimulated MDM in the absence (Fig. 5.3A) and in the presence of serum (Fig. 5.3B), suggesting that both of the peptides have anti-endotoxic capacity. The anti-endotoxic effects of these two peptides were not sensitive to the presence of serum, although LPS-induced MDM responses were much greater in the presence of serum. This observation implies that these two CHDP have overlapping immunomodulatory functions and may act redundantly in certain inflammatory responses.

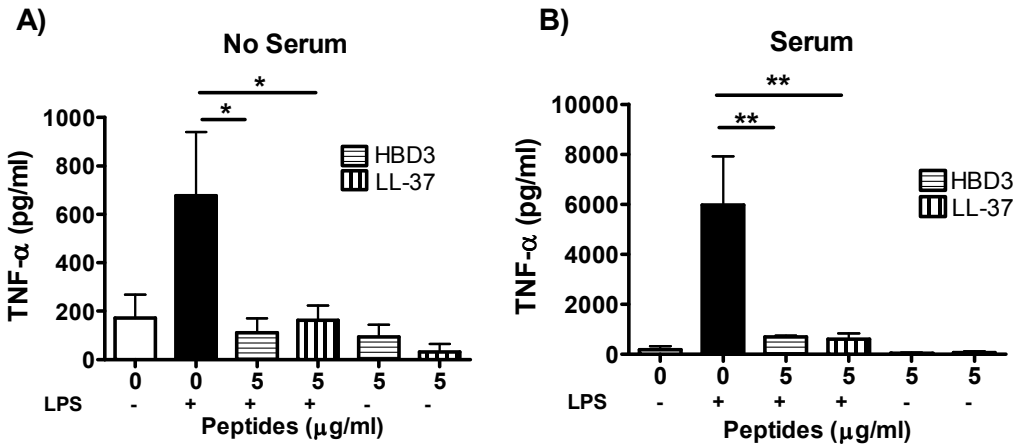


Figure 5.3 HBD3 (human β-defensin 3) has anti-endotoxic activity. MDM were incubated with 5 μg/ml LL-37 or 5 μg/ml HBD3 with concomitant exposure to 10 ng/ml *E.coli* 0111:B4 LPS for 18 hr in the absence (A) or presence (B) of 10% FCS. Untreated MDM and peptide-exposed MDM were also studied as controls. Supernatants were evaluated for TNF-α responses by ELISA. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test; data are shown as mean + SEM for $n \geq 4$ donors; *, $p \leq 0.05$; **, $p \leq 0.01$, compared with LPS-activated MDM.

5.2.2. Stimulation of cytokine release

5.2.2.1. LL-37 triggers IL-1β release from LPS-primed monocytes through $P2X_7$ receptor-independent pathway.

Elssner *et al* demonstrated that LL-37 induced IL-1β release from LPS-primed monocytes through $P2X_7$ receptor [29]. However, in collaboration with Dr. David Brough (University of Manchester), the Davidson lab has determined that LL-37 can promote IL-1β release from LPS-primed murine bone marrow-derived monocytes from $P2X_7$ receptor deficient mice, suggesting an alternative mechanism (data not shown). In order to further investigate this capacity of LL-37 in human cells, LPS-primed primary human monocytes were stimulated with LL-37, ATP or nigericin (the latter two compounds being knwon secondary signals in IL-1β processing and release), and the production of activated IL-1β was determined. Purinergic receptor inhibitors, oATP, KN-62, and a novel $P2X_7$ antagonist AZ-167 (a kind gift from Dr Keith Findlayson, University of Edinburgh) were utilised to clarify the involvement of P2 receptors. In addition, a potent caspase-1 inhibitor (YVAD-CHO), with YVAD as the specific recognition sequence for caspase-1, was exploited to try to define the role of caspase-1 in IL-1β processing in primed monocytes exposed to these secondary signals.

In agreement with previous reports, LL-37 increased IL-1β release from LPS-primed monocytes, as assessed by measuring the concentrations of total IL-1β (full length and cleaved forms) in supernatants by ELISA (Fig. 5.4A). However, this

increase could not be inhibited by specific $P2X_7$ receptor antagonists KN-62 and AZ-167. In contrast, the IL-1β release was significantly blocked by oATP, described as a $P2X_7$-inhibitor by Elssner *et al*. [29], but recently described as being a nonspecific P2 receptor antagonist [518], (Fig. 5.4B), suggesting that the induction of secretion was not via $P2X_7$ receptor. In contrast to the induction of IL-1β release by nigericin, LL-37-mediated IL-1β release was not observed to be dependent on the activation of caspase-1, with no effect observed from the addition of caspase-1 inhibitor on IL-1β secretion in LL-37-stimulated monocytes (Fig. 5.4B). However, caspase-1 inhibitor did not significantly reduce IL-1β release from ATP-stimulated monocytes in these studies as expected, raising the possibility that this inhibitor was poorly functional or that optimal concentrations had not been reached. As expected, the effects of ATP could be inhibited by either nonspecific P2 receptor antagonist or $P2X_7$ receptor antagonists (Fig. 5.4D), while nigericin acted as an potassium ionophore disrupting membrane potential and triggering IL-1β release via caspase-1 activation [379], and could not be blocked by purinergic receptor antagonists, but was sensitive to caspase-1 inhibition (Fig. 5.4F). Western immunoblotting was also conducted to analyse the maturation of IL-1β in supernatants and cell lysates and the activation of caspase-1 in cell lysates. Consistent with the ELISA data, LL-37, ATP, or nigericin did not induce IL-1β release from unprimed monocytes, but did promote mature IL-1β secretion by LPS-primed monocytes (Fig. 5.5A), albeit at very much lower levels when LL-37 was used as a second signal. In cell lysates, monocytes exposed to LPS alone, also had increased expression of proIL-1β as expected, but no cleaved IL-1β was observed in these cells (Fig. 5.5B). The activation of caspase-1 (10 kDa or 20 kDa)

was found in LPS-primed monocytes exposed to ATP or nigericin, while there was no active casapse-1 detected in LPS-primed monocytes exposed to LL-37 (Fig. 5.5C). In summary, these data demonstrate that LL-37, like ATP and nigericin, promote IL-1β release from LPS-primed monocytes, but indicate that the mechanism is independent of $P2X_7$ receptor and raising questions about the role of caspase-1.

5.2.2.2. LL-37 does not promote other pro-inflammatory cytokine release from LPS-primed monocytes.

To clarify whether LL-37-mediated promotion of cytokine release in LPS-primed monocytes was specific to IL-1β, TNF-α concentrations from the same samples were evaluated. Data revealed that LPS effectively activated monocytes, resulting in the increase of TNF-α. However, the subsequent addition of LL-37, ATP, nigericin, purinergic receptor antagonists, or caspase-1 inhibitor did not alter TNF-α response by monocytes (Fig. 5.6), indicating that LL-37, ATP, and nigericin specifically functioned as the second signals to promote IL-1β processing and release from monocytes.

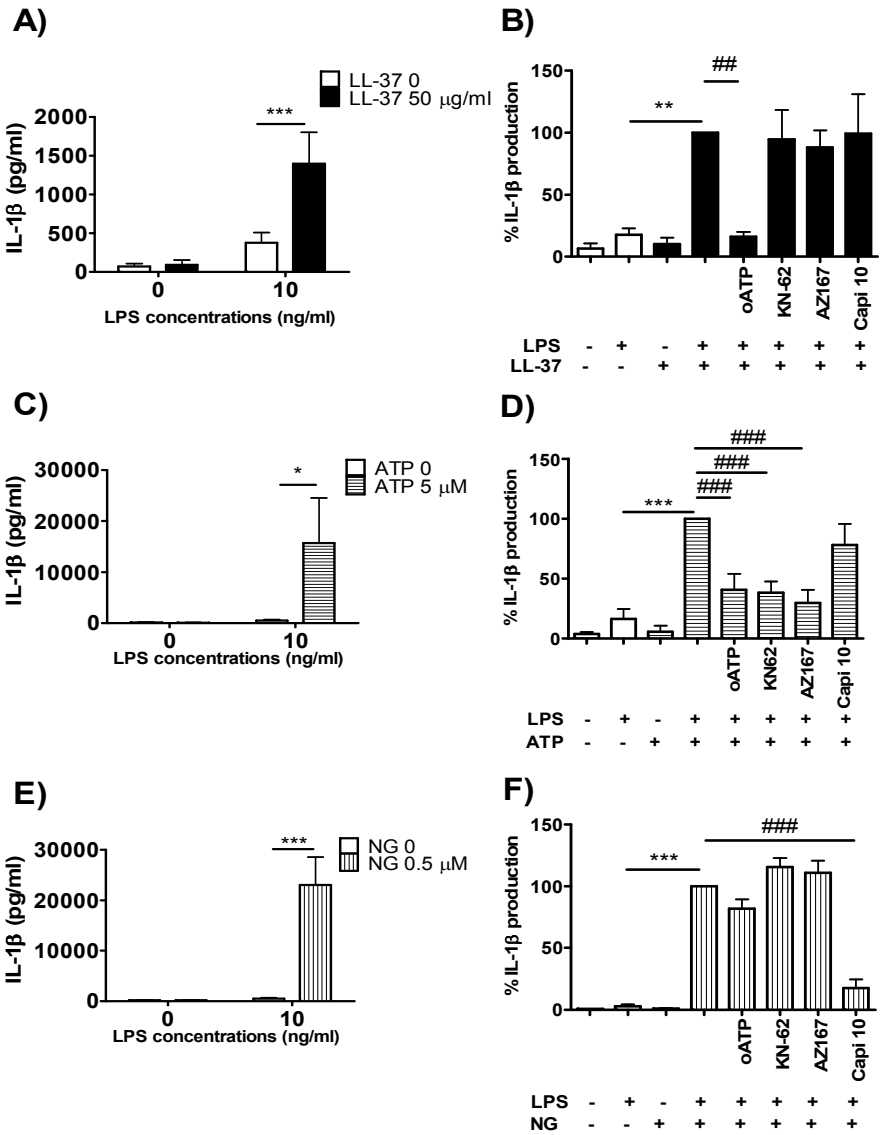


Figure 5.4 LL-37, ATP, and nigericin (NG) trigger IL-1β release from LPS-primed monocytes. Freshly isolated PBMC were purified by CD14 magnetic bead positive selection. CD14 positive monocytes were then stimulated with 10 ng/ml *E.coli* 0111:B4 LPS for 3 hr in IMDM +10 %FCS, followed by 1 hr LL-37 50 μg/ml (A), ATP 5 mM (C), NG 0.5 μM (E) stimulation concomitant with exposure to P2 receptor antagonists (oATP 900 μM; KN-62 1 μM; AZ-167 0.1 μM) or caspase-1 inhibitor ("Capi" 10 μM) (B, D, and F). Untreated monocytes and monocytes exposed to single stimulation of LL-37, ATP, or nigericin were also studied as controls. Supernatants were evaluated for IL-1β release by ELISA. Significance was assessed by two-way ANOVA (IL-1β, A, C, and E) or one-way ANOVA (B, D, and F) with Bonferroni's multiple comparison test; data are shown as mean + SEM for $n \geq 6$ donors; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared with LPS-primed monocytes; ###, $p \leq 0.001$, compared with monocytes exposed to LPS and secondary stimulus in the absence of inhibitors (expressed as a ratio due to donor variation in absolute levels).

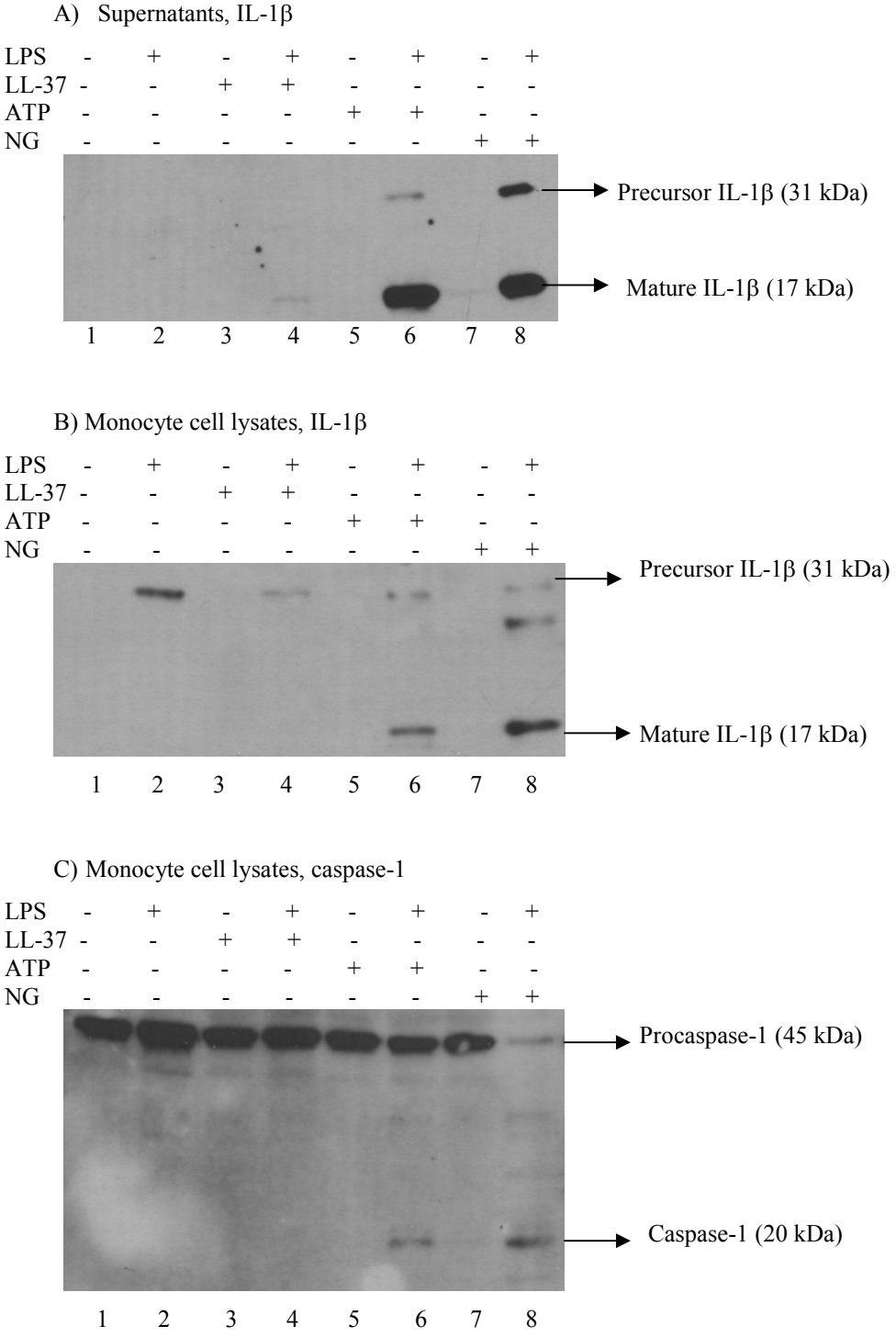


Figure 5.5 Representative images of Western immunoblotting for IL-1β and caspase-1. (A) Supernatants from monocytes were analysed using anti-IL-1β antibody. (B & C) Cell lysates were analysed using anti-IL-1β antibody (B) or anti-caspase-1 antibody (C). Lane 1: Untreated monocytes; lane 2: LPS-primed monocytes; lane 3: LL-37-stimulated monocytes; lane 4: LL-37-stimulated LPS-primed monocytes; lane 5: ATP-stimulated monocytes; lane 6: ATP-stimulated LPS-primed monocytes; lane 7: nigericin-stimulated monocytes; lane 8: nigericin-stimulated LPS-primed monocytes.

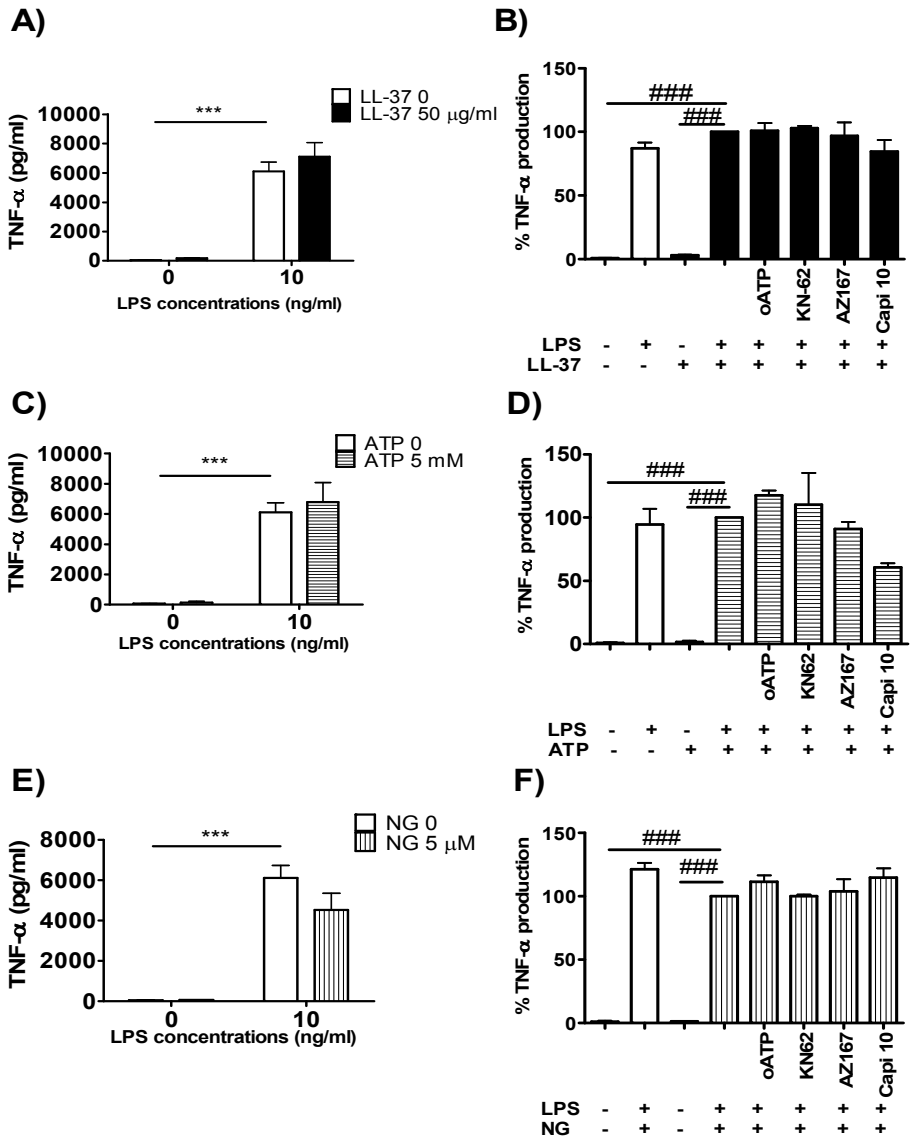


Figure 5.6 LL-37 does not promote other pro-inflammatory cytokine release from LPS-primed monocytes. CD14 positive monocytes were stimulated with 10 ng/ml *E.coli* 0111:B4 LPS for 3 hr in IMDM +10 %FCS, followed by 1 hr LL-37 50 μg/ml (A), ATP 5 mM (C), NG 0.5 μM (E) stimulation concomitant with exposure to P2 receptor antagonists (oATP 900 μM; KN-62 1 μM; AZ-167 0.1 μM) or caspase-1 inhibitor ("Capi" 10 μM) (B, D, and F). Untreated monocytes and monocytes exposed to single stimulation of LL-37, ATP, or nigericin were also studied as controls. Supernatants were evaluated for TNF-α release by ELISA. Significance was assessed by two-way ANOVA (A, C, and E) or one-way ANOVA (B, D, and F) with Bonferroni's multiple comparison test; data are shown as mean + SEM for n = 3 donors; ***, $p \leq 0.001$, compared with untreated monocytes; ###, $p \leq 0.001$, compared with monocytes exposed to LPS and secondary stimulus in the absence of inhibitors (expressed as a ratio due to donor variation in absolute levels).

5.2.2.3. IL-1β responses are not the results of monocyte death/ lysis.

Previous studies have found that the release of processed IL-1β takes place before there is significant cell death, which can occur after the formation of a non-selective pore by repeated or prolonged stimulation of $P2X_7$ receptor [519]. Nevertheless, to clarify whether the release of IL-1β resulted from cytolytic cell death, an MTT assay was performed to evaluate monocyte viability after stimulation with LPS and secondary stimuli. Data showed that none of these stimuli significantly altered viability of cells (Fig. 5.7), indicating that the release of cytokine was not the general result of cell death.

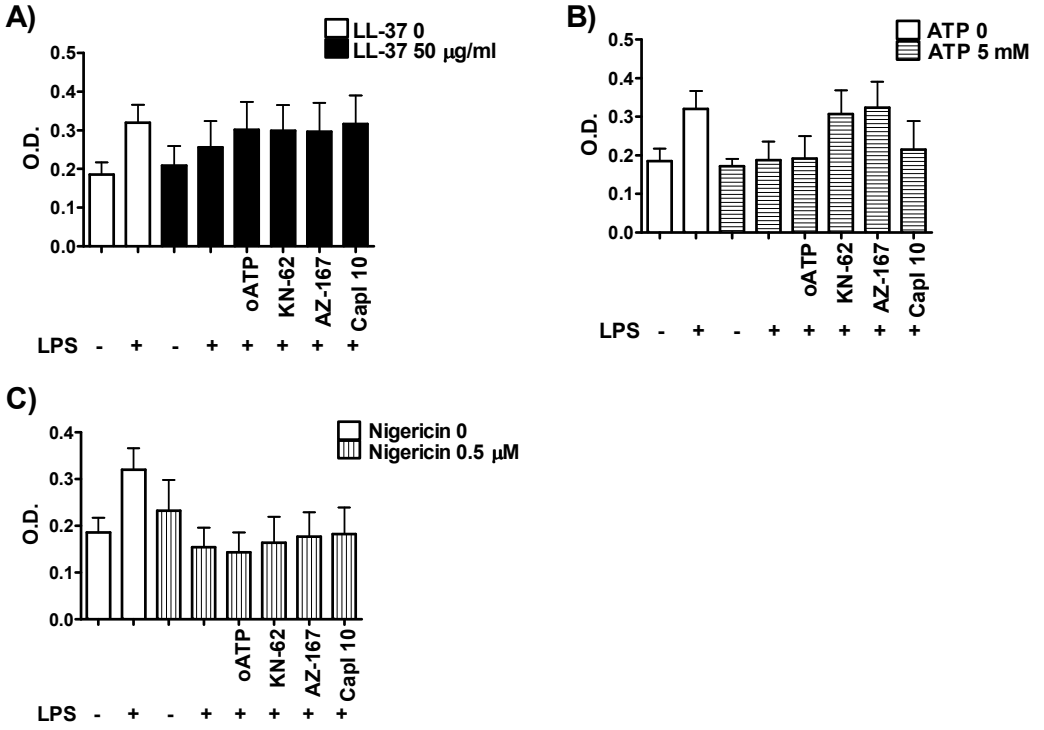


Figure 5.7 IL-1β responses are not the results of monocyte death/ lysis. CD14 positive monocytes were stimulated with 10 ng/ml *E.coli* 0111:B4 LPS for 3 hr in IMDM +10 %FCS, followed by 1 hr LL-37 50 μg/ml (A), ATP 5 mM (B), NG 0.5 μM (C) stimulation concomitant with exposure to P2 receptor antagonists (oATP 900 μM; KN-62 1 μM; AZ-167 0.1 μM) or caspase-1 inhibitor ("Capi" 10 μM). Untreated monocytes were also studied as controls. Supernatants were discarded followed by the addition of MTT reagent (volume of media: MTT solution = 5:1). Absorbance at 490nm wavelength was measured 1 hr after incubation. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test compared with untreated monocytes; data are shown as mean + SEM for n = 3 donors.

5.2.2.4. LL-37 promoted proIL-1β release from LPS-primed monocytes.

To determine whether the IL-1β released in these studies was the mature form or inactive precursor form, the proform IL-1β in supernatants was evaluated using a specific ELISA. Triton X100-lysed activated monocytes were used as a positive control for the release of substantial amounts of precursor proform IL-1β. Interestingly, both LL-37 and nigericin, promoted extensive release of proIL-1β (Fig. 5.8). However, studies by Western immunoblotting did not detect substantial proIL-1β release in supernatants of LPS-primed monocytes exposed to LL-37 (Fig. 5.5). This discrepancy may be due to the different sensitivity of antibodies from two different manufactures to detect mature or precursor form of IL-1β, which made it more difficult to interpret the data. Although there are still unsolved problems to delineate the mechanism by which LL-37 induces IL-1β release, the data on proIL-1β measured by ELISA raise the possibility that LL-37 may function similar to nigericin: perturbing the cell membrane and inducing the secretion of both proIL-1β and active IL-1β.

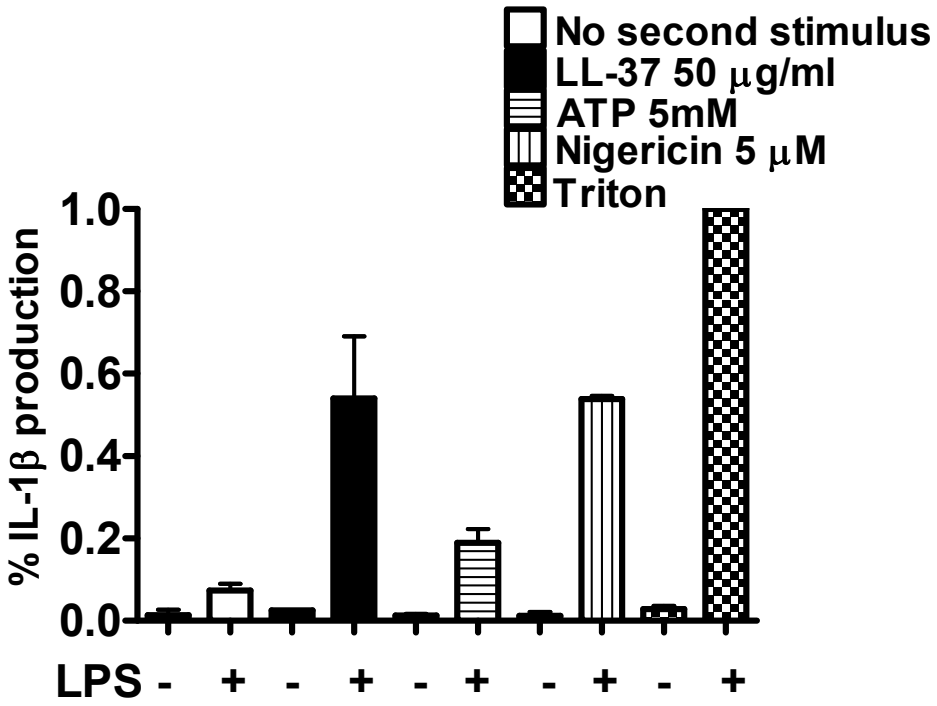


Figure 5.8 LL-37 and nigericin trigger precursor IL-1β release from LPS-primed monocytes. CD14 positive monocytes were stimulated with 10 ng/ml *E.coli* 0111:B4 LPS for 3 hr in IMDM +10% FCS, followed by 1 hr LL-37 50 μg/ml, ATP 5 mM, or nigericin 0.5 μM exposure. Untreated monocytes and 0.1% Triton X-lysed LPS-primed monocytes were studied as negative and positive control, respectively. Supernatants were evaluated for proIL-1β release by ELISA. Data were presented as ratio of pro-IL-β released by cells as a proportion of the Triton-treated positive control from the matched sample from the same donor; data are shown as mean + SEM for n = 2 donors.

5.2.2.5. Human monocyte-derived macrophages have diminished LL-37-induced IL-1β release compared with fresh monocytes.

LL-37 was shown to trigger IL-1β secretion from LPS-primed monocytes. In order to determine whether this peptide has similar effects on differentiated macrophages, studies were conducted in MDM. Exposure to LL-37, or positive controls ATP or nigericin, was used as a secondary signal to trigger IL-1β release from LPS-primed MDM. Similar to the results of observed in monocytes, ATP and nigericin induced IL-1β release, albeit at much lower concentrations (Fig. 5.9B & 5.9C). In contrast to monocytes, LL-37 could not promote IL-1β secretion from LPS-activated macrophages (Fig. 5.9A). These data raise the possibility that an as yet undefined receptor involved in LL-37-induced IL-1β release exists and is differentially expressed on these cell types, or that these two monocytic cells may use different mechanisms in IL-1β processing and release.

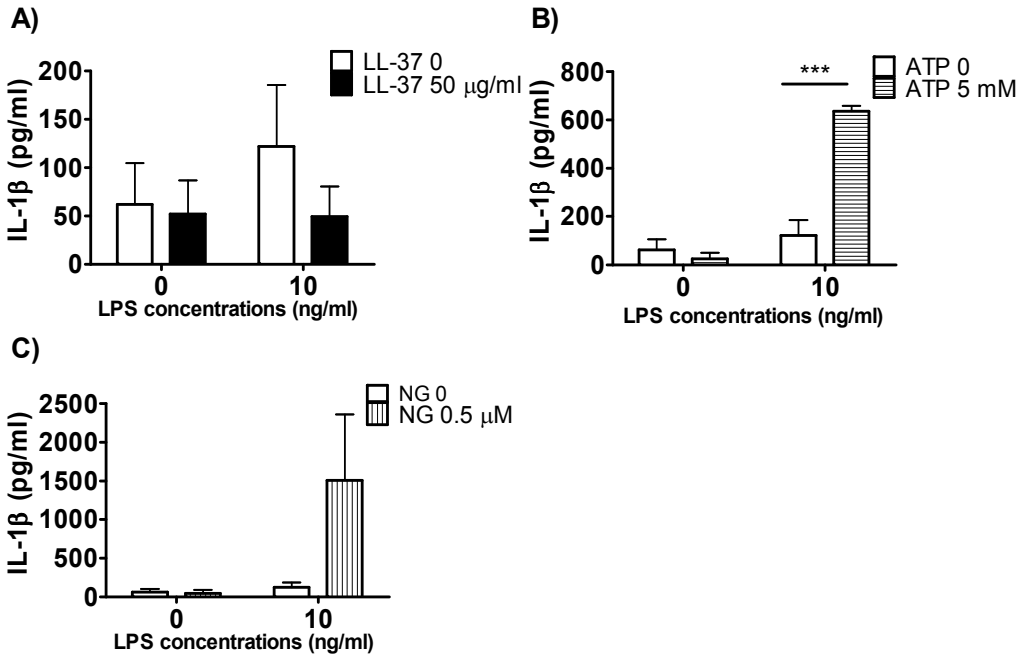


Figure 5.9 MDM have diminished IL-1β release compared with fresh monocytes. MDM were stimulated with 10 ng/ml *E.coli* 0111:B4 LPS for 3 hr in IMDM +10 %FCS, followed by 1 hr LL-37 50 μg/ml (A), ATP 5 mM (B), NG 0.5 μM (C) stimulation. Untreated MDM and MDM exposed to single stimulation of LL-37, ATP, or nigericin were also studied as controls. Supernatants were evaluated for IL-1β release by ELISA. Significance was assessed by two-way ANOVA with Bonferroni's multiple comparison test; data are shown as mean + SEM for n = 3 donors; ***, $p \leq 0.001$, compared with MDM without secondary stimulus under the same active status.

5.3. Discussion

CHDP have been shown to have multiple immunomodulatory properties and play critical roles in host defence. Here we further investigated these modulatory functions of CHDP in immune effector cells, demonstrating anti-inflammatory effects in response to TLR agonists and inhibiting pro-inflammatory cytokine production. In contrast, CHDP can also promote certain cytokine release in TLR-primed immune cells, indicating their pleiotropic roles in regulating inflammatory responses.

Previous studies have demonstrated that LL-37 can modulate inflammatory responses by affecting TLR signalling. For example, LL-37 has been reported to selectively neutralise the pro-inflammatory response to the TLR2, TLR4, and TLR9 ligands in human monocytic cells, THP-1, probably through directly acting on NF-κB and MAPK pathway [89, 131, 136]. However, the exact mechanism by which LL-37 inhibits pro-inflammatory cytokine responses to TLR ligands remains unclear. My study investigated cytokine responses which were dependent on MyD88 or TRIF pathways to differentiate the inhibiting effects of LL-37 on TLR signals. It is known that production of the pro-inflammatory cytokines TNF-α and IL-6 after LPS stimulation is regulated by both TLR-MyD88-dependent and TLR-MyD88-independent signalling pathways that result in the activation of NF-κB and p38, JNK, and p42/44 ERK MAPKs. Transcription of the CXCL10 (IP-10) gene downstream of TLR agonists is exclusively regulated by the TLR-MyD88-independent signalling pathway via interferon production [517]. Both TLR3 and

TLR4 agonists, which utilise the TRIF pathway, significantly induced IP-10 production as expected but LL-37 could not block this response, indicating that the inhibiting effect of LL-37 was not simply blocking TRIF-dependent signalling, and may not affect the interferon-regulated responses. LL-37 may have selective affinity/ interaction with different agonists, resulting in the inhibition of certain TLR signalling pathways. Taken together, LL-37 does not directly inhibit TRIF signalling pathways and further studies are required to clarify at which point of signalling pathway LL-37 has its inhibitory effects.

Similarly to LL-37, HBD3 demonstrates potent anti-endotoxic properties in the presence or absence of serum. These two peptides, although belong to different families of CHDP (cathelicidin and defensin, respectively), have overlapping immunomodulatory functions, including inducing secondary necrosis of apoptotic PMN and inhibiting activation of MDM *in vitro*. This observation may suggest the redundancy, of these two peptides *in vivo*, or that they may have additive, or even synergistic, roles in immunomodulation. Indeed, emerging evidence reveals that these two peptides are present simultaneously throughout the body at epithelial barriers. At these sites they may significantly contribute to host defence against pathogens, but could also play detrimental roles in chronic inflammatory conditions [520]. Future studies are required to determine the interactions of these peptides in physiological and pathological conditions.

LL-37 can also stimulate pro-inflammatory cytokine release [29]. Some reports using other CHDP, suggest that some of these peptides may act as $P2X_7$ agonists,

ionophores, or bacterial toxins, to induce pore formation on plasma membrane of cells, triggering inflammasome-mediated caspase-1 activation and IL-1β release [365, 379, 521, 522]. However, the exact mechanism is not fully characterised. It is well-known that LL-37, like most of CHDP, can insert into the membrane through their amphipathic propensity, which may eventually generate a perturbation of cell membrane and allow certain mediator leakage. LL-37 can also modulate the activities of receptors, transiting channel-gated receptor to pore formation [523], which may enhance the secretion of intracellular mediators. However, further work is needed to better characterise the mechanism by which LL-37 stimulate IL-1β cytokine release.

Elssner *et al.* demonstrated that LL-37 promoted IL-1β secretion by LPS-primed monocytes through $P2X_7R$-dependent pathway. However, Dr. David Brough (University of Manchester) showed that LL-37 could promote IL-1β release from LPS-primed murine bone marrow-derived monocytes from $P2X_7$ receptor deficient mice, suggesting an alternative mechanism. Consistent with Dr. Brough's finding, my data revealed that LL-37-induced IL-1β release could not be inhibited by specific $P2X_7$ receptor antagonists, suggesting the induction of secretion was not via $P2X_7$ receptor. Additionally, given that the activity was proposed to be dependent on $P2X_7$ receptor, it would be anticipated that LPS-primed macrophages would demonstrate greater IL-1β release in response to LL-37, as a consequence of their greater $P2X_7$ receptor expression on the cell surface [524]. In contrast to this assumption, my results showed that LPS-stimulated macrophages were less responsive to LL-37 than LPS-primed monocytes. Other studies also revealed

similar results, showing that macrophages had diminished IL-1β production compared with monocytes [525, 526]. Using fresh human blood monocytes and monocyte-derived macrophages, the study indicated that there is a differential requirement for processing and releasing IL-1β in these two different monocytic cells [525]. Different cell types are equipped with distinct machinery (e.g. the components of inflammasome) for IL-1β process and release. which may explain the discrete responses of these two cell types [527].

My data demonstrated that LL-37 induced IL-1β release by LPS-primed monocytes. Both mature form and precursor form of IL-β were detected by different experimental approaches (Western immunoblotting and specific proIL-1β ELISA, respectively). However, the underlying mechanisms by which LL-37 mediate IL-1β release remain unclear. The involvement of caspase-1 in this process was not fully studied. It has been shown that IL-1β can be released by two distinct pathways: the well-known caspase-1 cascade mediating release of processed IL-1β, and a calcium-independent, caspase-1-independent release of pro-IL-1β [527]. Future work is required to determine the activation of caspase-1, either by FLICA probe which enters cells and binds active caspase-1 enzyme with fluorescence, or by studying the response of monocytes from caspase-1 knockout mice.

Although the precursor form of IL-1β is biologically inactive [373], extracellular proteases than the intracellular activated caspase-1 can also mediate pro-IL-1β

processing. For example, protease-3 and elastase from PMN granules and proteases released from pathogens have been reported to contribute to the processing of IL-1β precursor into an active cytokine [369, 373, 528]. It is proposed that during pathophysiological conditions of inflammation or infection when monocytes are stimulated with pro-inflammatory cytokines or TLR ligands (eg. LPS), LL-37 can promote the release of mature and precursor forms of IL-1β by an undefined mechanism. Proteases released from recruited PMN or LL-37-mediated secondarily necrotic PMN may trigger the extracellular processing of proIL-1β into the active form. LL-37 released from PMN can further potentiate the release of this potent pro-inflammatory cytokines, altering the nature of the subsequent inflammatory response.

5.4. Conclusion

CHDP possess the capability of modulating inflammatory response, to inhibit pro-inflammatory cytokine production by interfering with TLR signaling in MDM, and to promote IL-1β secretion of activated monocytes. These observations indicate that CHDP play a complex role during inflammation, and tuning their modulatory activity may contribute to effective therapeutic for inflammatory disorders.

Chapter 6

Conclusion and future work

6. Conclusion and future work

PMN are important innate immune cells packed with toxic substances that allow effective microbial killing. PMN are rapidly recruited to sites of inflammation, where they play a key role in innate immune responses, before undergoing spontaneous apoptosis. This mode of cell death maintains their intact plasma membrane, preventing release of their cytotoxic contents, and promotes the resolution of inflammation. In contrast, necrotic PMN, which lose their cell membrane integrity, have been proposed to release their harmful intracellular substances, resulting in sustained inflammatory responses.

In this thesis I have demonstrated that the human cathelicidin LL-37 is a potent inducer of secondary necrosis in apoptotic PMN. The specific membrane alterations that make these apoptotic cells susceptible to permeabilisation remain unclear, but it is likely independent of known LL-37 receptors [500]. Whether, the interaction of LL-37 and apoptotic PMN is through a mechanism that differs from the interaction of LL-37 and live PMN, or simply has different consequences for cells in these different states remains unknown. Interestingly, although LL-37 can induce secondary necrosis in other apoptotic cell types (NK cells and Burkitt's lymphoma), it has not been reported to permeabilise apoptotic cells in all circumstances. Indeed, LL-37 can induce apoptosis in certain cell types, such as lung epithelial cells and Jurkat T leukemia [20, 108, 529], but did not further induce these apoptotic cells into secondary necrosis. Which specific property of

apoptotic cells determines the susceptibility to LL-37-induced secondary necrosis requires further studies.

The secondary necrosis-inducing property of LL-37 is conserved in the murine homologue mCRAMP, and is also retained in C-terminal fragments of the peptide. I have also demonstrated that HBD3 also possesses this property. The effect is neither dependent primarily on peptide charge (with no effects seen for a scrambled peptide, N-terminal partial peptides, and α-defensins HNP1-4 with positive charge), nor is dependent on alpha-helical structure (with no effects seen for Hp(2-20) with α-helical nature). A greater propensity of forming amphipathic structure, a critical character for the direct microbicidal effects of CHDP [530], may however be related to the capability of inducing secondary necrosis. Future research is required to unravel the structure determinants responsible for the permeabilisation of apoptotic cells.

In contrast to initial expectations, LL-37-mediated secondary necrosis does not affect PMN ingestion by human monocyte-derived macrophages and is not pro-inflammatory. Indeed the anti-inflammatory effects of apoptotic PMN on activated macrophages are retained, and even enhanced, by LL-37-induced secondarily necrotic PMN, and are independent on the anti-endotoxic activity of LL-37. Exposure to a concentration of LL-37 sufficient to induce PMN granule content releases, was necessary to generate potentiating the anti-inflammatory effects of the dead cells, suggesting a key role for the release of more anti-inflammatory PMN granule contents. Although the components released from secondary necrotic

PMN have not been well characterised, my data, and that of Dr Mohini Gray's group support this hypothesis, demonstrating that α-defensins in the membrane-free fraction of necrotic PMN have potent anti-inflammatory effects in the absence of serum [387]. Since the activity of α-defensins is inhibited by unknown serum factors, only PMN induced to maximal release of granule contents, including the most "serum-resistant" LL-37 (eg. secondary necrosis induced by higher concentrations of LL-37 or primary necrosis) retain their anti-inflammatory effects in the presence of serum. In addition to inhibiting the anti-inflammatory effects of α-defensins, undefined serum components may interact with secondarily necrotic PMN and engage alternative clearance pathways, resulting in altered inflammatory consequences. Studies by using fractionated serum to isolate important components which are involved in cell engulfment, such as complement components and immunoglobulins, or proteases which can degrade soluble mediators, may help address this question.

In vivo, a murine sterile peritonitis model confirmed elements of the findings from my *in vitro* studies, demonstrating anti-inflammatory effects of LL-37-mediated secondarily necrotic PMN, that were comparable to apoptotic PMN. Although the experiments were too simple to reflect the complexity of inflammatory diseases, with the interplay between numerous factors, they provided an initial confirmation of the *in vitro* observations. More *in vivo* studies are required to better characterise the biological significance of LL-37-induced secondary necrosis at inflamed sites and their interaction with pathogens, cytokines, peptides, and immune effector cells. In addition, although LL-37-induced

secondary necrosis of apoptotic PMN is anti-inflammatory to MDM, it is still unknown whether these cells are immunogenic or immunosuppressive to DC. The immune properties of secondarily necrotic PMN may have physiological significances, bridging innate immune cells to adaptive immune responses, and merit further investigation.

Clinical observations evaluating secondary necrosis in certain chronic disorders [509], suggesting that secondarily necrotic PMN may not exclusively serve an anti-inflammatory role. It is noteworthy that LL-37 also promotes PMN granule content release, which has the potential to induce the release of toxic intracellular substances and could have deleterious consequence of the host, particularly in chronic disease status. Additional *in vivo* experiments, and more clinical studies are necessary to determine the association of secondary necrosis with the pathogenesis of inflammatory disorders.

In addition to modulating cell death of PMN, CHDP have multiple modulatory functions in other immune effector cells. The anti-inflammatory capability of peptides has been demonstrated in response to a variety of TLR stimuli although the exact mechanisms remain unclear. It is likely that CHDP directly interact with TLR ligands or function on signalling pathways in immune cells, selectively inhibiting pro-inflammatory responses. On the other hand, LL-37 promotes specific pro-inflammatory cytokine IL-1β secretion by TLR-primed immune cells though undefined mechanisms. These findings indicate the pleiotropic roles of CHDP in immune system. Neither exclusively acting as a pro-inflammatory factor

nor as a purely anti-inflammatory mediator, CHDP modulate inflammatory responses in multiple aspects through complex mechanisms, to alter the balance of the inflammatory response, and thus the outcome.

In conclusion, I have demonstrated that the human cathelicidin LL-37 is a potent inducer of secondary necrosis of apoptotic PMN, with the potential to alter the consequent inflammatory responses. The data provide novel insight into the effects of PMN death, suggesting that LL-37-induced secondary necrosis of apoptotic PMN, in contrast to previous dogma, is not pro-inflammatory to MDM, and can even potentiate anti-inflammatory effects on activated MDM. *In vivo* murine sterile peritonitis studies have confirmed these data, demonstrating anti-inflammatory effects of secondarily necrotic PMN. However, LL-37-induced secondary necrosis has the potential to induce the release of potentially harmful granule contents, which may have detrimental consequences for the host. In addition to modulating cell death, CHDP possess other immunomodulatory capabilities to alter inflammatory responses and cytokine production, indicating some of the critical regulatory roles of this peptide in immune system. It is anticipated that further advancements in our understanding of the multiple modulatory mechanisms of CHDP in innate and adaptive immune responses in the future, will help inform the development of novel therapeutics for infectious and inflammatory diseases.

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Appendix

Publication：

Meeting abstract

- ✓ Li HN, Barlow PG, Haslett C, Rossi AG, and Davidson DJ (2007) The human cationic host defence peptide LL-37 modulates cell death pathways in human neutrophils. *Scottish Immunology Groups* **Poster presentation.**

- ✓ Li HN, Barlow PG, Bylund J, Bjorstad A, Hiemstra PS, Haslett C, Simpson AJ, Rossi AG, and Davidson DJ (2008) The human cationic host defence peptide LL-37 modulates cell death in human neutrophils. *10th Biennial IEIIS meeting* **Poster presentation.**

- ✓ Li HN, Barlow PG, Bylund J, Björstad Å, Mackeller A, Hiemstra PS, Conlon J, Haslett C, Gray M, Simpson AJ, Rossi AG, and Davidson DJ (2009) Secondary necrosis of apoptotic neutrophils induced by the human cathelicidin LL-37 is not pro-inflammatory to phagocytosing macrophages. *Tri-society Annual meeting (Society for Leukocyte Biology, International Cytokine Society, & International Society for Interferon and Cytokine research)* **Poster presentation.**

- ✓ Li HN, Barlow PG, Bylund J, Björstad Å, Mackeller A, Hiemstra PS, Conlon J, Haslett C, Gray M, Simpson AJ, Rossi AG, and Davidson DJ (2009) Secondary Necrosis of Apoptotic Neutrophils Induced by LL-37 Has Anti-inflammatory Effects on Macrophages. *Euroscicon meeting (Antimicrobial Peptides: New Challenges for Science)* **Oral and poster presentation.**

- ✓ Li HN, Barlow PG, Bylund J, Björstad Å, Mackeller A, Hiemstra PS, Conlon J, Haslett C, Gray M, Simpson AJ, Rossi AG, and Davidson DJ (2010) Secondary Necrosis of Apoptotic Neutrophils Induced by LL-37 Has Anti-inflammatory Effects on Macrophages. *Keystone Symposia (Cell death Pathways: Apoptosis, Autophagy and Necrosis)* **Poster presentation**

Paper publication

- ✓ Semple F, Webb S, Li HN, Patel HB, Perretti M, Jackson IJ, Gray M, Davidson DJ, and Dorin JR (2010) Human beta-defensin 3 has immunosuppressive activity in vitro and in vivo. *Eur. J. Immunol.* 40(4): 1073-8

- ✓ Li HN, Barlow PG, Bylund J, Mackellar A, Björstad A, Conlon J, Hiemstra PS, Haslett C, Gray M, Simpson AJ, Rossi AG, and Davidson DJ (2009) Secondary necrosis of apoptotic neutrophils induced by the human cathelicidin LL-37 is not proinflammatory to phagocytosing macrophages. *J. Leuk. Biol.* 86(4): 891-902

- ✓ Björstad A, Askarieh G, Brown KL, Christenson K, Forsman H, Onnheim K, Li HN, Teneberg S, Maier O, Hoekstra D, Dahlgren C, Davidson DJ, and Bylund J (2009) The host defense peptide LL-37 selectively permeabilises apoptotic leukocytes. *Antimicrob Agents Chemother.* 53(3): 1027-38

European Journal of Immunology

SHORT COMMUNICATION

Human β-defensin 3 has immunosuppressive activity *in vitro* and *in vivo*

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β-defensins are antimicrobial peptides with an essential role in the innate immune response. In addition β-defensins can also chemoattract cells involved in adaptive immunity. Until now, based on evidence from dendritic cell stimulation, human β defensin-3 (hBD3) was considered pro-inflammatory. We present evidence here that hBD3 lacks pro-inflammatory activity in human and mouse primary Mϕ. In addition, in the presence of LPS, hBD3 and the murine orthologue Defb14 (but not hBD2), effectively inhibit TNF-α and IL-6 accumulation implying an anti-inflammatory function. hBD3 also inhibits CD40/IFN-γ stimulation of Mϕ and *in vivo*, hBD3 significantly reduces the LPS-induced TNF-α level in serum. Recent work has revealed that hBD3 binds melanocortin receptors but we provide evidence that these are not involved in hBD3 immunomodulatory activity. This implies a dual role for hBD3 in antimicrobial activity and resolution of inflammation.

Key words: Anti-inflammatory · cAMP · Defensin · TNF-α

Supporting Information available online

Introduction

β-defensins are broad spectrum, cationic, antimicrobial peptides. They are expressed predominantly at mucosal surfaces and believed to be important components of innate immunity although their precise *in vivo* role has not been clarified [1]. Human β-defensins are a multigene family and the main cluster on chromosome 8p23 has been shown to be copy number variable [2]. Increased copy number in humans is associated with psoriasis and decreased copy number with Crohn's disease, suggesting involvement in these autoimmune diseases [3, 4]. Human β defensin-3 (hBD3) is one of the most cationic of the β-defensins with broad spectrum, salt insensitive, antimicrobial activity [5]. It is highly expressed in psoriatic skin and the reproductive tract [6, 7]. Defensins have been considered pro-inflammatory as their expression increases in response to TLR ligands, TNF-α, IL-1β, IFN-γ and PMA, and following infection or injury [5, 8, 9]. In addition, they have been shown to chemoattract CD4 T cells and immature dendritic cells through CCR6, suggesting that they link innate and adaptive immunity [10]. hBD3 and 4 also chemoattract monocytes and Mϕ [8, 11], and hBD3 has been shown to activate monocytes and myeloid dendritic cells through TLR-1/2 by inducing expression of co-stimulatory molecules and NF-κB [12]. Recently, human α-defensins present in neutrophil granules have been shown to display anti-inflammatory properties [13].

In this paper we show that hBD3 does not induce TNF-α or IL-6 in Mϕ and in fact has potent anti-inflammatory effects on both human and mouse primary Mϕ. The anti-inflammatory effect was also

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evident *in vivo* and in the THP-1 human monocytic cell line and RAW264.7 mouse Mϕ cell line. hBD3 effectively inhibited the inflammatory effects of both LPS and CD40 ligand (CD40L). Recently it has been shown that hBD3 can interact with melanocortin receptors *in vitro* [14] and a dominant mutation in this gene in dogs and arctic wolves is causative for black coat colour [15]. Despite melanocortin 1 receptor (MC1R) and melanocortin 3 receptor (MC3R) being expressed on Mϕ and having known immunomodulatory activity, we show here that these receptors do not mediate the novel, potent anti-inflammatory effect displayed by hBD3.

Results and discussion

hBD3 is anti-inflammatory *in vitro*

In contrast to the assumed pro-inflammatory effect of hBD3 summarised above, we show here that synthetic hBD3 inhibits production of TNF-α by the human myelomonocytic cell line THP-1 in a concentration-dependent manner (Fig. 1A). The effect was maximal at 2.5 μg/mL, and comparable in magnitude to the cationic antimicrobial peptide LL37, which is a known immunomodulatory peptide [16–18]. This same effect was also evident using human peripheral blood monocyte derived Mϕ (Fig. 1B). Treatments did not affect cell viability as MTT assay measurements were comparable between treated cells and untreated controls.

Addition of hBD3 to the mouse Mϕ cell line RAW264.7 also led to inhibition of TNF-α and IL-6 production (Fig. 1C and D). In our experimental settings hBD3 did not induce TNF-α or IL-6, in contrast to the recent report that this defensin activates monocytes and myeloid dendritic cells *via* TLR1/2, up-regulating the co-stimulatory molecules CD80, CD86 and CD40 [12]. We observe our anti-inflammatory effect with 5 μg/mL (~1 μM) of synthetic hBD3 by directly measuring the attenuation of pro-inflammatory cytokine production, whereas Funderburg *et al* observe their effects on co-stimulatory molecules with 20 μg/mL of recombinant hBD3 (and do not measure pro-inflammatory cytokines). We did, however, observe a slight increase in TNF-α with hBD3 at 10 μg/mL but only in RAW264.7 cells (Fig. 1D), not primary Mϕ or THP-1 cells, suggesting that in specific cells at higher concentrations of hBD3 there may also be a pro-inflammatory effect of hBD3.

The anti-inflammatory effect of both hBD3 and the mouse orthologue Defb14 [19] was observed in mouse primary BM-derived Mϕ (BMDM), reducing the TNF-α response to LPS (Fig. 1E). hBD2 was not an effective suppressor of the TNF-α response to LPS in mouse cells (Fig. 1E), whereas hBD3 was more effective than LL37 in all mouse strains tested (Fig. 1F). hBD2 has only approximately

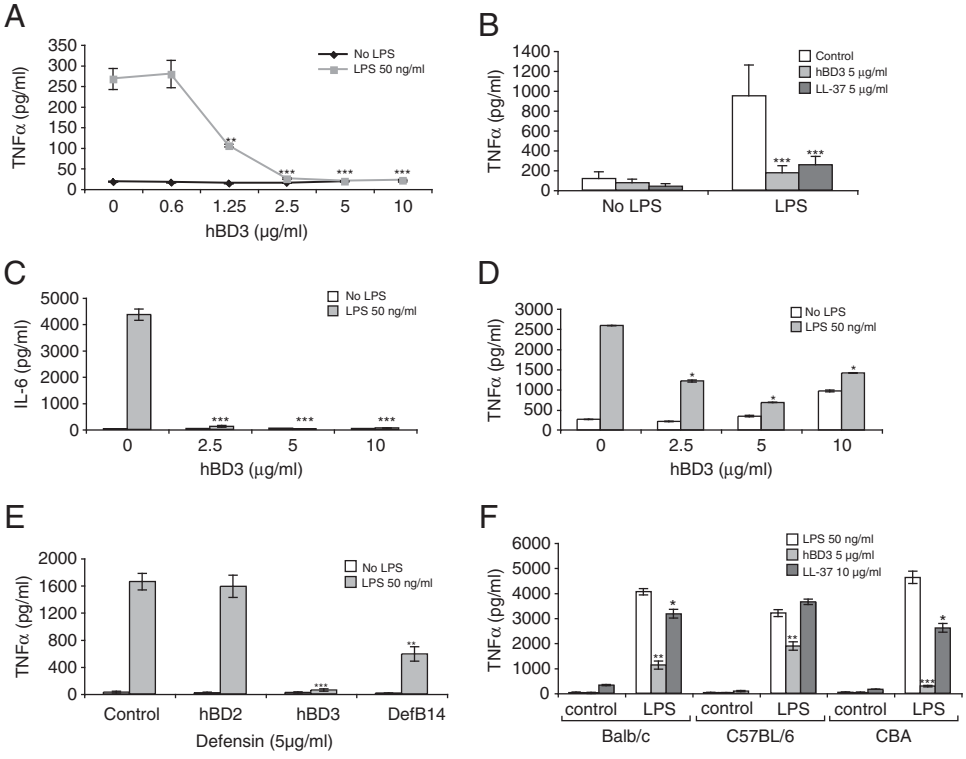

Figure 1. hBD3 down-regulates cytokine production in response to the TLR4 agonist LPS in human and mouse Mϕ. (A) THP-1 monocyte cell line, (B) human monocyte-derived Mϕ, (C and D) RAW264.7 mouse Mϕ cell line and (E) BMDM from CBA mice were exposed to β-defensin or control peptides at 5 μg/mL, in the presence or absence of LPS for 18 h in serum-free media. TNF-α (or IL-6) in the supernatant was measured by ELISA, $n = 6$ donors for the human primary cells, $n = 3$ THP-1, $n = 3$ RAW264.7 and $n = 3$ mouse BMDM (from three separate mice). Figure shows means ± SEM, significance assessed by two-way ANOVA, ***$p<0.001$, **$p<0.01$ was calculated by comparing LPS plus peptide to LPS alone. (F) TNF-α levels in supernatant in BMDM from different mouse strains, treated with hBD3 or LL37 at indicated concentrations, with and without LPS for 18 h, $n = 3$. Statistical comparisons are between LPS alone *versus* LPS with peptide in each strain, ***$p<0.001$, **$p<0.01$, *$p<0.05$.

30% amino acid similarity to hBD3, which may explain lack of anti-inflammatory effects. Conversely, Defb14, which is 64% identical to hBD3 [20], did demonstrate anti-inflammatory activity.

The anti-endotoxic effects of LL37 have been shown to be partly due to direct binding of LL37 to LPS [16, 21]. It has previously been shown that hBD3 does not inhibit endotoxin binding in a Limulus assay [22] and we confirmed this finding (Supporting Information) to demonstrate similar endotoxin activity in the presence and absence of hBD3. However, the Limulus assay is not a direct measure of LPS-hBD3 binding; so we also investigated hBD3 effects after LPS stimulation of cells. Figure 2A shows that TNF-α levels were significantly reduced even when hBD3 was added to Mϕ 1 h after LPS. This suggests that even if hBD3 binds LPS to some extent, most of the hBD3 inhibitory effect is occurring downstream of TLR4 activation by LPS.

Further evidence that hBD3 is endowed with general anti-inflammatory properties is shown in Fig. 2B. Stimulation with IFN-γ and CD40L results in Mϕ activation and increased TNF-α, but here we show that hBD3 inhibited this pro-inflammatory cytokine response in mouse BMDM. This effect was also evident in C3H/HeJ Mϕ, which lack functional TLR4, demonstrating that hBD3 is not simply inhibiting stimulation by endotoxin contamination. The anti-inflammatory effect was not evident when cells were exposed to PAM_3CSK_4 a TLR1/2 agonist (Fig. 2C). This suggests that hBD3 has an effect on signalling molecules that are used by TLR4 and CD40 but not TLR1/2. This differs from LL-37, which has been shown to inhibit pro-inflammatory responses *via* both TLR4 and TLR1/2. [16]. As TLR4 and TLR1/2 signalling both involve MyD88 it is possible that hBD3 is affecting components of the non-MyD88 pathway (such as TRAM and TRIF) downstream of TLR4.

hBD3 is anti-inflammatory *in vivo*

Next, we wished to see whether hBD3 could reduce the accumulation of TNF-α in mice following exposure to LPS. We injected 16 mg/kg LPS into male Balb/c mice with and without 10 μg of hBD3 and measured serum TNF-α levels 1 h later. We found that the group injected with hBD3 and LPS had significantly reduced levels of TNF-α compared with mice receiving LPS alone (Fig. 2D). This result demonstrates that hBD3 inhibits LPS-stimulated TNF-α production *in vivo* as well as *in vitro*. The extent of inhibition afforded by hBD3 was comparable to that conferred by 1 μg IL-10, which protects mice from endotoxic shock [23], so hBD3 may provide similar protection.

Melanocortin receptors are not involved in hBD3 anti-inflammatory function

hBD3 is a promiscuous ligand which interacts with CCR6 and another unknown Mϕ receptor [14, 24]. In addition, Candille *et al* elegantly show that overexpressing the dog orthologue of hBD3

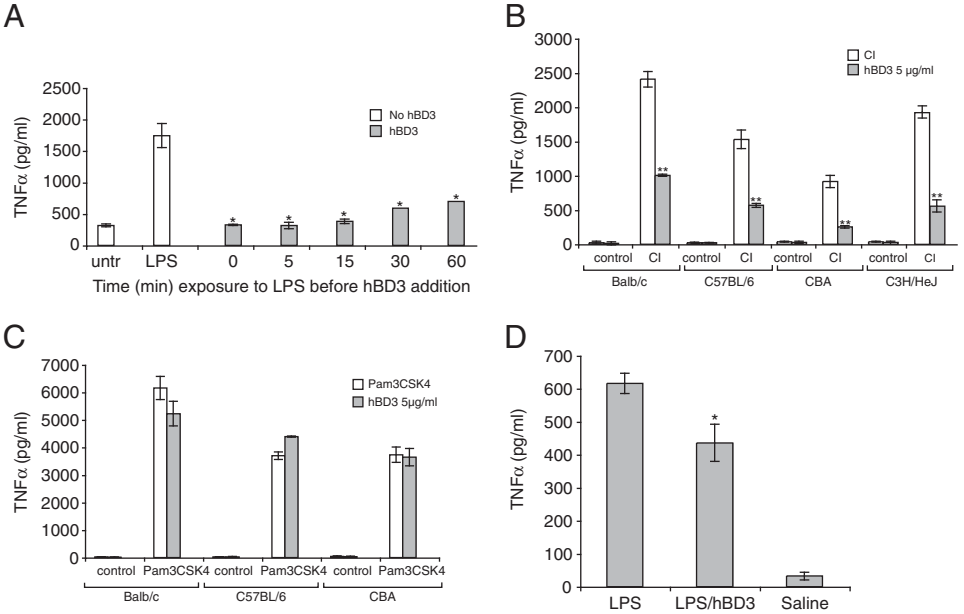

Figure 2. Dissection of hBD3 anti-inflammatory function and *in vivo* effects of hBD3. (A) The mouse Mϕ cell line RAW 264.7 was exposed at various times up to 1 h with LPS before treatment with hBD3, followed by 18 h incubation. The inhibitory effect was still evident at all time points ($n = 2$, $^*p<0.05$). (B) BMDM from different mouse strains were activated with CD40L (5 μg/mL) and IFN-γ (5 ng/mL) (CI) with and without hBD3 for 18 h. TNF-α levels in supernatants were measured. BMDM were prepared from at least three separate mice for each strain. Statistical comparisons are between agonist alone versus agonist with hBD3 within each strain, $^{**}p<0.01$ by two-way ANOVA. (C) TNF-α levels in supernatant in BMDM from different mouse strains, treated with hBD3 at indicated concentrations, with and without Pam_3Csk_4 for 18 h, $n = 3$. (D) Balb/c male mice were injected i.p. with 16 mg/kg LPS with or without 10 μg hBD3 in 200 μL saline. After 1 h animals were killed and exsanguinated and serum TNF-α measured. Figure shows means ± SEM and comparison between LPS alone ($n = 18$) and LPS with hBD3 ($n = 21$) is statistically different $^*p = 0.032$ by unpaired *t*-test.

alters hair colour in transgenic mice *via* binding to murine MC1R [14]. We tested whether hBD3 might mediate its anti-inflammatory effect through MC1R or MC3R, as these receptors are expressed in Mϕ, and the known ligand α-melanocortin stimulating hormone is an anti-inflammatory mediator [25]. The absence of either receptor has also been reported to influence the response to inflammatory agents [26, 27]. We tested the naturally defective *Mc1r* mutant mouse strain (recessive yellow *Mc1r*^{e}) [28] and an *Mc3r* knockout mouse [29]. We found no statistically significant difference between the ability of hBD3 to reduce TNF-α levels following stimulation of TLR4 or CD40 in BMDM from WT controls or mutant mice (Fig. 3A and B). This demonstrates that the anti-inflammatory properties of hBD3 are not mediated by MC1R or MC3R.

hBD3 Anti-inflammatory effect is not mediated by IL-10 or cAMP

IL-10 is a well-known anti-inflammatory cytokine that inhibits co-stimulatory molecule expression on Mϕ and limits the production of pro-inflammatory cytokines and chemokines [30]. We investigated the ability of hBD3 to induce IL-10 in BMDM and established that IL-10 levels were not altered by hBD3 in the presence or absence of LPS (Fig. 3C), suggesting that the hBD3 anti-inflammatory effect is not mediated by IL-10.

cAMP is an important controller of the innate immune system, with a wide range of functions including up-regulation of IL-10 and reduction of TNF-α [31]. Using the membrane permeable cAMP analogue, 8-Bromoadenosine-cAMP (8Br-cAMP), we examined similarities between cAMP and hBD3 anti-inflammatory activity. TNF-α levels induced by LPS were markedly reduced by 8Br-cAMP or hBD3 alone, however a combination of 8Br-cAMP and hBD3 reduced TNF-α levels further. This effect was evident at low concentrations of hBD3, where hBD3 alone shows minimal inhibition of TNF-α (Fig. 3D). Similarly induction of IL-10 by 8Br-cAMP was inhibited by hBD3 (Fig. 3C). These results suggest that cAMP and hBD3 act through distinct mechanisms.

Concluding remarks

In conclusion, hBD3 is a potent inhibitor of the accumulation of pro-inflammatory cytokines TNF-α and IL-6, secreted in response to the TLR4 agonist LPS and following activation with CD40L. This effect was not due to direct peptide binding of LPS and was not mediated through the anti-inflammatory receptors MC1R or MC3R. In support of this finding hBD3 anti-inflammatory action was independent of cAMP levels and not controlled by an increase in IL-10. In addition, administration of hBD3 to mice reduced LPS-induced serum levels of TNF-α, indicating that hBD3 may be important in controlling inflammation and septic shock. The copy number variation of β-defensins at the 8p23 cluster may lead to subtle variation in expression levels in the human population [2]. Up-regulation of hBD3 is critical to antimicrobial activity in the epithelia; however, the novel hBD3 functions presented here suggest a role in the resolution of inflammation, which is necessary to avoid tissue damage by effectors of antimicrobial action.

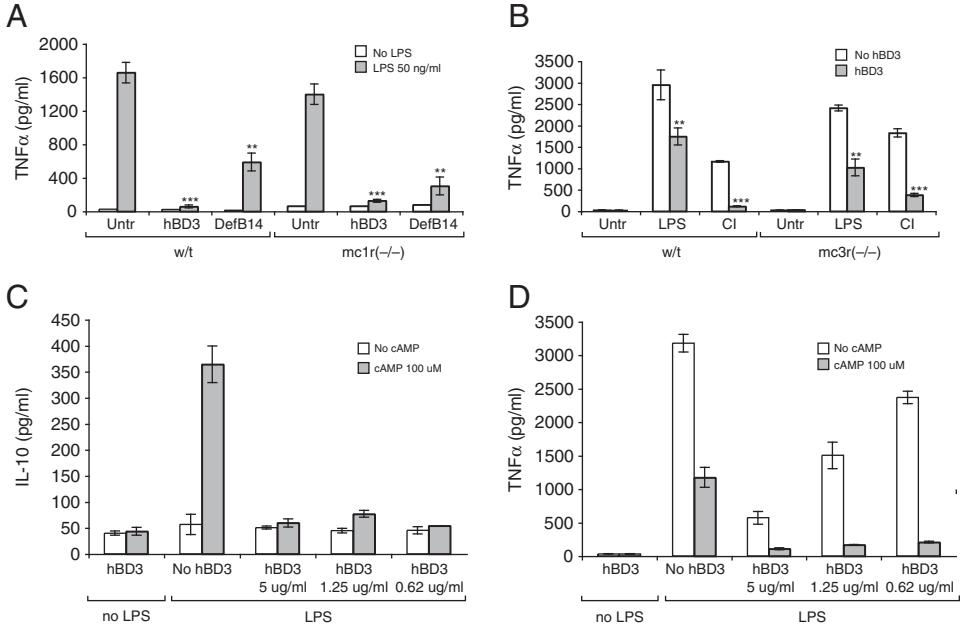

Figure 3. hBD3 anti-inflammatory effect does not act through MC1R or MC3R and has mechanisms distinct from cAMP. BMDM from (A) MC1R mutant and (B) MC3R mutant were stimulated with LPS alone or LPS in combination with hBD3 or DefB14 (5 μg/mL) or (B only) CD40/IFN-γ (CI) alone or in combination with hBD3 as described. After 18 h, concentrations of TNF-α in the supernatants were measured, $n = 3$. Statistical comparisons are between LPS alone *versus* LPS with peptide in each strain, ***$p<0.001$, **$p<0.01$ by two-way ANOVA. BMDM from Balb/c mice were cultured with LPS (50 ng/mL) in the presence of 8Br-cAMP (100 μM) and decreasing concentrations of hBD3, as shown, for 18 h. Concentrations of (C) IL-10 and (D) TNF-α in the supernatants were measured by ELISA.

Materials and methods

Reagents

Ultra pure LPS from *E. coli* 0111:B4, Pam_3CSK_4 and IFN-γ were purchased from InvivoGen (San Diego, USA), pertussis toxin, polymixin B and 8Br-cAMP (B7880) from Sigma Dorset, UK and QCL-1000® Endpoint Chromogenic LAL Assay from Lonza Group, Basel, Switzerland. Mouse CD40L was kindly provided by Dr. David Gray (University of Edinburgh). hBD3 (GIINTLQKYYCRVRGGR-CAVLSCLPKEEQIGKCSTRGRKCCRRKK) and hBD2 (GIGDPVTCLKS-GAICHPVFCPRRYKQIGTCGLPGTKCCKKP) were purchased from Peptides International Louisville, USA and are oxidised so the disulfide connectivities are of the canonical β-defensin arrangement [32]. Defb14 (FLPKTLRKFFCRIRGGRCAVLNCLGKEEQIGIRCSNS-GRKCCRKKK) and LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLR-NLVPRTES) were synthesized as previously described [20, 33].

Cells and mice

RAW264.7 cells were maintained in DMEM (GIBCO Paisley, UK) and THP-1 cells in RPMI containing 10% FBS, essential amino acids and antibiotics. Balb/c, CBA and C57 Black/6 mice were obtained from Charles River (UK) and *Mc1r e/e* and *Mc3r* KO mutants were bred in-house. C3H/HeJ OlaHsd-Tlr4 mutants and C3H/HeN controls were obtained from Harlan Laboratories, UK. Primary Mϕ were generated from femur BM and grown in DMEM containing 10% FBS and 20 ng/mL M-CSF (R&D Systems, Abingdon, UK) for 7 days. Cells were seeded at 1.25×10^5 into 48-well plates and grown without growth factor for 24 h prior to treatment. Replicate experiments were done with separate Mϕ preparations from at least three mice for each experiment.

Human PBMC preparation

Human venous blood was collected according to Lothian Research Ethics Committee approvals ♯08/S1103/38, using sodium citrate anticoagulant (Phoenix Pharma, Gloucester, UK), and cells were separated by Dextran sedimentation, followed by discontinuous, isotonic Percoll gradient centrifugation as previously described [33]. PBMC were incubated at 4×10^6/mL in IMDM (PAA Laboratories, Somerset, UK) at 37°C, 5% CO_2, for 1 h. Non-adherent cells were removed and adherent monocytes cultured for 6 days in IMDM with 10% autologous serum to generate monocyte-derived Mϕ.

Cell treatment and ELISA

Cells were treated with LPS (50 ng/mL), Pam_3CSK_4 (100 ng/mL), CD40L (3 μg/mL) IFN-γ (5 ng/mL), hBD3, Defb14, LL-37, 8Br-cAMP (at concentrations shown) or combinations of these as described, in serum free media then incubated at 37°C, 5% CO_2 for 18 h. Supernatants were collected and centrifuged to remove particulate debris. Levels of TNF-α, IL-6 and IL-10 in the supernatants were measured using human or mouse DuoSet ELISA (R&D Systems) according to the manufacturer's instructions. Cell viability was measured using TACS™ MTT assay (R&D Systems).

LPS delivery *in vivo*

Balb/c male mice (5–8 wk) were injected with 16 mg/kg of LPS (approx. 200 μg/mouse) with or without 10 μg of hBD3 in 200 μL of PBS. After 1 h mice were killed by cervical dislocation, exsanguinated and serum TNF-α levels measured by ELISA. All experiments were covered by Project License PPL 60/3787 granted by the Home Office under the Animal Scientific Procedures Act 1986, and locally approved by the University of Edinburgh Ethical Review Committee.

Statistical analysis

GraphPad Prism 5 statistical software was used to determine statistical significance. One or two-way ANOVA with Bonferroni's multiple comparison post-tests were performed. Where appropriate, statistical significance was determined by an unpaired *t*-test using GraphPad software. For all statistical analyses $p<0.05$ was considered significant. Values are expressed as mean ± SEM.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **8Br-cAMP:** 8-Bromoadenosine-cAMP · **BMDM:** BM-derived Mϕ · **CD40L:** CD40 ligand · **hBD3:** human β defensin-3 · **MC1R:** melanocortin 1 receptor · **MC3R:** melanocortin 3 receptor

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Article

Secondary necrosis of apoptotic neutrophils induced by the human cathelicidin LL-37 is not proinflammatory to phagocytosing macrophages

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ABSTRACT

Cathelicidins are CHDP with essential roles in innate host defense but also more recently associated with the pathogenesis of certain chronic diseases. These peptides have microbicidal potential and the capacity to modulate innate immunity and inflammatory processes. PMN are key innate immune effector cells with pivotal roles in defense against infection. The appropriate regulation of PMN function, death, and clearance is critical to innate immunity, and dysregulation is implicated in disease pathogenesis. The efferocytosis of apoptotic PMN, in contrast to necrotic cells, is proposed to promote the resolution of inflammation. We demonstrate that the human cathelicidin LL-37 induced rapid secondary necrosis of apoptotic human PMN and identify an essential minimal region of LL-37 required for this activity. Using these LL-37-induced secondary necrotic PMN, we characterize the consequence for macrophage inflammatory responses. LL-37-induced secondary necrosis did not inhibit PMN ingestion by monocyte-derived macrophages and in contrast to expectation, was not proinflammatory. Furthermore, the anti-inflammatory effects of apoptotic PMN on activated macrophages were retained and even potentiated after LL-37-induced secondary necrosis. However, this process of secondary necrosis did induce the release of potentially harmful PMN granule contents. Thus, we suggest that LL-37 can be a potent inducer of PMN secondary necrosis during inflammation without promoting macrophage inflammation but may mediate host damage through PMN granule content release under chronic or dysregulated conditions. *J. Leukoc. Biol.* 86: 891–902; 2009.

Introduction

PMN are important first-line innate immune cells, which are mobilized rapidly in response to infection and injury. However, the arsenal of products used by PMN to destroy microbes is also potentially deleterious to host cells. Appropriate regulation of PMN influx, activation, death, and removal is therefore critical, and dysregulation of these processes has been implicated in the pathogenesis of chronic lung diseases [1, 2].

PMN have a short half-life and undergo spontaneous apoptosis. This programmed and regulated form of cell death enables recognition, ingestion, and removal by macrophages or DCs, a process involving multiple receptors and adaptors [3]. Efferocytosis (the uptake of apoptotic cells) can protect the host from the release of toxic PMN intracellular contents and induce anti-inflammatory and immunosuppressive effects. These include dampening the LPS-induced, proinflammatory cytokine response and enhancing anti-inflammatory responses by macrophages [4–6]. These processes, in addition to an active switch in the lipid mediators generated to the production of lipoxins, resolvins, and protectins [7], can stimulate the resolution of inflammation. However, in the absence of efferocytosis, apoptotic PMN will undergo secondary necrosis with loss of membrane integrity. Although necrosis is typically regarded as proinflammatory [1], the effect of PMN secondary necrosis on inflammation and the mechanisms of clearance of these

Abbreviations: AV=Annexin V, BALF=bronchoalveolar lavage fluid, CD40L=CD40 ligand, CHDP=cationic host defense peptide(s), DC=dendritic cell, hCAP-18=human cationic antimicrobial protein, LAL=Limulus amoebocyte lysate, mCRAMP=mouse cathelicidin antimicrobial peptide, MDM=monocyte-derived macrophage(s), MPO=myeloperoxidase, PI=propidium iodide, PMN=polymorphonuclear granulocytes, TEM=transmission electron microscopy

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cells are relatively poorly understood. Further understanding of these issues is of great significance to evaluate the impact of delayed PMN clearance in the resolution of inflammation.

The survival and death of PMN can be modulated significantly by the inflammatory milieu, including cytokines such as TNF-α and GM-CSF, TLR agonists (e.g., LPS), and bacterial products (e.g., pyocyanin) [8], in addition to potential therapeutic modulators [9]. The human CHDP (also known as antimicrobial peptide) LL-37 can also modulate PMN death pathways in vitro [10, 11]. We have demonstrated that LL-37 promotes PMN necrosis at the expense of apoptosis [10]. However, the mechanisms involved and the consequences for inflammation remain to be determined.

CHDP are key evolutionarily conserved components of innate host defense systems, which are being modified and developed as novel, antimicrobial therapeutic agents. The two major CHDP families in vertebrates are defensins and cathelicidins. LL-37 is the predominant, mature cationic peptide fragment of the sole human cathelicidin hCAP-18, which is found at high concentration in neutrophil specific granules and is cleaved to its active form by proteinase-3 [12, 13]. LL-37 is also produced by epithelial cells from a variety of tissues in response to infectious and inflammatory stimuli and is expressed by macrophages and other leukocytes [14]. In addition to direct microbicidal potential, LL-37 has a broad range of immunomodulatory functions including antiendotoxic activity and chemotactic function, modulation of chemokine and cytokine responses, promotion of cell wound-healing and angiogenesis, modulation of DC differentiation and function, and the capacity to modulate cell death pathways [10, 11, 15–27]. In vivo evidence in humans and mice supports a critical role in innate defense [28–31], hCAP-18/LL-37 concentration in BALF is increased significantly in human lung infections [32], and overexpression of LL-37 in the murine lung enhanced the clearance of infection [33]. However, increased levels of hCAP-18/LL-37 have also been associated with and implicated in chronic disease processes [34–36]. Thus, despite a fundamental role in innate defense, dysregulated control of LL-37 may be detrimental to the host. The key immunomodulatory properties that contribute to the physiological role of LL-37 in host defense and how these might contribute to disease pathogenesis when dysregulated remain unresolved.

We hypothesized that LL-37-induced necrosis of PMN could alter efferocytosis and the nature of the subsequent inflammatory response and could lead to the release of potentially damaging PMN intracellular contents. To examine the role of LL-37-induced PMN necrosis on the inflammatory response, we characterized the dynamics of LL-37-induced PMN necrosis before evaluating the effect of LL-37-induced PMN necrosis on efferocytosis and macrophage inflammatory responses and the consequences for release of PMN intracellular contents.

MATERIALS AND METHODS

Reagents

rhGM-CSF was purchased from Research Diagnostics Inc. (Flanders, NJ, USA). *R*-Roscovitine was supplied by A. G. Scientific (San Diego, CA, USA). Ultra-pure LPS from the *Escherichia coli* 0111:B4 strain was purchased from InvivoGen (San Diego, CA, USA). Dexamethasone was purchased from Organon Laboratories Ltd. (Cambridge, UK). rhCD40L and IFN-γ were purchased from PeproTech ED Ltd. (London, UK). LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and mCRAMP (GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ) were synthesized by N-(9-fluorenyl) methoxycarbonyl chemistry at the Nucleic Acid/Protein Service Unit at the University of British Columbia (Canada), as described previously [10]. Peptides were purified by reverse-phase HPLC and were at least 98% pure. The concentration of the peptides in solution was determined by amino acid analysis. Scrambled LL-37 (RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL) was purchased from CSS-Albachem Ltd. (Gladsmuir, Scotland, UK), and the panel of 16 overlapping 22-mer partial LL-37 peptides (from N-terminal peptide LLGDFFRKSKEKIGKEFKRIVQ through to C-terminal peptide EFKRIVQRIKDFLRNLVPRTES) was synthesized by Jan W. Drijfhout at Leiden University Medical Center (The Netherlands) as described previously [37]. Peptides were dissolved in endotoxin-free water (Sigma-Aldrich, Poole, UK) and stored at –20°C until further use. All reagents were tested using a LAL kinetic-quantitative chromogenic LAL assay (Cambrex, Walkersville, MD, USA) to ensure they were free of endotoxin and reconstituted in endotoxin-free water.

Isolation of human blood neutrophils

Human venous blood was collected according to Lothian Research Ethics Committee approval #08/S1103/38 or #1702/95/4/72 using sodium citrate anticoagulant (Phoenix Pharma Ltd., Gloucester, UK), and cells were separated by Dextran sedimentation, followed by discontinuous, isotonic Percoll gradient centrifugation as described previously [10]. Granulocytes were washed in PBS without calcium or magnesium (PAA Laboratories, Somerset, UK) and resuspended in IMDM (PAA Laboratories) with 10% FCS (Biosera, East Sussex, UK). Purity was assessed by morphological criteria using cytocentrifuge preparations and FACS analyses and antibodies against human CD16 (Caltag-Medsystems Ltd., Towcester, UK) and CD66b (BD Biosciences, San Diego, CA, USA) to distinguish PMN from eosinophils and against CD14, CD4, CD8, and CD19 (Caltag-Medsystems Ltd.). Granulocyte purity of >98% was yielded by this method, and granulocytes were typically 95–98% PMN. However, high eosinophil donors were also studied, demonstrating no significant effect of eosinophil number on LL-37-induced secondary necrosis of apoptotic PMN. Total cell number was assessed by hemacytometer counts and by NucleoCounter YC-100 (ChemoMetec, Allerød, Denmark) automated cell number counting.

Assessment of PMN death

Freshly isolated PMN were incubated at 37°C, 5% CO_2, at 5×10^6/ml in IMDM with 10% (v/v) FCS in the presence of LL-37, GM-CSF, *R*-Roscovitine, LPS, mCRAMP, scrambled LL-37, or partial LL-37 peptides at the stated concentrations or in control media in triplicate over the time periods detailed. To induce primary necrosis, freshly isolated cells were heated at 65°C for 30 min. Cell death was assessed by light microscopic evaluation of apoptotic morphology as described [10] and also by examining cells stained at 4°C with FITC-labeled AV (Roche Applied Sciences, West Sussex, UK), diluted 1:2000 in HBSS with 5 mM $CaCl_2$, and 3 μg/ml PI (Invitrogen Ltd., Paisley, UK) for flow cytometric evaluation using a FACSCalibur, counting ≥10,000 cells, and analyzed using FloJo software (TreeStar Inc., Ashland, OR, USA). High concentrations of LL-37 can lead to a complete loss of cells from analysis [10, 38]. To evaluate this accurately, the concentration-dependent induction of necrosis was determined by FACS, and the total detectable PMN numbers were determined using a NucleoCounter YC-100 (ChemoMetec) after 20 h culture over a range of LL-37 concentrations (≤25 μg/ml). An approximate EC_{50} = 3.8 μg/ml was determined for LL-37-induced necrosis, and LL-37 ≥10 μg/ml (~2 μM) was found to induce a significant and concentration-dependent loss in total detectable cell number (total cell number as a proportion of control sample=81%±5% at 20 h, LL-37 10 μg/ml; $P<0.01$; $n=20$). In contrast, incubation with ≤5 μg/ml LL-37 did not result in a significant decrease, although a degree of cell loss was observed sometimes. LL-37 was therefore used at ≤5 μg/ml for further studies examining LL-37-mediated modulation of PMN cell death.

TEM

Freshly isolated PMN were incubated with or without 5 μg/ml LL-37 for 20 h, as described above, centrifuged at 250 *g* for 5 min at room temperature, and resuspended gently in 1 ml 2.5% glutaraldehyde (Sigma-Aldrich) in 0.1 M sodium cacodylate buffer, pH 7.3 (Sigma-Aldrich), for 1 h. Cells were washed in three 10-min changes of 0.1 M sodium cacodylate and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 45 min before three 10-min changes of 0.1 M sodium cacodylate. Samples were then dehydrated in 50%, 70%, 90%, and 100% normal grade acetones before two 10-min changes in analar acetone and embedded in Araldite resin. Toluidine blue-stained sections (1 μm) were previewed before 60 nm ultrathin sections were cut from selected areas, stained in uranyl acetate and lead citrate, and then viewed in a Philips CM120 transmission electron microscope (FEI UK Ltd., Cambridge, UK). Images were taken on a Gatan Orius charged-coupled device camera (Gatan UK, Oxon, UK).

Western immunoblotting

Freshly isolated PMN were incubated as described above over a range of LL-37 concentrations. At Time 0 h or after 20 h, cells were washed with PBS without Ca^{2+} and Mg^{2+}, and proteins were extracted using Mammalian Protein Extraction Reagent (Pierce/Perbio Science UK, Cheshire, UK) containing 30 μl/ml Halt™ protease inhibitor cocktail, 30 μl/ml Halt™ phosphatase inhibitor cocktail, 10 μl/ml EDTA solution (all Pierce/Perbio Science UK), 30 μM pepstatin (Sigma-Aldrich), and 10 μM lactacystin (Calbiochem/Merck Biosciences Ltd., Nottingham, UK). Total protein concentrations were determined by bicinchoninic acid assay (Pierce/Perbio Science UK). Equivalent total protein (20–50 μg) was resolved in Pierce Precise gels (Pierce/Perbio Science UK) transferred to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA) and immunoblotting performed as described [10] with anti-human cleaved caspase-3 rabbit polyclonal antibody and reprobed with anti-pan-actin antibody (both Cell Signaling Technology, Beverly, MA, USA) for protein loading correction via densitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Phagocytic studies

Human PBMC were isolated by isotonic Percoll gradient centrifugation as described above and incubated at 4×10^6/ml in IMDM at 37°C, 5% CO_2, for 1 h. Nonadherent cells were removed and adherent monocytes cultured for 5–7 days in IMDM and 10% autologous serum to generate MDM. For light microscopic analyses of phagocytosis, PMN were incubated previously for 20 h, with or without LL-37 (or scrambled LL-37) at the stated concentrations (as described above), and the level of apoptosis was assessed as described. PMN (2.5×10^6; ~2.5:1 ratio of PMN:MDM) were overlaid onto HBSS-washed MDM monolayers for 1 h at 37°C. MDM were then washed gently four times with IMDM to remove noningested PMN and fixed with 2.5% paraformaldehyde (Sigma-Aldrich) for 10 min. PMN MPO was stained with 0.1 mg/ml dimethoxybenzidine (Sigma-Aldrich) and 0.03% (v/v) H_2O_2 (Sigma-Aldrich) before analysis by light microscopy, counting at least 200 MDM in five randomly selected fields of view to evaluate the proportion of MDM-containing peroxidase-positive cells, with $n = 2$ replicates/experiment. Only MDM that had engulfed PMN clearly were scored as positive. For flow cytometric analyses of phagocytosis, freshly isolated PMN were stained with Cell Tracker Green (Molecular Probes, Eugene, OR, USA) at 37°C for 20 min, according to the manufacturer's instructions, and then washed twice with PBS before incubation for 20 h in the presence or absence of LL-37, as described. The effects of LL-37 were not altered by Cell Tracker Green staining (data not shown). MDM were exposed to PMN as described above, but after 1 h incubation, cells were detached and collected using 0.05% trypsin/0.02% EDTA solution (Cambrex). Samples were analyzed by FACSCalibur as described previously [39], with $n = 2$ replicates/experiment. The number of Cell Tracker Green-positive events in the macrophage gate (based on forward- and side-scatter) represented the number of macrophages that had ingested PMN and was evaluated as a proportion of total number of MDM. As a positive control, wells of adherent monocytes were also cultured in the presence of 1 μM dexamethasone for 5 days, a process demonstrated previously to up-regulate MDM phagocytosis of dead PMN [40].

MDM cytokine production

Freshly isolated PMN at 5×10^6/ml were incubated for 20 h in the presence or absence of LL-37, scrambled LL-37, or partial LL-37 peptides p1 (LLGDFFRKSKEKIGKEFKRIVQ), p2 (KIGKEFKRIVQRIKDFLRNLVP), or p3 (EFKRIVQRIKDFLRNLVPRTES) at the concentrations indicated in 10% FCS as described above. Samples (1 ml) were collected and each split in two, and half of each sample was centrifuged at 230 *g* before collection of the supernatant and resuspension of the cells in 0.5 ml X-vivo 10 media (Lonza Biologics, Slough, UK), with or without 10% FCS. Washed MDM, with or without concomitant exposure to 10 ng/ml *E. coli* 0111:B4 LPS or 3 μg/ml rhCD40L/5 ng/ml rhIFN-γ, were then incubated at 37°C, 5% CO_2, for 18 h with 0.5 ml PMN in fresh X-vivo 10, PMN in the original IMDM with 10% FCS, IMDM with 10% FCS supernatant collected from overnight-incubated PMN, or media alone. MDM alone and PMN alone in the presence of absence of LPS and/or LL-37 were also studied as controls. Supernatants were collected, centrifuged at 230 *g* for 6 min to remove cells and particulate debris, and then stored in aliquots at –70°C. The concentrations of TNF-α, IL-6, IL-10, IL-12p70, and IL-1β in the supernatants were measured using BD Cytometric Bead Array human inflammation kits with a BD FACSArray (BD Biosciences) or Human TNF-α DuoSet ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

MPO release

Freshly isolated PMN were incubated with different concentrations of LL-37 or scrambled peptide for 20 h as described above, centrifuged at 230 *g* for 6 min, and placed immediately on ice, and the supernatants were collected. To detect MPO, 100 μl supernatant samples (used neat or diluted in PBS) were incubated in the dark for 20 min at room temperature with 100 μl substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine; R&D Systems), followed by addition of 50 μl 2 N H_2SO_4 stop solution and colorimetric analyses at 450 nm using a microplate reader. The concentration of MPO in the supernatants was evaluated as a percentage of MPO release after complete lysis of fresh control PMN using 0.1% Triton X-100 (Sigma-Aldrich).

Statistical analysis

GraphPad Prism 5 statistical software was used to determine statistical significance. One-way or two-way ANOVA with Bonferroni's multiple comparison post-tests or Student's *t*-tests was performed as appropriate. $P < 0.05$ was considered significant. Values are expressed as mean ± SEM.

RESULTS

LL-37 induces the rapid secondary necrosis of apoptotic PMN

We and others [10, 11] have demonstrated that the proportion of apoptotic (AV^+PI^-) cells detected in an in vitro model of spontaneous apoptosis of human PMN was decreased significantly when cells were cultured with LL-37. The most prominent consequence of this was an increased proportion of necrotic (AV^+PI^+) PMN [10]. To carefully characterize LL-37-induced, necrotic PMN, with which to examine the consequences for macrophage inflammatory responses, a large donor pool (n=28 different donors) was evaluated. This demonstrated the concentration-dependent capacity of LL-37 to promote PMN necrosis significantly at the expense of apoptosis, with no signifi-

cant impact on the proportion of live (AV^-PI^-) cells at ≤5 μg/ml LL-37 using FACS (**Fig. 1A**) and morphological analyses. LL-37-induced necrotic PMN were observed to be similar to "late apoptotic" cells observed in control samples at 20 h, with less-severe loss of membrane integrity than primary necrosis induced by heat treatment of cells (Fig. 1B). TEM was used to confirm the capacity of LL-37 to promote PMN necrosis after 20 h incubation when compared with control cells (Fig. 1, C and D).

We have demonstrated previously that LL-37 was not lytic to PMN [10]. Furthermore, no significant necrosis was observed at 0, 1, 4, or 6 h (ref. [10], and data not shown). However, significant ($P \leq 0.001$) induction of necrosis was observed at 12 h (data not shown) and thereafter, occurring at time-points at which substantial apoptosis was observed. In keeping with recent publications [38, 41], this suggested prior induction of apoptosis may be necessary and that LL-37 might induce secondary necrosis. To confirm this in our model system, PMN were exposed to early or late pulses of LL-37. PMN incubated with LL-37 (≤5 μg/ml) for 4 h, followed by washing and incubation in control media for 16 h, had proportions of apoptotic, necrotic, and live cells identical to untreated controls (data not shown). In contrast, 20 h incubation of cells in control media, followed by exposure to LL-37 for just 10 min before FACS analyses, completely replicated the dose-dependent effect of 20 h incubation with LL-37 (Fig. 1E), demonstrating that LL-37 induced a rapid secondary necrosis of apoptotic PMN without affecting live cells.

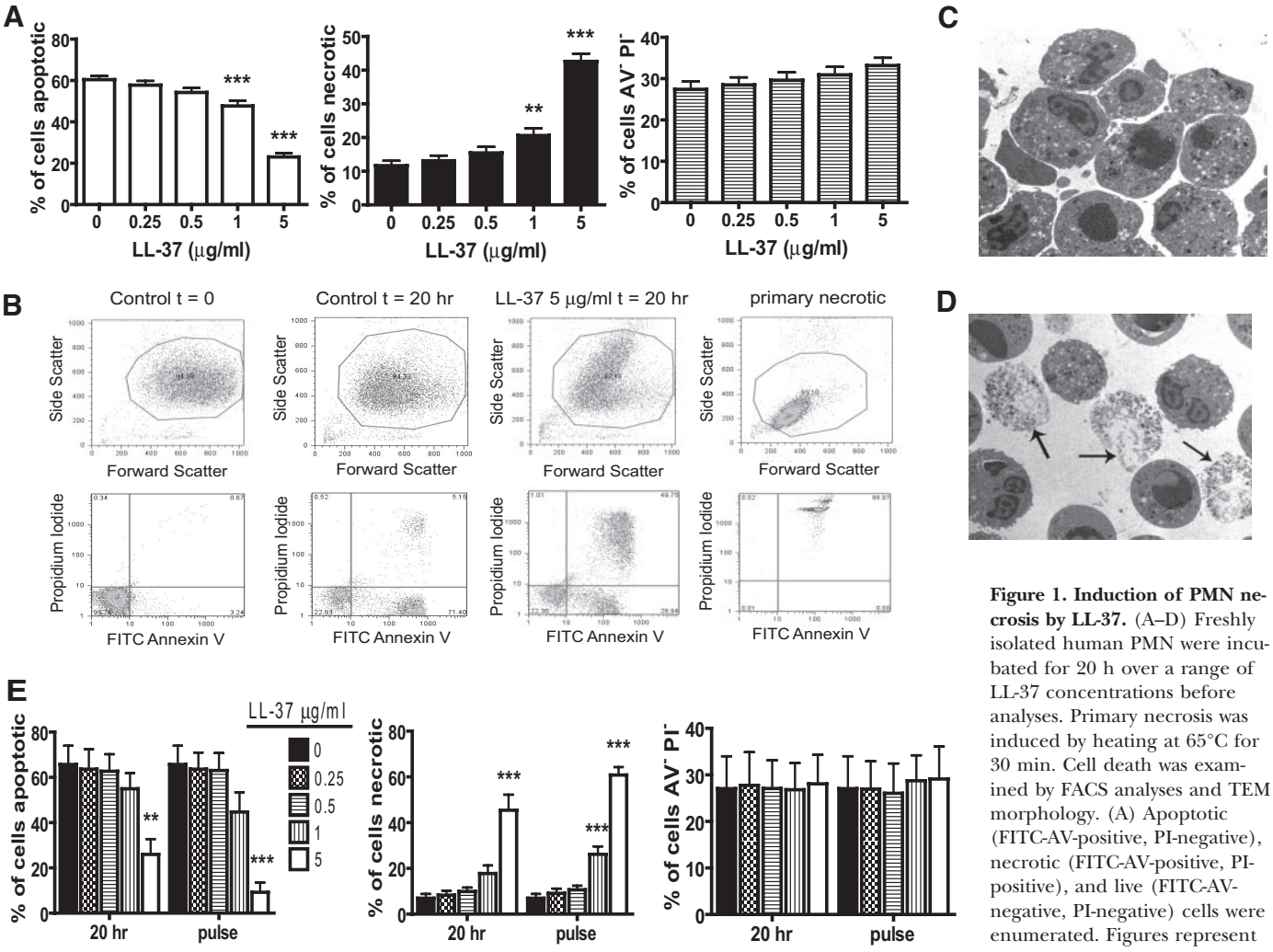

Figure 1. Induction of PMN necrosis by LL-37. (A–D) Freshly isolated human PMN were incubated for 20 h over a range of LL-37 concentrations before analyses. Primary necrosis was induced by heating at 65°C for 30 min. Cell death was examined by FACS analyses and TEM morphology. (A) Apoptotic (FITC-AV-positive, PI-negative), necrotic (FITC-AV-positive, PI-positive), and live (FITC-AV-negative, PI-negative) cells were enumerated. Figures represent mean values ± SEM for $n = 28$ different donors for each condition, performed in triplicate. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control; **, $P \leq 0.01$; ***, $P \leq 0.001$. (B) Representative FACS plots. (C and D) Representative TEM images of (C) untreated, apoptotic PMN and (D) PMN exposed to 5 μg/ml LL-37. Arrows indicate examples of necrotic PMN. (E) Freshly isolated human PMN were incubated for 20 h over a range of LL-37 concentrations (20 h samples) or incubated for 20 h in the absence of LL-37 followed by exposure to a range of LL-37 concentrations for 10 min (pulse samples) before FACS analyses of apoptotic, necrotic, and live cells. Panels represent mean values ± SEM for $n = 3$ donors performed in triplicate. Significance was assessed by two-way ANOVA with Bonferroni's post-test; **, $P \leq 0.01$; ***, $P \leq 0.001$, compared with untreated control.

Interaction of LL-37 with known modulators of PMN apoptosis

To investigate whether LL-37 can interact with and modulate the effects of known modifiers of PMN apoptosis pathways, cells were incubated with LL-37 in the presence or absence of GM-CSF (a PMN survival factor [42]) or *R*-Roscovitine (a cyclin-dependent kinase inhibitor and inducer of PMN apoptosis [9]). GM-CSF reduced PMN apoptosis significantly at 20 h, increasing the proportion of live cells with no effect on necrosis (**Fig. 2A**). Concomitant treatment with 5 μg/ml LL-37 had no effect on the capacity of GM-CSF to promote cell survival but significantly ($P<0.05$) increased the proportion of necrotic cells with a reciprocal loss of apoptotic cells when compared with GM-CSF alone (Fig. 2A). *R*-Roscovitine induced PMN apoptosis significantly at 6 h with minimal necrosis (Fig. 2B). LL-37 alone ($\leq$25 μg/ml) had no effect on cell death at this timepoint. Concomitant LL-37 treatment had no effect on the capacity of *R*-Roscovitine to induce cell death but significantly ($P<0.01$) increased the proportion of necrotic cells in a dose-dependent manner with a reciprocal loss of apoptotic cells when compared with *R*-Roscovitine alone (Fig. 2B). These data demonstrate no interaction with these direct modifiers of PMN apoptosis, but LL-37 induced secondary necrosis, relating in magnitude to the extent of induction of apoptosis.

In addition, studies were performed to assess the impact of LL-37 (a well-characterized antiendotoxic agent [15]) on the antiapoptotic effects of LPS in PMN [43]. LPS promoted PMN survival (Fig. 2C) significantly, inhibiting apoptosis without inducing necrosis (Fig. 2D). Concomitant incubation with LL-37 inhibited this effect significantly in a dose-dependent manner. LL-37 (5 μg/ml) blocked the pro-survival effects of LPS completely and significantly increased the proportion of dead cells that were necrotic ($P<0.01$). However, the capacity of 5 μg/ml LL-37 to induce secondary necrosis was diminished significantly by LPS ($P<0.001$). These data demonstrate LL-37-mediated inhibition of the antiapoptotic effects of LPS and suggest a mutually inhibitory interaction with LPS diminishing LL-37-induced secondary necrosis.

LL-37-mediated induction of secondary necrosis is conserved in mCRAMP and C-terminal partial peptides and is not primarily charge-dependent

To examine the important characteristics of LL-37 for the capacity to induce secondary necrosis, FACS-based studies were performed using a scrambled LL-37 peptide (with conserved charge but altered amino acid sequence), the murine homologue mCRAMP, and a panel of sixteen 22-mer partial peptides spanning the sequence of LL-37 [37]. Incubation of PMN with scrambled LL-37 had no effect, whether exposed over 20 h or as a final 10-min pulse (**Fig. 3A**). In contrast, mCRAMP exposure closely replicated the effects of LL-37 with significant, dose-dependent induction of secondary necrosis (Fig. 3B). Exposure to N-terminal 22-mer partial LL-37 peptides (from the peptide incorporating aa 1–22 of LL-37 through that spanning aa 10–31) had no significant effects (Fig. 3C). In contrast, C-terminal partial peptides (from the peptide incorporating aa 11–32 through that spanning aa 16–37) induced significant secondary necrosis ($P<0.01$) in the absence of effects on live cells but to a lesser extent than full-length LL-37. This identified a putative minimal core functional region for induction of secondary necrosis spanning aa 16–32. The effects of these peptides showed no correlation with charge, which ranged from +3 to +6. These data demonstrate that the capacity to induce PMN secondary necrosis is evolutionarily conserved between mouse and human cathelicidin, is not primarily dependent on peptide charge, and is retained by C-terminal but not N-terminal-truncated peptides of LL-37.

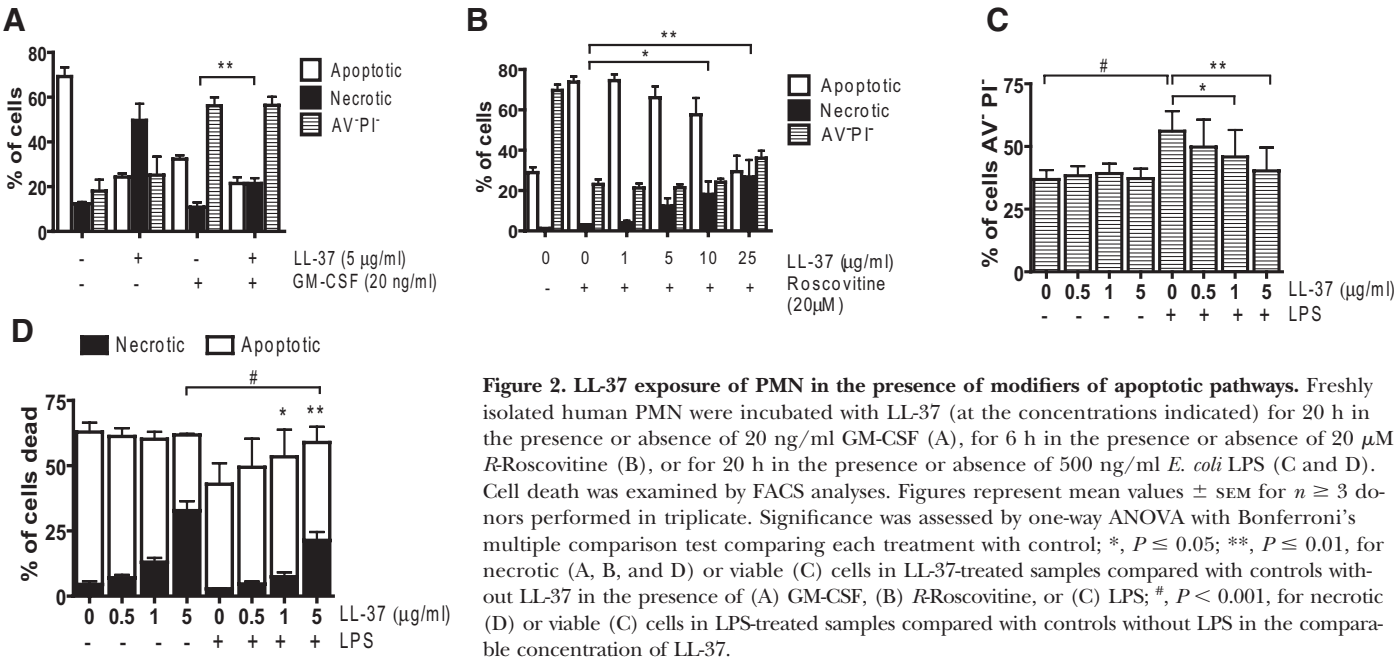

Figure 2. LL-37 exposure of PMN in the presence of modifiers of apoptotic pathways. Freshly isolated human PMN were incubated with LL-37 (at the concentrations indicated) for 20 h in the presence or absence of 20 ng/ml GM-CSF (A), for 6 h in the presence or absence of 20 μM *R*-Roscovitine (B), or for 20 h in the presence or absence of 500 ng/ml *E. coli* LPS (C and D). Cell death was examined by FACS analyses. Figures represent mean values $\pm$ SEM for $n \geq 3$ donors performed in triplicate. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control; *, $P \leq 0.05$; **, $P \leq 0.01$, for necrotic (A, B, and D) or viable (C) cells in LL-37-treated samples compared with controls without LL-37 in the presence of (A) GM-CSF, (B) *R*-Roscovitine, or (C) LPS; #, $P < 0.001$, for necrotic (D) or viable (C) cells in LPS-treated samples compared with controls without LPS in the comparable concentration of LL-37.

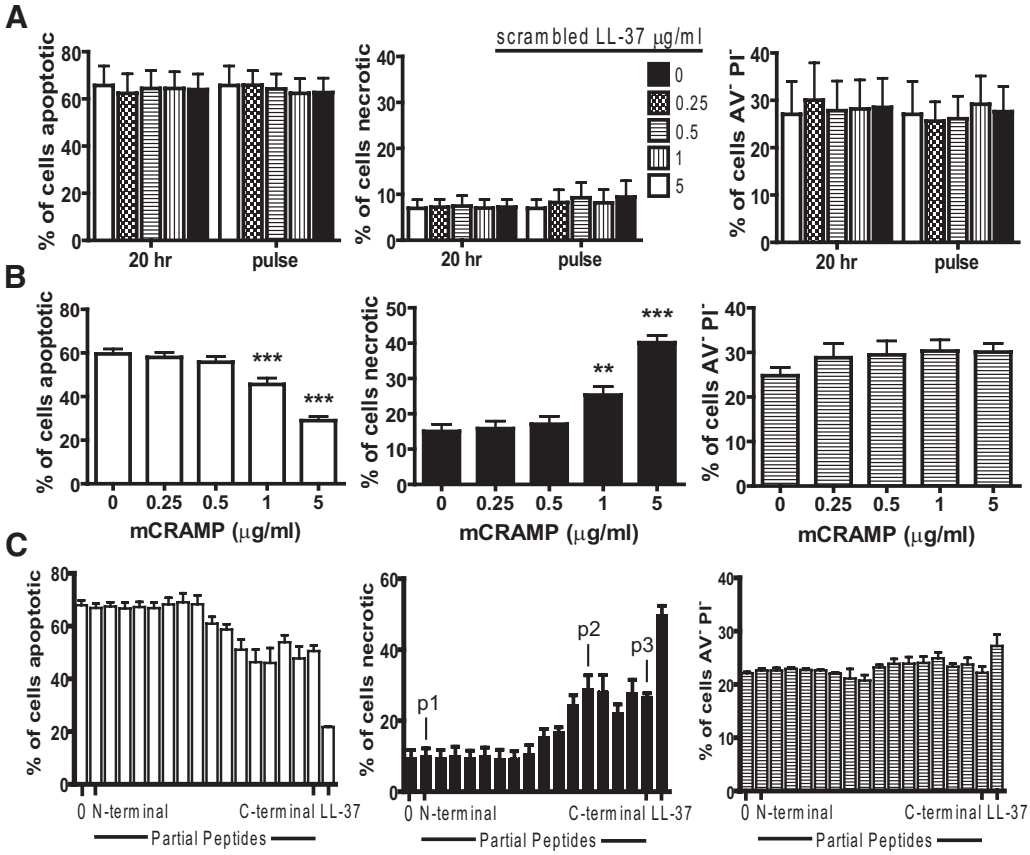

Figure 3. Peptide specificity in the induction of PMN secondary necrosis. Freshly isolated human PMN were incubated with (A) a range of concentrations of scrambled LL-37 peptide for 20 h, or for 10 min after 20 h incubation in the absence of peptide (pulse); $n = 3$ donors performed in triplicate; (B) a range of concentrations of mCRAMP peptide; $n = 16$ donors performed in triplicate; or (C) 5 μg/ml partial LL-37 peptides from a panel of 16 overlapping 22-mers using N-terminal peptide (aa 1–22), then peptide spanning aa 2–23, and so on through C-terminal peptide (aa 16–37) or full-length LL-37; $n = 3$ donors performed in triplicate. Peptides p1, p2, and p3 are identified. Cell death was examined by FACS analyses as described. Figures represent mean values ± SEM. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control; **, $P \leq 0.01$; ***, $P \leq 0.001$.

LL-37-induced secondary necrosis of apoptotic PMN does not inhibit macrophage efferocytosis

To determine whether macrophage ingestion of dead PMN was altered by LL-37-mediated secondary necrosis, ingestion of LL-37-treated PMN was studied using MDM by light microscopic enumeration (**Fig. 4A**). Confirmatory studies were performed using FACS analyses of MDM to determine ingestion of labeled PMN (Fig. 4B). In both studies, positive control dexamethasone-primed macrophages (shown previously to have increased capacity for apoptotic PMN ingestion [40]) were used. LL-37-mediated induction of secondary necrosis had no significant effects on the magnitude of PMN ingestion by MDM, even at 25 μg/ml LL-37 (a concentration at which ~80% of dead cells are necrotic [10]).

LL-37-induced secondarily necrotic PMN do not induce proinflammatory macrophage responses

The interaction of apoptotic PMN and macrophages can induce anti-inflammatory macrophage responses and inhibit LPS-induced proinflammatory cytokine responses [5]. In contrast, necrotic cells are suggested to induce proinflammatory responses [44]. To examine the effects of LL-37-mediated PMN secondary necrosis, MDM cytokine responses to these cells were evaluated at 18 h after exposure. To separate the effects of any residual, functional LL-37 in the media from the effects of the dead PMN and of the cell bodies from the cellular contents released from secondarily necrotic cells, PMN were incubated for 20 h at a range of concentrations of LL-37, and MDM were then exposed to LL-37-treated PMN, resuspended in fresh serum-free media, unwashed LL-37-treated PMN in their original media, supernatant from LL-37-treated PMN alone, or media without PMN as a control.

MDM exposed to control apoptotic PMN, PMN incubated for 20 h with LL-37 (≤25 μg/ml), or the supernatants from these cells did not produce detectable levels of TNF-α, IL-6, IL-10, IL-12p70, or IL-1β (**Fig. 5**, and data not shown), and there were no significant differences between responses to LL-37-treated and control apoptotic PMN. These data suggest that LL-37-induced secondarily necrotic PMN are not proinflammatory to macrophages nor release effective, proinflammatory contents into the supernatant.

LL-37-induced secondarily necrotic PMN have anti-inflammatory effects on macrophages

To determine the effect of these cells in the context of proinflammatory stimuli, MDM were exposed concomitantly to 10 ng/ml *E. coli* LPS. Treatment of MDM with LPS alone stimulated significant production of TNF-α, IL-6, and IL-10 (but not IL-12p70 or IL-1β). In keeping with previous reports [5], the LPS-induced proinflammatory response was reduced by the addition of control apoptotic PMN in serum-free media with significant inhibition ($P<0.01$) of TNF-α and IL-6 (**Fig. 6A**). However, these control apoptotic cells had no significant effect on LPS-induced IL-10 production (with considerable donor

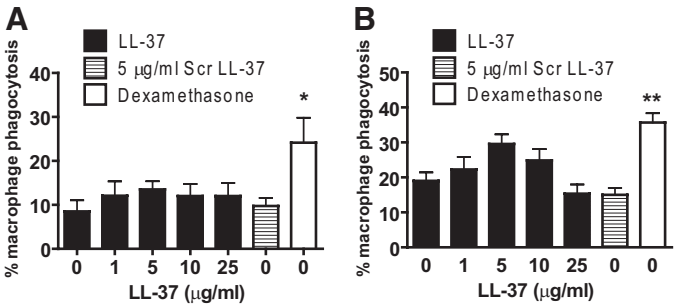

Figure 4. Macrophage phagocytosis of LL-37-induced secondarily necrotic PMN. PMN were incubated previously for 20 h, with or without LL-37 (or scrambled LL-37) at the stated concentrations, and then incubated with hMDM for 1 h before removal of noningested PMN. Phagocytosis was assessed by (A) light microscopy, counting the proportion of MDM peroxidase-positive cells; $n = 3$ donors for each condition and $n = 2$ replicates/experiment; or (B) flow cytometric analyses, evaluating the proportion of MDM ingesting Cell Tracker Green-stained PMN; $n \geq 3$ donors for each condition, with $n = 2$ replicates/experiment. As a positive control, wells of adherent monocytes were also cultured in the presence of 1 μM dexamethasone for 5 days to up-regulate MDM phagocytosis of dead PMN. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test comparing each treatment with control; *, $P \leq 0.05$; **, $P \leq 0.01$.

variation). Contrary to expectation, when PMN, incubated previously for 20 h with LL-37 to induce secondary necrosis, were resuspended in fresh, serum-free media and used, these cells also inhibited LPS-induced TNF-α and IL-6 responses. The effect of cells incubated previously with 5 μg/ml LL-37 to induce secondary necrosis was not significantly different than control apoptotic cells (Fig. 6A). However, significantly greater inhibition of LPS-induced TNF-α ($P<0.01$) was observed in response to cells incubated previously with 25 μg/ml LL-37 (Fig. 6A), in which the largest induction of secondary necrosis is evident. In addition, in contrast to control apoptotic cells, a trend toward diminished, LPS-induced MDM IL-10 responses was observed in response to these secondarily necrotic cells (Fig. 6A). Exposure of MDM to control apoptotic PMN in their original supernatant (Fig. 6B) had no effect on the LPS-induced production of TNF-α, IL-6, or IL-10 by MDM and was essentially the same as exposure to the supernatant alone (data not shown). In contrast, exposure to unwashed, LL-37-treated PMN in their original supernatant did inhibit the LPS-induced production of TNF-α ($P<0.001$), IL-6 ($P<0.05$), and IL-10 by MDM (Fig. 6B). This effect could be replicated using supernatants alone (data not shown) but was not observed using cells treated with scrambled LL-37 (Fig. 6B). In addition, fresh LL-37, added directly to LPS-stimulated MDM in the absence of PMN, was able to replicate this inhibitory effect (Fig. 6B), indicating that the antiendotoxic effects of remaining, active LL-37 in the original supernatant may be responsible for the results observed in these latter studies. To determine whether the loss of inhibitory activity of dead PMN in their original supernatant related to cellular products or serum within the original media, 20 h PMN, washed and resuspended in fresh serum-containing media, were used. These cells (control and LL-37-treated) lost the capacity to inhibit LPS-induced MDM TNF-α production (**Fig. 7A**), implicating an unidentified serum-factor and demonstrating that washed, LL-37-treated cells did not have sufficient residual LL-37 to be directly antiendotoxic.

To confirm that the anti-inflammatory effects of LL-37-induced secondarily necrotic PMN were independent of the antiendotoxic effects of the peptide, rCD40L and rIFN-γ treatment was used to activate MDM, rather than LPS. Control-apoptotic and LL-37-induced secondarily necrotic PMN were capable of significantly ($P<0.001$) inhibiting the resultant TNF-α production (Fig. 7B).

In addition, the impact of three 22-mer partial LL-37 peptides was evaluated. N-terminal peptide p1 induced no PMN secondary necrosis (Fig. 3C) and had no direct antiendotoxic effects when applied to LPS-treated MDM (Fig. 7C). C-terminal peptides p2 and p3 induced significant PMN secondary necrosis (Fig. 3C), but whereas p2 had direct, significant antiendotoxic effects ($P<0.05$), p3 did not (Fig. 7C). When PMN, incubated previously for 20 h with these peptides, were resuspended in fresh serum-free media, these cells were all capable of mediating a significant ($P<0.01$) inhibition of LPS-induced MDM TNF-α production, identical to control cells (Fig. 7D). However, when used in their original media, the effects of these cells replicated the direct, antiendotoxic capacity of the peptide with which they had been treated (Fig. 7C).

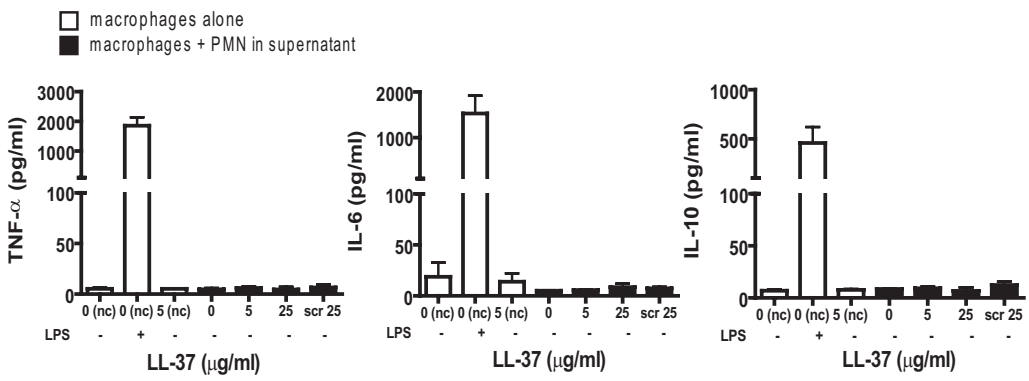

Figure 5. LL-37-induced secondarily necrotic PMN are not proinflammatory for macrophages. Human PMN were incubated for 20 h in the presence or absence of LL-37 or scrambled LL-37 at the concentrations indicated and used unwashed in the IMDM + 10% FCS media supernatant, in which they had been incubated. hMDM were incubated with these PMN or incubated without PMN (nc) in the presence or absence of LL-37 or 10 ng/ml *E. coli* 0111:B4 LPS as a positive control. Supernatants were evaluated for cytokine responses.

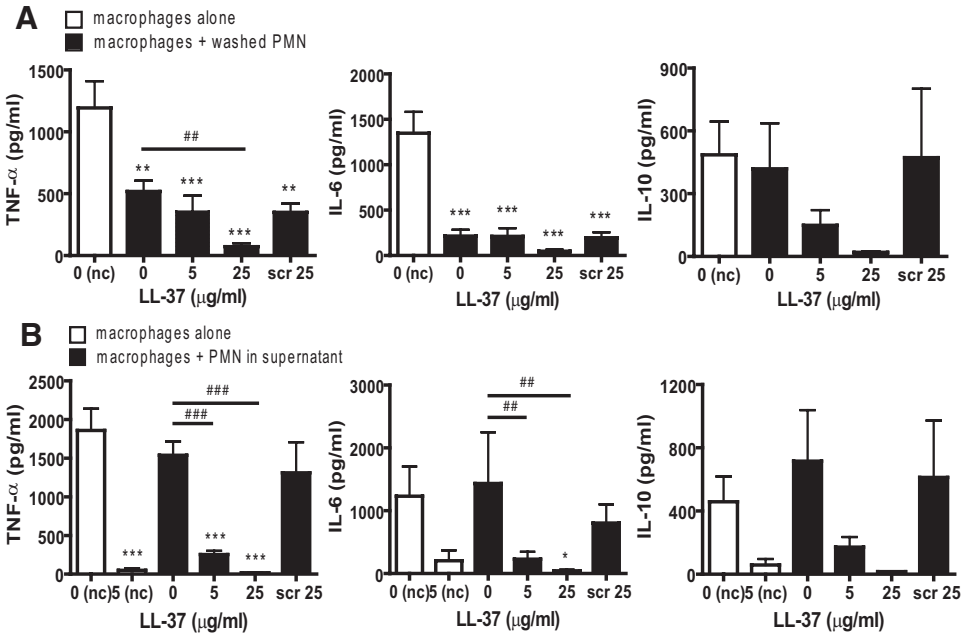

Figure 6. LL-37-induced secondarily necrotic PMN retain anti-inflammatory properties. Human PMN were incubated for 20 h in the presence or absence of LL-37 or scrambled LL-37 at the concentrations indicated. PMN were resuspended in serum-free X-vivo 10 media (A) or used unwashed in the IMDM + 10% FCS media supernatant, in which they were incubated overnight (B). MDM were incubated with these PMN, with concomitant exposure to 10 ng/ml *E. coli* 0111:B4 LPS for 18 h. LPS-treated MDM without PMN, in the presence or absence of LL-37, were also studied as controls. Supernatants were evaluated for cytokine responses. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test; $n = 9$ donors (TNF-α); $n = 5$ donors (IL-6/IL-10); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, compared with MDM exposed to LPS in the absence of PMN under the same conditions; ##, $P \leq 0.01$; ###, $P \leq 0.001$, compared with MDM exposed to control apoptotic PMN untreated with LL-37.

These data demonstrate the effect of residual peptide in the original media, show that functional peptide is not carried over in washed cells, and demonstrate that these dead PMN can inhibit LPS-induced MDM cytokine production, irrespective of the degree of peptide-induced secondary necrosis. These data demonstrate that the inhibition of activated MDM proinflammatory cytokine production mediated by apoptotic PMN is not inhibited and may even be enhanced by LL-37-mediated induction of secondary necrosis of these apoptotic PMN. Furthermore, the intracellular contents released by these cells are not actively proinflammatory or can be inhibited by the effects of LL-37.

LL-37-mediated secondary necrosis can induce the release of PMN granule contents

To determine the effect of LL-37-induced secondary necrosis of apoptotic PMN on the azurophilic (primary) granules, the release of MPO in response to LL-37 was quantified at 20 h. MPO release was not detected above background for this timepoint following exposure to ≤10 μg/ml LL-37 (concentrations at which substantial secondary necrosis occurred) or in response to scrambled LL-37 (**Fig. 8**). However, significant levels of MPO were observed in response to ≥25 μg/ml LL-37 ($P<0.01$) at levels approaching 40% of total MPO released by

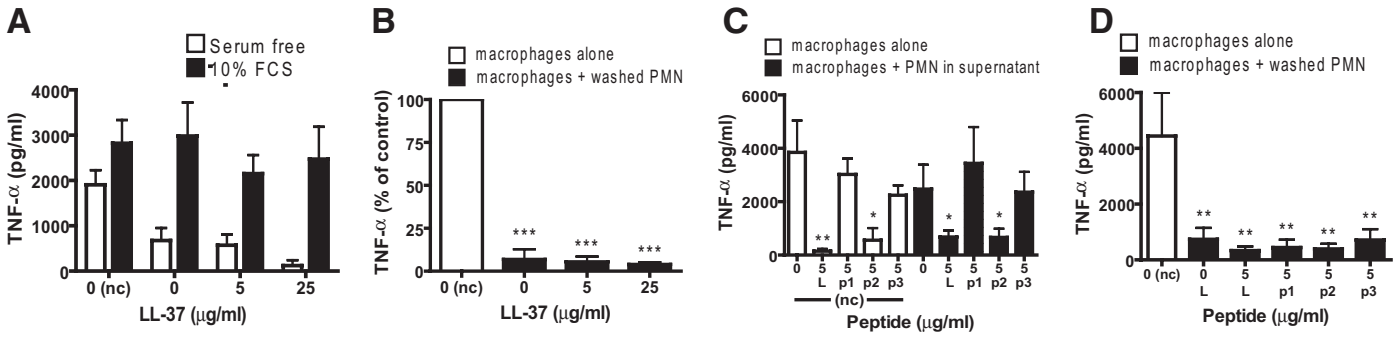

Figure 7. The anti-inflammatory properties LL-37-induced, secondarily necrotic PMN are independent of peptide antiendotoxic activity. Human PMN were incubated for 20 h in the presence or absence of LL-37 or partial peptides p1, p2, or p3 at the concentrations indicated. PMN were resuspended in X-vivo 10 media with 10% FCS (A), resuspended in serum-free X-vivo 10 media (B and D), or used unwashed in the IMDM + 10% FCS media supernatant, in which they were incubated overnight (C). MDM were incubated with these PMN, with concomitant exposure to 10 ng/ml *E. coli* 0111:B4 LPS (A, C, and D) or 3 μg/ml rhCD40L + 5 ng/ml rhIFN-γ (B) for 18 h. Activated MDM without PMN, in the presence or absence of peptides, were also studied as controls and to evaluate direct antiendotoxic activity. Supernatants were evaluated for cytokine responses. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test; $n \geq 3$ donors; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, compared with activated MDM in the absence of PMN under the same conditions.

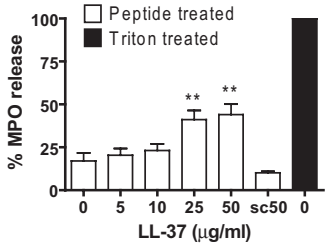

Figure 8. LL-37-induced secondary necrosis of PMN can release granule contents. Isolated human PMN were incubated for 20 h with a range of concentrations of LL-37 or 50 μg/ml scrambled LL-37 (sc50). The concentration of MPO in the supernatants was evaluated as a percentage of total MPO release after lysis of fresh control PMN using 0.1% Triton X-100. Significance was assessed by one-way ANOVA with Bonferroni's multiple comparison test; $n = 7$ donors; **, $P \leq 0.01$, compared with untreated PMN.

lysis with Triton X-100. These data suggest that although LL-37-induced secondary necrosis of apoptotic PMN does not lead directly to the release of granule contents over this time scale, higher concentrations of LL-37 are able to destabilize granules and lead to the release of their potentially damaging contents.

DISCUSSION

PMN are key cells of the innate immune system. The appropriate regulation of PMN function, death, and clearance is critical to effective control of inflammation, and dysregulation of these processes has been implicated in disease pathogenesis. Cell death by apoptosis, followed by efferocytosis by professional phagocytic cells, is proposed to enable safe PMN removal without the release of harmful intracellular contents to inhibit proinflammatory responses and to promote anti-inflammatory responses to enhance resolution [3]. In contrast, cell death by necrosis (whether primary necrosis or secondary necrosis of apoptotic PMN that have not been cleared) is proposed to be proinflammatory. We demonstrate that the human cathelicidin LL-37 is a potent inducer of secondary necrosis in PMN, which have undergone prior apoptosis, and evaluate the impact of this process on macrophage inflammatory responses.

We demonstrate that the ingestion of LL-37-induced secondarily necrotic PMN was not a proinflammatory event. This process did not promote the release of proinflammatory cytokines by macrophages and was indistinguishable from the macrophage response to apoptotic cells. Furthermore, these secondarily necrotic PMN were actively anti-inflammatory, demonstrating that the capacity of apoptotic PMN to inhibit LPS-induced proinflammatory cytokine production by macrophages was undiminished by LL-37-induced secondary necrosis. Indeed, this anti-inflammatory effect was actually enhanced in response to PMN with the most extensive secondary necrosis. Although the anti-inflammatory activity of unwashed, LL-37-treated apoptotic PMN was a result of the antiendotoxic properties of the remaining, functional LL-37 in the supernatant, washed cells retained the anti-inflammatory capacity of control apoptotic cells, even after treatment conditions (25 μg/ml LL-37 for 20 h) in which ~80% of dead PMN have undergone secondary necrosis [10]. These data suggest functional equivalence, irrespective of membrane integrity, of apoptotic PMN and LL-37-induced secondarily necrotic cells in this system. A previous report has demonstrated potential functional dominance of apoptotic cells over primary necrotic cells [45]. We cannot exclude a dominant role for the remaining membrane-intact apoptotic cells in our system. However, the significantly enhanced anti-inflammatory effects induced by PMN, with the greatest proportion of secondary necrosis, indicate that the anti-inflammatory activity of apoptotic PMN can in fact be potentiated by LL-37-induced secondary necrosis. Whether this applies to PMN necrosis induced in other ways and the mechanisms involved remains to be determined. However, exposure to a concentration of LL-37, sufficient to induce PMN granule content release, appeared to be necessary to generate secondary necrosis capable of potentiating the anti-inflammatory effects. This indicates that induction of necrosis per se is not sufficient and implicates a possible role for the release of endogenous LL-37 or other anti-inflammatory agents from PMN granules. Irrespective, it is clear that LL-37-induced secondary necrosis did not compromise the anti-inflammatory effects of the dead cells nor confer proinflammatory properties on them. Furthermore, any proinflammatory mediators released by secondarily necrotic PMN into the supernatant were unable to initiate or potentiate macrophage production of the cytokines studied in the presence of LL-37.

Whereas apoptosis has been viewed as anti-inflammatory and inducing immune-tolerance, necrosis has been considered a proinflammatory and immunogenic form of cell death. However, recent studies have suggested that this axiom is oversimplified [46]. Apoptotic Jurkat T cells are reported to be equally potent in modulating macrophage MAPK pathways, irrespective of membrane integrity [47], and the membranes of apoptotic and primary necrotic PMN have been shown to inhibit TNF-α production by activated macrophages [44]. In addition, apoptosis can neutralize potential intracellular danger signals, such as high-mobility group box-1 protein, via caspase-activated mitochondrial reactive oxygen species production [48]. Thus, the release of intracellular contents from apoptotic cells having undergone secondary necrosis may not be equivalent to the proinflammatory contents of primary necrotic cells, an hypothesis that would be compatible with our data.

We demonstrate that LL-37-induced PMN secondary necrosis had no impact on the magnitude of ingestion of dead PMN by macrophages, consistent with previous reports [44, 45]. Efferocytosis is a complex process with multiple receptors and adaptors proposed [3], independent from the anti-inflammatory effects initiated by apoptotic cells [49]. We cannot exclude the possibility that the mechanism of ingestion of these secondary necrotic PMN and apoptotic PMN may be different, but comparable levels of uptake suggest that LL-37-mediated induction of PMN secondary necrosis in vivo would not inhibit the clearance of dead PMN.

Irrespective of the impact of secondary necrosis on the clearance of dead PMN or macrophage cytokine responses, the loss of PMN membrane integrity presents the additional

threat of the release of harmful intracellular contents, including granule contents such as neutrophil elastase, cathepsin G, MPO, and gelatinase [50]. In contrast to previous TEM-based observations [38], our data demonstrate that high concentrations of LL-37 induced the release of MPO from the azurophilic granules. Interestingly, this was not observed at lower LL-37 concentrations, at which significant induction of secondary necrosis was observed nevertheless. This suggests that LL-37-induced secondary necrosis did not disrupt granule integrity per se but that high concentrations of LL-37 ($\geq$10 μg/ml) had the capacity to damage granule membranes in addition to apoptotic outer membranes. The consequences of such an effect in a rapidly resolving acute infection may contrast with those in a chronic inflammatory process associated with high levels of LL-37. Indeed, increased hCAP-18/LL-37 concentrations in cystic fibrosis lung disease (detected at up to ~15 μg/ml in BALF from patients without recent infectious exacerbation) were correlated with increased lung damage [34], and raised pulmonary LL-37 levels are associated with bronchiolitis obliterans syndrome in the recipients of lung transplants [36]. In addition, LL-37 has been proposed recently to contribute to the pathogenesis of psoriasis [35], and the concentration of hCAP-18/LL-37 was reported to be ~1.5 mg/ml in psoriatic skin lesions [51]. These estimates of in vivo peptide concentrations do not take into account the recent observation that the ratio of active LL-37 to full-length precursor hCAP-18 varied considerably between donors in the inflamed lung [36]. Nevertheless, at these concentrations, extensive induction of secondary necrosis of apoptotic PMN, with the release of PMN granule contents, could be generated by exposure to LL-37 in vivo. Thus, the potentially detrimental effects of LL-37-mediated induction of PMN secondary necrosis could have significance in chronic disease processes.

The potential consequences of PMN granule release may be of particular significance for the ongoing development of CHDP as therapeutic agents for infectious and inflammatory diseases. A greater knowledge of the capacity of these peptides to modulate inflammation and the peptide attributes that confer them will have important implications. We have demonstrated recently that the capacity of LL-37 to induce secondary necrosis of apoptotic cells is not specific to PMN [41] and now additionally define a core functional region and describe interaction with other modifiers of cell death.

We demonstrate that LL-37-induced secondary necrosis of PMN occurred rapidly, within minutes of exposure of an apoptotic PMN to the peptide. The specific membrane alterations that make these apoptotic cells acutely susceptible to damage by LL-37 and the nature of this interaction remain unclear, but the process has been proposed recently to be energy-independent [38] and independent of known LL-37 receptors [41]. Our data demonstrate that this property was conserved in the murine ortholog mCRAMP, and the effect was not dependent primarily on peptide charge (with no effects seen for a scrambled sequence peptide with the same charge) and was retained partially by C-terminal, but not N-terminal partial LL-37 peptides. These latter studies, using truncated partial peptides, shown previously to be poorly antiendotoxic [37], identified a minimal core-functional region for induction of secondary necrosis by LL-37, spanning aa 16–32. This may indicate an important motif or be related to the more amphipathic nature of the peptides spanning this region. Interestingly, the region 17–32 has been identified previously as the core antimicrobial region [52]. This suggests a possible conservation of mechanism between the direct microbicidal effects of LL-37, for which amphipathicity is critical [52], and the capacity to induce PMN secondary necrosis. However, it is noteworthy that whereas core LL-37 peptide 17-32 had enhanced microbicidal function compared with full-length LL-37, our partial peptides had diminished capacity to induce secondary necrosis. This has important implications for the development of therapeutic peptides and in consideration of the possible in vivo activities of shorter-form cleavage products of hCAP-18, identified previously in the skin [53]. Further study of our partial peptides will enable dissection of the various properties of LL-37.

In conclusion, we demonstrate that the human cathelicidin LL-37 is a potent inducer of secondary necrosis of apoptotic PMN, with the potential to alter the profile of PMN cell death at sites of infection and inflammation. Our data give novel insights into the potential effects of PMN secondary necrosis, suggesting that LL-37-mediated secondary necrosis of PMN does not have proinflammatory effects on macrophages and can even potentiate the anti-inflammatory effects of efferocytosis. These data challenge the prevailing model that although apoptotic cells are anti-inflammatory to macrophages, necrotic cells (whether primary or secondary necrosis) are inherently proinflammatory. This has particular importance for the delayed clearance of the apoptotic cell hypothesis for chronic inflammation and implications for novel, anti-inflammatory strategies being developed based on our understanding the mechanisms controlling the resolution of inflammation. However, we also demonstrate that LL-37-induced secondary necrosis has the potential to induce the release of potentially harmful granule contents, which could have deleterious consequences for the host, particularly in chronic disease states. We demonstrate that this induction of secondary necrosis by LL-37 is not primarily charge-dependent, is partially retained by C-terminal partial peptides, and is conserved in the murine ortholog. Further understanding of this mechanism and its physiological significance will assist the appropriate modification of synthetic analogs as novel therapeutics, illuminate possible roles in the pathogenesis of chronic disease, and advance our understanding of the impact of different cell death processes in the promotion and resolution of inflammation.

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The Host Defense Peptide LL-37 Selectively Permeabilizes Apoptotic Leukocytes[▽]

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LL-37 is a cationic host defense peptide that is highly expressed during acute inflammation and that kills bacteria by poorly defined mechanisms, resulting in permeabilization of microbial membranes. High concentrations of LL-37 have also been reported to have cytotoxic effects against eukaryotic cells, but the peptide is clearly capable of differentiating between membranes with different compositions (eukaryotic versus bacterial membranes). Eukaryotic cells such as leukocytes change their membrane composition during apoptotic cell death, when they are turned into nonfunctional but structurally intact entities. We tested whether LL-37 exerted specific activity on apoptotic cells and found that the peptide selectively permeabilized the membranes of apoptotic human leukocytes, leaving viable cells unaffected. This activity was seemingly analogous to the direct microbicidal effect of LL-37, in that it was rapid, independent of known surface receptors and/or active cell signaling, and inhibitable by serum components such as high-density lipoprotein. A similar selective permeabilization of apoptotic cells was recorded for both NK cells and neutrophils. In the latter cell type, LL-37 permeabilized both the plasma and granule membranes, resulting in the release of both lactate dehydrogenase and myeloperoxidase. Apoptosis is a way for inflammatory cells to die silently and minimize collateral tissue damage by retaining tissue-damaging and proinflammatory substances within intact membranes. Permeabilization of apoptotic leukocytes by LL-37, accompanied by the leakage of cytoplasmic as well as intragranular molecules, may thus shift the balance between pro- and anti-inflammatory signals and in this way be of importance for the termination of acute inflammation.

The cathelicidins form a large family of microbicidal, cationic host defense peptides found in all mammalian species examined so far, including humans, cows, pigs, rabbits, sheep, mice, monkeys, and horses (60). Only one human cathelicidin, human cationic antimicrobial protein 18 (hCAP-18; unprocessed form), or LL-37 (the predominant, mature antibacterial form), has been isolated. LL-37 is mainly expressed by neutrophils and epithelial cells during acute inflammation. LL-37 is stored as a propeptide in the specific granules of neutrophils. Upon neutrophil activation, the proform is cleaved enzymatically by proteinase 3 to the active form during degranulation toward the extracellular compartment (49). The concentration of LL-37 can be severely increased at sites of inflammation, and levels up to about 15 to 25 μg/ml have been reported in bronchoalveolar lavage fluid from infants with pulmonary infections as well as from cystic fibrosis patients (14, 45); even higher levels (over 1 mg/ml) has been reported from psoriatic skin lesions (40).

LL-37 was originally identified as a cationic microbicidal peptide due to its ability to specifically permeabilize prokaryotic membranes (1, 16, 31); it possesses direct microbicidal activity against bacteria, fungi, and enveloped viruses (5, 33). Very high concentrations of LL-37 have also been reported to have cytotoxic effects against eukaryotic cells in vitro (28). The microbial killing mechanism is dependent on the membrane-active properties of the peptide, which has the ability to interact with both the inner and the outer membranes of gram-negative bacteria (22). The mode of action for the direct microbicidal activity of host defense peptides has not been determined conclusively, but the leading hypothesis is that these peptides are membrane active and have the ability to break the integrity of bacterial cell membranes more or less selectively over eukaryotic membranes (47). Bacterial membranes are composed of phospholipids different from those of eukaryotic cell membranes, with the bacterial membranes being more negatively charged than their eukaryote counterparts. The presence of surface molecules other than phospholipids, e.g., lipopolysaccharide (LPS; an outer membrane component of gram-negative bacteria), is also of importance to the overall negative charge of bacterial membranes. This negative charge is, in part, believed to be a determinant for the interaction with cationic peptides and gives the peptides selectivity for the microbial membranes (37).

Lately, LL-37 has been shown to have immunomodulatory

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properties, in addition to its microbicidal potential (10), and is also capable of activating various immune cells; thus, it possesses a functional dualism that is also displayed by certain other cationic peptides (7, 13, 51, 58). Among the numerous immunomodulatory actions displayed by LL-37, chemoattraction and the induction of chemokine release from leukocytes, the stimulation of angiogenesis, and the binding and neutralization of bacterial LPS can be found (9). LL-37 has been shown to be highly upregulated under inflammatory conditions, particularly those involving the skin, e.g., psoriasis (20, 40). During inflammation, it is of vital importance that the activities of the cells involved are tightly regulated. Many inflammatory leukocytes, especially neutrophils, are packed with a variety of cytotoxic substances aimed at microbial killing, and if these substances reach the extracellular environment in an uncontrolled fashion, the risk of tissue destruction and sustained inflammation are increased (25, 56). One important means of ridding the system from potentially harmful intracellular constituents is by apoptosis of the inflammatory cells, followed by phagocytic clearance of the corpse by viable phagocytes (34, 44). Apoptosis is often called the physiological, programmed form of cell death, characterized by the lack of proinflammatory actions and the minimization of damage to surrounding cells and tissue. Apoptotic cells are nonfunctional but, importantly, carry an intact cell membrane that prevents the uncontrolled leakage of intracellular molecules (25). Despite the integrity of the apoptotic cell membrane, its surface-exposed molecule composition differs substantially from that of the membranes of viable cells. One prominent example of surface changes relates to phospholipids. In viable cells, an uneven distribution of phospholipids between the two leaflets of the double-sided membrane is maintained by an energy-dependent process that keeps the negatively charged phosphatidylserine (PS) on the inside. In a nonfunctional apoptotic cell, the PS distribution is leveled out over time, with the PS also being on the outer leaflet as a result (35), making the membrane of apoptotic cells more negatively charged and, in this way, more like the membrane of bacteria. The surface-located PS is also an important "eat-me" flag that ensures phagocytic clearance of the apoptotic cell (18). Annexin V is a Ca^{2+}-dependent, cell-impermeant protein with a high affinity for PS. When it is conjugated to a fluorochrome, it is widely used for the detection and quantification of apoptosis by flow cytometry.

Given the ability of LL-37 to distinguish between prokaryotic and eukaryotic membranes and the fact that the composition of eukaryotic membranes changes when a cell becomes apoptotic, we investigated whether the peptide could distinguish between viable and apoptotic leukocytes. Using primary human neutrophils, a cell type that has a short half-life in vivo and that rapidly enters apoptosis in culture, we found that LL-37 selectively permeabilized the membranes of apoptotic cells under serum-free conditions, leaving viable cells unaffected in this respect. A specificity similar to that for apoptotic cells was also found for NK cells, another inflammatory leukocyte, which was rendered apoptotic by treatment with hydrogen peroxide. The selective permeabilization of apoptotic neutrophils was very rapid and seemingly independent of known LL-37 surface receptors and/or active cell signaling. Peptide-induced permeabilization in effect shifted apoptotic cells into a necrotic phenotype, whereby intracellular constituents were released into the extracellular milieu; leakiness was effectively counteracted by the presence of human serum or high-density lipoprotein (HDL) in a similar manner, as microbial killing by LL-37 was blocked by these substances. The mere surface exposure of the negatively charged PS, a consequence of apoptosis, was not responsible for the selective permeabilization of apoptotic cells. We concluded that LL-37 possesses selective permeabilizing activity on apoptotic leukocytes in a manner seemingly analogous to its activity against bacterial membranes. In pathological settings with high levels of cathelicidin expression in the absence of serum constituents (such as psoriatic lesions), the permeabilization of apoptotic leukocytes by LL-37 could be of importance in determining the eventual outcome of acute inflammation.

MATERIALS AND METHODS

Reagents. LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was synthesized by *N*-(9-fluorenyl)methoxycarbonyl chemistry at the Nucleic Acid/Protein Service Unit at the University of British Columbia (Vancouver, British Columbia, Canada). Scrambled LL-37 (sLL-37; RSLEGTDRFPFVRLK NSRKLEFKDIKGIKREQFVKIL) was purchased from CSS-Albachem Ltd. (East Lothian, United Kingdom). WKYMVM was from Alta Bioscience (Birmingham, United Kingdom), and WRW4 was from Genscript Corp. (Scotch Plains, NJ). Hp(2-20) was synthesized and purified by high-pressure liquid chromatography (Innovagen, Lund, Sweden). Ficoll-Paque was from Pharmacia (Uppsala, Sweden), and Ficoll-Hypaque was from Nycomed (Oslo, Norway). Dextran was purchased from Amersham Biosciences (Uppsala, Sweden). The A-6013 agarose used for the casting of all plates for the inhibition zone assays was from Sigma Chemical Co. (St. Louis, MO), as were oxidized ATP (oxATP), HDL, cytochalasin B, lidocaine, colchicine, and calcein. The antibody α-CD95 was from Nordic BioSite (Täby, Sweden). Annexin V-fluorescein isothiocyanate (FITC), annexin V-allophycocyanin (APC), 7-amino-actinomycin D (7-AAD), and the BD IMag NK cell isolation kit were supplied by BD Biosciences (San Jose, CA). RPMI 1640 medium was from PAA Laboratories (Pasching, Austria). The lipids used for liposome preparation (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine [DPPE], 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine [DPPC], 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl-*rac*-glycerol, sodium salt [DPPG], dioleoyl-*sn*-glycero-3-phosphatidylserine [DOPS], and cholesterol) were purchased from Larodan (Malmö, Sweden).

Isolation and culture of human cells. Human neutrophils were isolated by a standard technique (11, 12) from buffy coats obtained from the blood of healthy donors at the Sahlgrenska Hospital, Gothenburg, Sweden. In short, dextran sedimentation was performed at $1 \times g$, followed by hypotonic lysis of the remaining erythrocytes and centrifugation in a Ficoll-Paque gradient. Thereafter, the neutrophils were washed twice and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum to a density of 5×10^6 cells/ml. Cells (500 μl) were added to polypropylene tubes and incubated at 37°C in 5% CO_2 for 20 h for the induction of spontaneous apoptosis. For increased apoptosis, azide-free α-CD95 monoclonal antibody (10 μg/ml) was coincubated with the cells, which resulted in approximately 20% viable neutrophils and 80% apoptotic neutrophils.

For the isolation of NK cells, peripheral venous blood was incubated with Iscove's modified Dulbecco's medium, dextran, and acid citrate dextrose for 15 min at room temperature. The supernatant was centrifuged through a Ficoll-Hypaque (Lymphoprep) density gradient, and the mononuclear cells at the interface were collected and further processed by countercurrent centrifugal elutriation (57). In short, this technique allows cell separation on the basis of the cell sedimentation rate in a spinning rotor at 2,150 rpm balanced by a counter-directed flow through a chamber. By varying the flow rate, different cell fractions were separated on the basis of their sizes. The NK-cell-enriched lymphocyte fractions were collected at flow rates of between 14 and 15 ml/min, and the cells were then further isolated through negative selection with magnetic beads (BD IMag NK cell isolation kit), according to the instructions provided by the manufacturer. Finally, the isolated NK cells were incubated overnight in Iscove's modified Dulbecco's medium supplemented with 10% normal human serum at 37°C in 5% CO_2. For the induction of the early apoptotic characteristics in the NK cells, 100,000 to 200,000 cells incubated overnight were exposed to 200 μM H_2O_2 for 6 h at 37°C in 5% CO_2 (23, 24, 53).

Evaluation of apoptosis. The neutrophils that had been incubated overnight, as described above, were washed and resuspended in annexin V binding buffer (AVBB; 1 mM HEPES, 14 mM NaCl, 0.25 mM $CaCl_2$; pH 7.4) to 6.25×10^6 cells/ml. Samples (70 μl) were stained with 5 μl annexin V-FITC for 5 min at ambient temperature, whereupon 7-AAD was added, and the staining was continued for 10 min before the addition of AVBB to give a final volume of 500 μl. In some experiments, annexin V-FITC was exchanged for α-CD16–FITC: 100-μl samples were stained with 10 μl antibody for 10 min at 0°C, whereupon staining with 7-AAD was performed as described above. Peptides diluted in water were added 5 min prior to analysis, if not indicated otherwise. HDL (650 μg/ml), bovine serum albumin (0.5%; wt/vol), or 10% salt mimicking serum salt (11.3 mM NaCl, 2.4 mM $NaHCO_3$, 0.06 mM $MgCl_2$, 0.13 mM $CaCl_2$, 0.39 mM KCl) (28) was added to the indicated samples before the addition of peptide. Samples were analyzed by fluorescent-activated cell sorter analysis before and after the addition of peptides. For samples treated with the formyl peptide receptor-like 1 (FPRL1) antagonist, WRW4 (5 μM) was added 5 min (at 37°C) prior to the addition of peptide. The same procedure was used for the $P2X_7$ inhibitor oxATP (900 μM), but by the procedure with oxATP, the samples were preincubated with the inhibitor for 30 min. The concentrations and incubation times for the inhibitors have previously been shown to specifically block Ca^{2+} flux induced through FPRL1 and $P2X_7$, respectively (15). The inhibitors of membrane repair cytochalasin B (10 μg/ml), colchicine (10 μM), or lidocaine (5 mM) were coincubated with the cells overnight and were added to all buffers used for the subsequent experiments, as indicated.

The H_2O_2-treated NK cells were washed and resuspended in AVBB. Each sample was stained at ambient temperature with 5 μl annexin V-FITC for 5 min, followed by staining with 5 μl 7-AAD, for a final volume of 100 μl, for another 10 min. The samples were thereafter diluted to 300 μl with AVBB before analysis. Flow cytometry was performed before and 5 min after the addition of LL-37 (5 μg/ml was used for NK cells, as these cells are more susceptible than neutrophils to the cytotoxic action of LL-37).

Determination of direct microbicidal activity. *Escherichia coli* strain MG1655 was grown overnight in Luria-Bertani (LB) broth (3) at 37°C on a rotary shaker. A modified inhibition zone assay was used for the detection of direct microbicidal activity (21, 26, 27). In short, standard LB agar (LB broth supplemented with 1% [wt/vol] agarose) containing bacteria (approximately 5×10^5 CFU in logarithmic growth phase per milliliter agar) was poured into petri dishes (diameter, 92 mm). Wells (diameter, 3 mm; depth, 1 mm) were punched in the agar, and peptide preparations (3 μl) diluted in distilled water were added to the wells, whereupon the plates were incubated at ambient temperature for 45 min and then at 37°C overnight. The peptide was used in the assays at amounts ranging from 1.88 μg/well to 7.5 μg/well. Inhibition zones were calculated as the diameter of the clear zones, free of visible bacteria, surrounding the wells. HDL (650 μg/ml) was added to the agar plates before the plates were cast.

Liposome preparation. Lipids (DPPE, DPPC, DPPG, and cholesterol) were dissolved in chloroform-methanol (1:1) to a concentration of 20 mg/ml. DOPS was dissolved in chloroform to 25 mg/ml. The lipid mixtures (final concentration of total lipids, 2.5 mM) were dissolved in chloroform-methanol (1:1) and dried under reduced nitrogen pressure at 45°C, with subsequent drying under vacuum for 1 h. Liposomes containing the following were prepared: DPPE-DPPC-cholesterol (1:1:1; lipids typically found in eukaryotic membranes), DPPE-DPPG (7:3; phospholipids typically found in bacterial membranes), and DPPE-DPPC-cholesterol-DOPS (3:3:3:1; lipids typically found in eukaryotic plasma membranes with 10% PS). During the subsequent preparation steps, the liposomes were kept at 50°C at all times to avoid solidification of the dipalmitoyl lipids. The dried lipid film was dissolved in 1 ml Tris buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA; pH 7.4) containing self-quenching concentrations of calcein (80 mM), and the mixture was vigorously vortexed and then given a short sonication. Large unilamellar vesicles were obtained by passing the suspension 20 times through 400-, 200-, and 100-nm-pore-size polycarbonate filters (Eschborn, Germany) on a handheld extruder. Extraliposomal calcein was removed by filtration through Sephadex G-50 resin. The release of calcein to the extraliposomal medium upon peptide addition was monitored continuously by measuring the fluorescence intensity on a Perkin-Elmer LS55 fluorescence spectrometer (excitation λ, 494 nm; emission λ, 513 nm). The maximum fluorescence intensity, which corresponded to 100% leakage, was determined by adding Triton X-100 (0.1%) to the liposomes.

Intracellular Ca^{2+} measurements. Intracellular Ca^{2+} was measured by a previously described method (42). In short, neutrophils incubated overnight for the induction of apoptosis were resuspended in cell loading medium (1% fetal calf serum in Krebs-Ringer phosphate buffer containing glucose [10 mM glucose, 1 mM Ca^{2+}, 1.5 mM Mg^{2+}; pH 7.3]) and loaded with Fluo-3 (4 mg/ml) and Fura-Red (10 mg/ml) reagents at 37°C for 30 min. The cells were washed twice, resuspended in AVBB to a final concentration of 6×10^6/ml, and stained with annexin V-APC at ambient temperature for 15 min. The samples were diluted to 8.75×10^5 cells/ml, and 0.5 ml was prewarmed at 37°C for 5 min. The level of intracellular Ca^{2+} accumulation in individual cells upon the addition of WK YMVM (10^{-7} M) was assessed by flow cytometry over 5 min. The fluorescence emissions of the Fluo-3 and Fura-Red reagents were measured in the FL-1 and the FL-3 channels, respectively. The data were analyzed by using the kinetics mode of FlowJo software (version 5.7.1; FlowJo, LLC, Ashland, OR), and the relative intracellular Ca^{2+} concentration was presented as the ratio between the mean fluorescence intensity of the Fluo-3 reagent and that of the Fura-Red reagent over time. The analysis of Ca^{2+} accumulation was performed after gating of viable and apoptotic cells by the use of annexin V-APC staining.

To establish that the apoptotic neutrophils were properly labeled with the Fluo-3 and Fura-Red reagents, ionomycin (500 nM) was included as a positive control stimulus. This ionophore forms pores in the plasma membrane, giving rise to an artificial, signaling-independent rise in the intracellular calcium concentration. Addition of ionomycin resulted in an increase in the intracellular calcium concentration in the apoptotic cells as well as the viable cells (data not shown).

Measurements of LDH and MPO release from LL-37-treated neutrophils. Neutrophils (5×10^6/ml) were incubated overnight in 5% CO_2 at 37°C, and α-CD95 (10 μg/ml) was included for the induction of apoptosis or was not included. The cells were washed and diluted to 1×10^6/ml before treatment with LL-37 (50 μg/ml) for 10 min. All samples were divided in two, and one portion of each sample was lysed with Triton X-100 (1%) for 1 min, after which the cell debris was removed by centrifugation. The amount of the cytosolic enzyme lactate dehydrogenase (LDH) released was measured with a cytotoxicity detection kit from Roche Diagnostics GmbH (Mannheim, Germany), according to the manufacturer's directions. The myeloperoxidase (MPO) content in the neutrophils was measured by determination of the amount of enzyme activity by mixing 50 μl of the cell supernatants described above with 100 μl peroxidase substrate (1,2-phenylenediamine dihydrochloride [Dako Denmark A/S]) in a 96-well plate and incubation of the plate overnight at room temperature. The absorbance at 492 nm was then measured.

Statistical analysis. The results are expressed as means ± standard deviations (SDs). Unpaired and paired *t* tests were used for statistical analysis, and *P* values are two tailed with 95% confidence intervals. For comparisons of multiple data sets, one-way analysis of variance with Bonferroni's multiple-comparison tests was performed. The data were analyzed with GraphPad Prism software (version 4.02; GraphPad Software, San Diego, CA).

RESULTS

Direct microbicidal action of LL-37 and discrimination between membranes of different compositions. LL-37 is a host defense peptide with dual actions that both works directly by permeabilizing microbial membranes and has receptor-dependent, immunomodulatory actions on leukocytes as a means of protecting against infections. The direct microbicidal effects of LL-37 and the sLL-37 control peptide against *E. coli* were assessed in inhibition zone assays; LL-37 induced a clear zone around the well that was free of visible bacteria, while sLL-37 had no direct microbicidal effect (Fig. 1A). The lethal concentration was calculated to 6.3 μg/ml for *E. coli* (strain MG1655) under the specific medium conditions used in the assay. The lethal concentration was determined from the diameter of the inhibition zone by a previously described method (26) and was defined as the lowest concentration that just inhibited colony formation in the thin agar plates. Under the serum-free conditions used, LL-37 (100 μg/ml) had no effect on the integrity of the cell membranes of freshly prepared neutrophils, as shown by a lack of 7-AAD staining (Fig. 1B). This dye is incapable of passing intact cell membranes and therefore functions as a useful marker of membrane integrity (46). These experiments show that LL-37 can be directly microbicidal in the 5-μg/ml range (as determined by calculation of the lethal

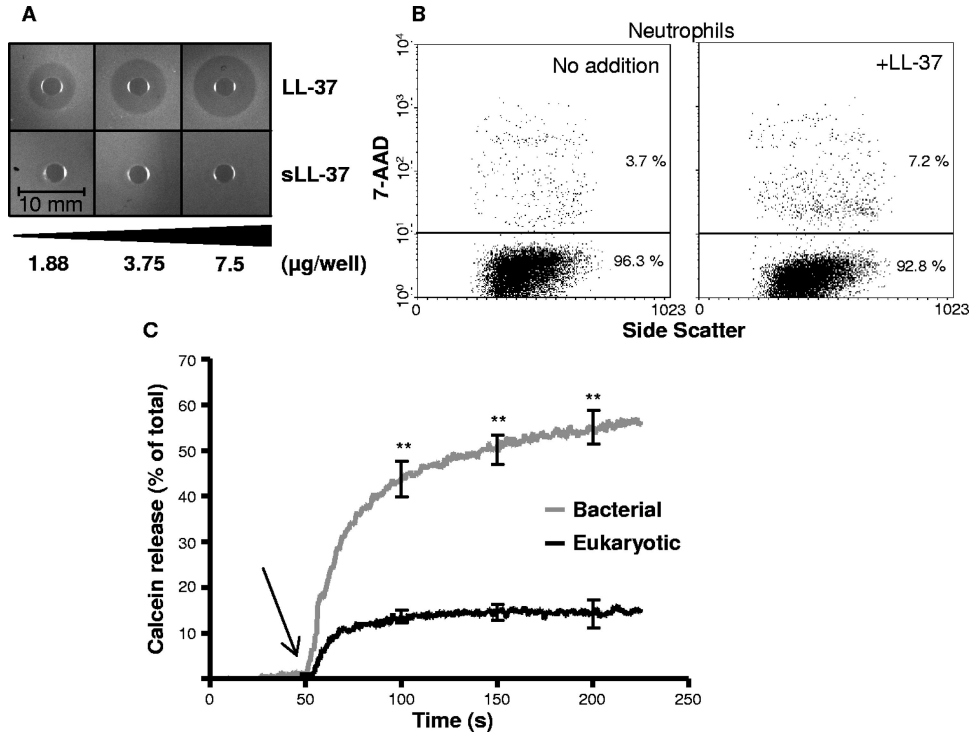

FIG. 1. LL-37 can distinguish between membranes with different compositions. (A) The direct microbicidal activity of LL-37 (1.88, 3.75, and 7.5 μg/well) against *E. coli* was determined in an inhibition zone assay. LL-37 gave rise to clear, bacteria-free zones around the wells (upper panel), while sLL-37 (1.88, 3.75, and 7.5 μg/well) was inactive (lower panel). (B) Freshly separated neutrophils were not sensitive to the direct microbicidal, lytic action of LL-37 (100 μg/ml), as shown by the lack of 7-AAD fluorescence intensity plotted against the forward scatter. 7-AAD is a nucleic acid stain used for the detection of the loss of membrane integrity in cells. The results of one experiment with the percentage of cells in each region are shown. (C) LL-37 (5 ng/ml) readily permeabilized bacterial membrane-like liposomes (gray line) composed of PE-PG (7:3), while the eukaryotic membrane-like liposomes (black line), composed of PE-PC-cholesterol (1:1:1), were more resistant to the permeabilizing effect of LL-37. The percentage of total calcein released, as assessed by the addition of Triton X-100, is plotted against time. The means ± SDs ($n = 3$) obtained at 0, 50, 100, 150, and 200 s are shown. The amounts of calcein released at 100, 150, and 200 s were compared; and significant differences between the amounts released were was established ($P < 0.01$ at each point, as indicated [**]; $n = 3$). The arrow indicates the time of addition of peptide.

concentration from the results obtained from the inhibition zone assays), whereas concentrations up to 100 μg/ml were not cytotoxic for neutrophils under serum-free conditions.

To further characterize the ability of LL-37 to discriminate between bacterial and eukaryotic membranes, we tested the effect of LL-37 on liposomes containing the lipids typically found in bacterial as well as in eukaryotic membranes in a calcein release assay. Bacterial membranes are slightly more negatively charged than eukaryotic membranes, and liposomes containing large amounts of neutral PE and the negatively charged phosphatidylglycerol (PG) were synthesized. For the eukaryotic membrane-like liposomes, cholesterol was incorporated together with the two neutral phospholipids PE and PC. As expected, LL-37 readily permeabilized the bacterial membrane-like liposomes (Fig. 1C). The eukaryotic membrane-like liposomes were permeabilized to a limited extent (approximately 15%), but they were obviously much more resistant than the bacterial membrane-like liposomes to the lytic action of LL-37. Taken together, these data show that LL-37 is clearly capable of differentiating between membranes with different compositions.

Selective permeabilization of apoptotic neutrophils by LL-37. LL-37 had no cytotoxic effect on freshly prepared human neutrophils (Fig. 1B). In order to study the effect of LL-37 on viable and apoptotic membranes of primary leukocytes, we employed human neutrophils, which gradually enter spontaneous apoptosis during in vitro culture. In this set of experiments, neutrophils incubated overnight spontaneously entered apoptosis, leaving a mixed population of cells with 66% viable cells (annexin V negative, 7-AAD negative) and 31% apoptotic cells (annexin V positive, 7-AAD negative), as assessed by flow cytometry (Fig. 2A, left panel). By use of this standardized protocol, very few cells (less than 3%) displayed a necrotic phenotype with permeable membranes, as indicated by the lack of 7-AAD-positive events. The use of this mixed culture enabled us to investigate viable and apoptotic cells simultaneously, and addition of LL-37 (50 μg/ml, 5 min of incubation) to the mixed population selectively permeabilized the apoptotic cells that acquired the 7-AAD stain under serum-free conditions (Fig. 2A, middle panel, upper right quadrant). The membranes of annexin V-negative cells were still intact (7-AAD negative) after the addition of peptide (Fig. 2A; middle panel, lower left quadrant). sLL-37 (50 μg/ml, 5 min of incubation) did not permeabilize any cell type (Fig. 2A, right panel), indicating that the cationic nature of LL-37 is not sufficient to permeabilize apoptotic neutrophils. sLL-37 at a

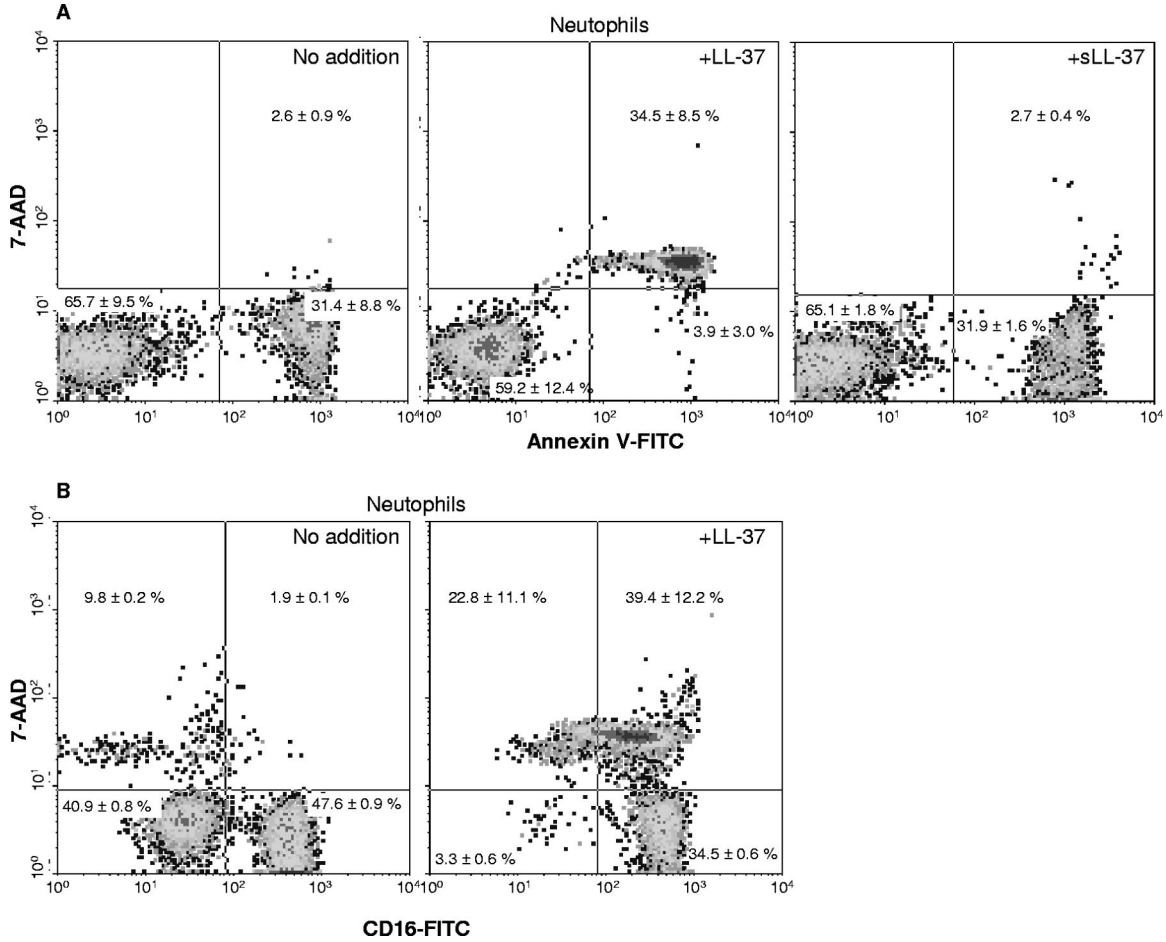

FIG. 2. Selective permeabilization of apoptotic neutrophils by LL-37. (A) Neutrophils incubated for 20 h spontaneously entered apoptosis, resulting in 66% viable cells and 31% apoptotic cells, as assessed by annexin V and 7-AAD staining and visualized by flow cytometry (left panel). When LL-37 (50 μg/ml) was added to these cells, all apoptotic (annexin V-positive) cells were permeabilized (7-AAD positive), while the viable (annexin V-negative) population was unaffected (middle panel) 5 min after addition of the peptide. sLL-37 (50 μg/ml) was inactive when it was added to the system (right panel). The results presented here represent those of one of three experiments performed for this experimental series. (B) Apoptosis was also assessed by CD16 shedding (left panel). Only the apoptotic neutrophils (CD16 negative) were permeabilized by the addition of LL-37 and stained positive for 7-AAD. The α-CD16 antibody also gained access to intracellular CD16 upon permeabilization, as shown by the increase in the fluorescence intensity of CD16 (right panel). The plots represent those from one of two independent experiments that were performed. Each plot shows the means ± SDs from one representative experiment.

concentration of 500 μg/ml was also ineffective (data not shown).

In order to rule out the possibility that an undefined interaction between LL-37 and annexin V caused the permeabilization, experiments were conducted with CD16 instead of annexin V as a marker for neutrophil apoptosis (Fig. 2B, left panel). CD16 is a surface protein present on viable neutrophils (Fig. 2B, left panel, lower right quadrant) that is lost upon apoptosis (Fig. 2B, left panel, lower left quadrant), and only cells negative for CD16 (i.e., apoptotic cells) became 7-AAD positive after addition of peptide (Fig. 2B, right panel). After addition of LL-37, the apoptotic cell population was permeant not only to the nuclear dye 7-AAD but also, apparently, to the α-CD16 antibody that gained access to intracellular CD16 and, as a result, conferred a CD16 signal to the permeabilized cells (Fig. 2B, right panel).

Selective permeabilization of apoptotic NK cells by LL-37. Inflammatory cells other than neutrophils have the ability to enter apoptosis as well. NK cells are cytotoxic, large granular lymphocytes important for eradicating viruses and combating tumor cells. One efficient way to drive NK cell apoptosis is to subject these cells to extracellular reactive oxygen species (23). To test whether the selective permeabilization of apoptotic cells applied only to neutrophils or if it represents a more general mechanism that acts on other leukocytes as well, NK cells were isolated and subjected to H_2O_2 treatment for the induction of apoptosis. Untreated NK cells incubated for 6 h displayed only 5% apoptotic cells, as assessed by annexin V and 7-AAD labeling (Fig. 3A). When the cells were coincubated with H_2O_2 (200 μM), a larger population (17%) became apoptotic (Fig. 3B), resulting in a mixed population of viable and apoptotic NK cells. A population of NK cells (16%) also appeared to be permeabilized after the addition of H_2O_2 (Fig. 3B, upper right quadrant). After this mixed population was washed, it was treated with LL-37 (5 μg/ml, 5 min of incubation), and with these leukocytes, the apoptotic cells were also

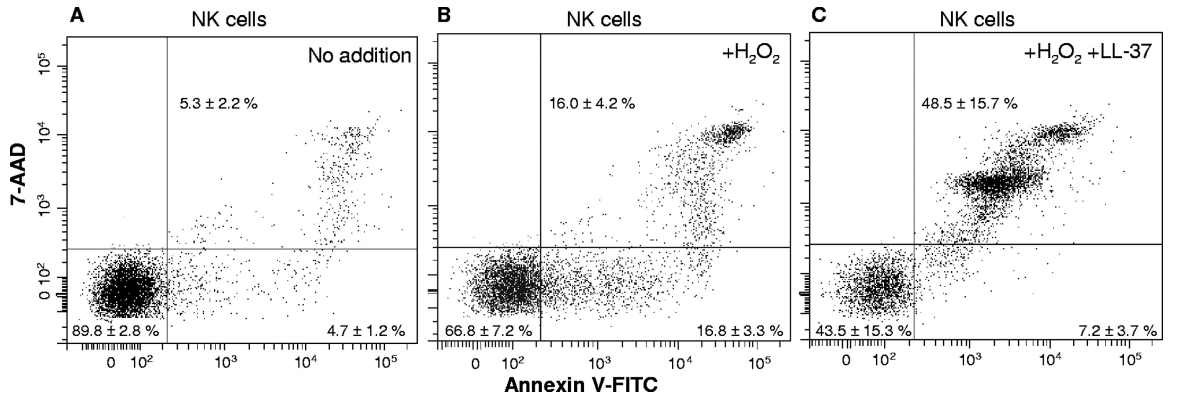

FIG. 3. Selective permeabilization of apoptotic NK cells by LL-37. (A) Isolated NK cells were incubated for 6 h and stained with annexin V and 7-AAD. Only a small amount of cells displayed apoptotic characteristics (4.7 ± 1.2%; $n = 3$). (B) When the cells were incubated in the presence of H_2O_2 (200 μM) for 6 h, the population of apoptotic NK cells increased (16.8 ± 3.3%; $n = 3$). The population of permeabilized NK cells also increased upon addition of H_2O_2 (from 5.3 ± 2.2% to 16.0 ± 4.2%; $n = 3$). (C) When LL-37 (5 μg/ml) was added to the H_2O_2-treated NK cells, the apoptotic population was permeabilized and stained positive for 7-AAD, in accordance with the results seen for neutrophils. At this concentration, no significant permeabilization of the viable population compared to that for the population treated with H_2O_2 alone was seen ($P = 0.07$; $n = 3$).

primarily permeabilized (Fig. 3C). When the results for the LL-37-treated viable population were compared to those for the H_2O_2-treated control cells, no significant difference was found ($P = 0.07$; $n = 3$). A lower standard concentration of LL-37 was used against the NK cells than against the neutrophils (5 μg/ml and 50 μg/ml, respectively), owing to the fact that NK cells were more susceptible to the cytotoxic action of LL-37. At 10 μg/ml, a substantial amount of cytotoxicity against NK cells was observed (the proportion of viable cells dropped from 82.6% to 38.5%; data not shown). At 5 μg/ml of LL-37, mainly apoptotic (annexin V-positive) NK cells were permeabilized, whereas the permeabilization of viable cells was not statistically significantly reduced. These data indicate that the selective permeabilization of apoptotic cells is independent of the type of leukocyte.

Characterization of selective permeabilization. Dose titration experiments revealed that the permeabilizing effect on apoptotic neutrophils was concentration dependent starting at approximately 10 μg/ml, and a full effect was reached at 50 μg/ml of LL-37. The half-maximal effective concentration (EC_{50}) was determined for the selective permeabilization of apoptotic cells by plotting the mean value of the percentage of apoptotic cells at concentrations 0, 5, 10, and 50 μg/ml for three separate experiments and fitting a straight line to the data set ($R^2 = 0.99$); the EC_{50} for the permeabilization of apoptotic neutrophils was 27 μg/ml. At 500 μg/ml, a more general cytotoxic effect was observed; in addition to the permeabilization of all apoptotic cells, viable neutrophils were also affected (Fig. 4A) ($P < 0.001$ compared to the results for viable neutrophils treated with buffer). The EC_{50} was also determined for the cytotoxic action of LL-37 against viable cells by plotting the mean value of the percentage of viable cells obtained after treatment with LL-37 at concentrations of 0, 5, 10, 50, 100, and 500 μg/ml from three separate experiments. A straight line was fitted to the data set ($R^2 = 0.97$), the trend line was extrapolated, and the EC_{50} was determined to be 700 μg/ml. Due to technical limitations, we could not use concentrations of LL-37 higher than 500 μg/ml. On the basis of EC_{50}s, the concentration needed for the permeabilization of viable neutrophils (general cytotoxicity) was found to be approximately 25 times higher than that needed for the permeabilization of apoptotic neutrophils. Time and titration experiments showed that the specificity was intact (i.e., viable cells were unaffected) for at least the first 5 min ($P > 0.05$ compared to the results obtained by treatment with buffer alone). Almost all annexin V-positive cells became leaky within 5 min of LL-37 addition (Fig. 4B). In an attempt to study the leakage within the first 5 min, we analyzed the process (7-AAD fluorescence) kinetically in a flow cytometer, having first gated the cells on the basis of annexin V staining (Fig. 4C, left panel). Increased 7-AAD staining was apparent immediately after addition of LL-37 (Fig. 4C, right panel) in the annexin V-positive cells (red), whereas the annexin V-negative population (green) never became 7-AAD positive. These results again showed that only apoptotic cells were permeabilized by LL-37 and that the leakage started immediately after addition of peptide.

Cationicity alone was not sufficient to confer direct microbicidal action (Fig. 1A) or specific permeabilization (Fig. 2A, right panel), since sLL-37 displayed no activity in these assays (at the highest concentrations used of 7.5 μg/well and 500 μg/ml, respectively). We next tested the direct microbicidal and immunomodulatory peptide Hp(2-20), which combines cationicity with an alpha-helical nature, i.e., classical features of cationic host defense peptides (13). This peptide was unable to permeabilize leukocytes (viable or apoptotic) up to concentrations of 2 mM (data not shown), showing that the selective action described for LL-37 is not a general feature of cationic, alpha-helical host defense peptides.

The effect of LL-37 on apoptotic neutrophils is independent of known surface receptors and Ca^{2+} signaling. LL-37 has been reported to employ a variety of surface receptors for the mediation of its immunomodulatory effects; for neutrophils, two of the most likely receptor candidates are FPRL1 and $P2X_7$ (17, 39). We used the antagonists WRW4 (4) and oxATP (38) to block FPRL1 and $P2X_7$, respectively. In the presence of these two antagonists, the apoptotic cells were still permeab-

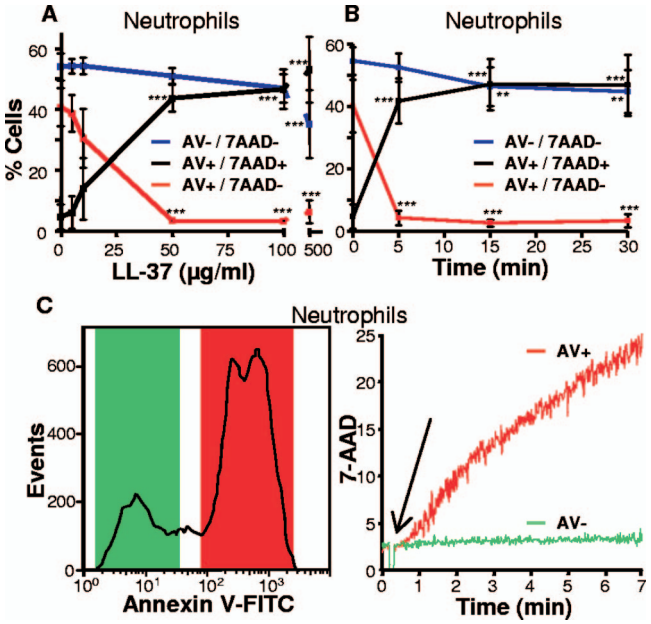

FIG. 4. The permeabilizing effect of LL-37 on neutrophils is concentration dependent and rapid. (A) Different concentrations of LL-37 ranging from 0 to 500 μg/ml were tested; increased concentrations led to an increased permeabilizing effect on the apoptotic cells, and at 500 μg/ml, a more general cytotoxic action that also affected viable neutrophils was observed. The percentage of cells is plotted against the LL-37 concentration; and the populations were viable (blue), apoptotic (red), and permeabilized (black). Mean ± SDs ($n = 3$) are shown. The results for each population were compared to those for an untreated sample, and significance was established, as indicated, by analysis of variance and Bonferroni's test for multiple comparisons. (B) Within 5 min after the addition of LL-37 (50 μg/ml), the permeabilizing action was complete. The membranes of viable cells retained their integrity for at least 5 min. The percentage of cells is plotted against time; and the populations were viable (blue), apoptotic (red), and permeabilized (black). Mean ± SDs ($n = 3$) are shown. Statistical significance was determined as indicated above for panel A. (C) After the gating of viable (green) and apoptotic (red) cells with the aid of annexin V staining (left panel), the increase in the intensity of 7-AAD staining after the addition of LL-37 (50 μg/ml) was monitored in real time. Only the apoptotic cells displayed an increase in 7-AAD intensity (right panel), and the permeabilization started immediately following addition of the peptide (arrow). The results represent those of one of two experiments that were performed. **, $P < 0.01$; ***, $P < 0.001$.

ilized (Fig. 5A), indicating that the permeabilization was independent of known surface receptors. According to the general dogma, apoptotic cells are nonfunctional and should thus be unable to transmit intracellular signals (2). When FPRL1 and $P2X_7$ are activated, they transmit signals that result in the mobilization of intracellular calcium (15). Using flow cytometry with mixed neutrophil samples containing both viable and apoptotic cells, we gated the cells on the basis of annexin V staining and measured the intracellular calcium mobilization in both types of cells simultaneously (Fig. 5B). Stimulation of the mixed population with the potent FPRL1 ligand WKYMVM resulted in a classic intracellular calcium flux (Fig. 5B, right panel) for the viable cells (green), whereas the apoptotic cells (red) were completely unresponsive. These data indicate that the permeabilizing effect of LL-37 on apoptotic cells is most likely independent of surface receptors and/or active cell signaling. In this respect, the permeabilizing effect was different from other (mostly receptor-dependent) immunomodulatory effects on leukocytes reported previously (10). In conclusion, the effect of LL-37 on apoptotic leukocytes is closely related to the direct microbicidal, permeabilizing effect of LL-37 rather than immunomodulating, receptor-dependent effects.

Inhibition by serum components. We hypothesized that the mechanism behind the observed specific permeabilization of apoptotic cells by LL-37 had more in common with its direct microbicidal effects than its immunomodulatory actions. The direct microbicidal effect of LL-37 is known to be inhibited by various serum components like HDL and apolipoprotein A1 (32, 55), and in our microbicidal assay, the presence of HDL also markedly inhibited bacterial killing by the peptide. When HDL was added to the inhibition zone plates at levels expected to be found in serum (650 μg/ml), the inhibition zone size generated by LL-37 was markedly decreased (Fig. 6A).

We next subjected a mixed population of neutrophils (viable and apoptotic) to LL-37 in the presence or the absence of either 10% human serum or HDL (650 μg/ml). Both serum and HDL completely blocked the LL-37-induced permeabilization of apoptotic neutrophils (Fig. 6B). The immunomodulatory, receptor-dependent effects of LL-37 have previously been shown to be intact in the presence of serum (17), in which HDL is a prominent protein. These data further strengthen the hypothesis that the observed effect is, in fact, receptor independent. When a salt solution composed to mimic the composition of 10% serum or bovine serum albumin (0.5%; wt/vol) was added, no inhibitory effects were observed (data not shown). These data imply that the presence of proteins in general and serum salts does not impair the function of LL-37.

Negatively charged PS is insufficient to mediate membrane permeabilization by LL-37. One plausible cause of the specific action of LL-37 on apoptotic cells could be the changes in membrane composition that accompany apoptosis, such as the surface exposure of the phospholipid PS. This negatively charged molecule could theoretically confer an affinity for the positively charged peptide, similar to the reported electrostatic interaction between LL-37 and LPS (30, 31). We checked whether LL-37 displays a direct affinity for PS by testing whether it binds to phospholipids and glycosphingolipids immobilized on a solid phase (50). When an attempt was made to bind LL-37 to total lipid extracts from human erythrocytes (40 μg) and control mixtures of acid glycosphingolipids from various sources (40 μg each), which were separated on thin-layer chromatograms, or to pure PE, PC, and PS (4 μg each), no specific binding of LL-37 to any of these compounds was observed (data not shown). In a microtiter well assay, the anionic GM1 ganglioside [Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer] and the neutral phospholipid PE were included as controls. LL-37 exerted no specificity toward PS compared to its specificity toward the other two lipids found in eukaryotic membranes included in this assay (data not shown).

We next checked whether the presence of PS could contribute to making membrances sensitive to the lytic action of LL-37 by incorporating PS into liposomes containing the phospholipids typically found in eukaryotic membranes and subsequently measuring the level of calcein leakage after addition of the peptide. The incorporation of PS into liposomes containing

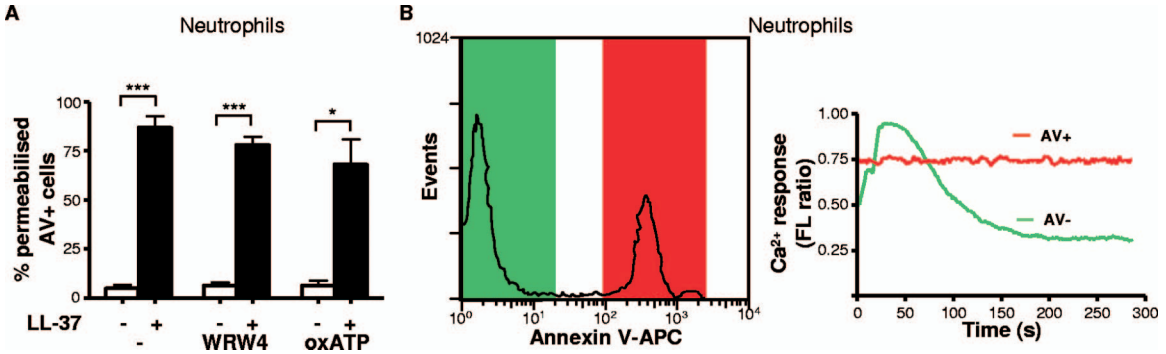

FIG. 5. The effect of LL-37 on neutrophils is independent of known receptors and Ca^{2+} signaling. (A) A mixed population of viable and apoptotic neutrophils was treated with the two antagonists WRW4 (5 μM) and oxATP (900 μM), which antagonize two known LL-37 neutrophil receptors, FPRL1 and $P2X_7$, respectively. In the presence of the antagonists, LL-37 (50 μg/ml) still permeabilized apoptotic, annexin V-positive cells. Paired *t* tests were performed to establish statistical significance ($n = 3$). (B) An overnight preparation of incubated neutrophils was assessed for annexin V staining, and a mixed population of both viable (green) and apoptotic (red) neutrophils (left panel) was shown. The mixed population was stimulated with the potent neutrophil FPRL1 ligand WKYMVM (10^{-7} M), and the transient Ca^{2+} release was monitored by flow cytometry. The viable cells (green line) responded with a rise in the intracellular Ca^{2+} concentration, while the apoptotic cells (red line) were totally irresponsive. The results of one representative experiment are shown. The FL-1/FL-3 ratio was plotted against time (right panel). *, $P < 0.05$; ***, $P < 0.001$.

phospholipids typically found in eukaryotic membranes did not confer any permeabilizing activity on LL-37 (Fig. 7). The total amounts of calcein released (determined by calculation of the area under the curve) for the two liposome preparations were determined and when the means were compared, no significant difference between the two was found ($P = 0.08$; $n = 3$).

The presence of PS did not change the susceptibilities of the liposomes to the lytic activity of LL-37, and the peptide did not seem to have any direct affinity for PS. We next hypothesized that LL-37 does not have any specific affinity for the apoptotic cells but instead interacts with both viable and apoptotic cells to the same extent and in this way induces limited membrane damage. The differences in the outcomes would then be due to the fact that the nonfunctional apoptotic cells are unable to repair the disturbed membrane, whereas the viable cells are quick to reseal the disruption. Viable cells are equipped with a complex machinery that allows them to repair membrane damage, and that machinery often involves a variety of cytoskeletal

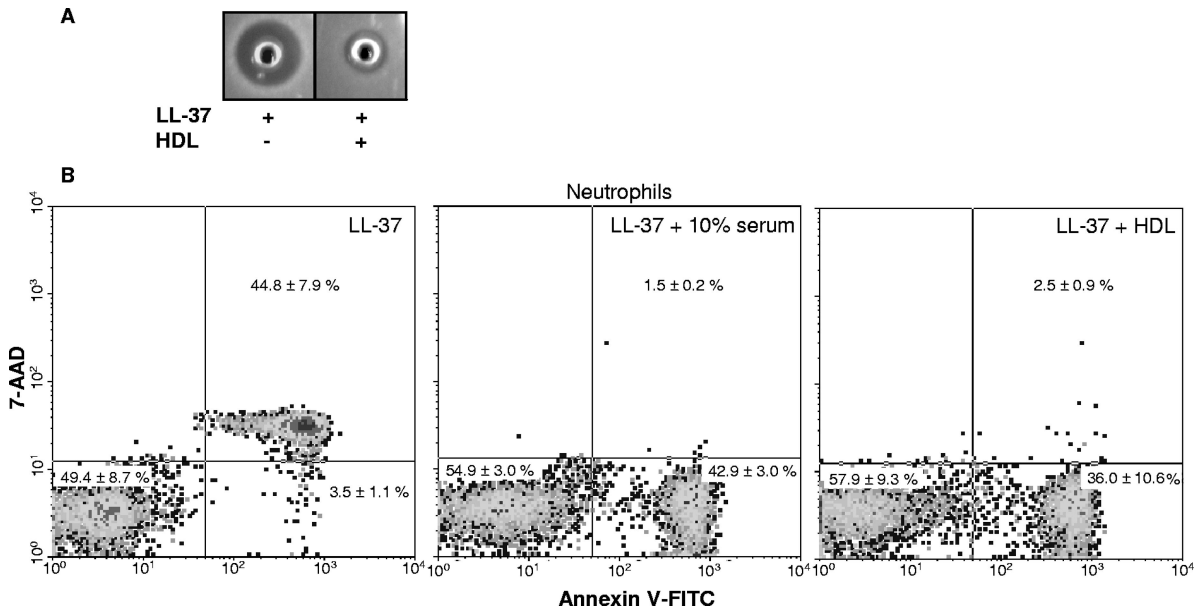

FIG. 6. The permeabilizing effect of LL-37 on apoptotic neutrophils is inhibited by human serum and HDL. (A) When HDL (650 μg/ml) was added to the inhibition zone plates, the clear zone surrounding the well after the addition of LL-37 (1.88 μg/well) decreased markedly. (B) In a control experiment, apoptotic neutrophils were readily permeabilized by the addition of LL-37 (50 μg/ml; left panel). When LL-37 was added to cells in a solution containing 10% normal human serum, the permeabilizing effect was totally blocked (middle panel). The addition of HDL (650 μg/ml) to the neutrophil population prior to the addition of LL-37 (50 μg/ml) also inhibited the permeabilizing effect of LL-37 on apoptotic neutrophils (right panel). Representative plots from three independent experiments and means ± SDs summarized from three independent experiments are shown.

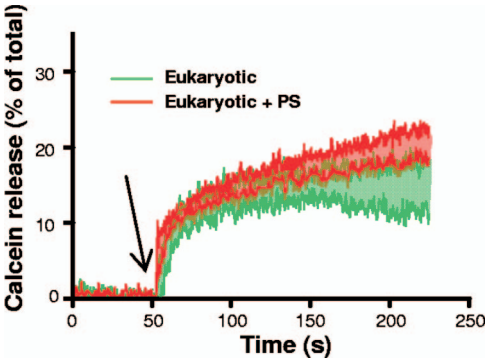

FIG. 7. The selectivity of LL-37 for lysis of apoptotic cells is independent of PS exposure. Liposomes containing typical eukaryotic lipids were prepared without PS (green line) or with 10% PS (red line). LL-37 (5 ng/ml) was added, as indicated by the arrow, to the different liposomes, and calcein leakage was monitored over time. The introduction of PS in liposomes containing the phospholipids typically found in eukaryotic membranes had no effect. The percentage of total calcein released, as assessed by the addition of Triton X-100, is plotted against time. The ranges for the two data sets are shown. The total amount of calcein released over 225 s (determined from the area under the curve) was established and did not differ significantly ($P = 0.08$; $n = 3$) between the two types of liposomes.

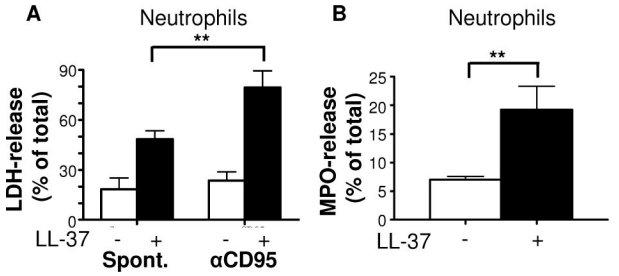

FIG. 8. Release of LDH and MPO from LL-37-treated apoptotic neutrophils. (A) Neutrophils were incubated together with or without the apoptosis inducer α-CD95; the result was 75% apoptotic cells and 45% apoptotic cells, respectively, in this set of experiments. These cells were treated with 50 μg/ml of LL-37 for 10 min, and the amount of the cytosolic enzyme LDH released was measured. It corresponded well with the fraction of apoptotic cells seen for each treatment, indicating the complete release of LDH from apoptotic neutrophils. Means + SDs ($n = 3$) are shown. Statistical significance was established by an unpaired t test. (B) The release of the intragranular matrix protein MPO was also measured in α-CD95-treated neutrophils. The amount of MPO released was significantly higher for LL-37-treated cells than for untreated cells. Means + SDs ($n = 3$) are shown. A paired t test was used for statistical analysis. **, $P < 0.01$.

filaments such as actin and microtubuli (36). We therefore subjected mixed neutrophil populations containing both viable and apoptotic cells to different inhibitors of cytoskeletal filament function in order to see whether such treatment would also enable the permeabilization of viable cells. Treatment of cells with cytochalasin B (which inhibits actin polymerization) (29) or colchicine (which perturbs microtubule assembly) (8) before addition of LL-37 did not make the viable cells sensitive to the lytic action of LL-37 (data not shown). In addition, the local anesthetic lidocaine, which is known to modify cell membrane fluidity (59), did not alter the pattern of susceptibility to LL-37; i.e., the peptide still permeabilized only the annexin V-positive cells (data not shown).

Permeabilization of apoptotic cells by LL-37 is associated with leakage of intracellular as well as intragranular constituents. Among the inflammatory leukocytes, neutrophils harbor an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an intact cell membrane retain their intracellular substances, and to see if permeabilization by LL-37 resulted in true leakage, we measured the extracellular presence of the cytoplasmic enzyme LDH. Peptide treatment of mixed neutrophil samples resulted in the release of LDH, and the levels of release correlated well with the level of apoptosis in the original cell sample; α-CD95-treated cells (74.8 ± 2.0% apoptotic cells; mean ± SD, $n = 3$) released significantly more LDH ($P = 0.009$) after addition of LL-37 than the spontaneously apoptotic cells did (35.5 ± 4.9% apoptotic cells; mean ± SD, $n = 3$) (Fig. 8A).

Most potent enzymes of neutrophils are, however, not stored in the cytoplasm but, rather in intracellular, membrane-enclosed granules such as the azurophil granules (19). We looked at the release of the azurophil granule protein MPO from α-CD95-treated neutrophils and found that LL-37 indeed provoked the release of significant amounts of MPO from these cells (Fig. 8B). Taken together, these data show that LL-37 is able to permeabilize the cell membranes of apoptotic neutrophils, leading to the leakage of cytoplasmic contents. In addition, the fact that MPO was released from LL-37-treated apoptotic neutrophils also indicates that the granular membranes are permeabilized, facilitating the extracellular release of various potentially deleterious substances.

DISCUSSION

The human cathelicidin LL-37 was originally identified as a cationic alpha-helical antibacterial peptide capable of microbial killing mediated by its interaction with the microbial membranes (1, 16, 31). Clearly, the peptide is capable of distinguishing between membranes with different compositions, and eukaryotic cells are much less susceptible to membrane attacks from LL-37 than, e.g., bacterial membranes. More recently, it has also been shown that LL-37 is capable of modulating the activity of several important immune cells (9). Similar to certain other peptides (7, 13, 51, 58), LL-37 displays a functional dualism, in that it directly kills microbes and modulates immune cell function; both of these activities are likely important for host defense. It is well known that leukocytes change the composition of their membranes during, e.g., apoptotic cell death. The differences between the membranes of viable and apoptotic cells relate not only to the expression of various surface proteins but also to their phospholipids compositions (35). On the basis of these facts and the ability of LL-37 to distinguish between different types of membranes, we reasoned that the peptide could possibly discriminate between viable and apoptotic leukocytes. Primary human neutrophils gradually enter apoptosis spontaneously during in vitro culture, enabling easy access to mixed cell samples containing both viable and apoptotic cells from the same donor. We thus used neutrophils as a model cell and showed that LL-37 selectively induced the permeabilization of apoptotic neutrophils, leaving the viable cells intact. In addition, a similar pattern of selective

permeabilization of apoptotic cells was seen for NK cells rendered apoptotic by hydrogen peroxide treatment, indicating that the effect was not limited to neutrophils. NK cells were more susceptible to the lytic action of LL-37, and at concentrations higher than 5 μg/ml, LL-37 displayed a more general cytotoxicity against purified NK cells. Viable neutrophils were not permeabilized until LL-37 concentrations exceeded 100 μg/ml, and it makes sense that neutrophils, being important reservoirs for LL-37, should be more resistant to peptide-induced membrane disruption.

The immunomodulatory actions of LL-37, as opposed to its direct microbicidal effects, are primarily mediated by receptor binding. The surface receptors most often implicated in LL-37-mediated responses in neutrophils are the chemoattractant receptor FPRL1 (17) and the nucleotide receptor $P2X_7$ (39). Antagonists for these receptors, WRW4 (an antagonist of FPRL1 [4] and oxATP (an antagonist of $P2X_7$ [38]), did not affect the LL-37-induced permeabilization of apoptotic neutrophils, nor did they influence the susceptibilities of viable cells. These findings suggest that neither FPRL1 nor $P2X_7$ is involved in the specific permeabilization of apoptotic cells. We also showed that the apoptotic cells in our mixed neutrophil samples were completely unable to mobilize intracellular calcium in response to FPRL1 activation, indicating that these cells are, in fact, nonfunctional and incapable of transmitting intracellular signals. Taken together, the permeabilization of apoptotic cells is highly unlikely to depend on the interaction of LL-37 with surface receptors or even active signal transduction. This makes the effect of LL-37 on apoptotic leukocytes appear to be much more like its direct microbicidal action than its immunomodulatory functions. The fact that the presence of blood constituents (serum or HDL) that inhibit LL-37's direct microbicidal effect was also effective at blocking the permeabilization of apoptotic cells supports this view.

LL-37 has previously been shown to inhibit apoptosis (39) as well as promote the necrosis (6) of human neutrophils, and it is likely that the induction of secondary necrosis could explain the former finding, as was proposed in a study published during the preparation of the manuscript (61). In line with this, our data presented here imply that in the presence of LL-37, cells that spontaneously enter apoptosis are rapidly permeabilized and are eventually lost from subsequent analyses. The viable cells, however, would be unaffected by the peptide, resulting in an apparent enrichment of viable cells at the expense of apoptotic cells, which could lead to the false impression that LL-37 inhibits apoptosis. We also show that the LL-37-induced permeabilization of apoptotic cells destroys the integrity of the plasma membrane, as seen by the leakage of the cytoplasmic enzyme LDH to the extracellular space. In addition, we could also see the leakage of MPO, which is stored in the azurophil granules (19). Since this granule type is responsible for storing most of the proteolytic enzymes (19) as well as the immunomodulatory molecules, this finding is important and indicates that LL-37-mediated permeabilization could be a potent means of getting such substances out to an extracellular location. The functional consequences of releasing tissue-destroying enzymes in combination with molecules that facilitate wound healing remain to be determined. Interestingly, the levels of MPO released were lower than anticipated, on the basis of the proportion of apoptotic cells; it is possible that LL-37 interacts electrostatically with anionic membrane components and sticks to the plasma membrane during permeabilization. The permeabilization of azurophil granules could also require a time longer than the time required for permeabilization of the plasma membrane. One must also take into account the possible release of hCAP-18 from the specific granules in neutrophils upon permeabilization. If LL-37 is cleaved extracellularly by proteinase 3, the accumulation of additional LL-37 could result in a positive-feedback loop.

The mere cationicity of a peptide is not sufficient to induce specific permeabilization, since sLL-37 was inert in terms of direct microbicidal activity as well as in the permeabilization of apoptotic cells. Cationicity combined with an alpha-helical structure was also not enough to permeabilize apoptotic cells, since Hp(2-20) did not possess this ability, even though it potently kills bacteria (43). This *Helicobacter pylori* peptide is in many ways similar to LL-37 and also shares specificity for FPRL1 as a neutrophil receptor (13). We first hypothesized that flipping of the negatively charged PS from the inner leaflet of the lipid bilayer to the outer leaflet could confer susceptibility to the cationic peptide LL-37. Several studies have shown that cationic peptides have an increased affinity (and killing capacity) for cancer cells compared to their affinity for healthy cells and that this is due to the fact that cancer cells have more negatively charged plasma membranes (54, 62). This charge difference partly originates from the various amounts of PS present on the outer leaflet of the plasma membranes (41). However, our data generated from liposome studies and chromatogram binding assays failed to support a direct role for PS. It is likely that apoptotic membrane changes other than the flipping of PS could explain the selective action of LL-37 on the apoptotic membranes.

In striking contrast to most immunomodulatory functions of LL-37 that are intact or that even depend on the presence of serum (48), we found that serum completely blocked the permeabilization of apoptotic cells. In this respect, the effect that we describe seems to be much more related to the direct microbicidal action of LL-37. With this in mind, it is reasonable to assume that the ability of LL-37 to permeabilize apoptotic cells in vivo would be of importance mainly at sites devoid of serum components, such as the skin or the lungs. LL-37 is very highly expressed in psoriatic scales of the skin and contributes to keeping the scales free of microbial pathogens (40). In addition, psoriatic scales are characterized by the continued presence of neutrophils, indicative of a chronic state of acute inflammation (52). Our findings that LL-37 induces permeabilization of apoptotic leukocytes, concomitant with the leakage of both cytoplasmic and granular proteins, could thus help explain why a state of acute inflammation fails to resolve at sites rich in LL-37 and deprived of serum components.

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