LL-37: An Immunomodulatory Antimicrobial Host Defence Peptide

Paula E. Beaumont, Hsin-Ni Li, and Donald J. Davidson

Abstract Cationic host defence peptides (CHDP) are conserved peptide components of the innate immune system. These peptides, also known as antimicrobial peptides, were originally discovered and described on the basis of their direct microbicidal properties. However, it has become increasingly clear that CHDP, such as the human cathelicidin hCAP18/LL-37, have an extensive range of immunomodulatory properties that can be complementary to microbicidal activity or may even represent their major antimicrobial function. As a result of its capacity to interact with cells involved in host defence LL-37 can modulate both innate inflammatory processes and interact with the generation of adaptive immunity. CHDP have been implicated in a variety of disease processes at diverse organ sites and are attracting increasing attention as templates for the development of novel immunomodulatory antimicrobial therapeutics. This chapter will focus on the antimicrobial and immunomodulatory properties of hCAP18/LL-37 and the underlying mechanisms involved in the bioactivity of this peptide.

Keywords Cationic host defence peptides • Antimicrobial peptides • Innate immunity • Host defence • Cathelicidins • LL-37 • mCRAMP

1 Mammalian Cationic Host Defence Peptides

Cationic host defence peptides (CHDP) are evolutionarily conserved, small, positively charged peptide components of innate host defences. In mammals, CHDP are represented by two main classes of peptide: the defensins and cathelicidins. The multiple different defensins are believed to share a common ancestral gene and can

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P.S. Hiemstra and S.A.J. Zaat (eds.), *Antimicrobial Peptides and Innate Immunity*, Progress in Inflammation Research, DOI 10.1007/978-3-0348-0541-4_4,

be subdivided into α-, β- and θ-defensins, based on the organisation of three characteristic cysteine disulphide bonds in the mature peptide fragment of the prepropeptide (reviewed in Taylor et al. 2008). In contrast, cathelicidins are not grouped as a family on the basis of the mature peptide structure, which displays considerable diversity, but rather by the presence of an evolutionarily conserved N-terminal cathelin domain in the propeptide (reviewed in Zanetti 2004). Mammals express a plethora of defensins [with humans expressing six α-defensin genes and having over forty predicted β-defensin genes (Taylor et al. 2008)], and multiple cathelicidins are seen in some species. However other species, including humans and mice, express only a single cathelicidin. Although steadily more reports detailing bioactivities of defensins are emerging, the immunomodulatory properties of cathelicidin peptides will be the focus of this chapter.

The defining features of cathelicidins are an N-terminal signal sequence, a conserved cathelin domain and a variable C-terminal domain which, upon cleavage, becomes the mature functional peptide. The cathelin domain was named on the basis of its capacity as a cathepsin L inhibitor, and the cleaved cathelin protein has been described as a cysteine protease inhibitor with some microbicidal properties in its own right (Zaiou et al. 2003). The mature cathelicidin peptides range from 12 to 88 amino acids in length and take various forms including linear peptides with the capacity to form amphipathic α-helical structures, disulphide bond-stabilised β-hairpin structures and proline-rich structures (Zanetti 2004). The sole human cathelicidin human cationic antimicrobial peptide of 18KDa (hCAP18) generates a 37-amino-acid peptide called LL-37 as its primary mature product (Gudmundsson et al. 1996), which adopts an α-helical structure in lipid membranes and in physiological ionic environments (Johansson et al. 1998).

2 Human Cathelicidin hCAP18/LL-37

hCAP18 is encoded by the *CAMP* gene on chromosome 3p21.3. After removal of the signal peptide, the propeptide may be initially stored or immediately cleaved by proteinase 3 to form LL-37, the 4.5 kDa mature peptide fragment (Sorensen et al. 1997a, 2001). Although LL-37 is the major mature form, smaller fragments such as KS-30, KS-22, LL-29, KR-20, RK-31, LL-23 and KS-27 may also be formed by serine proteases (e.g. kallikreins) in keratinocytes and sweat (Murakami et al. 2004; Yamasaki et al. 2006), and cleavage by gastricin in the semen can lead to the formation of the ALL-38 form (Sorensen et al. 2003). These alternatively processed forms have variations in their balance of microbicidal and immunomodulatory properties (Braff et al. 2005), demonstrating a mechanism of in vivo functional control and illustrating the therapeutic potential to modulate function through peptide sequence manipulation.

hCAP18/LL-37 is produced at highest concentration by neutrophils [~630 μg/10^9 cells (Sorensen et al. 1997b)] where it is stored in propeptide form in the secondary granules. However, expression can also be induced in epithelial cells, keratinocytes,

monocytes, macrophages, mast cells, NK cells, γδT cells and B cells (reviewed in Bowdish et al. 2006). hCAP18/LL37 can be detected in a broad range of tissues and bodily fluids including plasma, bone marrow, airway surface fluid, skin, sweat, reproductive tract, semen, urine, breast milk and vernix (reviewed in Bowdish et al. 2005a).

The expression of hCAP18 is subject to complex transcriptional and post-transcriptional control, with upregulation in response to inflammatory and infectious stimuli [such as lipopolysaccharide (LPS), IL-6 and IL-1α (Frohm et al. 1997; Erdag and Morgan 2002; Nell et al. 2004)] and to wounding (Dorschner et al. 2001). The precise mechanisms remain to be fully determined; however, recent studies have clearly shown the importance of the active vitamin D metabolite 1,25-dihydroxyvitamin D3 (1,25(OH)D3) as an inducer of hCAP18 expression, acting via a vitamin D response element in the *CAMP* gene promoter (Wang et al. 2004; Gombart et al. 2005; Martineau et al. 2007; Hansdottir et al. 2008). The observation that expression of the hydroxylase CYP27B1, which converts 25-hydroxyvitamin D3 to the active 1,25(OH)D3, can be upregulated by TLR stimulation (Liu et al. 2006) indicates a mechanism for vitamin D-dependent upregulation of *CAMP* expression in response to inflammatory and infectious stimuli. Other mechanisms of control include butyrate-enhanced histone acetylation at the *CAMP* promoter, resulting in AP-1-mediated transcription (Kida et al. 2006), recruitment of the PU.1 transcription factor to the *CAMP* promoter in response to vitamin D, butyrate or lithocolic acid (Termen et al. 2008), and the identification of nuclear factor for interleukin-6 expression sites (Gudmundsson et al. 1996).

The importance of hCAP18/LL-37-mediated protection against infectious diseases in vivo can be seen in patients with the rare condition morbus Kostmann, in whom neutrophils are deficient in hCAP-1/LL-37 and who are more susceptible to infection (Putsep et al. 2002), and in the association between hCAP18/LL-37 levels and susceptibility to infection in dermatological pathologies (reviewed in Schauber and Gallo 2008). Whereas expression is not increased in response to the inflammation in atopic dermatitis and increased susceptibility to infection is observed, high levels of hCAP18/LL-37 (Ong et al. 2002) are associated with a relative protection from skin infections in psoriasis. However, pathologically high LL-37 levels may be harmful to the host and have been proposed to contribute to the pathogenesis of psoriasis by conferring antigenicity to self-RNA and self-DNA (Lande et al. 2007; Ganguly et al. 2009). Increased levels of hCAP18/LL-37 have also been reported in pulmonary infection, cystic fibrosis (CF) lung disease and bronchiolitis obliterans syndrome (Schaller-Bals et al. 2002; Chen et al. 2004; Anderson et al. 2008). Although these could represent a protective microbicidal response, dysregulated LL-37-mediated immunomodulatory effects might contribute to pathology, with the severity of CF lung disease found to correlate with increased LL-37 levels in the lung persisting between exacerbations (Chen et al. 2004). Indeed altered posttranslational processing of hCAP18, associated with an increase in stratum corneum tryptic enzyme, contributes to disease pathogenesis in acne rosacea (Yamasaki et al. 2007), demonstrating a pathological role for the angiogenic properties of this CHDP (Koczulla et al. 2003). In addition, recent studies demonstrate that LL-37

bound to DNA, generated by neutrophil extracellular trap formation, may play a key role in the pathogenesis of systemic lupus erythematosus (Lande et al. 2011; Garcia-Romo et al. 2011). Thus, in common with other key controllers and modulators of inflammation, LL-37 has potential to protect the host from infection but to be detrimental when expression is excessive or dysregulated.

Additional evidence for the critical, non-redundant protective effects of cathelicidins in vivo comes from studies of mCRAMP (mouse cathelin-related antimicrobial peptide, encoded by *Camp*), the murine orthologue of hCAP18. Genetically modified mice deficient in mCRAMP expression ($Camp^{-/-}$) demonstrate increased susceptibility to infections of the skin, intestinal tract, cornea, urinary tract and lung (Nizet et al. 2001; Iimura et al. 2005; Chromek et al. 2006; Huang et al. 2007; Yu et al. 2010) and are more susceptible to dextran sulphate sodium-induced colitis (Koon et al. 2011; Tai et al. 2012). Interestingly, regulation of murine cathelicidin expression diverges from that observed in humans, as mCRAMP is not regulated by vitamin D (Gombart et al. 2005), but has been shown to be HIF1α (hypoxia-inducible factor 1 alpha)-responsive (Peyssonnaux et al. 2005). Nevertheless, these studies show considerably more severe effects upon host defence than knockout models deficient in single β-defensins (Morrison et al. 2002; Moser et al. 2002), where there may be considerable redundancy, and demonstrate multi-organ effects of cathelicidin deficiency in vivo. Additional evidence of in vivo antimicrobial function was demonstrated using gene augmentation with hCAP18/LL-37 to enhance the clearance of *Pseudomonas aeruginosa* from the murine lung (Bals et al. 1999), a study that also demonstrated the therapeutic potential of these peptides. Although this research clearly indicates the importance of cathelicidins to host defence, the precise mechanisms underpinning these observations remain uncertain.

As with many antimicrobial peptides, the minimum inhibitory concentrations (MIC) for LL-37 against microbes in vitro [in the range of 10–250 μg/ml (Travis et al. 2000; Johansson et al. 1998; Saiman et al. 2001; Li et al. 2006; Pompilio et al. 2011)] are much higher than the physiological concentrations that have been described in vivo at uninflamed mucosal sites (<2 μg/ml hCAP18/LL-37, of which it is unclear what proportion is mature peptide). In addition, LL-37 can be inhibited by the presence of cations (Bowdish et al. 2005b), serum apolipoprotein (Wang et al. 1998), DNA and F-actin (Weiner et al. 2003; Bucki et al. 2007). Indeed, in the presence of concentrations of divalent cations (Ca^{2+}, Mg^{2+}) found in the human body, even 100 μg/ml LL-37 (exceeding levels observed at inflammed mucosa) was not microbicidal for *Staphylococcus aureus*, or *Salmonella* typhimurium (Bowdish et al. 2005b) nor for *P. aeruginosa* (Barlow et al. 2010) against which cathelicidin-mediated in vivo protection has been observed (Bals et al. 1999; Yu et al. 2010). The question therefore arises as to how cathelicidins function as antimicrobial agents in vivo. While antimicrobial effects might be mediated through direct microbicidal properties at sites of localised high peptide concentrations or through synergy with other antimicrobial agents, perhaps the most important functions are indirect inflammomodulatory and immunomodulatory effects (Fig. 1).

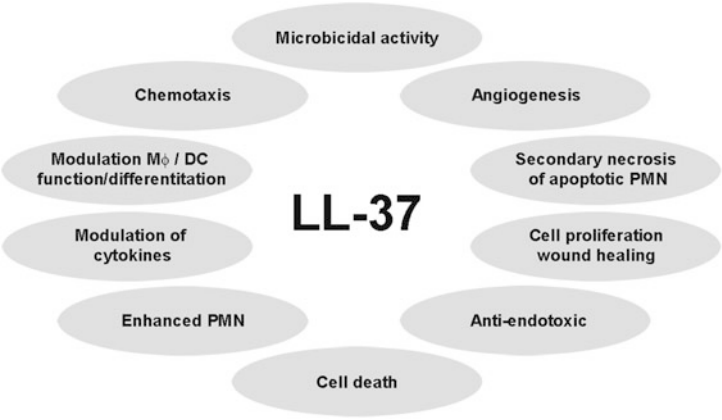

Fig. 1 LL-37 is a multifunctional component of host defence

3 Microbicidal Activity

LL-37 was initially described and characterised as an antimicrobial peptide, with the focus placed squarely on its microbicidal functions. LL-37 has been reported to be microbicidal against a broad range of gram-positive and gram-negative bacteria, including *P. aeruginosa*, *S. aureus* and *E. coli* (Travis et al. 2000; Johansson et al. 1998; Saiman et al. 2001; Li et al. 2006) and the yeast *Candida albicans* (den Hertog et al. 2005), and to inhibit biofilm formation by *P. aeruginosa* (Overhage et al. 2008). However, the capacity for direct bactericidal activity in physiologically relevant environments and lower concentrations has been questioned (Bowdish et al. 2005b; Barlow et al. 2010; Pompilio et al. 2011). Additional studies have also demonstrated antiviral activity of LL-37 in vitro and/or in vivo against HIV (Bergman et al. 2007), vaccinia virus (Howell et al. 2004), influenza virus (Barlow et al. 2011) and respiratory syncytial virus (Logermann et al. 2012), indicating broad-spectrum antimicrobial potential.

The microbicidal properties of CHDP have been variously attributed to three main mechanisms (Henzler Wildman et al. 2003), with the focus primarily on bacterial membranes: (a) a "barrel-stave" pore formation where hydrophobic surfaces interact with membrane lipid acyl chains while hydrophilic regions align to form a pore which may enlarge as more monomers are added, (b) a "carpet model" with transient toroidal pore formation induced through CHDP-mediated membrane curvature strain at sites of high local peptide concentration and (c) an alternative "carpet model" characterised by detergent-like bilayer disruption eventually leading to the formation of micelles at high peptide concentrations. LL-37 appears to function by the toroidal pore formation, binding to the negatively charged bacterial surfaces and adopting a stable α-helical conformation at the polar/nonpolar interface, aligned parallel to the membrane surface (Henzler Wildman et al. 2003). Studies evaluating the properties of LL-37 analogues and truncated peptides have

demonstrated that hydrophobicity and the propensity to form α-helices is critical to microbicidal function, but that the helical sense (using enantiomeric peptides) is not (reviewed in Burton and Steel 2009). In addition, the core microbicidal region has been defined as amino acids 17–32 (Li et al. 2006), with this truncated peptide having enhanced microbicidal activity in comparison to full length LL-37 (MIC ~100 μg/ml against *E. coli* K12, compared to 200 μg/ml for LL-37). The membrane defects induced by CHDP are proposed to allow leakage of intracellular contents, although whether this alone induces death or whether subsequent intracellular translocation of the peptide to interact with internal targets (Hancock and Rozek 2002) is also critical remains to be determined. Although bacteria appear less able to develop resistance to CHDP than to conventional antibiotics, various resistance strategies have been reported. These include the production of proteases capable of cleaving LL-37 (e.g. SpeB of *Streptococcus pyogenes*, metalloproteases of *Pseudomonas aeruginosa*, gelatinose by *Enterococcus faecalis* and ZapA from *Proteus mirabilis* (Nyberg et al. 2004; Schmidtchen et al. 2002)], membrane modifications (e.g. *Neisseria meningitidis* lipid A modifications (Jones et al. 2009), PmrA-PmrB-based modification of *P. aeruginosa* LPS structure (McPhee et al. 2003)) and the capacity of *Shigella* spp. to downregulate hCAP18/LL-37 production by host cells (Islam et al. 2001) [a process that could be counteracted by the therapeutic use of phenylbutyrate (Sarker et al. 2011)].

In specific protected environments, such as leukocyte phagolysosomes, high concentrations of peptide and controlled ionic environment may be well suited for direct effects on bacterial pathogens (Rosenberger et al. 2004; Martineau et al. 2007). In addition, alterations to in vitro bacterial culture conditions, designed to more closely mimic those present in mammalian tissues by increasing carbonate concentration, can alter the sensitivity of *S. aureus* and *E. coli* to LL-37 (Dorschner et al. 2006). This suggests that adaptations occurring in invading organisms may increase their susceptibility to innate microbicidal CHDP defences in vivo. Furthermore, LL-37 can act synergistically with other CHDP (Chen et al. 2005) and has been shown to synergise with conventional antibiotics (Leszczynska et al. 2010). However, the mechanisms of antiviral activity are less clear, and LL-37 had protective effects in a mouse model similar to that of a current first line anti-influenza therapeutic, despite very modest in vitro antiviral activity (Barlow et al. 2011). Furthermore, at mucosal surfaces in vivo, the capacity of LL-37 to play a fundamentally microbicidal role seems unlikely given the expression levels of LL-37, the presence of serum proteins, DNA and F-actin and the concentrations of cations. It is in these contexts that the additional bioactivities of LL-37 may prove to be of greatest significance to host defence.

4 Modulation of Cytokine Expression

Mammalian cells respond to a range of different microbial components or pathogen-associated molecular patterns (PAMPs) via innate pattern recognition receptors (PRR) including Toll-like receptors (TLR), RIG-I-like receptors (RLR)

and nucleotide-binding domain leucine-rich repeat containing receptors (NLR) [reviewed in (Kawai and Akira 2010)]. LPS and lipoteichoic acid (LTA) from gram-negative and gram-positive bacteria are powerful, well-studied proinflammatory PAMPs that can be released by dying bacteria. These PAMPs can activate leukocytes and epithelial cells to promote an initially protective inflammation, but can induce harmful inflammation and sepsis. The properties of LL-37 appear to extend beyond pathogen killing, to include mopping up and detoxifying liberated endotoxin upon microbial death to limit damage to host tissues. LL-37 has been shown to bind and neutralise both LPS and LTA and to modulate downstream TLR signalling, downregulating expression of PAMP-induced genes (Nagaoka et al. 2001; Scott et al. 2002; Rosenfeld et al. 2006; Mookherjee et al. 2006), even when the peptide was not applied for up to 90 min after PAMP stimulation (Scott et al. 2002). Interestingly these effects are observed at peptide concentrations lower than those required for microbicidal activity (typically 1–5 μg/ml). However, these modulatory effects of LL-37 appear to be PRR specific. LL-37 inhibited TLR4 and TLR2/1 agonists but not TLR2/6, TLR5, TLR7 and TLR8 agonists in peripheral blood mononuclear cells (Molhoek et al. 2009). Furthermore, LL-37 complexed with self-DNA and self-RNA can induce TLR7-, TLR8- and TLR9-dependent inflammatory responses in dendritic cells (Lande et al. 2007; Ganguly et al. 2009), LL-37 upregulated TLR9 expression and induced type I IFN responses in keratinocytes independent of DNA-LL-37 complex formation (Morizane et al. 2011), and LL-37 has been proposed to enhance (Lai et al. 2011) or inhibit (Hasan et al. 2011) TLR3-dependent responses to viral RNA or synthetic mimics. The precise points in the TLR signalling pathways at which LL-37 functions have not been clearly defined to explain all these functions, but the anti-inflammatory activities presumably underpin the protective effects of LL-37 in animal models of sepsis (Cirioni et al. 2006, 2008). The use of analogues and truncated peptides has demonstrated that the LPS-neutralising activity of LL-37 resides primarily in the C-terminal portion of the peptide and resulted in the generation of a 24-amino-acid peptide derivative with similar efficacy to LL-37 in terms of LPS and LTA neutralisation, but lower proinflammatory activity (Nell et al. 2006). These studies highlight the potential for development of cathelicidin-based peptides as novel anti-endotoxic therapeutics.

Inflammatory responses induced by PAMPS are driven by classic proinflammatory cytokines (e.g. TNF-α) and by chemokine-dependent recruitment of leukocytes. Interesting, while LL-37 can inhibit PAMP-induced TNF-α responses, it can also promote the production of chemokines [e.g. IL-8, MCP-1; (Scott et al. 2002; Tjabringa et al. 2003; Braff et al. 2005; Mookherjee et al. 2006; Filewod et al. 2009)] and has potent direct chemotactic properties for neutrophils, monocytes, memory T cells and mast cells in vitro and in vivo (Yang et al. 2000; Niyonsaba et al. 2002; Tjabringa et al. 2006; Kurosaka et al. 2005; Soehnlein et al. 2008). In addition LL-37 can induce degranulation in mast cells, resulting in the release of histamine, prostaglandin D2 and leukotriene B4, increasing vascular permeability and further promoting infiltration of leukocytes to the site of inflammation

(Niyonsaba et al. 2001). While optimal induction of chemokine production by monocytes, epithelial cells and keratinocytes occurs at ~25–50 μg/ml and involves activation of MAPK pathways (Tjabringa et al. 2003; Bowdish et al. 2004), the optimal direct chemotactic activity is observed in response to 2–25 μg/ml and functions through FPRL-1, CXCR2, MrgX2 and perhaps other unidentified G-protein-coupled receptors (Yang et al. 2000; Niyonsaba et al. 2002; Kurosaka et al. 2005; Zhang et al. 2009; Subramanian et al. 2011). Importantly, in contrast to the microbicidal properties, the chemotactic properties of LL-37 are not inhibited by serum (Yang et al. 2000).

LL-37 has also been shown to enhance responses to IL-1β and GM-CSF in peripheral blood mononuclear cells, but antagonise the responses to IFN-γ, IL-4 or IL-12 (Yu et al. 2007), to promote caspase-1-dependent posttranslational processing and release of IL-1β by LPS-primed monocytes (Elssner et al. 2004) and induce a caspase-1-independent processing of IL-18 from keratinocytes acting synergistically with β-defensins (Niyonsaba et al. 2005). These functions all suggest that, rather than being conventionally anti-inflammatory or proinflammatory, LL-37 can "rebalance" inflammatory responses in a concentration- and stimulus-dependent manner. Such complexity highlights the need to examine the effects of potential cathelicidin-based therapeutics in a pathogen-specific manner.

5 Leukocyte Differentiation and Function

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

Neutrophils are the key, innate immune effector cells that are the major cellular constituent of the early-phase response to inflammatory stimuli. In keeping with observations in other cells types, LL-37 can both promote neutrophil IL-8 responses in a MAPK p38 and extracellular signal-regulated kinase (ERK)-dependent manner (Zheng et al. 2007) and inhibit cytokine responses to Toll-like receptor (TLR) agonists and whole bacteria (Alalwani et al. 2010). However, in addition, recent studies have shown that exposure to 5–20 μg/ml of LL-37 can induce dose-dependent increases in neutrophil intracellular calcium mobilisation (Zheng et al. 2007; Zhang et al. 2009), induce the generation of reactive oxygen species [ROS; (Zheng et al. 2007)] and/or amplify ROS production in response to PMA or whole bacteria (Alalwani et al. 2010). Significantly decreased ROS production in *Camp*$^{-/-}$ murine neutrophils underscores the role of the endogenous peptide in this process (Alalwani et al. 2010). Given the importance of ROS as effector molecules in the

direct microbicidal function of neutrophils and the additional capacity of LL-37 to enhance neutrophil phagocytosis (Alalwani et al. 2010), these results suggest that LL-37 can prime and enhance neutrophil antimicrobial functions. Furthermore, LL-37 was shown to induce expression and release of human α-defensins (human neutrophil peptides 1–3) from live and apoptotic neutrophils (Zheng et al. 2007; Li et al. 2009). These α-defensins have recently been shown to also have effective anti-inflammatory properties in vitro and in vivo (Miles et al. 2009) and are likely to act in concert with the inflammomodulatory effects of LL-37 to modify the responses of macrophages and other cells.

LL-37 has been clearly shown to modulate the inflammatory responses of macrophages and monocytes, as described earlier; however, LL-37 is also capable of modulating macrophage differentiation (van der Does et al. 2010). While LL-37 exposure during the in vitro generation of human monocyte-derived macrophages (MDMs) promoted a more proinflammatory M1 phenotype, LL-37 could also redirect fully M2 phenotype-differentiated MDMs to produce more IL-12p40 and less IL-10. This bioactivity of LL-37 was localised to the C-terminus of the peptide, and LL-37 internalisation by the cells was necessary to modulate phenotype. In addition, the vitamin D-regulated antimycobacterial activity of human monocytic cells, attributed in part to the activity of CHDP (Martineau et al. 2007; Sonawane et al. 2011), has recently been demonstrated to involve LL-37-mediated autophagy of the infected cells (Yuk et al. 2009). Expression of LL-37 was shown to be critical both for the infection-induced transcription of autophagy-related genes Beclin-1 and Atg5, and for the colocalisation of mycobacterial phagosomes with autophagosomes. These studies demonstrate that both LL-37 expression by monocytic cells and the exposure of these cells to external sources of this peptide can modulate the antimicrobial and immunomodulatory properties of these cells.

In addition to their multiple roles in innate immunity, it is becoming clear that CHDP can modulate the adaptive immune response (reviewed in Bowdish et al. 2005a). Immunisation of mice with a plasmid fusing LL-37 to a tumour antigen generated enhanced antigen-specific humoral and cytotoxic responses and prolonged survival in a tumour model in vivo (An et al. 2005). LL-37 fusion plasmids were found to be significantly more effective than the tumour antigen plasmid alone, or co-administration of separately encoded plasmids for LL-37 and the tumour antigen, but the mechanisms remain unclear. Direct modulation of lymphocyte activity and/or proliferation, although demonstrated for defensins (Tani et al. 2000), is not a reported property of LL-37. Indirect mechanisms, such as alteration of the local cytokine environment should be considered, but a likely explanation may be found in the effects of LL-37 on dendritic cell (DC) differentiation and function. LL-37 has been shown to modulate DC differentiation from monocytic precursors in vitro, with LL-37-primed DC displaying significantly upregulated endocytic capacity, modified phagocytic receptor expression and function, upregulated co-stimulatory molecule expression (including CD86 expression in the absence of DC maturation) and enhanced Th-1 responses in vitro (Davidson et al. 2004), as well as modifying the nature of adaptive immune responses in vivo (Davidson, Schwarze, Wang, unpublished data). LL-37 therefore has the capacity to induce the differentiation

of immature DC "primed" to skew the nature of the adaptive response. Thus, LL-37/tumour antigen fusion proteins may function by delivering both the target for the adaptive immune response and a CHDP to generate a "primed" DC to same cell in a temporally appropriate manner for an enhanced adaptive response. These effects of LL-37 involved signalling via an unidentified G-protein-coupled receptor (Davidson et al. 2004), while related DC phenotype-modulating properties have been shown to require internalisation of LL-37 by the DC (Bandholtz et al. 2006). In addition to the effects of LL-37 on DC differentiation, LL-37 has been shown to inhibit LPS-induced maturation of differentiated DC (Kandler et al. 2006) in a manner consistent with its anti-endotoxic activities, but to promote DC activation in response to DNA and RNA (Lande et al. 2007; Ganguly et al. 2009). In the latter studies, LL-37 was demonstrated to bind non-inflammatory self-DNA and self-RNA and promote its uptake into DC in a manner that resulted in retention in early endocytic vesicles and activation of both plasmacytoid and myeloid DC, via TLR7, TLR8 and TLR9. These findings suggest a possible mechanism by which dysregulation of or exposure to high levels of LL-37 might be involved in breaking self-tolerance and driving autoimmunity in psoriasis and SLE. However, the initiation of LL-37 overexpression in psoriasis remains unclear as do the mechanisms by which tolerance is maintained in the context of inflammatory levels of LL-37 and dead cells in the healthy individual. These studies demonstrate the capacity of LL-37 to modulate DC differentiation and function in an inflammatory environment and reiterate the contrasting effects of this cathelicidin on cellular responses to diverse stimuli.

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

6 Modulation of Cell Death

Although CHDP can rapidly permeabilise prokaryotic membranes, most natural peptides are relatively less toxic to eukaryotic cells, an observation proposed to relate to the essentially neutral outer surface of eukaryotic membranes and their cholesterol content (reviewed in Lai and Gallo 2009). This affords host cells a degree of protection from the lytic effects of such peptides. However, negatively charged erythrocytes are more susceptible, presenting a challenge in the design of novel therapeutic derivatives (reviewed in Burton and Steel 2009), and CHDP can be cytotoxic to mammalian cells in a manner specific to cell type and its concomitant stimuli.

LL-37 has long been known to have cytotoxic effects on peripheral blood leukocytes at concentrations above 125 μg/ml, even in the presence of 10 % foetal bovine serum [FBS; (Johansson et al. 1998)], but it was unclear whether this death was due simply to primary necrosis resulting from peptide-induced membrane

damage or an induction of programmed cell death. LL-37 can enter eukaryotic cells by an active process requiring endocytic machinery (Lau et al. 2005) and can facilitate the cellular entry of nucleic acids (Sandgren et al. 2004; Zhang et al. 2010) and DNA dyes (Elssner et al. 2004; Tomasinsig et al. 2008) without inducing cell lysis, suggesting temporary membrane disruption or pore opening mediated by this cathelicidin in live cells. Exposure to higher concentrations of LL-37 can induce apoptosis of airway epithelial cells in a dose-dependent manner (with substantial cell death at $\geq$50 μg/ml) in vitro and in murine airway epithelial cells in vivo (Lau et al. 2006; Barlow et al. 2006). This induction of cell death by high concentrations of LL-37 involves Bax translocation to the mitochondria and is partially dependent on caspases (Barlow et al. 2006, 2010). The presence of high-density lipoproteins from human serum could block entry of LL-37 into the epithelial cells, inhibiting this LL-37-induced cell death and the IL-8 production by these cells (Lau et al. 2006). LL-37 has also been shown to induce death in Jurkat T leukaemia cells, although requiring exposure to higher concentrations of peptide (50–200 μg/ml). This was demonstrated to be mediated via a caspase-independent and calpain- and AIF-dependent apoptosis that involved Bax activation and translocation to the mitochondria (Mader et al. 2009), but also associated with significant levels of necrosis (with propidium iodide entry into the cells) at the higher peptide concentrations in another study (Aarbiou et al. 2006). However, no cell death was induced in primary human lymphocytes or monocytes, at more physiologically relevant levels of LL-37 (up to 50 μg/ml) in the presence of 10 % FBS (Davidson et al. 2004; Bowdish et al. 2004). Furthermore, LL-37 has been found to protect primary keratinocytes from induction of apoptosis by camptothecin, an effect mediated by a cyclooxygenase-2-dependent mechanism involving production of inhibitor of apoptosis 2 protein (Chamorro et al. 2009), and to inhibit tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in intestinal epithelial cells in vitro (Otte et al. 2009), demonstrating the cell-type specificity of cathelicidin-mediated effects on cell death.

The extent to which direct induction of eukaryotic cell death at high peptide concentrations might modulate innate or adaptive immune responses in vivo remains unclear. However, a recent study has demonstrated that more physiological, inflammatory concentrations of LL-37 (10–30 μg/ml) can preferentially induce death in airway epithelial cells that have been infected with *P. aeruginosa* (Barlow et al. 2010). This enhanced susceptibility of infected cells to peptide-induced death was associated with mitochondrial depolarisation and a later-stage activation of caspase-3 and caspase-9 that were only observed in the presence of both peptide and infection, and required invasion of the epithelial cell by live bacteria. The internalisation of *P. aeruginosa* by epithelial cells and the induction of apoptosis in infected pulmonary cells in vivo have been proposed to be important in innate host defence against this organism (Pier et al. 1997; Grassme et al. 2000). In addition, apoptosis in the context of infection has been proposed to modulate the nature of subsequent adaptive immune responses, promoting a TH17 response (Torchinsky et al. 2009). However, intriguingly LL-37 has also been shown to mediate increased epithelial cell stiffness, diminishing intracellular localisation of *P. aeruginosa*

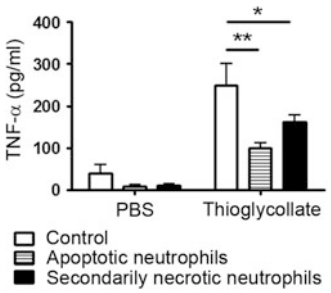

Fig. 2 *LL-37-induced secondarily necrotic neutrophils are anti-inflammatory in thioglycollate-induced sterile peritonitis*. 6–8-week-old Balb/c mice were injected intraperitoneally with 0.5 ml 10 % thioglycollate or 0.5 ml PBS concomitantly with human neutrophils, either (1) control-no neutrophils, (2) apoptotic neutrophils or (3) neutrophils previously induced to undergo post-apoptotic secondary necrosis by exposure to 25 μg/ml LL-37. Peritoneal lavage collected at 3 h post-injection was evaluated for TNF-α by ELISA. 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); *, $p \leq 0.05$; ***, $p \leq 0.001$, $n = 4$ per group

(Byfield et al. 2011a) Thus, LL-37 may contribute to innate defence against epithelial cell-invading microbes by diminishing epithelial cell invasion and by inducing the death of infected, compromised epithelial cells to both deny microbes a safe niche for replication and invasion of the host tissue and modulate the nature of subsequent adaptive immune responses.

The control of cell death is critical in maintaining homeostasis and in host responses to infection and inflammation but also for the resolution of inflammatory responses. Despite the key roles played by neutrophils in innate immunity, uncontrolled or persistent neutrophilia is detrimental to the host. Neutrophils undergo spontaneous apoptosis and have a short half-life that can be modulated by a broad range of substances, including bacterial products (e.g. LPS) and cytokines (e.g. GM-CSF) (Bianchi et al. 2006). Control of neutrophil death and the anti-inflammatory effect that apoptotic neutrophils have on phagocytosing macrophages are critical in the resolution of inflammatory responses (Savill et al. 2002). LL-37 can antagonise the effects of LPS on neutrophil survival (Li et al. 2009) and has been shown to modulate neutrophil death directly. Although initially proposed to be an inhibitor of neutrophil apoptosis (Nagaoka et al. 2006; Barlow et al. 2006), the principal effect of LL-37 is the rapid induction of secondary necrosis of apoptotic neutrophils, occurring at concentrations of peptides as low as 1 μg/ml (Li et al. 2009; Bjorstad et al. 2009; Zhang et al. 2008). This property was retained by C-terminal partial peptides and was also evident for mCRAMP. In contrast to expectation, LL-37-induced secondarily necrotic neutrophils had anti-inflammatory effects in vitro on activated macrophages (Li et al. 2009) and in vivo in a murine thioglycollate-induced sterile peritonitis (Fig. 2). The maximal anti-inflammatory effects were observed in association with LL-37-mediated release of granule contents from the apoptotic cells, induced by exposure to higher concentrations of LL-37 (25 μg/ml). These effects were independent of the anti-endotoxic activity of the

peptide used to induce secondary necrosis (Li et al. 2009) and may result from the release of both LL-37 and α-defensins from the apoptotic neutrophils (Miles et al. 2009). Although other granule contents could have deleterious effects, LL-37-mediated release of CHDP from apoptotic neutrophils may enhance the apoptosis-driven resolution of inflammation.

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

7 Cellular Proliferation and Angiogenesis

The expression of LL-37 is upregulated at sites of wounding and has been shown to play roles in cell proliferation, wound healing and angiogenesis. hCAP18/LL-37 was found to be strongly expressed in healing skin, but absent from chronic skin ulcers, and to promote re-epithelialisation of wounds in organ-cultured human skin (Heilborn et al. 2003). LL-37 and mCRAMP also enhanced re-endothelialisation, limiting neointima formation, after stent implantation (Soehnlein et al. 2011). Intriguingly this latter observation was still observed in mice lacking active forms of neutrophil-derived serine proteases (proteinase-3, cathepsin G and neutrophil elastase), raising interesting questions about the active product and proteolyic processing of mCRAMP in this system in vivo in these studies. However, mCRAMP has also been found to promote atherosclerosis, with deposition of this cathelicidin on inflamed endothelium mediating enhanced inflammation at these sites (Doring et al. 2012). LL-37 has been shown to induce keratinocyte migration in vitro at concentrations as low as 100 ng/ml [in the absence of serum (Carretero et al. 2008; Tokumaru et al. 2005)], associated with MAPK and matrix metalloproteinase-dependent epidermal growth factor receptor (EGFR) activation, and to enhance re-epithelialisation at skin wound sites in vivo (Carretero et al. 2008). This cathelicidin can also promote fibroblast proliferation (Tomasinsig et al. 2008), but inhibits collagen production by dermal fibroblasts and may have anti-fibrotic properties in wound healing, with the degree of fibrosis in dermal keloids found to be inversely correlated with the expression of hCAP18/LL-37 (Park et al. 2009). Furthermore, in studies using airway epithelial cells, LL-37 promoted wound healing in a dose-dependent manner by stimulating epithelial cell migration and proliferation at concentrations as low as 1 μg/ml, but interestingly only in the presence of serum (Shaykhiev et al. 2005). In addition to these wound healing properties, LL-37 has been shown to induce the proliferation of endothelial cells and neovascularisation in vitro and in vivo, with decreased vascularisation observed during wound repair in $Camp^{-/-}$ mice (Koczulla et al. 2003).

The capacity of LL-37 to modulate cell proliferation has stimulated a number of studies to evaluate the effects of this peptide on tumour growth and metastasis

(reviewed in Wu et al. 2010b). LL-37 derivatives have been proposed to have tumouricidal activity, via induction of apoptosis (Okumura et al. 2004). However, increased expression of hCAP18/LL-37 has been found in breast, ovarian and lung carcinomas (Heilborn et al. 2005; Coffelt et al. 2008; von Haussen et al. 2008), correlating with vascular density (Coffelt et al. 2008), and proposed to be mitogenic, with LL-37-dependent activation of the IGF-1R implicated as a possible mediator of increased migratory and invasive potential of malignant cells (Girnita et al. 2011). Transfection of epithelial cell lines (HEK293 and HaCaT cells) with hCAP18 enhanced cellular proliferation in vitro (Heilborn et al. 2005). Similarly, recombinant LL-37 stimulated proliferation of ovarian cell lines (Coffelt et al. 2008), although this occurred exclusively in the presence of serum and the enhanced proliferation observed at 1 μg/ml LL-37 was lost for two of the three cell lines at higher concentrations of peptide. The growth of anchorage-independent lung carcinoma cell lines in vitro was shown to be enhanced after the addition of ng/ml concentrations of LL-37, but significantly diminished by 20 μg/ml of peptide (von Haussen et al. 2008). In addition, LL-37 has been proposed to promote ovarian tumour progression by enhancing invasion, matrix metalloproteinase expression and the recruitment of multipotent mesenchymal stromal cells (Coffelt et al. 2008, 2009a), and tumours derived from transformed cells injected into nude mice showed significantly faster growth when engineered to overexpress hCAP18 (von Haussen et al. 2008). However, in contrast, exogenous LL-37 demonstrated anti-proliferative properties for gastric carcinoma cells, inducing cell cycle arrest, and had direct anticancer activity in vivo in a gastric cancer xenograft model (Wu et al. 2010a). Thus, although this cathelicidin can clearly impact upon tumour growth in model systems, the cell-type specificity and net effect of its properties in vivo remains to be determined.

8 Mechanisms of Immunomodulatory Activity

The pleiotropic effects of LL-37 in modulation of host defence responses raise questions about the mechanisms that could underpin such a broad array of bioactivities. At the simplest level, the anti-endotoxic properties of LL-37 are at least partly a consequence of direct, charge-based binding of LPS as discussed above, inhibiting interaction between LPS and its binding protein and/or receptor. However, even for this property, additional mechanisms are required to explain the selective LL-37-mediated inhibition of specific LPS-induced proinflammatory genes, without inhibition of LPS-induced genes that antagonise inflammation (Mookherjee et al. 2006), and a variety of receptor-specific and alternative mechanisms for LL-37-mediated immunomodulation have been proposed (Fig. 3).

A classical receptor-ligand mechanism has been proposed for LL-37, functioning through formyl receptor-like 1 (FPRL1), a G-protein-coupled receptor (GPCR). This receptor interaction was initially identified as the mechanism for LL-37-mediated chemotaxis of leukocytes (Yang et al. 2000). FPRL-1 has also been implicated in LL-37-mediated wound healing (Carretero et al. 2008), angiogenesis

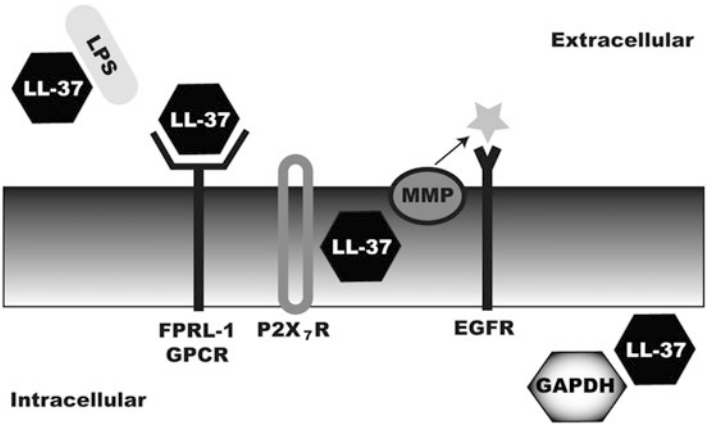

Fig. 3 *LL-37: mechanisms of immunomodulatory activity*. LL-37 may function via (a) direct sequestration of ligand (e.g. LPS), (b) classical receptor-ligand mechanisms, (c) interaction with diverse membrane-bound receptors and (d) intracellular mechanisms

(Koczulla et al. 2003), inhibition of neutrophil apoptosis [in one study (Nagaoka et al. 2006)] and in activating MAPK and enhancing invasiveness of ovarian carcinoma cells (Coffelt et al. 2009b). However, additional mechanisms occurring concomitantly have been implicated for many of these properties, while a recent study has described CXCR2 as an alternative receptor for LL-37-mediated neutrophil and monocyte chemotaxis (Zhang et al. 2009). The GPCR MrgX2 has been identified as a key LL-37 receptor on mast cells, and unidentified other GPCRs have also been proposed as receptors for LL-37 (Lau et al. 2005) and implicated in LL-37-mediated modulation of DC differentiation (Davidson et al. 2004) based on inhibition of LL-37-mediated effects by pertussis toxin. Furthermore, utilisation of GPCR by cathelicidins has been excluded in other studies, implicating alternative mechanisms and receptors, including $P2X_7R$, EGFR, IGF-1R and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The purinergic receptor $P2X_7R$ has important roles in the regulation of inflammatory processes (Lister et al. 2007). Activation by ATP (described as its principal ligand) reversibly opens large non-selective pores involving $P2X_7R$ and pannexin 1, which can enable ion flux across the cell membrane. The $P2X_7R$ has been identified as responsible for LL-37-mediated posttranslational modification and release of IL-1β from LPS-primed monocytes (Elssner et al. 2004), experimentally implicating LL-37 as an alternative direct ligand for this receptor. $P2X_7R$ activation has also been implicated in LL-37-mediated modulation of neutrophil apoptosis (Nagaoka et al. 2006; Barlow et al. 2006), endothelial cell stiffening (Byfield et al. 2011b) and the mitogenic properties of LL-37 on fibroblast proliferation (Tomasinsig et al. 2008). However, the latter study demonstrated that LL-37 could restore pore-forming activity to a truncated $P2X_7R$, which could not itself generate the classical non-selective pore (Tomasinsig et al. 2008). This activity, its independence from pannexin 1 and the equivalent mitogenic activity of similarly

structured orthologues and the D-enantiomer of LL-37, lead to a proposal of functional interaction between $P2X_7R$ and amphipathic peptides with appropriate helix-forming propensity, mediated by binding of transmembrane segments, and opening pores through mechanisms distinct from that of ATP-stimulated $P2X_7R$. LL-37 has also been proposed to function through activation of metalloproteinases in the cell membrane, by as yet undefined mechanisms, with consequent cleavage of soluble membrane-bound EGFR ligands and transactivation of EGFR. This mechanism has been implicated in LL-37-mediated induction of IL-8 expression (Tjabringa et al. 2003; Braff et al. 2005), wound healing, keratinocyte migration and enhanced cellular proliferation (Carretero et al. 2008; Tokumaru et al. 2005; Shaykhiev et al. 2005). Common to these and other studies is the activation of MAPK pathways by LL-37 (Bowdish et al. 2004); a downstream signalling event that can also be observed following FPRL-1 ligation by LL-37 (Coffelt et al. 2009b) and has can been implicated in LL-37-mediated modulation of TLR responses (Molhoek et al. 2009). The potential for LL-37 to modulate multiple signalling processes via interactions with transmembrane domains of diverse membrane-bound receptors may help to explain its pleiotropy and the apparently key nature of the amphipathicity of this peptide, irrespective of helical sense (Braff et al. 2005; Tomasinsig et al. 2008). However, a role for promiscuous receptors cannot be excluded and other properties of LL-37 require peptide entry into the eukaryotic cell. These include the induction of chemokine expression (Lau et al. 2005), altered MDM/DC differentiation (van der Does et al. 2010; Bandholtz et al. 2006) and peptide-mediated cell death (Lau et al. 2006). The identification of GAPDH as a novel intracellular receptor for LL-37 (Mookherjee et al. 2009) may be significant in this regard, but the full extent of intracellular effects mediated by this peptide and the mechanisms involved remain to be determined. Membrane integration of cathelicidin in the absence of peptide internalisation by the cell might also be fundamental to the cathelicidin-mediated induction of secondary necrosis in apoptotic membranes (Li et al. 2009; Bjorstad et al. 2009). Clearly the mechanisms of immunomodulation employed by LL-37 are complex and may be atypical, and elucidation will be important to furthering our understanding of these intriguing peptides.

9 Conclusions

The sole human cathelicidin hCAP18/LL-37 is a multifunctional CHDP with direct microbicidal potential and the capacity to modulate inflammation and immune responses through a broad range of mechanisms. It has been implicated in host defence and disease pathogenesis in multiple systems and conditions and represents both a fascinating target for clinical intervention and promising template for the development of novel antimicrobial, immunomodulatory therapeutics. Early clinical trials using synthetic analogues of CHDP were designed to maximise microbicidal activity, but achieved only moderate efficacy (Lipsky et al. 2008),

perhaps due to failure to recognise the importance of their immunomodulatory functions. A recent approach, using non-microbicidal analogues that retained other bioactive functions, has demonstrated effective host defence augmentation in mice (Scott et al. 2007). These studies suggest that realising the full therapeutic potential requires further research to more clearly understand the precise mechanisms of action underpinning the inflammomodulatory and immunomodulatory properties and the in vivo effects of these peptides' pleiotropic functions in specific clinical conditions.

Acknowledgments The University of Edinburgh is a charitable body, registered in Scotland, with registration number SC005336.

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