The roles of cathelicidin LL-37 in immune defences and novel clinical applications

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Purpose of review

LL-37 is the only member of the cathelicidin family of host defence peptides expressed in humans. It is primarily produced by phagocytic leucocytes and epithelial cells, and mediates a wide range of biological responses: direct killing of microorganisms, chemotaxis and chemokine induction, regulation of inflammatory responses, as well as adjuvant, angiogenic and wound healing effects. In this review we will cover the recent advances in the understanding of LL-37 biology: its activities, the mechanisms of its induction and roles in immune defence.

Recent findings

Recent studies advanced our understanding of the mechanisms controlling LL-37 expression, demonstrating the key involvement of the vitamin D_3 and the hypoxia response pathways, and the impacts of commensal and pathogenic microorganisms on its production. The synergistic and antagonistic interactions between LL-37 and other immune mediators have been further elucidated. Furthermore, studies in animal models and human patients further characterized the roles of cathelicidins in immunity, with roles in infectious and inflammatory conditions. The underlying properties of LL-37 have been exploited to create innate defence regulator peptides that represent a novel immunomodulatory approach to treating infections.

Summary

The understanding of the biological properties and functions of LL-37 and other host defence peptides advances our knowledge of innate immunity, the interactions of the host with pathogens and the microflora, as well as the pathology of infectious and inflammatory diseases, creating many strategies and opportunities for therapeutic intervention.

Keywords

cathelicidin, host defence peptide, LL-37

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Introduction

LL-37 is the only member of the cathelicidin family of host defence peptides expressed in humans [1,2]. It is a linear 37 amino acid peptide produced from the C-terminus of the hCAP18 precursor protein by a proteolytic cleavage. Like most host defence peptides, LL-37 is a cationic molecule (charge +6 at physiological pH), with a high content of basic and hydrophobic amino acids. It is relatively disordered in aqueous solution, but folds into an amphipathic α-helix in other environments, such as on contact with lipid membranes [3].

LL-37 is produced by phagocytic leucocytes, cells of the mucosal epithelium, and keratinocytes. It is a major constituent of the azurophilic granules of neutrophils, and is also present in mucosal secretions, sweat, and at low levels in the plasma. A wide range of biological activities have been attributed to LL-37 (summarized in Table 1) [4,5•,6,7,8••,9,10•,11–17,18••,19–22,23•,24–26], including direct antimicrobial action and diverse immunomodulatory properties. As outlined in Table 1, these LL-37 responses are elicited through the activation of a number of cell-surface receptors and signalling pathways, depending on the cell type being studied and the responses being assessed. Furthermore, LL-37 is also known to interact with cell membranes and to enter the cytosol of target cells [27], suggesting that it may also act by altering membrane dynamics [16] or by binding to intracellular target proteins.

The physiological significance of the different activities of LL-37 has been actively debated, and is ultimately dependent on the peptide concentration and the composition of the media at the specific site *in vivo*. Determination of LL-37 concentrations *in vivo* is challenging. Its levels in the airway fluids are estimated to be 0.4 μmol/l (2 μg/ml) in adults and 1 μmol/l (5 μg/ml) in

Table 1 Diverse biological activities of human cathelicidin peptide LL-37

Activity	Description	Signalling and mechanisms	References
Microbicidal activity	Acts on a broad spectrum of pathogens, the activity is highly sensitive to divalent cations.	Membrane disruption.	[2,4]
Inhibits biofilm formation	Inhibits formation of *Pseudomonas aeruginosa* biofilms.	Downregulation of genes essential for biofilm formation.	[5•]
Chemotaxis	Acts as a chemokine for neutrophils, monocytes, mast cells and T cells.	FPRL1 and other Gi-coupled receptors, Ca-flux.	[6,7,8••]
Mast-cell degranulation	Release of histamine and prostaglandins from mast cells.	Gi-coupled receptor, Ca-flux and phospholipase C.	[9]
Neutrophil antimicrobial functions	Stimulates phagocytosis, reactive oxygen species production and the synthesis of leucotriene B_4 by neutrophils.	Poorly understood, Ca flux, p38 and ERK are activated in neutrophils by LL-37.	[10•,11]
Induction of immune mediators	Induces many chemokines and other immune mediators in monocytes; induces IL8 in airway epithelial cells and keratinocytes.	Gi-coupled receptor, MAPKs, PI3K, NFκB in monocytes. $P2X_7$ and EGFR in epithelia.	[12–14]
Regulation of inflammatory response	Suppresses LPS-induced production of inflammatory cytokines, but not chemokines and other immune mediators.	Direct LPS binding, and the modulation of TLR and NFκB signalling pathways.	[15–17,18••]
IL1β processing	Promotes IL 1β processing and release from LPS-primed monocytes.	$P2X_7$ receptor and activation of caspase-1.	[19]
Apoptosis	Suppresses apoptosis of neutrophils, but promotes apoptosis of epithelial cells.	$P2X_7$, FPRL1 and another Gi-coupled receptor.	[20,21]
Wound healing	Promotes keratinocyte migration and wound healing.	Metalloprotease dependent transactivation of EGFR and STAT3. Also FPRL1 receptor and AKT pathway.	[22,23•]
Angiogenesis	Promotes vascularization by acting on the vascular endothelium.	FPRL1 receptor, Ca-flux, PI3K, PKC, phospholipase C and NFκB pathways.	[24]
Adjuvant activity	Promotes antibody production when administered with test antigens *in vivo*, complex effects on DCs *in vitro*.	Mechanisms unknown, LL-37 pretreatment promotes DC differentiation; chemotactic activities may play a role.	[25,26]

DC, dendritic cell; LPS, lipopolysaccharide.

neonates [28,29], with a further 2–3 fold upregulation in pulmonary infections. In contrast, in acute inflammation, LL-37 concentrations can dramatically increase, with levels estimated to reach 300 μmol/l (1.5mg/ml) in the skin lesions of psoriasis patients [30]. Considering such wide differences in LL-37 concentrations, the roles of LL-37 in these environments may also be widely different. Composition of the media is another factor that strongly impacts on LL-37 activity. Physiological levels of divalent cations and serum are strongly antagonistic for the direct microbicidal action of LL-37 [4], whereas some serum cytokines show synergistic effects on the immunomodulatory functions of the peptide [12]. On the basis of these factors, LL-37 is considered to be directly antimicrobial in the phagolysosomes of neutrophils and macrophages and at sites of acute inflammation, whereas the diverse immunomodulatory properties of the peptide can play a more profound role in a broad range of physiological settings.

Regulation of LL-37 production: signalling pathways and transcription factors

An understanding of the biological functions of LL-37 requires an in depth knowledge of its in-vivo concentrations and the mechanisms regulating its expression in different physiological settings. As already mentioned, LL-37 is known to be upregulated at sites of inflammation, such as in inflammatory skin disorders and in tracheal fluids during respiratory infections [29,31]. Mechanisms regulating LL-37 production are not fully understood, however important advances were recently made in establishing the roles of the vitamin D_3 and the hypoxia response pathways in the regulation of LL-37 production in leucocytes and keratinocytes.

Vitamin D_3 is naturally produced in the skin during exposure to sunlight, and is activated by hydroxylases CYP27A1 and CYP27B1 to generate the biologically active 1,25-dihydroxyvitamin D_3 ($1,25VitD_3$), which binds to the intracellular vitamin D receptor (VDR) to regulate gene expression (reviewed in [32••,33••]). The promoter of the hCAP18 gene contains multiple VDR-response elements, and stimulation with $1,25VitD_3$ *ex vivo* enhances hCAP18 expression in keratinocytes, monocytes and neutrophils [34]. Induction of LL-37 by $1,25VitD_3$ in these cells requires VDR, as well as the steroid receptor coactivator 3 (SRC3) and histone acetylation [35].

Importantly, the production of LL-37 in human primary monocytes in response to infection or TLR1/2 stimulation is also dependent on $1,25VitD_3$, either exogenously supplemented in ex-vivo systems or naturally present in the human serum [36,37••]. Furthermore, the $1,25VitD_3$-dependent induction of LL-37 contributes to the microbicidal activity of macrophages against *Mycobacterium*

tuberculosis, providing a likely explanation for the links between vitamin D deficiency and susceptibility to tuberculosis and for the therapeutic effects of sunlight against mycobacterial infections [32••]. The 1,25VitD_3 system is also implicated in the induction of hCAP18 and LL-37 in keratinocytes following wounding, with cytokine TGFβ1 upregulating the expression of the VitD_3-activating enzyme CYP27B1, which subsequently leads to VitD_3 activation and increased levels of hCAP18 and LL-37 in the wounded tissue [38].

In addition to the widely recognized role of 1,25VitD_3 in antimicrobial immunity, it is also known to have anti-inflammatory activity, suppressing the induction of TNFα, IFNγ and IL12p40 in human mononuclear leucocytes, at the same time as promoting the clearance of infection [39]. Thus the recently established role of 1,25VitD_3 in LL-37 induction further reinforces the notion that LL-37 and other host defence peptides may function in the context of 'nonclassical', noninflammatory responses to infection [15]. Possible contributions of LL-37 to the anti-inflammatory activity of the 1,25VitD_3 system remain to be investigated.

Hypoxia inducible transcription factor HIF1α is the key mediator of the hypoxia response pathway in mammalian cells, with critical roles in angiogenesis, tumourigenesis and in immune and inflammatory responses [40]. Recently, the regulation of cathelicidin expression has been added to the list of HIF1α functions. Thus, a selective inactivation of HIF1α in myeloid cells or keratinocytes results in a significant decrease in cathelicidin production: HIF1α-null mouse neutrophils show a dramatic reduction in the levels of mouse cathelicidin cathelin-related antimicrobial peptide (CRAMP) [41], and an siRNA knock down of HIF1α in human keratinocytes decreases the expression of hCAP18 [42••]. In both systems the loss of HIF1α and consequent reduced cathelicidin levels were associated with suppressed immunity to subcutaneous group A *Streptococcus pyogenes* (GAS) infections. The role of HIF1α in the induction of LL-37 raises several questions. Firstly, the links between the angiogenic functions of LL-37 and its induction by HIF1α warrant further investigation [24]. Furthermore, the recently established role of NFκB in HIF1α expression [43], together with the complex and context-dependent effects of LL-37 on NFκB signalling [15], suggest the possible existence of feedback loops involving NFκB, HIF1α and LL-37 signalling.

Regulation of LL-37 production: effects of commensals and pathogens

The mechanisms responsible for the different interactions of the immune system with pathogenic and commensal microorganisms remain one of the least explored areas in the field of innate immunity. Thus there is much interest in the effects of commensals and pathogens on LL-37 production, and conversely in the effects of LL-37 and other host defence peptides on these groups of microorganisms. Butyrate, a product of bacterial microflora in the colon, induces LL-37 production by colonic epithelia via TGFβ1-dependent, MAPK-dependent and VDR-dependent pathways [44,45]. In contrast infectious organisms such as *Shigella flexneri* suppress LL-37 production [46,47••]. Interestingly, oral delivery of butyrate in animal models could partly alleviate the symptoms of *Shigella* infection by restoring cathelicidin production [48]. Furthermore, mixed probiotic components derived from *Lactobacillus* sp., when delivered orally to mice, were shown to upregulate the expression of CRAMP in liver macrophages, increasing the bacterial clearance and improving survival in polymicrobial sepsis [49]. The induction of LL-37 by metabolites derived from commensal organisms highlights the poorly understood roles of LL-37 in the maintenance of immune homeostasis between the host and the microflora, and reinforces the notion that LL-37 and other peptides act to promote noninflammatory mechanisms of immune defence.

Pathogens targeted by host defence peptides have developed certain mechanisms for sensing and resisting peptide activity. Recent advances in our understanding of these mechanisms saw the identification of a three-component peptide sensing system in a Gram-positive opportunistic pathogen *Staphylococcus epidermidis* [50]. This system is unrelated to the PhoP/PhoQ system of Gram negative bacteria, and is responsible for sensing a wide range of peptides and for the induction of resistance mechanisms, like altering the charge of the bacterial surface to reduce peptide interactions. Other examples of resistance mechanisms include the downregulation of peptide expression by *Shigella flexneri*, recently shown to be mediated by the MxiE transcriptional regulator [47••]. Importantly, unlike the mechanism decreasing the bacterial susceptibility to direct peptide killing, the suppression of peptide production would also abolish the immunomodulatory effects of the peptides, and may have wider effects on immunity. For example, *Shigella* infection results in a MxiE-dependent suppression of dendritic cell recruitment to the lamina propria of the infected gut, which may be due to the suppression of the chemotactic activity of host defence peptides in the infected tissue [47••].

Biological activity of LL-37 and its interactions with other immune mediators

The immunomodulatory properties of LL-37 have been widely studied in highly controlled in-vitro systems (Table 1). However, the effects of LL-37 in the context

of a highly complex immune response in the presence of other immune mediators are only beginning to be explored. LL-37 was shown to act in synergy with GM-CSF in the activation of IL8 production by monocytes [12]. More recently, synergy between LL-37 and IL1β was also demonstrated, with a synergistic activation of AKT, CREB and NFκB pathways and a reinforced induction of a number of cytokines and chemokines [51•]. Furthermore, a functional interaction between LL-37 and leucotriene B_4 was recently reported in neutrophils, with a proposed positive feedback in the induction of expression and release of the two immune mediators *in vitro* and *in vivo* [11,52].

Others and we have previously shown that LL-37 is strongly antiendotoxic. This activity is mediated through both direct lipopolysaccharide (LPS) binding and complex modulation of TLR4 signalling, resulting in a selective downregulation of a subset of proinflammatory TLR4 target genes [13,15]. The potent antiendotoxic properties of LL-37 are further demonstrated by its protective effects in mouse models of endotoxaemia [13,53]. However, the effects of LL-37 on responses to other TLR ligands are variable, with an inhibition of responses to lipoteichoic acid (LTA), but an upregulation of some responses to CpG oligonucleotides [15]. This effect is analogous to the synergy between LL-37 and IL1β [51•], as the IL1β receptor utilizes a similar MyD88-dependent signalling pathway to most TLRs.

Further complexity in the effects of LL-37 on TLR responses is emerging from studies of dendritic cells. LL-37 was previously shown to modulate the process of monocyte differentiation into dendritic cells, with LL-37 pretreatment enhancing the expression of costimulatory molecules and Th1 cytokines by monocyte-derived dendritic cells [26]. The more recent studies focussed on the effects of cathelicidins on dendritic cell activation. Overall the activity of LL-37 on TLR responses in dendritic cells mirrors that in monocytes [15]. LL-37 was shown to suppress maturation and activation of human dendritic cells in response to a number of TLR ligands: LPS, flagellin and LTA, reducing expression of activation markers and pro-inflammatory cytokines, and suppressing *ex vivo* costimulation of T cells [17]. The suppression of LPS-induced dendritic cells maturation by cathelicidins was further confirmed in an independent study in both mouse and human systems [16]. The study also demonstrated the suppressive and anti-inflammatory functions of LL-37 *in vivo* in a mouse model of allergic contact dermatitis, and suggested that this is in part mediated via the activity of LL-37 on dendritic cells.

However, several studies report an LL-37-mediated enhancement of dendritic cell responses to TLR ligands. For example, LL-37 enhanced IL6 production by mouse and human dendritic cells in response to peptidoglycan and lipopeptides [16]. Furthermore, LL-37 was recently shown to strongly augment plasmacytoid dendritic cell IFNα responses to self-DNA, and this activity was implicated in the development of human psoriasis [18••]. In summary, the effects of LL-37 on inflammatory responses are complex and go beyond its antiendotoxic activity. This highlights the notion that LL-37 is a modulator rather than a suppressor of immune and inflammatory response. The effects of LL-37 on signalling by different TLRs in different cell types, their molecular basis and significance in the inflammatory responses *in vivo* clearly require further investigation.

Roles of LL-37 in the defence of epithelial surfaces

The skin remains one of the most widely studied systems for the investigation of cathelicidin-mediated immunity. The roles of cathelicidins in the immune defences of the skin are demonstrated by the strong negative correlation between LL-37 levels in the skin and the incidence of cutaneous infections in humans [33••], and by the impaired immunity of CRAMP-knockout mice to subcutaneous challenge with Group A *Streptococcus* [54].

One of the recent advances in the understanding of the role of LL-37 in the immunity of the skin has been the demonstration that mast cells are an important source of cutaneous LL-37, and also that LL-37 plays a significant role in mast cell mediated immunity. Mast cells express high levels of hCAP18 and LL-37, whereas CRAMP-deficient mouse mast cells are impaired in their *ex vivo* microbicidal activity [55]. Furthermore, recent studies showed that cathelicidin production by mast cells makes a significant contribution to the immunity of the skin, as the increased susceptibility of mast cell deficient animals to subcutaneous challenges with Group A *Streptococcus* could not be rescued by a reconstitution with CRAMP-null mast cells [56••].

Alterations in LL-37 production and processing have been implicated in the pathology of three distinct skin disorders: atopic dermatitis, psoriasis and rosacea (reviewed in [33••]). In atopic dermatitis, the levels of cathelicidins and the induction of cathelicidin production in the skin are dramatically reduced, with the Th2 polarized cytokine environment being implicated in this deficiency [57]. The low cathelicidin levels in the skin of individuals with atopic dermatitis are strongly associated with an increased incidence of bacterial and viral skin infections in the disease [57]. In contrast, extremely high hCAP18 expression and LL-37 levels are found in psoriatic skin [31]. In a recent study LL-37 from psoriatic

lesions was shown to complex with self-DNA, and such complexes were powerful inducers of IFNα production by plasmacytoid dendritic cells, possibly contributing to the loss of immunological tolerance in psoriasis [18••]. Interestingly, an increased copy number of β-defensin genes was also recently shown to predispose to psoriasis [58•], suggesting that peptides other than LL-37 may be involved in the aetiology of the disease. Another inflammatory skin disorder, rosacea, is also associated with an increased level of hCAP18-derived peptides. It was recently shown that an increased activity of proteases in the facial skin of rosacea sufferers results in an unusual pattern of proteolytic processing of hCAP18, and the role of the altered cathelicidin processing in the disease was supported in a mouse model of skin irritation [59••]. Although altered peptide processing may be involved, the high level of the hCAP18 gene expression in psoriasis and rosacea remains to be explained. Also the widely used practice of treating psoriasis with the LL-37-inducer vitamin D [32••] is surprising and suggests that the roles of the peptide in the disease may not be fully deleterious. Similarly a peptide derived from bovine indolicidin recently demonstrated statistically significant efficacy in phase II clinical trials against rosacea in reducing the number of inflammatory lesions (http://www.cutanealife.com/news-and-media.html).

The functions of LL-37 in the defences of other epithelial surfaces are less well explored. Its role in the urinary tract has recently been addressed [60]. The study showed that LL-37 and the mouse ortholog CRAMP are constitutively produced by the epithelia of the urinary tract and strongly upregulated in response to infections or bacterial products. CRAMP inhibited bacterial attachment to the epithelium, and CRAMP knockout mice showed increased susceptibility to and severity of urinary *Escherichia coli* infections. Studies of the roles of LL-37 in the gastrointestinal tract recently focused on the interactions between the mucosal surfaces of the gut and the microflora, and on the pathology of inflammatory bowel disease (reviewed in [61]). Dysregulation of LL-37 production in the gut epithelia was reported in patients with ulcerative colitis [61], and the rectal delivery of LL-37 in a mouse model of dextran sulphate induced colitis reduced disease severity [62]. The roles of cathelicidins in immune defences of the neonatal gut were highlighted in the recent study by Menard *et al.* [63••]. This study showed that CRAMP is highly expressed in the mouse gut epithelium during the first 2 weeks of life, prior to the development of α-defensin-producing Paneth cells, and during this period it plays a major role in the immune defences and immune homeostasis of the tissue. The roles of cathelicidins in the immunity of the airways and of the vaginal and cervical mucosa were recently reviewed [64,65].

Therapeutic uses of cathelicidin derivatives

Our understanding of the biology of host defence peptides suggests many strategies for therapeutic intervention in infectious and inflammatory diseases. One avenue is to boost immunity by stimulating the endogenous production of natural peptides, and the recent advances in the understanding of the control of LL-37 expression make an important contribution in this respect. This approach is exemplified by the use of butyrate to upregulate peptide expression in the gut [44,45,48], or vitamin D_3 and light to boost peptide expression in the skin and circulating leucocytes [36,37••]. Another example of this approach is the adenoviral transfer of the CAP18/LL-37 gene into a cystic fibrosis bronchial xenograft model, resulting in an increased LL-37 production and an improved microbicidal activity of the airway fluids [28].

An alternative approach utilizes natural peptides as templates for the development of artificial analogues with optimized biological activities [66,67]. This approach has gained much attention with the spread of bacterial resistance to conventional antibiotics, as the multidrug resistant pathogens are susceptible to the activities of peptides. Both the direct antimicrobial and the immunomodulatory properties of peptides are of interest. The peptide omiganan, an indolicidin derivative, has shown statistically significant prevention of catheter colonization and tunnel infections in phase III clinical trials [53], as well as recent success in treating an inflammatory condition, rosacea, in phase II clinical trials. Recently, we have shown that a short artificial innate defence regulator-1 (IDR-1) peptide lacking direct antimicrobial activity can provide protection in animal models of infection [68,69••], proving that the immunomodulatory activities of these peptides can on their own boost protective immunity. Indeed, the scope of the immunomodulatory activities of LL-37 suggests that peptides can be designed to enhance innate immunity through noninflammatory mechanisms [53], which is a unique and a highly desirable mode of action for a therapeutic agent. Furthermore, the potential angiogenic, adjuvant and wound-healing effects of peptides (Table 1) are also of therapeutic interest. However, many challenges need to be overcome in the transition of such peptides from the laboratory into the clinic, including the costs of peptide production, their stability and pharmacokinetics, and any possible toxicities that might accompany systemic administration.

Conclusion

LL-37 is a multifunctional host defence peptide, the full significance of which in the human immune defences is only beginning to be fully recognized. As well as having a direct antimicrobial activity, LL-37 elicits a complex array of responses in many cell types, either directly or

through modulation of cellular responses to microbial compounds and other immune mediators. The current challenges in the field are to analyse the significance of the different activities of LL-37 *in situ* in different physiological settings, to further characterize the receptors and signalling pathways mediating the different aspects of peptide activity, and to explore its interactions with other immune mediators in the wider context of immune response. Further advances in our understanding of the biological activity of LL-37 and other host defence peptides will create new avenues for therapeutic intervention in infectious and inflammatory diseases.

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Conflicts of Interest: REWH is developing peptides as potential immunomodulators and antimicrobial agents. In particular he is a shareholder and SAB member of Inimex Pharmaceuticals, which is developing IDR-1, and a minor shareholder of Migenix Inc that is developing omiganan.

References and recommended reading

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