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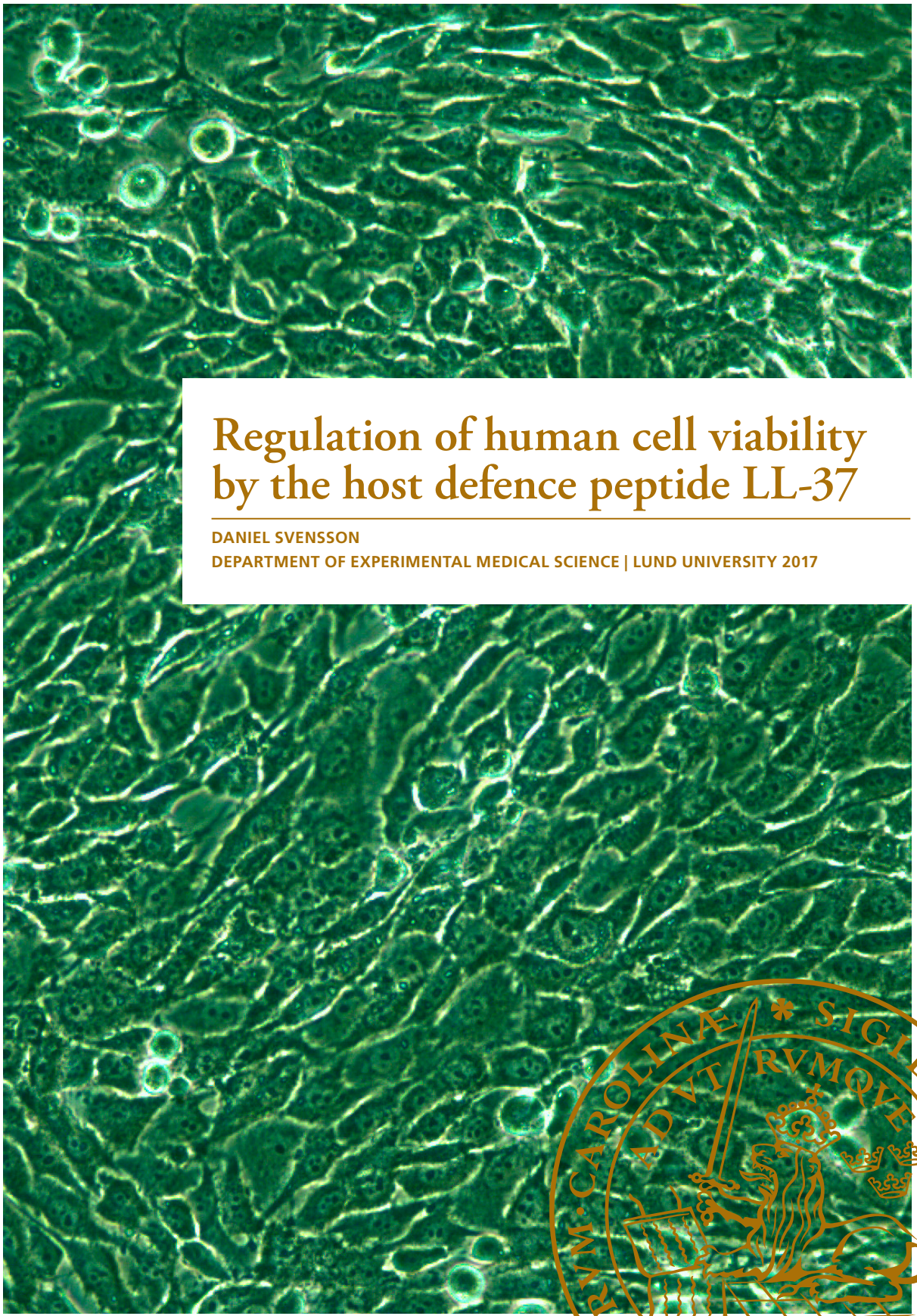

Regulation of human cell viability by the host defence peptide LL-37

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DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY 2017

Regulation of human cell viability by the host defence peptide LL-37

LL-37 induced cytotoxicity

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Accumulating evidence suggests that the main human cathelicidin peptide, LL-37, may influence host cell viability and pro-inflammatory signaling when locally overexpressed. LL-37 has pro-inflammatory properties linked to the development of for example psoriasis, rosacea, SLE and atherosclerosis. However, the importance of LL-37-induced host cell permeabilization and cytotoxic effects are poorly understood. We hypothesize that these effects are implicated in the tissue destruction associated with inflammatory diseases such as chronic periodontitis (CP). CP, the number one reason for adult tooth loss globally, is associated with elevated LL-37 levels. Here, tooth detachment is caused by degradation of the supporting bone. We aimed to investigate effects of LL-37 on osteoblast cell viability *in vitro* and the underlying mechanism in order to explore the detrimental effects of LL-37 and the possible involvement in tissue destruction associated with CP. In agreement with this, the peptide was found to permeabilize and kill osteoblasts at concentrations relevant for the *in vivo* situation. Cell permeabilization by LL-37 was associated with LDH release, Ca^{2+}-influx, attenuation of cell viability, accumulation of annexin V positive cells and caspase 3 activation, indicative of apoptosis in MG63 osteoblasts.

As LL-37 constitutes a possible drug target, we further investigated compounds and endogenous mechanisms that may serve to inactivate LL-37. We found that the protein p33 (gC1qR) may be added extracellularly to rescue osteoblasts from LL-37-evoked cytotoxicity. Additionally, endogenous p33 appears to be a mean by which host cells are protected from LL-37-induced cytotoxicity, as the extent of the toxicity correlates to p33 expression levels in various host cell types. Host cell sensitivity towards LL-37 may moreover be modulated through up- or down-regulation of p33 expression, mediated through transfection with a pcDNA3.1 expression vector and siRNA. p33 was found in the mitochondria, cytoplasm and in proximity to the cell membrane. It inactivates LL-37 intracellularly, as cell viability but not cellular LDH release is influenced by p33 expression, and additionally, LL-37 co-immunoprecipitates with cytoplasmic p33. This indicates that internalization of LL-37 is critical for host cell cytotoxicity. Furthermore, a comparison of LL-37's effects on various cell types indicates that LL-37-evoked cytotoxicity is not directly correlated to the degree of cell permeabilization.

The active form of vitamin D, 1,25D3, is a well-known mediator of LL-37 synthesis, although its possible role in dysregulated expression of LL-37 is yet to be elucidated. Stimulation of LL-37 expressing THP-1 monocytes with 1,25D3 was shown to attenuate cell viability both of co-cultured periodontal ligament (PDL) cells and the THP-1 cells themselves. This effect is reversed by addition of recombinant p33 to the cultures, implicating LL-37 as the mediator of the cytotoxic effect. In skin, RXRα protein levels were found to be critical for LL-37 expression: siRNA mediated silencing of RXRα attenuates 1,25D3-induced stimulation of LL-37 in HaCaT keratinocytes. Furthermore, gene expressions were analyzed in human skin and gingiva biopsies. Skin shows significantly higher LL-37 gene expression compared to gingiva and this difference correlates to RXRα mRNA levels.

In conclusion, our study contributes to the mechanistic understanding of LL-37 induced cytotoxicity in host cells, as well as the regulation of LL-37 expression by vitamin D. The *in vitro* results presented here support the hypothesis that LL-37 may be involved in the tissue destruction, observed locally in inflammatory diseases such as CP.

Key words: LL-37, cathelicidin, cytotoxicity, host defence peptides, autoimmune diseases, inflammation, vitamin D

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Front: Phase-contrast photography of MG63 cells
Back: LL-37 structure and amino acid sequence

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“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after”
– J.R.R. Tolkien

“An expert is one who knows more and more about less and less until he knows absolutely everything about nothing.”
– N. M. Butler

”Vem ska jag tro på
Tro på
Tro på när
Tro på när allt är såhär?”
– T. Di Leva

“Sometimes,' said Pooh, 'the smallest things take up the most room in your heart”
– A.A. Milne

CONTENT

LIST OF PAPERS

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. *The Antimicrobial Peptide LL-37 Alters Human Osteoblast Ca^{2+} Handling and Induces Ca^{2+}-Independent Apoptosis*. Johanna Säll, Martin Carlsson, Olof Gidlöf, Anders Holm, Johan Humlén, Jenny Öhman, **Daniel Svensson**, Bengt-Olof Nilsson, Daniel Jönsson, Journal of Innate Immunity 2013 Apr;5(3):290-300

II. *The human endogenous peptide p33 inhibits detrimental effects of LL-37 on osteoblast viability*. **Daniel Svensson**, Johannes Westman, Claes Wickström, Daniel Jönsson, Heiko Herwald, Bengt-Olof Nilsson, Journal of Periodontal Research 2015 Feb;50(1):80-8

III. *LL-37-induced host cell cytotoxicity depends on cellular expression of the globular C1q receptor (p33)*. **Daniel Svensson**, Laura Wilk, Matthias Mörgelin, Heiko Herwald, Bengt-Olof Nilsson, Biochemical Journal 2016 Jan;1;473(1):87-98

IV. *Vitamin D-induced release of hCAP18/LL-37 from monocytes attenuates viability of co-cultured human periodontal ligament cells.* **Daniel Svensson**, Daniel Nebel, Johannes Westman, Heiko Herwald, Bengt-Olof Nilsson, Manuscript

V. *Vitamin D-induced upregulation of human keratinocyte cathelicidin antimicrobial peptide expression involves retinoid X receptor α.* **Daniel Svensson**, Daniel Nebel, Ulrikke Voss, Eva Ekblad, Bengt-Olof Nilsson, Cell and Tissue Research 2016 Nov;366(2):353-362

PAPERS NOT INCLUDED

Additional peer-reviewed papers, not included in the thesis.

- *Vitamin D modulates the innate immune response through regulation of the hCAP-18/LL-37 gene expression and cytokine production*. **Daniel Svensson**, Daniel Nebel, Bengt-Olof Nilsson, Inflammation Research 2016 Jan;65(1):25-32
- *Inhibition of microRNA-125a promotes human endothelial cell proliferation and viability through an anti-apoptotic mechanism*. **Daniel Svensson**, Olof Gidlöf, Karolina M. Turczynska, David Erlinge, Sebastian Albinsson, Bengt-Olof Nilsson, Journal of Vascular Research 2014 Aug;51(3):239-45
- *1α,25-dihydroxyvitamin D3 promotes osteogenic activity and downregulates pro-inflammatory cytokine/chemokine expression in human periodontal ligament cells*. Daniel Nebel, **Daniel Svensson**, Karin Arosenius, Elisabeth Larsson, Daniel Jönsson and Bengt-Olof Nilsson, Journal of Periodontal Research 2015 Oct;50(5):666-73
- *Pyk2 inhibition promotes contractile differentiation in arterial smooth muscle*. Mario Grossi, Anirban Bhattachariya, Ina Nordström, Karolina Turczynska, **Daniel Svensson**, Sebastian Albinsson, Bengt-Olof Nilsson, Per Hellstrand, Journal of Cellular Physiology 2016 Dec 26, doi: 10.1002/jcp.25760 (Epub ahead of print)
- *A novel cytotoxic terpenoid from the flowers of Kaunia lasiophthalma Griseb*. Eliana Maldonado, **Daniel Svensson**, Stina Oredsson, Olov Sterner, Phytochemistry Letters 2014 May;8:105-108
- *Cytotoxic Sesquiterpene Lactones from Kauna lasiophthalma Griseb*. Eliana Maldonado, **Daniel Svensson**, Stina Oredsson, Olov Sterner, Scientia Pharmaceutica 2014 Jan;82(1):147-160
- *Detrusor induction of miR-132/212 following bladder outlet obstruction: association with MeCP2 repression and cell viability*. Mardjaneh Karbalaei Sadegh, Mari Ekman, Katarzyna Krawczyk**, Daniel Svensson**, Diana Dahan, Bengt-Olof Nilsson, Sebastian Albinsson, Bengt Uvelius, Karl Swärd, PLoS One 2015 Jan;10(1):e0116784

- *Vasopressin-induced mouse urethral contraction is modulated by caveolin-1*. Jianwen Zeng, Mari Ekman, Mario Grossi, **Daniel Svensson**, Bengt-Olof Nilsson, Chonghe Jiang, Bengt Uvelius and Karl Swärd, European Journal of Pharmacology 2015 Mar;750:59-65
- *The G Protein-Coupled Estrogen Receptor 1 (GPER1/GPR30) Agonist G-1 Regulates Vascular Smooth Muscle Cell Ca^{2+} Handling*. Anders Holm, Per Hellstrand, Björn Olde, **Daniel Svensson**, L.M Fredrik Leeb-Lundberg, Bengt-Olof Nilsson, Journal of Vascular Research 2013 Sep;50(5):421-9

BACKGROUND

Host defence peptides

Host defence peptides (HDPs) are effector molecules present across the evolutionary spectrum. In humans, they represent the first line of defense against invading pathogens by targeting the fundamental differences between microbial and mammalian cells such as membrane composition, structural features and energetics [1]. These host defense peptides are generally cationic; they consist of 12-100 amino acids and display a broad spectrum of biological activities including antibacterial, antiviral, antifungal and immunomodulatory properties. Accumulating evidence of host cell interactions with these peptides, including immunomodulatory functions, have led to the generally accepted classification of HDPs, rather than the still commonly used term antimicrobial peptides (AMPs) [2]. In humans, primarily, two distinct groups of HDPs are found: defensins and cathelicidins.

The two HDP families differ in many ways. Human cathelicidin is expressed from a single gene expressing the protein hCAP18 which, as a rule, generates a single active HDP, LL-37, upon cleavage by proteases. Defensins on the other hand are expressed from multiple genes, generating at least seventeen distinct HDPs in humans. These are classified as either α-defensins (four in human) or β-defensins (eleven in human) according to the organization of the characteristic conserved disulphide bonds [3, 4].

The structure and mode of action also differs between the defensins and LL-37. Defensins form compact peptides due to their three conserved disulphide bonds and have a characteristic β-sheet, while LL-37 lacks any disulfide bonds and adopts a α-helical structure in ionic solutions. LL-37 and defensins can act both pro- and anti-inflammatory, but LL-37 is in general considered more potent than defensins [2].

Cathelicidin

Cathelicidins are a family of polypeptides present in vertebrates including birds, fish, reptiles and mammals. Even very primitive vertebrates such as the jawless hagfish express the proteins [5-9]. The cathelicidins have been suggested to have evolved from the cystatins, the family of cysteine protease inhibitors, about 500 million years ago [10].

The first cathelicidin (then not known as such) was reported in 1988 after it had been isolated from bovine neutrophil granules. This protein, at that time referred to as bactenecin, was found to display antibacterial activities and it was hypothesized to play a role in the innate immune defense system [11]. Since then, the cathelicidins have been shown to display an extraordinary range of different functions and have proven to be critical in host defense systems in many mammals including humans. Their effects include activities against bacteria, virus, parasites and fungi, as well as host cells.

In humans, the cathelicidin gene, Cathelicidin Anti-Microbial Protein or CAMP encodes for the 16 kDa large protein hCAP18 [12]. The name was derived from its similarities to the rabbit cathelicidin CAP18, having a molecular weight of 18 kDa [13].

hCAP18

Human hCAP18 was first reported in 1995 by three independent research groups. In January 1995, Agerberth *et al.* reported on the pro-form of a peptide, which they called FALL-39 due to an error in the estimated cleavage site [14]. Later, in April, Larric *et al.* published the finding of hCAP18, discovered when looking for the human equivalent of CAP18 [15]. In June Cowland *et al.* published results on the isolation of hCAP18 and its corresponding cDNA [16].

hCAP18 is produced in various white blood cells (leukocytes): granulocytes such as neutrophils and mast cells, lymphocytes including NK-cells, and in monocytes. Further, the hCAP18 protein is also expressed and secreted by epithelial cells in skin [17], gut [18], lung [19], epididymis [20] and mammary glands [21]. Production and secretion has also been reported from dermal fibroblasts [22]. It is generally supposed that the hCAP18 protein is inactive against microorganisms in itself, even though there are conflicting reports on this [23, 24]. Instead, once it's secreted from the cells (or passively released when cells are dying), the protein is cleaved extracellularly by specific proteases, generating the active peptide. Only two proteinases have to date been found to generate LL-37: proteinase 3 from

white blood cells and kallikrein 5 expressed by keratinocytes in the skin. Both of these serine proteases cleave extracellular hCAP18 to LL-37, derived from the C-terminal, and a pro-region from the N-terminal referred to as the cathelin-like domain (hCLD): $hCAP18 \rightarrow hCLD + LL\text{-}37$ [23, 25].

Cathelin-like domain (CLD)

The cathelicidins are defined and named after the proteins' pro-region known as the cathelin-like domain (CLD), which, as opposed to the HDPs themselves, is highly evolutionarily conserved with the presence of two characteristic disulphide bonds constraining its structure [23, 26]. The name CLD is derived from the protein cathelin, to which CLD was shown to have high degree of sequence homology.

Cathelin is actually the ortholog of CLD in pigs, but was originally classified as a member of the cystatin family rather than a cathelicidin after being isolated from pig leukocytes in 1989 [27]. The name cathelin, in turn, was derived from its protease inhibiting properties, namely its ability to inhibit the protease cathepsin-L [27-29]. This is a capacity often observed in cystatin proteins (from which cathelicidins are believed to have evolved). The human cathelin-like domain (hCLD) folds, just like cathelin, in a cystatin like way, with a compact core formed by a N-terminal α-helix packed closely together with twisted antiparallel β-sheets by the two characteristic disulfide bonds (Fig. 1) [23]. Despite a cystatin-like structure, there are conflicting reports whether or not hCLD has any protease activity against cathepsin-L [23, 24].

The function of hCLD still remains to be concluded. It has been reported to express antibacterial activities on its own [24] although this has been disputed [23].

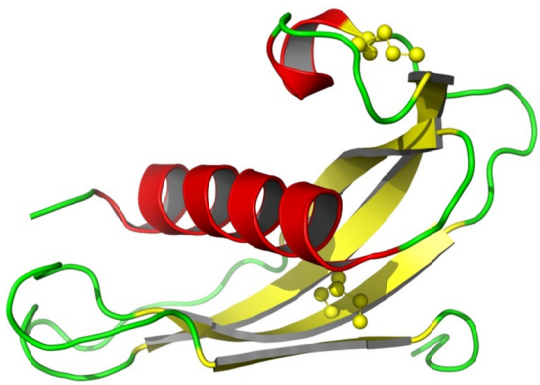

Figure 1. Structure of hCLD. Drawn as a cartoon diagram, yellow spheres indicates the two disulfide bonds. Figure created using PyMOL and the crystal structure obtained by Pazgier *et al.* [23], downloaded from RCSB PDB.

LL-37

The structure of the HDP fragment generated fromrom hCAP18 and the proteins' many orthologues vary extremely much between animal species. The HDP fragment LL-37 is found in humans but not in any other species with the exception of chimpanzees [30]. Since the discovery of the hCAP18 protein in 1995 there have been extensive studies on LL-37, showing that it exhibits many and varied properties. Besides possessing antimicrobial, antiviral and immunomodulatory properties, it has also gained a lot of attention for its stimulation of wound healing. In fact LL-37 possesses both cell type specific pro-proliferative and anti-proliferative/cytotoxic effects. It promotes cell migration, angiogenesis and arteriogenesis at the same time as it permeabilizes host cell membranes and triggers apoptosis [31]. Its host cell effects are mediated both by its membrane permeabilizing abilities and by its interactions with at least 16 different proteins and receptors, consecutively interacting with more than 1000 secondary effector proteins [2]. The peptide promotes the adaptive immunity response both by recruiting and activating immune cells and by initiating pro-inflammatory cascades. This has suggested LL-37 to be an "alarmin", which are endogenous molecules that signal tissue and cell damage [32, 33]. These different effects of LL-37 will be described and discussed in more detail in later sections of the thesis.

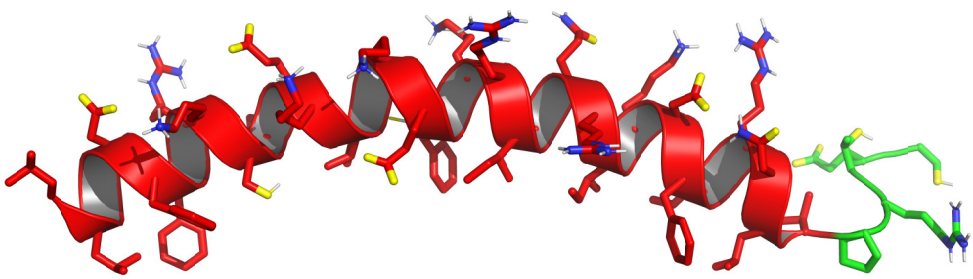

Figure 2. Structure of LL-37. Drawn as a cartoon diagram with sticks indicating amino acid side chains, nitrogen as blue, oxygen as yellow, polar hydrogens as white. Secondary structure is shown as elucidated by Wang ***et al.*** [34] using NMR spectroscopy of LL-37 in solution complex bound to deuterated SDS micelles. The structure is highly affected by enviromental factors. Here, it consists of one α-helix covering residue 2-31 followed by disordered C-terminal residues.

Expression and regulation of LL-37

hCAP18 is encoded by the CAMP gene located on chromosom 3, locus p21. The gene transcript has three introns and four exons, of which the first three code for hCLD while the fourth one codes for LL-37 [12].

The LL-37 protein is transcribed as a pre-pro-form, with a signaling sequence flanking the cathelin domain. The signaling sequence directs pre-hCAP18 to granules, a type of secretory vesicles found mainly in granulocytes such as neutrophils, and is there cleaved off. In keratinocytes, the protein is instead directed to lamellar bodies, where it is co-packed with lipids [35]. Upon stimulation, these hCAP18 filled intracellular bodies are released by exocytosis. Alternatively, the hCAP18 protein can also be secreted directly from the cells after the signaling sequence has been removed (for a schematic illustration see Fig. 5) [12].

Regulation of cathelicidin expression

Vitamin D regulates cathelicidin expression

The CAMP gene contains several transcription promotor regions, of which the vitamin D response element (VDRE) for the vitamin D receptor (VDR) is the most studied [36].

In vivo levels of active vitamin D_3, 1α,25-dihydroxyvitamim D_3 (1,25D3), depend on the dietary uptake and biosynthesis of its pro-form vitamin D_3 (D3). D3 can be synthesized from 7-dehydrocholesterol in skin keratinocytes through a UVB light-dependent mechanism (λ = 290–315 nm) [37]. The pro-hormone D3 is transported to the liver and hydroxylated at the C-25 position via catalysis by the cytochrome P-450 oxidase CYP27A1, yielding 25-hydroxyvitamin D_3 (25D3). A second hydroxylation of 25D3 on the C-1 position is catalyzed by CYP27B1, forming 1,25D3. The last reaction occurs primarily in the kidneys, although it can take place in other tissues as well (see Fig. 3) [38].

The second naturally available form of vitamin D, vitamin D_2 (D2), is unlike to D3 not synthesized in the human body, while it is produced by some algae and fungi such as chanterelles. D2 is commonly used in vitamin D supplements in several countries, including the USA. D2 is hydroxylated at the same positions and through the same pathway as D3, subsequently forming the active metabolite 1,25D2 (the structure is shown in Fig. 4). 1,25D2 has traditionally been considered to have similar effects as 1,25D3, but this has later come under debate [38, 39]. Still, 1,25D2 does induce CAMP gene expression similar to 1,25D3 [40].

The classical pathway for 1,25D3-induced gene regulation takes place after the vitamin binds to the vitamin D receptor (VDR), a nuclear receptor which in turn forms a complex with retinoid X receptors (RXRs). This 1,25D3/VDR/RXR complex binds to the VDRE promotor region on certain genes, such as CAMP, resulting in up or downregulation of the gene-transcriptional activity [41]. While 1,25D3 is considered to be the active metabolite, the more abundant precursor 25D3 also shows some affinity for VDR (about a hundred times lower than 1,25D3 [42]) and 25D3 may stimulate CAMP gene activity by itself [43].

The preferred method for assessing D3 status in serum or plasma is by measuring the concentration of 25D3 rather than 1,25D3. This is because the pro-form is much more stable and is present at much higher concentrations compared to 1,25D3, making detection easier. 25D3 is present at about a thousand fold higher concentrations than 1,25D3 in plasma (although this may not reflect the intracellular levels in proximity to VDR) [42].

Other ligands with affinity for VDR, e.g. the bile acid lithocholic acid (Fig. 4), may also activate VDR and upregulate CAMP in the same way as vitamin D [44].

The functional importance of 1,25D3-induced hCAP18/LL-37 expression is not well understood, but it appears to be an integral part of the antibacterial effects of vitamin D, e.g. CAMP induction in monocytes seems to be critical for vitamin D induced killing of *Mycobacterium tuberculosis* [45]. Interestingly, it is also reported that vitamin D supplementation reduces the need for antibiotics [46], although the role of LL-37 in this effect is unclear.

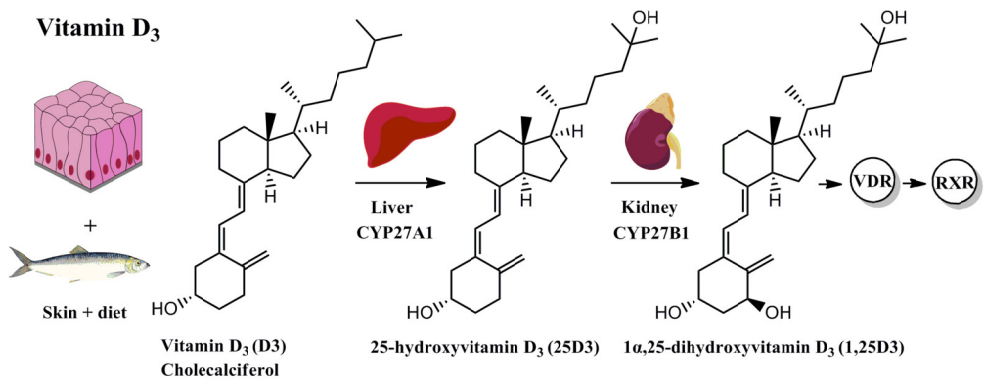

Figure 3. Metabolism and signaling of vitamin D. The main sources of the pro-hormone vitamin D3 are skin keratinocytes (formation of vitamin D3 is catalyzed by UVB light) and the diet. The pro-hormone is metabolically transformed primarily in the liver and the kidneys to the biologically active 1α,25-dihydroxyvitamin D3. Vitamin D2 is metabolized in the same way. After the hydroxylation steps, the steroid may bind to the VDR receptor, causing complex formation with RXRs, in turn regulating gene activity through VDRE binding.

Involvement of Toll-like receptors (TLRs) in vitamin D signaling

TLRs seem to be involved in CAMP regulation through modulation of the vitamin D hydroxylation pathway. TLR ligands such as bacterial endotoxins, e.g. lipopolysaccharide (LPS), activate pro-inflammatory cascades in human cells through receptor activation (TLR2 and TLR4 in the case of LPS). It was originally reported that LPS and 1,25D3 synergistically upregulate CAMP expression in neutrophils. However, stimulation of CAMP activity by LPS has since then proven to be difficult to replicate [36, 47-49]. In 2011 a plausible explanation was discovered: it was found that stimulation of TLR1/2/4 signaling in monocytes/macrophages enhanced CAMP expression through upregulating CYP27B1, promoting intracellular conversion of 25D3 to 1,25D3, and also by upregulation of VDR [50, 51]. Hence, for a full synergistic effect between LPS and vitamin D_3, the presence of 25D3 is required (1,25D3 is commonly used alone in *in vitro* studies), and, thus, LPS may promote CAMP expression through an indirect mechanism involving 1,25D3.

While 1,25D3 normally stimulates CAMP expression, it seems to do the opposite in keratinocytes under inflammatory stress. This in line with 1,25D3's potent anti-inflammatory effects which are unrelated to CAMP [52]. Inflammation stimuli subjected to keratinocytes, in the form of UVB irradiation and LPS treatment, causes upregulation of CAMP. This upregulation of CAMP was attenuated by 1,25D3 treatment, which also mitigated TLR2/4 expression correspondingly. Expression pattern of CAMP followed those of TLR2 and TLR4 [53]. Vitamin D signaling and CAMP regulation could in other words be altered in inflamed skin, e.g. in psoriasis, as compared to healthy tissue. The mechanism behind this is however not understood and some conflicting reports exist.

In relation to this, a negative correlation between TRL2 and VDR expression has been found in psoriatic skin, where LL-37 is heavily upregulated, but not in healthy skin [54]. This may represent some kind of feedback mechanism from the differential vitamin D regulation of CAMP activity at inflammation.

Another regulation mechanism of VDR induced CAMP expression can be observed when serum levels of vitamin D are high (25D3 <50 nM). When sufficient D3 levels are present, TLR induction of CYP27B1 diminishes as a feedback, thus impairing 1,25D3 synthesis. Hence, during TLR activation (e.g. bacterial infection), high levels of vitamin D may act as a negative regulator of the CAMP expression through this mechanism as well [50]. In line with this, Walker *et al.* showed that the cathelicidin gene expression in LPS stimulated monocytes from umbilical cord blood were significantly higher when serum levels of 25D3 were intermediate (50-70 nM) compared to serum with insufficient levels (<30 nM). However, in keeping with the described mechanism, they observed reduced induction of cathelicidin when 25D3 serum levels exceeded 75 nM [50]. Raqib *et*

al. [55] confirmed this adverse effect of high vitamin D levels on cathelicidin expression in a study where pregnant women received placebo or high doses of D3 (0.875 mg a week). This treatment significantly increased the cord blood plasma levels of 25D3 at delivery (from 30-40 nM to 100 nM), while it did not correlate to any changes in cathelicidin levels. Further, high, but not normal, 25D3 levels suppressed CAMP expression in LPS-stimulated white blood cells isolated from the umbilical cord blood [55]. The concentration dependent relationship between vitamin D and LL-37 expression has been seen in pathophysiological conditions as well, in asthma a positive correlation between 25D3 levels and LL-37 expression is seen exclusively in patients with vitamin D deficiency [56].

In conclusion, for a high induction of LL-37 during infection it is important that vitamin D levels are neither too high nor too low.

Other molecular regulators of LL-37 expression

Vitamin D-evoked regulation of LL-37 expression is a late evolutionary ability. While primates have it, most other species lack the VDRE promotor region on their cathelicidin genes. Instead, LL-37 expression seems to be induced by other stimuli in non-primates. In mice, for example, nitric oxide appears to be the main mediator of cathelicidin (mCRAMP) expression [51].

The human CAMP gene can also be regulated by pathways independent of VDR. Promotor regions on the CAMP gene has been identified for the transcription factors PU.1 [57], C/EBP [58], STAT3 and HIF-1α [59]. Thyroid hormones also have a response element at the promotor sequence allowing them to upregulate CAMP expression [60].

Other known CAMP- inducers includes various types of sugars, including lactose and trehalose [61], free fatty acids such as butyrate, phenylbutyrate and hexanoate [62, 63]. Some cytokines, e.g. IFN-γ, TNF-α, IL-17A and IL-36α/β/γ, have also been reported to stimulate CAMP expression. The IL-36 family of cytokines enhances CAMP through activation of MAPK and subsequently the NF-κB transcription factor which in turn activates C/EBPε [64, 65].

Resveratrol is a polyphenol common in plants that is commercially available as an antioxidant with anti-inflammatory properties. It protects keratinocytes from bacterial infection through upregulation of LL-37. Resveratrol stimulates production of the lipid molecule ceramide, and subsequently promotes its conversion to sphingosine-1-phosphate (S1P) which stimulates CAMP expression through a NF-κB-C/EBPα dependent pathway [66]. Similarly, the widely used plant metabolite curcumin has therapeutic uses as an anti-inflammatory and antioxidative drug. It has been found to induce CAMP expression in a VDR independent manner, perhaps accounting for some of its medicinal values [67]. Structures of resveratrol, S1P and curcumin can be found in Fig. 4.

Several aroylated phenylendiamines (ADPs), such as the histone deacetylase inhibitor entinostat, currently in clinical trials for cancer treatment, are potent stimulators of CAMP expression. They appear to upregulate LL-37 expression in a STAT3 and HIF-1α dependent way, where STAT3 induces HIF-1α expression, which in turn activates CAMP transcription [59, 68].

Several common drugs can also negatively modulate the expression of the CAMP gene. This includes anti-inflammatory glucocorticoids, cyclooxygenase (COX)-inhibitors and statins [60, 69]. Inhibition of hCAP18 exocytosis is an effective way to block LL-37 production *in vitro*. This can be done with for example protein phosphatase 1 [70].

Leukotriene B4 (LTB4, Fig. 4) is a lipid inflammatory mediator with properties similar to those of LL-37: it is a major endogenous chemotactic factor, it is antibacterial and a potent inducer of inflammation. LTB4 strongly induces hCAP18 exocytosis from granulocytes via activation of the BLT1 receptor at very low concentrations (1 nM) leading to elevated LL-37 levels extracellularly [70]. LL-37 also promotes the production of LTB4, seemingly through Ca^{2+} mobilization, indicating a positive feedback loop [70, 71]. In several inflammatory diseases where LL-37 is highly expressed, LTB4 levels are also elevated. The expression of LTB4 also correlates to the severity of the diseases. Medical conditions where both LL-37 and LTB4 are elevated, and where the former is indicated in the disease pathogenesis, includes psoriasis [72], asthma [73], atherosclerosis [74] and chronic periodontitis [75].

Chemerin, another signaling protein with potent chemotactic properties, also stimulates hCAP18 release from granulocytes at concentrations as low as 1 nM [76]. Similar to LTB4, the abundance of this protein has been correlated to high LL-37 expression in skin diseases, including early stages of psoriasis development [77]. In skin pathology, chemerin expression may be enhanced by an absence of the transcription factor FLI1. This was recently observed in the rare condition systemic sclerosis in which LL-37 is overexpressed and implicated in the pathogenesis [77].

Another factor besides LTB4 and chemerin that is able to stimulate exocytosis of hCAP18 is Zn^{2+} which promotes the release of the hCAP18 protein from epithelial cells [78, 79]. None of the studies on LTB4, chemerin or Zn^{2+} reported if the exocytosis-stimulatory treatments also affect the CAMP gene expression or the hCAP18 protein synthesis.

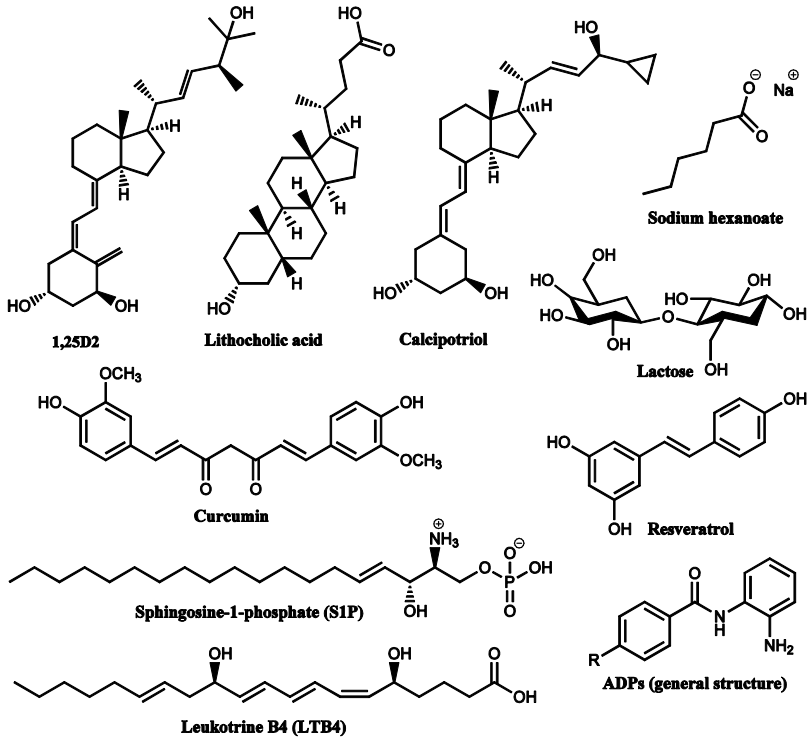

Figure 4. LL-37 expression regulators. Structures of different types of compounds known to upregulate hCAP18/LL-37 expression and/or exocytosis.

Light and ER stress regulate LL-37 expression

As previously mentioned, UVB at λ = 290–315 nm induces vitamin D3 synthesis in keratinocytes, which can consecutively induce the CAMP gene [37]. UVB irradiation of keratinocytes has also been shown to result in the opposite effect, treatment was able to downregulate the CAMP gene expression, possibly through effects on the *S*-nitrosylation pathway [80]. A range of factors such as light intensity and various regulatory systems could be involved in this inconsistency.

Light by other wavelengths than those of UVB may also affect the CAMP expression, seemingly without the involvement of vitamin D. Lee *et al.* [81] showed that LED light at λ = 630 and 940 nm caused downregulated expression of LL-37 both on gene and protein level in skin [81]. Similarly Kim *et al.* reported that light at λ = 410 nm downregulates the CAMP gene expression while 540 nm has the opposite effect [80].

CAMP upregulation may also be caused by sub-toxic ER stress in epithelial cells by a mechanism similar to that of resveratrol. The stress causes conversion of sphingosine, which is a part of many membrane lipids, to S1P. S1P in turn

upregulates CAMP via a NF-κB-C/EBPα dependent pathway. Stress that may trigger this pathway includes cell perturbation by UV irradiation, wounding and infection [82, 83].

Effects of age, weight and gender on LL-37 expression

Age also affects the LL-37 expression. In young (<18 years) asthmatic patients, there is a significantly stronger positive correlation between vitamin D levels and LL-37 expression, compared to that in adults [56]. In newborns, neutrophil expression of hCAP18 is about one third of that of adults. The hCAP18 expression by neutrophils increases gradually up till adulthood. Still, serum level of hCAP18 is similar in newborns and adults, probably due to newborns' higher white blood cell count [84]. At higher age, starting in middle aged individuals (30-59 years old), the circulating hCAP18 concentration decreases, correlating to a reduction in 25D3 levels as well as altered expression of several TLRs [85].

There may also be a gender difference in LL-37 production. hCAP18/LL-37 levels in saliva of children are significantly higher in girls compared to boys (median: 6 nM compared to 2 nM in boys) [86]. An explanation for this could be that testosterone inhibits CYP27B1, and thereby the formation of 1,25D3 [87].

Implications of the possibly higher LL-37 expression in females compared to men are not well studied. However, when looking at autoimmune diseases where elevated LL-37 expression has been implicated in the disease progression (these are discussed later in the thesis), the trend is that women are much more susceptible to these medical conditions compared to men. This includes palmoplantar pustulosis (affected women:men = 9:1, [88]), systemic scleroderma (9:1, [89]), systemic lupus erythematosus (9:1, [90]) and rosacea (3:1, [91]). The main exception is psoriasis, where both genders show similar frequencies [92]. The reverse could also be true in pathophysiological conditions with a deficiency of LL-37, e.g. in diabetic chronic foot ulcers, which may be caused by LL-37 deficiency, the prevalence is higher in men (~1:2). Men also tend to develop more serious consequences of the ulcers than women, including death (1:11 in a limited study [93]). Taken together, this could indicate that LL-37 is a key factor for the development of these autoimmune diseases and chronic wounds, and that the gender differential expression may be critical. Of course it may all be circumstantial as well, or secondary to other gender differences responsible for the correlation. Further, the gender-associated differential expression of LL-37 is to my knowledge not reported for any other types of samples than saliva.

Recently, a positive correlation between cathelicidin serum levels and body mass index (BMI) has been reported as well [94].

Processing of hCAP18 and LL-37

After secretion of hCAP18, proteinase 3, produced by white blood cells, may cleave hCAP18 to LL-37 and hCLD. Epithelial cells produce their own set of proteases acting on hCAP18. Kallikrein 5 from keratinocytes is able to generate LL-37 from hCAP18, but epithelial proteinases may do more than that. They are able to generate shorter cathelicidin peptides, using LL-37 as a substrate, and, moreover, they may even process hCAP18 to HDPs other than LL-37. This has been reported to occur in skin and sweat glands [95, 96] as well as in the vagina [79].

In skin, kallikrein 5 produces LL-37 from its pro-form. It also, probably together with kallikrein 7, 8 and 14, generates shorter peptides through further processing of the cleaved peptide. These products include KR-20, RK-31 and KS-30, all HDPs showing higher antimicrobial activity, and less pro-inflammatory effects, than LL-37. Some fragments are also more or less inactive against bacteria [95, 97, 98]. The human serine protease thrombin, highly expressed in liver, is also known to cleave LL-37 to shorter active peptides *ex vivo*, although I'm not aware of any study investigating the functional role of this for the *in vivo* situation [99]. KR-20, RK-31 and KS-30 show lower pro-inflammatory effect compared to LL-37 which may be due to loss of the hydrophobic C-terminal residues. The C-terminal residues are important for the peptides' ability to interact with eukaryotic cell membranes. Loss of this segment has also been shown to reduce host cell cytotoxicity [100]. Degradation of LL-37 to shorter HDPs, promoting antimicrobial effects while weakening immunomodulatory properties, can be an important mechanism for modulation of HDP activity in skin. Furthermore, the LL-37 derived peptides often act synergistically with LL-37 and each other, at least when it comes to killing of bacteria [98]. Fig. 5 schematically illustrates the processing of human cathelicidin to active HDPs.

In vesicles from lesions of palmoplantar pustulosis, a chronic inflammatory skin disease in which LL-37 has been suggested to promote the inflammatory process, a larger cleavage product of hCAP18 than LL-37 has been found. The peptide, called TLN-58, is of an estimated MW of 6858 Da and has similar pro-inflammatory potency as LL-37, but carries lower antibacterial efficiency [101]. Murakami *et al.* reported on TLN-58 in 2016 and suggested that the protease elastase 2 might be responsible for its generation [101].

The only other reported example of an HDP other than LL-37 endogenously being generated from hCAP18 was described by Sørensen *et al*. in 2003. They found that the slightly longer peptide than LL-37, ALL-38, could be formed in the vagina after intercourse [79]. Gastricsin is the protease implicated in the ALL-38 generation. It is an aspartic protease active only at low pH and it is produced in the

prostatic gland and seminal vesicles. The seminal fluid level of hCAP18 is very high (about 3-9 μM) [20]. hCAP18 is produced from epithelial cells in epididymis, and only at ejaculation hCAP18 and gastricsin are combined. The gastricsin protease is activated due to the low pH in vagina after intercourse (pH ~4). The antimicrobial effects of ALL-38 appear to be comparable to those of LL-37 [79, 102]. On a related note, LL-37 has potent spermicidal activity. The buffer capacity of the seminal plasma, however, causes a rise in vaginal pH for 2-6 h post-ejaculation, providing time for cells to migrate further before gastricsin is activated [102, 103]. ALL-38 generation is likely a mechanism evolved to protect against infection after intercourse, but perhaps it could also play a role in some cases of infertility i.e. where the pH and/or cell mobility is low for one reason or another.

The bioactivities of cathelicidin peptide fragments are greatly dependent on their ability to adopt a helical conformation in the C-terminal end. As long as this ability is retained after fragmentation of LL-37, the antimicrobial activity remains. Both RK-31 and KS-30 shows higher calculated helicity than LL-37, and furthermore a higher antibacterial activity. As long as the C-terminal α-helical region is preserved, the peptide can be cleaved down to a fragment as short as 12 residues (KR-12) and still retains the same antibacterial activity as LL-37 against *E. coli* (minimum inhibitory concentration (MIC): 40 μM) [104]. However, smaller fragments than this, almost completely lose their antimicrobial activity [100]. C-terminal helicity is however not the only factor influencing activity and helix stabilization may also result in oligomerization, attenuating the bactericidal activities.

Probably there are other, yet not identified, cleavage products of hCAP18 in humans. For example in gastric acid, a 6 kDa fragment is observed together with LL-37 [105] and in chronic periodontitis an 11 kDa fragment can be seen. Both of these structures remain to be elucidated. There is a possibility that bacterial proteolytic degradation of hCAP18 can be behind the formation of these fragments [106].

Citrullination is another way by which the LL-37 structure and activity may be modified *in vivo*. Peptidylarginine deiminases (PADs) converts the ketimine groups (=NH) present on LL-37's five arginine residues to ketone groups (=O). This process can vary in extent, PADI2 appears to citrullinate all arginine residues of LL-37, while PADI4 leaves two of them intact producing a partially citrullinated peptide [107].

Citrullination of LL-37 promotes the α-helix conformation, and the peptide may even adopt a β-sheet/β-turn. Consequently, citrullinated LL-37, and in particular the fully citrullinated variant, has significantly higher chemotactic properties compared to the native peptide. On the other hand, it lacks LPS-neutralizing properties. Furthermore, citrullination negatively affects LL-37-induced membrane permeabilization and it also sensitizes the peptide to proteinase-digestion [107, 108].

Sepsis and cigarette smoke are examples of factors that upregulate PADs and may thus promote citrullination of LL-37 [107, 108]. As of date, however, no study has been able to detect endogenous citrullinated LL-37, leaving the importance of this mechanism questionable.

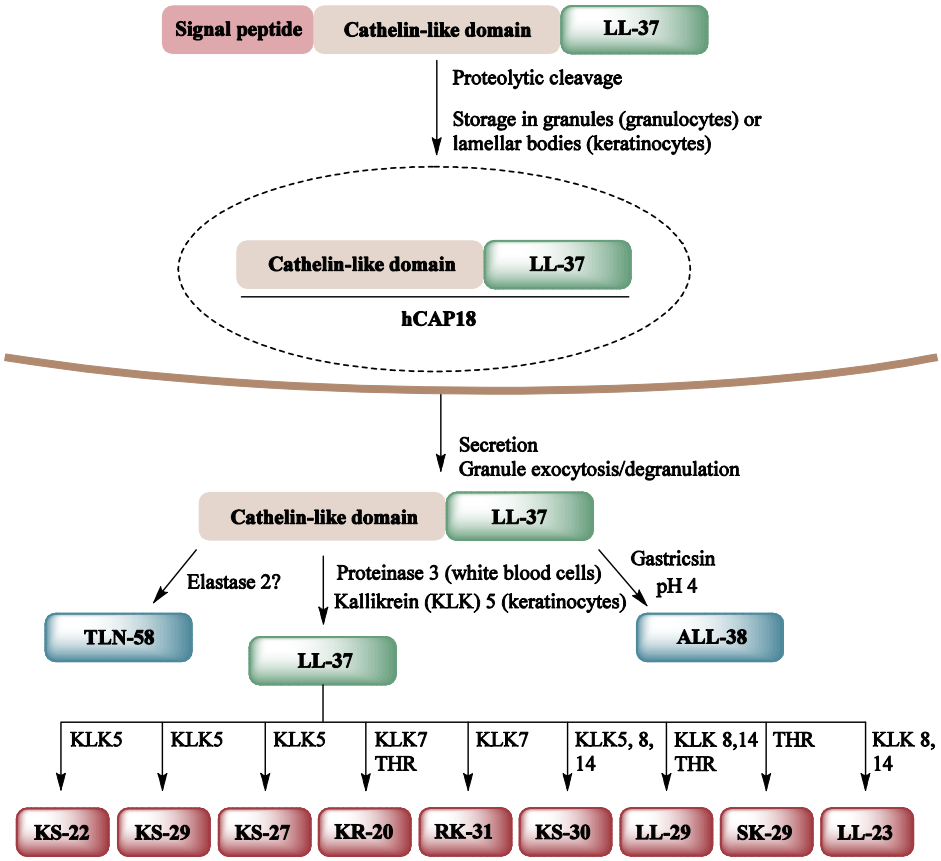

Figure 5. Human cathelicidin processing. Schematic figure showing the processing steps in the generation of LL-37 and other active peptides. KLK = kallikrein, THR = thrombin.

Structure-activity relationship

LL-37 consists of 37 amino acids of the sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES. It's a linear peptide with 11 basic residues (6 lysin, 5 arginin) compared to 5 acidic ones (3 glutamic acid, 2 aspartic acid) giving a net charge of +6 at physiological pH.

About one third of the amino acids in the LL-37 peptide are hydrophobic, prompting the amphiphatic peptide to adopt a helical structure at physiological ionic concentrations, compared to a random coil in water. A reason behind this change in conformation is that ions increase surface tension between the hydrophobic residues of LL-37, making the conformation energetically unfavorable. This is commonly referred to as the *Hofmeister effect*. By forming an α-helix, the peptide can shield the hydrophobic groups from the polar environment. The peptide has high electrostatic affinity for negatively charged membranes, and by interacting with such anionic membranes, the helical conformation is further stabilized [30].

Under eukaryotic-membrane-mimicking conditions, where LL-37 interacts with dodecylphosphocholine (DPC) bilayers of micelles, the α-helical peptide has been shown consists of three structural parts: an N-terminal α-helix which bridges to a C-terminal α-helix at residue K12, which in turn is flanked by a C-terminal hydrophobic tail, stretching from L31 to S37 [109]. These α-helical structures are dependent on the pH, anions present and peptide concentration [110]. The hydrophobic surface at the concave side of the helical peptide greatly affects LL-37's characteristics at physiological conditions. The hydrophobic surface promotes aggregation to mask itself from the polar environment, either via interactions with other hydrophobic molecules, or by interactions with the corresponding region on other LL-37 molecules, resulting in oligomerization. The antiparallel oligomer is further stabilized by intramolecular salt bridges between acidic and basic residues and intermolecular electrostatic interactions [12]. Oligomerization may also occur from the terminal ends. The oligomeric and monomeric confirmations of LL-37 is in equilibrium at physiological conditions [111] and the size of the oligomers has been shown to range from dimers to hexamers [112].

Oligomerization of the α-helical structure reduces LL-37's interaction with bacterial membranes and hence much of its antimicrobial activity according to most studies [12, 113]. There are exceptions however, e.g. Johansson *et al.*, stabilized the helical conformation with SO_4^{2-}, HCO_3^-, and $CF_3CO_2^-$ and thereby observed increased oligomerization and enhanced antimicrobial activity. These authors found a positive correlation between antibacterial activity and ion content, leading them to conclude that oligomerization results in higher antibacterial activity [110]. One alternative explanation for these findings is that the bacterial

susceptibility for LL-37 increases in the presence of these ions, to the extent that it out weights inactivation caused by the aggregation [12].

Although it might be safe to assume that oligomerization negatively affects the antibacterial properties, this may not be the case as it comes to host cell interactions. Helix stabilization can in fact promote effects of LL-37 on the host cells. One example of this interplay is that the α-helical conformation of LL-37 appears to be necessary for activation of the P2X purinoceptor 7 (P2RX7 or P2X7), through which the peptide exerts many of its effects [114]. Dimers of LL-37 can show higher cytotoxicity compared to the monomer against host cells: haemolytic activity (lysis of red blood cells) of both an antiparallel LL-37 dimer and a C-terminal dimer is higher than that of the monomer, while the N-terminal dimer has lower efficiency. The N-terminal appears to be of higher importance for the insertion into eukaryotic cell membranes than for bacterial ones [112]. Stabilization of the helical structure probably increases the activity against enveloped virus as well, since the envelopes are derived from eukaryotic cells [115].

Shifting the equilibrium between the different conformations could hence switch the properties of LL-37 from being antimicrobial to being more host cell modulating.

Oligomerization, or lack thereof, is likely why LL-37 fragments can exhibit higher antibacterial activities and at the same time less immunomodulatory effects [112]. This also, at least partly, explains why LL-37 activity is extremely dependent on medium composition.

The conformation changes may affect the mode of cell permeabilization. Both oligomeric LL-37 and the unstructured peptide interact with membranes. The unstructured peptide seems to cause permeabilization in a more unstructured, detergent-like, manner. Oligomeric LL-37, on the other hand, often binds to the surface as oligomers (after which it can disintegrate to some extent [12]). This results in more discrete, channel like, membrane pores [112, 113].

In PBS, 20-30% of the LL-37 molecules adopt a α-helical conformation, while in contact with large phosphatidylglycerol (PG) or phosphatidylcholine (PC) unilamellar vesicles, mimicking cell membranes, the α-helical conformation increases to 60 and 35%, respectively. PG is a negatively charged phospholipid promoting helicity to a larger extent than PC which doesn't carry a net charge on the surface [112, 113].

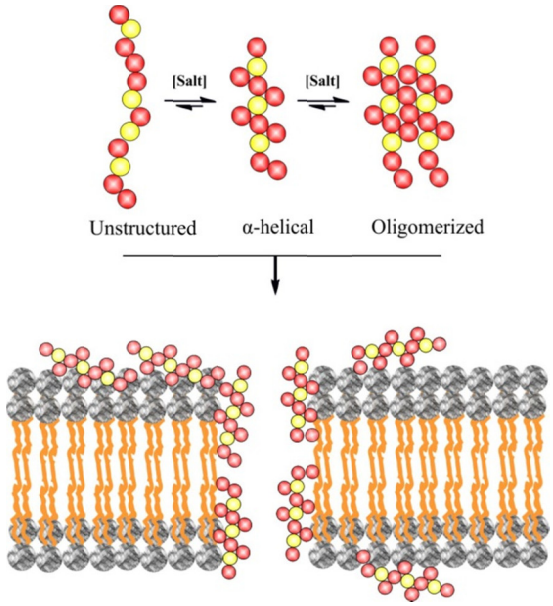

Figure 6. LL-37-induced plasma membrane permeabilization. Equilibrium exists between different structures of LL-37, high ionic concentrations drive this towards an α-helical structure which can form oligomers. All forms interact and permeabilize plasma membranes, although oligomerized peptide tends to form more narrow pores.

Effects of LL-37 on pathogens

Antibacterial effects

LL-37 was originally identified as an antibacterial peptide, and since then much focus has been on this effect of the peptide. LL-37's toxicity against bacterial cells is caused by its permeabilization of their cell walls, both that of gram-positive and gram-negative bacteria. This ability is dependent on the secondary structure of LL-37 rather than any specific receptor interactions exerted by LL-37. In line with this, replacing L-amino acids with the corresponding D-enantiomers, does not affect the antibacterial effect [112]. Due to the importance of the secondary structure, the antibacterial effect is also strongly dependent on medium components and ionic strength, and for this reason, NaCl is often added to *in vitro* experiments to mimic physiological conditions [19]. Surprisingly, LL-37 seems to lack capacity to permeabilize bacteria in tissue cultures, even at very high concentrations (around 200 μM), and, thus, the importance of direct LL-37-mediated killing of bacteria *in vivo* has been disputed [116, 117].

It is likely that LL-37's other antimicrobial properties, such as its inhibition of biofilm formation, inflammatory cell recruitment and neutralization of bacterial LPS, are of higher importance than the direct effect on bacterial cell viability [118, 119]. However, the bactericidal ability of LL-37 is still of interest, due to the peptide's activity against many antibiotic resistant bacterial strains [120].

LL-37-induced neutralization of bacterial endotoxins

One of the more well documented effects of LL-37 is its ability to reduce pro-inflammatory effects of bacterial products: LPS originating from the outer membrane of gram negative bacteria and lipoteichoic acid (LTA) from the cell wall of gram-positive bacteria [121].

The cationic LL-37 peptide, binds to the negatively charged LPS and LTA molecules with high affinity, shielding them from the surroundings. Further, LL-37 is able to disrupt the structure of LPS micelles [122, 123]. Both of these effects prevent interactions between LPS/LTA and their binding proteins, including cellular receptors such as CD14 and Toll-like receptors (most importantly TLR2 and TLR4 for LPS and TLR2 for LTA). The LPS-neutralizing effect, as opposed to the ability to bind to cells, does not seem to be directly dependent on the conformation of the peptide. Both the oligomers and the unstructured form of LL-37 bind and neutralize LPS, although they appear to bind to LPS in somewhat different manners [113].

The structure of LPS and LTA vary from different bacteria, but free LPS from both *P. aeruginosa* and *E. coli* as well as LTA from *S. aureus* bind LL-37 with very similar affinity (EC_{50} of about 100 nM). However, if LPS and LTA is immobilized, the affinity starts to vary, with EC_{50} values against LTA about 10 times higher than LPS, and higher affinity for *E. coli* LPS compared to *P. aeruginosa* LPS [124]. Higher affinity of LL-37 for bound *E. coli* LPS could be suspected to increase bacterial sensitivity to LL-37 through the increased affinity of the peptide to the surface. It could also have an opposite effect, with decreased sensitivity due to promoted binding/inactivation of LL-37 by surface bound LPS. The latter appears to be the case with surface expressed, LL-37 neutralizing, polysaccharides on eukaryotic cells [125]. Interestingly, LL-37 shows lower MIC against *E. coli* compared to *P. aeruginosa* under low salt conditions [119], supporting the first option, that LPS affinity renders bacteria exposed to LL-37, although this evidence is circumstantial.

LL-37-evoked anti-biofilm properties

Biofilms are aggregates or clusters of bacteria adhering to a surface, often embedded in an extracellular matrix composed of extracellular DNA, proteins and polysaccharides [126]. Biofilms severely reduce the bacterial susceptibility to antimicrobials, and are known to cause many types of persistent infections. It has been estimated that 80% of all microbial infections in humans involves biofilm formation [127]. LL-37 has been found to inhibit biofilm formation by several types of bacteria including *Pseudomonas aeruginosa* [128], Uropathogenic *Escherichia coli* [129], *Francisella novisida* [130] S*taphylococcus aureus* [131], *Burkholderia pseudomallei* [132] and *Aggregatibacter actinomycetemcomitans* [133].

The mechanism of LL-37's inhibitory effect on biofilms is still largely unknown, but there are reports showing that LL-37 downregulates autoinducers, which are signal molecules that allow bacteria to sense one another, as well as inhibits formation of flagella through gene suppression [128].

LL-37 inhibits biofilm formation at concentrations much lower than those required to kill the bacteria [128, 129, 133]. For instance, it reduces biofilm formation of *P. aeruginosa* at a concentration of around 100 nM while its MIC of the same bacteria is around 14 μM [128], suggesting that inhibition of biofilm formation can be a much more important function of LL-37 than direct bacterial killing.

LL-37 reduces bacterial viability through membrane interactions

LL-37 is attracted to anionic membranes due to its positive net charge. Bacterial cell wall membranes are rich in anionic groups and thus show high affinity for LL-37. The cell wall of gram-negative bacteria has LPS and gram-positive bacterial cell wall contains peptidoglycan, lipoteichoic acid, and wall teichoic acid, all of which LL-37 has high affinity for. LL-37 binds to the surface both as unstructured and as helical peptide. At the surface LL-37 will adopt, or retain, the α-helical conformation due to the surrounding anionic bilayer.

The four phenylalanine residues spread over the LL-37 sequence are important for the hydrophobic interactions with membranes. The arginine residue R23 also seems to be important for the internalization of LL-37 by interaction with the phospholipid head groups [134]. The LL-37 peptide positions in parallel to the membrane surface at the interface between the phospholipid head groups, to which it has electrostatic interactions, and the bilayer, which is made up of fatty acyl side chains that interact with hydrophobic residues of the peptide [12].

The structure of LL-37 is more rigid between the two α-helical domains partly due to the presence of S9, as compared to other primate orthologues and thus it lacks a continuous hydrophobic face against the acyl bilayer. This hinders a deep insertion into the membrane as seen with LL-37 orthologues such as rhesus monkey RL-37 [134].

How LL-37 acts on membranes has been under some debate. LL-37, just like other helical and amphipathic HDPs, has been proposed to interact with the surface according to the so called 'carpet' model. In this model the peptide coats the surface of the cell where it induces curvature on the membrane until a threshold concentration is reached. At this point LL-37 either permeabilizes the membrane in a detergent-like way, or form toroidal pores.

Most research supports a mode of permeabilization in which LL-37 forms toroidal pores on the cell surface, and not a detergent-like mechanism or the alternative 'barrel-starve' model [100, 135-137]. However, one study on DPC micelles has indicated that the permeabilization occurs in a non-pore carpet-like, detergent-like fashion [109]. Results in line with the detergent-like model have also been observed on fungal membranes, where LL-37 causes disintegration of the membrane into vesicles due to formation of large holes [138]. The different LL-37-evoked permeabilization pathways seem to be highly dependent on the experimental settings and the exact composition of the membrane lipid.

The toroidal pore formation by LL-37 is likely also favored by the oligomeric structure that LL-37 adopts in solution [111, 112]. The monomeric form would likely favor the detergent-like permeabilization [113].

Both the 'dough nut-like' toroidal pores, as well as those formed in detergent like manners, may cause loss of membrane potential, leakage of cytoplasmic components and osmotic stress, ultimately killing the bacterial cell.

LL-37 induced antiviral activity

As previously mentioned, LL-37 has virucidal activity. It is easy to see why LL-37 would be able to damage enveloped viruses as these often construct their envelope of phospholipids and other proteins from eukaryotic cell membranes. Once the virus envelope becomes disintegrated by LL-37, the virus is susceptible to antibodies for neutralization.

LL-37 also prompts autophagy of viruses, and its induction is critical for vitamin D induced virus-autophagy [139]. LL-37 enhances autophagy of viruses through a mechanism involving upregulation of autophagy-related proteins, namely Beclin-1 and Atg5. During autophagy, pathogens and viral antigens can be delivered to autolysosomes where they are degraded. However, some virus have developed the

capacity to instead use the autophagy process to promote their own replication, complicating the understanding of LL-37's effect on viral infection [140].

The LL-37 peptide inhibits replication of the HIV virus and during HIV infection the LL-37 expression is upregulated [141, 142]. Another virus known to be sensitive to LL-37's membrane disruption includes the skin-rash causing vaccinia virus, which can be very serious for patients suffering from atopic dermatitis [143, 144].

Influenza A is another common virus that is sensitive to LL-37's envelope disruptive effect, but it does not affect binding or initial uptake of the virus into host cells [145]. This can be corroborated by experiments in influenza A infected mice where LL-37-treatment effectively reduces viral replication and the production of pro-inflammatory cytokines in the lungs [146].

Infection with the mosquito-borne dengue virus has recently been found to cause an upregulation of hCAP18/LL-37, but the role of the peptide in this disease is yet unknown [147].

LL-37 also appears to be protective against respiratory syncytial virus (RSV), the most common viral pathogen affecting the airways. It inhibits spread of the infection, protects epithelial cells against virus induced cytotoxicity and inhibits virus replication. RSV infection upregulates serum hCAP18 expression and further hCAP18 levels positively correlates to the patient's recovery [148-150].

LL-37 has been shown to synergistically promote viral pro-inflammatory cascades through binding viral dsRNA and thereby enabling them to activate TLR3 in several types of human cells. This pro-inflammatory effect is proposed to allow the immune system to detect viral dsRNA which otherwise doesn't show high affinity for the pattern recognition receptors [151]. However, LL-37 may also have detrimental effects in some autoimmune diseases by promoting inflammation via dsRNA [152].

Interestingly, LL-37 may also facilitate viral infections through upregulation of virus receptors on host cells. As mentioned earlier in this section, LL-37 inhibits HIV replication, but endogenously expressed LL-37 have also been shown to strongly upregulate HIV receptors on Langerhans cells, a dendritic cell implicated as an initial target in HIV infection. In the same study, LL-37 was shown to promote cytokine production in rhinovirus-infected cells [153].

Almost all documented effects of LL-37 on viruses, including the ones mentioned above, are on enveloped viruses. One exception however is the virus Ad5, which is non-enveloped but still shows sensitivity to LL-37 (at >2 μM LL-37) via an yet unknown mechanism [150].

LL-37 constitutes an important immune defence factor against several common viral pathogens, and it could be part of the reason why vitamin D deficiency has been associated with increased severity and susceptibility of several viral infections including HIV, [154, 155] influenza [156, 157], rotavirus [140], and RSV [149].

LL-37 possesses antifungal activity

LL-37 also exhibits antifungal properties, including activity against *Candida albicans*, which is the main fungal pathogen in humans [158]. LL-37 kills *C. albicans* at micromolar concentrations (EC_{50} 0.8-8 μM) which is likely through membrane disruption, possibly via a detergent-like mechanism [138, 158]. Besides killing fungi, LL-37 also attenuates their ability to adhere to host cells, which is the first step of fungal infections. In *C. albicans,* LL-37 effectively inhibited fungi adhesion to a variety of cell surfaces at sub-toxic concentrations. It does so by binding to cell-wall carbohydrates including mannans, glucans, and chitin, but also glucanases (specifically the β-1,3-exoglucanase Xog1p in *C. albicans*). Glucanases appear to play a yet unknown role in fungal host recognition [158-160].

LL-37-induced activity against protozoan parasites

Protozoa are single cell eukaryotic organisms, some of which are parasites responsible for serious diseases such as leishmaniasis and malaria. The outer leaflet of the protozoan membrane is composed of anionic phospholipids, in some cases sensitizing them to membrane damage induced by HDPs. Only a few studies have been presented on LL-37 activity against protozoa parasites, showing that cathelicidins have activity against leishmania [161].

Blastocystis are another common genus of protozoan parasites, in fact they are considered to be the most commonly detected eukaryotic parasites [162]. Their presence has been implicated in a number of intestinal diseases. LL-37 is the only known HDP with a broad anti-blastocystis activity, causing membrane disruption and killing [162].

Pathogen clearance by neutrophil extracellular traps (NETs)

Another mechanism by which LL-37 clears bacteria as well as virus and other parasites, is by inducing formation of NETs. NETs can be produced by neutrophils [163] and mast cells [164] and is a fibrous extracellular matrix of self-DNA intertwined with cellular proteins and antimicrobial granules. The granules contain antibacterial agents such as citrullinated histones and LL-37 along with other HDPs [165, 166]. Many parasites have affinity for DNA, or alternatively certain proteins in the structure, such as calprotectin, resulting in pathogen scavenging. Once the pathogen is trapped they are effectively killed [167]. LL-37 induces the NET formation by permeabilization of the host cell plasma membrane and the nucleus thereby causing liberation of nuclear DNA (nDNA) which constitutes the backbone of the expelled NETs. Alternatively, mitochondrial DNA (mtDNA) can be utilized as the substrate [168], likely LL-37 causes release of mtDNA via mitochondrial pore formation. Reactive oxygen species (ROS) produced by the neutrophils are important for NET-induction. As LL-37 promotes ROS formation in neutrophils this could be another mechanism behind NET-induction by LL-37 [169, 170]. NETs appear to be an important part of the innate immune system, in particular as it targets microbes that are too large for phagocytosis [170]. The NET formation is counteracted by IL-10 and other inflammation mediators as well as by microbial nucleases which degrades DNA. Besides stimulating the production of NETs, LL-37 also protects the DNA from these nucleases via its binding to the DNA strands, rendering them unrecognizable to enzymes [165].

Prospects of LL-37 as an antibiotic drug

Due to the challenge of microbial resistance to conventional small molecule antibiotics, there is a growing interest in HDPs including LL-37. An important reason for this is that HDPs are less likely to lead to a general resistance compared to many conventional antibiotic drugs. This is supported by the fact that bacteria have evolved alongside these antimicrobial agents for millions of years without being able to develop a full protection against them [171]. Sensitivity towards LL-37, however, varies greatly for different microbes: amongst bacteria, a number of strains appear to have very low susceptibility to the bactericidal effects of LL-37 and further, certain bacteria have the ability to degrade LL-37 through secretion of proteolytic enzymes such as elastase and metalloproteases [172, 173]. Therefore, LL-37 can be expected to have very varied efficiency depending on the nature of the infection.

Upregulation of LL-37 through various pharmacological agents or sun light, factors commonly referred to as LL-37 elicitors, is currently of high interest as possible ways to combat infections [174]. For LL-37, sun exposure and vitamin D

supplements are likely the most commonly used elicitors, but as discussed earlier, supplementation and UVB-induced synthesis of vitamin D appears to only stimulate LL-37 expression in people with low vitamin D serum levels. Other compounds upregulating this peptide are also of interest in medicine, and some of these LL-37 elicitors show promising results against bacterial infections [175, 176]. LL-37 elicitors, as opposed to synthesized LL-37, are often affordable drugs, but they also carry properties independent of the peptide which may be either beneficial or detrimental. Further, targeting the treatment to infected areas may be difficult with elicitors. These are arguments for using LL-37, or derivatives thereof, as drugs.

Topical use of high LL-37 concentrations have been explored in a clinical trial on humans, with results indicating that relatively high concentrations of LL-37 can be applied locally without negative side-effects such as necrosis and severe inflammation which are only seen at very high doses of LL-37 [177]. Systemic administration is also a possibility but there are some complications to consider.

One is that LL-37 impairs not only microbial but also host cell viability, and furthermore it has potent pro-inflammatory properties, suspected to be a key in the initiation of several autoimmune diseases and may even promote cancer growth (this will be discussed later in the thesis). These properties may be modulated by modification of the peptide structure. The low selectivity against bacterial membranes compared to those of host cells can be increased by replacing some amino acids in LL-37 with their D-enantiomers or by replacing certain amino acids with others [104, 178]. Alternatively, fragments of LL-37 with less oligomeric properties can be used. Such derivatives show higher toxicity to bacteria compared to LL-37 with less host cell cytotoxicity and pro-inflammatory effects [95, 98, 100]. Most of LL-37 orthologues also have less pro-inflammatory effects and host cell cytotoxicity while displaying higher bactericidal activity. Both sheep and cow cathelicidin peptides, and derivatives thereof, show much better features as antibacterial drugs compared to LL-37 [179].

Another major issue in utilizing LL-37 as an antibiotic drug is its extremely short half-life. To increase stability of the peptide, as well as offering a successive release, nano-particles loaded with LL-37 have been used successfully in pre-clinical drugs against both cancer and to promote wound healing [180-183]. Another approach is to produce more stable analogues of LL-37, although this does not automatically solve the issue that free LL-37 rapidly binds up to its surrounding and gets inactivated due to its physical properties.

In cancer drug discovery, there is currently an interest in combining LL-37 with anti-cancer drugs. The peptides membrane disruptive effects sensitize even resistant cells to traditional drugs [181, 184]. A similar approach could be considered also in the treatment against bacterial infection, where properties such as anti-biofilm and bacterial membrane disruption can be of great value in sensitizing resistant bacteria to classical antibacterial agents.

Despite risks, the potential of LL-37 based drugs for treatment of infectious diseases is huge. The biggest deterrent to that could be the currently high production costs which could narrow down any usage to certain infections, i.e. antibiotic resistant infections, possibly as a co-treatment with other drugs.

Effects of LL-37 on host cells

LL-37 is considered a factotum peptide due to its plethora of different effects in host cells. It can recruit host immune cells including neutrophils [185], monocytes [186], eosinophils [187] T-cells and mast cells [188] through its chemotactic properties. Furthermore, LL-37 can activate these cells and stimulate their production of pro-inflammatory cytokines [116, 189, 190]. It may also initiate degranulation of mast cells [191], promote leukocyte extravasation, and promote angiogenesis [192]. LL-37 also enhances wound healing, probably via stimulation of cell migration and proliferation [31]. LL-37 is also cytotoxic to a range of host cells and acts in a pro-apoptotic manner. Properties as these are the reason LL-37 is considered an alarmin, a molecule that signals tissue and cell damage (alarmin is the endogenous equivalent of pathogen-associated molecular patterns, PAMPs) [33].

LL-37 can exhibit diverse effects in the host cells by activation of multiple receptors. LL-37 may interact with membrane receptors through different principles, e.g. both via direct receptor-activation following ligation to the binding sites, and through its membrane modulating effects. In the latter case, LL-37 can activate receptors by accumulating around them, interacting with their transmembrane regions or by incorporating the receptor in membrane lipid rafts [193]. LL-37 can also cause transactivation of receptors by releasing membrane-anchored factors which in turn liberates receptor ligands. In fact, LL-37 appears to not bind directly to several of its most well-known receptors, including P2RX7, the epidermal growth factor receptor (EGFR) and N-formyl peptide receptor 2 (FPR2), but instead activate them via other mechanisms not yet fully elucidated [12].

Several of LL-37's effects on host cells, whether they result in proliferation, inflammation or cytotoxicity, are in many cases highly intertwined. For instance, permeabilization and subsequent Ca^{2+} influx may trigger a range of different cellular events, and likewise activation of a membrane receptor can result in highly different signaling cascades depending on cell system. LL-37-induced pro-inflammatory effects may result in various detrimental cellular effects including lowered cell viability and apoptosis. While the effects of LL-37 on the host are intertwined, the most important ones will be discussed separately in this section.

LL-37 promotes wound healing

Increased expression of LL-37 is observed by keratinocytes and white blood cells at wound sites of the skin within hours after injury [194]. Importantly, low expression of LL-37 can be seen in difficult-to-heal ulcers and, interestingly, antibodies against LL-37 inhibit healing [195, 196]. Animal studies showed that topical application of LL-37 (50 µl of 45 µM LL-37 solution, two times a day on 0.2 cm^2 of wound) effectively promoted wound closure in mice [31]. Similar results were also seen in pigs [197]. Today there is a large consensus that HDPs, i.e. LL-37 and some β-defensins, play a crucial role in wound healing [198-200].

The mechanism behind LL-37-induced wound closure and re-epithelialization is not fully understood, but seems to involve LL-37 induced endothelial angiogenesis in the extracellular matrix as well as stimulation of epithelial cell proliferation and migration.

The pathway by which LL-37 promotes angiogenesis was first reported to act through activation of endothelial FPR2 receptors, consecutively stimulating endothelial tube formation/angiogenesis [201]. However, a later study on the same type of endothelial cells showed that the angiogenic properties did not involve FPR2 but rather a cytochrome C oxidase 1 dependent signaling cascade. This cascade is initiated through LL-37-induced Ca^{2+} influx and ERK activation, resulting in cPLA2 enzyme activity. Prostaglandin E2 is subsequently upregulated and activates the EP3 receptor, in turn signaling for angiogenesis [202].

LL-37 also upregulates the vascular endothelial growth factor, VEGFA, in keratinocytes and thereby promotes cell growth and migration. Further it suppresses keratinocyte and dermal fibroblast apoptosis through upregulation of anti-apoptotic proteins, i.e. cIAP2 and COX-2. At least in dermal fibroblasts, this effect is mediated through ERK activation [22, 203, 204].

Besides LL-37-evoked stimulation of skin cells and angiogenesis, LL-37 induced stimulation of immune cell chemotaxis and its anti-biofilm properties, maintaining

the epithelial barrier integrity against infections, may presumably also aid in the healing process of the skin.

LL-37 can promote wound healing in epithelial tissues other than skin. This includes airway epithelia, in which LL-37 exhibits similar pro-proliferative and pro-migratory effects on the epithelial cells as in skin keratinocytes. The effect is mediated by activation of EGFR and an unknown G protein–coupled receptor (GPCR) [205].

Corneal epithelium is another tissue where LL-37 is supposed to promote wound healing through EGFR [206]. Ocular complication from diabetes is today a common cause of blindness in the world, and this is caused by epithelial defects. One factor behind this is that high glucose levels inhibits EGFR and hence reduce healing of the corneal epithelium. Treatment with LL-37 restores high glucose impaired EGFR signaling in cornea and has thus been proposed as a possible remedy for ocular defects in diabetic individuals [206].

The first clinical trials with LL-37 on human wounds took place in Sweden 2012/2013 [177]. Patients with hard to heal leg ulcers were treated with a solution (25 μl/cm^2 of wound) twice weekly for four weeks, containing either vehicle or LL-37 at concentrations of 110, 350 and 710 μM. Both 350 and 710 μM are considerably higher doses than those used in the previously mentioned animal studies, and this could be a reason for the adverse effects reported here. These adverse effects, occurring after 710 μM treatments, include ulcer necrosis, edema and severe inflammation. Most patients in the study also experienced itching, with serious cases observed in patients receiving the two higher LL-37 doses. The results of the study showed that the lowest dose was the most effective in promoting wound closure: at the end of the four week study the placebo group's wound areas had healed to an average of 27%, while 110 μM treatments healed wounds to 68% closure. The 350 and 710 μM treatments resulted in healing of 50 and 19%, respectively, in other words high LL-37 levels attenuate the healing process. In conclusion, “low” doses of topical LL-37 appears to be safe and shows significant wound healing effects in ulcers, while the beneficial effect is lost at higher LL-37 concentrations where detrimental side effects may occur [177].

The wound healing properties of LL-37 have been improved by encapsulating it in different types of nanoparticles with other wound healing drugs. This offers slower release, as well as synergistic effects between the compounds. Both lactate and the elastase inhibitor Serpin A1 have wound healing properties, and both act synergistically with LL-37 when co-encapsulated [180, 183]. Other researchers have instead taken the approach of designing novel peptides which holds wound healing and antibacterial properties while showing lower host cell cytotoxicity compared to LL-37 [197, 207].

LL-37 may promote tissue fibrosis

Accumulating fibroblasts, often producing excessive amounts of extracellular matrix proteins (mainly collagen I), is detrimental in a range of diseases including several inflammatory conditions. In this process, called fibrosis, the fibrotic or "scarred" tissue replaces functional organ tissue, resulting in organ failure [208]. LL-37 has been suggested to stimulate fibrosis in pathological conditions, but the implications and underlying mechanisms remain unknown.

Systemic scleroderma (SSc) is a disease resulting in fibrosis of skin, as well as other organs such as kidney, heart and lungs. LL-37 levels are elevated in skin and serum of patients suffering from the disease, and furthermore the high LL-37 levels positively correlate to the fibrotic processes of lungs and gastrointestinal tract as well as skin thickness [22, 77]. The first study on the topic proposed that the anti-apoptotic effect of LL-37 on dermal fibroblasts (via ERK/COX-2) was the mechanism responsible for the accumulation of cells and subsequent fibrosis in SSc [22]. A later study instead suggested that LL-37 may promote the fibrosis through acting on the epithelium rather than direct actions on the fibroblasts [77].

LL-37 has also been proposed to promote fibrosis in the bladder, following urinary bladder inflammation in patients with spina bifida where urinary LL-37 levels may be extremely high. LL-37 was here suggested to promote the fibrosis through its chemotactic abilities, causing tissue infiltration of mast cells. Mast cell degranulation and enzyme activity are known fibrotic factors. The infiltration of mast cells occurred in a dose dependent way (10-320 μM) with relatively high concentrations of LL-37 needed for pronounced cell infiltration. Interestingly, LL-37 was also found to cause urothelial wounding and edema in the process [209]. The LL-37-induced effects on the urothelium may support the hypothesis that LL-37 induces fibrosis through acting on the epithelial layer. Commonly, fibrosis is induced by uncontrolled healing after tissue damage caused by a chronic inflammation releasing detrimental substances [208]. It's possible that elevated LL-37 levels may mediate tissue damage which in turn drives the fibrotic process.

LL-37 is also known to cause mast cell degranulation [191], and may thus contribute to fibrosis through this mechanism as well. Further studies are required to explain the correlation between LL-37 and fibrosis and its pathophysiological implications.

LL-37 induced membrane permeabilization of host cells

Cell permeabilization by LL-37 has been discussed previously in the aspect of bacterial cells. Eukaryotic cell membranes have a different composition than bacterial ones. The outer leaflet is composed mainly of zwitterionic phospholipids, sphingomyelin and PC, to which LL-37 has less or no electrostatic attraction. Further, cholesterol content of eukaryotic cells has been proposed to hindering LL-37 induced membrane permeabilization [210].

Despite these structural differences, LL-37 does not show higher selectivity for bacterial cell membranes over those of host cells when it comes to permeabilization. One reason for this has been proposed to be due to oligomerization of LL-37, but it is not clear how oligomerization promotes certain host cell interactions [12, 111, 114, 115].

LL-37 has been found to insert itself to a greater degree in bilayers of negatively charged phospholipids compared to those with neutral phospholipids and monolayers [111, 211, 212]. Besides electrostatic affinity for surface groups, the hydrophobic interactions with LL-37 is just as important and may facilitate its binding to the hydrophobic core of the bilayer, ultimately resulting in permeabilization [137].

We have reported that the pores formed in response to stimulation with low μM concentrations of LL-37 readily leak lactate dehydrogenase (LDH). Conversely, the permeabilization causes a rapid influx of Ca^{2+} into the cytoplasm from the extracellular space [213, 214]. Surprisingly this permeabilization occurs in all investigated eukaryotic cell types in similar dose interval, while the permeabilization does not completely correlate to the cytotoxicity. Certain cell types remain unaffected in terms of survival after permeabilization with relatively high concentrations of LL-37 (Results, Fig. 14, Fig. 27). Furthermore, human cells may close up the pores again in a matter of minutes after LL-37 exposure, suggesting that the cell membrane permeabilization *per se* may not be crucial for the cytotoxic effects of the peptide (Results, Fig. 15).

The increase in intracellular Ca^{2+} concentration following permeabilization may have important down-stream effects for the cells. In fact, LL-37-induced Ca^{2+} mobilization is involved in several of the peptides host cell modulating properties [71, 215].

LL-37-evoked cellular permeabilization probably results in release of many toxic and pro-inflammatory cytoplasmic components which may negatively affect the surrounding cells. This is illustrated by the fact that cellular LDH leaks following permeabilization, this is a protein larger than most cytoplasmic proteins (MW of unmodified LDH = 144 kDa [216]). These substances include proteolytic enzymes and various inflammation mediators. One case of deleterious leakage induced by

LL-37 has been observed in apoptotic leucocytes. Apoptotic cells, however, show a very different cell surface composition compared to non-apoptotic cells, and this may result in a more necrotic lysis by LL-37 which would not be representative of the toroidal pore formation observed elsewhere [217, 218].

With further permeabilization of intracellular organelles, LL-37 may cause release of self-DNA, which is found in several autoimmune diseases where LL-37 levels are high and promote inflammation. In line with this, this peptide's ability to induce NET formation, i.e. DNA release from leukocytes, appears to be highly dependent on the degree of LL-37-induced permeabilization [12]. However, taken together, the consequences of LL-37 induced permeabilization of host cells are poorly understood.

Internalization of LL-37 in host cells

LL-37 is known to internalize in cells, and the pore forming capacity of LL-37 suggests that the peptide can simply diffuse through the pores. However, a majority of the cellular uptake of LL-37 appears to be due to endocytosis. Tang *et al.* found that uptake of LL-37 into macrophages/monocytes is mainly through clathrin- and caveolae-lipid raft-mediated endocytosis, requiring activation of the P2RX7. After uptake, the clathrin/caveolin containing bodies remains around LL-37, and translocate the peptide to endosomal and lysosomal compartments, as well as the Golgi apparatus [215].

LL-37 uptake into peripheral blood mononuclear cells (lymphocytes and monocytes) is reported to be independent of P2RX7, but dependent on actin polymerization [219]. Studies on epithelial cells on the other hand suggest that FPR2 may be involved in cellular LL-37 uptake [220, 221]. The peptide, at least when complexed to DNA, has also been found to internalize via the CD32 receptor through a synergistic effect with other HDPs [222].

A recent study confirms that uptake of LL-37 and LL-37:DNA complexes in macrophages is at least partly mediated via P2RX7 and clathrin-dependent endocytosis. Following the uptake, LL-37 was found to accumulate in endosomes and phagolysosomes (the latter in the case of bacteria-infected cells) [223].

Sing *et al.* observed endosomal translocation in epithelial cells. Moreover, they found that LL-37 is degraded by cathepsin proteolysis in the endosomal compartments after endosome acidification. The half-life of LL-37, around 1 h the cell cultures, was prolonged to over 12 h by preventing endosome acidification with ammonium chloride treatment and through cathepsin inhibition [221].

In conclusion, these studies suggest that the main internalization of LL-37 occurs in an endocytic fashion. The mechanisms involved in LL-37 internalization appear

to be many, and are probably highly cell type specific. After endocytosis the peptide is co-localized to lipid rafts, and translocated to endosomes and lysosomes, the latter in which it gets degraded. Translocation to other organelles, such as the Golgi apparatus, may also occur. Intracellularly, endocytosed LL-37 exerts bioactivity, including antibacterial effects in infected cells [215], but exactly which of LL-37's cellular effects the endocytosed peptide is responsible for is not known. The association to cellular trafficking structures following endocytosis would limit LL-37-reactivity. There may very well be a smaller pool of pore-internalized peptide which has large implications on cellular function.

Pro-inflammatory properties of LL-37

Much of LL-37's pro-inflammatory effects are a result of it being able to activate a large number of different transcription factors, and consequently a range of signaling cascades. Many of these cascades result in promoted inflammation, largely by increased production and secretion of pro-inflammatory cytokines. The following section is focused on immunomodulatory effects such as these.

LL-37-evoked inflammation signaling by membrane receptor activation

While LL-37 receptor activation has very diverse effects, most receptor activations by LL-37 arguably results in the initiation of inflammatory signaling cascades through mitogen-activated protein kinases (MAPKs), e.g. ERK and p38, followed by activation of transcription factors and subsequent cytokine production.

The membrane disruptive property of LL-37 allows for diverse types of surface receptor interactions. It may thus activate MAPK through a number of different receptors- for example, in macrophages, the effect appears to be mediated by the P2RX7 [71] while In HepG2 hepatocellular carcinoma cells it triggers MAPK activation by FPR2 [224]. THP-1 monocytes and primary human monocytes/lymphocytes, on the other hand, display a surprising mode of MAPK activation, in which LL-37 is first internalized in the cells and then binds to GAPDH. GAPDH in turn dissociates from p38 activators to which it is normally bound, and these in turn initiate the MAPK signaling [219]. In MCF-7 breast cancer cells, LL-37 activates MAPK by binding to IGF-1R [225] while in other breast cancer cells, activation is triggered by the epidermal growth factor ErbB2 [226]. Similarly, induction in lung cancer cell lines may be initiated by stimulation of another epidermal growth factor receptor, EGFR (a.k.a. ErbB1) [227]. EGFR was found to be responsible for the activation in airway epithelial cells as well. In the case of EGFRs, the receptor activation occurs after an LL-37 induced liberation of membrane anchored MMPs. The MMPs cleave and release membrane-anchored EGFR-ligands, which in turn activate the receptor [189, 228].

Downstream of the cell-surface receptor activation, MAPK signaling is activated through phosphorylation. LL-37 is able to activate most of the MAPKs, specifically p38, ERK1/2 and JNK, in a cell type specific manner. MAPK are signal transduction pathways that regulate several processes including inflammation, differentiation, proliferation and cell death [229]. Not only can LL-37 activate MAPKs, but it also has the ability to inhibit MAPK induction/cytokine release induced by other pro-inflammatory mediators, i.e. LPS, LTA and the serum amyloid A protein family [230]. It has also been reported to inhibit ERK phosphorylation/activation in adipocytes and hepatocytes [94].

The LL-37-evoked MAPK signaling results in activation of transcription factors through a variety of mechanisms. One of these is the transcription factor NF-κB, regulating the expression of some of the most important pro-inflammatory cytokines in humans, and is therefore the principal target for many anti-inflammatory drugs. LL-37-induced MAPK-signaling effectively cause phosphorylation of the NF-κB inhibitor IκBα and NF-κB p105, resulting in degradation and subsequent activation of the NF-κB p65 and p50 transcription factor subunits [231]. Besides activating NF-κB, LL-37 also stimulates pro-inflammatory cytokine production via the AP-1 [232, 233] and Elk-1 transcription factors [234]. The peptide further activates a range of other transcription factors with other roles than immunological, contributing to the diverse effects that can be observed by LL-37. This includes the transcription factors AP-2 (critical for early development of the organism), EGR1 (important for cell differentiation and mitogenesis), CREB1 (regulates proteins involved in hormone response) and HIF1α (involved in e.g. proliferation) [2, 235]. In fact, MAPK activation mediates pro-proliferative and pro-migratory effects of LL-37. In particular EGFR and IGF-1R receptor activation of LL-37 may result in such MAPK driven effects, which are even suspected to promote tumorigenic activities of some cell types [227, 236, 237]. See Fig. 7 for a schematic illustration of receptor activations by LL-37.

Besides MAPK, PI3K is another family of signal transducers known to mediate cellular effects of LL-37 [233]. In epithelial cells, LL-37/EGFR induce PI3K signaling resulting in cytokine expression and cell-migration [238].

Furthermore, activation of receptors by LL-37 may not only enhance cytokine expression through MAPK/PI3K signaling, other mechanisms have also been reported. For instance, FPR2 stimulation may promote expression of specific cytokines such as CXCL10, as well as MMPs, exclusively on the protein level [239]. FPR2 is also highly involved in the chemoattractant properties of LL-37 in a variety of white blood cells [186, 187]. Besides FPR2, three other GPCRs are activated by and implicated in LL-37's pro-inflammatory signaling: MrgX2, EP3 and CXCR2. MrgX2 activation may cause cytokine (CCL4) production in mast cells, as well as chemotaxis and mast cell degranulation [191]. EP3, indirectly

activated by LL-37, results in production of pro-inflammatory LTB4 [202, 240]. Through CXCR2, known as the IL-8 receptor, LL-37 may by itself act as a cytokine, resulting in Ca^{2+} mobilization and chemotaxis [241].

Besides receptor activation of LL-37 itself, LL-37 modulates inflammatory cell signaling in other ways. Most importantly this involves TLRs, which LL-37 may modulate both in regards of expression and activity. These effects will be discussed in the following sections. Further details of LL-37's pro-inflammatory effects can be found in the last section of the Background where pathophysiological involvement of LL-37 is discussed for a number of medical conditions.

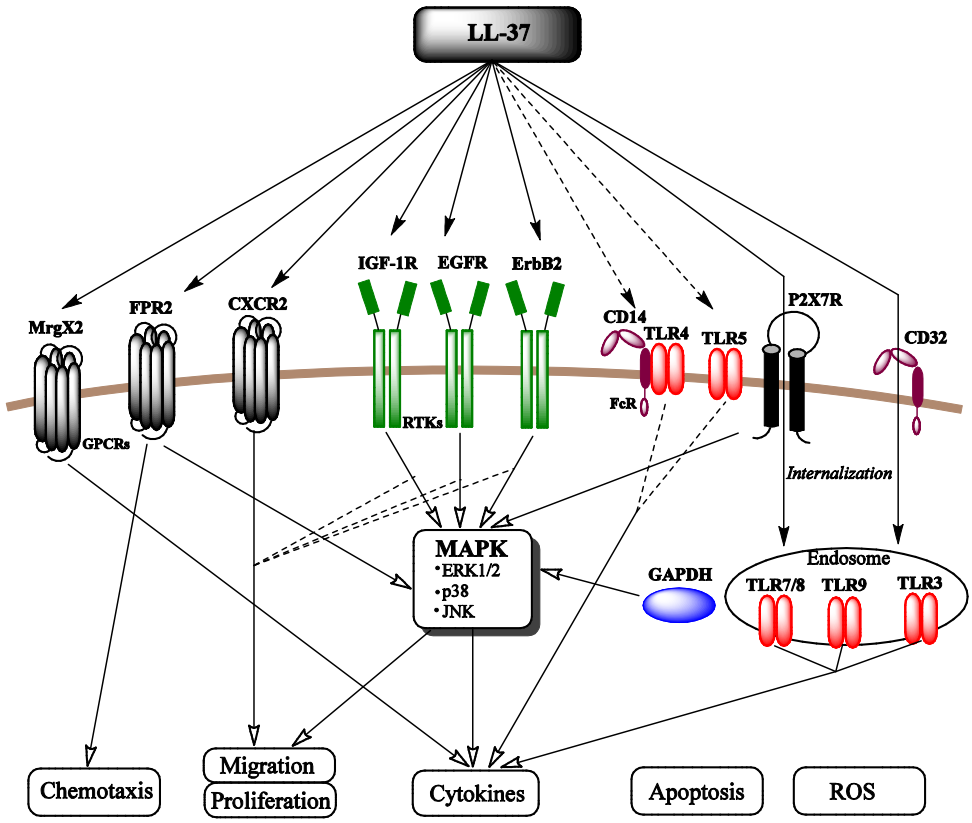

Figure 7. LL-37-induced receptor activation. Schematic figure over receptors involved in LL-37 signaling and some possible downstream events.

LL-37 may induce inflammation by interacting with nucleic acids

LL-37 can induce inflammatory signaling cascades via its ability to bind self-DNA, which can be released from dying cells and NETs, two cellular events which in fact may be caused by the peptide itself. Both forms of self-DNA can be either mtDNA or nDNA.

Self-DNA binds to α-helical LL-37, forming a stable complex as a result of reduced intramolecular charge interactions in the DNA helix. Hereby, LL-37 protects the DNA from enzymatic degradation [242]. LL-37 also allows for the DNA to be internalized by certain cells, e.g. B-cells, monocytes and plasmacytoid dendritic cells (pDCs). Intracellularly, the self-DNA, not least mtDNA, acts as powerful autoantigens [243]. The self-DNA, possibly after dissociation from LL-37, activates TLR9 with a subsequent inflammatory signaling cascade. One down-stream consequence of this is activation of interferon regulatory transcription factors (IRFs) resulting in production of the cytokine family type I interferons (IFNs), including IFN-α, -β and -γ [222, 242, 243]. IFNs, which are the cytokines particularly implicated in LL-37 driven pathogenesis, acts as potent recruiters of several immune cell types, infiltrating the area. IFNs further activate/mature said immune cells, promoting cytokine production, inflammation and even cytotoxicity [243, 244].

P2RX7 and CD32 have been implicated in mediating the cellular uptake of the LL-37:DNA complex. These receptors might allow for uptake of free LL-37 as well, but not of self-DNA on its own. The process involves endocytosis by both clathrin and caveolae/lipid rafts [117, 215, 223]. The internalization of LL-37:DNA of pDCs via CD32 is a process which may require the presence of the human neutrophil peptide group (HNPs). These are α-defensins which appear to synergize the LL-37-mediated DNA internalization [222].

LL-37's NET-inducing properties also result in other pro-inflammatory reactions as NETs releases a variety of pro-inflammatory and tissue destructive components. This release peaks when the neutrophils dies (a process called NETosis). The released factors include LL-37 in itself, which is found at high concentrations surrounding the dead and dying cells. Another abundantly released component is citrullinated histones, causing inflammation and potentially even tissue destruction following NETosis [245].

While NETs can be useful and beneficial against a range of different pathogens, the detrimental effect of NETs outweighs the positive ones in several diseases where the NETs are abundantly expressed. In several chronic inflammatory conditions LL-37 expression is elevated, and, moreover, NETs are implicated as a key factor in the pathogenesis. These diseases include psoriasis, atherosclerosis, rheumatoid arthritis and systemic lupus erythematosus (SLE) [222, 243, 246, 247].

By forming a complex with double stranded (ds) self-RNA, LL-37 is also able to activate TLR7/8 in pDCs resulting in cytokine (e.g. IFN-α) production [248]. In keratinocytes, LL-37:dsRNA may also cause cell activation and production of IFN-β, but not through TLR activation. Instead the complex activates MAVS (mitochondrial antiviral-signaling protein). This causes a downstream phosphorylation of Akt and a dephosphorylation of a TBK1 splice variant functioning as an IRF3 inhibitor, resulting in activation of IRF3 transcription and subsequent keratinocyte IFN-β production [152].

LL-37-induced immunomodulation via regulation of receptor expression

LL-37 promotes LPS induced pro-inflammatory cascades by upregulating TLR4 expression in macrophages [117] mast cells [249] and synergistically with LPS in epithelial cells [250]. It has also been shown to upregulate the LPS co-receptor CD14 in macrophages [117]. Additionally, LL-37 enhances TLR5 signaling in synergy with flagellin [251] and sensitizes TLR3 to viral dsRNA [151].

Moreover, the LL-37 peptide is also able to stimulate expression of certain Fc receptors such as CD32, CD64 and CD68. These membrane receptors recognize proteins of infected cells and invading pathogens and are involved in LL-37-induced macrophage mediated phagocytosis of bacteria [117].

The LL-37-evoked upregulation of these receptors may be of great importance since triggering of the receptors activates signaling cascades including activation of NF-κB/AP-1 transcription, and results not only in the production of pro-inflammatory cytokines [232, 233] but also other inflammatory mediators such as α-defensins [169].

LL-37 acts pro-inflammatory through ROS generation

LL-37 induces Ca^{2+} mobilization and NADPH oxidase activation, and although the exact mechanism is unknown, this results in increased ROS generation by neutrophils [169] and macrophages [252]. LL-37-induced ROS may play a role in the innate immune defence system where they regulate various signaling cascades in neutrophils and kill pathogens by means of oxidation [253]. ROS are also able to induce inflammation and tissue damage in diseases such as rosacea [254].

Cytotoxic effects of LL-37 on host cells

LL-37 exhibits a range of effects of host cells which may result in cytotoxicity and cell death. These effects include pro-inflammatory signaling, permeabilization and ROS generation.

In healthy individuals, where HDP levels often are relatively low, LL-37 cytotoxicity is likely not an issue. For reference, normal concentrations of LL-37/hCAP18 in different bodily fluids are reported to be 0-6 μM in saliva, 0.2-0.5 μM in plasma and 0.02-0.7 μM in cerebrospinal fluid [86, 255].

On the other hand, in several pathophysiological conditions, the concentration of LL-37 can radically increase to very high (mM) levels [256, 257]. However, it should be emphasized that few studies distinguish between hCAP18 and LL-37 when the authors calculate LL-37 concentration.

LL-37-evoked cytotoxicity can be buffered at least partly, by different endogenous systems. In saliva, much of LL-37 seems to be bound-up and inactivated by glycosylated proteins such as mucins. In serum, on the other hand, lipoproteins may fill the same function [258-260].

LL-37-induced cytotoxicity through membrane disruption and internalization

In red blood cells, the membrane composition is unusually rich in negative charges due to an abundance of sialic acid residues. This promotes electrostatic LL-37 interactions, which has been suggested to sensitize the cells to LL-37-induced membrane disruption, resulting in hemolysis [261].

As briefly mentioned in the paragraph regarding membrane permeabilization of host cells, LL-37 may selectively attack apoptotic cells (annexin V-positive, propidium iodide (PI)-negative), while leaving others. This has been seen in apoptotic neutrophils and NK-cells, which are put into a necrotic stage following LL-37-induced membrane permeabilization [217, 218]. This selective effect of LL-37 on apoptotic cells is probably due to altered membrane properties in apoptotic cells. One example of this is that the negatively charged end of the phospholipids, which is kept inwards in non-apoptotic cells by an energy consuming process, becomes mobile. This results in a negatively charged surface which facilitates electrostatic interactions with LL-37. The necrotic cells are quickly cleared by lysis, and hence an increased proportion of non-apoptotic cells may be detected after LL-37 treatment, something that may have been interpreted as an anti-apoptotic effect [217, 218]. The explanation of membrane composition for the selective effect on apoptotic cells may however not be the whole picture. For instance, Barlow *et al.* found that activation of PI3K was a critical factor for the selective effects by LL-37 on neutrophils [262].

Results from us strongly suggest that internalization of LL-37 is critical for its cytotoxic effects in human cells. These cells show modulated sensitivity against LL-37 depending on the expression of the intracellular LL-37 scavenger p33. Despite the differences in LL-37 induced cytotoxicity between different human cell types, all of the cell types that we have assessed show similar sensitivity to LL-37 induced permeabilization (measured as LDH release), suggesting that

membrane composition is not a critical factor in LL-37 induced host cell cytotoxicity [214].

A comparison between toxicity of LL-37 on bacteria and host cells by Li *et al.* [104] may support our hypothesis that resistance to permeabilization is not critical for LL-37 induced cytotoxic effects. Li and co-workers switched certain amino acids from L- to D-configuration in LL-37 fragments, not resulting in any effect on toxicity against bacteria, indicating that the disruptive effect of the cell membrane is a key step in LL-37-induced bacterial toxicity. While toxicity was retained against bacteria, switching L-amino acids for their D-enantiomers resulted in loss of LL-37-evoked cytotoxicity in both primary and immortalized host cells. The increased selectivity towards bacteria by a modified stereochemistry is suggested to be because D-residues disrupt the canonical helical structure of the peptide, resulting in less hydrophobicity, which may be more critical for interactions with host cells than with bacteria [104, 263]. An alternate explanation, in line with our results, is that that a stereospecific cell receptor activation, or even stereo-selective binding to cellular LL-37 scavenging proteins, is critical for host cell sensitivity towards LL-37-induced cytotoxicity.

LL-37-induced cytotoxicity mediated by NETs and self-DNA

Another distinct mechanism, by which LL-37 could promote cytotoxicity and tissue damage, is by NET induction. When NETs become very abundant, the carrier cells, as a direct consequence, undergoes cell death via a unique mechanism called NETosis. Consequently the nuclear and granular membranes of the NETs dissolves, releasing chromatin and granular proteins into the surrounding environment [264]. Because of the LL-37-induced cell-lysis seen in apoptotic neutrophils, it has been suspected that LL-37 may be involved in this release of granular proteins [265]. The released components include high levels of cytotoxic compounds, in particular LL-37 and citrullinated histones, both of which can induce inflammation as well as tissue toxicity [266].

The self-DNA released from NETs has an indirect killing capacity as well through the formation of LL-37:DNA complexes, internalized by monocytes and pDCs. This, results in activation of the immune cells, consequently producing/secreting type I IFNs with the capability to initiate signaling events leading to cell death. This has been demonstrated by Pinegin *et al.* who treated co-cultures of NK cells and monocytes with sub-toxic levels of LL-37 complex bound to DNA. This resulted in killing of the NK-cells via IFNs released from activated monocytes [244].

LL-37-induced apoptosis

LL-37 has been shown to induce apoptosis in both an intrinsic, caspase-dependent, fashion and by a caspase-independent pathway. In MG63 cells, we observed apoptosis correlating to caspase 3 cleavage [213]. LL-37-induced apoptosis is caused by a caspase-dependent, intrinsic, pathway in human lung epithelial cells and in airway epithelial cells when infected with the pathogen *Pseudomonas aeruginosa*. In both of these cell types, caspase 3 is cleaved and caspase inhibitors are able to attenuate the apoptosis. Furthermore, in the infected airway cells, cleavage of caspase 9 (a key cytochrome c–activated initiator caspase), but not 8 (a death receptor caspase, extrinsic pathway) was observed [267, 268].

Interestingly, the same airway epithelial cells without the bacterial infection are much more resilient against LL-37. In the non-infected cells, the LL-37-induced apoptotic pathway did not involve cleavage of caspase 3, yet it still resulted in apoptosis with membrane depolarization and cytochrome c release to the cytoplasm. This alternative pathway was completely dependent on the pro-apoptotic Bax protein, as opposed to the caspase dependent signaling seen in infected cells treated with LL-37. This fascinating ability of LL-37 to induce apoptosis in infected cells, but not in healthy ones at the same concentrations, is suggested to represent another mechanism by which the peptide can clear pathogens [268]. Caspase-independent apoptosis by LL-37 has been described in other cell types as well. Okumura *et al.* showed that the peptide induced caspase-independent apoptosis in squamous cell carcinoma cells [269].

Mader *et al.* further explored the mechanism behind the LL-37 caspase-independent apoptotic pathway which they had observed in Jurkat T leukemia cells [270]. They found that Bax and calpain activation, for which Ca^{2+} is required, was critical for the signaling cascade in which AIF (apoptosis-inducing factor) translocates to the nucleus. Here, AIF initiates chromatin condensation and DNA fragmentation resulting in apoptosis [270]. Ren *et al.* shed further light on what appears to be the same mechanism by showing that LL-37 induced apoptosis in colon cancer cells is independent of caspase 3 but dependent on p53 upregulation and translocation to the nucleus [271]. The nuclear translocation of p53 causes downregulation of Bcl2 protein and upregulation/activation of Bax and Bak. Bax/Bak activation results in nuclear translocation of AIF and EndoG (endonuclease G), both of which initiate chromatin condensation and DNA fragmentation. Importantly, Ren *et al.* could block the apoptosis by treating the cells with pertussis toxin (PTx) which disrupts Gi-coupled GPCRs such as FPR2, but not with a specific FPR2 inhibitor [271].

While this suggests that LL-37 initiates the caspase-independent apoptotic cascade through activation of a GPCR, the identity of the GPCR is still a mystery. Some possible candidates which may be activated by LL-37 can be found in the

literature. This includes MrgX2, a GPCR that acts in synergy with PTx sensitive elements and that has been implicated to cause mast cell degranulation [191]. CXCR2 is another PTx sensitive GPCR receptor that could be activated by LL-37, with unknown consequences on cell death [241]. EP3 is also of relevance as it is a PTx sensitive receptor which may be activated indirectly by LL-37, and it has recently been found to induce apoptosis [202, 240]. LL-37 may also activate the purinergic GPCR P2RY11, as LL-37's rat orthologue has been reported to do [272].

Fig. 8 schematically illustrates key steps in the intrinsic and caspase-independent signaling cascade, assuming that all the cell types are similar and share the same apoptotic pathways. How the intrinsic pathway is initiated is not known. Here it is hypothesized to be a result of internalization of LL-37 followed by organelle damage, i.e. mitochondrial permeabilization/depolarization or ER-stress.

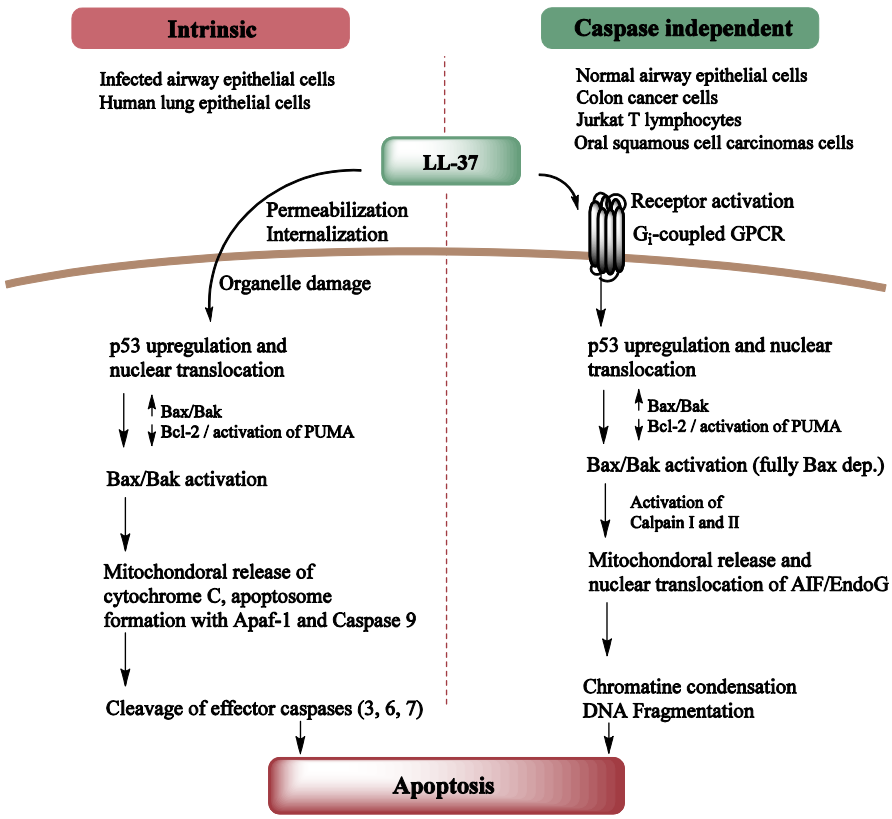

Figure 8.LL-37-induced apoptotic signaling. Schematic figure of two known apoptotic signaling cascades for LL-37: the intrinstic pathway and the caspase independent pathway.

Concluding remarks on cytotoxicity

Despite the well-documented cytotoxic effects of LL-37, there are only indications and hardly any solid evidence for its clinical importance. One exception is in colon cancer, where cytotoxicity of endogenous LL-37 appears to be an important factor in suppressing the cancer cells (involvement of LL-37 in cancer is discussed later). Possible reasons for the lack of proof in other pathologies, in which LL-37 is upregulated, may be due to inherent, methodological complications in the type of experiments needed. One example is that only humans and chimpanzees express LL-37, while other species express orthologues which generally show less host cell interactions [100, 179]. This complicates relevant *in vivo* studies. Another issue is that LL-37 has a very short half-life, both *in vitro* and *in vivo*. After addition of LL-37 to endothelial cells *in vitro,* it was shown that LL-37 accumulates in the cells only for a few minutes, after which cellular degradation of the peptide reverses the process [221]. Another factor limiting LL-37-induced cytotoxicity is that the peptide quickly binds up to particles and cells in order to shield its hydrophobic surface and to achieve electrostatic stabilization [12]. Experiments in which a single dose of LL-37 is used as treatment, no matter if it is *in vitro* or *in vivo*, does not correspond well to the pathophysiological condition in humans were we observe a consecutive, long-term release of LL-37, allowing for cellular accumulation and a constant stress on the cells.

Endogenous mechanisms impairing LL-37 activity

Lipoproteins

The cytotoxic effects of LL-37 are greatly reduced in human blood plasma because of a non-covalent binding of the peptide to apolipoprotein AI (apo-AI) with a 1:1 stoichiometry [259, 273]. Only LL-37 with an α-helical conformation is able to bind to apo-AI, which may also be able to scavenge hCAP18 through this binding [260, 273]. Affinity chromatography of LL-37 and apo-AI indicates a dissociation constant (Kd) of 1-2 μM, implying that 50 μM of apo-AI, a physiologically relevant concentration in serum (22-69 μM [274]), would scavenge >90% of 25 μM α-helical LL-37. It was further shown that 50 μM of apo-AI reduces the antibacterial activity of 50 μM of LL-37 to about 50%. These results suggests that apo-AI is a highly important protective mechanism against LL-37-induced cellular toxicity [259]. It has also been suggested that apo-AI-bound LL-37 may act as a reservoir of LL-37 to be released at infection.

To my knowledge, the proportion of LL-37 in serum which is in a helical conformation is not known. In phosphate-buffered physiological salt solution

(PBS), about 20-30% of LL-37 appears to form an α-helix ([112, 113]), a requirement for apo-AI binding. The helicity is likely more pronounced in plasma than in PBS, but the unstructured pool may still constitute a large proportion of LL-37, unless the apoAI-interaction switches the equilibrium, and this unstructured peptide still carries membrane permeabilizing properties.

Many biological fluids besides blood contain apo-AI, although usually at lower concentrations [275], suggesting that LL-37's biological effects are reduced by apo-AI in various types of body fluids. The inactivation of LL-37 by serum proteins would also suggest that host cell cytotoxicity from LL-37 could be more relevant in low-serum containing tissues, for example in skin as compared to inside the vasculature.

It is worth mentioning that Lau *et al.* were not able to block the cytotoxic effects of LL-37 with purified apo-AI [267]. Instead high density lipoproteins (HDL) particles, of which apo-AI is the main protein component, inactivated the peptide. This led the authors to the conclusion that full HDL particles rather than apo-AI in itself is responsible for the inactivation of LL-37 by serum [267]. Sørensen *et al.* reported that most serum LL-37 and hCAP18 is bound to low and very low density lipoproteins (LDL/VLDL), which lacks apo-AI [260]. LL-37 binds to phospholipid bilayers components, such as PC present in HDL/LDL/VLDL, and thus it seems plausible that phospholipids associated with lipoproteins contributes to the scavenging of LL-37.

Glycosaminoglycans

Even though wound fluid exudate contains serum to different degrees, Barańska-Rybak *et al.* could not find a correlation between inhibition of LL-37 activity and the protein content of the wound liquid. Instead inhibition of LL-37 was correlated to the fluid content of glycosaminoglycans (GAGs). Wound liquids with high concentrations of GAGs, 50-25 μg/ml, completely inhibited antibacterial effects of 100 μM LL-37 against *S. aureus* [276].

GAGs are polysaccharides comprising one repeated disaccharide unit, forming long unbranched chains. The disaccharide contains one type of amino sugar and one uronic sugar, or alternatively galactose, and carries sulfate substituents in various positions. GAGs are often attached to a core protein *in vivo*. Carboxyl groups from the uronic sugars and sulfate groups attributes to the molecules having a high negative charge, allowing them to bind LL-37, at least in part, by electrostatic interactions. Most eukaryotic cells express GAGs to different degrees, often bound to proteoglycan proteins on the cell surface [125]. Cell bound GAGs, such as heparin sulfate and chondroitin sulfate have capability to inactivate LL-37,

thereby providing protection against LL-37-induced cytotoxicity for cells with a rich cell surface expression of GAGs [277].

Plasma GAGs such as dermatan sulfate, CS-C and heparin all significantly inhibit LL-37's antibacterial activity at concentrations as low as 1 μg/ml *in vitro*, which is relevant for the *in vivo* situation (~2 μg/ml in blood plasma) [278]. LL-37 inactivation by GAGs is also suggested to be of relevance in lungs of individuals with cystic fibrosis [279].

Besides GAGs, also other polyanionic polysaccharides including cyclodextrins and dextran sulfate are able to inactivate LL-37 [124].

Ionic strength and pH

Activity of HDPs is affected by the presence of ions such as Na^+, Mg^{2+} and Ca^{2+}. The concentration of Na^+ is around 100 mM in most biological fluids, including plasma, which also contains Mg^{2+} and Ca^{2+} at concentrations of 1-2 mM.

The antimicrobial activity of LL-37 decreases 2-8 times in the presence of 100 mM Na^+ [119]. Na^+ does not influence the aqueous conformation of LL-37 [110]. Instead a high ionic strength inhibits various important ionic interactions by LL-37. Although effects on host cells are less studied than in bacteria, ionic strength can safely be assumed to play a role in LL-37 cytotoxicity on host cells as well. Ionic interactions are important for binding of LL-37 to both membranes and proteins [112, 280].

Another ion which at high concentrations impairs LL-37 activities is H^+. A low pH (pH 5-2) shifts its conformation equilibrium towards an unstructured peptide [110, 281]. A reduction in oligomerization could result in lower host cell cytotoxicity [112, 280].

Besides inactivating LL-37, a low pH also promotes dissociation between the peptide and its electrostatically bound ligands (e.g. nucleic acids). For example, during endosome acidification when intracellular pH locally drops to ~pH 6, LL-37 bound to self-DNA will release the DNA cargo which subsequently is able to activate intracellular TLR3 [221, 282].

Actin

Actin is the most abundant protein in eukaryotic cells and contributes to the formation for the cytoskeleton as a main component of microfilaments. It is also present in other cellular structures and as free proteins in the cytoplasm. It exists as a monomer (G-actin) or as polymers/microfilaments (F-actin). The protein is

negatively charged and both G-actin and F-actin has affinity for LL-37. The peptide is able to bind to the protein at its DNase I binding loop with electrostatic, and to some extent, hydrophobic interactions. Complexes between LL-37 and actin have been observed extracellularly both in cystic fibrosis (CF) sputum and in granular tissue at necrotizing regions of oral lesions [283, 284].

The binding of LL-37 to F-actin is stronger than to G-actin, and LL-37 also promotes bundle formation of the F-actin [285]. Although LL-37 activity is lost when bundled to F-actin, this may not be the case for interactions between LL-37 and other actin complexes. As previously discussed, the binding of LL-37 to ligands by electrostatic interactions is highly dependent on ionic strength and pH, LL-37/actin interactions is no exception. Sol *et al.* found in *in vitro* studies with physiological ion concentrations, that LL-37 remains active against bacteria in the presence of F-actin. Not only did actin not significantly interfere with LL-37's antibacterial effect but it also protected the peptide from degradation by bacterial proteases [283].

LL-37 also affects cellular actins, something that has been observed in endothelial and epithelial cells. Treatment with LL-37 (0.5-5 μM) increased endothelial cell stiffness through actin polymerization. This process, which requires activation of the P2RX7, results in cells with lower permeability [255]. The same effect has also been observed in lung epithelial cells, although the actin modulation was mediated by activation of FPR2 rather than P2RX7 in this experimental system [286]. Increased stiffness promotes epithelial integrity towards pathogens, and possibly also protects against permeabilization by LL-37, but that remains to be shown.

Cholesterol and sphingomyelin

As previously discussed, LL-37 has the ability to insert itself into various types of lipid bilayers with little distinction between negatively charged or zwitterionc membranes. In the outer leaflet of eukaryotic plasma membranes, cholesterol and sphingolipids can be present to various extents. Both of these affect the membrane in major ways where a high content of sphingomyelin results in a more rigid bilayer with less liquid properties. The rigidity of the cholesterol molecule prompts the lipid acyl chains to isomerize, from *gauche* to *trans*, causing an increased bilayer thickness [287].

Probably due to such properties, membranes containing high amounts of these components show higher resilience to LL-37 induced permeabilization as opposed to those lacking them [136, 287]. It should be mentioned that these studies are done on artificial, model lipid bilayers. There is to my knowledge no study showing that cholesterol or sphingolipids influence LL-37-induced permeabilization of host cells.

Mucins

Mucins are proteins synthesized and secreted by epithelial cells. They constitute the main protein content of mucus where they are responsible for mucus viscosity and various mucus functions. Certain mucins, including mucin 1, are also able to bind and inactivate LL-37 [288]. The mucin proteins are heavily glycosylated by *O*-glycosidic bonds. To these oligosaccharides, other groups such as sialic acid is attached, providing negative charge which is critical for the interactions with LL-37 [258]. Mucins appear to be an important factor for inactivation of LL-37 both in the oral/intestinal and the respiratory systems.

p33 (gC1qR)

p33, or the globular C1q receptor (gC1qR), is another candidate for protecting host cells from LL-37 induced cytotoxicity. p33 was first isolated in 1994 [289]. The protein appears to be expressed in all somatic cells with the exception of red blood cells [290]. A mitochondrial signaling sequence on the precursor protein results in localization of p33 to this organelle but mature p33 is also commonly observed at the cell surface, in the cytoplasm and in the nucleus. The protein is comprised of 209 amino acids and forms a trimeric structure under non-reducing conditions. The doughnut shaped trimer (Fig. 9) has one of its two surfaces negatively charged due to a high number of glutamic and aspartic acids (28 and 20 amino acids, respectively) [289, 291]. Intracellularly, p33 is not glycosylated, while this has been reported for the cell surface bound p33-pool [292].

Early on, p33 was described as a binding partner for C1q, the first subcomponent in the C1 complex, in the complement system [290, 293]. In the classical complement system, C1q activates the C1 complex by binding to immune complexes, or other C1q receptors, which initiates signaling cascades resulting in a series of inflammatory responses aimed to fight infection [294]. The implications of p33 as a C1q receptor is to date somewhat unclear, since there has been contradictory suggestions for the function of this protein, either activating C1, facilitating complement activation [295], or antagonizing the activation of the receptor by hindering binding of other ligands to C1q [296]. p33 binds C1q only with moderate affinity [297] and in light of this, the role of p33 in complement system activation is uncertain.

On the other hand, the protein is involved in a range of other biological processes, potentially with more important implications than its role in the complement system. p33 has been shown to modulate inflammation independent of C1q and can be upregulated at sites of inflammation [298, 299]. For example, it may suppress inflammation by inhibiting cytokine signaling, but also act as a pro-

inflammatory agent by associating with high molecular weight kininogen (HK), causing increased production of the pro-inflammatory mediator bradykinin [300, 301]. Besides being involved in inflammation and infection, p33 also plays a role in tissue remodeling and as a pro-apoptotic factor. Furthermore, it promotes angiogenesis and metastasis in cancer. In fact p33 is significantly upregulated in most tumors where it is also associated with high mortality [290, 302]. Some of these effects could be derived from p33's affinity to a number of proteins and by its Ca^{2+} modulating properties as it has been proposed to act as a Ca^{2+}-channel on the mitochondrial surface [300, 302].

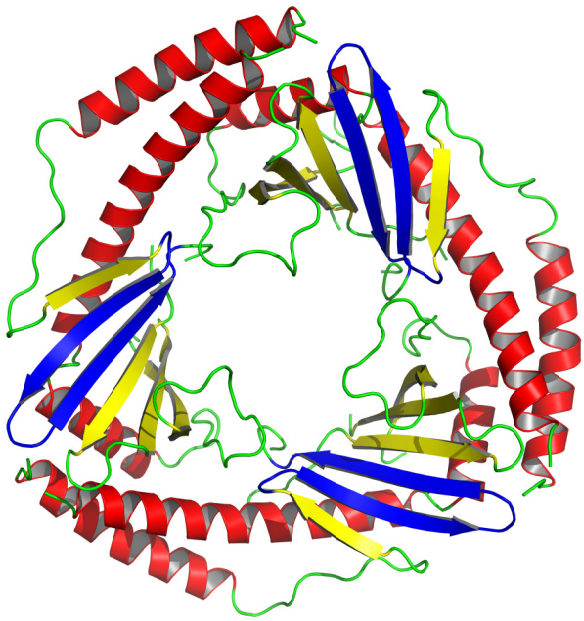

Figure 9. Structure of p33. Trimeric conformation of p33 drawn as a cartoon diagram. Blue area in each of the subunits represents the proposed LL-37 binding domain (aa 115-135). Figure created using the crystal structure by Jiang *et al.* [291], downloaded from RCSB PDB.

In solution, p33 binds LL-37 and other cationic HDPs, showing high affinity for the negatively charged β-sheet (residue 115-135) of the protein (Fig. 9, blue) [303]. Our results show that exogenous p33 (1-10 μM) antagonizes LL-37-induced apoptosis in human osteoblasts in a concentration-dependent manner, and that cellular p33 expression-level corresponds to sensitivity against LL-37 cytotoxicity. Endogenously expressed p33 does not however affect LL-37 induced permeabilization, suggesting that the intracellular protein, but not surface bound p33, inhibits the cytotoxic effect of LL-37. In other words, p33 may represent an intracellular defence mechanism that protects host cells from LL-37 toxicity without inactivating extracellular LL-37 [214, 304].

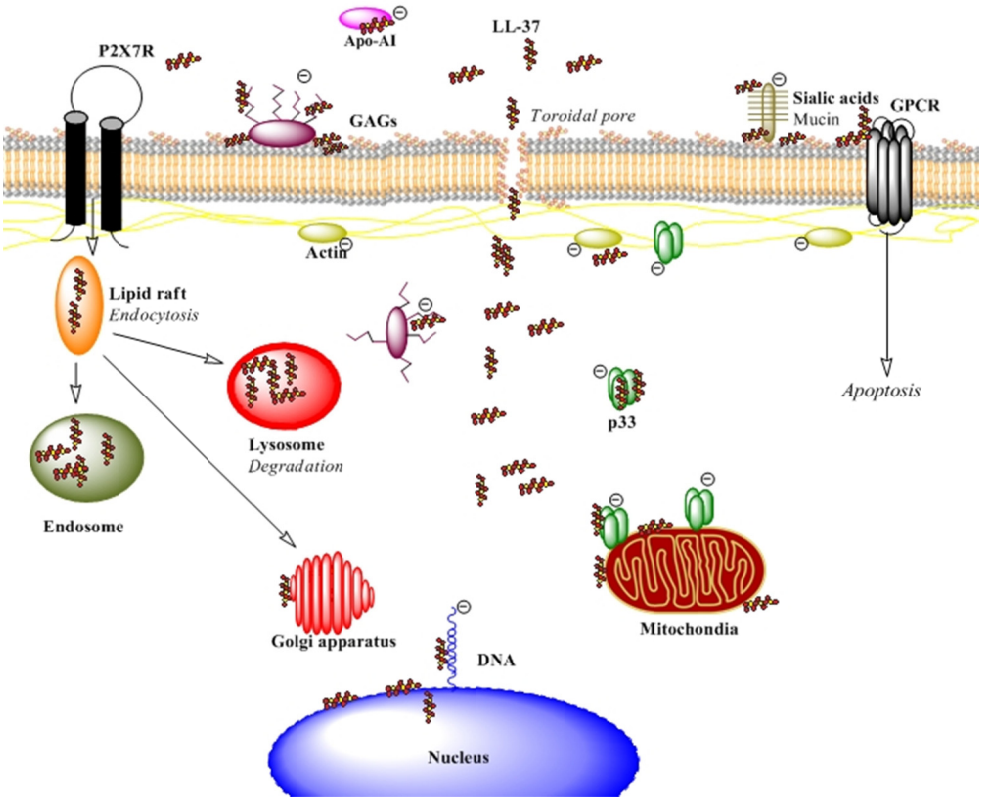

Figure 10. Schematic figure illustrating cellular uptake, and some possible faits, of LL-37. The peptide is internalized by endocytosis and diffusion over plasma membrane through pore formation. Intracellularly, it may bind to organells, causing cellular stress and cytotoxicity. Negatively charged proteins such as p33, actin and various glycosylated proteins, either bound to organells or free, binds and inactivates LL-37, intracellularly or extracellularly, and hence protects the cell from cytotoxicity. Negatively charges surface proteins however also increase LL-37 affinity for the cell.

LL-37 in periodontitis

Saliva is rich in LL-37 and LL-37 is reported to be expressed by the salivary glands, but also produced by the epithelium lining the inside of the mouth and at the back of the tongue. Infiltrating white blood cells may further contribute to the LL-37 production in mouth during an infection [106, 305]. Several studies have been conducted on LL-37 which shed light on its critical role in the oral immune defence system (for a review see [306]). The importance of LL-37 in sustaining oral health and preventing oral disease is demonstrated by the fact that syndromes associated with LL-37 deficiency correlate to periodontal diseases. Aggressive periodontitis (AP) is an infectious/inflammatory disease leading to periodontal

tissue destruction, loss of tooth-attachment and in its final stage, loss of teeth [106, 306]. The prevalence of AP is high in the two genetic disorders morbus Kostmann and Papillon–Lefèvre syndrome, which are both associated with low LL-37 expression [307, 308]. In morbus Kostmann syndrome, the affected individuals lacks neutrophils which are an important source of LL-37 [307] while in Papillon–Lefèvre syndrome neutrophils lack serine protease activity, and consequently lose the ability to generate mature LL-37 from hCAP18 [308]. Furthermore, a common missense mutation on the CAMP gene, p.S34N, seems to be a risk factor for generalized AP [309].

The most common form of periodontitis is chronic periodontitis (CP), affecting close to half the adult population of Sweden, and being the number one reason for tooth loss in this age group [310]. It is characterized by gingival inflammation and gradual loss of tooth attachment, and because the periodontal tissues gradually recess and are lost, it may appear as if the teeth are getting longer (Fig. 11).

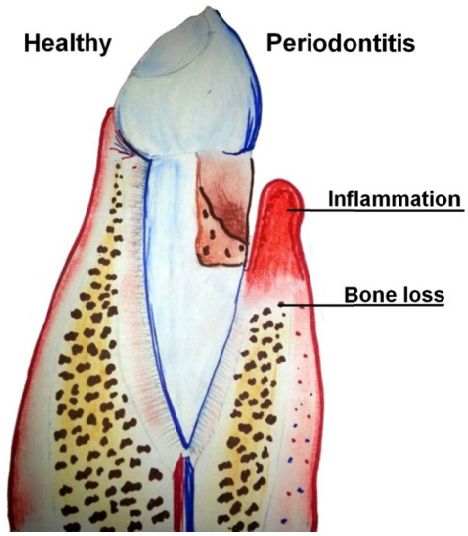

Figure 11. A tooth with chronic periodontitis. Section of a tooth with healthy gingiva to the left and periodontitis to the right. The inflammation results in the formation of a gingival pocket and loss of alveloar bone.

Both AP and CP can further be classified as localized or generalized depending on the % of area that is affected [311]. The clinical distinction between AP (in particular generalized form) and CP is not clear cut, but can be found in the following reviews [312, 313].

The pathogenesis of AP and CP is not fully clarified, but is largely attributed to detrimental effects of host immune/inflammatory response on the periodontal tissues. This is initiated by a microbial challenge to the immune system. The

infection causes a host immune response (more so in CP than in AP) where pro-inflammatory mediators such as cytokines stimulate bone loss through promoting differentiation and activation of bone resorbing osteoclasts [314].

The levels of LL-37 are reported to be elevated locally in patients suffering from CP [106, 315, 316] but the involvement of LL-37 in osteoclast-activation and bone degradation is not well studied. One publication suggests that LL-37 inhibits the osteoclast differentiation *in vitro*, arguing against a direct stimulatory effect of LL-37 on osteoclast activity [317]. Something worth considering is LL-37:self-DNA-induced activation of TLR9. In this context, extracellular DNA levels in the gingival crevicular fluid (GCF) of patients having CP are several times higher than in GCF of healthy individuals [318]. The GCF is an inflammatory exudate in the crevicular pocket [319].

The hCAP18/LL-37 concentrations in the GCF are elevated in patients with CP and this may reflect LL-37 produced by infiltrated white blood cells, mainly neutrophils, as well as by epithelial cells lining the pocket [106]. Collecting GCF, which is done by paper strips or capillaries, is a difficult technique and LL-37 has been found to bind strongly to the strips [316]. Therefore reported levels of LL-37 in GCF could be compromised by such factors.

Elevated LL-37 in GCF is however not present in all cases of CP as reported by Puklo *et al.* [106]. These authors compared levels of hCAP18 and LL-37 in GCF between healthy individuals and patients suffering from AP or CP. They found that some individuals in both AP and CP patient groups seemed to completely lack the hCAP18 and LL-37 proteins (5 out of 16 patients suffering from AP and 3 out of 17 suffering from CP). The lack of hCAP18 is believed to be due to a local degradation by bacterial proteases in the GCF, as peripheral blood of the same individuals show normal hCAP18 levels. In spite of these LL-37 negative cases, Puklo and co-workers could show a significant increase of both hCAP18 and LL-37 in CP, reaching concentrations in the low μM range. In GCF from AP-patients, hCAP18 but not LL-37 was upregulated compared to healthy subjects [106]. Interestingly, Türkoğlu *et al.*, have reported that CAMP mRNA is upregulated in CP [320] as well as in generalized AP [321]. These authors also demonstrate that hCAP18/LL-37 levels are increased in AP, and may reach 1 μM in the GCF (based on the MW of LL-37) [321]. A similar upregulation of cleaved LL-37 in GCF of both generalized AP and CP patients has been reported by Makeudom *et al.* [316]. Interestingly, both Makeudom *et al.* and Türkoğlu *et al.* found that LL-37 was significantly upregulated in gingivitis, but to a lesser extent than in periodontitis [316, 321]. Higher expression of LL-37 in generalized AP compared to gingivitis was also observed by Ertugrul *et al.* These authors also showed that smoking significantly enhanced LL-37/hCAP18 protein levels in GCF of AP and gingivitis patients vs. that of healthy controls [322].

One could speculate what causes the LL-37 elevation, for instance, LTB4 and chemerin are inflammatory mediators that could be suspected to be involved. Both cause rapid upregulation of LL-37 and both are significantly elevated in GCF of CP (but not AP) patients [75, 323].

Our group has observed that osteoblast cells are sensitive to LL-37-induced cell death at concentrations similar to those observed in CP GCF. Tissue destruction by LL-37 in pathological conditions has not yet been proven, but we have hypothesized that LL-37-stimulated cytotoxicity could be yet another mechanism by which the host-immune response can lead to bone destruction in CP [213].

This hypothesis may be in line with the occurrence of a positive correlation in CP between the LL-37 levels in inflamed gingival tissue and the depth of the gingival crevice [324], as tissue-destructive effects of LL-37 can contribute to deepening of the pocket. An alternative explanation to the correlation between pocket depth and LL-37 concentration is that when the periodontium is inflamed, the oxygen content is reduced. This represses oxygen-dependent antibacterial effects, for instance phagocytosis by neutrophils for which oxygen is critical. In these conditions non-oxidative clearance of pathogens, such as by that of LL-37, becomes all the more important, and perhaps this could cause an upregulation in deep gingival pockets [306].

A positive correlation has been observed between LL-37 levels and the concentration of glycosaminoglycans (GAGs) in CP GCF. These GAGs originate from the degraded bone, indicating that bone degradation is associated with LL-37 levels. While GAG and LL-37 concentrations correlate in CP GCF, the same was not seen in AP [316]. This suggests that bone destruction in AP is less likely to involve LL-37-induced toxicity than in CP. The lack of correlation between GAGs and LL-37 in AP also clears the suspicion that the positive correlation between GAGs and LL-37/hCAP18 levels observed in CP could be a result of GAGs ability to inhibit proteolytic degradation of LL-37 [325].

Although LL-37 levels have been found to be elevated in both generalized AP and in CP, LL-37 driven bone degradation appears less probable in AP. Another fact which suggests another mechanism behind bone loss in AP than in CP is that the progression rate of bone destruction is several times faster in AP compared to CP [312]. Moreover, the fact that a mutation in the CAMP gene correlates to increased susceptibility to AP but not CP, and that other genetic alterations causing lack off LL-37 are linked mainly to AP but not CP prevalence, also suggest a differential role of LL-37 in the two classes of periodontitis [106, 309].

In CP, neutrophils produce very high levels of ROS, and ROS has been proposed to contribute to tissue degradation in CP [245, 326]. Since LL-37 has been shown to enhance ROS, this pathway may represent another mechanism by which LL-37 can drive tissue destruction in CP.

Another way by which LL-37 may drive tissue destruction in CP is via its induction and stabilization of NETs. NETs have been proposed to play a role in the pathophysiology of CP where they appear to be resilient to enzymatic digestion, in keeping with LL-37's protective effects on NETs [245].

As earlier discussed, LL-37 activity is attenuated *in vivo* by endogenous proteins neutralizing its effects. In saliva for example, activity of LL-37 is reduced by the presence of high concentration of the negatively charged mucins [258], while in blood cytotoxicity of LL-37 is buffered by apoA-I [259, 260, 273]. It is not clear if any such inactivating system is present to a significant degree in the GCF. Under normal (healthy) conditions the GCF will not be in contact with mucin in saliva, and the concentration of apoA-I appears to be negligible (<1 ng/ml) [327, 328]. The most likely LL-37 neutralizing factor in the gingival pocket is the GAGs released into the GCF during bone degradation [276]. It is possible that a lack of adequate protection against LL-37 in the GCF renders cells in the surrounding periodontal tissue more susceptible to LL-37-induced cytotoxicity compared to cells in other environments.

LL-37 in inflammatory skin diseases

In skin, hCAP18/LL-37 is produced by keratinocytes of the uppermost part of epidermis where LL-37 may serve as a protective barrier to the skin by enhancing wound healing and combating microorganisms synergistically with other HDPs such as dermcidin and lactoferrin. hCAP18/LL-37 is also produced and secreted in epithelial cells lining both eccrine glands and ductal epithelium, resulting in release in sweat [329, 330]. hCAP18/LL-37 levels in sweat are variable but often surprisingly high, with average concentrations measuring around 1 μM reported in one study [96]. Infiltrating white blood cells, and perhaps even dermal fibroblasts, contribute further to the LL-37 expression in skin [77].

While LL-37 provides an important protective function to healthy skin, an upregulated expression is often observed in pathological conditions. Here, LL-37 has been implicated in the pathogenesis of several chronic skin conditions. This is often attributed to the peptide's pro-inflammatory effects, including its ability to internalize self-DNA, and its vasoactive properties [331]. Demonstrating the pro-inflammatory effects, Yamasaki *et al.* subcutaneously injected LL-37 into mice

and observed vascular dilatation, erythema, neutrophil infiltration, thrombosis and hemorrhage in the skin, with observed skin-inflammatory effects at LL-37 doses as low as 40 µl of 3 µM solution [257]. Skin keratinocytes express kallikreins, serine proteases responsible for cleaving hCAP18 to mature LL-37 and degrading the LL-37 peptide to multiple shorter cathelicidin-peptides in skin. These shorter peptides have different biological activity on both host and bacterial cells. In terms of pro-inflammatory effects, LL-37 appears to be the most potent of these peptides [95, 98, 257].

Kallikrein 5 process hCAP18 to LL-37 and this effect seems to outweigh its ability to degrade LL-37 into inactive fragments, the kallikrein appears to have a pro-inflammatory net effect in skin inflammation which is mediated by LL-37 [257]. Other kallikreins have not been shown to process hCAP18 into LL-37, while a few other kallikreins uses LL-37 as a substrate and produces biologically active LL-37 metabolites (these can be found in Fig. 5).

Psoriasis

Increased expression of host defence factors such as LL-37, psoriasin, β-defensin 2/3, RNase 7 and calprotectin, is a hallmark of psoriatic skin [332, 333]. The fact that psoriatic skin lesions are less susceptible to infections has been attributed to this. However, elevated levels of LL-37, have also been implicated in the progression of the disease [17, 329, 334]. In fact, LL-37 has been identified as a key factor in the activation of the auto-inflammatory cascade in psoriasis [246].

Psoriatic keratinocytes contain high concentrations of cytosolic nucleic acids as opposed to healthy cells, and extracellularly there is also an abundance of endogenous self-DNA and self-RNA, released by dying/leaking cells and NETs. This can activate various inflammatory cascades, dependent or independent of LL-37, resulting in cytokine production, including type I IFNs which are also implicated in the pathogenesis [152, 334]. LL-37 promotes psoriatic inflammation by forming complexes with self-DNA or self-dsRNA, and then internalizing itself with the nucleic acids into immune cells and keratinocytes where the nucleic acids may activate TLR9 and other receptors, initiating cytokine production. The resulting IFNs activate and mature a range of immune cells, further enhancing the pro-inflammatory signaling cascades [152, 246]. The ability of LL-37 to bind self-DNA can alternatively also suppress inflammation: free cytosolic DNA but not DNA:LL-37 activates the AIM2 receptor in psoriatic keratinocytes, and subsequently promotes the production of certain pro-inflammatory mediators [334].

In fact, it is still debated if the pro- or anti- inflammatory properties of LL-37 are predominant in psoriasis. The most common arguments in support of net-beneficial effects of LL-37 involve vitamin D in effective treatments. In the commonly used narrowband UVB (NB UVB) treatment, the skin is exposed to UVB light of ~311-313 nm. This include wavelengths by which D3 synthesis occurs (290-315 nm) and clinical studies has also shown that this treatment may boosts D3 levels as well as increase CAMP gene activity/hCAP18 protein expression [335, 336]. However, this appears to be the case only in patients suffering from vitamin D deficiency. Patients with sufficient D3 serum levels showed no increase in 25D3 concentrations after NB UVB-treatment [337]. Further, besides vitamin D synthesis many other mechanisms are activated by NB UVB, including photochemical reactions with DNA, which are likely responsible for many of the positive effects [338]. The implications of D3/LL-37 induction in NB UVB treatment in psoriasis is still not well understood and requires further studies on patients with sufficient vitamin D levels. Another argument in support of LL-37 attenuating psoriasis inflammation is the efficiency of calcipotriol treatment. Calcipotriol is a 1,25D3 derivate which has been found to upregulate CAMP gene activity. There are however conflicting reports regarding the drug's upregulation of LL-37 in psoriatic lesions. One study showed that calcipotriol indeed upregulates hCAP18 in the lesions, although they did not measure mature LL-37 levels or report 25D3 status of investigated subjects [339]. On the other hand, in keratinocytes, inflammatory stimuli such as LPS, TNFα or UVB irradiation caused upregulation of LL-37 protein, and here calcipotriol was found to reverse the enhanced expression of the peptide [340]. This study suggests that calcipotriol is able to both stimulate keratinocyte LL-37 expression in the absence of inflammation and suppress the same under pro-inflammatory conditions. 1,25D3 also relieves inflammation in psoriatic lesions and displays the same inflammation-dependent differential regulation of keratinocyte LL-37 expression, as shown by Jeong *et al*. [53]. Interestingly, the 1,25D3 treatment in this study also counteracted stimulation of TLR2 and 4 expressions, indicating yet another mechanism by which 1,25D3 relives inflammation in psoriatic lesions [53].

LL-37 may also be suspected to have beneficial effects on epidermal barrier recovery in psoriatic lesions, in analogy with its wound healing properties. One should however keep in mind the incredibly high concentrations of LL-37 present in psoriatic skin, with reported levels of hundreds of μM (LL-37/hCAP18), even reaching over the mM mark [256]. This could be compared to the LL-37 concentrations needed for cytotoxicity and cell permeabilization of skin cells *in vitro*, with EC_{50} values of 4 and 9 μM LL-37 for dermal fibroblasts and keratinocytes, respectively, and cell permeabilization at 1-4 μM [214]. While EC_{50} values may be different for the *in vivo* situation, these concentrations are markedly lower than those observed in the pathology. The role of LL-37 in the abundance of

self-DNA/RNA seen in psoriasis is not yet fully understood, but our data suggest that permeabilization of keratinocytes and keratinocyte organelles are likely to contribute to the release of nucleic acids. This is also the case for LL-37-mediated killing of host cells, causing leakage of self-DNA together with other deleterious components. LL-37-induced NET-induction likely also contributes to the abundance of extracellular self-DNA in psoriasis. Hence, these effects may complement the deleterious LL-37-mediated uptake of self-DNA/RNA in immune cell.

Rosacea

Rosacea is a common and chronic inflammatory skin disease characterized by inflammation in face or eyes, involving both vascular and connective tissues. However, the pathogenic mechanisms behind the disease have not been clearly shown yet.

Yamasaki *et al.* found that patients suffering from this disease expressed abnormally high levels of cathelicidin in epidermis, not only hCAP18/LL-37, but also several other cathelicidin peptides identified by SELDI-TOF-MS. The ten other identified peptides are suggested to be produced from hCAP18/LL-37 by kallikrein 5, whose expression is elevated in the disease [257].

The researchers further did a quantification of skin surface levels of cathelicidin peptides by pulling tape strips from lesions and healthy skin. Proteins were extracted and subjected to dot blot with LL-37 as standard. The immunoreactivity correlated to around 12 μmol cathelicidin/kg of protein, compared to just below the 1 μmol/kg mark for healthy skin [257]. Moreover, they claim that cathelicidin concentrations in rosacea may be as high as 1 500 μM, but unfortunately they show no data in support of this. In a mouse model of rosacea they also observed that cathelicidin knock-out mice showed less inflammation compared to wild type mice, further indicating a deleterious role of LL-37 in the disease [257]. Additionally, Zhang *et al.* showed that scavenging of LL-37 in murine and *in vitro* rosacea models efficiently elevated signs of inflammation. This was achieved by a semi-synthetic GAG, showing high affinity for LL-37 [341]. The antibiotic doxycycline is another drug commonly used in the treatment of rosacea. Although its mechanism of action is not fully understood, doxycycline is associated with the inhibition of MMPs in skin. This indirectly attenuates kallikrein activity and by doing so also the maturation of LL-37 from hCAP18. This could be speculated to be one mechanisms behind doxycycline's anti-inflammatory effects in rosacea [342].

Besides promoting inflammation through the previously discussed pathways, e.g. chemotaxis and cytokine production, LL-37 induced generation of ROS has been proposed to aggravate rosacea. Stimulated ROS production is believed to be an important part of the disease pathogenesis where oxidations induce tissue damage and inflammation [254, 343]. An example of tissue damage in rosacea is on the lesional blood vessels which may result in abnormal leakage of fluids and proteins from capillaries in the skin, manifesting itself as a swelling.

The high expression of hCAP18 in the disease has been proposed to be vitamin D3 dependent. Vitamin D serum levels appear to be elevated in patients suffering from rosacea, and 1,25D3 upregulates expression of not only CAMP but also kallikrein 5 [344, 345]. Based on the circumstances of the regulation, it could be suspected of being TLR2/CYP27B1 mediated. The LL-37 expression in rosacea has also been associated to the GPCR PAR-2: a significant correlation between cathelicidin and PAR-2 expression has been found in lesions. PAR-2 is activated by kallikreins and stimulates CAMP gene expression [346, 347].

In conclusion, high expression of LL-37 and kallikrein 5 attributes to the pathophysiology of rosacea, possibly in synergy with other kallikrein-derived LL-37 fragments found in the lesions [98, 257]. Interestingly, involvement of hCAP18 peptides could explain why tetracyclines (e.g. doxycycline), which inhibits HDP formation through attenuation of serine protease activity, represents an effective treatment in rosacea unlike other antibiotics [257, 348].

Atopic dermatitis

The most common inflammatory skin disease, atopic dermatitis (AD), develops by an accumulation of microbial skin pathogens [349]. The skin concentrations of LL-37 and other host defence peptides are low in AD compared to psoriasis, and it has been a common opinion that the lack of LL-37 is the reason behind the patients' high susceptibility to skin pathogens [256]. Later studies, however, have shown that the levels of LL-37 in AD are comparable to those seen in healthy skin, and that the keratinocytes in AD lesions are capable of responding normally to CAMP stimulation. This suggests that the lack of LL-37 might not be the reason behind the AD infections [350]. Two other research groups have found that LL-37 in fact is upregulated in the AD lesions, both on gene and protein level, while non-lesional AD appears to have an LL-37 expression comparable to that of healthy skin [54, 351]. One proposed explanation for the low LL-37 levels sometimes reported in this condition is that the induction of LL-37 expression could be repressed by wounding, something often present in AD due to scratching [350]. In conclusion, the involvement of LL-37 in the AD pathogenesis is today uncertain, but a deficiency of the peptide might enable infection in non-lesional AD.

Acne and inverted acne

LL-37 expression appears to be upregulated in acne (*acne vulgaris*) together with other HDPs [346, 352]. Acne is an inflammatory condition in the sebaceous glands, often occurring after a bacterial infection, in particular by *Propionibacterium acnes*. Elevated LL-37 levels, in synergy with other HDPs such as psoriasin, could assist in clearing *P. acnes* infections [353]. It could also be suspected to be involved in the elevated production of pro-inflammatory mediators and the recruitment of inflammatory cells which are promoting the inflammatory process in and around acne pustules.

Inverted acne (*acne inversa*), is another a chronic inflammatory skin disease causing painful skin lesions around skin folds [354]. Similarly to acne, a histological study revealed that hCAP18/LL-37 is significantly upregulated in these skin lesions as well suggesting that LL-37 may be involved in the pathogenic process [355].

The potential role of LL-37 in the pathogenesis of these conditions remains to be investigated.

Palmoplantar pustulosis

Palmoplantar pustulosis or pustulosis palmaris et plantaris (PPP) is a common chronic inflammatory skin disease characterized by formation of intraepidermal pustules on the palms of the hands and soles of the feet (palmoplantar) [356]. PPP is difficult to treat and the pathogenesis of the condition is not well understood.

Early in the disease progression, vesicles form in proximity to the intraepidermal duct of sweat glands. These vesicles contain fluid of high hCAP18/LL-37 concentrations (3±1 μM) as well as proteinase 3 derived from infiltrating white blood cells and kallikreins originating in keratinocytes [357-359]. Here, the inflammation is established which promotes vesicles to develop into pustules, e.g. the chemotactic cytokine IL-8 is upregulated in surrounding keratinocytes and in *stratum conium*, stimulating leukocyte accumulation in the vesicles [360].

Murakami *et al.* suggested that LL-37 may be a principal trigger of the inflammation in PPP as LL-37 upregulates pro-inflammatory cytokines associated with the disease including IL-8, IL-1α and IL-1β [358]. The researchers removed LL-37 from the inflammatory vesicle fluid, using antibody column purification, and this resulted in loss of its IL-8-inducing abilities in a human skin model (a co-culture of ECM-expressing dermal fibroblasts, overlaid with keratinocytes forming a layered epidermis). IL-1α and IL-1β was on the other hand still induced to a similar extent by the fluid [358]. The importance of IL-8 in the pathogenesis of

PPP is undisputed and, in fact, a monoclonal antibody against IL-8 has already been in clinical phase 2 trials as a PPP treatment, showing good results [361]. Taken together, it appears as if LL-37 is involved in the pathogenesis of PPP, perhaps in synergy with other hCAP18 derived HDPs, for instance TLN-58 found in PPP vesicles [101].

Systemic scleroderma

Systemic scleroderma (SSc) is a relatively rare (~100 new cases in Sweden per year), but serious, autoimmune disease. It affects skin, causing modulated inflammatory response, vascular damage such as obstructed capillaries and serious fibrosis which may be observed as thickening of the skin. Other organs than skin are however also affected in SSc, and the fibrosis may ultimately result in organ failure. The cause of the disease remains to be elucidated. In an effort to do so, Kim *et al.* [22] isolated SSc dermal fibroblasts and found that they secreted hCAP18/LL-37. This is surprising as fibroblasts, to my knowledge, have not previously been known to express hCAP18. The elevated expression of hCAP18/LL-37 in dermal fibroblasts was confirmed by positive staining for the proteins in biopsies of SSc dermis. These authors further reported that LL-37 (0.1-1 μM) may protect the fibroblasts from drug-induced apoptosis (in an ERK/COX-2 dependent fashion), leading to the conclusion that LL-37 may cause SSc fibrosis through fibroblast accumulation via this anti-apoptotic mechanism [22].

Although studies on the importance of LL-37 in SSc are lacking, Takahashi *el al.* [77] recently confirmed the upregulation of hCAP18/LL-37 in SSc, which they identified not only in the fibroblasts, but also in epithelial cells, white blood cells infiltrating the skin, and in the systemic serum levels of SSc patients. The levels of hCAP18/LL-37 were increased around 6 times in serum from SSc patients compared to serum from healthy controls. Takahashi and co-workers further found that LL-37 expression positively correlates to the severity of the fibrotic processes and proposed this to be an effect of LL-37 on epithelial tissue [77].

Infiltrating pDCs are commonly observed in the skin of SSc patients and evidence suggests that pDC derived IFN-α play a critical role in SSc progression where it can be responsible for effects such as the vascular damage. There is a positive correlation between hCAP18/LL-37 and IFN-α transcription levels in the lesions, implicating LL-37 in the enhanced cytokine expression observed in SSc [77]. Thus, LL-37 may promote disease progression in SSc through activation of pDCs (i.e. via self-DNA), although this has not yet been reported. Takahashi *et al.* further found that a deficiency of Fli1 expression resulted in elevated chemerin levels in SSc, a hallmark of the disease [77]. As chemerin is a powerful pDC chemoattractant as well as an inducer of LL-37 expression [76] it may be

responsible for both the observed pDC infiltration and the high LL-37 levels of the disease.

In conclusion, evidence suggests an important role of LL-37 in SSc tissue fibrosis, and the peptide may further promote the inflammatory effects observed in the disease, although additional studies are needed to clarify this issue.

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE), frequently called lupus, is a common chronic autoimmune disease, affecting skin as well as other organs. What causes the disease is still unknown, but it has been found to be triggered by external stimuli, in particular sunlight, as well as genetic factors and exposure to estrogens [362]. The disease initiation involves a loss of host cell tolerance against self-antibodies. Immune cells are consequently activated which produce additional antibodies, targeting self-nucleic acids and proteins. The antibodies form complexes with their ligands, importantly with self-DNA released from NETs and cells. These complexes may be deposited at, in principle, any part of the body, where they result in inflammation as well as in tissue damage [222]. Just as in several other LL-37 associated autoimmune diseases, including psoriasis, the activation of pDCs and their subsequent release of type I IFNs have been identified as an essential step early in the inflammation progression [222, 363, 364].

White blood cells from SLE patients have been show to overexpress the cathelicidin gene (about three-fold compared to healthy controls) [365]. This, and the mechanistic similarities to psoriasis, led Lande *et al.* [222] to investigate the role of LL-37 in SLE pathogenesis. To do this they first purified DNA-containing immune complexes from patients and confirmed that these contain LL-37. They further showed that by adding an antibody against LL-37 to the complexes, they completely removed their ability to induce IFN release from pDCs. Moreover, inhibition of LL-37 production in NET-forming neutrophils by a protease 3 inhibitor resulted in self-DNA without pDC activity, as opposed to NETs without the inhibitor. The pDC activities of complexes were also lost in presence of a TLR9 inhibitor, as well as with an inhibitor against the Fc receptor CD32. This indicates that the CD32 surface receptor is responsible for pDC internalization of the LL-37-DNA-immune complex [222]. Furthermore, Lande *et al.* show that LL-37 protects the self-DNA complexes from a rapid DNAse I digestion. Interestingly, LL-37 apparently acts in synergy with a group of HNP α-defensins. HNPs are themselves unable to form complexes with DNA, but somehow they appear to be critical in promoting pDC uptake of these complexes. SLE patients were also found to produce elevated levels of LL-37 antibodies [222].

Besides the study by Lande *et al.,* Garcia-Romo *et al.* [166] published results from a similar study in parallel (in the same journal volume as the Lande study). Garcia-Romo *et al.* confirmed many of the key findings discussed above, and supports the hypothesis that LL-37 plays a critical role in SLE inflammation by allowing for self-DNA internalization/TLR9 activation in pDCs.

Kienhöfer *et al.* later confirmed increased levels of LL-37 and LL-37 antibodies of patients with SLE, however they argue that LL-37 does not promote the disease progression. They could not find any correlations between disease parameters of SLE and LL-37 antibody levels and they used a SLE mouse model where no differences in SLE disease development could be seen between $CRAMP^{+/+}$ and $CRAMP^{-/-}$ mice. These authors (boldly) conclude that there is no evidence of a pathogenic involvement of cathelicidins in SLE [366]. However, mouse CRAMP is known to affect TLR signaling to a much lesser extent than LL-37 [179].

Time will hopefully tell if the discrepancies between the results are due to model/species differences or if it has other explanations. The current literature, although limited and with one exception, strongly suggests an important role of LL-37 is in SLE pathogenesis.

Role of LL-37 in other pathologies

Asthma

Asthma is a common chronic inflammatory condition of the airways, and another disease where LL-37 has been suspected to play a pathogenic role. Sun *et al.* [367] showed that eosinophils from asthma patients expressed higher levels of hCAP18 than those from healthy controls. Furthermore, they showed that LL-37 itself activates these white blood cells through FPR2. This results in release of pro-inflammatory factors including leukotrienes (e.g. LTB4), which are important mediators of inflammation in the asthma pathogenesis [367].

A study on bronchoalveolar lavage fluid could however not see any increase in hCAP18/LL-37 protein in fluid from asthmatic lungs compared to healthy controls [368]. Further studies are needed in order to elucidate the role of LL-37 in asthma pathogenesis.

Atherosclerosis

Atherosclerosis is a chronic disease and a specific form of arteriosclerosis where the artery wall thickens due to inflammation, resulting in accumulation of white blood cells. Here, atherosclerotic lesions, or placks, are formed and these can be more or less stable depending on composition. Unstable placks (often rich in macrophages) can rupture and induce thrombus, which blocks the lumen and often causes strokes or heart infarcts. Placks growing thick can also narrow the blood vessel to such an extent that the blood supply to downstream tissues is insufficient, causing ischemia, although the risk of this is lower.

LL-37 is upregulated in atherosclerotic blood vessels compared to healthy ones. The peptide is there synthesized in placks by accumulating macrophages, neutrophils and endothelial cells. LL-37 is able to accumulate in these placks through aggregation to NETs [369-371]. It has been proposed that LL-37 may promote a pDC-driven pro-inflammatory signaling cascade in atherosclerosis which is similar to that reported in psoriasis. The LL-37 orthologue in mice (CRAMP) has been found to bind to self-DNA in placks, causing activation of pDCs and subsequent release of IFN-α, aggravating inflammation [372]. Recently, Zhang *et al.* [243] implicated specifically mtDNA:LL-37 as the main inflammatory factor aggravating atherosclerotic lesions. LL-37 protects the DNA from degradation and autophagy, promotes internalization, and subsequently allows mtDNA to activate pDCs, neutrophils and endothelial cells via TLR9. Consequently, levels of pro-inflammatory cytokines involved in atherosclerosis pathogenesis are elevated [243].

Interestingly there is a well-known, but poorly understood, positive correlation between periodontitis and atherosclerosis [373]. One possible explanation to this is that circulating bacteria and pro-inflammatory bacterial products, originating from an infection, can promote vascular inflammation [373]. Perhaps contradictory to this mechanism is the fact that antibiotic treatment does not demonstrate any long term benefits in patients with this cardiovascular disease, and only to a limited extent in the treatment of CP [374, 375]. LL-37/hCAP18 upregulation in GCF during AP does not appear to increase blood serum levels of the peptide [321], suggesting that the correlation between the diseases are not due to a systematic upregulation of LL-37 following periodontitis. Alternatively, perhaps other factors upregulating or expressing LL-37 could enter circulation from one inflammation and initiate/promote another one. For instance, inflammation mediators such as chemerin and LTB4 are both elevated in CP [75, 323] as well as in atherosclerosis [74, 376, 377], and as discussed in the section regarding cathelicidin regulation, both efficiently promote LL-37 secretion.

Inflammatory myopathies

Inflammatory myopathies are a group of chronic inflammatory conditions with unknown etiology affecting and weakening muscles. Two of the main classes are polymyositis and dermatomyositis. In these pathologies, inflammatory cell infiltration of the affected muscles has been reported, and furthermore gene expression of type I IFNs have been found to correlate to disease activity [378, 379]. Recently, Lu *et al.* reported that infiltrating neutrophils in muscles of both dermatomyositis and polymyositis produce high levels of hCAP18/LL-37 compared to those of healthy controls. The authors propose that LL-37 may induce muscle inflammation through pDC activation and subsequent type I IFN production. Additionally, LL-37/hCAP18 expression level in muscles correlates to the level of muscle creatine kinase, indicating that LL-37 might damage muscle fibers [378]. This calls for additional studies to further examine the involvement of LL-37 in the pathogenic mechanisms of inflammatory myopathy.

Bladder inflammation and fibrosis

Bladder inflammation (BI) is a common problem which can affect adults as well as children. Several disorders may cause the syndrome, sometimes resulting in a fibrotic dysfunctional bladder with severe consequences. One such disorder is the birth defect spina bifida, where there is a lack of connection between the backbones and the spinal cord. This often results in a neurogenic bladder (lack of bladder control) in children and chronic BI in adults [209]. The fibrotic process, in which connective tissue is accumulated in the urinary tract, results in a non-compliant bladder with increased bladder pressure, organ damage through urine infiltration and bladder failure as possible consequences [380-382].

LL-37 has been found to be upregulated during urinary tract infection (UTI), and markedly so in pediatric patients with spina bifida [209, 383, 384]. The study on spina bifida patients, conducted by Oottamasathien *et al.* [209], found that hCAP18/LL-37 urinary levels was 20 ± 132 ng/mg in patient group, compared to 0.23 ± 0.39 ng/mg in the control group. This corresponds to means of 4.5 μM and 0.05 μM of LL-37 in urine of respective groups, assuming full conversion to LL-37, with urine levels reaching as high as 220 μM in the patient group [209]. Oottamasathien and co-workers hypothesized that elevated LL-37 may cause the fibrosis through mast cell chemotaxis. Mast cell infiltration and degranulation has previously been shown to induce fibrosis. Furthermore, the authors suggest that LL-37 could be responsible for urothelial cell injury seen in UTIs through induction of apoptosis. They have shown in a mouse model that LL-37 induced bladder inflammation with erythema, hemorrhage and tissue edema, as well as mast cell recruitment in a dose dependent manner (10-320 μM) [209, 382].

LL-37 is also known to cause mast cell degranulation [191], which along with epithelial wounding, represents possible mechanisms through which it could induce bladder fibrosis.

This warrants for further research into the involvement of LL-37 in epithelial damage and fibrosis associated with BI.

Cystic fibrosis

Cystic fibrosis (CF) is the most common fatal inherited disease in the western world. It is caused by mutation of the CFTR gene, resulting in dysregulated epithelial sodium channels. Consequently a thick sputum is formed with reoccurring serious chronic airway infections as a consequence [285]. LL-37 is upregulated in CF, levels up to over 3 μM have been reported in CF patient sputum, but evidence suggests that local concentrations likely exceed this [285].

The viscous CF sputum promotes infections in the airways and is the main cause of death in the disease. Dying immune cells leads to release of high concentrations of molecules into the sputum, including self-DNA and actin. The free F-actin is known to form actin bundles, a main contributor to the high viscosity in CF sputum. LL-37 has been implicated in this bundle formation: the peptide forms stable complexes with F-actin, and this may result in bundling through an unknown mechanism. There is an interest in dissolving the LL-37-actin bundles as a CF treatment. This would both be a way to decrease the viscosity of the sputum and to free up the bound/inactivated LL-37 which may help in pathogen clearance, something which is critical in CF management [285, 385].

The severity of the disease have been correlated to the detected levels of hCAP18/LL-37, but the role of the peptide in the pathogenesis is not well understood [284].

Chronic wounds and wound exudates

As previously discussed, LL-37 plays an important role in wound healing. Normally, LL-37 is upregulated in wounds, although stimulation of LL-37 expression could be inhibited by wounding in other cases [194, 350]. In chronic wounds (CW) such as chronic ulcers the LL-37-induction is dysfunctional and levels are instead comparable to those of healthy skin. The lack of LL-37 is likely a reason behind the impaired healing, and in line with this, treatment of CW with LL-37 has in a number of studies been found to promote healing.

The CAMP gene expression, however, seems to be upregulated in CW, at least in some cases. Possibly, an impairment of hCAP18 translation or altered degradation of the peptide in the epithelium could be reasons behind this [195, 196]. A study by Grönberg *et al.* on LL-37 in wound liquids from CW showed that the peptide is very stable in the exudate, supplemented LL-37 remained stable after 24 h of incubation. This could imply that low LL-37 levels in CW are in fact due to a decreased expression, rather than a rapid degradation of LL-37 [325]. Schmidtchen *et al.*, however, found rapid proteolytic degradation of LL-37 in (non-chronic) wound liquids infected with elastase-producing bacteria. Without infection, LL-37 remained stable for around 4 hours in the wound exudate [386]. The most important LL-37 scavenger in wound liquids appears to be GAGs which are released from the connective tissues. Due to prolonged detrimental effects on the tissues in CW, GAG discharge is more abundant here [276, 387, 388]. Schmidtchen and co-workers were able to inhibit the proteolytic degradation of LL-37 by a GAG of relevance [325]. This raises the question if the stable peptide in Grönbergs wound liquids are in fact bound GAGs and subsequently inactivated, and that this is a more pronounced phenomena in CW as compared to wounds investigated by Schmidtchen *et al.* GAGs have been suggested to have important functions in wound healing [389] but perhaps high levels in CW wound liquid could inhibit the healing properties of LL-37.

Alginate is often employed as a wound dressing to manage exudates through absorption [390-392]. Interestingly, the anionic alginate has previously been known to interfere with LL-37 activity *in vitro* [393]. It appears to do so by neutralizing the positive charges of LL-37, rendering the peptide more hydrophobic which promotes helicity, self-aggregation and aggregation on the alginate [394]. Through this mechanism, one could suspect that alginate wound dressing may decrease healing rates, although this does not appear to have been researched. Studies comparing wound healing rates with alginate and other dressings have however been performed. While inconclusive, several of these indicate that higher healing rates may be seen with other absorption-dressings as compared to alginate, and even higher healing rates may be seen with vacuum-assisted wound closure as an alternative to wound dressings [395-397]. A study investigating clinical implications of LL-37 inactivation by alginate could be considered called for.

Diabetes, fat and hepatic steatosis

Several studies indicate that LL-37, or deficiency thereof, could play an important role in the development of diabetes complications and in obesity.

A positive correlation between serum cathelicidin levels and BMI has been found in non-diabetic individuals [94]. However, once diabetes type 2 develops, the expression of LL-37 is suppressed. This has been reported in both peripheral blood and in foot ulcers of diabetic individuals [195, 398]. In foot ulcers, the percentage of positive immunostained area for LL-37 was almost 95% lower than in that of healthy dermis. Therefore, LL-37 deficiency is proposed to be a reason behind the lack of healing in these wounds [195]. In support of this, mice model of type 2 diabetes showed improved wound healing after local treatment with LL-37 expressing adenoviruses [399]. In a rat model for obesity, it was shown that chronically high blood sugar levels (hyperglycaemia) caused a reduction in their cathelicidin expression. This could be an effect from the reduction of IGF-1 observed in hyperglycaemia, as IGF-1 is a hormone known to stimulate LL-37 expression. Interestingly, LL-37 may also initiate IGF-1 receptor signaling cascades [225, 400, 401].

To study the role of increased cathelicidin expression in obesity, Tran *et al.* [94] used diabetic and non-diabetic mice in which they overexpressed CAMP through IV injection of lentivirus vectors. After three weeks, no significant changes from the overexpression could be seen on the body weight or fat mass of the non-diabetic mice. In the diabetic mice, too, the body weight was unaltered, but the percentage of fat mass was significantly reduced while the proportion of lean mass, consisting of muscles and bones, had increased by the LL-37 expressing virus. This could indicate that LL-37 inhibits muscle loss or muscle wasting, two common consequences of untreated diabetes. While LL-37 did not affect glucose metabolism in the study, it did inhibit lipid accumulation in adipocytes. This effect was dependent on the downregulation of the fat receptor CD36 mediated by a (somewhat surprising) LL-37-induced ERK-inhibition [94].

Tran *et al.* further noted that liver fat content was reduced by LL-37 lentivirus in both diabetic and non-diabetic mice, suggesting that LL-37 may be an important factor in protecting against hepatic steatosis (fatty liver disease), a highly prevalent condition in type 2 diabetic individuals [94].

In conclusion, loss of LL-37 expression in type 2 diabetes could be a key factor for some of the disease consequences, e.g. suppressed wound healing, muscle loss and hepatic steatosis, making it an interesting drug target.

Cancer

Studies on involvement of LL-37 in cancer pathogenesis demonstrate a complex role of the peptide, which may both be able to stimulate or suppress cancer growth in different forms of the disease.

As mentioned earlier, LL-37 induces apoptosis selectively in squamous and colon cancer cells, while sparing healthy ones in the same dose range, suggesting that it may have potential as an anti-cancer drug in these diseases [269, 271]. In support of this, cathelicidin deficient mice were shown to have an increased susceptibility to colon cancer [271]. In gastric cancer, LL-37 is downregulated and has been proposed to act as anti-tumorigenic through inhibiting cancer cell proliferation, as well as stimulating the tumor-suppressing BMP signaling [402]. The selectivity of LL-37 towards cancer cell membranes over others has been proposed to be due to an overexpression of anionic membrane components such as glycoproteins in cancer cells [403].

On the other hand, LL-37 has been found to be upregulated in tumors of several cancer types including ovarian [404], breast [405], lung [406], and prostate [407]. In these diseases, LL-37 has instead been implicated to promote tumorigenesis: in ovarian cancer, LL-37 acts pro-proliferative and pro-angiogenic via FPR2, subsequently promoting a more aggressive cancer cell phenotype [228, 239, 404]. Similarly, in breast cancer epithelial cells LL-37 stimulates proliferation and migration, possibly via ErbB2 signaling [228, 405]. In lung cancer, LL-37 has been proposed to promote cancer cell proliferation, angiogenesis, invasion and metastasis while causing resistance to apoptosis through EGFR [228, 406]. In prostate cancer, a mouse model of the disease were subjected to downregulation of cathelicidin, resulting in decreased proliferation and invasion of tumors [407].

While LL-37 may both promote tumorigenesis through receptor activation and suppress it by cytotoxicity, there is still an interest for LL-37 as a drug to combat cancer. In immunotherapy, for example, LL-37 is of interest for its ability to internalize compounds into host cells. CpG oligodeoxynucleotides are immunotherapeutic drugs which promote tumor suppressing properties of host immune cells via TLR9 activation. LL-37 was found to enhance these effects when co-administered with the drug. It activates immune cells including B-cells, pDCs and NK cells, promoting anti-tumor effects as well as causing high tumor cytotoxicity with involvement of type I IFNs. LL-37 was found to synergize CpG treatment in mice ovarian cancer (a LL-37 overproducing cancer type) [184, 408]. Fascinatingly, NK cells apparently completely lack anti-tumor effects without the presence of cathelicidin - perhaps explaining the protective effects of vitamin D to several cancer forms [407, 409]. Similarly, co-treatment of LL-37 with the

classical anticancer drug docetaxel also shows promise, resulting in increased tumor inhibition and survival in colon cancer mice [181].

In conclusion, LL-37 shows both potential as a novel therapeutic target for various types of cancer, and as an anti-cancer drug.

LL-37 as a possible drug target

LL-37 upregulation seems to be involved in the pathophysiology of several different inflammatory diseases, and thus LL-37 represents a possible drug target.

Coincidentally, or not, several effective remedies in these diseases do in fact suppress LL-37 expression. In psoriasis, etanercept, a TNFα inhibitor, is an effective treatment which also significantly downregulates LL-37-expression in lesions [410]. Similarly, oral doxycycline and topical azelaic acid are common treatments for rosacea, both of which inhibit LL-37 [342]. All three of these drugs act on LL-37 expression through inhibition of skin serine protease activity (i.e. kallikrein 5), while azelaic acid additionally attenuates the CAMP gene expression [254, 411]. It would be of interest to evaluate if regulation of LL-37 expression is a key mechanism of action for these drugs, or of limited importance.

Recently it was found that skin exposed to LED light at 630 and 940 nm showed reduced expression of LL-37, both on gene and protein level. Furthermore, this treatment decreased the expression of kallikrein 5 and TLR2 [81]. A decrease in keratinocyte CAMP expression has also been observed after skin irradiation at 410 nm [80]. Irradiation at these wavelengths could thus have potential as an effective treatment of certain inflammatory skin disorders through attenuation of LL-37 expression.

An alternative strategy to combat unwanted LL-37-effects could be to directly inhibit the peptide with scavenging molecules. As discussed earlier, there are a variety of relatively large, negatively charged, molecules which bind to and inactivate LL-37. This approach has been employed on rosacea models, where it was seen that a semi-synthetic GAG was able to relieve LL-37 induced inflammation [341]. Another possible candidate is sodium alginate, a cheap and commercially available substance that promotes LL-37 aggregation and inactivation [394].

As pointed out earlier, the use of LL-37 as an alternative to conventional antibiotics and to heal wounds could be other obvious strategies for therapeutic use of this peptide.

AIMS

We hypothesize that LL-37 may be responsible for the tissue destruction observed in inflammatory diseases through inducing host cell cytotoxicity and our aim was to investigate these effects and the underlying mechanism. Specific aims for the different sub-projects are presented below.

Sub-project I. Chronic periodontitis is characterized by destruction of the alveolar bone supporting the teeth, leading to tooth loss. Interestingly, the LL-37 concentrations are high in the gingival crevicular fluid from patients suffering from chronic periodontitis [106, 315]. The aim of this study was to investigate if LL-37 could take part in bone degradation through studying the effects of LL-37 on osteoblast cell viability and function.

Sub-project II. LL-37 was found to permeabilize and induce apoptosis in osteoblasts at pathophysiologically relevant concentrations, and could therefore be involved in tissue destruction in periodontitis as well as in other autoimmune diseases. Hence, endogenous mechanisms which may attenuate this effect could be of interest as drug targets. Here, we investigate compounds that could serve to inactivate LL-37, and further, constitute endogenous protective mechanisms against the deleterious effects of the peptide.

Sub-project III. LL-37 induced cell death is a process that exclusively has been studied by applying the peptide exogenously to cells. Endogenously, a range of factors are likely to influence these detrimental effects, and thus more complex models are needed. We aim to evaluate any possible cytotoxic effects of endogenously released peptide on host cells *in vitro*. Vitamin D is an important regulator of LL-37 gene expression, however less is known about the functional importance of vitamin D-induced LL-37 expression. Here, we stimulate LL-37 production of human monocytes and evaluate possible cytotoxic effects both on the monocytes themselves as well as co-cultured human tissue cells.

Sub-project IV. Dysfunctional expression of LL-37 in epithelial tissue is implicated in the pathogenesis of a range of autoimmune diseases as well as to the susceptibility of microbial infections. A deep understanding of the regulatory mechanisms involved, is important for treatment and drug development. The most well studied enhancer of LL-37 is vitamin D, and the nuclear VDR. Here, we wish to compare VDR and LL-37 expression in human epithelium from gingiva and skin and investigate regulatory pathways involved in vitamin D-induced LL-37 expression.

METHODS

This section contains descriptions of the main methods and techniques used in the thesis. Methods for other techniques used may be found in their respective papers (Appendix I-V). This concerns methods for immunogold electron microscopy, co-immunoprecipitation, co-cultures of cells, immunohistochemistry, annexin V flow cytometry, ELISA for cleaved caspase 3 and bacterial expression/purification of recombinant p33 and the pcDNA 3.1-p33 vector. More specific methodological details may also be found in the method-section of the papers.

Cell culture

The cells were cultured in DMEM/Ham's F12 (1:1) or RPMI 1640 cell culture medium supplemented with antibiotics (penicillin 50 U/mL, streptomycin 50 µg/mL), L-glutamine and 10% fetal bovine serum (FBS). Cells studied in this thesis can be found in Table 1.

Cells were cultured at 37 °C in 5 % CO_2 in air using a water-jacketed cell incubator. Primary cells were used in passage 3-10, while cell lines were used up to passage 50 and. Cell detachment was performed by covering cells in 0.25% Trypsin-EDTA solution. RAW 264.7 cells were subjected to scraping in trypsin-EDTA solution for complete detachment.

To promote osteogenic differentiation of PDL cells, an osteogenic differentiation medium was prepared by supplementing the normal culture medium with 10 mM β-glycerophosphate disodium salt hydrate and 0.05 mM L-ascorbic acid 2-phosphate. Mineralization was assessed after three weeks by staining of fixated cells for 45 min with Alizarin Red S solution. Cells were fixed with 10% paraformaldehyde (PFA) for 30 min. The osteogenic cells stained orange-red while normal PDL cells did not retain any color.

Tabel 1. Cells used in the studies

Cell	*Origin*	*Source*	*Culture medium*
MG63	Osteoblast-like cell line	A.T.C.C.	DMEM/Ham's F12 (1:1), 10% FBS
hFOB 1.19	Osteoblast cell line	A.T.C.C.	DMEM/Ham's F12 (1:1), 10% FBS
PDL	Primary periodontal ligament cells	Primary cells isolated from teeth, 14-15 year old individuals	DMEM/Ham's F12 (1:1), 10% FBS
HaCaT	Keratinocyte cell line	CLS Cell Lines Service GmbH	DMEM/Ham's F12 (1:1), 10% FBS
HDFa	Primary Neonatal dermal fibroblasts	Cascade Biologics	DMEM/Ham's F12 (1:1), 10% FBS
HeLa	Epithelial cancer cell line from cervix	A.T.C.C.	DMEM/Ham's F12 (1:1), 10% FBS
THP-1	Monocytic cell line	A.T.C.C.	RPMI 1640 medium, 10% FBS
RAW 264.7	Mouse macrophages, cancer	A.T.C.C.	DMEM/Ham's F12 (1:1), 10% FBS

Cell number and morphology

For determination of cell number by counting, cells were cultured in 12-well plates at appropriate cell densities (for the most part achieving subconfluency). For 24 h LL-37 treatments, cell culture medium was replaced by DMEM medium (0.8 ml) supplemented with 2% FBS and antibiotics (same composition as for culture medium). In some cases this medium was also modified, i.e. Ca^{2+} exclusion, $MgCl_2$/EGTA inclusion. LL-37 or DMSO vehicle was added and cells were incubated for 24 hours before cells were trypsinized. Trypsinized cells were collected with culture medium, stained with trypan blue, and counted in either a Bürker chamber or a LUNA™ automatic cell counter (Logos Biosystems).

A Nikon TMS microscope equipped with a digital camera (Pixelink, Nikon) was used to assess morphology by phase-contrast microscopy. In some experiments, dead cells were detected by first incubating cells with trypan blue (0.4 %, 2 min) followed by repeated washing with aqueous NaCl (0.9%).

Besides the concentration of LL-37, the volume of medium used during treatment and the cell density has high impact on LL-37-cytotoxicity. These factors were kept constant for all compared experiments. To illustrate the volume dependence of LL-37, the degree of induced permeabilization of MG63 cells (assessed as LDH-release) at different volumes and constant LL-37 concentration, is shown in Fig. 12.

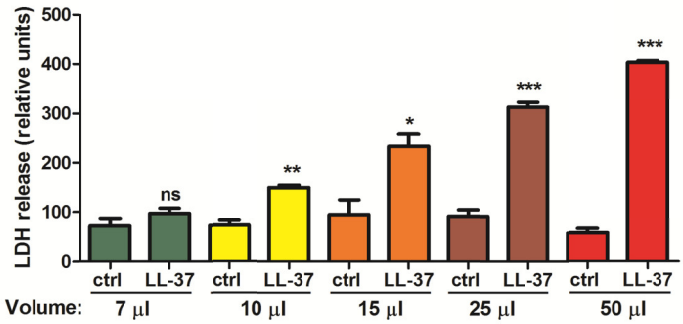

Figure 12. LL-37 effects depend on medium volume. The impact of volume for LL-37 efficiciency was illustrated by assessing permeabilization, as LDH release, from MG63 cells in a 368 well plate. Cells, of constant density, was treated with the same concentration of LL-37 (4 µM) in different volumes. Effects of LL-37 assessed here were much more pronounced in 50 µl of medium compared to 7 µl. Analyzed medium were proportional to the treatment-volumes and diluted with fresh medium to equal volumes for all samples.

DNA synthesis assay

RAW 264.7 and MG63 cells were seeded at a concentration of 40 000 cells/well and incubated for 24 h. The medium was replaced with DMEM containing 2% FBS and cells were treated with LL-37 (5 µM) for 72 h. After this, the cells were labeled with 1 µCi [^{3}H]-thymidine (1 µl/well, PerkinElmer) for 2 hours followed by PBS wash and collection of cells by trypsinization. Cells were dispersed in cold PBS and centrifuged at 4000 rpm for 5 min at 4°C. The cell pellets were lysed in NaOH (5 mM, 175 µl) by sonication on ice. Sonicate (125 µl), NaOH solution (125 µl, 5 mM) and trichloroacetic acid (250 µl, 0.5 M) were mixed in an Eppendorf tube by vortex. The homogenate was centrifuged at 13200 rpm for 2 min, the supernatant discarded and the pellet washed with trichloroacetic acid (300 µl, 0.5 M). The remaining pellet was dissolved in Soluene® 350 (500 µl) for 1.5 h at room temperature (RT) for 1.5 h with mixing by vortex every 30 min. Tubes were then cut open and placed in scintillation vials followed by Optiphase Hisafe2 scintillation liquid (8 ml). The tubes were shaken vigorously and left over night after which each sample was read for 2 min each in a liquid scintillation counter (Wallac Guardian, PerkinElmer).

The remaining cell lysates were used for protein determination by Bio-Rad *DC*™ Protein Assay (BioRad) according to the manufacturer's instructions. DPM-values were normalized to total protein concentration in each sample.

LDH assay

We have estimated LL-37-induced permeabilization in eukaryotic cells by the release of cytoplasmic lactate dehydrogenase (LDH). In this assay, NADH and pyruvate is added to the culture medium. LDH activates the ketone-carboxylic group of pyruvate, thereby functioning as a catalyst for its reduction by NADH. End products will be NAD^+ and L-lactate. Oxidation of NADH to NAD^+ cancels NADHs aromatic character, moving the absorption maximum from 340 nm to 260 nm. LDH concentration can thus be assessed by monitoring the reduction in NADH absorbance at 340 nm [412].

LDH in humans is present as five different tetrameric-isoenzymes, all able to catalyze the reaction [413]. The subunits, of 38 kDa each, gives unmodified LDH a molecular weight of 144 kDa. This is a relatively large protein, with a diameter of approximately 7.4 nm [216]. Pores smaller than the LDH diameter should not be detected providing that no other NADH reducing molecules are present. Morgera *et al.* treated anionic phospholipid membranes (PG lipids) with LL-37 and observed that the toroidal pores had a diameter of approximately 10 nm, suggesting that LL-37 pores may be just large enough to release the LDH protein [414].

LDH release was measured in cells cultivated in 96-well plates. After reaching confluence the cells were washed in PBS and phenol-red free DMEM (D5921, Sigma) supplemented with antibiotics was added (200 µl) followed by LL-37. The cells were incubated at 37 °C for 30 min after which the plate was centrifuged at 1200 *g* for 10 min. Medium (30 µl/well) were collected and transferred to a new 96-well plate for measurement. To each medium sample, a freshly prepared NADH solution was added (180 µl/well, 170 µM β-NADH, 5.6 mM EGTA, 56 mM TRIS) followed by sodium pyruvate (20 µl, 14 mM). The plate was agitated for 5 min at 30 °C in a Multiskan GO Microplate Spectrophotometer (Thermo Scientific) and the absorbance was measured at 340 nm. The LDH release was calculated by the equation:

$$\text{LDH release (\% of total)} = \frac{\text{Abs(sample)} - \text{Abs(blank)}}{\text{Abs(sonicated)} - \text{Abs(blank)}} * 100$$

For LDH measurements of recovery after cell injury by LL-37, cells were treated with LL-37 at different time points before LDH measurement (360, 240, 150, 90, 60 and 35 min). After 30 min of LL-37 treatment in FBS-free medium cells were washed with PBS and fresh culture medium was added in which LDH content was assessed as previously described.

Measurements of intracellular Ca^{2+} levels

LL-37–mediated effects on the intracellular Ca^{2+} concentration were assessed in Fluo-4 AM loaded MG63 and PDL cells. The Ca^{2+}/ Fluo-4 fluorescence was determined at 505 nm (excitation at 488 nm) by laser-scanning confocal microscopy (LSM 510 PASCAL, Carl Zeiss AG). The cells were cultured on glass bottom cell culture Petri dishes (MatTek), washed with HEPES and loaded with Fluo-4 AM (3 μM). After one wash with HEPES the cells were incubated with or without $CaCl_2$ (2.5 mM) for 25 min. For some experiments EGTA (2 mM) was included. The L-type Ca^{2+}-channel antagonists nifedipine (1 μM), the T-type Ca^{2+} channel blocker $NiCl_2$ (100 μM) and the P2RX7 antagonist AZ11645373 (10 μM) were added approximately 15 min previous to LL-37 (4 μM). For effects by conditioned medium, the HEPES was removed during the measurements and instantaneously replaced with cell culture medium after removal of any cells by centrifugation (800 *g*, 5 min).

The Intracellular Ca^{2+} levels were measured as an integrated signal of the Fluo-4 AM emission from all cells in the visual field (x100 magnification).

MTT assay

Cell viability was assessed using the tetrazolium dye MTT. Tetrazolium based assays, the most common of which is MTT, is bench mark technique for assessment of cell viability and probably most common method for evaluation of cytotoxicity *in vitro*. The MTT is taken up by viable cells and reduced in proximity to the cell membrane through the transmembrane electron transport and in the cytoplasm by various electron carriers (e.g. NADPH). Contrary to common belief, most of the reduction is non-mitochondrial [415]. The reduction yields a blue, water insoluble, salt which correlates to the number and integrity of cells.

In our experiments, cells were seeded in 96-well plates. Cells adhering to the bottom of the well (all but THP-1) were allowed to attach for 24 hours in culture medium (10% FBS) before medium was replaced with medium for the treatment, containing 0-2% FBS depending on the cells and duration of treatment. LL-37, 1,25D3 and MBP-p33 were added in DMSO or PBS and appropriate vehicles were added to control groups. LL-37 cytotoxicity was evaluated after 3 or 24 h. Effects of 1,25D3 were monitored after 3-14 days.

After treatments, cells were incubated with MTT (0.5 mg/ml) in culture medium for 1 h. Next, medium was removed and the formazan dissolved in DMSO, and thereafter the absorbance was monitored at 540 nm in a Multiscan GO Microplate Spectrophotometer (Thermo Scientific) and the background absorption was subtracted.

RNA extraction and PCR

For RNA extraction, cells and biopsies were briefly washed with PBS. Tissues were then frozen and pulverized in liquid nitrogen before lysis with QIAzol on ice. RNA was extracted using miRNeasy or RNeasy Minikit (Qiagen) according to manufacturer's instructions. RNA concentration and quality was measured using NanoDrop 2000C (Thermo Scientific).

The RNA samples were analyzed by subjecting them to one-step quantitative real-time RT qPCR using QuantiFast SYBR Green RT-PCR kit (Qiagen), QuantiTect primer assays (Qiagen) and a Step One Plus realtime thermal cycler (Applied Biosystems). Gene expression was calculated using the ΔΔCt method [416]. For cells, GAPDH was normally employed as reference gene after confirming stability with other housekeeping genes. Due to comparably low gene expression in our tissue samples, the sensitivity was increased by using a geometric mean between GAPDH and 18S ribosomal RNA (18S) as the housekeeping reference.

Immunoblotting

For western blotting and dot blotting, samples were lysed in SDS sample buffer (Tris-HCl 62.5 mM, pH 6.8, 2% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitor cocktails (Sigma-Aldrich) and when appropriate, Halt™ phosphatase inhibitors (Thermo Scientific). The samples were sonicated for 10 s, heated (5-10 min, 70 or 100 °C) and centrifuged. Supernatants were collected and total protein determined by a Bio-Rad protein determination kit to assure equal loading. The samples were supplemented with 2-mercaptoethanol (5%) and bromophenol blue (10‰). For dot blotting, proteins were loaded on nitrocellulose membranes (0.5-1µl per dot). For western blotting, proteins were loaded at an amount of 10-80 µg per lane on Criterion TXG ´any kD´,´4-15%´ or ´18%´ precast gels (Bio-Rad) depending on the proteins molecular weight. In some experiments, cell culture medium was loaded on gels and membranes without denaturation or reduction for detecting LL-37/hCAP18.

After gel electrophoresis, proteins were transferred to nitrocellulose membranes by electro blotting overnight at 4 °C or by a Trans-Blot Turbo transfer system (Bio-Rad). The membranes from both western and dot blots were blocked using 0.5% casein in TBS-T and incubated with primary antibodies at 4 °C over night or up to three days. Antibodies used can be found in Table 2. For increasing the immunoreactivity of LL-37/hCAP18 by western blotting, the membranes were pre-treated over night with another LL-37 antibody, a goat polyclonal LL-37 (C-14) antibody (sc-21578, Santa Cruz). In our hands, this antibody did not allow for detection of LL-37 or hCAP18 by itself, while it significantly increased immunoreactivity of the other antibody. The membranes were washed repeatedly in TBS-T and the immunoreactive bands or dots were visualized by chemiluminescence using HRP-conjugated secondary anti-mouse, anti-rabbit or anti-goat antibodies followed by SuperSignal West Femto chemiluminescence reagent (Thermo Fisher Scientific). The immunospecific signals were acquired using a LI-COR Odyssey Fc instrument (LI-COR Biosciences) and analyzed by photo densitometric scanning. The immunoreactive signal of the protein of interest was normalized to GAPDH or Hsp90 immunoreactivity for western blots, serving as internal controls. Normalization to either GAPDH or Hsp90 showed identical results.

Tabel 2. Primary antibodies used for immunoblottning

Protein	Antibody	Source
LL-37/hCAP18	Cathelicidin Antibody OSX12	Novus Bio
p33	p33 clone 60.11, MMS-606R	Nordic BioSite
Vitamin D receptor	VDR Antibody (D-6), sc-13133 B	Santa Cruz
Mucin 1	Mucin 1 Antibody (VU4H5), sc-7313	Santa Cruz
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GAPDH clone 6C5, MAB374	Merck Millipore
Heat-shock protein 90	Monoclonal Hsp90	BD Transduction Laboratories

Immunocytochemistry

For immunocytochemistry, cells were cultured on coverslips in 24 well plates. After reaching appropriate confluence, the cells were rinsed with PBS and fixed in 4% paraformaldehyde (PFA) for 10 min at RT. Alternatively, cells were fixed with either 2% glutaraldehyde (GA) at the same conditions or with methanol at -20 °C for 15 min. Cells were then permeabilized with 0.2% Triton-X100 for 10 min on ice. Interestingly, Triton-X100 has been found to selectively bind to LL-37 though I am unaware of what consequences this could have for LL-37 (immuno-) reactivity [99]. When blocking with GA, residual reactive aldehyde groups present

causing unspecific antibody binding, was neutralized by sodium borohydride reduction after permeabilization (5% *w/v* $NaBH_4$ in PBS, 30 min at RT). Other non-specific binding sites were always blocked with 2% BSA (60 min) before incubation with antibodies overnight at 4 °C. For p33 detection, polyclonal antiserum to p33 raised in rabbits (custom made antibody, Innovagen AB) was used, VDR was detected by the monoclonal D-6 antibody (sc-13133 B, Santa Cruz), both at dilutions of 1:200-1:400 in 2% BSA. After incubation with primary antibodies, cells were incubated with secondary anti-rabbit or anti-mouse Alexa Fluor 555 or Alexa Fluor 488 (Invitrogen) at dilutions of 1:500 for 2 h at RT. The coverslips were then repeatedly washed with PBS, rinsed with water and mounted on microscope slides. To visualize nucleus, the cells were either stained with SYTOX Green (500 nM, Invitrogen) or by mounting coverslips with DAPI loaded histological mounting medium (Fluoroshield, Sigma Chemicals). To stain mitochondria, cells were incubated with MitoTracker (MitoTracker Orange CM-H2TMRos, 0.5 µM, Molecular Probes) for 45 minutes in serum-free medium prior to fixation. Immunoreactivity and organelle staining was analyzed using either an Olympus BX60 fluorescence microscope or a laser scanning confocal microscope (LSM5 PASCAL, Carl Zeiss AG). No immunoreactivity was observed after omission of the primary antibodies.

Protein downregulation with siRNA

Gene expression was transiently suppressed using short interfering RNA (siRNA) constructs purchased from Qiagen. Transfections were performed in Opti MEM medium using Oligofectamine (Invitrogen) transfection reagent. Oligofectamine (40 µl/ml) and siRNA or non-targeting siRNA control (0.1-1 µM) were incubated individually for 5 minutes in medium after which the two components were mixed 1:1. Complex formation was allowed for the following 20 minutes by incubation at RT. Culture medium on cells were replaced with Opti MEM (66 $\mu l/cm^2$) and the RNA/Oligofectamine mixture (41 $\mu l/cm^2$) was added dropwise to the cells. Cultures were then incubated for 24-96 h after which DMEM supplemented with antibiotics and 20% FBS was added in a 1:1 ratio, giving final siRNA concentrations of 20-200 nM. The duration of the serum free period were adjusted according to the cells sensitivity to serum free conditions. During the growth stimulatory period, lasting 48-72h, cells were treated with agents as appropriate. In some cases transfection was repeated a second time for higher efficiency. Before seeding transfected cells for further experiments, variations in cell number were adjusted for when appropriate. For p33, MUC1 and RXRα the Hs_C1QBP_6, Hs_MUC1_7 and Hs_RXRA_3 FlexiTube siRNAs were utilized.

Upregulation of p33 by expression vector

For achieving a transient overexpression of cellular p33, HeLa cells were seeded in 96 and 12-well plates (15 000 or 200 000 cells/well, respectively). After adhesion overnight, the cells were transfected in Opti MEM medium. This was done using Lipofectamine 3000 (6 µl/ml) and P3000 reagent (8 µl/ml) (Thermo Scientific) with either a pcDNA3.1(+)-p33 vector coding for mature p33 without the mitochondrial signaling sequence or a non-coding pcDNA3.1(+) vector (4 µg/ml of either). The transfection medium was applied in volumes of 43 µl per well of 96-well plates and 400 µl per well of 12-well plates. Cells were left under these conditions for 16 h before DMEM culture medium supplemented with 20% FBS was added in a 1:1 ratio and the cells were incubated for another 8h. Cells transfected with the p33-expressing vector contained about two times more of the protein than the control cells.

Statistics

Summarized data presented in column graphs represents means ± SEM. Each culture well, or individual when tissues are analyzed, represents one biological replicate, i.e., one observation (N = 1). Statistical significance was calculated using ANOVA for unpaired multiple comparisons with Tukey´s Multiple Comparison test for post hoc analysis. For single comparisons between two groups, Student´s t test was employed. For the quantitative real-time RT qPCR data from human biopsies, statistical significance was analyzed by using the log-transformed expression data. P values <0.05 were considered significant. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ are indicated in graphs.

RESULTS AND DISCUSSION

Subproject I: LL-37 reduces osteoblast cell viability

LL-37 has detrimental effects on cell viability in osteoblast cell lines at pathophysiologically relevant concentrations

Treatment of both MG63 and hFOB 1.19 human osteoblasts with ≥ 4 µM LL-37 was cytotoxic. The LL-37-induced cytotoxic effect was observed through phase contrast microscopy, showing a reduction in cell number as well as cell shrinkage and membrane blebbing, indicative of apoptosis (Fig. 13B, D). For illustrating the osteoblast-specific attenuation of cell viability by LL-37, RAW 264.7 mouse macrophages were included as negative control where LL-37 (8 µM) had no effect on cell morphology (F). The difference in LL-37-sensitivity between human osteoblasts and mouse RAW macrophages may reflect cell type specific mechanisms but also human vs. mouse cell specific properties.

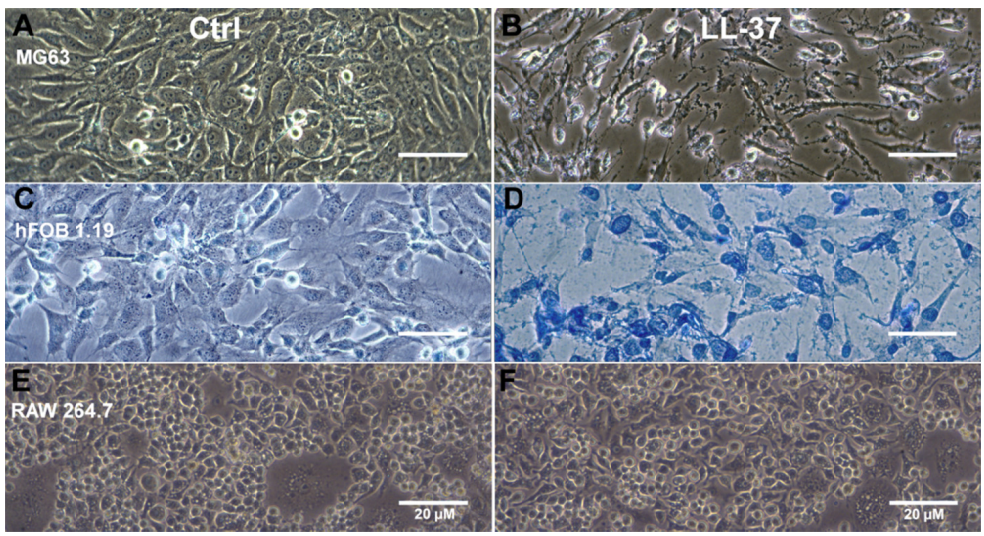

Figure 13. Treatment with LL-37 reduces cell number and alters cell morphology in osteoblasts but not RAW 264.7 macrophages. Treatment of osteoblasts (**A**,**C**) with LL-37 (**B**, **D**) for 24 hours at concentrations of 4 µM for MG63 and 8 µM for hFOB 1.19 causes osteoblast cell shrinkage and membrane blebbing as assessed by phase contrast microscopy. These morphological changes are characteristic for apoptosis. Treatment with 8 µM LL-37 has no effect on RAW 264.7 macrophage morphology (**F**) compared to untreated control (**E**).

Effects of LL-37 on cell viability are highly cell type specific

The MG63 osteoblast cell line was selected for further studies. As assessed by cell counting, treatment with 4 and 8 μM of LL-37 reduced cell number by about 30 and 80%, respectively (Fig. 14A). In RAW 264.7 cells no reduction in cell number could be observed (B).

DNA synthesis was assessed by measuring [^{3}H]-thymidine incorporation into newly synthesized DNA. LL-37 (5 μM) attenuated DNA synthesis in the osteoblasts by about 50%, while, on the other hand, 5 μM LL-37 stimulated DNA synthesis by about 9 times compared to controls in the RAW macrophages, indicating that LL-37 is pro-proliferative in these cells (Fig. 14C, D). In murine fibroblasts, pro-proliferative effects of LL-37 have been reported earlier, an effect thought to be mediated through P2RX7 [114]. To investigate if P2RX7 activation is critical for the stimulation of DNA synthesis, the effect of LL-37 was assessed in the presence of 10 μM AZ11645373, a P2RX7 antagonist attenuating most P2RX7 mediated effects in mouse cells at the used dose [417]. Inhibition of P2RX7 did not attenuate LL-37 stimulated DNA synthesis, suggesting a mechanism independent of P2RX7 (data not shown).

Since LL-37 is known to form pores in cell membranes, the degree of permeabilization was assessed by measuring release of the intracellular protein lactate dehydrogenase (LDH). As can be seen in Fig. 14E and F, both cell types are permeabilized to the same extent by LL-37, hence membrane disruption is not necessarily correlated to cytotoxicity. Staurosporine (0.5 μM) was used as a positive control for apoptosis in the macrophages, resulting in a >90% reduction in cell number after 24 hours (data not shown).

The lack of cytotoxicity in macrophages following LL-37 permeabilization is likely due to the nature of pores formed by LL-37, described as being well defined toroidal pores of limited diameter ([112, 418]). A general membrane disruption, i.e. detergent-like permeabilization, would assumedly be more difficult for cells to cope with.

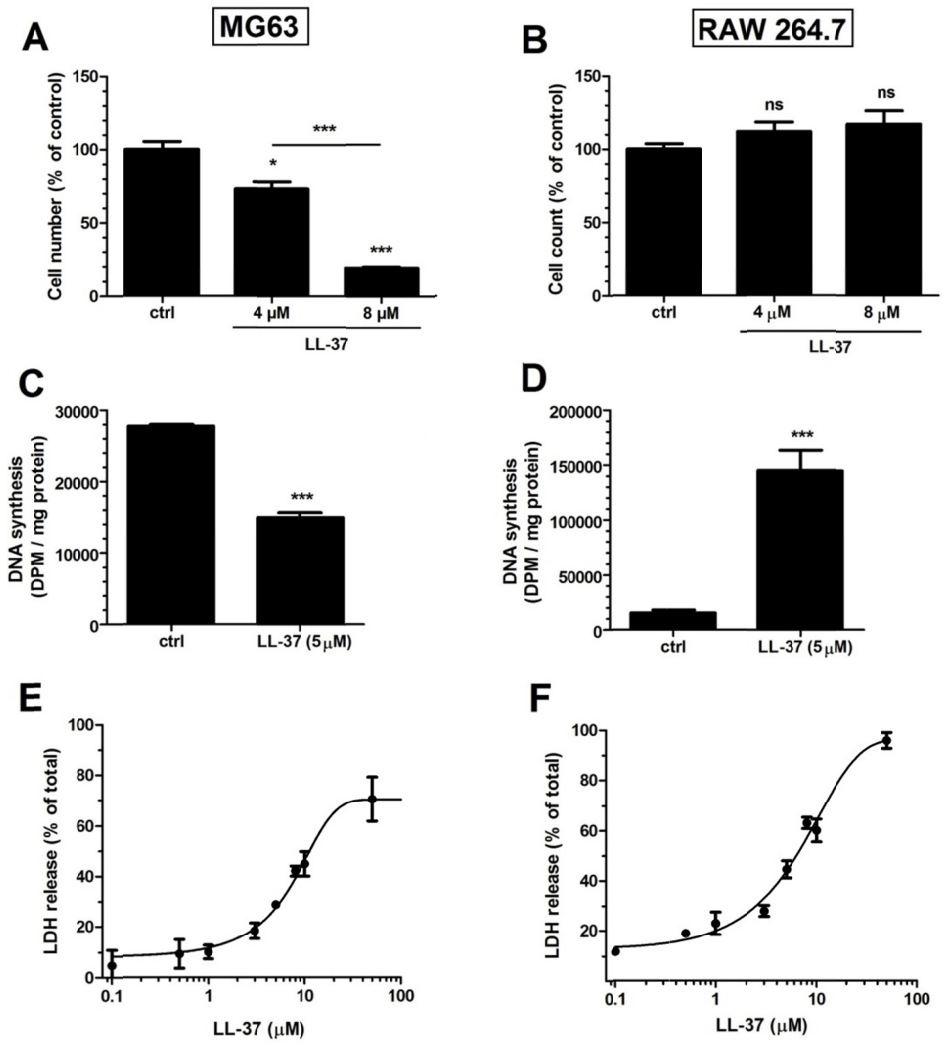

Figure 14. LL-37 reduces cell viability in MG63 osteoblasts but not in RAW macrophages, despite permeabilizing both celltypes. Treating MG63 with LL-37 causes a dose dependent reduction in cell number with an estimated EC_{50} value of around 5 µM (**A**). The same treatment in RAW macrophages has no detrimental effects, rather showing a trend of increased cell number (**B**). LL-37 (5 µM) attenuates DNA synthesis in MG63 osteoblasts to about half of that in control cells, assessed by incorporation of radio-labeled thymidine (**C**). Conversley, treatment with LL-37 stimulates DNA synthesis by about 9 times in RAW 264.7 cells (**D**). Both cell types are permeabilized in a dose dependant manner by LL-37 to about the same extent as determined by measuing release of LDH during 30 min of treatment (**E**-**F**).

LL-37-induced apoptosis in MG63 cells was confirmed by annexin V flow cytometry. Only cells in early stages of apoptosis were counted, necrotic cells were excluded through nuclear staining with 7-Aminoactinomycin D (7-AAD). Stimulation with LL-37 (4 µM) for 6 h caused an 80% increase in the proportion

of apoptotic (annexin V^+, 7-AAD^-) cells compared to controls. No effect was seen after a 2 h treatment. In line with caspase dependent apoptotic signaling, LL-37 (4 μM) increased the cellular levels of cleaved caspase 3 by about 2.5 times, as determined by ELISA after 24 h of treatment (Appendix I, Fig. 4).

The lack of correlation between cytotoxicity and permeabilization prompted us to look at cell membrane recovery after LL-37 treatment. This was done by treating cells with LL-37 at a dose only causing release of a minor fraction of the total cellular LDH, for a short time. After treatment with LL-37, the peptide-containing medium was replaced with fresh culture medium, allowing the cells to equilibrate for 5-550 min. This procedure was followed by a second medium replacement, into which LDH was allowed to leak for 30 min before analysis.

LDH measurements suggest that 5 min of LL-37-free conditions is enough for the cells to recover, as all induced LDH release is abolished by this incubation. The pores formed by LL-37 are apparently rapidly reversed to sizes smaller than those required for LDH to escape (Fig. 15A). The cell membrane integrity was confirmed after 2 h of LL-37 treatment using flow cytometry of MG63 cells incubated with the DNA staining dye 7-AAD. Treated cells did not show any increased 7-AAD internalization (B). Staurosporine (STS) was included as a positive control.

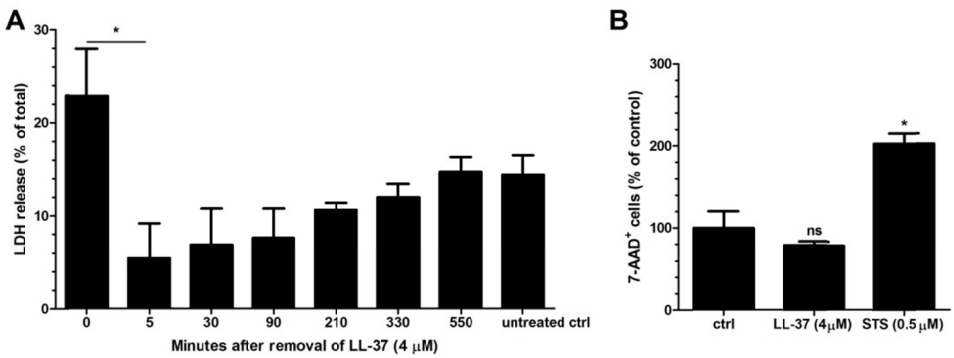

Figure 15. Cell permeabization of osteoblasts by LL-37 is a reversible process. Pores caused by LL-37 on MG63 osteoblasts are quickly reversed after LL-37 is removed. MG63 cells were stimulated with LL-37 (4 μM) for 30 min before LL-37 medium was replaced by fresh medium. LDH release was measured at specific time points 5–330 min after the removal of LL-37 (**A**). Five minutes after the removal of LL-37, LL-37-evoked LDH release had decreased to a value lower than that of the untreated control. Thereafter, LDH release showed a slight increase with time after the removal of LL-37 until reaching that of the untreated control. Flow cytometry of cells after 2 h of LL-37 treatment (4 μM) confirmed that there were no attenuation of membrane integrity in the LL-37 treated group at this time (**B**). Membrane integrity was assesed by 7-AAD nuclear staining and staurosporine (STS, 0.5 μM) was included as a positive control.

LL-37 mediates Ca^{2+} influx in osteoblasts without affecting cell viability

In Ca^{2+} containing HEPES, LL-37 induces an acute increase in intracellular Ca^{2+} concentration in MG63 osteoblasts. This is demonstrated by laser-scanning confocal microscopy measuring emission from Fluo-4 AM, a dye sensitive to intracellular Ca^{2+} (Fig. 16A). The increase in $[Ca^{2+}]$ is dose-dependent, 4 μM of LL-37 causes a more pronounced influx than 0.4 μM. The thromboxane A_2 analogue U46619, known to causes a rapid rise of intracellular $[Ca^{2+}]$, was included as positive control (B). A rise in intracellular Ca^{2+} could be due to an inflow of Ca^{2+} from the extracellular space but Ca^{2+} could also be released from intracellular stores such as the endoplasmic reticulum [419]. Under Ca^{2+}-free conditions, no increase in Ca^{2+} could be seen, indicating that Ca^{2+} mobilization is caused by influx from the extracellular space and not by release from intracellular compartments (C).

Activation of the P2RX7 is known to stimulate Ca^{2+} influx [420], but the LL-37-induced Ca^{2+} influx observed here appears independent of this receptor, as shown by unaltered LL-37-stimulation of Ca^{2+} inflow in the presence of the P2RX7 antagonist AZ11645373 (Fig. 16D). MG63 cells have been reported to express voltage-sensitive Ca^{2+} channels [421] and therefore some experiments were performed in the presence of nifedipine (L-type Ca^{2+} channel blocker) and $NiCl_2$ (T-type Ca^{2+} channel blocker). Neither of these drugs was able to attenuate the LL-37-induced Ca^{2+} influx (E, F).

These results strongly suggests that the LL-37-induced Ca^{2+} influx is due to membrane permeabilization, in accordance with the observed LDH release.

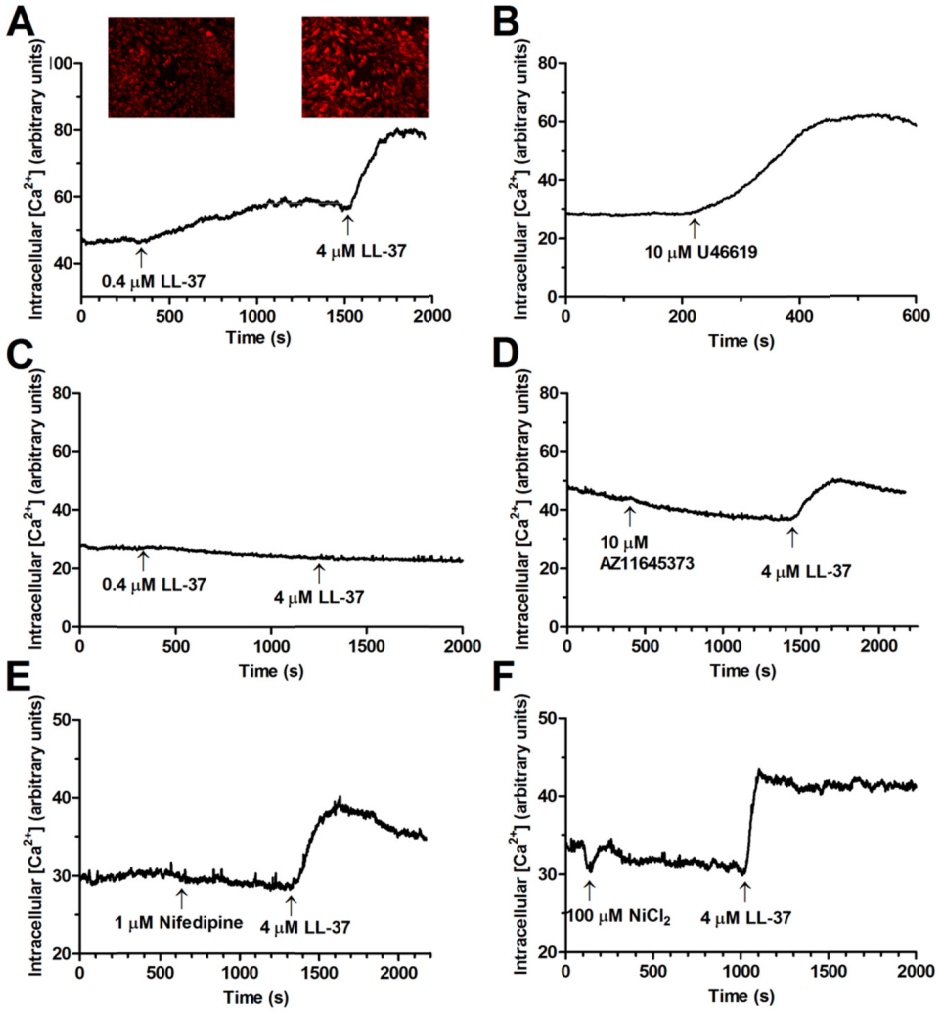

Figure 16. LL-37 causes a rapid increase in intracellular Ca^{2+} levels through permeabilization of the cell membrane. Treating MG63 with 0.4 or 4 μM of LL-37 causes a rapid, dose-dependant, increase in intracellular Ca^{2+} concentration. Panels on top illustrates Fluo-4 AM emission before (left) and after (right) addition of LL-37 (4 μM) (**A**). Ca^{2+} influx was also observed after treating cells with U46619, employed as a positive control (**B**). No increase in intracellular Ca^{2+} levels was observed in Ca^{2+} free surroundings (**C**). Influx of Ca^{2+} was not blocked by the P2RX7 antagonist AZ11645373 (**D**), nor by the Ca^{2+}-channel blockers nifedipine (**E**) or $NiCl_2$ (**F**).

The Ca^{2+} ion is critical for a range of cellular events, and a dysfunctional regulation of Ca^{2+} homeostasis has been known to result in apoptosis [419, 422]. For instance, LL-37 induced apoptosis in Jurkat T cells may be Ca^{2+}-dependent as Ca^{2+} is critical for facilitating calpain activation, a key step in the apoptotic signaling cascade [270].

Hence, we wished to find out if LL-37-evoked reduction of osteoblast cell viability is dependent on the Ca^{2+} influx. To investigate this, effects of LL-37 on MG63 cells were evaluated in the presence or absence of extracellular Ca^{2+}. Reduction in cell number by LL-37 was not weakened by exclusion of Ca^{2+} (Fig. 17A). These results suggest that LL-37-induced cytotoxicity occurs independent of Ca^{2+} influx.

Modulation of Ca^{2+} levels in the culture medium by other means supports this conclusion. Supplementation with the chelator EGTA (1.8 mM), which binds Ca^{2+} ions present at equimolar concentration in the medium, had no effect on LL-37-evoked cytotoxicity. One experimental group was further supplemented with an excess of $CaCl_2$. LL-37 (4 µM) for 24 h reduced the MG63 cell number to the same extent under both conditions (Fig. 17B).

Since LL-37 has been known to induce apoptosis in certain cells through a (yet unknown) pertussis toxin (PTx) sensitive GPCR, we pre-treated MG63 cells with PTx for 2 h prior to LL-37 treatment, through which PTx was present. Cell viability was here assessed by the MTT assay after 3 h with LL-37, showing identical response with or without PTx (Fig. 17C). The absence of effect by PTx (40 ng/ml) on the reduction in MG63 viability was confirmed at 24 h of LL-37 treatment as well (data not shown).

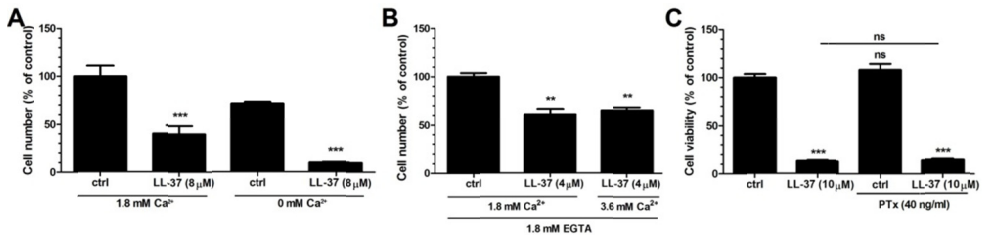

Figure 17. LL-37 reduces MG63 cell number irrespective of exclusion (A) or chelation (B) of Ca^{2+} or PTx (C). Ca^{2+} depletion impacts the cell number negatively after 24 h as can be seen on control groups of **A**. This promotes the reduction in cell number by LL-37, 8 µM of the peptide attenuates cell number by 60% in the presence of Ca^{2+} and 85% in its absence (**A**). After chelating Ca^{2+} with EGTA in equimolar concentration, reduction in cell number by LL-37 (4 µM) was 60%. The reduction in cell number was not influenced by adding an excess of Ca^{2+} (**B**). Pretreatment of MG63 by PTx (40 ng/ml) with LL-37 (10 µM) did not influence the LL-37 induced attenuation of cell viability, assessed by the MTT assay (**C**).

Both the intrinsic and caspase-independent apoptotic signaling cascades reported for LL-37 require an upregulation and nuclear translocation/activation of the transcription factor p53 (as shown in Fig. 8). LL-37-treatment of MG63 cells causes a rapid and substantial increase in p53 protein expression, as determined by western blotting (data not shown). However, pifithrin-α (15-40 µM), an inhibitor of p53 transcription, was not able to rescue cells from LL-37-induced cell death, either at 3 or 24 h of LL-37 treatment (data not shown). p53 mediated transcription is critical for p53s classical apoptotic effect, although transcription-independent p53-mediated apoptosis has also been reported which cannot be ruled out. To

investigate involvement of P2RX7, MG63 cells were treated with or without the antagonist AZ11645373 (20 μM). This did not alter the outcome of 3 or 24 h treatment with LL-37 (data not shown). Furthermore, LL-37 may activate NF-κB, a well-known response to stimulation of this transcription factor is apoptosis [423]. The NF-κB inhibitor santamarin (10 μM, [424]), did not modulate LL-37-induced cytotoxicity in MG63 cells, either at 3 or 24 h of LL-37 treatment.

In conclusion, these data indicates that LL-37-evoked cytotoxicity in MG63 osteoblasts is independent of Ca^{2+} influx as well as GPCR and P2RX7 activation and p53/NF-κB transcription.

Mineralized osteoblast-like cells are sensitive to LL-37

To investigate if osteoblast mineralization might protect cells against LL-37 cytotoxicity in bone, primary periodontal ligament cells (PDL cells) were differentiated towards an osteogenic osteoblast-like phenotype [425]. The osteogenic differentiation resulted in mineralized nodule formation and abundant calcium deposits (detected by Alizarin Red S staining) after 3 weeks.

PDL cells were cultured in either osteogenic differentiation medium or normal cell culture medium in a 96 well plate for three weeks. Cells were then treated with 2 or 4 μM of LL-37 for 3 h in culture medium without FBS. LL-37 reduced cell viability in cells cultured under bot normal and osteogenic conditions (Fig. 18).

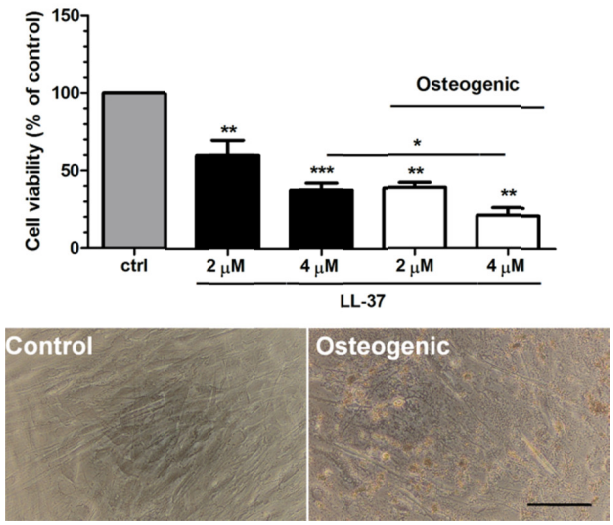

Figure 18. Osteoblast-like PDL cells are sensitive to LL-37-induced cytotoxicity. PDL cells were cultured for three weeks in either osteogenic differentiation medium or normal culture medium and treated with LL-37 (2 and 4 μM, 3 h). Cell viability, assessed by the MTT assay, shows that LL-37-treatment causes an attenuation of cell viability under both culture conditions. Osteogenic and non-osteogenic groups are nomalized to their respective control, set as 100%. Morphology of cells prior to LL-37 treatment are shown in the lower panel. Bar = 50 μm.

Subproject II: Defence systems against LL-37-induced cytotoxicity

It is reasonable to suggest that LL-37-induced cytotoxicity is balanced by endogenous protective systems. For example, due to the high cytotoxicity of LL-37 in osteoblasts at patophysiologically relevant concentrations, it appears plausible that LL-37 takes part in the degradation of alveolar bone during chronic periodontitis. The LDH assay was used to assess LL-37 permeabilization in the presence of compounds suspected to attenuate the effect of LL-37 on cell integrity. A submaximal dose of 4 μM LL-37, resulting in a significant but far from complete release of LDH, was used in these experiments.

Permeabilization by LL-37 is not affected by selected steroids or ions

The polyamines spermidine and spermine were investigated with the hypothesis that organic cations such as polyamines would compete with LL-37 binding sites on the outer membrane of the cell. These experiments were done in the presence of aminoguanidine to prevent the formation of toxic catabolites due to oxidation of polyamines [426]. A third type of polycations, poly-L-lysine HBr (MW: 4000-15000 kDa), was also investigated. None of the compounds affected LL-37-induced permeabilization (Fig. 19A), and furthermore, polyamines had no effect in LL-37-induced cytotoxicity assessed by measuring cell number (data not shown).

We went on to test a number of steroids by the same experimental design, since steroids are known to influence plasma membrane characteristics. 17β-estradiol (E2), for example, has been shown to increase membrane fluidity [427]. Pretreatment for 1 h with E2, 17α-estradiol (E3) or progesterone (P4) had no effect on LL-37-evoked LDH release as shown in Fig. 19B. Lack of effect by E3 on LL-37-induced cell death was confirmed by assessment of cell number (0.1-5 μM E3, 24 h pretreatment followed by treatment with LL-37 for 24 h, data not shown).

In model bilayers, cholesterol content has been shown to regulate LL-37-evoked membrane permeabilization [287, 428], but this has to my knowledge not been confirmed in cells. We treated MG63 cells with various concentrations of cholesterol–methyl-β-cyclodextrin, a commonly used substance for loading cells with cholesterol, for 24 h previous to LL-37 treatment. This had no impact on LL-37-induced LDH release (Fig. 19C). We further observed unaltered effects of LL-37 (24 h) on the number of MG63 cells treated with or without complexed cholesterol (data not shown). However, to rule out that cholesterol is protective against LL-37 permeabilization in our cells, quantification of incorporated cholesterol would be needed.

Next, trisodium citrate was tested in various concentrations. Citrate is a potent anion changing the hydrophobic nature of proteins according to the Hofmeister series [429]. Citrate was therefore suspected to cause increased helicity and oligomerization of LL-37, but no changes in cellular LDH release were observed (Fig. 19D).

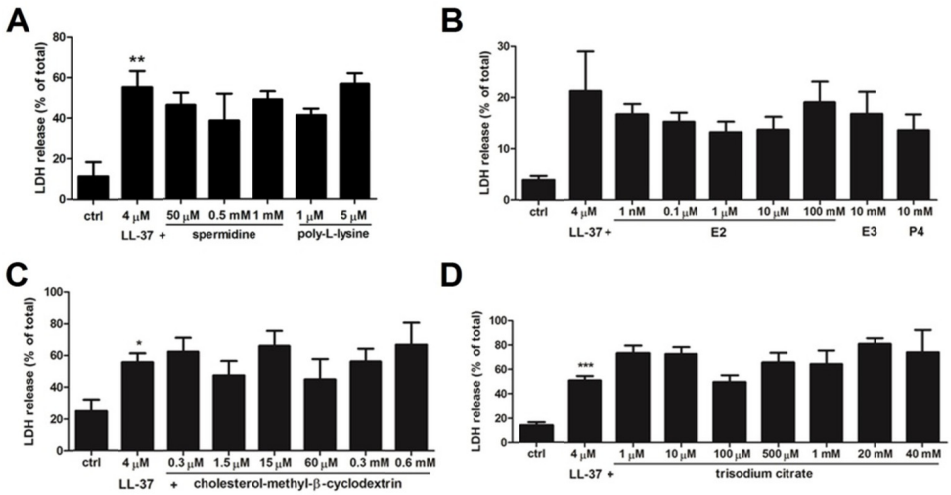

Figure 19. Neither polyamines nor steroids or citrate affects LL-37 induced permeabilization. Presence of polyamines did not affect LL-37 induced LDH release in MG63 cells (**A**), and neither did the common steroids 17β-estradiol (E2), 17α-estradiol (E3) or progesterone (P4) affected LL-37-induced LDH release (**B**). Cholesterol loading of cells by cholesterol-methyl-β-cyclodextrin for 24 h prior to LL-37 treatment (**C**) or trisodium citrate (**D**) also left the LL-37-induced cell perforation unaltererd.

Mucin 1 attenuates LL-37-induced cytotoxicity and is expressed in osteoblasts

Mucins are abundant salivary proteins reported to inactivate LL-37 [288]. We also observed this effect, using commercially available bovine mucin I-S. This mucin dose dependently (0.1–5 mg/mL) inhibits LL-37-induced LDH release (Fig. 20A). Sialic acid moieties attached to the mucin protein is implied to be critical for this interaction, forming ionic bonds to the cationic peptide [258]. The most abundant of the active N-linked sialic acids in mucin 1 is N-acetylneuraminic acid (NANA). In Fig. 20B, we show that NANA has no effect on LL-37-induced LDH release, indicating the importance of the protein backbone, perhaps affecting structure conformation or supplying alternative interactions between LL-37 and mucin.

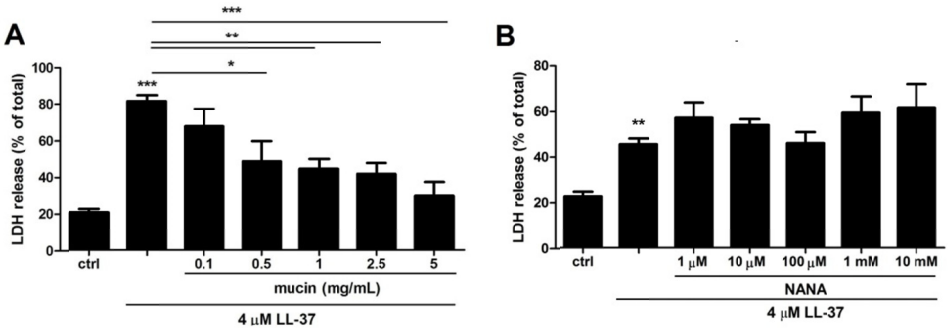

Figure 20. Mucin 1, but not N-acetylneuraminic acid, protects cells from LL-37-induced permeabilization. Bovine mucin I dose-dependently attenuates LL-37 induced LDH release of MG63 cells (**A**). The mucin moiety N-acetylneuraminic acid (NANA) has no effect on LL-37-induced LDH release at concentrations of 1 μM-10 mM (**B**).

Mucin can bind to the cell surface and here it has several important functions, particularly in the airway epithelium [430]. We looked at the cellular expression of mucin 1 (MUC1), the most abundant human mucin with documented effects on LL-37, to investigate if this could be an endogenous protective system against LL-37-induced cytotoxicity. We found that osteoblasts, in particular MG63 cells, express MUC1, both on transcriptional and protein level (Fig. 21A, B). The antibody only detects one band of MUC1 with a high molecular weight (>250 kDa). The native unmodified form is 122 kDa, suggesting that the observed protein is heavily glycosylated.

Neuraminidase (NA) are enzymes which cleave the glycosidic linkages of neuraminic acids such as NANA. By pre-treating the MG63 cells with one such enzyme (N2876, Sigma-Aldrich), the cells appear to become slightly, but significantly, sensitized to LL-37 (Fig. 21C), possibly by removing sialic acids from surface proteins including MUC1. At higher concentrations of NA the effect is reversed. One explanation for this could be that the cell gets depolarized when the acids are lost; a negatively charged surface facilitates LL-37 interactions.

To find out if MUC1 is involved in the cellular resistance against LL-37 we went on to knock down the expression by employing siRNA. As seen in Fig. 21D the MUC1 mRNA expression was attenuated by almost 90% by this treatment. However, this did not correlate to an increased sensitivity against LL-37, as illustrated by the MTT viability assay (Fig. 21E) and LDH assay (F), suggesting that cellular MUC1 does not act as a scavenger for LL-37.

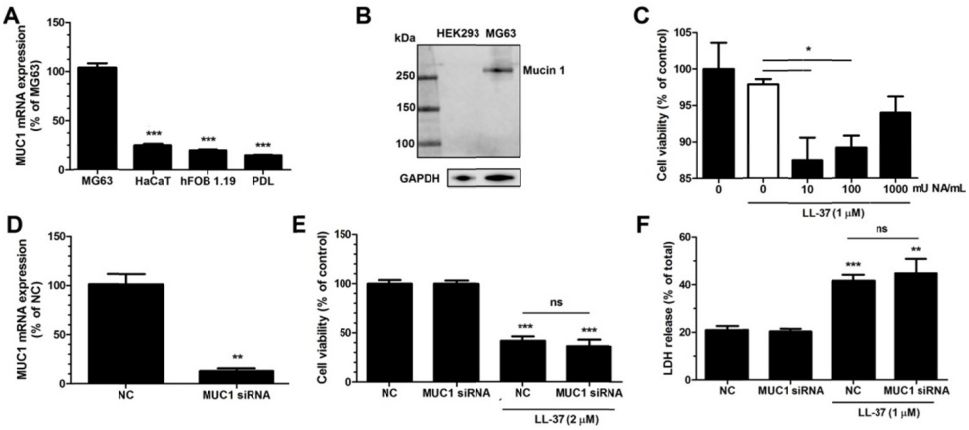

Figure 21. MUC1 is expressed in MG63 cells but does not appear to protect them against LL-37. MUC1 expression is highly expressed in MG63 cells compared to other human cell types on the transcriptional level (**A**). Western blotting detects one band at >250 kDa from MG63 lysate, while HEK293 lysate, serving as negative cotrol shows no immunoreactivity (**B**). Pretreatment of MG63 with a neuraminidase (NA, 10-100 mU/ml), cleaving off neuraminic acid groups on the surface, increases sensitivity against LL-37 (**C**). Downregulating MUC1 expression by siRNA (**D**), however, has no effect on LL-37 sensitivity assessed by the MTT (**E**) and LDH (**F**) assays.

While MUC1 does not protect MG63 cells from LL-37, the peptide was found to negatively modulate the protein expression in MG63 cells, both on transcriptional and protein level (Fig. 22A, B). The downregulation was not affected by the presence of AZ11645373 (20 μM), proving the effect to be P2RX7 independent (data not shown). The downregulation of MUC1 by LL-37 is cell type specific, in HaCaT keratinocytes the same treatment had an opposite effect on the gene expression (C).

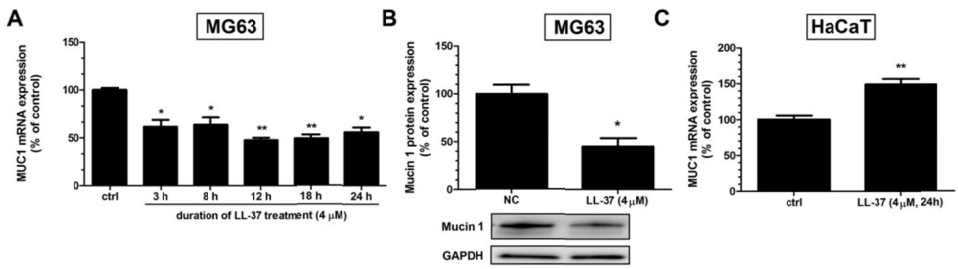

Figure 22. LL-37 downregulates MUC1 expression in MG63 and upregulates it in HaCaT. LL-37 (4 μM) attenuates MUC1 mRNA levels in MG63 cells at 3-24 h (**A**). This correlates to reduced MUC1 levels after 48 h of treatment (**B**). In HaCaT cells, LL-37 on the other hand stimulates the MUC1 mRNA expression (**C**).

Exogenously applied p33 protects host cells from LL-37-induced cytotoxicity

p33 (qC1qR), a 33 kDa protein expressed in almost all host cells, was recently found to bind cationic host defence peptides [303]. To investigate the effects of p33 on LL-37-induced permeabilization and cytotoxicity, p33 was recombinantly produced in *E. coli* and fused to maltose binding protein, giving it its native trimeric conformation. We found that 10 µM p33 (molarity counted as monomers), was able to completely abolish the LL-37 induced LDH release from MG63 and hFOB1.19 cells (Fig. 23A, B). Furthermore, the Ca^{2+} influx caused by 4 µM LL-37 was strongly reduced and delayed in the presence of 10 and 20 µM of p33 in MG63 cells (C).

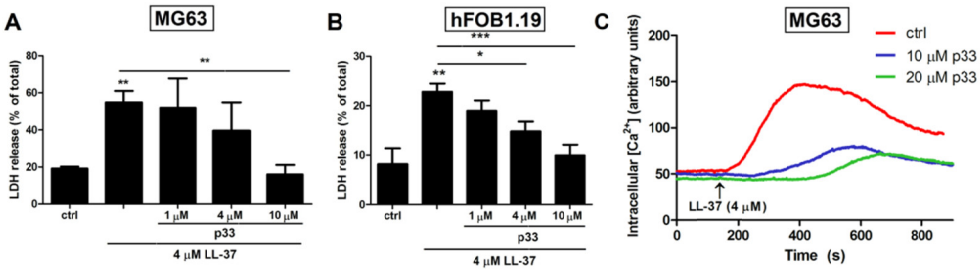

Figure 23. p33 blocks LL-37 induced permeabilization of osteoblasts. LDH release from MG63 (**A**) and hFOB 1.19 (**B**) cells after treatment of LL-37 (4 µM) in presence of p33 protein. Treatment with LL-37 (4 µM) causes an acute and sustained rise in the intracellular Ca^{2+} concentration, as demonstrated by laser-scanning confocal microscopy of MG63 cells loaded with Fluo-4 AM Ca^{2+} dye (**C**, ctrl). In contrast, p33 dose-dependently delays and reduces the LL-37-induced Ca^{2+} response.

The protective effect of p33 was confirmed by assessing cell number and morphology. Treatment with 8 µm LL-37 for 24 h reduced the number of MG63 cells by about 50%. LL-37-induced attenuation of cell number was completely reversed by 10 µM p33 (Fig. 24A). The LL-37-treatment causes cell shrinkage as indicated by arrows in (C), an early sign of cell death, compared to untreated controls (B). MG63 cell morphology was rescued by p33, no impairment of cell structure could be observed in cells co-treated with p33 and LL-37 (D).

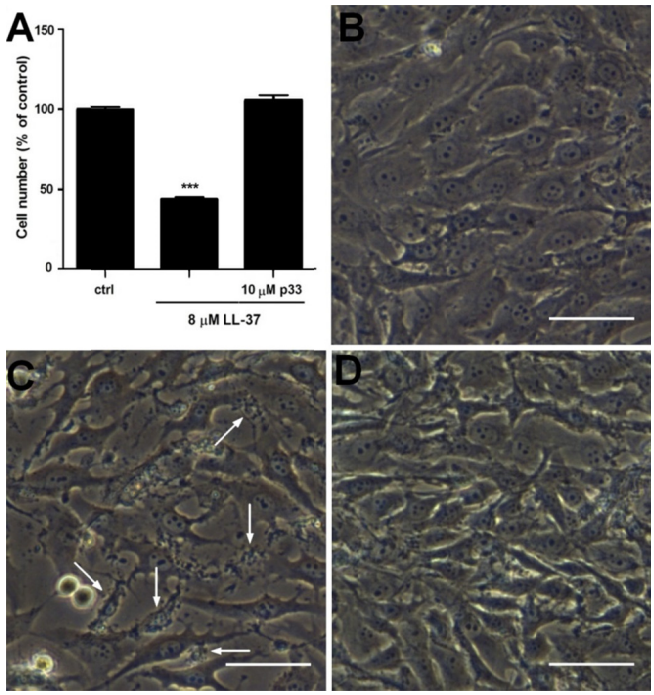

Figure 24. Reduction of MG63 cell number after 24 h of treatment with 8 μM LL-37 is completely reversed by 10 μM of p33. MG63 cells treated with vehicle control (**B**), compared to cells treated with 8 μM LL-37 (**C**) and cells treated with 8 μM LL-37 in the presence of 10 μM p33 (**D**). Cells treated with LL-37 alone (**C**) display shrinkage and membrane blebbing representative for apoptosis (arrows). Bars = 20 μm.

p33 is found in the cytoplasm, mitochondria and at the cell membrane of host cells

We went on to study endogenous p33 in host cells to investigate if it could protect against LL-37-induced cytotoxicity. For this we employed MG63 cells but also three other human cell types with both ectodermal and mesodermal origin and different sensitivity to LL-37: HaCaT keratinocytes, human dermal fibroblasts (HDFa) and periodontal ligament cells (PDL cells). Both HDFa and PDL are primary fibroblast-like cells while the cell line HaCaT represents an epithelial, LL-37 producing, cell type.

Initially, the endogenous p33 expression was analyzed through visualization by microscopical techniques. p33 immunoreactivity in MG63 cells detected by laser scanning confocal microscopy indicates mainly cytoplasmic p33, with less nuclear staining (Fig. 25A). Double-staining for p33 immunoreactivity and a mitochondria-selective dye (MitoTracker, B) indicates that p33 is mainly localized to the mitochondria (C). HaCaT, PDL and HDFa cells all showed the same degree of mitochondrial staining for p33 demonstrated by immunocytochemistry (HaCaT shown in Appendix III, Fig. 1C-E).

The sub-cellular distribution of p33 was further analyzed by immunogold labelling electron microscopy (EM). p33 immunoreactivity, seen as black dots, was here found at the mitochondrial periphery, at the plasma membrane and in some parts within the cytosol of MG63 (Fig. 25D). A similar distribution pattern for p33 was recorded in HaCaT (E) as well as in PDL and HDFa cells (Appendix III, Fig. 3).

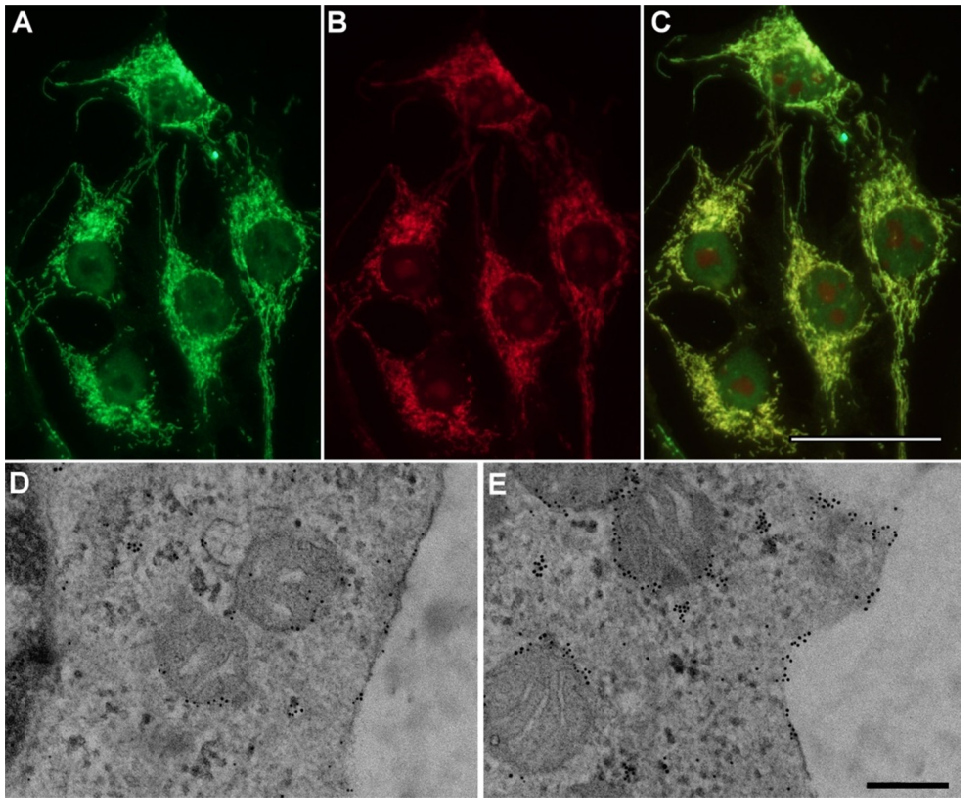

Figure 25. p33 localization in MG63 and HaCaT cells. p33 is localized to the mitochondria in human MG63 cells, as seen by laser scanning confocal images of MG63 cells stained for p33 immunoreactivity (**A**, green) and MitoTracker (**B**, red), overlay (**C**) indicates co-localization by yellow. Immunogold EM of MG63 (**D**) and HaCaT (**E**) reviels p33 pools also in the cytoplasm and in periphery to the plasma membrane. Scale bars = 10 µm (C) and 250 nm (E).

While immunogold EM shows expression of p33 in mitochondria, cytoplasm and in proximity to the cell surface, immunoreactivity observed by confocal or conventional fluorescence microscopy rather indicates more or less complete co-localization to the mitochondria. In fact, there has been a long-lasting debate regarding p33 localization outside of the mitochondria, and in particular its cell surface expression [431]. The inconsistency of data regarding this has been suggested to be due to varied antibody selectivity for the membrane associated protein [432]. Here, our two techniques overall show similar subcellular distribution of p33 but also indicate some different distribution patterns in the same cells, using the same antibody. While this may reflect different sensitivity of

fluorescence microscopy and immunogold EM, I propose that it could also be due to chemical translocation of p33. It has been reported that some proteins may translocate during chemical fixation with paraformaldehyde (PFA) and alcohols [433]. While we employed PFA fixation for fluorescence microscopy, glutaraldehyde (GA) in sodium cacodylate buffer was utilized for EM fixation. When p33 immunoreactivity is detected by fluorescence microscopy after fixation with PFA, a somewhat different distribution compared to that of GA fixation can be observed (Fig. 26A and B compared to C), indicating less non-mitochondrial protein. This data is inconclusive, but may suggest that p33 is redistributed during certain types of chemical fixation, and that this would explain why we see an almost exclusive mitochondrial localization by immunocytochemistry but also specific localization to the membrane and cytoplasmic bodies by EM. Perhaps this may also explain the discrepancies reported in previous studies. In fact, reports describing preferentially mitochondrial localization and an absence of membrane associated p33 employ PFA, or in one case alcohol, as cell fixatives [431, 434-437]. In our cells, a similar pattern as with PFA was observed by methanol fixation (D).

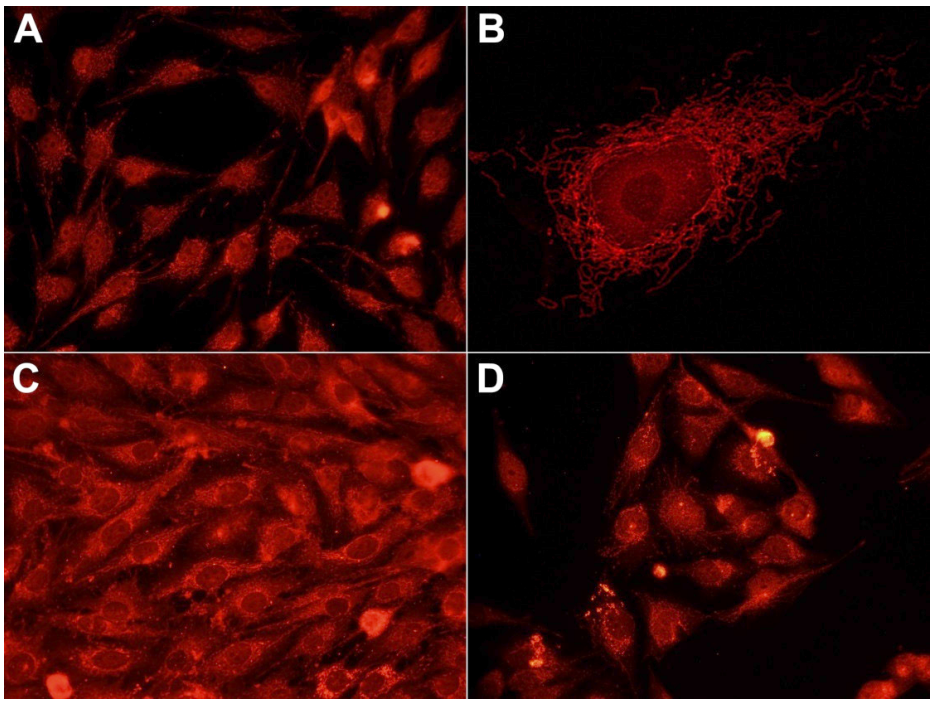

Figure 26. p33 immunoreactivity in MG63 cells detected after fixation with 4% PFA (A, B), 2% GA (C) and 100% methanol (D). These different fixation procedures results in different subcellular p33 staining. Strong mitochondrial and weak nuclar staining was observed after all preparations. **B** shows a high resolution magnification of one cell after PFA fixation by confocal microscopy, indicating no immunoreactivity outside of the mitochondrial network. Unlike PFA and MeOH fixation, GA fixation (**C**) also indicate cytoplasmic and/or membrane associated immunoreactivity, as seen by immunogold EM. Nonspecific antibody binding to introduced aldehyde groups after GA fixation was avoided by reduction of the fixed cells (5% $NaBH_4$ in PBS for 30 min).

LL-37-induced cytotoxicity correlates to cellular expression of p33

Four human cell types were evaluated for their LL-37 sensitivity both in regards of cell viability and permeabilization. Treatment with LL-37 for 3 h causes a concentration-dependent attenuation of cell viability assessed by the MTT assay in all cell types but they show high variability in sensitivity, HaCaT were the least sensitive while PDL showed the highest sensitivity (Fig.27A). EC_{50} values were as follows: HaCaT = 9 μM, MG63 = 5 μM, HDFa = 4 μM and PDL cells = 1.7 μM. There was a prominent LDH release in all four cell types (B), suggesting that LL-37 causes a similar degree of membrane permeabilization, and furthermore, may point to an intracellular mechanism for LL-37-induced cytotoxicity. This is in agreement with the previous observations on RAW 264.7 macrophages, where LL-37 induces permeabilization but not cytotoxicity. The LL-37-evoked decrease in cell viability was also reflected in morphological changes, such as cell shrinkage, and a reduction in cell number. LL-37-evoked cell shrinkage was more pronounced in PDL compared to HDFa fibroblasts (Appendix III, Fig. 5).

The p33 expression, quantified by western blotting, demonstrated that the sensitivity to LL-37-induced cytotoxicity negatively correlates to p33 expression: HaCaT cells showed the highest p33 level and PDL cells the lowest. HDFa and MG63 cells exhibited similar expression (Fig. 27C).

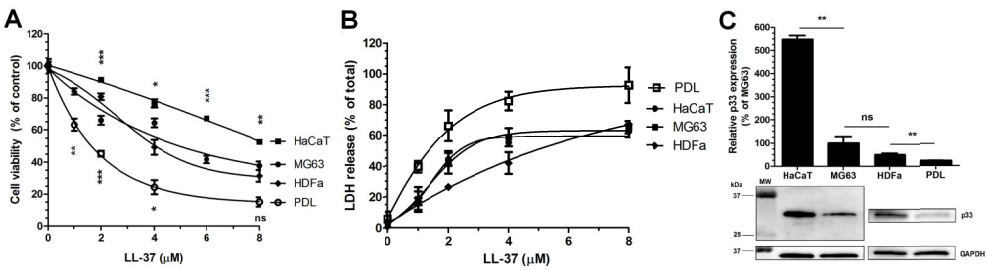

Figure 27. LL-37 attenuation of cell viability does not correlate to the degree of permeabilizaton but rather the cellular expression of p33. MG63, HaCaT, PDL and HDFa cells were incubated with LL-37 and cell viability was assessed after 3 h of treatment by the MTT assay (**A**). LDH release was analyzed after 30 minutes (**B**). p33 expression was determined in lysed cells through analysis by western blotting (**C**). For western blotting, p33 immunoreactivity was normalized to that of GAPDH serving as internal control.

Next, our aim was to show that LL-37-induced cytotoxicity depends on the level of p33 expression. We downregulated p33 mRNA and protein through transfection of cells with p33 siRNA. Treatment with p33 siRNA reduced p33 mRNA and protein level in MG63 and HaCaT cells by 80-95% (Appendix III, Fig. 6), affecting all cellular compartments of p33 as demonstrated by EM (Appendix III, Fig. 2B, D).

The p33 siRNA increased sensitivity to LL-37 in the HaCaT cells (Fig. 28A) but not in MG63 cells (Appendix III, Fig. 7A). A likely explanation for this is that HaCaT cells contain approximately six times higher levels of p33 compared to MG63 cells (Fig. 27C) and therefore the absolute decrease in p33 is greater in HaCaT compared to MG63 cells. Interestingly, the p33 knockdown had no effect on LL-37-induced permeabilization in neither of the cells, in line with similar LDH release observed for all cell types. This suggests that p33 does not protect cells from LL-37-evoked permeabilization, but protects the cells from LL-37-induced cytotoxicity via an intracellular mechanism.

Next, we confirmed the importance of intracellular p33 for regulation of LL-37-induced cytotoxicity by a gain of function experiment. Upregulation of p33 protein expression was achieved by transfection of HeLa cells with a p33 expressing pcDNA3.1 vector. Neither of our previously used human cell types turned out to be susceptible to this transfection but instead we employed HeLa cells as models for upregulation. HeLa cells show a similar p33 expression compared to MG63 and HDFa cells (data not shown). In cells transiently transfected with pcDNA 3.1-p33 vector for 24 h, p33 mRNA level was increased by approximately 200 times, corresponding to a 2-fold increase in p33 protein, as determined by western blot (Appendix III, Fig. 8A, B) compared to cells transfected with an empty pcDNA 3.1 vector as control.

As expected, the increased p33 expression protected cells from LL-37-induced toxicity. As seen in Fig. 28C, transfected p33 overexpressing cells but not control cells became completely resistant against an LL-37 attack. p33 overexpression did not influence LL-37-induced LDH release, supporting an intracellular mechanism of action (D).

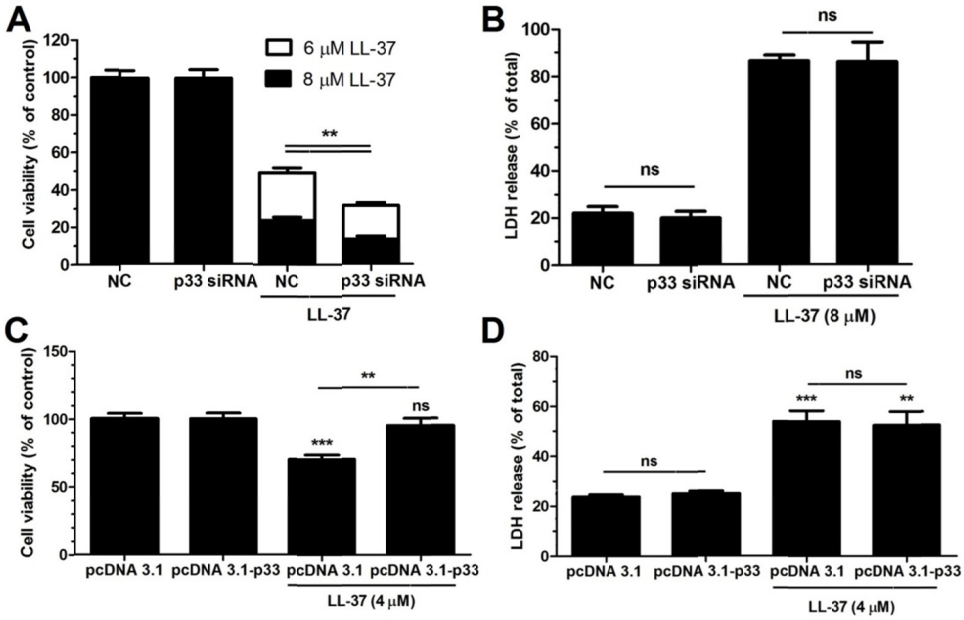

Figure 28. Downregulation of p33 by siRNA increases LL-37-induced cytotoxic effects while overexpression of p33 reduces LL-37-induced cytotoxicity. HaCaT cells were transfected with p33 siRNA or NC construct. Control cells were not treated with LL-37. Transfected cells were treated with 6 and 8 μM LL-37 and cell viability (**A**) or LDH release (**B**) was assessed after 3 h or 30 min respectively. Downregulation of p33 in HeLa cells promotes attenuation of cell viability by LL-37 but not LDH release. p33 protein was upregulated 2 times by transfecting with a pcDNA 3.1-p33 vector, compared to cells transfected with an empty pcDNA 3.1 vector. Reduction of cell viability by LL-37 (4 μM) was attenuated by overexpression of p33 (**C**) while LDH release was unaffected (**D**).

LL-37 interacts with p33 in the cytoplasm and upregulates p33 expression

The role of p33 as a scavenger of intracellular LL-37 was supported by showing binding between the two proteins. HaCaT cells were treated with LL-37 (10 μM) for 10 min and, after a lysis step, the interaction between p33 and LL-37 was investigated by co-immunoprecipitation. p33 was precipitated from lysates using a designated antibody, and the precipitates were analyzed on dot blots. Immunoreactivity for LL-37 in the precipitated p33 protein preparations demonstrates interaction between the two proteins (Fig. 29A). In order to provide further evidence of intracellular binding, HaCaT cells were subjected to sub-cellular fractionation after LL-37 treatment (10 μM, 10 min). Co-immunoprecipitation of the cytosolic fraction (containing both mitochondria and cytoplasm) showed strong LL-37 immunoreactivity, similar to that of whole cells (Fig. 29A, dot 6). Cytosolic interaction between LL-37 and p33 was further demonstrated by co-immunoprecipitation of cytosolic fractions from HeLa cells after vector transfection. Upregulation of p33 results in a higher abundance of cytosolic p33:LL-37 complexes as seen in panel B.

Our hypothesis that p33 is an endogenous defence system against LL-37 was further strengthened by data showing that LL-37 stimulates p33 protein expression in MG63 (Fig. 29C). Additionally, siRNA mediated downregulation of p33 in HaCaT cells resulted in a decrease of LL-37 gene expression (D). This may indicate a feedback mechanism in which the keratinocytes are forced to decrease cathelicidin production when the defence against LL-37 is low.

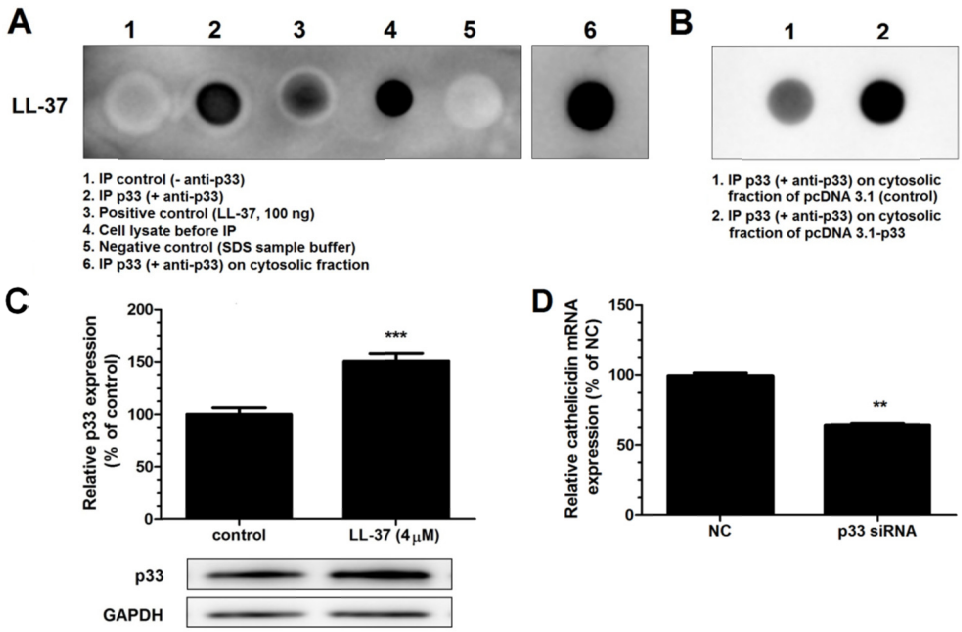

Figure 29. Co-immunoprecipitation shows cytoplasmatic binding of LL-37 to p33. Co-immunoprecipitation (IP) of p33 and LL-37 in HaCaT cells incubated with LL-37 (10 µM) for 10 min. After LL-37 treatment, cells were lysed and p33 were immunoprecipitated with an antibody. LL-37 immunoreactivity was detected in the precipitated p33 by dot blot (**A**). Dot 1 and 2 represent immunoprecipitate without and with p33 antibody, respectively. Dot 3 shows synthesized LL-37 (100 ng) as positive control. Dot 4 visualizes the cell lysate used for the IP. Dot 5 shows SDS sample buffer as background control. Dot 6 represents immunoprecipitate with p33 antibody in a cytosolic fraction of HaCaT. IP of p33 and LL-37 in cytosolic fractions of HeLa cells transfected with either pcDNA 3.1-p33 vector (dot 2) or pcDNA 3.1 vector as control (dot 1) for 24 h indicates that overexpressed p33 binds internalized LL-37 in the cytoplasm (**B**). Western blot analysis of MG63 cells shows an upregulation of p33 expression in response to treatment with LL-37 (4 µM) for 48 h (**C**). Conversely, when p33 is downregulated in HaCaT cells, the LL-37 gene expression subsides (**D**).

Subproject III: Cytotoxicity of LL-37 released in response to vitamin D

Accumulating evidence suggests that LL-37 may have detrimental effects in disease progression when overexpressed. Vitamin D is a well-known mediator of LL-37 synthesis, although its possible role in dysregulated expression of LL-37 is yet to be elucidated. In this subproject, we aim at assessing cytotoxicity of endogenous LL-37 *in vitro* using co-cultures of LL-37 producing monocytes and co-cultured PDL cells. We hope this approach could bring further insight into LL-37-induced cytotoxicity, and indicate if endogenous LL-37 could be responsible for tissue destruction observed locally in inflammatory diseases.

1,25D3 stimulates THP-1 monocyte CAMP gene expression but attenuates proteinase 3 (PRTN3) transcript

Treatment with 1,25D3 (1 μM) for 8, 24 and 48 h increased THP-1 CAMP mRNA by 39, 48 and 187 times, respectively (Fig. 30A). The 1,25D3-induced upregulation of CAMP was associated with downregulation of proteinase 3 (PRTN3) transcript at 24 and 48 h (B). Downregulation of proteinase 3, thereby inhibiting processing of mature LL-37, may represent a feedback mechanism to limit LL-37-induced cytotoxicity.

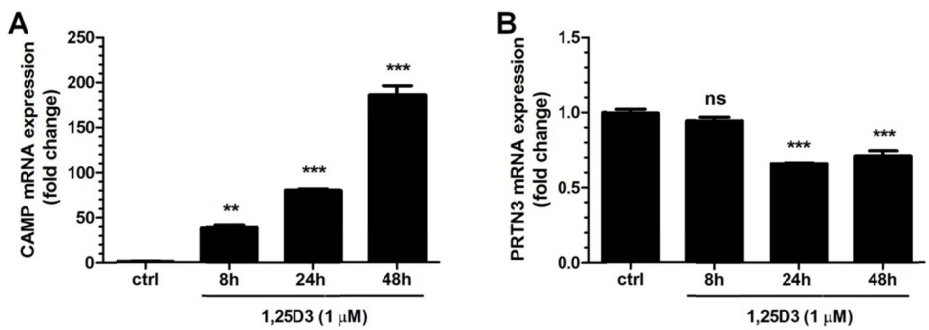

Figure 30. Treatment with 1,25D3 increases CAMP mRNA expression but attenuates proteinase 3 (PRTN3) in human THP-1 monocytes. The mRNA expression for both CAMP (**A**) and proteinase 3 (**B**) were determined by quantitative real-time RT PCR.

1,25D3-induced reduction of monocyte cell viability is reversed by p33

1,25D3 treatment for 10 days causes a reduction in THP-1 cell viability when THP-1 cells are seeded at a high (15 000 cell/ml), but not at low (10 000 cells/ml) density (Fig. 31). We suspect that this observation can be due to a sufficient release of LL-37 to promote cytotoxicity in wells with high, but not low cell number. In these experiments, the LL-37 scavenger p33 (1 μM) was included every third day throughout the treatment. p33 inhibited 1,25D3-induced cytotoxicity, suggesting a role of LL-37 in the attenuation of THP-1 viability. At low cell numbers, p33 on the other hand mediated a pro-proliferative effect by 1,25D3. This suggests that 1,25D3 may act pro-proliferative in THP-1 monocytes, although this effect is suppressed by 1,25D3-induced production of LL-37. The LL-37-induced THP-1 cell death is not dependent on GPCR induced apoptosis: attenuation of cell viability by 4 μM of LL-37 for 24 h was not significantly affected by pretreatment with 50 ng/ml PTx (data not shown).

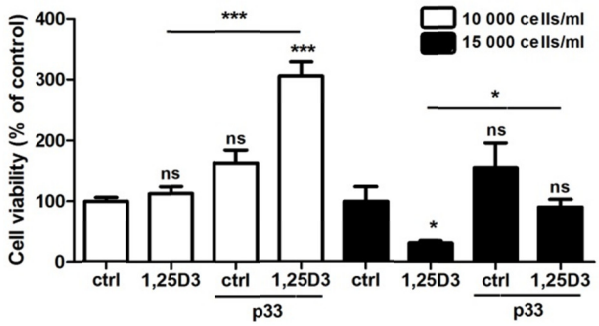

Fig. 31. 1,25D3-induced downregulation of THP-1 cell viability is antagonized by the LL-37 scavenger p33. In THP-1 cells seeded at low-density (10 000 cells/ml), stimulation with 1,25D3 enhances cell viability in the presence of p33. In THP-1 cells seeded at higher density (15 000 cells/ml), stimulation with 1,25D3 reduces cell viability and this effect is reversed by p33. The THP-1 cells were treated with 1 μM 1,25D3 for 10 days in the presence or absence of p33. p33 (1 μM) was included to the cells at day 3, 6 and 9 of the 10 days incubation period. Cell viability was assessed by the MTT assay.

Treatment with 1,25D3 reduces cell viability of human PDL cells co-cultured with THP-1 cells

Next, we investigated if the cytotoxic effects of 1,25D3 attenuate cell viability of primary tissue cells in proximity to monocytes. For this, PDL cells were co-cultured with THP-1, separated by a membrane fine enough to allow for protein, but not cell diffusion. Administration of 1,25D3 to the monocytes (100 nM, every third day) caused a reduction in co-cultured PDL cell viability observed at day 14. The treatment resulted in a reduction in PDL cell viability of about 80%, assessed by the MTT assay (Fig. 32A). Moreover, the PDL cells co-cultured with 1,25D3-stimulated monocytes showed cell shrinkage and membrane blebbing (Fig. 32B, right panel) and association to monocyte-derived hCAP18 protein (Appendix IV, Fig. 5B). The 1,25D3-induced cytotoxicity seemed to be dependent on the presence of the monocytes, as treatment with 1 µM 1,25D3 for 14 days had no effect on PDL cell viability in the absence of THP-1 cells (Fig. 32C). LL-37:DNA has in an earlier study been found to cause cytotoxicity in monocyte co-cultures through induction of type I IFN [244]. However, treatment with 1,25D3 markedly reduced THP-1 IFN-β protein expression (data not shown), ruling out this as the underlying mechanism.

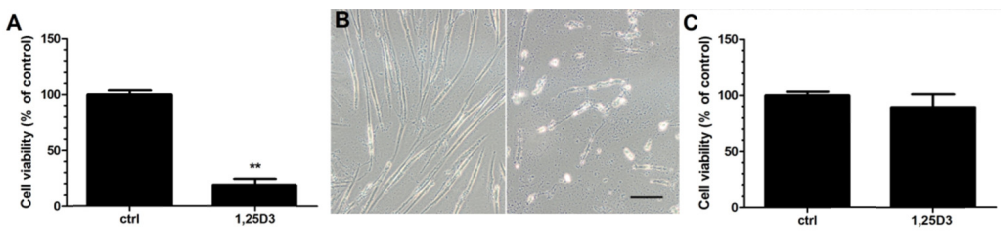

Figure 32. Treatment with 1,25D3 for 14 days reduces human PDL cell viability in a coculture system with THP-1 monocytes. The THP-1 cells in the insert (the upper chamber) were treated with 100 nM 1,25D3 every third day of the 14 days experimental-period. Cell viability was assessed by the MTT assay (**A**) and cell morphology by phase contrast microscopy (**B**). Photos are representative of co-cultured PDL cells without (left) and with (right) 1,25D3, bar = 20 µm for both panels. Treatment with 1,25D3 (1 µM as a bolus dose at the start of the experiment) for 14 days has no effect on PDL cell viability in the absence of THP-1 cells (**C**).

Subproject IV: Regulation of LL-37 expression by retinoid X receptor α

LL-37 is highly expressed in epithelial tissues, not least in skin where some of the highest cathelicidin levels in humans have been reported [256]. Here, vitamin D3 and its active metabolite 1,25D3 can be synthesized by keratinocytes from UVB light. 1,25D3 is able to bind to the vitamin D receptor (VDR) which in turn forms a heterodimer with retinoid X receptors (RXRs), a complex that can bind to VDR responsive elements on genes including CAMP, thereby regulating their transcription. It is possible for VDR to activate VDR responsive elements also without RXRs [438], and further, RXR can activate CAMP expression without binding to VDR [60]. The aim of this subproject was to investigate the CAMP expression in human gingiva and skin, and investigate the important of RXRα for 1,25D3-induced CAMP expression.

VDR is only expressed in a few cells of the stratum basale in human skin and gingiva

We began by studying the VDR expression in biopsies of human skin and gingiva. A few VDR-positive cells were observed within the *stratum basale* of both skin (Fig. 33A) and gingiva (B). VDR positive keratinocytes were very scarce in both tissues, less than 1% of the keratinocytes exhibited VDR immunoreactivity. No positive cells could be seen in the *lamina propria* or in the *muscularis mucosae* and importantly no difference in expression was observed between skin and gingiva. The low number of VDR positive cells in skin is a different VDR expression pattern compared to that of other studies showing that many or most of the skin keratinocytes express VDR [439-445].

Differences in human skin VDR immunoreactivity reported by different groups can be due to variations in antibody specificity, often recognizing different epitopes of VDR. It has been shown that different functional modifications of the receptor molecule, for example hormone, DNA or protein binding, can influence antibody affinity [442]. For this reason we use the VDR antibody D-6 in our experiments, in the most extensive comparison between VDR antibodies yet, D-6 proved to be the superior choice for immunoassays [446]. D-6 binds to the 344-358 epitope, in other words the hormone binding domain of VDR [442], but the immunoreactivity is not influenced by binding of 1,25D3 [446]. It is puzzling that different VDR expression pattern in skin has been reported even with the same antibody [439]. One possibility for the discrepancies could be variations in the preparation methods. Very few VDR positive cells were observed in arm skin biopsies of all individuals and the VDR staining seemed independent of the different degrees of tanning of the arm. Rat colon was employed as a positive

control, showing strong VDR immunoreactivity in a majority of the enterocytes (Appendix V, Fig. 1C).

VDR expression was also assessed in HaCaT keratinocytes, a commonly used transformed keratinocyte cell line from adult skin which retains full epidermal differentiation capacity [447]. As opposed to the keratinocytes of the skin biopsies, the HaCaT keratinocytes show abundant nuclear VDR immunoreactivity, indicating that these cells may represent the VDR positive cell population of the human skin *stratum basale* in the biopsies (Fig. 33C, D).

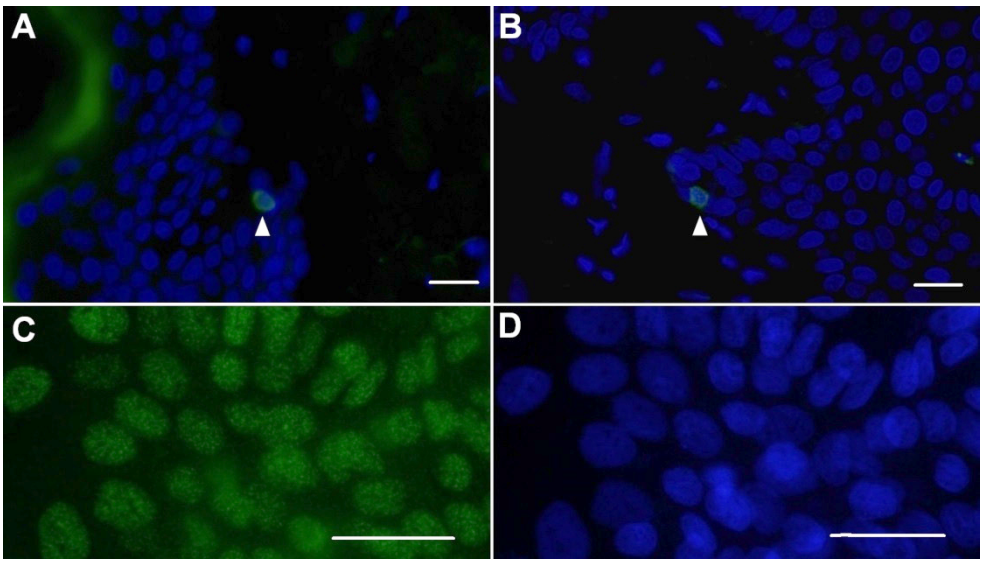

Figure 33. Occasional keratinocytes within the stratum basale of human skin (A) and human gingiva (B) display VDR immunoreactivity (arrowheads). Nuclei are stained blue. Human skin HaCaT keratinocytes on the other hand show abundant nuclear VDR immunoreactivity with no VDR-negative cells (**C**, **D**). Scale bars = 20 µm.

CAMP and RXRα expression is higher in human skin compared to gingiva

Expression of genes involved in the vitamin D/CAMP signaling pathway was compared in skin and gingiva biopsies by RT qPCR. VDR mRNA expression was similar between the two tissues (Fig. 34A), in line with the protein expression results. Nevertheless, the CAMP mRNA expression was about eight times higher in skin compared to that of gingiva (B). This correlated to the expression of the RXRα gene, showing three times higher expression in skin vs. gingiva. RXRβ mRNA showed similar expression in both tissues (C, D).

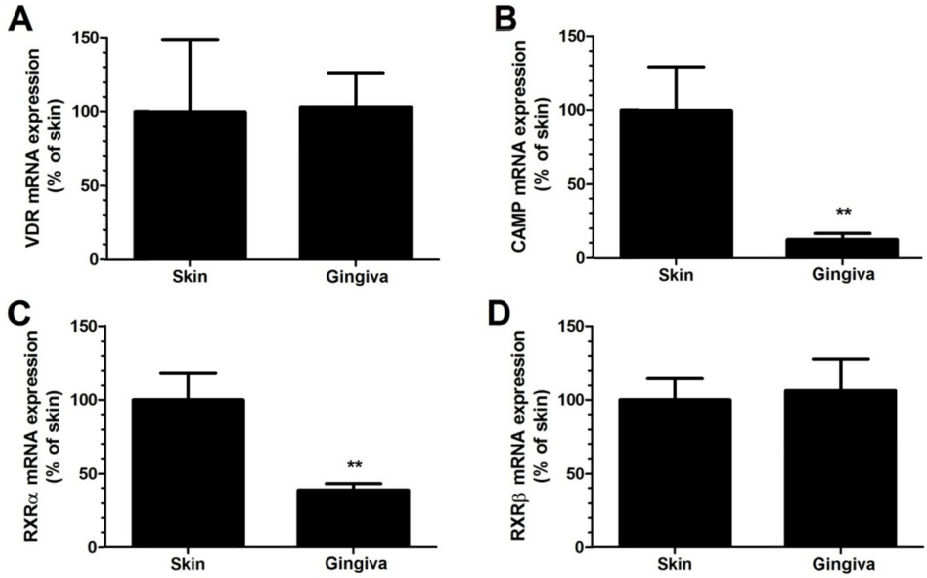

Figure 34. Gene expression of VDR, CAMP, RXRα and RXRβ in human skin and gingiva. VDR expression is similar in both skin and gingiva (**A**). Cathelicidin and RXRα have higher gene expression in skin compared to gingiva (**B, C**), while the RXRβ gene shows similar expression in the two tissues (**D**).

RXRα is crucial for 1,25D3-induced CAMP expression

The PCR data suggest that RXRα may be critical for the CAMP gene expression. To assess the importance of RXRα, we studied cathelicidin expression induced by 1,25D3 in HaCaT keratinocytes (Fig. 35). A siRNA mediated downregulation of RXRα mRNA by about 60% was sufficient to attenuate 1,25D3 induced CAMP expression to the same extent (A, B). The reduction of 1,25D3 induced CAMP gene expression by RXRα siRNA was confirmed on the protein level by western blot for hCAP18 (C). On a side note, some reports indicate that dermal fibroblasts also express hCAP18 in skin ([77]). We observe, however, low (1/20) CAMP gene expression in HDFa dermal fibroblasts vs. HaCaT cells (data not shown).

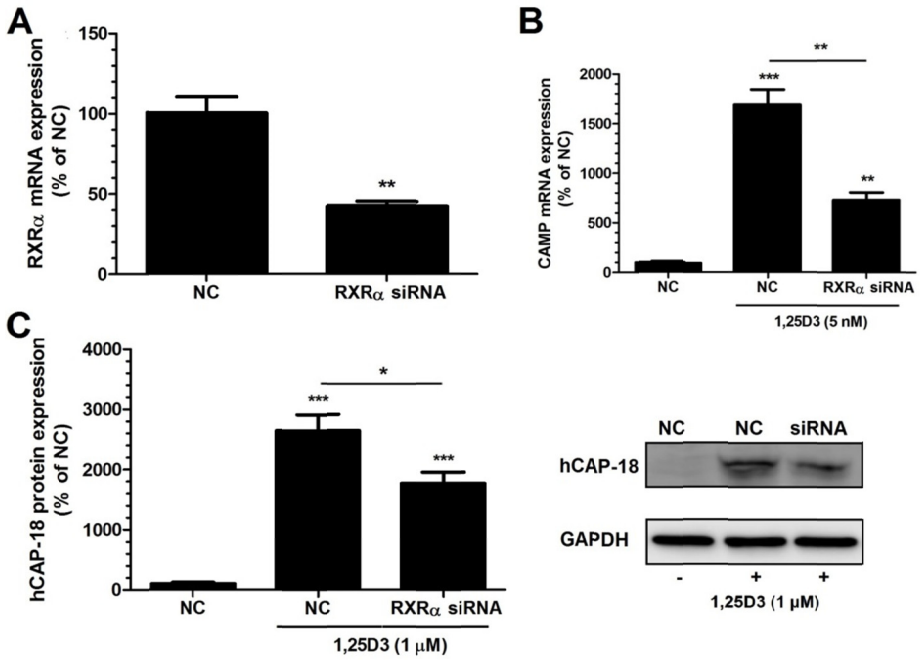

Figure 35. 1,25D3-induced expression of cathelicidin is dependant on RXRα levels in keratinocytes. Treatment with RXRα siRNA reduces mRNA expression for RXRα by about 60% compared to cells transfected with a control construct (NC) (**A**). The 1,25D3-evoked increase in CAMP mRNA was attenuated to the same extent (60%) in the siRNA treated cells (**B**). The was further reflected in a decreased 1,25D3 stimulated hCAP18 protein expression (**C**). Protein was quanified by western blotting, the immunoreactive hCAP18 band appeared at 16 kDa and was normalized to GAPDH, serving as a loading control.

1,25D3 is pro-proliferative and downregulates VDR expression

1,25D3 promotes proliferation of HaCaT cells. Stimulation with 1 μM of 1,25D3 for 72 h increased the cell viability/cell number by 90% as assessed by the MTT assay, while 5 nM of the steroid had no effect (Appendix V, Fig. 4). Pro-proliferative effects of 1,25D3 on keratinocytes have been reported at low 1,25D3 levels, in a cell density and Ca^{2+}/serum concentration dependent manner, but high concentrations of 1,25D3 (e.g. 1 μM) have been known to be anti-proliferative in keratinocytes [448, 449]. Recently, it was found that transformed keratinocytes are less sensitive to the anti-proliferative effect of 1,25D3 compared to primary cells [450], suggesting differences between HaCaT cells and primary keratinocytes in terms of 1,25D3-evoked pro-proliferation.

We further noted that 1,25D3 treatment significantly downregulated VDR expression, both on the transcript and protein levels (Appendix V, Fig. 5). 1,25D3 have previously been shown to enhance VDR levels in a variety of cells through protecting the protein from degradation [451-454]. One study recently reported that 1,25D3 does not alter the VDR protein levels in HaCaT cells in any direction, although this can be explained by the fact that the protein levels here were assessed after only 24 h of treatment with 1,25D3 [450]. We required 72 h of 1,25D3 stimulation to observe reduction of VDR protein expression. 1,25D3 likely protects VDR from degradation to some extent in HaCaT cells as well, while the downregulation of the gene expression appears to counteract this.

CONCLUSIONS

Subproject I, paper I. We show that LL-37 induces apoptosis in human osteoblast-like MG63 cells. This is associated with elevated intracellular Ca^{2+} concentrations, but the LL-37-induced cytotoxicity is not dependent on the Ca^{2+} influx. The cellular Ca^{2+} influx is neither reliant of L- or T-type voltage dependent Ca^{2+} channel activity, nor activation of P2RX7. Instead the Ca^{2+} influx is a result of LL-37-induced membrane permeabilization, which also results in release of cytosolic proteins such as lactate dehydrogenase to the extracellular space. The pore-formation by LL-37 is not necessarily correlated to apoptosis or cytotoxicity, as illustrated by comparisons with mouse macrophages. Furthermore, the LL-37-induced pores appear to rapidly close after removal of LL-37, showing that LL-37-evoked membrane permeabilization is reversible.

LL-37 levels are elevated locally in chronic periodontitis in proximity to the alveolar bone. Our data suggests that LL-37-induced osteoblast cell death may represent a mechanism by which the immune system causes degradation of the jaw bone, resulting in detachment and finally loss of teeth.

Subproject II, paper II-III. We have assessed possible endogenous defence systems against LL-37. Mucin 1 inactivates LL-37-induced cytotoxicity and is expressed by MG63 osteoblasts, but does not appear to protect the cells in a significant way. We show that p33, another endogenous protein, can be added extracellularly to rescue osteoblasts from LL-37-evoked cytotoxicity. Furthermore, LL-37 stimulates p33 expression. In HaCaT keratinocytes, expressing high levels of the p33 protein, downregulation of p33 results in an increased susceptibility to LL-37-induced cytotoxicity. Conversely, upregulation of intracellular p33 renders cells insensitive to LL-37-induced toxicity. Expression of endogenous p33 appears to be a way by which host cells can protect themselves from LL-37-evoked toxicity, through binding of internalized LL-37. We suggest that internalization is critical for the cytotoxic effects of LL-37.

Subproject III, paper IV. To assess the cytotoxic effects of endogenously released LL-37, CAMP expression and LL-37/hCAP18 release from THP-1 monocytes, in co-culture with human PDL cells, was stimulated with 1,25D3. Our results indicate that endogenous LL-37 may attenuate cell viability of both co-cultured PDL cells and the THP-1 cells themselves. Co-treatment with the LL-37 scavenger p33 reverses the 1,25D3-mediated attenuation of cell viability, suggesting that the downregulation of cell viability indeed involves LL-37. Additionally, we also reveal a pro-proliferative effect of 1,25D3. Furthermore, 1,25D3 negatively regulates proteinase 3 gene expression, possibly representing a compensatory mechanism that may limit LL-37-induced cytotoxicity.

Subproject IV, paper V. VDR is expressed only by a small fraction of the keratinocytes in *stratum basale* of the skin and gingiva. Cathelicidin gene expression is however much higher in skin than gingiva, an effect correlating to the expression of RXRα. We further show that cellular RXRα level is critical for vitamin D-induced stimulation of cathelicidin expression, and that RXRα is an important factor in the regulation of endogenous LL-37 levels. We propose that RXRα may represent a future drug target for modulation of LL-37 expression.

FUTURE PERSPECTIVES

No doubt much research has been conducted on the topic of LL-37 over the last twenty years. Its multifaceted role, however, still leaves many important questions left to answer. For example the involvement of LL-37 in disease pathogenesis is still under debate. In some inflammatory diseases there is to date strong evidence supporting that LL-37 may have a role in the pathophysiological process of these pathologies. LL-37 can have an impact both via its pro-inflammatory properties but also through its detrimental effects on host cell viability. While LL-37-induced cytotoxicity may be of benefit in suppressing certain types of cancer, LL-37-evoked cytotoxic effects could also contribute to tissue damage in several inflammatory diseases, as well as causing release of destructive intracellular components which are often characteristic of autoimmune diseases.

In my opinion, the most vital future projects regarding detrimental effect of LL-37 in humans, would entail evaluation of the pathophysiological implications of LL-37-induced permeabilization and cytotoxicity in host cells. These effects are anticipated where the highest local tissue levels of LL-37 has been reported (psoriasis, rosacea and bladder inflammation). The peptide may here be responsible for the abundant cell death and tissue destruction associated with these conditions. The LL-37-induced cytotoxicity should not be ruled out in the pathophysiology of other diseases showing elevated LL-37 concentrations. LL-37 may for instance promote bone destruction in chronic periodontitis.

Many drugs used to treat psoriasis and rosacea today, e.g., doxycycline, azelaic acid, glucocorticoids and etanercept, are known to downregulate LL-37, either via protease inhibition, transcriptional suppression or through an unknown mechanism. While this suppression of LL-37 may represent a mechanism of action and point to the pathophysiological importance of LL-37, the suppressed expression may be secondary, as the case is for the protease inhibiting drugs, and subsequently not necessarily involved in the drugs beneficial effects. In my view, the most direct way to study the pathophysiological importance of LL-37 would be to selectively inhibit or silence the LL-37 expression locally in patients (e.g. by RNA interference or antibodies) and assess consequences of this intervention, for example on tissue integrity and cytokine expression.

In animal models the reverse approach, to locally induce LL-37 expression, may be used to assess the role of LL-37 in pathogenesis. For instance in the case of periodontitis, CAMP coding lentiviral vectors could be injected into the GCF of animals, to assess effects on bone integrity. Comparing the LL-37-induced cytotoxic effects of exogenously applied peptide with those of endogenous LL-37, for example by co-culture systems such as those used in this thesis, will also shed some light on the physiological importance of endogenous LL-37.

The mechanism by which LL-37 induces apoptosis and/or cytotoxicity is not well understood. For instance the receptor by which LL-37 induces caspase independent apoptosis remains to be defined. This could be of importance in several regards, for instance the GPCR-induced apoptotic signaling activated by LL-37 are indicated to be selective for cancer cells. In my view, this line of research represents an important task for the future.

With respect to LL-37 induced cytotoxicity in host cells and p33-evoked inactivation of LL-37, our result leaves some questions left to be answered. We see that intracellular LL-37 attenuates host cell viability. It has been reported that most LL-37 is internalized through endocytosis, but it is not known if this pool of LL-37, or perhaps LL-37 internalized through permeabilization, is responsible for the cytotoxicity. This may be elucidated by assessing cell viability after LL-37-treatment with and without endocytosis inhibitors. Based on earlier reports, clathrin inhibitors may be appropriate for this purpose. Intracellular LL-37 scavengers (e.g. p33) may also be useful in such a study to distinguish between intracellular and extracellular effects of LL-37.

Furthermore, our work does not answer which of the cellular p33 pools that acts as a LL-37 inhibitor. This can be studied by cellular fractionation and co-precipitation of p33 and LL-37. An alternative way would be to use gold labeled LL-37 and EM to study the intracellular localization and its co-localization to p33. A third way to evaluate involvement of cytoplasmic p33 may be to use protease inhibitors prior to LL-37 treatment. This would rapidly elevate the protease-sensitive vesicular p33 fraction but much less so the mitochondrial pool of p33 [436].

SAMMANFATTNING PÅ SVENSKA

Antimikrobiella peptider (AMP) eller värdskyddande peptider (HDP), som de också kallas, utgör människans första försvarslinje mot invaderande patogener. En av de viktigaste av dessa peptider är LL-37, vilket produceras och frisätts av vita blodkroppar och barriärceller (epitelceller), bland annat i hud, luftvägar och kärlväggar. Tillverkningen av peptiden stimuleras vid bland annat infektion, men även av D-vitamin. Produktion av LL-37 verkar ligga bakom en del av vitamin D:s funktioner i immunförsvaret. LL-37 utövar ett brett spektrum av betydelsefulla biologiska effekter, inklusive antibakteriella, inflammatoriska och sårläkande egenskaper. Peptidens celldödande effekter omfattar mer än bara patogener som bakterier och virus, även våra egna värdceller är mer eller mindre känsliga för LL-37.

Höga halter av LL-37 har kopplats till flera autoimmuna/inflammatoriska sjukdomar, i vilka immunförsvaret skadar den egna organismen, bland annat till hudsjukdomarna rosacea och psoriasis. I båda dessa har det visat sig att LL-37 har en aktiv roll i sjukdomsförloppet, där peptiden verkar trigga de inflammatoriska utslagen. En annan sjukdom som kopplats till LL-37 är kronisk parodontit, ett tillstånd som är den vanligaste orsaken till förlust av tänder hos vuxna. Här har höga halter av LL-37 upptäckts i tandköttsvätskan som omger tänderna.

Vi misstänker att LL-37 kan bidra till nedbrytning av ben vid kronisk parodontit, resulterande i tandlossning, men även till vävnadsskador som ses vid andra inflammatoriska sjukdomsförlopp. Ett av våra syften har därför varit att studera bakomloggande mekanismer till LL-37:s skadliga egenskaper på våra celler. Därför har vi odlat diverse olika typer av celler från människa, inklusive celler som påminner om de i tandben, och behandlat dessa med LL-37 för att studera effekterna.

Vi kan se att LL-37 bildar porer i cellens membran, oavsett celltyp. Samtidigt är vissa celler, så som benceller, känsliga och dör vid låga LL-37 koncentrationer, medan andra celler är relativt resistenta. Nivåerna av LL-37 kan lokalt, vid t.ex. psoriasis, vara långt högre än de som krävs för celldöd i vår undersökning. Detta styrker att LL-37 skulle kunna vara vävnadsnedbrytande vid sjukdom. Av denna anledning har vi därför valt att även leta efter molekyler som inaktiverar LL-37 och som därmed skulle kunna vara värdefulla som skydd mot LL-37:s skadliga effekter.

En sådan skyddsmekanism är ett protein kallat p33. Vi har visat att detta protein har förmågan att binda och inaktivera LL-37. p33 finns i praktiskt taget alla celler, men i olika mängder. Vi har visat att celler som producerar relativt lite p33 är extra känsliga för LL-37.

Vi har även intresserat oss för kopplingen mellan LL-37 och D-vitamin och möjligheten att LL-37 kan ligga bakom en del av vitamin D:s effekter. En ökad förståelse av detta samband kan vara viktigt och då inte minst idag när vi har ett utbrett vardagligt bruk av D-vitamin.

Vi har studerat hur D-vitamin reglerar produktion av LL-37 i barriärvävnaderna hud och tandkött med hjälp av vävnadsprover från människa samt odlade hudceller. Vi har även försökt att utvärdera skadliga effekter av kroppseget LL-37 efter D-vitaminbehandling genom att odla vita blodkroppar, med eller utan D-vitamin, i närhet men inte i kontakt med andra värdceller. Vi ser att D-vitamin leder till celldöd bland värdcellerna, en effekt förmedlad av de LL-37 producerande blodcellerna. Detta är viktigt, då ingen tidigare påvisat celldödande effekter av kroppseget LL-37 producerat i cellsystem. I regel används syntetiskt LL-37 för studier av peptidens effekter på celler och andra biologiska system.

Denna avhandling stärker bilden av att LL-37 kan vara skadligt vid överproduktion, inte enbart via sin förmåga att skapa inflammation, utan troligen också genom att orsaka perforering och celldöd av värdceller. Detta skulle kunna möjliggöra för nya typer av behandlingar vid vissa inflammatoriska sjukdomar. Våra rapporterade effekter av LL-37 på celler från människa kompletterar bilden av LL-37 som en viktig komponent i vårt immunförsvar.

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ABBREVIATIONS

AD	Atopic dermatitis
AIF	Apoptosis-inducing factor
AIM2	Melanoma 2 receptor
Akt	Protein kinase B
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
AP	Aggressive periodontitis
AP-1/2	Activator protein 1/2
Bax	Bcl-2-associated X protein
BI	Bladder inflammation
BMP	Bone morphogenetic protein
C/EBP	CCAAT-enhancer-binding protein
CAMP	Cathelicidin anti-microbial protein (gene name)
CCL	Chemokine (C-C motif) ligand
cDNA	Complementary DNA
CF	Cystic fibrosis
cIAP2	Inhibitor apoptosis protein-2
CLD	Cathelin-like domain (hCLD = human CLD)
COX-2	Cyclooxygenase-2
CP	Chronic periodontitis
CRAMP	Cathelicidin-related antimicrobial peptide
CREB1	CAMP responsive element binding protein 1
CW	Chronic wounds
CXC	C-X-C chemokine
CYP27B1	Cytochrome P450 family 27 subfamily B member 1
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DPC	Dodecylphosphocholine
EC50	Half maximal effective concentration
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

EGFR	Epidermal growth factor receptor
EGR1	Early growth response protein 1
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EM	Electron microscopy
EndoG	Endonuclease G
EP3	Prostaglandin E2 receptor 3
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal–regulated kinase 1 and 2
FBS	Fetal bovine serum
FLI1	Friend leukemia integration 1 transcription factor
FPR2	N-formyl peptide receptor 2
GA	Glutaraldehyde
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gC1qR	Globular C1q receptor
GCF	Gingival crevicular fluid
GPCR	G protein–coupled receptor
HDFa	Human dermal fibroblasts
HDP	Host defence peptide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1	Hypoxia-inducible factor 1
HRP	Gorseradish peroxidase
Hsp90	Geat shock protein 90
ICC	Immunocytochemistry
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IP	Co-immunoprecipitation
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LED	Light emitting diode
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase

mtDNA	Mitochondrial DNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide, reduced
nDNA	Nuclear DNA
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
P2RX7	P2X purinoceptor 7
p38	Extracellular signal-regulated kinase 1
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
pDC	Plasmacytoid dendritic cells
PDL	Periodontal ligament
PFA	Paraformaldehyde
PG	Phosphatidylglicerol
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PPP	Pustulosis palmaris et plantaris
PTx	Pertussis toxin
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
RT	Room temperature
RT PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
S1P	Sphingosine-1-phosphate
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SSc	Systemic scleroderma
STAT3	Signal transducer and activator of transcription 3
TBK1	TANK binding kinase 1
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRIS	Tris(hydroxymethyl)aminomethane
VDR	Vitamin D receptor
VDRE	Vitamin D response element

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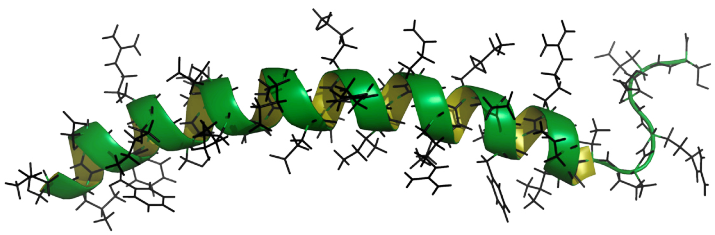

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