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#### Immunomodulation and cytotoxicity

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# Effects of the host defense peptide LL-37 on human cells

Immunomodulation and cytotoxicity

#### SARA BODAHL

EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY





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Department of Experimental Medical Science

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# Effects of the host defense peptide LL-37 on human cells

Immunomodulation and cytotoxicity

Sara Bodahl



#### DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden. To be defended in Segerfalksalen, BMC A10, Lund. Friday the 13<sup>th</sup> of May 2022 at 9.00 a.m.

> Faculty opponent Docent Katrin Pütsep Department of Physiology and Pharmacology Karolinska Institutet, Stockholm

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| Title: Effects of the host defense peptide LL-3  | 7 on human cells: Immunomodulation and cytotoxicity   |
| Abstract   |   |
| The human host defense peptide LL-37 has ar<br>This cathelicidin is mainly produced by immun-<br>upregulated upon infection and inflammation<br>pathogens, but also modulates the immune res-<br>inflammatory mediators and by affecting imm<br>been linked to inflammatory diseases, such as<br>is known about the role of LL-37 in the devel-<br>and more research is needed to clarify and es-  | e essential role in the first line of defense against invading pathogens.<br>e cells and epithelial cells aligning the mucosal areas and is normally<br>n. LL-37 displays direct antimicrobial activity against a variety of<br>sponses by acting as a chemoattractant, influencing the production of<br>nune receptor signaling. Abnormally high local levels of LL-37 have<br>s psoriasis, atherosclerosis, periodontitis and asthma. However, little<br>opment and progression of inflammatory and autoimmune diseases<br>tablish how LL-37 mediates host cell functions in these conditions.  |
| In this thesis we have studied LL-37-induced ce<br>in several types of human cells. We show t<br>permeabilizes the cell membranes in all humar<br>the cell viability and generates cell membrane<br>clathrin-mediated endocytosis is prevented. In<br>release of both cytosolic and nucleic compone<br>as the nucleus. Furthermore, we show that LL-<br>cells and bronchial epithelial cells. More spec<br>upregulation of TLR3 expression and downstr<br>NF-kB. Finally, we show that LL-37 induces a<br>that this mechanism of action is associated with | Il toxicity, cell membrane permeability and immune receptor signaling<br>that LL-37 reduces cell viability in a dose-dependent manner and<br>n cell types studied. Interestingly, we demonstrate that LL-37 reduces<br>permeabilization in osteoblast-like cells even though LL-37 import via<br>mast cells, LL-37-induced cell membrane permeabilization results in<br>ents, indicating that LL-37 permeabilizes cellular compartments such<br>-37 influences TLR3 signaling in both coronary artery smooth muscle<br>ifically, LL-37 enhances viral dsRNA signaling on TLR3, resulting in<br>eam pro-inflammatory signaling that involves the transcription factor<br>n increased import of dsRNA in bronchial epithelial cells, suggesting<br>th upregulated expression of TLR3. |

Overall, the work in this thesis provides new insights on underlying mechanisms behind LL-37-induced host cytotoxicity and cell membrane permeability and proposes a novel LL-37-driven pro-inflammatory mechanism of action involving potentiation of dsRNA-stimulated TLR3 expression and signaling.

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# Effects of the host defense peptide LL-37 on human cells

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Cover photo by Sara Bodahl

Front: Exogenous LL-37 in human coronary artery smooth muscle cells. Back: LL-37 amino acid sequence and structure (Created with BioRender.com).

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*Till min Anton* ♥

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## List of papers

The following articles are included in the thesis:

- I. Anders E, Dahl S, Svensson D, Nilsson B-O. *LL-37-induced human* osteoblast cytotoxicity and permeability occurs independently of cellular *LL-37 uptake through clathrin-mediated endocytosis*. Biochemical and Biophysical Research Communications. 2018; 501(1): 280-285. DOI: 10.1016/j.bbrc.2018.04.235.
- II. Dahl S, Anders E, Gidlöf O, Svensson D, Nilsson B-O. The host defense peptide LL-37 triggers release of nucleic acids from human mast cells. Peptides. 2018; 109: 39-45. DOI: 10.1016/j.peptides.2018.10.001.
- III. Dahl S, Cerps S, Rippe C, Swärd K, Uller L, Svensson D, Nilsson B-O. Human host defense peptide LL-37 facilitates double-stranded RNA proinflammatory signaling through up-regulation of TLR3 expression in vascular smooth muscle cells. Inflammation Research. 2020; 69: 579-588. DOI: 10.1007/s00011-020-01340-2.
- **IV. Bodahl S**, Cerps S, Uller L, Nilsson B-O. *LL-37 and double-stranded RNA synergistically upregulate bronchial epithelial TLR3 involving enhanced import of double-stranded RNA and downstream TLR3 signaling.* Biomedicines. 2022; 10 (2): 492. DOI: 10.3390/biomedicines10020492.

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## Papers not included

Oldén K, Fält F, **Dahl S**, Aidoukovitch A, Ericson D, Nilsson B-O, Hedenbjörk-Lager A. *Odontoblast-like MDPC-23 cells produce pro-inflammatory IL-6 in response to lipoteichoic acid and express the antimicrobial peptide CRAMP*. Acta Odontologica Scandinavia. 2019; 78(3): 210-216.

Aidoukovitch A, Anders E, **Dahl S**, Nebel D, Svensson D, Nilsson B-O. *The host defense peptide LL-37 is internalized by human periodontal ligament cells and prevents LPS-induced MCP-1 production*. Journal of Periodontal Research. 2019; 00: 1-9.

Aidoukovitch A, **Dahl S**, Fält F, Nebel D, Svensson D, Tufvesson E, Nilsson B-O. *Antimicrobial peptide LL-37 and its pro-form, hCAP18, in desquamated epithelial cells of human whole saliva*. European Journal of Oral Sciences. 2019; 00: 1-6.

Bankell E, **Dahl S**, Gidlöf G, Svensson D, Nilsson B-O. *LL-37-induced caspase-independent apoptosis is associated with plasma membrane permeabilization in human osteoblast-like cells*. Peptides. 2021; 135: 170432.

# Abbreviations

| AMP    | Antimicrobial peptide                       |
|--------|---|
| AP     | Aggressive periodontitis                    |
| CAMP   | Cathelicidin antimicrobial protein gene     |
| CLD    | Cathelin-like domain                        |
| CLP    | Chlorpromazine                              |
| CLTCL1 | Clathrin heavy chain like 1                 |
| COPD   | Chronic obstructive pulmonary disease       |
| СР     | Chronic periodontitis                       |
| DCs    | Dendritic cells                             |
| DMEM   | Dulbecco's modified eagle's medium          |
| dsRNA  | Double-stranded RNA                         |
| EGFR   | Epidermal growth factor receptor            |
| ELISA  | Enzyme-linked immunosorbent assay           |
| ETs    | Extracellular traps                         |
| FBS    | Fetal bovine serum                          |
| GAPDH  | Glyceraldehyde 3-phosphate dehydrogenase    |
| GPCR   | G-protein coupled receptor                  |
| hCAP18 | Human cathelicidin antimicrobial peptide 18 |
| hCASMC | Human coronary artery smooth muscle cell    |
| HDP    | Host defense peptide                        |
| IL     | Interleukin                                 |
| LDH    | Lactate dehydrogenase                       |
| LPS    | Lipopolysaccharide                          |
| LTA    | Lipoteichoic acid                           |
|        |   |

| MAPK     | Mitogen-activated protein kinase                               |
|----------|--|
| MCP-1    | Monocyte chemoattractant protein-1                             |
| NADH     | Nicotinamide adenine dinucleotide                              |
| NF-κB    | Nuclear factor kappa-light chain-enhancer of activated B cells |
| PAMPs    | Pathogen-associated molecular patterns                         |
| PMA      | Phorbol-12-myristate-13-acetate                                |
| Poly I:C | Polyinosine-polycytidylic acid                                 |
| RHSF     | Recombinant human steel factor                                 |
| RPMI     | Roswell Park memorial institute                                |
| RT qPCR  | Reverse transcriptase quantitative polymerase chain reaction   |
| SDS      | Sodium dodecyl sulfate   |
| siRNA    | Small interfering RNA  |
| SMCs     | Smooth muscle cells  |
| SMGS     | Smooth muscle growth supplement                                |
| TF       | Transcription factor   |
| TLR      | Toll-like receptor   |
| VDR      | Vitamin D receptor   |
| VDRE     | Vitamin D response element                                     |

# Populärvetenskaplig sammanfattning

Kroppens immunsystem är ett mycket effektivt maskineri med många olika delar som arbetar tillsammans för att skydda oss mot sjukdomsframkallande mikrober, så som bakterier och virus. Antimikrobiella peptider är små proteiner som har många betydelsefulla funktioner i vårt immunförsvar, där LL-37 är en av de allra viktigaste. Peptiden bildas naturligt i kroppen av olika vita blodkroppar som rör sig i vårt blodsystem, men också av barriärceller, så kallade epitelceller, som beklär huden och olika slemhinnor. LL-37 verkar direkt på bakterier genom att borra hål i bakteriecellen, vilket orsakar läckage och celldöd, men peptiden fungerar även som en kommunikationslänk mellan immunförsvarets olika delar. I denna avhandling har jag studerat en rad olika effekter av LL-37 på olika humana värdceller, där jag har fokuserat på LL-37s skadliga egenskaper, hur LL-37 kan göra hål i våra egna celler, samt hur LL-37 kan öka immunförsvarets aktivitet.

I flera typer av inflammationssjukdomar har man kunnat uppmäta ovanligt höga nivåer av LL-37. Då ett område i kroppen blir inflammerat ökar blodflödet och fler ansamlas Vid vita blodkroppar kommer att i området. kroniska inflammationssjukdomar till exempel hudsjukdomen psoriasis. som tandlossningssjukdomen parodontit och luftrörsinflammationen astma bevaras inflammationen i lokala områden under en längre period, och där finns även höga halter LL-37 peptid. Det har rapporterats att höga nivåer LL-37 inte bara dödar mikrober utan även orsakar celldöd i humana celler. Då LL-37 också har visat sig vara drivande vid inflammation misstänker man att peptiden spelar en aktiv roll i dessa sjukdomar. Likväl, mer forskning behövs för att fastställa hur dessa höga nivåer av LL-37 påverkar våra egna celler.

Det är allmänt känt att LL-37 kan ta sig in i humana celler och kan därmed utföra många olika funktioner. Det har dock visat sig att peptiden tar sig in i cellerna med hjälp av olika mekanismer. Vi har därför undersökt hur olika typer av import av peptiden hänger ihop med LL-37s toxiska egenskaper och LL-37s förmåga att borra hål i värdceller. Vi har också demonstrerat att dessa hål i cellens yttre vägg, så kallad perforering, gör så att joner, proteiner och andra beståndsdelar läcker ut/in i cellen. Intressant nog har vi sett att denna perforering inte nödvändigtvis innebär att cellerna dör. Det har visat sig att olika typer av humana celler är olika känsliga för LL-37s skadliga effekter.

Fortsättningsvis har vi undersökt hur LL-37 tillsammans med RNA från virus kan öka immunförsvarets signalering och bidra till inflammation. Bakterier och virus innehåller många delar som vårt immunförsvar känner igen som främmande. Tolllika receptorer, ofta förkortade till TLR, är proteiner som känner igen olika bakteriella och virala beståndsdelar och signalerar till andra delar av immunförsvaret att skadliga mikroorganismer finns i närheten. Vi har visat att LL-37 förstärker signaleringen mellan RNA från virus och receptorn TLR3. Mer specifikt så har vi sett att LL-37 gör så att mer RNA från virus tar sig in i cellerna. Detta tror vi är en viktig del i mekanismen, då mer tillgängligt RNA i cellerna gör så att mer RNA kan bind till sin TLR3 receptor och därmed öka immunsystemets svar. Tillsammans visar detta på en ny cellulär mekanism för hur LL-37 kan öka immunförsvarets aktivitet och bidra till inflammation i humana celler.

Sammanfattningsvis, så presenterar den här avhandlingen flera nya upptäckter om LL-37s effekter på humana celler. Dessa studier bidrar till ökad förståelse och kunskap om hur antimikrobiella peptider som LL-37 kan bidra till vävnadsdöd och kronisk inflammation i många inflammatoriska/autoimmuna sjukdomar.

## Background

## Host defense peptides

Host defense peptides (HDPs) are small cationic peptides that have an essential role in innate immunity [1]. They are produced in all complex organisms, including animals, plants, and insects and display a broad activity against a variety of microorganisms, which is why they are also known as antimicrobial peptides (AMPs) [2]. However, due to their involvement in host defense and immunomodulation they are nowadays generally called HDPs instead of AMPs. HDPs are around 12-100 amino acids in length and are usually amphipathic, which means that they have both hydrophilic and hydrophobic properties.

In mammals, two different groups of HDPs have been identified, defensins and cathelicidins [3]. Defensins are a large family of HDPs with a small compact structure, containing a  $\beta$ -sheet core [4]. They are expressed from multiple genes and generates a various number of peptides. In humans, at least seventeen defensins have been identified, and based on their chemical properties they are subdivided into  $\alpha$ - and  $\beta$ -defensins. Cathelicidins, in contrast to defensins, are expressed from a single gene and form an  $\alpha$ -helical shape in physiological conditions [5]. They have been well studied in mammals, such as rabbits [6], mice [7], rats [8], and guinea pigs [9], but also in chickens [10] and several types of fish [11] (Table. 1). They contain the cathelin-like domain (CLD), which is a highly conserved structure both within a single species but also among different species [12]. This has made it easier to establish the genetic origin and phylogenetic relationships of cathelicidins.

In humans only one cathelicidin have been identified, the human Cathelicidin Antimicrobial Protein 18 (hCAP18), which is extracellularly processed into the active LL-37. In humans, LL-37 represents the first line of defense against invading pathogens and is upregulated upon infection and disease. In addition, LL-37 can modulate immune responses and has an essential role in inflammation. However, the mechanisms behind how LL-37 interacts with host cells and what signaling events are involved is still largely unknown. High levels of LL-37 are found locally in lesions of inflammatory diseases, and more research is needed to know how these high concentrations affect surrounding tissues and influence the inflammatory environment.

**Table 1. Selected cathelicidin-derived peptides.** The peptides are listed in order according to the closest resemblance to the human LL-37. The amino acid sequence is listed as one-letter abbreviations where all peptides listed form an  $\alpha$ -helix structure. "AA" stands for the number of amino acids. This table is modified from Dürr *et al* [12].

| Peptide      | Origin        | Amino acid sequence  | AA | Ref  |
|--------------|---------------|--|----|------|
| LL-37        | Human         | LLGDFFRKSK-EKIGKEFKRI-VQRIKDFLRN-LVPRTES   | 37 | [13] |
| RL-37        | Monkey        | RLGNFFRKVK-EKIGGGLKKV-GQKIKDFLGN-LVPRTAS   | 37 | [14] |
| CAP18        | Rabbit        | GLRKRLRKFR-NKIKEKLKKI-GQKIQGLLPK-LAPRTDY   | 37 | [6]  |
| CRAMP        | Mice          | GLLRKGGEKI-GEKLKKIGQK-IKNFFQKLVP-QPE   | 33 | [7]  |
| rCRAMP       | Rat           | RFKKISRLAG-LLRKGGEKFG-EKLRKIGQKI-KDFFGKLAPE-<br>IEQ                                | 43 | [8]  |
| CAP11        | Guineapig     | (GLRKKFRKTR-KRIQKLGRKI-GKTGRKVWKA-WREYGQIPYY-<br>CRY) <sup>2</sup>                 | 43 | [9]  |
| Fowlicidin-1 | Chicken       | RVKRVWPLVI-RTVIAGYNLY-RAIKKK   | 26 | [10] |
| rtCATH_1     | Rainbow trout | RRSKVRICSR-GKNCVSRPGV-GSIIGRPGGG-SLIGRPGGGS-<br>VIGRPGGGSP-PGGGSFNDEF-IRDHSDGNRF-A | 71 | [11] |

## hCAP18/LL-37

The human cathelicidin hCAP18 was initially identified and sequenced in 1995 [15-17]. The name of the peptide originates from similarities to the rabbit cathelicidin CAP18. The peptide can be found at low concentrations in various cell types and tissues in the body and is usually upregulated upon inflammation and various disease processes [12]. hCAP18 is mainly expressed in neutrophils [17], but also in other leukocytes such as mast cells [18], lymphocytes [19] and monocytes [20]. It is also produced in epithelial cells in various parts of the body, such as the skin [21], the gastrointestinal [22-25], respiratory [24, 26], and genitourinary tracts [24]. Furthermore, hCAP18 has been found to be expressed in salivary glands, the gingiva and detected in human saliva [27, 28].

#### hCAP18 processing

hCAP18 is expressed from the Cathelicidin Antimicrobial Protein (CAMP) gene located on chromosome 3 [29]. The gene contains four exons, where exon 1-3 encode the pre-pro-regions and exon 4 encodes the active LL-37 peptide (Fig. 1). The mRNA is translated into a pre-pro-LL-37 peptide, which is transferred into granular compartments (granulocytes) or lamellar bodies (keratinocytes) with the help of a signal peptide (~ 30 residues) [12]. The pro-LL-37/hCAP18, containing the CLD, is also referred to as the N-terminal domain (101 residues), whereas the LL-37 sequence is known as the C-terminal domain (37 residues). The pro-peptide is excreted via exocytosis and the CLD is extracellularly cleaved off to release the active LL-37. Depending on cell type, the enzymes proteinase 3 or kallikrein 5 are responsible for the catalytic extracellular cleavage [30, 31] (Fig. 1). hCAP18 is not considered to have antimicrobial activity, though this has been under debate [32, 33]. The same applies to the CLD which functions remains to be concluded.



Figure 1. Expression and processing of human cathelicidin LL-37. The CAMP gene is transcribed into a pre-mRNA consisting of 4 exons and 3 introns. The mRNA is spliced and translated into a pre-pro-LL-37 peptide, which is transferred into cellular granules/lamellar bodies with the help of a signal peptide. The hCAP18 protein is exported via exocytosis and extracellularly cleaved into the active LL-37. This image was created with BioRender.com.

#### Structure of LL-37

The LL-37 peptide owes its name to the fact that it has 37 amino acids and that the sequence starts with two leucine residues. 16 out of 37 residues are charged, providing an overall net charge of +6 (Fig. 2). This positive charge is an important feature since it provides LL-37 with a high affinity for negatively charged elements, such as bacterial membranes, nucleotides and other negatively charged molecules [34]. The peptide forms an  $\alpha$ -helix in physiological conditions by enabling hydrogens bonds between the hydrogen in the amino group and the oxygen in the carboxyl group, thereby generating a more energetically favorable conformation [13]. In pure water, the peptide forms a more coil-like shape. The secondary structure of the peptide can be divided into three parts, the N-terminal  $\alpha$ -helix, the C-terminal  $\alpha$ -helix, and the C-terminal tail [35] (Fig. 2). The two helices are divided by a break, creating a helix-bend-helix formation. The  $\alpha$ -helix formation provides the peptide with an amphipathic structure, featuring both a hydrophobic and a hydrophilic surface (Fig. 2). Here, the majority of hydrophobic residues (green) are localized on the concave side, whereas the majority of charged residues (yellow and blue) are located on the opposite side. The hydrophobic surface can protect itself in a water-based environment by binding to the hydrophobic part of other molecules present, or to other LL-37 molecules, so called oligomerization [36]. The confirmational state of LL-37 is thought to affect LL-37 function and its interactions with biological membranes. For example, the oligomeric peptide has been shown to have a reduced antimicrobial activity compared to the monomeric structure [37]. On the other hand, oligometric LL-37 has also been shown to promote host cell effects [38]. It is believed that both LL-37 structures are present under physiological conditions [39].

#### **Regulation of LL-37 expression**

The transcriptional mechanisms of hCAP18/LL-37 are complex, and it is assumed that both expression and regulatory mechanisms are cell type dependent and stimulus specific. The peptide is often constitutively expressed, where hCAP18 remains stored in intracellular compartments such as granules or lamellar bodies [40, 41]. External stimuli like infection or tissue damage activates toll-like receptors (TLRs) and other inflammatory mediators, leading to extracellular release of the pro-peptide [42]. LL-37 gene expression can also be directly induced, where vitamin D is one of the most studied transcriptional regulators of CAMP. Here, the gene expression is upregulated by the binding of vitamin D to the vitamin D receptor (VDR), and this complex in turn binds to the vitamin D response element (VDRE) located in the CAMP promoter region [43, 44]. Even though vitamin D upregulates LL-37 expression at a non-inflammatory state it can also downregulate the expression during inflammation [42], suggesting that the effects of vitamin D on

LL-37 expression may depend on the inflammatory conditions and environment. Interestingly, the VDRE is present in primate CAMP, but not in other mammalian cathelicidin genes, showing that this is not an evolutionary conserved regulatory element [39]. Furthermore, there are numerous additional molecules and inflammatory pathways affecting LL-37 production. To mention a few, LL-37 expression is stimulated by endoplasmic reticulum stress in epithelial cells [45]. This pathway involves nuclear factor kappa-light chain-enhancer of activated B cells (NF-kB) signaling, but not VDR, advocating an VDR-independent pathway. Also, fatty acids such as butyrate, lactose and other sugars, and a various range of cytokines all upregulate the expression of CAMP [46-51].



**Figure 2. LL-37 structure.** The amino acid sequence presented in order in one-letter abbreviations. The peptide forms an  $\alpha$ -helix in a helix-bend-helix structure, which is coupled to a tail in the C-terminal. The helical wheel illustrates the peptide's amphipathic properties. The amino acid coloring represents their chemical properties, with non-polar (white and green), hydrophobic (green), polar (pink), potentially positively charged (blue) and potentially negatively charged (yellow) amino acids. This image was created with BioRender.com.

### The effects of LL-37 on pathogens

LL-37 shows activity against a wide range of pathogenic microorganisms (Fig. 3). This activity has been shown to be dependent on the secondary structure and the positive net charge of the peptide, but also on environmental composition such as ions, pH and other media components [13]. In this section the most common pathogenic effects are discussed in more detail.



Figure 3. An overview of selected LL-37 functions. LL-37 displays a variety of functions, here divided into antimicrobial and host cell effects. The image was created with BioRender.com.

#### Antibacterial

#### Membrane permeabilization

LL-37 induces toxicity in bacterial cells by permeabilizing their cytoplasmic membranes. The peptide is attracted to the negatively charged phospholipids of the bacterial cell wall and orients itself in a parallel position along the membrane surface [52]. Permeabilization leads to loss of membrane potential, thus also leakage of cytoplasmic components, leading to cell lysis and death of the bacteria. There are several theories on how LL-37 permeabilizes biological membranes (Fig. 4). In the "carpet" model the peptide coats the membrane surface until a threshold concentration is reached [53]. Here, the peptide either disrupts the membrane in a detergent-like manner by the formation of micelles or inserts itself into the lipid bilayer by forming so called "toroidal pores". In this last model, the peptide does not bind to each other but instead affects the curvature of the membrane, inducing a wormhole-like pore [54]. The third theory is the "barrel-stave" model where the peptide instead accumulate together to form a specific structure resembling an ion channel, which is then inserted into the bilaver [55]. The peptide seems to bind to bacterial membranes both as an unstructured coil and as an  $\alpha$ -helix, though the helix conformation is further stabilized when bound to membranes [56]. Oligomerization of LL-37 is thought to favor the "toroidal pore" formation, whereas the primate cathelicidin RL-37 (see Table 1) that mainly forms monomers in salt solutions favors the "carpet" model [57]. Hence, it seems that polymeric and monomeric LL-37 differentially affect pore formation.

#### Bacterial endotoxins and biofilms

LL-37 also displays antibacterial activity by binding to bacterial endotoxins and neutralize their effects [58]. The peptide can bind to lipopolysaccharides (LPS) present in the cell wall of Gram-negative bacteria and to lipoteichoic acid (LTA) in the peptidoglycan layer in Gram-positive bacteria with high affinity due to their negative charge. Furthermore, LL-37 has been shown to have activity against bacterial aggregates, so called biofilms [59, 60]. Bacteria found in biofilms are less susceptive to immunological responses and the aggregates have been shown to inhibit wound healing. LL-37 has been shown to inhibit the formation of biofilms by preventing the attachment of bacteria to each other and to other surfaces.



Figure 4. Proposed models of cell membrane permeabilization induced by LL-37. In the "carpet" model cells are permeabilized in a detergent-like manner, inducing the formation of micelles. In the "toroidal pore" model the peptide is inserted between the lipids, thereby forming a mixed pore, whereas in the "barrel-stave" model the peptide is inserted into the bilayer, clustered in a barrel-like shape with the hydrophilic face facing the pore lumen. This image was created with BioRender.com.

#### Antibiotic resistance and drug use

The increasing problems of bacterial antibiotic resistance has generated an interest in using LL-37 as an antibacterial drug. Since HDPs are evolutionary evolved alongside bacteria it has been suggested that they are less likely to induce resistance, though recent studies suggest otherwise [61]. Even if LL-37 displays a broad range of antimicrobial activity against a variety of bacterial strains, the peptide has not yet been approved for therapeutic use. There are many challenges remaining to be solved, such as induced host cell toxicity, decreasing antimicrobial activity over time and the fact that the peptide is easily degraded. Therefore, more research is needed to be able to use HDPs, such as LL-37, as an antibiotic drug.

#### Antiviral, antifungal and antiparasitic

LL-37 have direct antiviral activity against a variety of enveloped viruses, including influenza virus, adenovirus and HIV [62-64]. The peptide interacts and permeabilizes the membrane envelope, leading to viral disruption and exposure of its nucleic acids, which enables other immune cells to continue the immunological process. LL-37 can also indirectly induce antiviral activity by binding and delivering viral double stranded (ds) RNA to TLRs in host cells, resulting in an increased expression of type I interferons and other pro-inflammatory mediators [65]. This is an example of LL-37's immunomodulatory properties, and its interactions with host cells will be more elaborately discussed in the next section. Furthermore, LL-37 has been shown to have direct activity against fungi, including *Candida albicans*, which is one of the most common fungal pathogens in humans, but also against single cell parasites, such as protozoa [66, 67].

#### **Extracellular trap formation**

Extracellular traps (ETs) are web-like structures composed of DNA, histones, HDPs and cellular proteins that trap, neutralize, and kill different types of pathogens [68]. They were initially discovered to be produced in neutrophils but has later been found to also be generated in mast cells, macrophages and eosinophils [69-72]. The overall process, generally called ETosis, involves nuclear membrane disruption, decondensation of chromatin, followed by mixing of the nuclear, cytosolic, and granular components in the cytosol [73]. The disruption of the cell membrane results in cell death and release of the web into the extracellular space. An alternative more explosive mechanism has also been observed in neutrophils that does not result in cell death [74, 75]. ETosis has been characterized as an alternative programmed cell death mechanism that is separated from apoptosis and necrosis [73]. LL-37 is abundantly present in ETs and has also been shown to in itself be able to induce the formation of ETs in neutrophils [76]. Since the majority of research has been conducted in neutrophils, more research on the role of LL-37 in other ETosisforming cells is needed. Overall, the underlying mechanism behind the formation of ETs is still largely unknown.

## The effects of LL-37 on host cells

LL-37 is multifunctional and displays a range of different effects on host cells. The peptide acts as an immune system modulator by recruiting and activating immune cell responses. It can also activate a number of cellular receptors, leading to various pro- and anti-inflammatory downstream signaling. At high concentrations, LL-37 displays cytotoxicity and has been shown to induce cell death and apoptosis to a number of human cells. On the other hand, LL-37 can promote wound healing, angiogenesis and has an evident role in cancer development. All of these properties combined demonstrates the complexity and pleiotropic actions of the peptide. An overview of selected LL-37 host cell functions is presented in figure 3, and the most relevant features are discussed in more detail in this section.

#### Membrane permeabilization

The cell membranes of eukaryotes contain high levels of cholesterol and has a different phospholipid composition compared to those of the procaryotic cell, making eukaryotic membranes less electrostatically attractive to HDPs. Interestingly enough, LL-37 does not show a higher selectivity for bacterial membranes, and permeabilizes host cell membranes to a similar extent [77]. Instead, differences in LL-37-induced permeabilization seems more likely to be cell type specific/dependent on type of bacteria. The mechanistic models purposed for LL-37-induced permeabilization in host cells are considered to be similar to those previously described for the bacterial cell (Fig. 4) [39]. It has been demonstrated that permeabilization of host cells stimulates release of lactate dehydrogenase (LDH), which is a cytosolic enzyme responsible for converting lactate to pyruvate [78, 79]. LL-37 increases the extracellular LDH levels in several human cell types and LDH has been shown to be released in a dose dependent manner [80]. The fact that LDH is a relatively large cytosolic protein (144 kDa) provides an indication on the size of the LL-37-induced pores. Interestingly, it has been suggested that these pores are reversable, though this it likely dependent on LL-37 concentration [80].

The release of cellular components and/or influx of extracellular elements is likely to be an unspecific mechanism rather than a controlled one. For example, LL-37induced permeabilization generates a cellular influx of  $Ca^{2+}$  ions from the extracellular space due to its gradient, thereby increasing the cells' intracellular  $Ca^{2+}$ concentration [78, 79]. It has been suggested that this mobilization of  $Ca^{2+}$  can activate different receptors, leading to downstream effects, though this has not been fully established [38, 81, 82]. In general, it is not clear exactly what downstream effects are generated by LL-37-induced cell membrane permeabilization.

#### **Cellular internalization**

LL-37 has been shown to be internalized into several types of host cells [83-86], though the overall mechanism and how this relates to intracellular signaling is not entirely clear. The majority of LL-37 is considered to be imported through endocytosis, but the peptide can probably also enter cells via its self-made pores in the cell membrane. The endocytic pathways are made up by a complex protein machinery that transport a variety of molecules and receptors into internal membrane compartments, such as endosomes or the Golgi apparatus. LL-37 has been shown to be imported in macrophages via clathrin-mediated endocytosis, which is a well-characterized endocytic mechanism [81]. Here, cellular uptake is initiated by cargo assembly in the plasma membrane, which usually includes transmembrane receptors and their ligands (Fig. 5) [87]. A variety of cytosolic adaptor proteins, including clathrin, are recruited and bind to the inner leaflet of the specific area. The coating of the inner surface together with actin filaments promote bending of the membrane and initiates the formation of so called clathrin-coated pits. To separate the vesicle from the membrane, proteins such as dynamin assists in the scission process. The vesicle is then uncoated and fused together with endosomes or other cellular compartments.

LL-37 is also thought to be imported via caveolae/lipid raft-dependent endocytosis, though this pathway has not been as well-characterized [81]. Caveolae, which are invaginations of the plasma membrane, are mainly made up by integral membrane proteins called caveolins, which are directly bound to membrane cholesterol [88]. In caveolae-mediated endocytosis, caveolae vesicles are budded off from the cell membrane and further directed to membrane compartments of the cell. On the whole, more research on LL-37 internalization is needed to better understand how different mechanisms of import may affect cellular functions.

#### Cytotoxicity

LL-37 has been shown to induce toxicity and apoptosis in several human cell types, though the precise apoptotic mechanisms involved are not fully understood. The apoptotic pathways are highly regulated and often involves the activation of proteolytic enzymes called caspases, which mediate a complex cascade of events that leads to cell death [89]. LL-37-induced apoptosis has been shown to involve caspase activation in a number of cell types, inducing cleavage of caspase 3 and 9 [78, 90, 91]. Interestingly, LL-37 has also been shown to induce apoptosis in a caspase-independent manner [92-94]. This mechanism is suggested to involve the release of apoptosis-inducing factor from the mitochondria, indicating that LL-37 also permeabilizes mitochondrial membranes [95]. It is likely that LL-37-induced membrane permeability have a role in LL-37-induced cytotoxicity, but this remains

to be concluded. On the other hand, reports show that LL-37 can display both proand anti-apoptotic effects, which indicates that apoptotic events specifically depend on cell type and environmental stimuli [93, 96-98]. For example, neutrophils have been shown to express the globular C1q receptor p33 which can bind to LL-37 and reduce its toxic effects, making these cells less sensitive to peptide toxicity [79, 99]. A similar mechanism has been reported in endothelial cells for the plasma protein apolipoprotein A-I that bind to LL-37 and attenuates its effects [100]. Overall, LL-37-induced cytotoxicity in host cells needs further investigation to determine how it implements different types of cells and tissues upon inflammation and disease.



**Figure 5. Schematic illustration of clathrin-mediated endocytosis**. (1) Cargo proteins are recruited to the outer membrane (e.g., receptor and their ligand), with cytosolic coat proteins (adaptor proteins and clathrin) assembling on the inner leaflet. (2) Coating promotes membrane bending, initiating transformation into clathrin-coated pits. (3, 4) Dynamin assists in the scission process to separate the clathrin coated vesicle from the cell membrane. (5) Uncoating releases the cargo vesicle, (6, 7) enabling endosome fusion. This image was created with BioRender.com.

#### Immunomodulation

LL-37 can modulate both innate and adaptive immune responses by functioning as a chemoattractant, inducing the production of inflammatory agents in surrounding cells and manipulate microbial components. LL-37 has also been shown to activate a number of surface bound and intracellular receptors, leading to both pro- and anti-inflammatory responses.

#### Immune cells recruitment and activation

LL-37 is a chemoattractant and recruits granulocytes and other immune cells, including neutrophils [101], eosinophils [102], monocytes [103], mast cells [104], and T-cells [105] to infected sites. LL-37 has been shown to directly attract neutrophils and eosinophils by activating formyl-peptide receptors, which leads to additional LL-37 release since neutrophils are a stable source of the peptide [102]. LL-37 can also stimulate the production of pro-inflammatory cytokines in various immune and epithelial cells [106, 107]. For example, LL-37 induces release of interleukin 8 (IL-8) in monocytes and epithelia, which further initiates immune cell recruitment by promoting pro-inflammatory responses, demonstrating that LL-37induced chemotaxis can be both directly and indirectly activated [58]. Also, several reports show that LL-37 triggers release of IL-6, a cytokine known to be expressed in the initial stages of inflammation, in immune cells such as mast cells and dendritic cells (DCs), but also in airway epithelia, gingiva fibroblasts and keratinocytes [108-112]. On the other hand, LL-37 has been shown to inhibit pro-inflammatory mediators such as tumor necrosis factor  $\alpha$  in monocytes and interferon  $\gamma$  in immune cells, leading to reduced activation of lymphocytes [113, 114]. Together, these selected reports provide evidence of the peptide's pro- and anti-inflammatory activities.

#### TLR receptor modulation

TLRs are transmembrane sensor receptors of innate immunity that are expressed on a variety of cell types [115]. The family consists of around 10 receptors located intracellularly or on the cell surface. They recognize conserved microbial components known as pathogen-associated molecular patterns (PAMPs) (Fig. 6). Some TLRs like TLR2/TLR1 and TLR2/TLR6 heterodimers recognize a diverse range of PAMPs, such as lipoproteins, LPS, and peptidoglycans [116]. Both LPS and LTA can bind to TLR4 [117], whereas TLR5 is considered to be specific for bacterial flagellin [118]. TLRs that are intracellularly located has been shown to bind viral and/or bacterial RNA/DNA (TLR3, 7, 8 and 9) [119-121].

LL-37 has been shown to modulate multiple TLR-mediated responses [122, 123]. The peptide can synergize with flagellin to regulate TLR5 [124] and bind to single stranded RNA and DNA to enhance pro-inflammatory signaling by TLR7, 8 and 9 [125-127]. Interestingly, LL-37 inhibits the activation of TLR4 by binding to LPS,

which represents an anti-inflammatory effect [128]. Furthermore, it has been shown that LL-37 enhances TLR3 signaling by enabling viral dsRNA to serve as a TLR3 agonist, initiating downstream expression of IL-6 and other pro-inflammatory mediators [65]. The synthetic dsRNA polyinosine-polycytidylic acid (poly I:C) is often used as a TLR3 ligand to mimic viral infections *in vitro* [129]. It has been suggested by Singh *et al* that LL-37 binds to poly I:C at normal pH and upon endosomal acidification releases the dsRNA, thereby increasing its bioavailability [130]. However, the mechanisms of how LL-37 enhances poly I:C-induced TLR3 signaling still needs to be further elucidated.



Figure 6. TLRs and their selected ligands. TLRs mainly expressed on the cell surface recognizes a variety of PAMPs such as tricylated or diacylated lipoproteins (TLR2/TLR1, TLR2/TLR4), LPS (TLR4) and flagellin (TLR5), whereas intracellularly expressed TLRs recognize viral RNA (TLR3, 7 and 8), or bacterial/viral DNA (TLR9). This image was created with BioRender.com.

#### Other receptors and downstream pro-inflammatory signaling

Apart from TLRs, LL-37 has been shown to activate a number of cellular receptors located on the cell surface [131]. G protein coupled receptors (GPCRs) mediate a multitude of cellular functions in a wide range of cell types [132]. LL-37 in known to activate several GPCRs, including the *N*-formyl peptide receptor 2, inducing cell specific responses such as immune cell recruitment and neutrophil ETosis [103, 133]. Apart from GPCRs, LL-37 activates several receptor tyrosine kinases, including the subfamily of epidermal growth factor receptors (EGFR) which are enzymes involved in cell growth and homeostasis [131]. Also, LL-37 has been

shown to activate  $P2X_7$  in macrophages, a ligand-gated ion channel, initiating LL-37 internalization by endocytosis [81].



**Figure 7. Schematic illustration of transcription factor NF-kB signaling.** LL-37 can activate a range of cell surface receptors initiating downstream signaling that involves MAPK cascade signaling and the NF-kB pathway. In the classic NF-kB pathway the inhibitory IkB $\alpha$  protein is degraded after being phosphorylated and released from the NF-kB complex. NF-kB p105 is processed into p50, and the activated p50/p65 dimer is translocated into the nucleus where it can initiate gene transcription.

The fact that LL-37 activates receptors from different receptor families is surprising since ligand/receptor bindings are considered to be quite specific. How LL-37 do interact with different receptors is poorly understood. One theory is that LL-37 can accumulate around membrane receptors and initiate activation, which in turn triggers a wide range of signaling cascades [134]. It has been shown that LL-37/receptor signaling often involves mitogen-activated protein kinases (MAPKs) that leads to the activation of transcription factors (TFs) and transcription of immune response genes (Fig. 7) [106, 135, 136]. One of the most important TFs involved in pro-inflammatory signaling is NF- $\kappa$ B, which consists of DNA binding proteins of the Rel family, usually formed into homo or heterodimers [137]. The inhibitory protein IkBa controls NF- $\kappa$ B activity and is initially bound to the NF- $\kappa$ B complex. It is released after phosphorylation by the IkB kinase complex (IKK $\alpha$ - $\gamma$ ), followed by its degradation. This results in the activation of NF- $\kappa$ B p65 (also called RelA) and p105, where p105 is processed into p50. The p50/p65 complex is translocated

to the nucleus and can there bind to DNA and activate gene transcription. Overall, the majority of LL-37-induced functions are considered to be mediated by activation of multiple receptors, both cell surface and intracellularly expressed, though the underlying mechanisms need further investigation.

## LL-37 in disease

In several types of autoimmune disorders and other types of diseases abnormal levels of LL-37 have been detected. However, in most cases the relationship between LL-37 and disease is not fully known, although inflammation seems to be a common factor. In this section, a selected number of diseases where LL-37 has been suggested to be involved will be more elaboratively discussed (shown in Fig. 8). LL-37 involvement in cancer has been widely studied and is considered quite complex since LL-37 enhances tumor progression in some types of cancer, but also displays anti-cancer effects in other types [138]. Due to this, LL-37's involvement in cancer will not be more elaborated on in this chapter.



Figure 8. Abnormally high LL-37 levels are found in several autoimmune disorders and other inflammatory diseases.

#### **Skin conditions**

LL-37 has many important functions in healthy skin. It is produced by keratinocytes and other epithelial cells but is also provided by parading immune cells. It protects the skin barrier by fighting pathogens and modulates the immune responses. The peptide has been found at high concentrations in lesions of several types of inflammatory skin diseases [139]. Psoriasis is a chronic inflammatory skin condition that is characterized by red itchy skin patches that can be located all over the body. To date, no cure has been found, though treatments are available to manage and relieve symptoms. Abnormally high levels of LL-37 has been found in psoriatic lesions with concentrations ranging from  $\sim 100 \mu$ M to over 1 mM [140, 141]. Due to these high peptide levels the lesions appear to be less susceptive to infections. LL-37 has been shown to have a critical role in mediating inflammatory cascades in psoriasis [127]. In fact, LL-37 can bind to self-DNA and this complex induces T cell activation by TLR9 in DCs. On the other hand, LL-37 neutralizes cytosolic DNA and blocks inflammatory signaling in keratinocytes [142]. Hence, whether LL-37 mainly influences pro- or anti-inflammatory responses in psoriasis is still to be determined.

Rosacea is another though fairly common chronic skin disorder that mainly affects the face, resulting in flushing, redness, and swelling. The disease primarily affects people with lighter skin and is predominant in women [143]. LL-37 has been found at high levels in lesions of rosacea [144]. The enzyme kallikrein 5, known to process hCAP18 to LL-37 in keratinocytes, is also found to be upregulated in rosacea. Though, the mechanisms behind enhanced LL-37 production in rosacea and if this contributes to the inflammatory state is not known.

On the other hand, LL-37 has been shown to promote wound healing in both skin and other epithelia, making it suitable as a treatment of persistent wounds [145-147]. Reports have shown increased LL-37 expression at wound sites and lower levels of LL-37 was found in chronic wounds [148, 149]. The mechanism of LL-37-enhanced wound healing is not fully understood but may involve LL-37s' chemoattractive properties [146]. It is also possible that LL-37-evoked antibacterial effects indirectly may promote proliferation of epithelial cells and thereby stimulate wound healing.

#### Periodontitis

LL-37 has an essential role in the oral immune system and has been shown to be crucial in preventing periodontal infections [150]. In the healthy periodontium the peptide is produced by neutrophils and epithelia of the mouth and is found in saliva. Periodontitis is a chronic infectious and inflammatory disease that involves inflammation of the gingiva as a response of microbe infection [151]. If progressed, it eventually leads to tissue destruction and tooth loss. The disease is classified into

chronic periodontitis (CP) and aggressive periodontitis (AP) [152]. CP is prevalent in a large percentage of the adult population and is characterized by a slow progression compared to AP, which instead has a fast more sever progression, and mainly affects adolescents. High levels of LL-37 have been found in the gingiva, the gingival crevicular fluid, and in saliva of patients with CP [153-157]. The mechanisms of LL-37-upregulation in oral tissues and its role in periodontitis is not entirely clear.

Interestingly, in some cases of AP LL-37 levels have instead been found to be downregulated [157]. AP has been linked to patients suffering from congenital recessive disorders involving LL-37 deficiency such as Kostmann and Papillon-Lefèvre syndrome [158, 159]. In Kostmann's syndrome patients are born with a neutrophil deficiency [160]. Though, even if regular treatments with granulocyte colony growth factors can enhance the neutrophil count these neutrophils are still deficient in hCAP18/LL-37 [158]. In contrast, neutrophils in patients with Papillon-Lefèvre syndrome do express the hCAP18 protein, but cannot process the precursor into LL-37 due to the lack of serine protease activity [161]. Also, a missense CAMP mutation has been reported to be prevalent in patients with AP, but not in CP patients [162]. Together these reports suggests that LL-37 may have different roles in different forms of periodontitis [163].

#### **Respiratory disorders**

Asthma is a common inflammatory condition of the airways characterized by airflow obstruction and is often triggered by allergens. The role of LL-37 in asthma is suggested to be pro-inflammatory. LL-37 has been found to be upregulated in eosinophils in asthmatics and can activate eosinophil signaling, which induces the production of inflammatory mediators known to contribute to asthma [164, 165]. Also, LL-37 is known to induce chemotaxis of asthma-related immune cells [102, 166]. Though, the role of LL-37 in asthma progression still needs to be further studied.

LL-37, including many other types of HDPs, have been shown to be elevated in chronic obstructive pulmonary disease (COPD) [167, 168]. COPD is another type of chronic lung disorder, usually caused by heavy smoking, that results in obstructed airflow. COPD is in contrast to asthma a progressive disease, whereas asthma can be more daily regulated with medications. Reports have suggested that LL-37 is involved in the pathogenesis of COPD by inducing inflammation, apoptosis and mucus production in the airways [169, 170].

#### Atherosclerosis

Atherosclerosis is a chronic inflammatory disease where atherosclerotic plaques are formed in the arteries [171]. It is the most prevalent cause of cardiovascular disease in the world and can in worst cases cause stroke and heart attacks that result in death. The plaques are made up by different cells such as macrophages, other immune cells, and smooth muscle cells (SMCs), together with connective tissues, lipids, and proteins. LL-37 has been shown to be expressed in atherosclerotic plaques, and its activity has mainly been linked to macrophages [91, 172]. It has been suggested that LL-37 assists in the recruitment of inflammatory cells to the plaque and hence also modulates the cytokine environment [173]. LL-37 has been shown to stimulate atherosclerotic development by complex binding to self-DNA in DCs [174]. Also, LL-37 in complex with mitochondrial DNA has been shown to have an important role in immune cell activation and progression of atherosclerosis [175]. Though, more research is needed to fully establish the role of LL-37 in atherosclerosis.

# Aims

The general aim of this thesis was to further gain understanding in LL-37-mediated host cell effects, more specifically regarding LL-37-induced cell membrane permeability, cytotoxicity, and immunomodulatory effects.

The specific aims for each paper were to:

- I. Investigate LL-37 internalization in human MG63 osteoblast-like cells and assess how LL-37 import influences cell viability and cell membrane permeability.
- **II.** Examine the effects of LL-37 on cell viability, nucleic acid release and extracellular trap formation in human LAD2 mast cells.
- **III.** Assess LL-37 and dsRNA-induced upregulation of inflammatory signaling in human coronary artery vascular smooth muscle cells.
- **IV.** Study the mechanism behind how LL-37 elevates dsRNA-induced upregulation of TLR3 expression in human bronchial epithelial cells.
# Material and Methods

In this chapter I briefly describe and reflect upon the methods and techniques I have used in this thesis. Detailed descriptions can be found in the method section of each paper.

# Cell culture

The advantage of using cell culture is that it allows you to investigate molecular mechanisms in a controlled environment. In my case, working with a human peptide favors the use of human cells and tissues. In this thesis I have used four different types of human cells, all with different properties and culture requirements (see Table 2). I mainly chose to work with cell lines, more specifically in paper I, II and IV, since they provide an easy, inexpensive, and stable model to perform experiments in. Though, it is important to reflect on the limitations of cell lines and to consider how they compare to the original cell type. In paper III, I worked with the commercially available primary human coronary artery SMCs (hCASMCs), which have been directly isolated from tissue using enzymatic digestion. The main advantage of working with primary cells is the fact that they more closely resemble the original tissue, though you can only work with them for a limited period and their expression pattern might change over time. All cells were cultured in a waterjacked incubator at 37 °C with 5 % CO2 and seeded one day prior to each experiment. The LAD2 cells, which are grown in suspension, were attached to a surface using poly-L-lysine. All cells were treated in their regular growth medium if nothing else was stated. Exceptions were made when the cells were transfected, or the assay required specific buffers or transparent media without phenol-red.

| Cell    | Growth     | Origin                    | Туре      | Culture media                                    | Source                       | Paper |
|---------|------------|---------------------------|-----------|--|------------------------------|-------|
| MG63    | Adherent   | Osteoblast-like cell      | Cell line | DMEM F12<br>+10% FBS                             | A.T.C.C.                     | I     |
| LAD2    | Suspension | Mast cell                 | Cell line | StemPro-34<br>+StemPro-34<br>supplement<br>+RHSF | Gift from Dr.<br>Kirchenbaum | II    |
| hCASMC  | Adherent   | Coronary artery SMC       | Primary   | M231<br>+5% SMGS                                 | ThermoFisher<br>Scientific   | Ш     |
| BEAS-2B | Adherent   | Bronchial epithelial cell | Cell line | RPMI Glutamax<br>+10 FBS                         | A.T.C.C.                     | IV    |

Tabel 2. Human cells used in the different studies.

# Assessing cell viability, cell number and cell morphology

In all studies it was important to examine the cytotoxic effects of LL-37 since this varies depending on cell type. I used the tetrazolium dye MTT to assess the cell viability in paper I-IV. This assay is based on measuring the cells metabolic activity, meaning that the number of viable cells correlates with the amount of MTT substrate being reduced into a formazan product. This product is then dissolved, and the absorbance spectrophotometrically analyzed. However, the results from this assay may also reflect the number of cells present in each culture well, which is an important factor to consider. I also complemented the cell viability assay with counting the number of cells per well in paper III, using an automated cell counter, and with morphology assessments in paper II and III, using a phase contrast microscope.

### Extraction of RNA and real-time RT-qPCR

Reverse transcriptase quantitative polymerase chain reaction (RT qPCR), also known as real-time RT-qPCR, was used to assess mRNA expression of specific target genes in paper I, III and IV. For all targets RNA was extracted from cell lysates and concentration and purity evaluated using NanoDrop measurements. I used a one-step reaction, which means that the conversion from RNA to complementary DNA and the qPCR reaction occurs in the same well/tube. The advantages of a one-step reaction are that the contamination risks are reduced due to fewer laboratory steps, that it saves reagents and is less time consuming compared to a two-step reaction, though it can be less sensitive and problematic when there is a need to troubleshoot. I used a fluorescence-based assay to detect the real-time amplifications, more specifically a SYBR green based assay. SYBR green is a sensitive dye that only emits fluorescence when bound to dsDNA. However, the SYBR green dye is not sequence specific, so to ensure specificity a melt curve analysis was necessary. For all experiments, the target gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a protein which gene expression was not expected to change after treatment.

# Transfection using siRNA

To downregulate the protein expression of a certain target, I transfected cells using short interfering RNA (siRNA). This is a type of transfection where the siRNA binds to the target mRNA and initiates its degradation, thereby preventing translation. This method was used in paper I and III, and in both cases oligofectamine transfection reagent was used to transfer the siRNA more efficiently into the cells. To ensure specificity of the target siRNA, a scrambled negative control siRNA was used, where the target siRNA nucleotide sequence had been randomly rearranged. For both paper I and III, cells were transfected for a total of 72 h. Regular growth medium was added 1:1 after 24 h, followed by another 48 h of transfection. The knockdown was confirmed by using real-time qPCR.

### Determination of protein expression

#### Immunoblotting

Western blot, also called immunoblotting, is a technique used to separate and detect proteins in a sample using specific antibodies. In this thesis we used Western blot analysis in paper I, III and IV. Initially, cells were lysed, and proteins purified in a buffer containing sodium dodecyl sulfate (SDS), which denatures and binds the protein, giving it a negative charge. Total protein concentration in each sample was determined in a colorimetric assay, using a bovine serum albumin protein standard. The proteins were separated according to size, using SDS-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane. The efficiency of the transfer was confirmed by staining the gel with Coomassie stain. The membrane was blocked for unspecific antibody-binding with casein solution, and then incubated with a primary antibody, specific for the target protein. Next, the membrane was incubated with a secondary antibody conjugated with the enzyme horse-radish peroxidase, which generates a chemiluminescent signal after incubation with the substrate West Femto, enabling detection of the target protein. For all blots, the immunoreactive band for the target was normalized to the band intensity of GAPDH, which was used as a reference protein to ensure equal loading. Even though the western blot method is considered specific it can provide falsepositive results, and it is therefore important to include the right type of controls and to use validated antibodies. It is also important to consider that the membrane can get saturated with protein when using highly expressed targets, which will provide incorrect results.

#### ELISA

Enzyme-linked immunosorbent assay (ELISA) is used to quantify the amount of a specific protein in a sample. Specifically, I used a sandwich ELISA to determine the target of interest protein levels in paper I and III. The word sandwich refers to the process in which the target protein is bound between two antibodies, one bound to the surface of the well and one conjugated with an enzyme. Since ELISA kits have ready-made solutions and protocols, I followed the protocol according to the manufacturer's instructions. In general, cells were lysed in phosphate buffered saline by sonication and the total protein concentration in each sample was determined. The samples and a protein standard were loaded to a microplate that had previously been pre-coated with antibodies against the specific target. After protein binding, the plate was incubated with an enzyme-linked antibody, and after adding a substrate, this led to color development. The reaction was then stopped and analyzed in a microplate spectrophotometer. To eliminate incorrect results, samples and standard was always analyzed in duplicates. The advantage of using ELISA is the use of a protein standard, in that the amount of target protein in each sample can be specifically determined, compared to relative measurements. For each sample the target protein concentration was normalized to the total protein concentration.

### Immunofluorescence staining

Immunocytochemistry is a method used to localize specific targets in cells. This method was used in paper II and III to visualize the internalization of LL-37. Cells were cultured on glass slides and initially fixed and permeabilized before being incubated with bovine serum albumin to block for unspecific binding. Here, I used indirect immunofluorescent staining, meaning that a primary antibody initially binds specifically to the target, followed by the binding of a secondary antibody, conjugated with a specific fluorophore. The slides were mounted using a medium

containing the nuclear marker DAPI and analyzed in a fluorescence microscope. To evaluate import of poly I:C in paper III and IV, I treated cells with poly I:C that was directly labeled with the fluorophore rhodamine. After treatment, cells were fixed and mounted using DAPI before being analyzed. In paper II, cells were seeded on glass-bottom culture dishes and incubated with the plasma membrane fluorescent dye SYTOX green during treatment to visualize intracellular and extracellular nucleic acids. The fluorescent signal was immediately analyzed in live cells using confocal microscopy.

# Measuring permeabilization

#### LDH release assay

It has been shown that LL-37 permeabilizes human cell membranes. To measure LL-37-induced plasma membrane permeabilization, I evaluated the release of the cytoplasmic enzyme LDH in paper I and II. Cells were seeded in a microplate and treated at 37 °C for 30 min in phenol-red free medium to eliminate optical interference. Then, supernatants were collected and incubated with sodium pyruvate and a nicotinamide adenine dinucleotide (NADH)-solution, allowing LDH to catalyze a redox reaction, thereby converting pyruvate to L-lactate and NADH to NAD<sup>+</sup>. The LDH concentration was indirectly quantified by measuring the amount of NADH remaining in each sample, evaluating the efficacy of the redox reaction. The amount of LDH was normalized to the maximum LDH release, which was obtained by sonicating cells.

#### Measuring intracellular Ca<sup>2+</sup>

We have previously demonstrated that LL-37 alters the intracellular  $Ca^{2+}$  concentration in host cells by permeabilizing the cell membrane. In paper I, the intracellular  $Ca^{2+}$  levels were evaluated in MG63 cells cultured on glass-bottom culture dished and incubated with the  $Ca^{2+}$  sensitive dye Fluo-4. The binding of Fluo-4 to the  $Ca^{2+}$  ions will increase the fluorescence signal. In this case, the dye was used in the acetoximethyl ester form, which loads faster and is cleaved inside the cells to release the free Fluo-4 dye. The measurements were performed in a HEPES-buffered salt solution containing  $Ca^{2+}$  and analyzed immediately after treatment in a confocal microscope. The ionophore ionomycin was later added to obtain the maximum intracellular  $Ca^{2+}$  concentration. To reset the baseline, cells were incubated with a  $Ca^{2+}$  free solution.

#### Measuring nucleic acid release

In paper II, I investigated nucleic acid release from LAD2 mast cells as a result from LL-37-induced membrane permeabilization and extracellular trap formation. Cells were seeded in a microplate and treated in 37 °C in a HEPES-buffered salt solution. DNase was added to digest the released nucleic acids, and the reaction stopped using EDTA. Supernatants were transferred to a black microplate and incubated with the nucleic acid dye SYTOX green. The binding between SYTOX green and nucleic acids results in an enhanced fluorescent emission that can be analyzed in a microplate reader. The fluorescent signal was normalized to the concentration of total protein in each sample.

# **Results and Discussion**

In the following chapter, the results obtained in paper I-IV are presented and the main findings are generally discussed. A more elaborated description of the results and specific figures can be found in the result section of each paper.

# Paper I

*LL-37 causes cytotoxicity and cell membrane permeabilization in human osteoblastlike cells independently of LL-37 internalization via clathrin-mediated endocytosis* 

It is suggested that LL-37 can be internalized into human cell by both endocytosis and self-made pores formed in the cell membrane [35, 78, 81]. However, it is not clear if LL-37 imported via these two mechanisms have different or similar cellular effects. The aim in paper I was to investigate how cellular import of LL-37 affects cell viability and cell membrane permeabilization in MG63 osteoblast-like cells. Importantly, these cells do not endogenously express LL-37, which was essential to ensure that all internalized LL-37 had been imported from the outside. Clathrinmediated endocytosis was prevented both pharmacologically with chlorpromazine (CLP), which prevents clathrin lattices to assemble/dissemble, and by downregulating clathrin heavy chain like 1 (CLTCL1), a major protein of the clathrin-coated pits, using siRNA.

We show that blocking clathrin-mediated endocytosis reduces LL-37 import by around 30% in osteoblast-like cells. Previous reports demonstrate that inhibiting clathrin-mediated endocytosis with CLP reduce the LL-37 import in a similar manner in macrophages, indicating that LL-37 is similarly imported via clathrin-mediated endocytosis in both of these cell types [81]. We also pharmacologically blocked caveolin-mediated endocytosis using filipin, though this treatment did not reduce the LL-37 uptake, suggesting that LL-37 is not imported into osteoblast-like cells via caveolin-mediated endocytosis.

The sensitivity of human cells to LL-37-induced cytotoxicity has previously been shown to be cell specific, though majority of cells seems to be affected in a dose dependent manner [80]. LL-37-induced cytotoxicity in MG63 cells have previously been reported [78, 99]. We demonstrate that LL-37 reduces the cell viability in a

similar manner whether or not LL-37 import via endocytosis is partly prevented (Fig. 9A), indicating that the LL-37 internalized via clathrin-mediated endocytosis does not have a cytotoxic effect. One might argue that blocking the import by 30% would not be enough to visualize changes in cell viability. To address this, we performed a dose response curve to ensure system sensitivity. Importantly, neither CLP treatment nor CLTCL1 knockdown displayed cytotoxicity on their own.

Furthermore, we show that LL-37 permeabilizes the cell membrane in a similar manner both with and without blockage of clathrin-mediated endocytosis (Fig. 9B). We evaluated membrane permeability in two ways, by measuring an immediate  $Ca^{2+}$  influx and by assessing the release of LDH protein. It has previously been established that measuring influx of cellular  $Ca^{2+}$  is a useful method to determine LL-37-induced cell membrane permeabilization [78]. We show that intracellular  $Ca^{2+}$  increases similarly, both with and without endocytic prevention. Also, we found that LDH was released in a similar manner in both control cells and in cells where endocytosis was blocked (Fig. 9B). Together, these data indicate that LL-37-induced membrane permeability occurs independently of clathrin-mediated endocytosis.

Summarizing the results from paper I, we show that both pharmacological inhibition of clathrin-mediated endocytosis and knockdown of CLTCL1 reduce LL-37 import, and that this reduction in uptake do not have an impact on LL-37-induced cytotoxicity and cell membrane permeability in MG63 osteoblast-like cells.



**Figure 9.** (A, B) MG63 osteoblast-like cells were treated with chlorpromazine (CLP, 10  $\mu$ M) for 1 h, before treatment with LL-37 (4  $\mu$ M). Cell viability was assessed after 3 h, using the MTT assay (A) and release of LDH after 30 min (B). Data are presented as ± SEM. \*\*\* p<0.001, ns represents "non-significant". This data was originally published as part of Fig. 2a (A) and Fig. 3c (B) in paper I [176].

# Paper II

#### LL-37 induces release of nucleic acids and proteins in mast cells

ETosis has been shown to be an important immunological process, in which antimicrobial webs are released from immune cells as a response to infection [73]. Apart from DNA and cellular proteins, HDPs such as LL-37 are essential components of ETs. ETosis predominantly takes place in neutrophils and LL-37 has been shown to in itself be able to initiate the formation of ETs in these cells [76]. Mast cells are also ET forming cells, though the ability of LL-37 to directly induce ETosis in mast cells have not previously been investigated. In paper II, we assessed the ability of LL-37 to induce ETosis in mast cells, but also the effects of LL-37 on mast cell toxicity and cell membrane permeability. In this study we used LAD2 mast cells, which is a prominent mast cell line commonly used *in vitro* [177].

Here, we show that LL-37 increases release of nucleic acids and proteins into the extracellular space (Fig. 10A and B). This release is suggested to be a result of cell membrane permeabilization, which was confirmed by measuring the release of LDH protein. Interestingly, LL-37 also induced release of nucleic acids at concentrations where the cell viability remained unaffected, suggesting that LL-37-induced permeabilization does not always result in a reduction of cell viability. Treatment with phorbol-12-myristate-13-acetate (PMA) was included as a control for ETosis since this is a well-known inducer of ETs in both neutrophils and mast cells. PMA caused release of nucleic acids, but not proteins (Fig. 10A and B), suggesting that LL-37 and PMA stimulates nucleic acid release by different mechanisms.

Furthermore, confocal microscope images illustrated that nucleic acids were present in both the nucleus and the cytosol (Fig. 10C-E). In these experiments we used the nucleic acid dye SYTOX green, which is impermeant to intact cells. Also, internalized LL-37 was detected in both the nucleus and in the cytosol. Together these data suggest that LL-37 permeabilizes both the nuclear and the cellular membranes. However, LL-37 did not induce the formation of extracellular trap-like structures in contrast to PMA treated cells, though these PMA-induced webs were produced at a relatively low percentage. In fact, mast cells are generally considered to induce ETosis in 40% of cells, compared to neutrophils where 90% of cells have been shown to produce ETs [178]. Overall, this data provides further evidence that LL-37 and PMA induce release of cellular components using different mechanisms.

In summary, we show in paper II that LL-37-induced permeabilization of the nuclear envelope and the cellular membrane promotes release of nucleic acids and proteins in human mast cells.



**Figure 10.** (A-B) LAD2 mast cells treated with LL-37 (10  $\mu$ M) or PMA (250 nM) for 4 h release nucleic acids (A) and protein (B). Data are presented as ± SEM. \*\* p<0.01, \*\*\* p<0.001. (C-E) Nucleic acids are labeled with SYTOX green in control cells (C) and cells treated with LL-37 (10  $\mu$ M) (D-E) for 4 h. The bar in panel D represents 70  $\mu$ m and in panel E 10  $\mu$ m. This data was originally published as part of Fig. 3a-b (A,B) and Fig. 5a-c (C-E) in paper II [179].

# Paper III

*LL-37 potentiates poly I:C-induced signaling and TLR3 expression in human coronary artery smooth muscle cells* 

LL-37 has been found to be upregulated in atherosclerotic plaques, though the precise role of LL-37 in the progression of atherosclerosis needs more investigation [91, 172]. Macrophages are considered the main producers of LL-37 in the plaques, and reports suggest that LL-37 mediates a pro-inflammatory role in the inflammation and progression of atherosclerosis [173-175]. Vascular SMCs have a prominent role in atherosclerosis, though the effects of LL-37 on vascular immunity have not been established. In paper III, we investigated the pro-inflammatory effects of LL-37 and dsRNA signaling in hCASMCs, including the expression of cytokines IL-6 and monocyte chemoattractant protein 1 (MCP-1), and the innate immune receptor TLR3. Here, we used the synthetic dsRNA poly I:C since it is known be effective in activating immunological pathways *in vitro*.

We found that co-treatment with poly I:C and LL-37 for 24 h increased both mRNA and protein expression of IL-6 and MCP-1 compared to treatment with poly I:C alone. We used two different concentrations of poly I:C (10 and 30  $\mu$ g/ml) to examine the concentration/response relationship and found that 30  $\mu$ g/ml poly I:C generated a more enhanced IL-6 and MCP-1 protein expression compared to 10  $\mu$ g/ml. Though, the co-treatment induced a more pronounced upregulation of IL-6 and MCP-1 levels compared to poly I:C alone at both poly I:C concentrations used.

Importantly, the LL-37 concentration used in this study did not display cytotoxicity. Even so, LL-37 was shown to be internalized into the SMCs using fluorescent imaging, suggesting an intracellular mechanism of action. Moreover, we found that the LL-37-induced potentiation of poly I:C signaling was not due to enhanced import of poly I:C, but instead a result of increased mRNA (Fig. 11A) and protein expression of TLR3. This data showed that LL-37 enhances the poly I:C-induced TLR3 expression compared to poly I:C alone (Fig. 11A). Furthermore, knockdown of TLR3 reduced both IL-6 and MCP-1 transcripts, thereby confirming loss of function (Fig. 11B). Recent new data also reveal that the co-treatment with poly I:C and LL-37 is most effective in upregulating TLR3 expression at 15 h and 24 h (Fig. 12), indicating that LL-37 enhancement of TLR3 expression in hCASMCs increases with time.

Summarizing paper III, we show that poly I:C-induced expression of the inflammatory mediators IL-6 and MCP-1 in hCASMCs is a result of enhanced TLR3 expression, and that this immunological response induced by dsRNA can be enhanced by LL-37.



**Figure 11.** (A) Transcript levels of TLR3 are upregulated in human coronary artery smooth muscle cells after treatment with LL-37 (1  $\mu$ M) and poly I:C (10 or 30  $\mu$ g/ml) in combination for 24 h. (B) The upregulated IL-6 mRNA expression induced by treatment with LL-37 (1  $\mu$ g/ml) and poly I:C (10  $\mu$ g/ml) after 24 h was reduced by TLR3 knockdown, using siRNA. (A-B) Data are presented as ± SEM. \*\*\* p<0.001. This data was originally published as part of Fig. 6a (A) and Fig. 7a (B) in paper III [180].



Figure 12. TLR3 mRNA expression in human coronary artery smooth muscle cells treated with LL-37 (1 μM) and poly I:C (10 μg/ml) for 3, 6, 15 and 24 h. Data are presented as ± SEM. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

# Paper IV

*LL-37 enhances poly I:C-induced upregulation of TLR3 due to LL-37-stimulated import of poly I:C in BEAS-2B epithelial cells* 

LL-37 has been shown to mediate TLR signaling and is able to bind to a wide variety of TLR ligands, such as bacterial and viral nucleic acids [122-127]. Reports suggest that LL-37 can bind to the synthetic dsRNA poly I:C and enhance TLR3 signaling in epithelial cells [130]. We recently found (paper III) that this enhanced TLR3 signaling may be a result from LL-37/poly I:C-stimulated expression of TLR3, though this study was performed in SMCs and not epithelial cells [180]. Therefore, in paper IV, we investigated LL-37 and poly I:C co-induction of TLR3 expression in the human bronchial epithelial BEAS-2B cell line, and if upregulation of TLR3 could be pharmacologically blocked using the glucocorticoid dexamethasone.

Here, we show that LL-37 potentiates poly I:C-induced mRNA and protein expression of TLR3 compared to treatment with poly I:C alone. At a short time (6 h), a lower LL-37 concentration (1  $\mu$ M) was enough to potentiate the TLR3 expression (Fig. 13A). However, at a longer treatment time (24 h), a higher LL-37 concentration (4  $\mu$ M) was required to obtain similar results. Reports suggest that LL-37 have a short half-life *in vitro*, (around 1 h) [130], which would explain why a higher concentration of LL-37 was necessary at a longer treatment time. It is important to mention that neither one of the LL-37 concentrations used displayed cytotoxicity.

Furthermore, we demonstrate that LL-37 generates an enhanced import of poly I:C (Fig. 13B). The immunofluorescent signal was analyzed in cells treated with a rhodamine-labelled poly I:C, either alone or in combination with LL-37 for 6 and 24 h. A higher cellular fluorescence was detected after co-treatment compared to poly I:C alone at both 6 and 24 h, suggesting that LL-37 stimulates poly I:C import in a rapid process where the internal poly I:C levels remain unchanged after 24 h. This LL-37-enhanced import of poly I:C may be a novel mechanism of action to explain LL-37/poly I:C-induced upregulation of TLR3 expression in BEAS-2B cells.

In addition, we show that the glucocorticoid dexamethasone reduces the TLR3 expression generated by LL-37 and poly I:C, demonstrating the importance of dexamethasone as an anti-inflammatory treatment. Dexamethasone also enhanced the expression of the NF- $\kappa$ B inhibitory protein I $\kappa$ B $\alpha$ , whereas the co-treatment induced upregulation of the NF- $\kappa$ B protein p105. Together these data suggest that LL-37/poly I:C-induced TLR3 upregulation involves NF- $\kappa$ B signaling. Also, the endosomal acidification inhibitor chloroquine abolished the induced TLR3 levels, suggesting that acidification of the endosomes is important for upregulating TLR3 expression.

In summary, we show that upregulated TLR3 expression induced by LL-37 and poly I:C is associated with LL-37-enhanced import of poly I:C. Also, our data demonstrates that LL-37/poly I:C-induced TLR3 expression involves downstream signaling and elevated NF- $\kappa$ B activity.



**Figure 13.** (A) TLR3 mRNA expession of human BEAS-2B cells treated with poly I:C (0.2 and 2  $\mu$ g/ml) and LL-37 (1  $\mu$ M) for 6 h. (B-C) The average relative cell fluorescence (red) and DAPI staining (blue) in BEAS-2B cells treated with rhodamine-labeled poly I:C (4  $\mu$ g/ml) for 24 h. The bar in the bottom image represents 40  $\mu$ m for all panels. (A-B) Data are presented as ± SEM. \*\* p<0.01, \*\*\* p<0.001. This data was originally published as part of Fig. 3a (A), Fig. 5d (B) and Fig. 5c (C) in paper IV [181].

# Summary and Reflection

The multifunctional role of LL-37 has been widely studied, though there are still many aspects of LL-37-mediated functions that remain to be investigated. High levels of LL-37 are found locally in tissues of many inflammatory and autoimmune disorders. If we are to better understand the progression and maintenance of such diseases, the mechanisms of LL-37-induced host cell effects needs to be further investigated. In this thesis I have elaborated on various LL-37-mediated effects with my main focus on LL-37-induced host cell toxicity, cell permeability and TLR mediated pro-inflammatory functions.

In paper I-IV we observe that LL-37 reduces host cell viability in a dose dependent manner in all cell types tested, though the general level of sensitivity seems to be cell specific. In paper I and II we intended to induce a medium level of toxicity, whereas in paper III and IV we wanted to avoid cytotoxic effects from the peptide. However, since these different cell types have dissimilar morphologies and grow in different patterns it is challenging to directly compare their sensitivity to LL-37-induced toxicity. As previously described, the pathways involved in LL-37-induced toxicity and apoptosis have been shown to be many and the mechanisms behind this diversity are not completely understood.

In paper I and II we show that the LL-37-induced permeabilization results in influx of Ca<sup>2+</sup> and release of both nucleic and cytosolic components. The fact that LDH is a relatively large protein provides us with information regarding the size of cellular components being able to diffuse through the LL-37-induced pores. We hypothesize that the release of nucleic acids, caused by LL-37-stimulated membrane permeabilization, may be an important mechanism in modulating pro-inflammatory signaling in innate immunity. On the other hand, the results from paper I indicate that LL-37-induced membrane permeability may be an important factor in mediating host cell cytotoxicity. Although, data in paper II demonstrates that LL-37 can induce cell membrane permeability, but not cytotoxicity. Hence, exactly what cellular functions LL-37-induced permeabilization mediates remains to be concluded.

In paper I-III we demonstrate that LL-37 is internalized into several types of human cells. It is generally believed that LL-37 import is a necessary process to allow LL-37 to execute many of its cellular effects. LL-37 has been shown to mediate TLR responses by interacting with PAMPs and other TLR ligands. In paper III and IV we demonstrate that LL-37 mediates TLR3 signaling and enhance downstream pro-

inflammatory responses in both hCASMCs and bronchial epithelial cells. We found that LL-37 enhanced poly I:C-evoked TLR3 expression in both cell types, though poly I:C seemed to generate an overall quicker and larger immunological response in the epithelial cells compared to the SMCs. In paper IV we found that LL-37 enhanced the import of poly I:C in epithelial cells and we propose that this mechanism is responsible for the LL-37/poly I:C-induced TLR3 upregulation in these cells. We suspect that the increased poly I:C import is a result from both enhanced endocytic import and increased diffusion through LL-37-mediated pores. Notably, we did not detect a LL-37-enhanced import of poly I:C in hCASMCs in paper III, though this might be due to numerous factors, such as different experimental conditions and the cells' responsiveness to dsRNA. Interestingly, LL-37 modulation of TLR mediated effects has been observed as both pro- and anti-inflammatory, advocating the opinion that LL-37 can both increase or revoke inflammatory signaling depending on cell type and microenvironment.

In summary, the general conclusions from paper I-IV are as follows:

- LL-37 reduces the cell viability in a concentration dependent manner in human cells, though the level of cytotoxicity varies with cell type.
- LL-37 permeabilizes host cell membranes, resulting in Ca<sup>2+</sup> influx, release of nucleic acids and proteins, such as LDH.
- LL-37-induced cell membrane permeability does not always result in cytotoxicity, but permeabilization also occurs at low LL-37 concentrations where the cell viability remains unaffected.
- LL-37 is internalized into human osteoblasts, mast cells and SMCs, indicating that LL-37 might trigger intracellular signaling in these cells.
- LL-37 is imported by clathrin-mediated endocytosis in osteoblast-like cells, and this import does not affect LL-37-induced cell cytotoxicity and cell membrane permeability.
- LL-37 potentiates poly I:C-induced TLR3 expression and downstream proinflammatory signaling in hCASMCs and bronchial epithelial cells.
- LL-37 induces an increased import of poly I:C in bronchial epithelial cells, and this effect is associated with downstream TLR3 signaling and increased NF-κB activity.

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