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Between good and evil: Complexation of the human cathelicidin LL-37 with nucleic acids

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ABSTRACT   The innate immune system provides a crucial first line of defense against invading pathogens attacking the body. As the only member of the human cathelicidin family, the antimicrobial peptide LL-37 has been shown to have antiviral, antifungal, and antibacterial properties. In complexation with nucleic acids, LL-37 is suggested to maintain its beneficial health effects while also acting as a condensation agent for the nucleic acid. Complexes formed by LL-37 and nucleic acids have been shown to be immunostimulatory with a positive impact on the human innate immune system. However, some studies also suggest that in some circumstances, LL-37/nucleic acid complexes may be a contributing factor to autoimmune disorders such as psoriasis and systemic lupus erythematosus. This review provides a comprehensive discussion of research highlighting the beneficial health effects of LL-37/nucleic acid complexes, as well as discussing observed detrimental effects. We will emphasize why it is important to investigate and elucidate structural characteristics, such as condensation patterns of nucleic acids within complexation, and their mechanisms of action, to shed light on the intricate physiological effects of LL-37 and the seemingly contradictory role of LL-37/nucleic acid complexes in the innate immune response.

INTRODUCTION

Different classes of antimicrobial peptides (AMPs) have specialized roles in the human innate immune system in protecting the body against a broad range of microbial infections (1–3). One of the most important and well-studied members of this group is LL-37, which is the only cathelicidin peptide within the human proteome (4). LL-37 has broad-spectrum antiviral, antifungal and antibacterial properties as well as antibiofilm activity (5). The peptide consists of 37 amino acids (6), forming an α-helical motif throughout residues 2–31 with a disordered tail at the C-terminus, in a physiological solvent environment (Fig. 1) (7,8). Active LL-37 peptide is generated through extracellular cleavage of this peptide tail from its precursor protein hCAP18 by either proteinase 3 in neutrophils (9) or serine proteases in keratinocytes (10). LL-37 is utilized by neutrophils, macrophages, epithelial cells, keratinocytes, B cells, and natural killer (NK) cells to target and kill infected cells (4,11). The peptide is immunostimulatory (12,13) and plays an important role in inflammation and autoimmune diseases (14) and in the killing of pathogens and infected host cells (15).

The expression of LL-37 is, among others, modulated by vitamin D3 (cholecalciferol) and butyrate and is triggered by infection, physical wounding, and/or an endoplasmic reticulum stress response (16–19). LL-37 is beneficial and necessary for human health (20,21), and upregulation of the peptide has been suggested to play an important role in reducing pathology of several infectious diseases, including severe coronavirus disease 2019 (COVID-19) infections (22).

LL-37 can drive cellular regeneration via direct involvement in autophagy and enhances progenitor cell chemotaxis in wound healing. Typically, unbound peptide would rapidly be degraded by proteases, but due to its ability to form complexes with other biomolecules in solution, including plasma proteins, amyloid-β (23), islet amyloid polypeptide (24), and α-synuclein (25) as well as lipid bilayers/phospholipid membranes (8,26,27), LL-37 is well protected from proteolytic degradation. LL-37 can also form complexes with F-actin (28) and glycosaminoglycan (29,30).

LL-37 has a positive net charge of +6, promoting electrostatic interactions with negatively charged molecules and structures (e.g., the above-mentioned phospholipid membranes (8,31) or different types of nucleic acids). LL-37 has been shown to form complexes with, among others, mitochondrial DNA (32), genomic DNA explosively expelled from neutrophils in neutrophil extracellular traps

a LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

b

FIGURE 1 (*a*) LL-37 amino acid sequence. (*b*) Structure of LL-37 (PDB: 2K6O) (7). To see this figure in color, go online.

(NETs) (33), and RNA (34). Complexes of LL-37 and different nucleic acids have been shown to trigger toll-like receptors (TLRs) including TLR3 (35,36), TLR7 (34), TLR8 (34), and TLR9 (37,38), which all play key roles in the innate immune system.

Contrary to the reported beneficial immunomodulatory effects of complexes of LL-37 with nucleic acids, several studies have also identified these complexes as disease drivers with detrimental effects on human health. Thorough work to elucidate the consequences of the interactions of LL-37 and nucleic acids has been performed, especially by Wong, Gilliet, Gallo, and colleagues (34,36–42). In the following sections, we will try to connect the functions of the complexes and their roles in the human body to their structural appearance.

LL-37 FACILITATES GENOMIC DNA ENTRY INTO CELLULAR COMPARTMENTS

In several studies, LL-37 showed affinity to bind to DNA to form complexes of significance for the human innate immune system (e.g., references (32–38)). Helical, double-stranded genomic DNA presents a negatively charged backbone due to its phosphate groups, which based on electrostatic interactions makes it an ideal binding partner for the cationic LL-37. Lande et al. (42) showed how LL-37 and inert self-DNA together formed condensed structures. It was concluded that LL-37 converts inert self-DNA into a trigger of IFN production through condensation of DNA, and these complexes subsequently trigger TLR9 to break the innate tolerance to self-DNA, driving autoimmunity in psoriasis. As described by the authors, the DNA in that complex is not fully condensed by LL-37 but only an inner node with three DNAs arranged around the node. The resulting complexes were able to migrate into early endocytic compartments. Studies have shown LL-37 penetrates cell membranes and is internalized by human macrophages and osteoblasts via endocytosis; however, inhibition of the endocytic pathways did not completely prevent LL-37 from entering cells (43,44). It was postulated that LL-37 can form pores in cell walls and plasma membranes (6), and it uses these openings to transport cargo into the cells (35). The mechanism of how LL-37 functions as a cargo delivery vehicle to transport extracellular DNA into cells through membrane perturbation was previously investigated by Zhang et al. (45). The authors tested the "membrane disruption capacity" of LL-37 using unilamellar vesicles prepared from different lipids (1-palmi-toyl-2-oleoyl-sn-glycero-3-phosphocholin, 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoglycerol, cholesterol) and found that the induced α-helical element of LL-37 orients parallel to the lipid membrane surface, enabling penetration into the membrane. In addition, they observed via confocal microscopy that LL-37 mediated the delivery of oligonucleotides into eukaryotic cells. Complexes of LL-37 and fluorophore-labeled, 21-mer DNA oligonucleotide, at a ratio of one peptide per DNA basepair, resulting in a net positive charge, were found in eukaryotic cells after incubation, whereas experiments without LL-37 did not show any significant DNA uptake by the cell. Based on their results, the authors suggested endocytosis as major pathway of membrane translocation, whereas general cytoplasmic delivery/endosomal escape did not appear to be an efficient pathway.

LL-37/NUCLEIC ACID COMPLEXES FORM DISTINCT STRUCTURES THAT MAY EITHER BE BENEFICIAL OR DETRIMENTAL TO HUMAN PHYSIOLOGY

To fully understand the mechanism of action of LL-37-induced nucleic acid condensation, detailed biophysical structural investigations are necessary. Accordingly, Schmidt et al. (38) investigated the impact of different kinds of condensation agents, including LL-37, on double-stranded DNA (dsDNA). Their results revealed how LL-37 and dsDNA can under some conditions assemble into a structured liquid-crystalline ordered columnar square lattice, where the peptide controls the inter-DNA spacing. Compared with other condensing agents, LL-37 in complexation with DNA showed a greater induction of interferon-α (IFN-α) production in dendritic cells (DCs), an effect that is connected to TLR9 structure. The specific inter-DNA spacing resembles the distance between two TLR9s, which can accommodate binding of multiple TLR9s and further amplify their activation, whereas smaller spacings discourage activation due to the sterically reduced accessibility of complexed DNA to receptors (Fig. 2).

In another study, complexes were formed with the monodisperse λ DNA (48.5 kbp, dsDNA) or genomic dsDNA from *Escherichia coli* (*E. coli*) (37). The authors used small-angle x-ray scattering (SAXS) to elucidate the complexation mechanisms and suggested that LL-37 assembles into a cationic protofibril in the presence of an anionic DNA backbone to form a square columnar lattice. (This study used centrifugation to concentrate the LL-37 complexes to a substantial degree, which may not be physiologically relevant.)

As mentioned earlier, LL-37 is utilized by NK cells to target and kill infected cells. A study by Chuang et al. found that complexes of LL-37 with subunits of DNA can also enhance the proliferation and activation of NK cells in the peritoneal cavity, implying a distinct role of the complexes
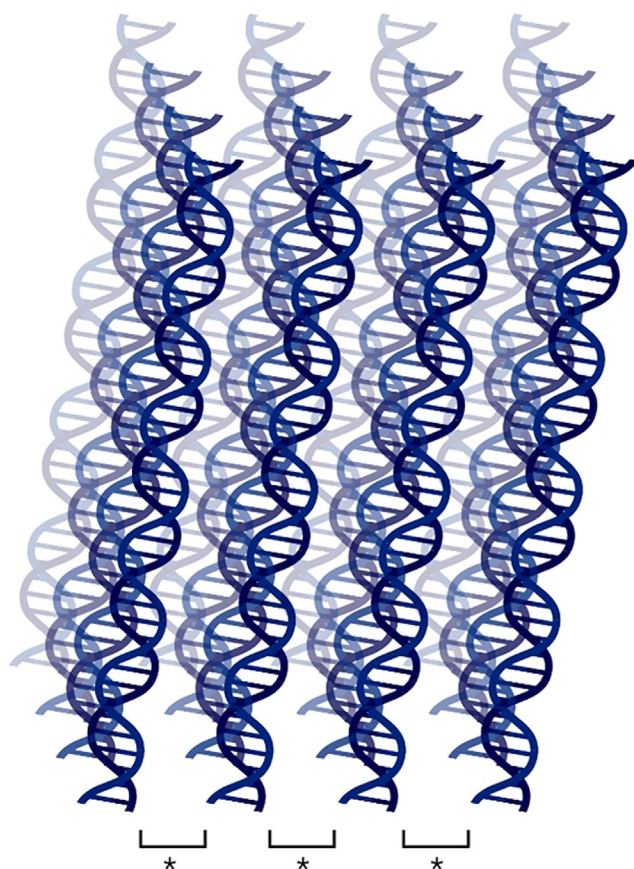
FIGURE 2 Schmidt et al. (38) identified a structured liquid-crystalline ordered columnar square lattice for dsDNA, with LL-37 controlling the inter-DNA spacing. Inter-DNA spacing is indicated by *. Figure was created with BioRender.com. To see this figure in color, go online.

within the innate immune system (46). In this study, which was focused on ovarian cancer, the peritoneal NK cells played a critical role in the observed antitumor effects. The presented data suggest that the combination of DNA subunits with LL-37 may lead to the control of ovarian tumors through the activation of innate immunity. Evidence that LL-37-DNA complexes cause an increase in the number of NK cells within a biological system is in itself significant, because NK cells not only clear infectious agents (47) and have antimicrobial properties (48), but they also are critical immune cells in the fight against cancer and have recently been explored as a new therapeutic alternative to T cell-based approaches (49).

Not directly implicated with but related to human health, Khairkhah et al. showed the interaction between LL-37 and plasmid DNA, recombinant and empty PX330 and PX458 CRISPR-Cas9 vectors (50). The complexes were able to overcome limitations of viral vectors for the delivery of CRISPR-Cas9 vectors for gene editing, such as restricted insertion size and provoking immune responses (51). In conclusion, the complexes contributed to the nontoxic, effective, and specific CRISPR-Cas9-mediated gene editing

of pre-existing cervical tumors. Here, LL-37 showed as well its potential to protect nucleic acids from nuclease degradation (50). Complexes at a nucleic acid-peptide ratio of 5 were imaged using scanning electron microscopy and identified to be spherical shapes of 120–190 nm. The authors did not indicate if w/w or molar ratios were mixed.

Besides the beneficial effects the complexes appear to have on the human body, a study by Lande et al. identified LL-37 and its ability to bind DNA as the key factor mediating the activation of plasmacytoid dendritic cells (pDCs) in psoriasis, an autoimmune skin disease (42). LL-37 and DNA formed aggregated and condensed structures, resulting in the conversion of inert self-DNA into a trigger for interferon production. This means that LL-37 is able to break innate tolerance to self-DNA, which the authors suspected enhanced autoimmunity in psoriasis. Using atomic force microscopy, the authors showed that three DNA molecules form a flower-like structure around one LL-37 "node," suggesting a disproportionate level of DNA to LL-37 and potential underregulation or underexpression of LL-37 (see Fig. 3 a). The authors followed up on their work on the role of antimicrobial peptides and self-DNA in autoimmune skin inflammation in a comprehensive opinion piece (52).

COMPLEXES OF LL-37 AND MITOCHONDRIAL DNA ENHANCE INFLAMMATION

Inflammation is often connected to oxidative stress and injured cells, releasing elevated levels of molecules called damage-associated molecular patterns (DAMPs) into their direct environment. Mitochondrial DNA (mtDNA) has been identified as a DAMP. It is considered a major player in the activation of the innate immune system (53) and is mainly known to cause maternally inherited mitochondrial diseases (54). mtDNA originates in the mitochondria and encodes essential protein subunits to maintain oxidative phosphorylation. It is a small, double-stranded, and circular molecule with 16,569 basepairs and extensive methylation at non-CpG sites (55) that is able to encode 37 genes (56). mtDNA regulation is believed to be controlled basally by cell-specific mechanisms and in response to intrinsic or environmental stresses (57), but mtDNA copy number is overly abundant in many cells and tissues, suggesting other factors might be causing a high cellular mtDNA occurrence (58). In recent years, mtDNA has gained more attention as a potential activator of human receptors influencing antimicrobial responses and inflammatory pathologies within the innate immune system (59,60). On the other hand, abnormal responses of the innate immune system are tightly connected to pathologies such as autoimmune diseases, metabolic syndrome, or neurodegeneration, and, considering mitochondrial dysfunction and/or damage is a shared feature in the aforementioned diseases, upregulation of mtDNA might either cause or propagate the pathologies.
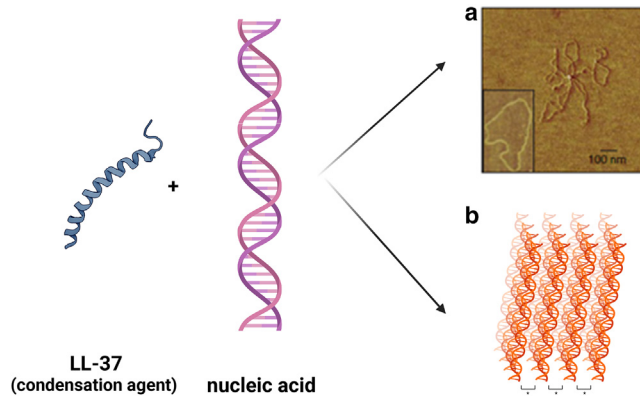
FIGURE 3 LL-37 is a condensation agent shown to interact with nucleic acids to form (*a*) loosely condensed, flower-like complexes (42) (Copyright Springer Nature) and (*b*) crystalline columnar square lattice structures (38,129). Structure of LL-37 (PDB: 2K6O) (7). Figure was created with BioRender.com. To see this figure in color, go online.

The literature shows that naturally upregulating LL-37 could help maintain low mtDNA concentrations while at the same time inducing IFN-α production (61). For example, mtDNA was highlighted as an inflammatory mediator in cardiovascular diseases (62,63) and atherosclerosis. Considering LL-37's ability to complex with nucleic acids, it is logical that upregulation of LL-37 provides more binding partners to eliminate mtDNA and prevent mtDNA from stimulating receptors and causing immune reactions. One might assume that complexation of LL-37 with mtDNA would therefore reduce inflammation leading to cardiovascular disease and atherosclerosis.

However, Zhang et al. (32) have shown that complexes formed between LL-37 and mtDNA actually seemed to promote atherosclerosis. They found high amounts of LL-37 in complexation with mtDNA in atherosclerotic plaques and plasma, causing increased atherosclerotic lesions in apolipoprotein E-deficient mice. Furthermore, the formed complexes were resistant to autophagy and seemed to protect the mtDNA from DNase II degradation. However, TLR9 still recognized the mtDNA and thereby activated and mediated inflammatory responses causing autoimmune activation of chemokines and cytokines (see (37)). This means that complexes of cathelicidin and mtDNA within a cell will be difficult to clear and may result in continuous activation of TLR9 signaling pathways. Antibody treatment targeting the LL-37-mtDNA complex mitigated lesion formation, leading the authors to conclude that the complexes drive atherosclerotic plaque formation, and disruption of these complexes may offer strategies for atherosclerosis prevention and treatment (32). By preventing mtDNA degradation-induced autophagy, another study showed that LL-37-mtDNA complexes aggravate local inflammation in sepsis-induced acute lung injury (64). Just as in the atherosclerosis study, elevated levels of LL-37 in serum were found in severe sepsis patients compared with individuals with mild sepsis. Neutrophils isolated from septic mice treated with cathelicidin-related antimicrobial peptide (CRAMP) (the LL-37 equivalent for mice)-mtDNA showed excess proinflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8, MMP-8, and TNF-α. On the other hand, antibody treatment against the CRAMP-mtDNA complexes resulted in an improvement in inflammatory symptoms. LL-37 complexation with mtDNA seems to cause autophagy dysfunction in proinflammatory cells, causing tissue damage and triggering inflammatory responses of the human body.

Hitherto, no information about complexation mechanism or condensing patterns of mtDNA and LL-37 can be found in the literature.

THE ROLE OF LL-37 IN NEUTROPHIL EXTRACELLULAR TRAPS

NETs are part of the innate immune response in which neutrophils respond to inflammatory stimulation by migrating to infected tissue and then releasing a web-like structure capable of trapping and eliminating microbes. These traps consist of a backbone of DNA/histones (65,66), which are decondensed nuclear and mtDNA associated with granule proteins, and LL-37, which together enable NETs to engulf and kill pathogens such as bacteria (67). After "NETosis" where neutrophils experience plasma rupture and NET release, NETs can be cleared by DNase I or, presumably, macrophages. NET clearance is an important part of preventing NET-related detrimental effects (67,68). For example, uncleared or slowly cleared NETs are associated with progression of different diseases including cancer (69), chronic rhinosinusitis (70), chronic lupus erythematosus (71), and COVID-19 (72,73). Further, NETs are suspected to cause atherosclerosis and thrombosis (74,75). One AMP found in NETs is LL-37, which notably increased the resistance of NETs to *Staphylococcus aureus* nuclease degradation (33,76). Neumann et al. described this phenomenon through the cationic character of LL-37, which binds to neutrophil DNA and hence protects it from degradation by the nuclease. By contrast, they found association with chromatin in NETs reduced the antimicrobial activity of LL-37 (76). However, Stephan et al. (77) showed in their 2016 study that LL-37 extracted from NETs maintained its antimicrobial activity. Specifically, they found that when separated from the complexes, LL-37 is active against mycobacteria in BCG-infected macrophage phagolysosomes (77). Using fluorescence microscopy, the authors were able to follow the uptake and intracellular procession of the LL-37-DNA complexes within macrophages and the subsequent release of the peptide to attack and kill the intracellular mycobacteria. Their study suggests that LL-37-DNA complexes contribute to the human host defense against intracellular bacterial infections in human macrophages. Another study pointed to a physiologically relevant

role of RNA, instead of DNA, within NETs in psoriasis (78). Herster et al. showed a correlation between plaque psoriasis, the most dominant form of psoriasis, and NETs. They showed that self-propagating NET and cytokine response occurred independently of DNA, but complexes of LL-37 and RNA triggered TLR8- and TLR13-mediated cytokine and NET release in vitro and in vivo, augmenting inflammation and amplifying a self-sustaining disease progression (78). In other words, NET-associated RNA/LL-37 furthers the development of psoriasis by self-propagating in a "vicious cycle" contributing to chronic inflammation.

The binding of LL-37 into biomacromolecular complexes has been proposed as the key factor in other autoimmune diseases. In particular, dysregulation of LL-37 has been suggested to play a role in systemic lupus erythematosus (SLE) (14), which is an autoimmune disease accompanied by severe organ manifestations due to inflammation and immune complex depositions. Anti-double-stranded DNA antibodies (anti-dsDNA Abs) and atherosclerosis are hallmarks of SLE (79). Even though the exact cause of SLE is unclear, type I IFN pathways have been suggested as main contributors to disease progression and severeness (80). Furthermore, SLE neutrophils were shown to undergo NETosis (81–83), and LL-37 has been suggested as a strategy to stabilize these DNA-immunostimulatory molecule complexes in SLE patients (84) instead of clearing them. In this study, complexes formed between DNA from NETosis, LL-37, and anti-dsDNA Abs were shown to stimulate TLR9 in pDCs and hence upregulate type I IFN production. In addition, SLE patients presented autoantibodies to LL-37, which induced NET production. Both phenomena contribute to an inflammation cycle, creating more NETs and stimulating further type I IFN production.

Type I IFN production of pDCs due to LL-37-DNA complexation in NETs has also been connected to atherosclerosis (79). Döring et al. demonstrated that pDCs can be stimulated by LL-37-DNA complexes to produce IFN-α in atherosclerotic arteries, causing overexpression of LL-37 and increased formation of NETs. LL-37-DNA complexes were shown to increase atherosclerotic lesion formation, whereas CRAMP deficiency in the bone marrow of apolipoprotein E-deficient mice resulted in a reduction of atherosclerosis symptoms and hallmarks such as anti-dsDNA-Abs. The authors suggested that overexpressed LL-37 and self-DNA may stimulate a pDC-driven pathway of autoimmune activation in atherosclerotic lesions generating anti-dsDNA Abs, which in turn enhance atherosclerosis lesion formation.

Within this section, it should be mentioned that not only neutrophils cause extracellular traps (ETs) contributing to microbial defense, but mast cells do as well (85). Both neutrophils and mast cells are part of the innate immune response, and mast cells have been shown to initiate recruitment of neutrophils in wounds (86). ETs from mast cells, so called MCETs, were similar to NETs, characterized as web-like DNA strands decorated with histones and AMPs (87). Dahl et al. (88) used immunofluorescence microscopy to illustrate how LL-37 permeabilizes nuclear and plasma membranes of human mast cells to be internalized within the cytoplasm and nucleus, triggering an ET-like release or "export" of nucleic acids without the actual formation of ETs. We hypothesize that LL-37 plays an important role in ET clearance while using the DNA (and potentially RNA) in these traps to degrade the larger molecular network through formation of complexes and to help in the fight against pathogens. Therefore, the exact influence and mechanism of LL-37 during ETosis should be investigated to gain more insight into the cellular processes through which NETosis (66) and other forms of ETs (89) occur.

LL-37-NUCLEIC ACID INTERACTION MIGHT YIELD INSIGHTS INTO LL-37 ANTIBACTERIAL ACTIVITY

Traditionally the mechanism of action for bacterial killing of AMPs has been attributed to membrane effects; however, recent evidence has shown that the ability of AMPs in general, and LL-37 specifically, to permeabilize the bacterial membrane and to bind bacterial nucleic acids or ribosomes intracellularly may be essential. Barron and co-workers showed by electron microscopy and soft x-ray tomography that LL-37 triggers extensive and rapid nonspecific aggregation of intracellular biomacromolecules, including DNA and ribosomes, when added to *E. coli* bacteria (Fig. 4) (90). This was later supported by *Zhu* et al. who found bacterial DNA (bacDNA) and a subset of ribosomes to be rigidified on a length scale of ∼30 nm, seconds after LL-37 permeated into the *E. coli* cytoplasm (91). In this experiment the bacteria were located in a flow cell and exposed to about 90 mM of LL-37 in solution. The authors used super-resolution, single-particle tracking to show the loss of "jiggling" motion of specific DNA and ribosome markers after contact with LL-37. In this study, it was implied that LL-37 inhibits bacterial material growth through rigidification of the entire cytoplasm, which after subsequent washing, growth, and function was not able to recover. It was further suggested that the polyanionic nature of the cytoplasm (due to e.g., DNA, ribosomes, RNA, and most globular proteins) renders it susceptible to fast and impactful adsorption of polycationic agents once penetration of the membranes occurs and that the ability to alter the cytoplasm by electrostatically linking dsDNA is a criterion to design peptides that kill gram-negative bacteria.

Wang et al. (92) synthesized and tested LL-37 analogs as a means to identify a more specific working mechanism of action. The authors found the DNA binding and retarding abilities (as measured by gel electrophoresis) of the analogs to be proportional to their ability to kill bacteria from *Pseudomonas aeruginosa*, which they proposed was the basis for the mechanism of LL-37 in bacterial killing, through hindrance of DNA replication.
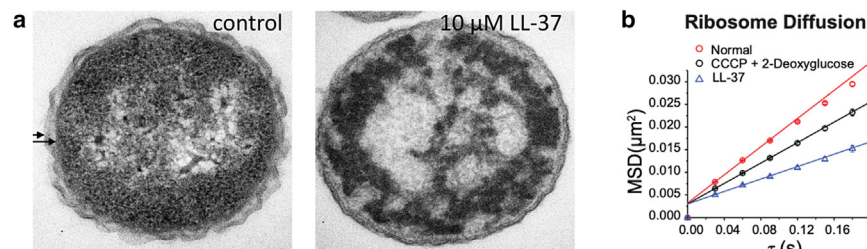
FIGURE 4 (*a*) Comparison of transmission electron micrographs of transverse thin sections of representative *E. coli* bacteria without (control) and with treatment of 10 μM LL-37 for 1 h indicating intracellular biomass flocculation upon LL-37 treatment. Figure was reproduced from Chongsiriwatana et al. (90). (*b*) Comparison of the average ribosome diffusive motion, represented by the ribosome mean square displacement versus lag time τ, in normal cells, in cells treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP) as a positive control, and in cells treated with LL-37 (at t > 15 min). Compared with normal cells, treatment with CCCP decreases the mean diffusion coefficient by a factor of 1.5, whereas LL-37 treatment leads to a decrease by a factor of 2.3. Figure was reproduced from Zhu et al. (91). To see this figure in color, go online.

Although these studies showed clear evidence for the interaction between LL-37 and bacDNA, the exact mechanisms of interaction and how these intertwine with the antibacterial function of the AMP need further investigation. Although human DNA is always linear, highly coiled, and attached to histone proteins, bacDNA can have a circular form and is less tightly packed as it is typically not bound to histones. bacDNA has been identified in a series of places within the human body, including in serum of ulcerative colitis (93) and Crohn's disease (94) patients. However, it is unclear how bacDNA escapes from immune reactions to translocate into the blood stream. Duan et al. (95) demonstrated that through complex formation with LL-37, bacDNA was able to avoid degradation while inhibiting the antibacterial properties of LL-37. These complexes were then found in abundance in plasma and lesions of patients with ulcerative colitis while promoting inflammation by inducing T helper (Th) 1 cell, Th2 cell, and Th17 cell differentiation and activation of TLR9. It was suggested that through increased cellular permeability bacDNA utilized LL-37 as a vehicle to migrate into the blood stream and to evade immune elimination (as seen elsewhere, e.g., (45)).

LL-37 expression is induced as a reaction to bacterial infections, and LL-37 is able to lyse the invading microbial cells. Sandgren et al. demonstrated that LL-37 also targets the released extracellular bacterial DNA plasmids and can transport them into mammalian cells to trigger further LL-37 expression (96). Physiological LL-37 concentrations killed bacterial cells without showing cytotoxic or growth-inhibitory effects on mammalian cells (96). LL-37 was able to protect DNA from serum nuclease degradation processes, whereas DNA seemingly protected LL-37 from proteolytic degradation, with LL-37 maintaining its antimicrobial activity. The process, observed through confocal fluorescence microscopy to show localization of internalized DNA, was described as being time and temperature dependent. Another influence on the cargo transportation ability of LL-37 was the presence of cholesterol-depleting agents to hinder the formation of cholesterol-rich rafts. Here, cholesterol—which has been previously discussed as a binding partner of LL-37 (97)—was mentioned as being necessary to transport nucleic acids into cell compartments.

STRUCTURED BUNDLES OF LL-37 AND RNA TRIGGER IMMUNE REACTIONS

RNA, just as DNA, features a negatively charged backbone due to phosphate groups, making it an excellent binding partner for LL-37, and similarly, LL-37 can transport RNA into different kinds of cells. Macleod et al. (98) showed that intracellular uptake of ssRNA oligonucleotides is facilitated by cathelicidin. The group used RNA aptamers, which are synthetic single-stranded RNA oligonucleotides with functions similar to antibodies, and tested them as therapeutics in inflammatory skin diseases through topical administration. These aptamers formed complexes with LL-37 and were able to enter keratinocytes and fibroblasts, whereas the free aptamers were hindered from entering. The aptamers that were internalized through LL-37 complexation remained immunologically inert in keratinocytes, fibroblasts, and peripheral blood mononuclear cells including infiltrating DCs and monocytes, and, hence, they did not cause unwarranted inflammation, as shown through inflammatory mediator- and interferon-stimulated gene measurements.

Beyond these effects, LL-37 was shown to transport dsRNA into cells to induce growth factor expression from keratinocytes and endothelial cells (39), and Bodahl et al. (35) reported that LL-37 and dsRNA synergistically upregulated bronchial epithelial TLR3 due to an increased amount of imported dsRNA and downstream TLR3 signaling.

Further, LL-37 was shown to promote inflammation by enabling binding of self-RNA to cell surface scavenger receptors. Takahashi et al. (40) saw in their 2018 publication that cathelicidin enabled keratinocytes to recognize self-noncoding U1 RNA via interaction with SR-B1 surface scavenger receptors of the cells. Similar to the work by Schmidt et al. (38), Takahashi et al. (40) were able to connect the square columnar lattice structure as observed by SAXS and inter-RNA spacing of LL-37-RNA complexes to similar distances in receptor distance. A derivative of cathelicidin, consisting of the N-terminal 34 amino acids

of LL-37 with substitution of amino acid 20 from isoleucine to proline, called LL-34(I20P), was not able to localize with SR-B1 even though LL-34(I20P) condensed dsRNA into comparable square lattices with LL-37 with comparable first peak positions (40).

In a related study, complexes of nonmodified LL-34 (truncated version of LL-37 without the three C-terminal amino acids) with U1 dsRNA were investigated using SAXS (36). To understand the proper working mechanism of LL-34, Kulkarni et al. (36) modified the LL-37 variant with different alanine substitutions clustered on its hydrophobic face, which subsequently inhibited type 1 interferon expression when RNA was presented to keratinocytes and created a loss of inflammatory activity in keratinocytes and epithelial and endothelial cells. It was shown that the amino acid residues 24 (isoleucine) and 31 (leucine) of LL-34 are responsible for binding of U1 RNA to cell surface scavenger receptors. However, the authors did not expand their study to investigate the structure of RNA in complexation with the cathelicidin in its natural form.

In another study, it was found that dsRNA forms disordered columnar complexes with an average inter-dsRNA spacing of a = 3.63 nm in the presence of LL-37, and the authors pointed out that the inter-dsRNA spacing is a valuable tool for predicting IL-6 production in certain cells via TLR3 activation (41). This was related to the similarity of inter-RNA spacing and inter-TLR spacing, which matches when LL-37 is in complexation with the RNA, but not when other condensation agents are used (such as spermine). Comparable spacing led to activation of several TLRs at the same time without hindering neighboring TLRs.

DOES ANTIVIRAL ACTIVITY OF LL-37 RESULT FROM INTERACTION WITH VIRAL NUCLEIC ACIDS?

A very comprehensive recent review by Pahar et al. (99) illustrated the role of LL-37 in viral infections in terms of interaction of the cathelicidin with both DNA and RNA viruses, where the feasibility of LL-37 as antiviral agent was highlighted. In terms of DNA viruses, the antiviral activity of LL-37 was shown for vaccine viruses (100) and herpes simplex virus type 1 (101,102), whereas in regards to RNA viruses, the beneficial effects were described for human immunodeficiency virus (103), severe acute respiratory syndrome coronavirus 2 (22,104), dengue virus type 2 (105), hepatitis C virus (106), Ebola virus (107), Zika virus (108), and others (109–111). Although much effort has been put into investigating LL-37-viral interactions, no research can be found directly elucidating structural characteristics and potential mechanisms of action of LL-37-nucleic acid complexes in their fight against viral infections. Additionally, LL-37 was identified to not only have favorable but also unfavorable roles in virus replication and disease pathogenesis.

For example, a study involving over 100 infants reported that the infants with the highest amount of LL-37 in their bloodstream showed lower sensitivity to respiratory syncytial virus bronchiolitis but were more prone to human rhinovirus bronchiolitis (112). In patients with herpes simplex virus type 2 infections, it was found that LL-37 level was increased in normal human epithelial cells, which caused increased human immunodeficiency virus susceptibility of Langerhans cells (113). The underlying mechanisms of the effects are unknown as mainly activation pathways and (up-)regulation patterns of LL-37 in the fight against viral infections were investigated. The above-mentioned study on dengue virus type 2 (105) did take a deeper look into structural characteristics and potential molecular interactions and found through in silico experiments that binding of LL-37 to the E protein might prevent the binding of the latter to its receptor. A decrease in viral and genomic RNA levels was observed when cultures were pretreated with 10–15 μM LL-37 compared with cultures with virus control. It was not discussed if LL-37-RNA complexation might be a cause for these observations. Other publications speculated about interactions between LL-37 and viral RNA in relation to their results but did not further characterize the structure of the complexes (111,114).

Polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analog of dsRNA similar to the one present in some viruses, is used as a molecular pattern associated with viral infections. Using poly(I:C), LL-37 was shown to augment antiviral activity induced by the dsRNA in keratinocytes (115). Using gel electrophoresis, LL-37 and poly(I:C) were shown to interact directly through complex formation (116). Notably, others have reported poly(I:C) in complexation with LL-37 was able to enter through cell membranes (96). An important finding was that LL-37 prevented poly(I:C)-induced glucocorticoid resistance by stimulating phosphorylation and nuclear translocation of a glucocorticoid receptor; and glucocorticoid-induced expression of the antiinflammatory protein's promyelocytic leukemia zinc finger was increased by the complex, compared with poly(I:C) alone. The authors did not further investigate the macromolecular structure of the complexes (116).

EFFECT OF CITRULLINATION ON LL-37 AND DNA COMPLEXES

A common naturally occurring posttranslational modification of LL-37 is citrullination, where peptidyl arginine residues undergo enzymatic deimination through conversion of the natural amino acid arginine (Arg) into citrulline, thereby converting ketimine groups into ketone groups (Fig. 5) (117). A catalyst for this process are PAD enzymes (peptidylarginine deiminases, EC 3.5.3.15) (118,119) with calcium as a key regulator of PAD activity (120,121). There are five isoenzymes of PAD (122), although only PAD2 and
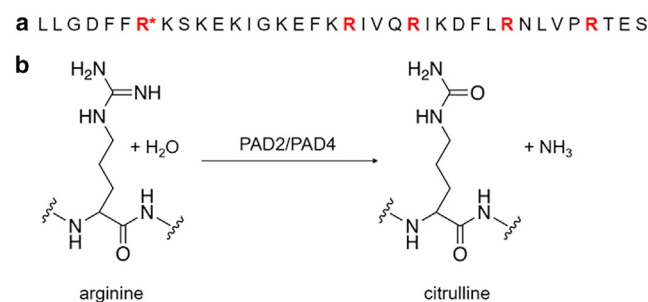
FIGURE 5   (*a*) LL-37 Amino acid sequence with R arginines in bold. R* arginine 7 is the most susceptible to citrullination, and (*b*) citrullination chemical reaction converts arginine to citrulline. To see this figure in color, go online.

PAD4 have been clearly identified as essential in enzymatic deimination of LL-37 (123,124).

Through citrullination, LL-37 with its five Arg groups can potentially lose positive charges, and the α-helical structure can be altered, thus affecting LL-37's molecular interactions with other biomolecules. Citrullination by PADs occurs gradually with sequential deimination of arginines (with Arg 7 the most susceptible residue) likely resulting in partially citrullinated peptides (123). As the function of LL-37 is driven by the cationic charges and α-helical structure, citrullination causes LL-37 to lose its antiinflammatory and bactericidal effects (123,124). Citrullinated LL-37 showed a reduced ability to quench proinflammatory activities of lipopolysaccharides, which are well known to trigger acute inflammatory responses by activating the release of a vast number of inflammatory cytokines in various cell types (123). Furthermore, citrullinated LL-37 cannot prevent mortality and morbidity due to septic shock in the same way as LL-37, and the ability to downregulate harmful cellular responses to host inflammatory mediators and TLR ligands is lost. As citrullination of LL-37 can happen during inflammatory responses, care should be taken when developing drugs based on LL-37 itself, as they might contribute to the severity of a sepsis (123).

The binding characteristics of citrullinated LL-37 and nucleic acids are not well studied. However, Wong et al. (125) used PAD-citrullinated LL-37 to investigate its function and complexation pattern with cell-free *Tannerella forsythia* genomic DNA. They showed that citrullination of LL-37 hindered binding to DNA and peptide-dependent nucleic acid uptake by pDCs, a consequence of nullifying the immunostimulatory effects of the peptide. Additionally, LL-37 is citrullinated by PADs during NET formation, which further affects the inflammatory potential of NETs. As mentioned previously, we suggest that LL-37 supports ET clearance through binding to nucleic acids and subsequent nucleic acid condensation. Since PAD-induced citrullination affects the working mechanisms of LL-37, and PADs are a common denominator in ETs, investigating the impact of LL-37 citrullination is essential.

DISCUSSION AND OUTLOOK

As we can see, complexes formed by LL-37 and different nucleic acids appear to be powerful immunomodulators that can have significant impact on the human innate immune system. We showed that LL-37 acts as a nucleic acid condensing agent with the potential to form different types of complexed structures.

LL-37 was suggested to form a cationic protofibril in the presence of an anionic DNA backbone to form a square columnar lattice with nonnatural, monodisperse λ DNA (37,38,41). However, published findings (126) indicate that LL-37 without nucleic acids present can form tetramers in solution, and LL-37 in the presence of membrane-mimicking detergents was identified as an oligomer with functional adaption mechanisms (127). This might indicate that the presence of other biomolecules could influence the complexation and condensation mechanisms and functions of the LL-37 in regard to nucleic acids, since different peptide assemblies can interfere with electrostatic and aromatic interactions, the main complexation mechanisms of LL-37 and nucleic acids. Most likely, experimental conditions such as in vitro buffers, other biomolecules present, and in vivo conditions can drastically change the complexation behavior of LL-37 and nucleic acids.

It is possible that the negative effects that Lande et al. observed in their study of the partially condensed DNA, seen as aggregation and coiling of three plasmid DNA molecules around one LL-37-DNA node through atomic force microscopy (Fig. 3) (42), are caused by not forming fully condensed particles, which then cause and continue inflammation and negatively influence the innate immune system.

A potential major factor in differentiating between positive and negative effects of LL-37/nucleic acid complexes that must be considered is the regulation of the expression of LL-37. For example, and as discussed earlier, the literature shows that a higher concentration of LL-37 in the human body could help maintain low mtDNA concentrations (61). Additionally, when present in bundles with LL-37, both native and oxidatively modified mtDNA forms were shown to provoke enhanced IFN-α secretion from pDCs with the native form causing significantly more IFN-α secretion than the oxidized form (61). IFN-α is important in the suppression of viral infections in the human body through mediation and regulation of the immune response. Furthermore, simultaneous administration of a TLR9 antagonist completely inhibited the secretion of IFN-α from pDCs previously induced by mtDNA in the presence of LL-37. Similarly, complexes of LL-37 and inert self-nuclear DNA were shown to induce IFN-α secretion from pDCs (128). Through protection of the extracellular DNA and the formation of aggregated and condensed complexes, LL-37 delivered DNA to and retained within early endocytic compartments to trigger TLR9 (42), which would not occur to this extent with less LL-37 present. Furthermore, it has

been hypothesized that LL-37 supports the clearing of NET formation through complexation of these expelled DNA molecules, and it was reasoned that upregulation of LL-37 expression could reduce the occurrence of microthrombosis in COVID-19 caused by NETs (22).

Dysregulation of LL-37 has been suggested to be involved in several autoimmune diseases (14,79,129,130). We hypothesize that dysregulation of LL-37 in the human body can cause low levels of the peptide, which results in a different condensation pattern of DNA within such complexes. It is apparent from this review that a drastic change in complexation structure (fully condensed nucleic acids versus only partially condensed nucleic acids) could influence their functionality and mechanism of action. However, to date, no published research has elucidated the impact of under- or overrepresentation of LL-37 during nucleic acid condensation. Concentrations, mixing ratios, and approaches on investigating the functionality of the complexes vary greatly in literature, and no consistent approach to making the results comparable or on how to identify their mechanism of action can be found. From the presented literature, it is not straightforward to draw conclusions of over- or underrepresented LL-37 in the studies. Over- or underrepresented LL-37 in a system can be controlled through the molar mixing ratios of both LL-37 and nucleic acid and is influenced by the size, structure (methylation, CpG sites), and origin of the nucleic acid and, hence, their charges. These characteristics will not only have influence on the complex formation kinetics but may also influence their intricate biological behavior and seemingly contradictory role within the innate immune response.

We suggest that the complexation ratio is important to define the state of condensation of the nucleic acid within the complex. For example, as discussed earlier, LL-37 was shown to be able to transport DNA into cells through pores (45,96) and to fight intracellular bacterial infections (77). Due to pore size and occupied area (entropic barriers), LL-37 may not be able to transport only partially condensed DNA into cell compartments, in the same way as it transports fully condensed DNA. Further, conclusions from in vitro or ex vivo experiments may be hard to interpret as other biomolecules and crowding effects are present in biological systems that may interfere with the condensation process, as seen previously for bacDNA in *E. coli* where rigidification was observed instead (91).

CONCLUSION

This review has attempted to collect, discuss, and appreciate the contradictory research results and literature with regard to complexation of the human cathelicidin LL-37 with different kinds of nucleic acids. The complexes have been shown to present diverse structures, to be immunostimulatory, and to exhibit a myriad of beneficial health effects in the human body while also showing detrimental effects on

human health. We reasoned that the beneficial or detrimental health effects may depend on the regulation of LL-37 in the human body, giving way to different condensation states of nucleic acids in complexation. It is apparent that a thorough understanding of the complexation ratios and patterns of these two biomolecules needs to be established to gain clarity on the actual effects of the complexes in the human body.

AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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