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LL-37 Promotes Rapid Sensing of CpG Oligodeoxynucleotides by B Lymphocytes and Plasmacytoid Dendritic Cells

Plinio Hurtado*† and Chen Au Peh*‡∗

LL-37 is a cationic antimicrobial peptide derived from neutrophils and keratinocytes. It plays an important role in protection against bacterial infection in the skin and mucosal surfaces. However, its role within the blood compartment remains unclear given that serum inhibits its bactericidal property. In this study, we show that LL-37 promotes very rapid and highly efficient sensing of CpG motifs in bacterial DNA by human B lymphocytes and plasmacytoid dendritic cells (pDCs) in serum-containing media and in whole blood. LL-37 allowed detection of CpG oligodeoxynucleotide (ODN) within minutes of exposure. Without LL-37, 20–30 times more CpG was required to produce the same effect. The promotion of CpG detection by LL-37 was independent of the backbone of the ODN, as the effect was observed not only in ODNs with modified phosphorothioate backbone, but also in ODNs with natural phosphodiester backbone, as found in genomic DNA. Unmethylated CpG motifs within the phosphodiester ODN and LL-37-mediated delivery are required for pDCs to respond. In keeping with the above, cells responded to CpG-rich bacterial DNA and LL-37, but not to human DNA and LL-37. The ability of LL-37 to enhance delivery of CpG to stimulate immune cells is independent of its amphipathic structure and its bactericidal property. LL-37 aids the delivery of CpG to B cells and pDCs, but not T cells. These findings are pertinent to rapid recognition of microbial DNA and are highly relevant to contemporary studies of CpG/TLR9 agonists in vaccines and cancer therapy. The Journal of Immunology, 2010, 184: 1425–1435.

Prompt recognition of infection by the innate immune system is vital to survival. TLRs are one of the biological systems that have evolved to detect pathogens. They recognize pathogen-derived ligands collectively known as pathogen-associated molecular patterns. For example, TLR4 recognizes LPSs (1), and TLR9 recognizes unmethylated CpG motifs commonly found in bacterial DNA (2, 3).

Another biological system that has evolved to fight infection involves the release of antimicrobial peptides like cathelicidins from neutrophils (4–6). In mammals, ~30 members of the cathelicidin family of proteins have been reported. Although other mammals have retained several cathelicidins, only one (hCAP18) is present in humans (7). These proteins are characterized by having a conserved N-terminal cathelin domain and a biologically active C-terminal domain that contains the cationic antimicrobial peptide. hCAP18 is mainly expressed in neutrophils and keratinocytes (8, 9). In neutrophils, hCAP18 is stored within specific granules. The content of these granules is released upon neutrophil activation in response to infection (10). Cleavage of hCAP18 by the serine protease proteinase-3 generates the 37 aa cationic and amphipathic peptide LL-37 (11). LL-37 has potent and broad-spectrum bactericidal activity and plays an important role in protection against bacterial infection in the skin and mucosal surfaces (12, 13). Recently, the relationship between infection, TLRs, and LL-37 was also aptly demonstrated in Mycobacterium tuberculosis infection (14).

However, because serum inhibits the bactericidal function of LL-37 (15, 16), the main activity of LL-37 in the blood compartment remains in question. In this regard, several other functions have been described for LL-37. It is known to bind to LPS (6), act as a chemotactic factor for neutrophils, monocytes, and T cells (17), and increase the expression of costimulatory molecules on dendritic cells (18).

In a recent paper on the pathogenesis of psoriasis, Lande et al. (19) found that LL-37 from skin keratinocytes was able to complex with human DNA by virtue of their opposite charges. Overnight incubation of plasmacytoid dendritic cells (pDCs) with the resultant complex led to release of IFN-α via stimulation of TLR9, suggesting an involvement of LL-37 and human DNA in the pathogenesis of psoriasis (19). In a separate paper, Sandgren et al. (20) reported that LL-37 was able to transfer extracellular DNA plasmids into mammalian cell lines. These reports stimulated us to explore the possibility that in blood, LL-37, which has been released by neutrophils in response to infection, might interact with bacterial DNA and facilitate its delivery to stimulate TLR9 possessing cells like B cells and pDCs. If this is true, LL-37 might be important for efficient recognition of microbial infection.

Materials and Methods

Reagents

CpG-A with phosphorothioate backbone (5’-ggGGGACGATCGTCggggg-gg-3’) and CpG-B with phosphorothioate backbone (5’-tgcggttttgcggtttt-gg-3’) (lowercase letters, phosphorothioate (PS) linkage; capital letters, phosphodiester (PD) linkage) of the base; bold letters, CpG dinucleotides), and CpG-B conjugated to FITC (CpG-FITC) were synthesized by Gencworks, Hindmarsh, South Australia. Variants of CpG-A oligodeoxynucleotides (ODNs) were as follows: guanine cytosine oligodeoxynucleotide (GpC)-A with PS backbone (5’-ggGGGACGATCGTCggggg-gg-3’); CpG-A with PD backbone (5’-GGGGGACGATCGTCggggg-gg-3’); GpC-A with

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2. Czerkinsky C, Kalaydjieva L, Willems M, Ouyang X, Cao D, Zhang X, et al. CPG motifs in bacterial DNA and LL-37, but not to human DNA and LL-37. The ability of LL-37 to enhance delivery of CpG to stimulate immune cells is independent of its amphipathic structure and its bactericidal property. LL-37 aids the delivery of CpG to B cells and pDCs, but not T cells. These findings are pertinent to rapid recognition of microbial DNA and are highly relevant to contemporary studies of CpG/TLR9 agonists in vaccines and cancer therapy. The Journal of Immunology, 2010, 184: 1425–1435.

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PD backbone (5′-GGGGGACATGCTGGGGGC-3′), and CpG-A with methylated cytosine residue and PD backbone (5′-GGGGGACmCmATC (methyl)GTC(methyl)GGGGG-3′). Variants of CpG-B ODNs were as follows: GpC-B with PS backbone (5′-tgctggttgctggttgctg-3′); CpG-B with PD backbone (5′-TGCGTGGTTTCGCGTTGGTGTG-3′); GpC-B with PD backbone (5′-TGTTGTCGmGTGTGTCmTGTTGTCG-3′). LL-37 (LGDDFRFRKSEKEKEKFRVRIKDFKLRNVPRTES) and the scrambled peptide version of LL-37 named GL-37 (GLKLRF-EFSKIGKFLKPTEVFRDKILDNKVISQR) were purchased from Invivogen, San Diego, CA. DNase I was from Roche Applied Science, Indianapolis, IN. Human genomic DNA isolated from leukocytes was purchased from BioChain, Hayward, CA. The following mAbs were obtained from BD Biosciences, San Jose, CA: anti–CD3-PE-Cy7, anti–CD86-PE, anti–CD69-PE-Cy7. Mouse IgG1 conjugated with Alexa 546 was from Molecular Probes, Eugene, OR.

**Cell culture**

PBMCs were isolated fromuffy coats kindly donated from healthy individuals (Australian Red Cross Blood Bank, Adelaide, Australia). PBMC separation was performed by density gradient over LymphoPrep (Axis-Shield, Oslo, Norway). After extensive washing, cells were resuspended in culture media (RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine) and adjusted to a final concentration of 2 × 10⁶ cells/ml. LL-37 dDCs were purified from PBMCs with BDCA-2-coated microbeads (Miltenyi Biotec, Auburn, CA) and adjusted to 50,000 cells in 200 μl/well. Cells were stimulated with CpG-B (final concentration 1.3 μg/ml), CpG-A (final concentration 1.3 μg/ml), LL-37 (final concentration 6 μg/ml), or the combinations of CpG-B plus LL-37 (1.3 μg/ml plus 6 μg/ml) or CpG-A plus LL-37 (1.3 μg/ml plus 6 μg/ml). In Fig. 4, cells were stimulated with variant ODNs of CpG-A or CpG-B (1.3 μg/ml unless indicated otherwise) with or without LL-37 (6 μg/ml). CpG plus LL-37 complexes were formed by mixing the relevant CpG with LL-37 in PBS at room temperature for 10 min prior to being added to cells to achieve the final concentrations listed above. Cells were incubated with these reagents for 15 min (unless indicated otherwise) at 37°C, washed three times in PBS, and cultured in RPMI 1640 supplemented with 10% FCS in an incubator at 37°C, 5% CO₂. Where indicated, cells were stimulated with increasing concentrations of CpG-B or CpG-A (3.9, 13, 26, and 39 μg/ml, labeled as ×3, ×10, ×20, and ×30, respectively). Where indicated, cells were treated with bacterial DNA (16 μg/ml), bacterial DNA plus LL-37 (16 μg/ml plus 30 μg/ml), human DNA (16 μg/ml), or human DNA plus LL-37 (16 μg/ml plus 50 μg/ml). Complexes were formed by mixing the relevant DNA with LL-37 in PBS at room temperature for 10 min prior to being added to cells to achieve the final concentrations listed above. To digest bacterial DNA, bacterial DNA (16 μg/ml) was treated with DNase I (10 μg) overnight at 37°C prior to being used. Complete digestion of DNA was confirmed by gel electrophoresis.

**Flow cytometry**

To determine the percentage of lymphocytes that stained for CpG-FITC, PBMCs were treated with either CpG-FITC alone or CpG-FITC plus LL-37 for 15, 30, or 45 min at 37°C prior to washing three times in PBS. Lymphocytes were gated on the basis of forward and side scatter characteristics and FITC fluorescence intensity was measured. Cells were analyzed using the FC500 Tepsihore (BD Biosciences) and CellQuest Acquisition and Analysis Software (BD Biosciences). B cell activation was measured by double staining with CD19-FITC and CD69-PE. The expression of CD40 and CD86 on B cells were defined as the percentage of B cells whose respective levels of expression of CD40 and CD86 were above isotype control. To determine the uptake of CpG-FITC by T cells and B cells in whole blood, peripheral venous blood was obtained in heparinized tubes. Red cells were lysed, and the remaining WBCs were stained with CpG-FITC or CpG-FITC plus LL-37 for 15 min at 37°C. Cells were washed and then incubated with anti–CD3-PE-Cy7 or anti–CD19-PE-Cy7 for 30 min on ice. Cells were washed and fixed for analysis by flow cytometry.

**Cytokine production by B cells and pDCs**

Cell culture supernatant was collected, and various cytokines released by B cells (IL-8, IL-6, IL-10, and TNF-α) were analyzed by cytometric bead array (BD Biosciences) according to the manufacturer’s instructions. Cytometric analysis was performed by FACSAria (BD Biosciences), and data analysis was achieved using the software cytometric bead analysis (BD Biosciences). IFN-α was measured using a commercial ELISA kit (Mabtech, Nacka Strand, Sweden).

**Measurement of IgS**

Total IgG and IgM concentrations in cell culture supernatants were measured using an ELISA quantification kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer’s instructions. Samples were diluted 1:10 in PBS for IgG and 1:20 for IgM before testing.

**Immunofluorescence**

PBMCs and dDCs were incubated with CpG-B conjugated to FITC (1.3 μg/ml), LL-37 peptide (6 μg/ml), or a mixture of CpG-B-FITC and LL-37 at similar concentrations. Cells were incubated at 37°C for 15 min. Cells were then washed twice in PBS and incubated with anti–CD3, anti–CD19, or anti–DR molecules for 30 min on ice. Cells were washed, and goat anti-mouse IgG conjugated with Alexa 546 came from Molecular Probes, Eugene, OR.

**Bacterial culture**

*E. coli* ATCC strain 25922 was grown to log phase in Luria-Bertani broth at 37°C. Thereafter, bacteria was inoculated either into 500 μl RPMI 1640 media (Invitrogen, Carlsbad, CA) with no serum or into 500 μl heparinized fresh whole blood at 1:1,000,000 dilution, and expanded for 3 h at 37°C on a rotating platform. At the 3-h time point, bacteria was treated with either LL-37 (5 μM) or GL-37 (5 μM) or left untreated. Bacterial culture was allowed to proceed for another 1 h, at which time 100 μl bacterial culture was plated onto standard bactoagar plates. Plates were incubated overnight at 37°C. Where indicated, LL-37 was mixed with 20 μg bacterial genomic DNA isolated from *E. coli* (Invivogen) for 10 min prior to being added to bacteria.

**Statistics**

The graphs and the statistical data analysis (unpaired t test) were carried out using GraphPad Prism Software (GraphPad, San Diego, CA).

**Results**

In initial flow cytometry studies using CpG ODN (CpG) conjugated to FITC, we found that ~10% of lymphocytes among human PBMCs became FITC positive when PBMCs were incubated with CpG-FITC complexed to LL-37 for 15 min, but not with CpG-FITC alone (Fig. 1). CpG was used as a synthetic analog of bacterial DNA. The remarkable feature of this finding was that only a brief period of time was required for cells to take up the complex. Henceforth, we wanted to see if this rapid delivery of CpG by LL-37 within 15 min was sufficient to evoke an immunological response.

First, we studied the effects on B lymphocytes within PBMCs. PBMCs were incubated with CpG-B (21) in the presence or absence of LL-37 for 15 min. We chose to use 1.3 μg/ml of CpG and 6 μg/ml of LL-37 after dose-titration experiments had shown that these concentrations and their ratio were optimal (Supplemental Fig. 1). The cells were washed extensively and then cultured for 16 h. B cell activation was assessed by the level of expression of the costimulatory molecules CD40 and CD86 on CD19-positive gated B cells. The expression levels of CD40 and CD86 on cells treated with CpG alone or LL-37 alone for 15 min were not significantly different to cells that had not been treated. However, stimulation with CpG-B complexed to LL-37 (CpG-B+LL-37) led to significant upregulation in the expression of CD40 (p = 0.004) and CD86 (p = 0.013) on B cells (Fig. 2A, 2B). Remarkably, this
effect was evident already after only 5 min of exposure (Supplementary Fig. 2). It has been reported in the past that administration of CpG DNA to mice led to upregulation of IL-6 mRNA within 30 min and IL-6 production within 1 h (22). Our results suggest that the presence of LL-37 significantly minimizes the time of exposure required for CpG to activate cells.

To explore if naive and memory B cells respond differently to CpG-B/LL-37, we analyzed the expression of CD40 and CD86 among CD19+CD27− naive B cells and CD19+ CD27+ memory B cells after 15 min exposure to CpG-B+LL-37. We found that both subsets responded to CpG-B+LL-37. However, CD19+CD27+ B cells showed higher baseline levels of CD40 and CD86, and they achieved greater absolute levels of CD40 and CD86 after CpG-B+LL-37 (Supplemental Fig. 3). This observation is interesting given that memory B cells have been shown to express higher levels of TLR9 (23).

We also measured cytokine and Ig production by PBMCs in response to 15 min of CpG-B/LL-37. The concentration of IL-6 was significantly higher (p = 0.0013) in the supernatant of PBMCs that had been stimulated with CpG-B+LL-37 compared with those stimulated with CpG-B alone (37.33 ± 25.87 versus 11.36 ± 7.458 pg/ml). IL-10 concentration also increased in response to CpG-B+LL-37, although the difference did not reach significance (Fig. 2E, 2F). IL-8, TNF-α, and IFN-α could not be detected in any of the conditions. The finding of increased production of IL-6 is of particular interest because the binding site for the NFκB IL-6 resides in the promoter region of the hCAP18 gene (7). Similar to cytokines, Ig production was significantly higher in cells stimulated with CpG-B+LL-37 compared with CpG-B alone, and this applied to both IgG (516 ± 373 versus 114 ± 58 ng/ml; p < 0.001) and IgM production (2781 ± 771 versus 951 ± 263 ng/ml; p < 0.001) (Fig. 2C, 2D).

To explore the efficiency of this process, we examined the effect of LL-37 on the concentration of CpG that is required to stimulate B cells and pDCs. Following the same 15 min duration of stimulation, B cells were stimulated with either CpG-B alone, CpG-B+LL-37, or increasing concentrations of CpG-B alone. We found that for all the individuals studied, at least 20 times more of CpG-B alone was required to reach the same stimulatory effect that was achieved when CpG-B was delivered with LL37 (Fig. 2G–2J). Of note, without LL-37, CpG-B required at least 3 h to reach a similar level of activation as CpG-B+LL-37 after 15 min. Prolonged incubation with CpG-B+LL-37 did not increase the level of activation substantially beyond what had been achieved at 15 min (Supplemental Fig. 4A–4D).

To stimulate pDCs, we used CpG-A instead of CpG-B (21). Again, we found that LL-37 allowed rapid sensing of CpG-A by pDCs (Fig. 2K). When complexed to LL-37, 15 min exposure to CpG-A was sufficient to stimulate pDCs to release IFN-α, whereas CpG-A alone was not. This phenomenon was demonstrable as quickly as after 5 min exposure (Supplemental Fig. 4F). For pDCs, 30 times more of CpG-A alone was not enough to equal the effect generated by 15 min exposure to 31 concentration of CpG-A when it was delivered with LL-37 (Fig. 2K). Continuous overnight exposure to CpG-A+LL-37 led to ∼2-fold increase in the release of IFN-α compared with 15 min exposure to CpG-A+LL-37 (Supplemental Fig. 4E). Remarkably, continuous overnight exposure to 10 times concentration of CpG-A alone was also not enough to equal the effect generated by 15 min exposure to ×1 concentration of CpG-A when it was delivered with LL-37 (Fig.
FIGURE 2. LL-37 allows rapid detection of otherwise limiting quantities of CpG by B cells and pDCs (A–F). PBMCs (2 × 10⁶ cells/ml) from eight healthy individuals were incubated with LL-37 (6 μg/ml), CpG-B (1.3 μg/ml), CpG-B+LL-37, or left untreated (NT) for 15 min. Thereafter, cells were washed three times in PBS. Cells were incubated overnight in A, B, E, and F, or 7 d in C and D prior to assay. CD40 (A) and CD86 (B) expression on B cells was analyzed by flow cytometry with gating on CD19⁺ B cells. The production of IgG (C) and IgM (D) Ab was assayed by ELISA. The concentration of IL-6 (E) and IL-10 (F) in the culture supernatant was assayed by cytometric bead array. G–J, PBMCs (2 × 10⁶ cells/ml) were incubated with LL-37 (6 μg/ml), CpG-B (1.3 μg/ml), CpG-B+LL-37, or with increasing concentrations of CpG-B (3.6, 13, and 26 μg/ml, labeled as ×3, ×10, and ×20, respectively) for 15 min, whereas untreated cells (NT) were used as control. Thereafter, cells were washed three times with PBS and cultured overnight in G and H or 7 d in I and J. CD40 (G) and CD86 (H) expression on B cells was analyzed by flow cytometry with gating on CD19⁺ B cells. Concentration of IgG (I) and IgM (J) Ab was assayed in culture supernatant by ELISA. G and H show the results from three healthy individuals. I and J graphs are representative results out of four individuals tested. K, Purified pDCs were stimulated with LL-37 (6 μg/ml), CpG-A (1.3 μg/ml), CpG-A+LL-37, or with increasing concentrations of CpG-A (labeled as ×3, ×10, and ×30, respectively) for 15 min, whereas untreated cells (NT) were used as control, pDCs were then washed and cultured overnight. In addition, pDCs were also cultured overnight in the presence of ×10 concentration of CpG-A continuously (arrow). The concentration of IFN-α in the overnight culture supernatant was determined by ELISA. E, The graph is a representative result out of four individuals tested.
These observations show that LL-37 allows rapid sensing of otherwise limiting quantities of CpG containing DNA sequences by B cells and pDCs.

Having observed the effects of LL-37 facilitating the detection of CpG by pDCs and B cells, we wanted to see if LL-37 would have the same effect with bacterial DNA. Hence, we tested LPS-free bacterial DNA derived from *E. coli* and LL-37 using the same experimental system. We found that 15 min exposure to bacterial DNA+LL-37 stimulated B cells more so than bacterial DNA by itself (Fig. 3A, 3B). For B cells, we detected a measurable effect with exposure time as short as 5 min. For pDCs, 15 min of bacterial DNA alone was inadequate to generate response, whereas 15 min of bacterial DNA+LL-37 stimulated production of IFN-α (Fig. 3C). Notably, prior treatment of *E. coli* DNA with DNAase completely abolished the response that was seen in conjunction with LL-37, which indicated that the stimulating effect was due to bacterial DNA (Fig. 3C).

Human DNA is generally thought to be poorly recognized by TLR9 (24). Nevertheless, Lande et al. (19) showed that LL-37 rendered human DNA capable of stimulating pDCs via TLR9. Because these experiments had been based on overnight stimulation, we wondered if the same effect would take place when the stimulation time is shortened to 15 min. Firstly, we confirmed that indeed, overnight incubation of human DNA complexed to LL-37 led pDCs to release IFN-α (data not shown). However, we could not reproduce this effect when pDCs had been exposed to human DNA and LL-37 for only 15 min (Fig. 3C). Put together, when exposure time is limited to minutes, LL-37 enhances the immunostimulatory effect of bacterial DNA but not human DNA.

The CpG-A and CpG-B ODN sequences used thus far have modified PS backbone, as opposed to natural PD backbone as found in genomic bacterial and human DNA. The substitution of the oxygen atom in the PD backbone with a sulfur atom in the PS backbone renders the DNA sequence much more resistant to degradation by...
nucleases (25). Given our finding that LL-37 enhances the immunostimulatory effect of bacterial DNA when exposure time is limited to minutes, it is important to explore if LL-37 promotes rapid sensing of CpG sequences with PD backbone. At the same time, we also wanted to test the importance of CpG motifs and unmethylated cytosine residues within the ODN sequence, given that when compared with bacterial DNA, human DNA contains methylated cytosine residues generally and a paucity of CpG motifs.

With this in mind, pDCs were tested with variants of CpG-A, and B cells were tested with variants of CpG-B respectively for 15 min, with and without LL-37 (Fig. 4). These variants compared PS versus PD backbone, CpG versus GpC motifs, and unmethylated versus methylated cytosine residues within ODN sequences. pDCs responded to CpG motif in ODN with PS backbone as expected and also slightly less well to CpG motif in ODN with PD backbone (Fig. 4A). LL-37 was required for this to occur. Importantly, substitution of CpG with GpC within ODNs with either PS or PD backbone led to complete loss of stimulatory activity (Fig. 4A). We could not elicit any response from pDCs despite increasing the concentration of PD GpC-ODN up to 20 times (26 μg/ml) and extending the time of coculture with PD GpC-ODN overnight (Fig. 4B). Lastly, methylation of cytosine residues within the CpG ODN with PD backbone also led to loss of stimulatory activity (Fig. 4A). In summary, pDCs can respond after brief exposure to DNA sequences with PD backbone, provided they contain unmethylated CpG motifs, and as long as LL-37 is available. This is thus in keeping with our finding that pDCs responded to CpG-rich bacterial DNA and LL-37, but not to human DNA and LL-37.

The effect of CpG-B variants on B cells is slightly different. Firstly, B cells failed to respond to CpG motifs in ODN with PD backbone (Fig. 4E). Secondly, B cells failed to respond to CpG motifs in ODN with PD backbone despite increasing the concentration of PD GpC-ODN up to 20 times (26 μg/ml) and extending the time of coculture with PD GpC-ODN overnight (Fig. 4B). Lastly, methylation of cytosine residues within the CpG ODN with PD backbone also led to complete loss of stimulatory activity (Fig. 4A).

In summary, pDCs can respond after brief exposure to DNA sequences with PD backbone, provided they contain unmethylated CpG motifs, and as long as LL-37 is available. This is thus in keeping with our finding that pDCs responded to CpG-rich bacterial DNA and LL-37, but not to human DNA and LL-37.

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In summary, pDCs can respond after brief exposure to DNA sequences with PD backbone, provided they contain unmethylated CpG motifs, and as long as LL-37 is available. This is thus in keeping with our finding that pDCs responded to CpG-rich bacterial DNA and LL-37, but not to human DNA and LL-37.
backbone either with or without LL-37 (even when exposure time was extended overnight), whereas the response to CpG in ODN with PS backbone was reproducibly robust (Fig. 4C, 4D). Secondly, substitution of CpG with GpC within the PS ODN led to partial though not total loss of stimulatory activity.

We wanted to explore reasons for the different patterns of behavior to CpG variants by pDCs and B cells—namely, why pDCs responded to CpG-A PD ODNs, but B cells did not respond to CpG-B PD ODN. One possibility may be that CpG-B PD ODN lacked biological activity. To test this, we exposed pDCs to CpG-B PD ODN, and B cells to CpG-A PD ODN with and without LL-37. pDCs are known to respond to CpG-B PS ODN after overnight stimulation by way of upregulation of activation markers (26). In response to CpG-B variants, we found to our surprise that pDCs responded with a similar pattern as they had to CpG-A variants—namely dependence on CpG motifs, dependence on assistance from LL-37, but independence of backbone (Fig. 4E, 4F). B cells did not respond to any of the CpG-A variants, even after extended overnight incubation with LL-37 (data not shown). This is not unexpected given that past researchers have shown that purified B cells did not respond to CpG-A PS ODN (27).

These findings would suggest that the slightly different patterns of responses of pDCs to CpG-A variants and B cells to CpG-B variants, respectively, may be due to cell-specific differences in their handling of PD ODNs (e.g., differential rates of degradation of PD ODNs within these cells). We had noted that B cells were capable of responding to bacterial DNA+LL-37 (Fig. 3A, 3B). The apparent disparity in B cell responses to bacterial DNA and CpG-B PD ODN with PD backbone may be due to several possibilities, including different sensitivities of these DNA sequences to DNA nucleases and perhaps the different number of CpG motifs in bacterial DNA compared with CpG ODNs.

Within the limits of this experimental assay system and at least as far as pDCs are concerned, the above results suggest that LL-37 can promote rapid sensing of not only PS ODNs, but also PD ODNs. CpG motifs and unmethylated cytosine residues within the ODN are important in this regard.

Given the significance of an efficient system for detecting infection, we wondered if CpG+LL-37 complexes might preferentially target TLR9-expressing cells. Although B cells and pDCs express TLR9 constitutively, T cells express very little or no TLR9 (28). To analyze the uptake of CpG by these cellular subsets, WBCs were incubated with either CpG-FITC or CpG-FITC+LL-37 for 15 min, followed by labeling of B cells and T cells with anti-CD3 and anti-CD19 mAbs, respectively, and analysis by flow cytometry. We found that the CpG-FITC+LL-37 complex targeted B cells but not T cells. T cells did not take up any CpG-FITC regardless of LL-37 (Fig. 5A–5D).

The selective targeting of B cells is consistent with our earlier finding that ~10% of cells among lymphocytes stained positive for CpG-FITC in the presence of LL-37 (Fig. 1). Confocal immunofluorescent staining of PBMCs confirmed this observation; CpG-FITC+LL-37 targeted CD19-positive B cells but not CD3-positive T cells (Fig. 5E, 5F). Staining of purified pDCs also showed enhanced uptake of CpG-FITC only in the presence of LL-37 (Fig. 5G–5I).

That the entry into these cells segregated with the expression of TLR9 is probably not a coincidence. The mechanism by which these cells are targeted is not known, but a receptor-mediated mode of entry is a possibility. It most likely contributed to the rapid uptake of CpG DNA in the presence of LL-37. An obvious possibility is cell surface expression of TLR9, but it is generally thought that the expression of TLR9 is largely confined to the endosomal compartment (29).

The bactericidal property of LL-37 is crucially dependent on its amphipathic and α-helical conformational features. It is known that the bactericidal property of LL-37 is lost in the presence of serum (15, 16). Given that its ability to enhance CpG detection is independent of its amphipathic and α-helical conformational features, we can conclude that the ability of LL-37 to enhance CpG delivery to stimulate immune cells is independent of its amphipathic and α-helical conformational features.

It is known that the bactericidal property of LL-37 is lost in the presence of serum (15, 16). Given that its ability to enhance CpG detection is independent of its bactericidal property, we wanted to see if LL-37 could still enhance delivery of CpG in whole blood. Fresh whole blood was stimulated for 15 min with either CpG-B alone or CpG-B+LL-37. It was then washed before PBMCs were isolated and cultured overnight. Thereafter, B cell activation markers were measured. We found that the whole blood environment did not inhibit the ability of CpG-B+LL-37 to induce expression of CD40 and CD86 on B cells (Fig. 6F, 6G). In regards to its bactericidal activity, whereas 5 μM of LL-37 was sufficient to totally inhibit bacterial growth in serum-free media, this concentration of LL-37 was ineffective at killing bacteria in whole blood (Fig. 6H). In fact, we were unable to overcome this inhibition in whole blood with concentrations of LL-37 as high as 30 μM.

We also wondered if bacterial DNA that has been released following bacterial lysis would interfere with the bactericidal activity of LL-37. To test this idea, LL-37 was mixed with or without bacterial DNA prior to being added to E. coli bacterial culture. The result showed that the presence of bacterial DNA completely inhibited the ability of LL-37 to kill bacteria (Fig. 6F). This observation suggests that once LL-37 has fulfilled its bactericidal function, the presence of bacterial DNA will block further bactericidal activity of LL-37 in the local vicinity. Instead, the newly formed complex may take on the immunostimulatory role of priming immune cells for rapid recognition of microbial DNA.

Discussion

Neutrophils represent the major source of LL-37 within the vascular compartment. Neutrophils play a key role in the innate immune response to bacterial infection. The relatively constant number of neutrophils in peripheral circulation in a normal individual can rapidly increase upon bacterial infection. On the other hand, patients with neutropenia are known to face a great risk of developing bacterial infections (30). Neutrophils have the ability to sense bacteria and their components and to migrate across epithelia in response to chemotactic gradients generated from the site of infection (31, 32). Although neutrophils are best known for their phagocytic and bactericidal capacity, it is becoming increasingly evident that they also play an active role in assisting other immune cells to mount an effective adaptive immune response (33, 34).

TLRs are one of the biological systems that have evolved to detect pathogens by immune cells. For example, TLR9 recognizes unmethylated CpG motifs commonly found in bacterial DNA (2, 3). Our results suggest that the binding of LL-37 to bacterial DNA brings about a significant reduction in the time that is required by B
FIGURE 5. LL-37 enhances targeting of CpG to B cells and pDCs but not T cells. Whole blood that had been depleted of RBCs was treated with either CpG-FITC or CpG-FITC+LL-37 for 15 min prior to extensive washing (A–D). Cells were then labeled with either anti–CD3-PE or anti–CD19-PE. The uptake of CpG-FITC was analyzed by gating on CD3-positive T cells in C and CD19 positive B cells in D. The histograms in red represent the FITC intensity after treatment with CpG-FITC, whereas the histograms in blue represent FITC intensity after CpG-FITC+LL-37 treatment. E and F, PBMCs were treated with CpG-FITC+LL-37 for 15 min prior to extensive washing and staining with either mAbs to CD3 for T cells in E or CD19 for B cells in F. E shows that CD3-positive T cells (red) did not stain with CpG-FITC (green). In contrast, most CD19-positive B cells (red) stained with CpG-FITC (F). G–I, Purified pDCs were stained with anti-DR (red) and either left untreated (G) or treated with CpG-FITC (H) or CpG-FITC+LL-37 (I) for 15 min prior to extensive washing. Only pDCs treated with CpG-FITC+LL-37 stained with CpG-FITC (I). Original magnification (E–I) ×80.

cells and pDCs to sense the presence of bacterial DNA via TLR9, especially when limiting quantities of DNA are present. This observation is all the more relevant given that we confirmed that this function is preserved in the whole blood setting, as opposed to its bactericidal function, which is inhibited by whole blood.

Given the intrinsic avidity of LL-37 for lipid membranes (35, 36), we had expected that LL-37/CpG complexes would bind indiscriminately to all cells. Therefore, to our surprise, we found that CpG-B/LL-37 complexes showed preferential binding to B cells and pDCs and not T cells. We have also examined monocytes and monocyte-derived dendritic cells. We generated immature monocyte-derived dendritic cells with GM-CSF and IL-4 from cultured overnight. Despite uptake of CpG/LL-37 complexes, our observation that immature monocyte-derived dendritic cells, just like monocytes, incorporated vast amounts of the CpG-FITC/LL-37 complex and also GFP used as a control. The uptake of these substances is likely a manifestation of their inherent ability to phagocytose. It is known that human monocytes do not express TLR9 (28). It is also known that human myeloid dendritic cells do not express TLR9. The failure of CpG ODN to activate myeloid dendritic cells has been reported previously (37, 38). A recent paper reported GAPDH as a monocyte cell receptor for human LL-37 (39). We incubated immature monocyte-derived dendritic cells with CpG/LL-37 for 15 min to see if CpG delivered to LL-37 could activate these cells. After 15 min, cells were washed and cultured overnight. Despite uptake of CpG/LL-37 complexes, our results show that neither CpG-A nor CpG-B was able to induce expression of activation markers on immature monocyte-derived dendritic cells (data not shown).

The mechanism by which bacterial DNA reaches the endosomal compartment of cells to stimulate TLR9, and the factors that influence its delivery have not been well established. Our paper suggests that LL-37 may be one of these factors. Several mechanisms may be involved in this process. For example, LL-37 may allow the delivery of more bacterial DNA given its ability to condense it (19). LL-37 could facilitate the binding of DNA to TLR9 once in the endosome/lysosomal compartment. Furthermore, the fact that the complex targets cells bearing TLR9 such as B cells may help deliver the bacterial DNA to the relevant cells.

The finding that LL-37’s function of enhancing the response to DNA was independent of its amphipathic and bactericidal property represents another surprising observation. This opens up the possibility that in the detection of bacterial DNA, other cationic peptides with similar charge properties to LL-37, such as neutrophil-derived defensins (40), may also contribute to the process. In addition, during bacterial infection, a great number of peptides are generated (41, 42) as a result of degradation and remodeling of the extracellular matrix by proteases released by microorganisms and infiltrating neutrophils and monocytes (42–44). It is possible that some of these peptides might share similar properties with LL-37 and also contribute to detection of bacterial DNA.

Neutrophil extracellular traps (NETs) generated in response to bacterial infection are known to contain cationic neutrophil proteins like myeloperoxidase (45). We had recently put forward the concept that these cationic molecules may complex with CpG DNA derived from bacteria caught in NETs (46). Even though the primary function of NETs is to fight infection, encounters with such CpG complexes formed in NETs might unexpectedly have
the undesired side effect of stimulating autoimmune B cells in patients with autoimmune diseases (46). Coincidental with the submission of this paper, it has been reported that LL-37 is also present in NETs (47). Given our experimental findings in regards to LL-37 and CpG, we would therefore predict that LL-37 as found in NETs may complex with bacterial CpG DNA and stimulate autoimmune B cells.

Stimulation of the immune system by CpG sequences have profound effects in the magnitude and quality of adaptive immune responses, ranging from enhancement of Ag presentation, cell activation and maturation (48), and Ig class switching (49) to maintenance of B cell memory repertoire (50). Our data suggest that LL-37 may enhance at least some of these functions. However, the precise contribution that LL-37 may have in vivo on the immune stimulatory effects of CpG remain to be assessed. In this regard, it would be interesting to perform a comprehensive study of the adaptive immune response in patients known to have LL-37 deficiency (51, 52) and in LL-37 knockout mice (13).

The potent immunostimulatory effects of TLR9 activation by PS CpG ODNs have resulted in enormous interest in the potential uses
of these TLR9 agonists as adjuvants for clinical therapy (53). Our finding that LL-37 enhances the efficiency by which the immune system responds to CpG is therefore potentially relevant to contemporary studies of TLR9 agonists as adjuvants for vaccines and cancer therapy.

Besides TLR9, human B cells and pDCs also express TLR7. TLR7 detects RNA sequences (54), which are also negatively charged. We would predict that LL-37 could potentially interact with RNA sequences and enhance delivery of RNA to stimulate TLR-7–expressing cells. A recent report suggests that this may indeed be the case (55). Lastly, it has been reported that TLR7 and TLR9 may be implicated in the pathogenesis of autoimmune diseases such as systemic lupus (56, 57) and antineutrophil cytoplasmic Ab-associated vasculitis (46), respectively. Hence, our findings in relation to LL-37 may be relevant to understanding the pathogenesis of these diseases.

For many years, it had been accepted that only bacterial DNA, but not vertebrate DNA, is capable of stimulating immune cells (58). Compared to bacterial DNA, human DNA contains methylated cytosine residues generally and a paucity of CpG motifs with favorable flanking sequences. These features, together with the intracellular endosomal location of TLR9 (29), are thought to contribute to our ability to distinguish bacterial from self-DNA. More recently, however, it has been reported that under certain circumstances, human immune cells can be activated by self-DNA. For example, chromatin-IgG complexes have been shown to activate B cells expressing surface IgM rheumatoid factors, whereby these complexes get internalized via BCR-mediated translocation into endosomes, followed by TLR9 stimulation by self-DNA (59). Another potential route of cellular entry occurs when human DNA is mixed with LL-37, and overnight incubation of pDCs with these complexes leads to stimulation of these cells to produce IFN-α (19). These reports have reignited renewed interest in finding out more about the conditions under which human DNA with PD backbone and suboptimal CpG motifs may become immunostimulatory. This has obvious and important implications for understanding diseases with autoimmune basis such as lupus.

Compared to DNA with PS backbone, DNA with PD backbone is firstly more sensitive to nucleases (25) and secondly shows poorer cellular uptake (60). To overcome these limitations, various experimental methods have been used to increase translocation of PD DNA sequences into cells. They include receptor-mediated uptake via anti-DNA IgG Ab (59, 61), complex formation with LL-37 (19) or DOTAP (62, 63), and extension of a poly-G tail to the ODN (62). These papers have reported that provided PD ODNs can be translocated into endosomes, PD DNA with unmethylated CpG is capable of stimulating immune cells. Furthermore, some of them have also reported that PD DNA with either suboptimal CpG motifs (61) or no CpG motifs (62, 63) is also capable of stimulating immune cells. However, close inspection of the dose-response data in these papers reveals that PD DNA with suboptimal CpG motifs or no CpG motifs is far more inferior to PD DNA with optimal CpG motifs in stimulating immune cells. Allowing for methodological differences, our data in regards to LL-37–mediated delivery of PD DNA are in broad agreement with the concept that PD DNA with CpG motifs can stimulate immune cells. However, we found that PD DNA lacking CpG motifs failed to elicit any response from pDCs. This shortfall could not be overcome by either increasing the concentration of the ODN or extending the time of stimulation overnight.

It would be relevant and interesting to test in the LL-37 system the behavior of PD ODNs with CpG motifs flanked by suboptimal bases, given that this mimics human DNA more closely than ODNs with no CpG motifs. Nevertheless, our LL-37–related findings would seem to suggest that in circumstances where exposure time and the quantity of DNA is limiting, immune cells are skewed toward the detection of bacterial DNA more so than human DNA. This may hold potential implications not only for understanding immune responses against pathogens, but also autoimmune diseases like lupus where the threshold of recognition of self-DNA may be lower than normal.

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Disclosures

The authors have no financial conflicts of interest.


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Supplementary Figure 1  Dose-response curve for CpG and LL-37.

(A) PBMCs (2x10^6 mL^-1) from a healthy individual were incubated with varying concentrations of CpG-B overnight. CD40, CD80 and CD86 expression on B cells was analysed by flow cytometry with gating on CD19^+ cells. This dose range was based on the commonly used concentrations in the literature. We noted that the dose-response curve approached saturation around 1.3 μg mL^-1 to 1.6 μg mL^-1. (B) We wanted to see if LL-37 could drastically reduce the exposure time to CpG-B that is required to stimulate B cells. For this reason and based on the above dose-response curve, cells were incubated for only 15 min with a fixed concentration of CpG-B but varying concentrations of LL-37 as indicated. Thereafter, cells were washed three times in PBS. Cells were incubated for 7 days prior to assay. The production of IgG and IgM antibodies was assayed by ELISA. We chose to use 1.3 μg mL^-1 of CpG and 6 μg mL^-1 of LL-37 as optimal concentrations for experimentation. The DNA weight : LL-37 weight ratio of ~1:5 is similar to that reported by Lande et al (19).

Supplementary Figure 2  The presence of LL-37 minimises the time of exposure required for CpG to activate B cells

PBMCs (2x10^6 mL^-1) from four healthy individuals were incubated with CpG-B (1.3 μg mL^-1), or CpG-B+LL-37 (6 μg mL^-1) for 5, 10, or 15 minutes, or left untreated (NT) for 15 min. Thereafter, cells were washed three times with PBS. Cells were incubated overnight prior to assay. (A) CD40 and (B) CD86 expression on B cells was analysed by flow cytometry with gating on CD19^+ B cells.
Supplementary Figure 3  LL-37 allows rapid detection of CpG by both naïve and memory B cells.

PBMCs (2x10^6 mL^-1) from eight healthy individuals were incubated with LL-37 alone (6 μg mL^-1), CpG-B (1.3 μg mL^-1), CpG-B+LL-37, or left untreated (NT) for 15 min. Thereafter, cells were washed three times with PBS and cultured overnight. CD40 (A,C) and CD86 (B,D) expression was analysed by flow cytometry with gating on CD19^+CD27^- naïve B cells (A,B), and CD19^+CD27^+ memory B cells (C,D).

Supplementary Figure 4  LL-37 increases the efficiency of CpG recognition

(A-D) PBMCs (2x10^6 mL^-1) from four healthy individuals were incubated with LL-37 (6 μg mL^-1), CpG-B (1.3 μg mL^-1), CpG-B+LL-37, or left untreated for 15 min or 16 hrs in A and B, and varying times as indicated in C and D. Thereafter, cells were washed three times in PBS and cultured overnight prior to assay. (A,C) CD40 and (B,D) CD86 expression on B cells was analysed by flow cytometry with gating on CD19^+ B cells. (E,F) Purified pDCs were stimulated with CpG-A alone (1.3 μg mL^-1) or CpG-A+LL-37 for 15 min or 16 hrs in E, and varying short periods of time in F. Thereafter, pDCs were washed and cultured overnight. The concentration of α-interferon in the overnight culture supernatant was determined by ELISA. The data in E and F is representative of results from three healthy individuals.
A

% of B cells Positive

CpG-B  NT  0.1  0.2  0.4  0.8  1.6 μg mL⁻¹

CD86

CD80

CD40

B

Ab (mg mL⁻¹)

CpG-B  LL-37  NT  1.6  2  4  6 μg mL⁻¹

IgM

IgG
CD19\(^{+}\) B cells

CD27\(^{-}\)

A

\[\text{CD40}^{+} \text{ B cells (%)}\]

P = 0.0014

B

\[\text{CD86}^{+} \text{ B cells (%)}\]

P = 0.0314

CD27\(^{+}\)

C

\[\text{CD40}^{+} \text{ B cells (%)}\]

P = 0.0327

D

\[\text{CD86}^{+} \text{ B cells (%)}\]

P = 0.0393

Conditions