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## Neutrophils Activate Plasmacytoid Dendritic Cells by Releasing Self-DNA–Peptide Complexes in Systemic Lupus Erythematosus

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**Author contributions:** R.L. performed and analyzed most of the experiments in this study and participated in their design. D.G. performed microscopy studies of DNA particles. V.F. performed immunoblot experiments. L.F. performed the neutrophil stimulation experiments in Fig. 6. C.C. performed the DNA complexation assays described in Figs. 2 and 3 and helped with the statistical analyses. J.G. purified immune complexes by high-performance liquid chromatography. S.M. participated in the confocal microscopy experiments. G.C. participated in the DNA complexation experiments. R.S. participated in the measurement of autoantibodies by ELISA. V.R., H.A., S.F., and T. I. provided patient samples and data. R.B. helped with statistical analyses. Y.-J.L. provided critical suggestions and discussions throughout the study. M.G. conceived and supervised this study, was involved in the design and evaluation of all experiments, and wrote the manuscript with comments from co-authors.

**Competing interests:** R.L. and M.G. hold a patent, “Inhibitors of LL-37 mediated immune reactivity to self nucleic acids” (WO/2008/076981, application # PCT/US2007/087787), related to this work.

### SUPPLEMENTARY MATERIAL

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## Abstract

Systemic lupus erythematosus (SLE) is a severe and incurable autoimmune disease characterized by chronic activation of plasmacytoid dendritic cells (pDCs) and production of autoantibodies against nuclear self-antigens by hyperreactive B cells. Neutrophils are also implicated in disease pathogenesis; however, the mechanisms involved are unknown. Here, we identified in the sera of SLE patients immunogenic complexes composed of neutrophil-derived antimicrobial peptides and self-DNA. These complexes were produced by activated neutrophils in the form of web-like structures known as neutrophil extracellular traps (NETs) and efficiently triggered innate pDC activation via Toll-like receptor 9 (TLR9). SLE patients were found to develop autoantibodies to both the self-DNA and antimicrobial peptides in NETs, indicating that these complexes could also serve as autoantigens to trigger B cell activation. Circulating neutrophils from SLE patients released more NETs than those from healthy donors; this was further stimulated by the antimicrobial autoantibodies, suggesting a mechanism for the chronic release of immunogenic complexes in SLE. Our data establish a link between neutrophils, pDC activation, and autoimmunity in SLE, providing new potential targets for the treatment of this devastating disease.

## INTRODUCTION

Systemic lupus erythematosus (SLE) is the second most common human autoimmune disease affecting between 40 and 100 per 100,000 people in the United States. SLE is caused by the breakdown of tolerance to nuclear self-antigens, which leads to activation of autoreactive B cells that produce autoantibodies against self-nucleic acids and associated proteins. These autoantibodies bind self-nucleic acids released by dying cells, and form immune complexes that are deposited in different parts of the body, leading to detrimental inflammation and tissue damage. A key early event that triggers autoimmunity in SLE is the chronic innate activation of plasmacytoid dendritic cells (pDCs) to secrete type I interferons (IFNs) (1–4). The high levels of type I IFNs induce an unabated differentiation of monocytes into dendritic cells that stimulate autoreactive B and T cells (5), and lower the activation threshold of autoreactive B cells (6–8), thereby promoting autoimmunity in SLE.

Neutrophils have also been suspected to play a pathogenic role in SLE. SLE patients have increased numbers of apoptotic neutrophils in the blood, which correlates with the development of autoantibodies against DNA and disease activity (9, 10). SLE neutrophils were found to be activated intravascularly (11–13), displaying a tendency to form aggregates, expressing high levels of adhesion molecules, and producing reactive oxygen species (ROS) (11, 14, 15). High numbers of immature neutrophils present in the blood of SLE patients are associated with an increased expression of neutrophil-associated genes. These findings, which may reflect increased neutrophil turnover, were found to strongly correlate with the expression of type I IFNs (16), suggesting a link between neutrophils and the chronic pDC activation in SLE.

Previous studies have shown that self-nucleic acids contained in circulating immune complexes trigger pDC activation in SLE patients (17–20). Self-nucleic acids are normally nonimmunogenic because they are rapidly degraded in the extracellular environment. However, when contained in immune complexes, they somehow acquire the ability to access

intracellular compartments of pDC and trigger activation of Toll-like receptors TLR7 and TLR9. Although the exact mechanism is still unclear, the ability of autoantibodies to facilitate internalization of self-nucleic acid complexes via binding to Fc $\gamma$  surface receptor II (Fc $\gamma$ RII) has been proposed to contribute to the immunogenicity of self-DNA (19, 21). We have recently found that in psoriasis, self-DNA becomes immunogenic and triggers TLR9 in pDCs by forming a complex with an antimicrobial peptide LL37 (22, 23) overexpressed in the skin of these patients (24). LL37, produced by neutrophils, is highly expressed in the blood of SLE patients (16), raising the possibility that this peptide is involved in the immunogenicity of self-nucleic acids in immune complexes.

Here, we demonstrate that self-DNA in immune complexes of SLE patients contains neutrophil antimicrobial peptide LL37 and HNP. These antimicrobial peptides were required for the ability of self-DNA to trigger TLR9 in pDCs by forming complexes with the DNA that is protected from extracellular degradation. Such immunogenic self-DNA–antimicrobial peptide complexes were released by dying neutrophils undergoing NETosis, a cell death process in which activated neutrophils extrude large amounts of nuclear DNA into the extracellular space in the form of web-like structures called neutrophil extracellular traps (NETs) (25–27). NETs were abundantly released by neutrophils of SLE patients and were found to directly activate pDC to produce IFN- $\alpha$ . SLE patients developed autoantibodies to both the DNA and the antimicrobial peptides present in NETs, suggesting that NETs also trigger activation of autoreactive B cells. Thus, we identify the ability of neutrophils to activate pDCs through the release of NETs and suggest that a dysregulation of this pathway drives chronic pDC activation and autoimmunity in SLE.

## RESULTS

### Neutrophil antimicrobial peptides are present in circulating DNA-containing immune complexes of SLE patients

The antimicrobial peptide LL37 is overexpressed in keratinocytes of psoriatic skin lesions and triggers chronic pDC activation and disease development by forming complexes with extracellular self-DNA (24). Because LL37 represents one of the top signature genes expressed by peripheral blood mononuclear cells of SLE patients (16), we investigated whether the antimicrobial peptide LL37 is also present in immunogenic DNA-containing immune complexes of SLE patients. First, we purified total immunoglobulin G (IgG) antibodies from sera of SLE patients with high levels of anti-DNA antibodies to enrich for DNA-containing immune complexes. Immunoblot analysis revealed that LL37 was present in these purified IgG antibodies (Fig. 1A) but not in purified IgG from the healthy donors or SLE patients with low anti-DNA antibody titers (Fig. 1A and fig. S1). Then, we separated the IgG antibodies of SLE patients with high levels of anti-DNA antibodies into immune complexes (molecular weight >300 kD), which strongly activated pDCs to produce IFN- $\alpha$ , and IgG fractions consisting principally of monomeric IgGs (molecular weight <300 kD), which lacked this ability (Fig. 1B). LL37 was found exclusively in immune complexes, not in IgG fractions containing monomeric antibodies (Fig. 1C). Electrospray ionization mass spectrometric analysis of the LL37-containing band identified the signature peptide of another family of cationic antimicrobial peptides, the human neutrophil peptides (HNPs) belonging to the  $\alpha$ -defensin family and comprising three isoforms that differ in a single N-terminal residue (fig. S2) (28). Depletion of both LL37- and HNP-containing immune complexes using antibodies against LL37 and HNP completely abrogated induction of IFN- $\alpha$  in pDCs, confirming the presence of neutrophil antimicrobial peptides in immunogenic immune complexes of SLE patients with high anti-DNA antibody titers (Fig. 1B). These immune complexes also contained self-DNA, because they were detected as insoluble particles that stained with a DNA-specific dye (Fig. 1D). On the basis of the presence of anti-DNA antibodies, we also isolated DNA–anti-DNA immune complexes and confirmed

that they specifically activate pDCs through TLR9, because IFN- $\alpha$  induction was completely abrogated when pDCs were preincubated with a specific TLR9 inhibitor (Fig. 1E). Together, these data indicate that neutrophil antimicrobial peptides LL37 and HNPs are present in immunogenic DNA-containing immune complexes of SLE patients.

### **Neutrophil antimicrobial peptides are required for the ability of DNA contained in immune complexes to activate pDCs**

To determine whether neutrophil antimicrobial peptides play a role in the immunogenicity of DNA-containing immune complexes, we mixed fragments of human genomic DNA with LL37, HNPs, and anti-DNA antibodies and used these complexes to stimulate pDCs. Consistent with our previous study (24), we found that free human DNA was unable to enter and activate pDCs, but acquired this ability when complexed with LL37 (Fig. 2, A and B). HNPs alone did not have similar effects; however, these peptides promoted DNA uptake and pDC activation when the DNA was complexed with LL37, in particular at suboptimal LL37 concentrations (Fig. 2, A and C). Surprisingly, anti-DNA antibodies were also unable to promote DNA uptake and pDC activation when complexed to human DNA alone, but acquired this ability when the DNA was in complex with neutrophil antimicrobial peptides (Fig. 2, A and D). Activation of pDC occurred through TLR9 and was entirely dependent on the human DNA in the complexes (Fig. 2, E and F). Thus, self-DNA in immune complexes can access intracellular compartments of pDCs and trigger TLR9 when complexed with antimicrobial peptides. Anti-DNA antibodies alone are not sufficient to confer immunogenicity to self-DNA, but can promote this process by binding to self-DNA complexed to neutrophil antimicrobial peptides.

To assess whether the antimicrobial peptides are also required for the immunogenicity of DNA-containing immune complexes from SLE patients, we took advantage of the fact that binding of DNA to antimicrobial peptides, but not to antibodies, is driven by charge interactions. These interactions occur between the anionic phosphate backbone of the nucleic acid and the cationic residues of the antimicrobial peptide and can be reversed by an excess of anionic charges (24, 29). Accordingly, treatment of *in vitro*-generated DNA complexes with polyanionic polymers efficiently detached the cationic peptides from the human DNA (fig. S3) and abrogated the ability of the immune complexes to trigger IFN- $\alpha$  in pDCs (Fig. 2G). Similarly, treatment of DNA-containing immune complexes isolated from SLE patients completely abrogated IFN- $\alpha$  induction in pDCs, confirming that the cationic antimicrobial peptides are essential for the immunogenicity of DNA-containing immune complexes of SLE patients (Fig. 2G).

### **Neutrophil antimicrobial peptides protect DNA from nuclease degradation**

Previous studies have shown that autoantibodies in immune complexes can interact with Fc $\gamma$ R2 on pDCs and trigger receptor-mediated endocytosis of self-DNA (19, 21). Indeed, IFN- $\alpha$  induced by DNA immune complexes purified from SLE patients or generated *in vitro* was completely blocked by neutralization of Fc $\gamma$ R2 on pDCs (Fig. 3A). These results confirm that autoantibodies can internalize self-DNA via Fc $\gamma$ R2, but raise the question why this process requires the presence of antimicrobial peptides. We found that LL37 induces aggregation of self-DNA fragments into insoluble particles (Fig. 3B and fig. S4) that are protected from extracellular degradation by nucleases (Fig. 3C and fig. S5). This process was found to be a prerequisite for the ability of the DNA to enter intracellular TLR9 compartments of pDCs, because only DNA complexes containing LL37 (DNA-LL37, DNA-LL37/HNP, or DNA-LL37/HNP + anti-DNA), but not those without LL37 (DNA alone, DNA-anti-DNA, and DNA-HNPs), formed such particles and stimulated pDC to produce IFN- $\alpha$  (Figs. 2, A to E, and 3, B and C). HNPs alone were unable to form such DNA particles, but promoted this process in the presence of suboptimal concentrations of LL37

(fig. S4 and Fig. 2C), providing an explanation for the synergistic effect between HNPs and LL37 in pDC activation.

Like in vitro-generated DNA complexes, DNA complexes purified from the sera of SLE patients were detected as insoluble particles resistant to nuclease degradation (Figs. 1D and 3D). Detachment of antimicrobial peptides from these complexes using polyanionic polymers rapidly solubilized these particles (Fig. 3D), resulting in the rapid degradation of the DNA (Fig. 3E) and the inhibition of IFN- $\alpha$  induction (Fig. 2G). Together, these findings suggest that aggregation and protection of self-DNA by the antimicrobial peptides is required for the ability of autoantibodies to promote the transport of the DNA across membranes of pDCs into intracellular TLR9 compartments via the process of receptor-mediated endocytosis.

### **SLE patients develop autoantibodies against both DNA and neutrophil antimicrobial peptides**

Because activation of autoreactive B cells requires co-engagement of B cell receptor and TLR9 (30), immunogenic self-DNA-antimicrobial peptide complexes may represent the key trigger of autoreactive B cell activation in SLE patients, potentially leading to the development of antibodies against both the DNA and antimicrobial peptides. To test this possibility, we measured anti-LL37 and anti-HNP antibody levels in the sera of 38 SLE patients compared to those of 30 scleroderma patients and 12 healthy individuals (characteristics listed in tables S1 to S3). We found significant anti-LL37 antibody titers in the sera from SLE patients but not in those of scleroderma donors or healthy individuals (Fig. 4A, left panel). The specificity of the antibodies for LL37 was confirmed by the inhibition of reactivity when sera were preincubated with LL37 but not an irrelevant peptide (fig. S6). Consistent with previous reports (31), we also found significant levels of antibodies against HNPs in the sera from SLE patients but not from patients with scleroderma or healthy individuals (Fig. 4A, middle panel). We did not find any antibodies against human  $\beta$ -defensin (hBD), an epithelial cell-derived cationic antimicrobial peptide (Fig. 4A, right panel), confirming that SLE patients selectively develop antibodies against antimicrobial peptides derived from neutrophils. The levels of anti-LL37 and anti-HNP antibodies significantly correlated with the anti-DNA antibody titers (fig. S7), suggesting that the neutrophil-derived antimicrobial peptides serve as B cell autoantigens in conjunction with DNA. Forty-one percent of SLE patients were found to have anti-LL37 antibodies, and 58% of patients had anti-HNP antibodies. The presence of these antibodies was significantly associated with the detection of IFN- $\alpha$  in the sera of SLE patients (Fig. 4B); anti-LL37 antibodies were even found to correlate directly with the serum IFN- $\alpha$  levels (table S4). Consistent with previous studies, we found that serum IFN- $\alpha$  significantly correlated with the disease activity score (table S4), suggesting that the development of autoantibodies against antimicrobial peptides is linked to disease activity in SLE through IFN- $\alpha$  induction. One of the mechanisms for this appears to be the ability of autoantibodies to enhance the uptake of DNA-antimicrobial peptide complexes into pDCs. Indeed, anti-LL37 and anti-HNP antibodies strongly increased IFN- $\alpha$  production by enhancing Fc $\gamma$ RII-mediated internalization of DNA complexes into pDCs (Fig. 4C), similar to the phenomenon described for anti-DNA antibodies (Fig. 3A).

### **NETs released by activated neutrophils contain DNA-antimicrobial peptide complexes that trigger TLR9 in pDC**

Because our data suggested that self-DNA-antimicrobial peptide complexes represent the immunogenic core of DNA-containing immune complexes of SLE, we sought to determine the origin of these complexes. LL37 and HNP are constitutively expressed by neutrophils and stored in cytoplasmic granules, from where they are released during the process of



degranulation (Fig. 5A) (28). Neutrophils can also release nuclear DNA in the form of long chromatin filaments that form web-like structures (25, 26). These structures, called NETs, were shown to contain granule-derived antimicrobial peptides, which are believed to play a major role in killing bacteria trapped inside the NET structures (27). To test whether self-DNA–antimicrobial peptide complexes are formed in the context of NET formation, we analyzed NETs released by neutrophils activated with phorbol 12-myristate 13-acetate (PMA). These NETs appeared as long stretches of smooth DNA filaments containing numerous globular domains consisting of DNA and antimicrobial peptides LL37 and HNP (Fig. 5B). Treatment of NETs with low concentrations of deoxyribonuclease (DNase) I induced a rapid disappearance of the smooth DNA stretches but not of the globular DNA domains containing antimicrobial peptides (Fig. 5C). These residual DNA–antimicrobial peptide complexes appeared as small insoluble particles resembling purified DNA complexes isolated from SLE patients (Fig. 1D).

To test whether the DNA–antimicrobial peptide complexes in NETs could activate pDCs, we stimulated pDCs with cell-free supernatants of PMA-activated neutrophils. These supernatants induced substantial levels of IFN- $\alpha$  in pDCs (Fig. 5D), and IFN- $\alpha$  induction was completely abrogated by the TLR9 inhibitor, indicating that pDC activation is triggered by self-DNA. IFN- $\alpha$  production was greatly enhanced by the addition of anti-LL37, anti-HNP, or anti-DNA antibodies but not of an irrelevant antibody (Fig. 5D), indicating that the DNA that triggers pDC activation is complexed to neutrophil antimicrobial peptides. Another evidence for this is the fact that IFN- $\alpha$  induction was not abrogated by treatment of supernatants with low concentrations of DNase I, which efficiently eliminates the smooth DNA stretches in NETs but not the DNA in complex with antimicrobial peptides (fig. S8). Three findings allowed us to confirm that these DNA–antimicrobial peptide complexes are components of NETs and are not formed during externalization of cellular material by dying neutrophils: first, the concentrations of LL37 and HNPs measured in the supernatants of activated neutrophils were found to be too low to bind free DNA and induce pDC activation (fig. S9 and Fig. 2B); second, supernatant of neutrophils cultured for 24 hours in the presence of an agonistic anti-FAS antibody to induce apoptosis (32) did not stimulate pDC (Fig. 5E); finally, treatment of neutrophils with *N*-acetyl-L-cysteine (NAC), which blocks the generation of ROS required for NET formation (33, 34), abrogated the ability of the neutrophil supernatants to stimulate IFN- $\alpha$  in pDCs (Fig. 5E). Because LL37 is required for the immunogenicity of DNA immune complexes, we next assessed whether this peptide is also required for the ability of NET-derived DNA complexes to activate pDCs. Unlike HNPs, LL37 is expressed in neutrophils as an inactive 18-kD precursor protein called hCAP18, and requires cleavage into its active 4-kD form (28, 35) by granule-derived serine proteases released by activated neutrophils. We used the serine protease inhibitor chloromethyl ketone (CMK) to inhibit the cleavage of LL37 from hCAP18 (Fig. 5F, upper panel) without affecting significantly the release of NET-DNA (fig. S10). We found that NETs released by neutrophils treated with CMK completely failed to induce IFN- $\alpha$  production (Fig. 5F, lower panel), suggesting that LL37 is required for the ability of the self-DNA in NETs to trigger activation of pDCs. Together, these data demonstrate that NETs released by activated neutrophils contain immunogenic self-DNA–antimicrobial peptide complexes that trigger IFN- $\alpha$  in pDC. The particulate nature of these complexes, their resistance to DNase degradation, and their ability to trigger TLR9 in pDCs, which can be enhanced by autoantibodies to DNA, LL37, and HNPs, suggest that NETs represent the source of self-DNA–antimicrobial peptide complexes present in the sera of SLE patients.

## NETs are induced by autoantibodies against antimicrobial peptides and are released in higher amounts by neutrophils of SLE patients

Because anti-neutrophil antibodies have been shown to trigger the formation of NETs (36), we sought to determine whether antibodies against antimicrobial peptides present in SLE sera can trigger NET formation. We found that both anti-LL37 and anti-HNPs but not antibodies against DNA or isotype control antibodies were able to activate neutrophils isolated from healthy donors to release NETs (Fig. 6, A to C, and figs. S11 and S12). NETs were also induced when neutrophils were stimulated by a F(ab)<sub>2</sub> fragment against LL37, suggesting the involvement of a specific binding of the antibodies to antimicrobial peptides on neutrophils (Fig. 6, A and C). Accordingly, we found low levels of LL37 and HNP exposed on the surface of cultured neutrophils (Fig. 6, D and E). IFN- $\alpha$  enhanced the surface expression of both LL37 and HNP on cultured neutrophils (Fig. 6, D and E) and promoted the release of more NETs by neutrophils stimulated with anti-LL37 and anti-HNP antibodies (Fig. 6F). Neutrophils isolated from SLE patients displayed high levels of surface LL37 and HNP, even before culture (Fig. 6, D and E), and consequently released much more NET-DNA than neutrophils from healthy donors in response to anti-LL37 and anti-HNP antibodies (Fig. 6F). This indicates that neutrophils in SLE patients are poised to release large quantities of NETs in response to anti-LL37 and anti-HNP antibodies due to the surface exposure of antimicrobial peptides. We also found that, compared to neutrophils from healthy donors, neutrophils from SLE patients released significantly higher amounts of NET-DNA even in the absence of in vitro stimulation (Fig. 6G), suggesting that circulating SLE neutrophils are chronically activated to release NETs, potentially through circulating antibodies against antimicrobial peptides.

## DISCUSSION

The chronic activation of pDCs by circulating immune complexes is central to the pathogenesis of SLE. Although a role for neutrophils in this process had been suggested, the mechanisms involved remained unclear. Here, we have identified a key link between neutrophils, pDC activation, and autoimmunity in SLE. First, we found that neutrophils can directly stimulate pDC activation by releasing NETs containing complexes of self-DNA with antimicrobial peptides. Second, we found that these self-DNA–antimicrobial peptide complexes are abundantly released by neutrophils of SLE patients and that they form the immunogenic core of immune complexes required for TLR9 activation of pDCs. Finally, we demonstrated that SLE patients develop autoantibodies against the antimicrobial peptides present in these self-DNA complexes. We propose that these antibodies promote chronic pDC activation by facilitating the uptake of the self-DNA complexes into pDC and by triggering the release of additional NETs by neutrophils.

The immunogenicity of self-DNA–containing immune complexes has been classically attributed to the ability of antibodies to internalize DNA into TLR9 compartments (19, 21). Here, we demonstrate that this process requires the presence of neutrophil antimicrobial peptides that protect the DNA from enzymatic degradation. Only LL37 but not HNPs have the ability to protect the DNA from degradation, probably due to the unique  $\alpha$ -helical structure of LL37, which efficiently stabilizes charge interactions with the DNA (37). This suggests that therapeutic targeting of LL37 may be sufficient to abrogate the immunogenicity of self-DNA in immune complexes of SLE patients.

The immunogenic self-DNA–antimicrobial peptide complexes were found to originate from neutrophils undergoing NETosis, a cell death process in which neutrophils extrude large amounts of nuclear DNA into the extracellular space in the form of web-like DNA structures (the NETs). NETs are typically released in the context of infections and are believed to play an important role in the fight against extracellular bacteria through their ability to entrap and

kill these microbes (25, 27). We now show that NETs can trigger activation of pDC via TLR9, providing a potentially important new role of NETs in initiating antimicrobial immune responses.

In contrast to those from healthy donors, neutrophils from SLE patients were found to release abundant levels of NETs. This finding is in accordance with previous studies showing that neutrophils from SLE patients are activated to release ROS (11, 38), a central factor in the formation of NETs (26, 39). Abundant NET production may drive the chronic pDC activation in SLE patients, providing an explanation for how dying neutrophils lead to IFN- $\alpha$ -mediated auto-immunity. SLE patients also develop autoantibodies against both the DNA and the antimicrobial peptides in NETs. In addition to pDC activation, therefore, abundant NET production may also trigger autoreactive B cell activation, probably through the ability of NETs to engage both B cell receptor and TLR9 in B cells (30). This is in line with the previous finding that LL37 also enables the entry of DNA into TLR9 compartments of B cells (40). A recent study described the development of antibodies against NETs in a subset of SLE patients with renal disease. This was explained by the inability of this group of SLE patients to efficiently degrade NETs (41). In our study, however, we were unable to establish a significant correlation between the development of antibodies against antimicrobial peptides and renal involvement (table S4).

Antibodies against DNA and antimicrobial peptide both directly bind to NET-derived self-DNA-antimicrobial peptide complexes and promote their transport into pDCs, leading to the amplification of IFN- $\alpha$  production. Therapeutic immunosuppression with oral corticosteroids significantly inhibited the development of these autoantibodies. Because corticosteroids do not inhibit the ability of neutrophils to release NETs (fig. S13) and are unable to inhibit IFN- $\alpha$  production by TLR-activated pDCs (42), this treatment modality most likely affects B cell activation directly.

Intriguingly, autoantibodies against antimicrobial peptides were found to directly trigger activation of neutrophils, providing a mechanism for the continuous release of NETs in SLE patients even in the absence of infections. We also found that neutrophils of SLE patients are poised to release large quantities of NETs in response to these autoantibodies due to an increased surface exposure of granule-derived antimicrobial peptides. It was previously demonstrated that granule-derived protein antigens are not on the surface of quiescent neutrophils, but can be exposed on the surface of cytokine-primed neutrophils as well as neutrophils that have become apoptotic (43, 44). In SLE, the increased exposure of antimicrobial peptide may result from both the increased numbers of apoptotic neutrophils as well as priming with IFN- $\alpha$ , which is present in the sera of SLE patients with active disease neutrophils. Another mechanism for the exposure of granule-derived proteins on live neutrophils could be the transfer of granular proteins released by dying neutrophils onto the surface of nonactivated neutrophils (45).

It is unknown whether other factors in addition to autoantibodies trigger neutrophil activation and the release of NETs in SLE. Intriguingly, a genetic basis for the activation of neutrophils in SLE has been suggested with the identification of a major susceptibility gene involving the complement receptor 3 (CR3 or CD11b/CD18) (46, 47), which mediates neutrophil activation and has been associated with disease activity in SLE (13, 15, 48).

Another unanswered question is whether self-RNA-containing complexes also contain neutrophil antimicrobial peptides. This appears particularly relevant because LL37 has been also shown to bind RNA fragments and to promote activation of TLR7 in pDCs (49).

In conclusion, we identify a link between neutrophils and the innate activation of pDCs and suggest that dysregulation of this pathway leads to chronic IFN- $\alpha$  production and



autoimmunity in SLE. This study reveals a fundamental component of the pathogenesis of SLE, providing new potential targets for the treatment of this life-threatening autoimmune disease.

## MATERIALS AND METHODS

### Human subjects

Studies were approved by the Institutional Review Board for human research at the University of Texas M. D. Anderson Cancer Center in Houston, the Kansai Medical University in Osaka, and the University La Sapienza in Rome. Sera were collected from 38 SLE patients and 30 scleroderma patients. The diagnosis of SLE was based on the American College of Rheumatology criteria for SLE; the diagnosis of scleroderma was based on the American College of Rheumatology criteria for scleroderma. Sera from 12 healthy donors were used as a control. For characterization and stimulation of SLE neutrophils, patients with active disease based on clinical criteria (rash, renal disease, and high anti-DNA titers) were selected.

### Purification of DNA immune complexes from SLE patients

Total IgG from 8 to 10 ml of sera of SLE patients (The Binding Site Inc.) was isolated with a HiTrap Protein G affinity column (GE Healthcare). Immune complexes were then separated from monomeric IgGs with an Amicon centrifugal filter device with a 300,000 nominal molecular weight limit cutoff. DNA/anti-DNA complexes were further purified from total immune complexes by MACS-based separation with DNA-coated microbeads generated by biotinylation of calf thymus DNA (BD Pharmingen) followed by coating with streptavidin beads (Miltenyi Biotec).

### In vitro generation of DNA immune complexes

Purified human genomic DNA ( $3 \mu\text{g ml}^{-1}$ ) was mixed with LL37 (Innovagen, 3 or  $10 \mu\text{M}$ ), HNP<sub>(1-3)</sub> (Hycult Biotechnology,  $10 \mu\text{M}$ ), and anti-double-stranded DNA (dsDNA) antibody H241 (a gift from D. Stollar, Tufts University,  $1 \mu\text{g ml}^{-1}$ ) in  $20 \mu\text{l}$  of phosphate-buffered saline (PBS). For pDC stimulation, complexes were diluted into  $200 \mu\text{l}$  of complete medium. For visualization, complexes were spun down and stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich,  $0.1 \text{ ng/ml}$ ) before confocal microscopy analysis. In some experiments, suspensions were treated for 1 hour with DNase I (Roche,  $600 \text{ U ml}^{-1}$ ) or with polyaspartic and polyglutamic acids (both 5000 to 15,000 molecular weight, Sigma-Aldrich,  $10 \mu\text{M}$ ).

### Isolation and stimulation of pDC

pDCs were enriched from peripheral blood mononuclear cells of healthy donors with anti-BDCA4-conjugated microbeads (Miltenyi Biotec) and further purified as lineage<sup>-</sup>CD11c<sup>-</sup>CD4<sup>+</sup> cells with fluorescence-activated cell sorting (FACSaria Cell Sorting System, BD Biosciences) to yield >99% purity. pDCs were then seeded at  $5 \times 10^4$  per well in  $200 \mu\text{l}$  of complete medium and stimulated overnight before IFN- $\alpha$  was measured in the supernatants by enzyme-linked immunosorbent assay (ELISA) (PBL Biomedical Laboratories).

### Purification of neutrophils and induction of NETosis

Neutrophils were isolated from peripheral blood of healthy donors or SLE patients with anti-CD15-conjugated microbeads (Miltenyi Biotec) to yield 95 to 97% purity (the remaining cells were eosinophils and monocytes expressing low levels of CD15). Purified neutrophils were seeded at  $50 \times 10^4$  cells in  $200 \mu\text{l}$  of complete medium and stimulated with PMA

(Sigma-Aldrich, 20 ng ml<sup>-1</sup>), anti-LL37 (clone 8A8, IgG2b, 10 µg ml<sup>-1</sup>), anti-HNP (clone 3G9, IgG2a, from Novus Biological, 10 µg ml<sup>-1</sup>), anti-dsDNA antibody H241, or IgG control antibodies (10 µg ml<sup>-1</sup>). After 3 hours of stimulation, cells were spun down and cell-free supernatant was collected and used at 1:3 dilution for overnight stimulation of pDC.

### Quantification of NETosis

To visualize NETs by confocal microscopy, we seeded neutrophils on coverslips pretreated with polylysine and stimulated them to induce release of the NETs. After 3 hours, cells were gently washed and stained with a DNA dye (YOYO-1 or DAPI), washed, and mounted in Prolong Gold antifade media (Molecular Probes) before analysis by Leica SP2 RS SE confocal microscope. For detection of LL37 and HNP expression in neutrophils, slides were fixed in 4% paraformaldehyde and permeabilized with 0.1% saponin (Sigma-Aldrich) and 1% normal goat serum (Jackson ImmunoResearch Laboratories). Slides were stained with either biotin-conjugated anti-LL37 (Innovagen) or biotin-conjugated anti-HNP antibodies (Hycult Biotechnology) followed by Alexa568-labeled streptavidin (Molecular Probes). The release of NET-DNA was also measured in cell-free supernatant collected after 3 hours of activation with the PicoGreen assay kit (Invitrogen).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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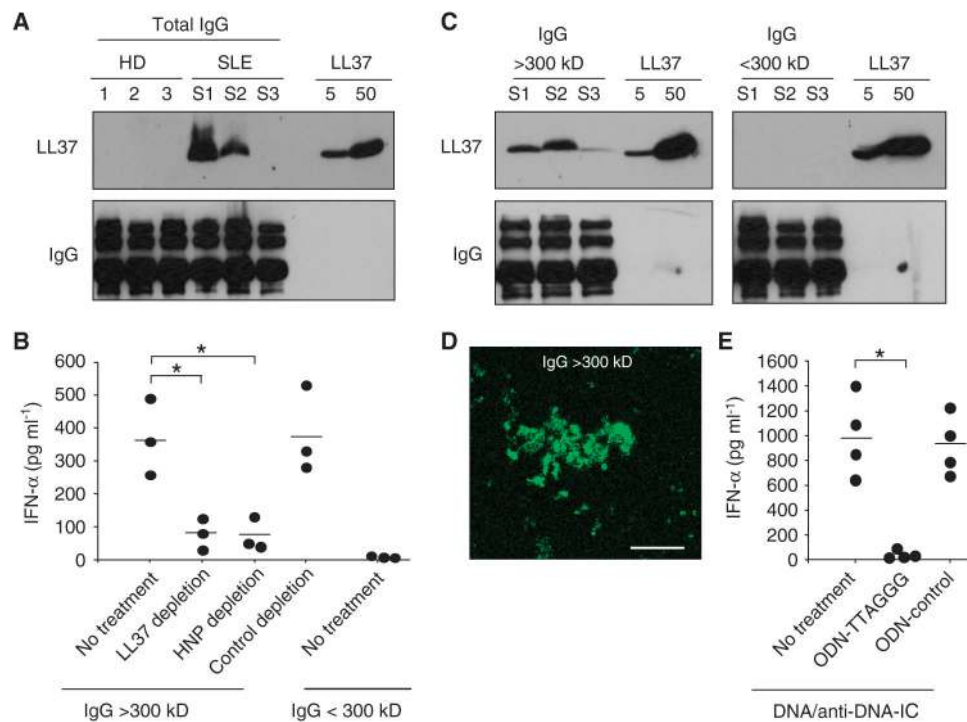
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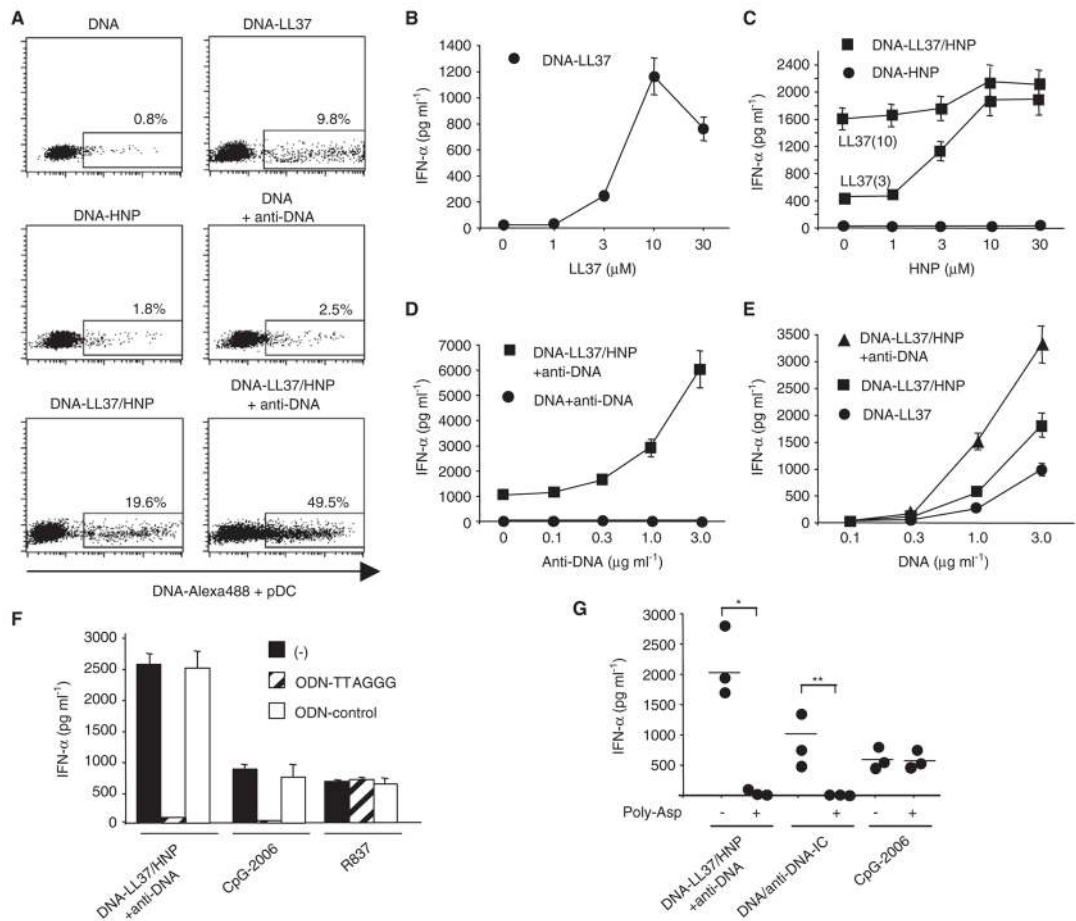
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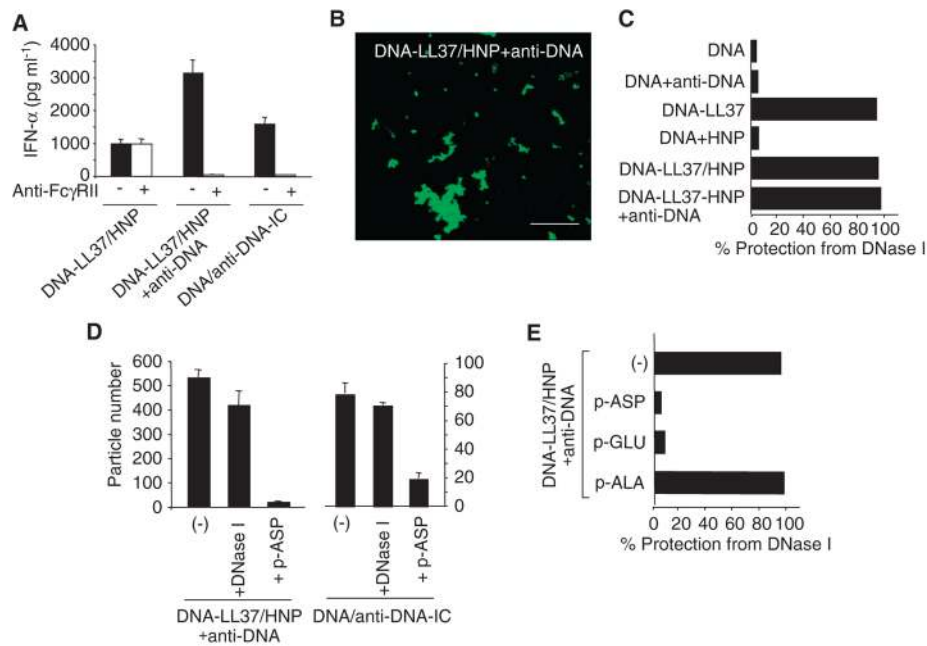




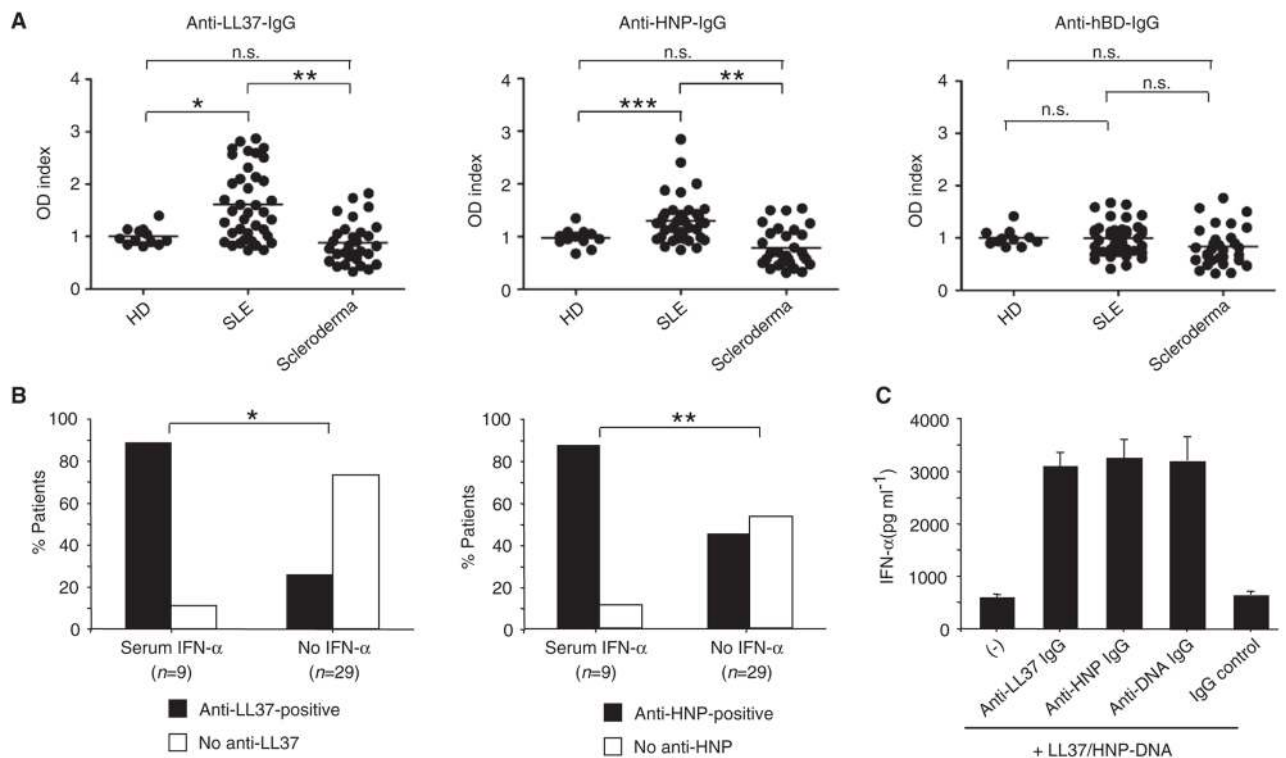
**Fig. 1.** DNA immune complexes from SLE patients contain neutrophil antimicrobial peptides. (A) Immunoblot detection of LL37 and IgG in total IgG antibodies purified from sera from healthy donors (HD) 1 to 3 or systemic lupus erythematosus (SLE) patients with high anti-DNA antibody titers (S1 to S3). Five and 50 ng of LL37 peptide were used as a positive control. (B) IFN- $\alpha$  produced by pDCs after stimulation with either immune complexes (>300 kD) or monomeric Ig (<300 kD) isolated from total IgGs of SLE patients S1 to S3. In some experiments, immune complexes containing LL37 or HNP were depleted. Each symbol represents an independent experiment, and horizontal bars represent the mean. \* $P < 0.01$ , analysis of variance (ANOVA) (adjusted for Dunnett's test). (C) Immunoblot detection of LL37 and IgG in immune complexes (>300 kD) and monomeric Ig (<300 kD) isolated from IgGs of SLE patients S1 to S3. The LL37 peptide is used as a control. (D) Representative immunofluorescence microscopy image of SLE immune complexes (>300 kD) detected as insoluble particles that stained with DAPI. Bar, 10  $\mu$ m. (E) IFN- $\alpha$  produced by pDCs after overnight stimulation with purified DNA/anti-DNA immune complexes (IC) after pretreatment of pDC with either a TLR9 inhibitor (ODN-TTAGGG) or a control oligonucleotide (ODN-control). Each symbol represents an independent experiment, and horizontal bars represent the mean. \* $P < 0.001$ , Student's  $t$  test.



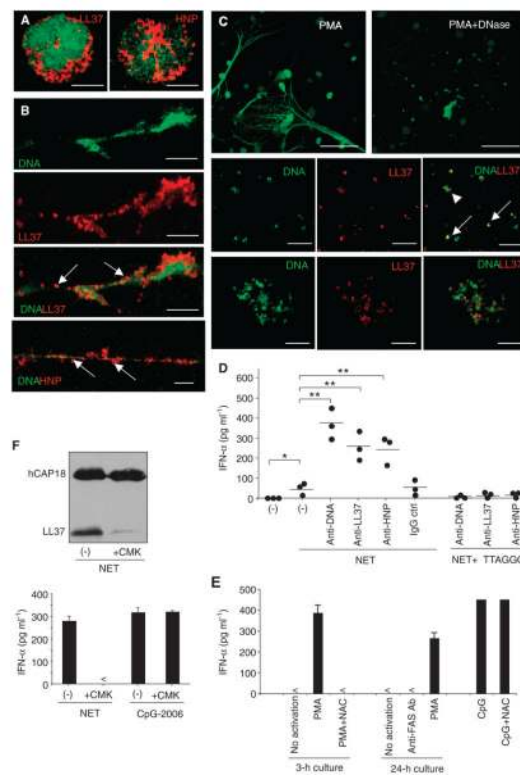
**Fig. 2.** Neutrophil antimicrobial peptides enable the self-DNA in immune complexes to trigger pDC activation. (A) Flow cytometry of pDCs stimulated with human DNA<sup>Alexa488</sup> alone or human DNA<sup>Alexa488</sup> in complex with LL37, HNP, and anti-DNA antibodies. Fluorescent cells (depicted on the x axis) represent pDCs that have taken up the DNA. (B to E) IFN- $\alpha$  produced by pDCs stimulated with DNA plus increasing concentrations of LL37 (B); DNA plus increasing concentrations of HNP alone or in the presence of LL37 (3 or 10  $\mu$ M) (C); DNA plus increasing concentrations of anti-DNA antibodies alone or in the presence of LL37 and HNPs (D); and increasing concentrations of DNA in the presence of LL37, LL37 plus HNP, or LL37/HNP plus anti-DNA antibodies (E). (F) IFN- $\alpha$  production by pDC stimulated with DNA-LL37/HNP-anti-DNA complexes after pretreatment with TLR9 inhibitor ODN-TTAGGG or ODN-control. TLR9 agonist CpG-2006 and TLR7 agonist R837 were used as controls. Data in (A) to (F) are representative of at least five independent experiments. Error bars in (B) to (F) represent the SD of triplicate wells. (G) IFN- $\alpha$  production in pDCs stimulated with DNA-LL37/HNP-anti-DNA complexes, natural DNA-anti-DNA immune complexes, or CpG-2006 after pretreatment with polyaspartic acid (Poly-ASP). Each symbol represents an independent experiment, and horizontal bars represent the mean. \* $P$  = 0.002; \*\* $P$  = 0.034, ANOVA (Bonferroni adjustment).



**Fig. 3.** Neutrophil antimicrobial peptides protect DNA in immune complexes from extracellular degradation. **(A)** IFN- $\alpha$  produced by pDCs after stimulation with DNA-LL37/HNP complexes, DNA-LL37/HNP-anti-DNA complexes, or DNA-anti-DNA complexes purified from SLE patients with or without neutralization of Fc $\gamma$ RII with a blocking antibody. **(B)** Representative immunofluorescence microscopy image of DNA-LL37/HNP-anti-DNA complexes detected as insoluble particles that stained with DAPI. Bar, 10  $\mu$ m. **(C)** Percentage of DNA remaining after treatment of DNA alone or DNA in complex with LL37, LL37/HNP, HNP, anti-DNA, or LL37/HNP + anti-DNA with DNase I for 30 min. Fluorimetric measurement of DNA was done at 480 nm after staining with PicoGreen dye. **(D)** Number of in vitro-generated DNA-LL37/HNP + anti-DNA complexes and purified DNA/anti-DNA complexes counted as insoluble DAPI<sup>+</sup> particles with or without treatment with DNase I or polyaspartic acid (p-ASP). **(E)** Percentage DNA remaining after treatment of DNA-LL37/HNP + anti-DNA complexes with polyaspartic acid, polyglutamic acid (p-GLU) (both anionic), or polyalanine (p-ALA) (no charge, used as control) followed by 30 min of incubation with DNase I. Data in (A) to (E) are representative of at least five independent experiments. Error bars in (A) represent the SD of triplicate wells, and in (D) the SD of counts by two independent investigators.

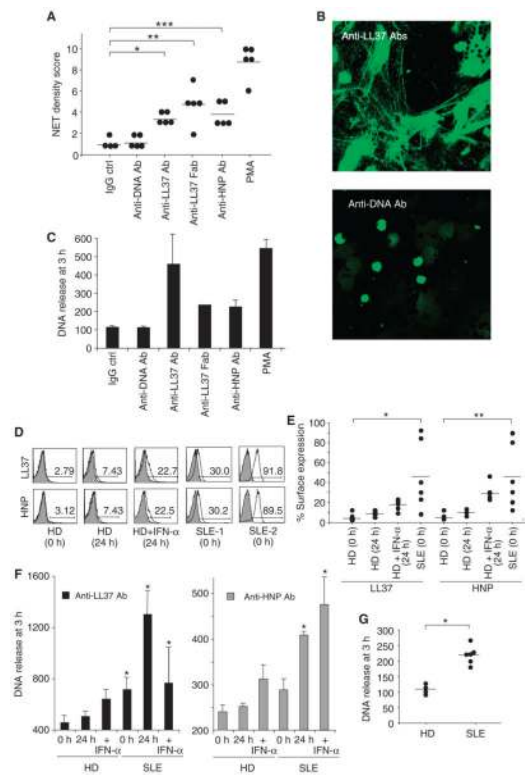
**Fig. 4.**

SLE patients develop autoantibodies against neutrophil antimicrobial peptides. (A) Antibodies to LL37, HNPs, and hBD in sera from patients with SLE ( $n = 38$ ), scleroderma ( $n = 30$ ), or healthy controls (HD;  $n = 12$ ). Results are expressed as optical density (OD) index, which is the ratio of the OD in the patient serum to the mean OD in healthy control sera. Each symbol represents an independent patient, and horizontal bars represent the mean. \* $P = 0.002$ ; \*\* $P < 0.0001$ ; \*\*\* $P = 0.023$ , ANOVA (adjusted for Dunnett's test). n.s., not significant. (B) Percentages of SLE patients with or without significant anti-LL37 antibody titers (cutoff value OD index, 1.611; sensitivity, 41%; specificity, 100%; left panel) or SLE patients with or without anti-HNP antibody titers (cutoff value OD index, 1.15; sensitivity, 59%; specificity, 91%; right panel) among patients with detectable IFN- $\alpha$  ( $n = 9$ ) and patients without IFN- $\alpha$  in the serum ( $n = 29$ ). \* $P = 0.001$ ; \*\* $P = 0.05$ , Fisher's exact test. (C) IFN- $\alpha$  produced by pDC stimulation with in vitro-generated DNA-LL37/HNP complexes alone or with anti-LL37, anti-HNP, anti-DNA, or IgG control antibodies. One representative of at least three independent experiments is shown. Error bars represent the SD of triplicate wells.



**Fig. 5.** NETting neutrophils release self-DNA–antimicrobial peptide complexes that activate pDCs. (A to C) Confocal microscopy of unstimulated neutrophils (A), NETting neutrophils activated for 3 hours with phorbol 12-myristate 13-acetate (PMA) (B), or NETting neutrophils activated with PMA followed by treatment with DNase I (C), stained for DNA (green), LL37 (red), or HNPs (red) as indicated. Representative images are shown. Bars, 5  $\mu$ m (A), 10  $\mu$ m (B), 50  $\mu$ m (C, upper panel), 20  $\mu$ m (C, middle panel), and 4  $\mu$ m (C, lower panel). Arrows indicate DNA–antimicrobial peptide complexes contained in NETs before (B) and after (C) DNase treatment. A high-power image of a complex indicated by the arrowhead is provided in the lower panel of (C). (D) IFN- $\alpha$  produced by pDCs stimulated with supernatants of NETting neutrophils alone or in the presence of anti-LL37, anti-HNP, anti-DNA, or control antibodies. In some experiments, the pDCs were pretreated with the TLR9 inhibitor ODN-TTAGGG. Each symbol represents an independent experiment, and horizontal bars represent the mean. \* $P = 0.023$ ; \*\* $P < 0.04$ , ANOVA (Bonferroni adjustment). (E) Supernatants of neutrophils activated for 3 hours with PMA after pretreatment with *N*-acetyl-L-cysteine (NAC; 5 mM) to block NET formation, and supernatants of neutrophils stimulated for 24 hours with an anti-FAS antibody (Ab) to induce apoptosis, were used to stimulate pDCs. (F) Supernatants of NETting neutrophils pretreated with the serine protease inhibitor chloromethyl ketone (CMK) were used for immunoblot detection of LL37 and hCAP18 (upper panel) and to stimulate pDC for IFN- $\alpha$  production (lower panel). TLR9 agonist CpG-2006 was used as a positive control to exclude NET-independent effects of CMK on pDC activation. (E to F) One representative of at least three independent experiments is shown. Error bars represent the SD of triplicate wells.



**Fig. 6.**

Autoantibodies to neutrophil antimicrobial peptides trigger abundant NET release in neutrophils of SLE patients. (A to C) Purified neutrophils from healthy donors were stimulated as indicated. NET-DNA release was quantified after 3 hours either by confocal microscopy (A and B) or by fluorimetry of the supernatants after PicoGreen staining (C). In (A), NET density was quantified in multiple experiments and scored to reflect the image area covered by NET structures.  $*P=0.024$ ;  $**P<0.001$ ;  $***P=0.006$ , ANOVA (adjusted for Dunnett's test). In (B), representative confocal images of anti-LL37- and anti-DNA-stimulated neutrophils stained for DNA. In (C), NET-DNA is given as nanogram of DNA released by  $10^6$  neutrophils. Mean  $\pm$  SD of four independent donors is shown. (D and E) Flow cytometry analysis of LL37 and HNP surface expression on freshly isolated neutrophils (0 h) and neutrophil cultured for 24 hours with or without IFN- $\alpha$  (24 h) from healthy donors in comparison to freshly isolated neutrophils (0 h) from SLE patients. Representative plots are shown in (D); results from multiple independent donors are given in (E).  $*P=0.030$ ;  $**P=0.025$ , Student's *t* test. (F) Freshly isolated neutrophils (0 h) or cultured for 24 hours with or without IFN- $\alpha$  (24 h) were stimulated for 3 hours with anti-LL37 and anti-HNP antibodies. DNA release was measured by fluorimetry in cell-free supernatants, and data are given as nanogram of DNA released by  $10^6$  neutrophils. The mean  $\pm$  SD of at least four independent donors is shown.  $*P<0.05$ , ANOVA (Bonferroni adjustment). In the absence of stimulation, the levels of DNA released into the supernatant by neutrophils cultured for 24 hours ( $110 \pm 25$  for healthy neutrophils, and  $240 \pm 80$  for SLE neutrophils) were similar to the levels released by freshly isolated neutrophils cultured for 3 hours. This indicates that the free DNA detected in the supernatant is actively released by neutrophils activated by anti-LL37 or anti-HNP antibodies and does not represent DNA passively released in the context of neutrophil apoptosis and secondary necrosis induced during the 24-hour culture. (G) Freshly isolated neutrophils (0 h) of healthy donors and SLE patients were cultured for 3 hours without any stimulus. DNA release was measured in the

cell-free supernatants by fluorimetry. Each dot represents an independent donor.  $*P=0.03$ , ANOVA (Bonferroni adjustment).