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Netting Neutrophils Are Major Inducers of Type I IFN Production in Pediatric Systemic Lupus Erythematosus

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Abstract

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SUPPLEMENTARY MATERIAL

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Fig. S1. IFN- α does not increase healthy neutrophil apoptosis.

Fig. S2. SLE serum or type I IFN reproduces many of the transcriptional alterations of SLE neutrophils.

Fig. S3. SLE serum and IFN- α up-regulate TLR7 expression in healthy neutrophils.

Fig. S4. Healthy and SLE neutrophils display different susceptibility to anti-RNP antibody-induced death as measured by the TUNEL assay.

Fig. S5. Elastase does not colocalize with LL37 and HMGB1 within NETs.

Fig. S6. Apoptotic and necrotic neutrophils fail to activate pDCs.

Fig. S7. SLE NETs induce pDC activation and survival.

Fig. S8. SLE NET activation of pDCs is independent of Fc γ RIIa.

Fig. S9. Release of extracellular DNA is specific to NETosis-associated death.

Fig. S10. IFN- α and anti-RNP antibody-induced NETs from healthy donors activate pDCs in a DNA-dependent manner.

Table S1. Demographic characteristics and disease activity of the SLE patients whose neutrophils were used for gene expression profiling.

Table S2. Demographic characteristics and disease activity of the SLE patients whose sera were used to culture healthy neutrophils in vitro for gene expression profiling.

Table S3. Characteristics of the SLE patients whose neutrophils were activated in vitro.

Table S4. Transcripts differentially expressed in freshly isolated SLE neutrophils.

Table S5. Transcripts differentially expressed in healthy neutrophils exposed to SLE sera in vitro.

Table S6. Transcripts differentially expressed in healthy neutrophils exposed to recombinant IFN- α 2 in vitro.

Table S7. Corticosteroid therapy does not have an effect on the capacity of SLE neutrophils to undergo NETosis upon exposure to anti-RNP antibodies.

Author contributions: G.S.G.-R. performed and analyzed most of the experiments, participated in their design, and provided critical discussions. S.C. designed, performed, and analyzed several experiments and provided critical discussions. B.V. designed, performed, and analyzed experiments included in the supplementary section. J.C. participated in microscopy studies of NETs. C.G., R.L.C., and F.J.B. provided purified anti-RNP antibodies, participated in the design of some of the experiments, and provided critical suggestions and discussions. F.A. and Z.X. did microarray analyses. M.P. provided patient samples and data. J.B. coordinated the sample drawing and Institutional Review Board-related issues. D.B. helped with statistical analyses. J.B. provided critical suggestions and discussions throughout the study while at the Baylor Institute for Immunology Research in Dallas, Texas. V.P. conceived and supervised this study, was involved in the design and evaluation of all experiments, and wrote the manuscript with comments from co-authors.

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Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a breakdown of tolerance to nuclear antigens and the development of immune complexes. Genomic approaches have shown that human SLE leukocytes homogeneously express type I interferon (IFN)-induced and neutrophil-related transcripts. Increased production and/or bioavailability of IFN- α and associated alterations in dendritic cell (DC) homeostasis have been linked to lupus pathogenesis. Although neutrophils have long been shown to be associated with lupus, their potential role in disease pathogenesis remains elusive. Here, we show that mature SLE neutrophils are primed in vivo by type I IFN and die upon exposure to SLE-derived anti-ribonucleoprotein antibodies, releasing neutrophil extracellular traps (NETs). SLE NETs contain DNA as well as large amounts of LL37 and HMGB1, neutrophil proteins that facilitate the uptake and recognition of mammalian DNA by plasmacytoid DCs (pDCs). Indeed, SLE NETs activate pDCs to produce high levels of IFN- α in a DNA- and TLR9 (Toll-like receptor 9)-dependent manner. Our results reveal an unsuspected role for neutrophils in SLE pathogenesis and identify a novel link between nucleic acid-recognizing antibodies and type I IFN production in this disease.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by breakdown of tolerance to nuclear antigens, immune complex (IC) deposition in tissues, and multiorgan involvement (1). The skin, blood vessels, kidneys, central nervous system, and joints become targets of inflammation at onset or during the course of the disease. Common hypotheses about SLE pathogenesis suggest that environmental triggers, such as infectious agents, operate in the context of both susceptibility genes and epigenetic modifications, resulting in alterations in antigen presentation, lymphoid signaling, apoptosis, and antigen/IC clearance (2, 3).

SLE patients display both innate and adaptive immune alterations that affect blood cell composition. Thus, decreased numbers of neutrophils, dendritic cells (DCs), and lymphocytes are common features of SLE (4). Although plasmacytoid DCs (pDCs) are significantly decreased in the blood of patients (5), they accumulate at sites of inflammation such as the skin and the kidney, where they secrete type I interferon (IFN) (6–8). Upon exposure to SLE serum, healthy monocytes differentiate into mature DCs in an IFN- α -dependent fashion (5). Genomic approaches have shown that >95% of children and 50 to 70% of adults with SLE display a type I IFN signature as measured by peripheral blood mononuclear cell (PBMC) gene expression profiling (9, 10). The second most prevalent PBMC transcriptional signature in children with SLE corresponds to neutrophil-specific genes, and differential expression of these genes correlates with disease activity (9). This signature is due to the presence in SLE blood of low-density neutrophils that copurify with mononuclear cells during gradient centrifugation. Their presence in the blood may reflect an early neutrophil release from the bone marrow due to SLE-specific cytokine alterations [that is, increased interleukin-8 (IL-8), IL-17, etc.] (11, 12) that recruit mature neutrophils to sites of inflammation, where ICs are also deposited. Early neutrophils might also enter the blood due to accelerated mature neutrophil death. Notably, SLE neutrophils undergo accelerated spontaneous apoptosis in vitro, and SLE sera induce the apoptosis of healthy neutrophils, both of which correlate with disease activity (13).

Upon encountering extracellular pathogens, neutrophils die by apoptosis, necrosis, or by releasing NETs (neutrophil extracellular traps) (14–16). NETs contain proteins from azurophilic (primary), as well as secondary and tertiary granules. However, nuclear material such as DNA and histones comprises the major structural components of NETs. Fittingly, cytokines, including IFN- α , can prime mature neutrophils in vitro and allow the formation of NETs (15).

The presence of neutrophils in lesions from lupus patients is well documented (17, 18) and represents one of the criteria used to define active lupus nephritis (19). Furthermore, neutrophil-specific proteins are found in the urine of SLE patients and can be used as a surrogate marker of disease activity (20). Nevertheless, the role of these cells in SLE pathogenesis has not been elucidated. Here, we asked whether neutrophils might contribute to the dysregulation of IFN production in SLE through the release of NETs. Our results support a model that positions this unique type of neutrophil death linked with pDC activation and type I IFN production at the core of SLE pathogenesis.

RESULTS

Mature SLE neutrophils undergo accelerated death in vitro

Mature neutrophils isolated from the blood of pediatric SLE patients undergo spontaneous death at a higher rate than healthy neutrophils, as assessed by trypan blue exclusion at 2, 6, and 18 hours after blood collection (Fig. 1A). To understand the pathways underlying this accelerated death, we analyzed the gene expression profiles of 21 neutrophil samples isolated from the blood of 19 pediatric SLE patients [age, 14.8 ± 2.5 years (mean $\pm$ SEM); SLE Disease Activity Index (SLEDAI) range, 0 to 24; 7.5 ± 6.6 (mean $\pm$ SEM); table S1] and 12 age- and ethnicity-matched healthy children using Illumina oligonucleotide microarrays. A remarkably homogeneous pattern of differentially expressed genes encompassing 1564 transcripts was observed in 20 of 21 patient samples (nonparametric *t* test, Benjamini and Hochberg multiple testing correction, $P < 0.01$). These transcripts could be assigned to a few predominant pathways, including type I IFN and Toll-like receptor (TLR) signaling, ubiquitination, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling, and cell death (Fig. 1B and table S4).

To ascertain whether these SLE neutrophil transcriptional changes result from intrinsic cell alterations or from exposure to soluble factors, we cultured neutrophils from two healthy children for 6 hours with autologous serum (AS), sera from 10 active pediatric SLE patients [age, 14.8 ± 2.2 years (mean $\pm$ SEM); SLEDAI range, 4 to 24; 12.2 ± 7.9 (mean $\pm$ SEM); table S2], or recombinant IFN- α 2. Neutrophil death was assessed by annexin V and propidium iodide staining, and RNA was extracted for microarray analysis. As opposed to the reported proapoptotic effects of SLE sera (13), IFN- α did not increase neutrophil death in vitro (fig. S1). Gene expression analysis of the neutrophils cultured with SLE sera showed significant dysregulation (more than a twofold change) of 638 transcripts (Fig. 1C and fig. S2). Of these, 243 overlapped with transcripts differentially expressed in freshly isolated SLE neutrophils. Overall, 461 of 638 transcripts could in fact be assigned to the predominant networks (that is, type I IFN and TLR signaling) found in SLE neutrophils (Fig. 1C, fig. S2, and tables S5 and S6). Mature neutrophils express all TLRs except TLR3 (21), but the transcript level of some of these receptors is barely detectable using oligonucleotide microarrays. Thus, we performed real-time polymerase chain reaction (PCR) using TLR-specific primers on healthy neutrophils exposed to SLE sera or IFN- α . A consistent increase in TLR7 mRNA levels was found after 6 hours, whereas expression of other TLRs did not change significantly under either culture conditions (Fig. 1D and fig. S3). Accordingly, SLE neutrophils respond to TLR7 agonists in vitro with significant production of IL-8 despite their spontaneous accelerated death rate (Fig. 1E).

Thus, SLE neutrophils display a gene expression profile compatible with in vivo type I IFN- and TLR-induced activation that can be reproduced by SLE serum factors and might contribute to their accelerated death.

SLE neutrophils die by NETosis upon exposure to anti-ribonucleoprotein antibodies

SLE patients characteristically develop antibodies against DNA and RNA/protein complexes, and ICs generated by incubating these antibodies with apoptotic and/or necrotic cells activate pDCs and B cells in vitro (22, 23). Because TLR7 expression is induced by SLE serum, we sought to assess whether RNA-recognizing antibodies could activate neutrophils through the engagement of this receptor. Thus, SLE neutrophils were exposed in vitro to a low concentration of anti-ribonucleoprotein (RNP) immunoglobulin (Ig) (10 µg/ml) purified from SLE serum. Using lipopolysaccharide (LPS) as a positive control, we did not observe neutrophil activation, because IL-8 secretion was not detected in the supernatants and there was no up-regulation of CD66b surface expression (Fig. 1F).

We next explored whether anti-RNP antibodies might induce neutrophil death. First, the proapoptotic effects of ultraviolet light and anti-RNP antibodies on healthy and SLE neutrophils were compared using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays. Whereas both conditions increased the apoptosis rate of neutrophils from healthy controls, anti-RNP antibodies reduced the apoptosis rate of neutrophils from SLE patients (fig. S4). To determine whether a different form of death not leading to DNA fragmentation might be induced by these antibodies, we cultured neutrophils for 3 hours with AS alone or in the presence of SLE-derived anti-RNP IgG (10 µg/ml) or control phorbol 12-myristate 13-acetate (PMA). Using a DNA-specific dye that penetrates apoptotic cells (Sytox Red), untreated healthy neutrophils appeared viable after 3 hours in culture, whereas SLE neutrophils were predominantly apoptotic, as DNA remained contained within the condensed nuclei (Fig. 2, A and B, a to c). Surprisingly, SLE neutrophils, but not healthy neutrophils, consistently died by releasing NETs after 3 hours of culture with anti-RNP IgG (Fig. 2, A and B, d to f). Addition of PMA, a known inducer of NET formation, triggered NETosis in both healthy and SLE neutrophils, as evidenced by the extrusion of extracellular DNA and by fluorescent DNA quantification of the culture supernatants (Fig. 2, A, B, g to i, and C). Overall, about two-thirds of SLE neutrophils tested ($n = 17$; table S3) but none of the healthy controls ($n = 16$) were susceptible to this form of death upon anti-RNP IgG triggering. This process was accompanied not only by the release of large amounts of DNA into the extracellular space (Fig. 2C) but also by the increased secretion of LL37 (Fig. 3A), a cationic antimicrobial peptide that converts self-DNA into a TLR9 activator in human pDCs and self-RNA into TLR7/TLR8 activator in pDCs and mDCs (myeloid DCs) (24, 25). Anti-RNP IgG also induced SLE, but not healthy neutrophils, to secrete HMGB1 (Fig. 3A), a protein that functions as a universal sentinel for nucleic acids and leads to type I IFN and inflammatory cytokine secretion by targeting DNA or RNA to cytosolic nucleic acid-sensing receptors (26). Thus, exposure to anti-RNP IgG drives SLE but not healthy neutrophils to die by releasing large amounts of potentially immunogenic DNA into the extracellular space. Although some of the medications used to treat patients with SLE might alter their neutrophil propensity to undergo cell death, oral steroid therapy did not seem to have an effect on NETosis, as we observed no difference in the NETosis propensity of neutrophils from patients on and off therapy (table S7).

We next investigated whether LL37 and HMGB1 secreted by SLE neutrophils would bind to NET DNA. Neutrophils from healthy children and SLE patients were activated with PMA (25 nM) for 3 hours to induce NETosis, and DNA, LL37, and HMGB1 were visualized by immunofluorescence using DNA counterstaining (Hoechst 33342) and anti-LL37- and HMGB1-specific antibodies. LL37 and HMGB1 colocalize within globular structures along NETs from SLE patients (Fig. 3B). Healthy neutrophils, however, tend to retain both proteins within cytoplasmic remnants (Fig. 3B and fig. S5), which contrasts with elastase, a neutrophil protein that diffusely follows the trajectory of extracellular DNA (fig. S5) (14).

SLE NETs are potent activators of pDCs

Apoptosis is normally a tolerogenic form of cell death, because it does not lead to activation and/or maturation of DCs (27). Indeed, culturing pDCs with apoptotic and/or necrotic neutrophils does not induce pDC activation (fig. S6). To determine whether anti-RNP IgG–induced SLE NETosis might have a different effect, we exposed pDCs isolated from the blood of healthy controls to the supernatants of healthy or SLE neutrophils that had been cultured in the presence or absence of anti-RNP antibodies as described above. As opposed to unstimulated pDCs, pDCs cultured with CpG survived in overnight cultures and expressed activation (CD80 and CD86) and maturation (CD83) markers (Fig. 4, A, a and b, and B, and fig. S7). This was accompanied by secretion of IFN- α , IP-10, tumor necrosis factor- α (TNF- α), and IL-6 (Fig. 4C). pDCs exposed to the low concentration of anti-RNP IgG (10 μ g/ml) used to induce NETs did not up-regulate the expression of activation markers (Fig. 4, A, c, and B) or the production of IFN- α (Fig. 4C). Addition of supernatants from about two-thirds of SLE but not healthy neutrophils exposed to anti-RNP IgG led to pDC activation (Fig. 4, A, d to g, and B), as well as to significantly high IFN- α secretion (Fig. 4C). Meanwhile, supernatants from anti-RNP IgG–treated SLE neutrophils also induced pDCs to produce IP-10, TNF- α , and IL-6 (Fig. 4C). Supernatants from SLE neutrophils that failed to produce NETs upon activation with anti-RNP IgG did not activate pDCs. Furthermore, blocking Fc γ RIIa on pDCs did not abrogate their activation in response to SLE NETs (fig. S8). Thus, we conclude that NET components, and not an IC carryover, are responsible for pDC activation. Together, these findings support that SLE NETs are remarkable pDC activators and IFN inducers.

Anti-RNP antibody–induced SLE NETosis is Fc γ RIIa-, NADPH-, and TLR7-dependent

To investigate the mechanism(s) responsible for the pro-NETotic effect of anti-RNP antibodies on SLE neutrophils, we induced NETosis in the presence or absence of specific inhibitors of Fc γ RIIa (anti-CD32 antibodies), NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase [diphenylene iodonium (DPI)], or TLR7 (IRS-661). Blockade of each of these receptors and/or pathways abrogated the release of NETs as assessed by extracellular DNA quantification and by the lack of pDC activation (Fig. 5A and fig. S9). Thus, Fc γ RIIa internalization and endosomal TLR7 activation are required for SLE neutrophils to release NETs in response to anti-RNP antibodies. Furthermore, this process is reactive oxygen species (ROS)–dependent, because it can be blocked by NADPH oxidase inhibitors. pDC activation by SLE NETs is independent, however, of Fc γ RIIa engagement on pDCs (fig. S8) but dependent on the NET DNA content. Thus, blocking pDC TLR9 abrogates IFN- α production, and treating SLE NETs with deoxyribonuclease (DNase) I blocks pDC activation (Fig. 5B).

IFN- α renders healthy neutrophils susceptible to anti-RNP antibody–induced NETosis

NETosis, an activation-induced form of cell death, can be elicited in vitro by a variety of stimuli, including PMA, cytokines, and live microbes (14, 15). As shown here, anti-RNP IgG elicits NETosis of SLE, but not of healthy neutrophils, without a need for previous activation. SLE neutrophils display a robust type I IFN signature (Fig. 1B and fig. S1), and IFN- α , in combination with other factors, has been shown to induce healthy neutrophil NETosis (15). Thus, we asked whether type I IFN would make healthy neutrophils susceptible to death by NETosis upon ensuing exposure to anti-RNP IgG. As described above, treatment of healthy neutrophils with IFN- α 2 in vitro did not result in increased cell death (fig. S1) but induced the up-regulation of TLR7 transcription (fig. S3B). Addition of anti-RNP IgG to healthy neutrophils after exposure to IFN- α for 6 hours resulted in NETosis. Furthermore, the supernatants from these cultures activated pDCs in a DNA-dependent manner (as shown by the up-regulation of CD80, CD86, and CD83 expression; prolonged survival; and increased production of cytokines) (Fig. 6, A and B, and fig. S10).

DISCUSSION

Neutrophils are very important in shaping both innate and adaptive immune responses (28) and have long been suspected to play a role in SLE pathogenesis. In 1948, Hargraves *et al.* described mature bone marrow neutrophils containing intracytoplasmic nuclear material in 25 SLE patients at the Mayo Clinic (29). This phenomenon, which they called the “LE cell,” helped develop the first diagnostic test for this disease (30). In 1949, Haserick and Bortz found that plasma from 50 to 75% of patients with SLE reproduced the LE cell phenomenon in vitro, with the formation of clumps of neutrophils around amorphous masses of nuclear material (31). Subsequent reports described the LE factor binding to nuclear components, including RNPs and histones. The identification of anti-nuclear antibodies (32) later replaced the LE cell as a diagnostic test, and switched the focus of lupus research from neutrophils to B cells.

Recent genomic approaches have identified SLE blood transcriptional signatures composed of type I IFN-induced (9, 10, 33) and granulopoiesis genes (9) that correlate with disease activity. The granulopoiesis signature could be attributed to the presence in SLE blood of low-density, including immature, neutrophils. Thus, we asked whether these cells might be released from the bone marrow in response to increased mature neutrophil activation and/or death. Here, we show that ex vivo mature lupus neutrophils die promptly and express prominent transcriptional signatures related to IFN and TLR signaling, as well as to cell death pathways. Furthermore, these signatures could be reproduced in vitro by incubating healthy neutrophils with serum from active SLE patients.

Both IFN- α and SLE serum up-regulate neutrophil TLR7 expression. In addition, sera from ~40% of SLE patients contain TLR7 ligands in the form of ICs derived from antibodies recognizing small nuclear RNA/RNA binding protein complexes. These ICs have been shown to activate pDCs and induce type I IFN secretion. Anti-RNP antibodies are not efficient activators of pDCs in vitro, however, unless combined with dying cells (34–36), which provide the substrate to form ICs that might be internalized via Fc γ RIIa (37) and reach the endosomal compartment. Here, we asked whether anti-RNP antibodies might also activate neutrophils in a TLR7-dependent manner. We found that exposure to low concentrations of anti-RNP IgG switches SLE neutrophil death from apoptosis to NETosis, a unique form of death characterized by nuclear disruption and release of extracellular traps highly enriched in DNA, histones, and neutrophil-specific proteins. Anti-RNP Ig-induced SLE NETosis requires Fc γ RIIa and endosomal TLR7 signaling and depends on the formation of ROS. Furthermore, anti-RNP antibodies induce SLE but not healthy neutrophils to secrete high levels of LL37 and HMGB1, two endogenous proteins that contribute to increase the immunogenicity and uptake of mammalian DNA by pDCs (38, 24). Accordingly, we show that anti-RNP-induced SLE NETs activate pDCs and lead to the secretion of high levels of IFN- α .

Healthy neutrophils die by NETosis upon exposure to extracellular pathogens, but they do not undergo this type of cell death in response to anti-RNP antibodies unless previously primed by cytokines such as IFN- α , which has been shown in vitro to act as a priming factor for the induction of NET formation (15). IFN- α up-regulates the transcription of RNA receptors including not only TLR7 but also LGP2, a member of the cytoplasmic DExD/H box-containing RNA helicase family that mediates RNA conformational changes promoting recognition by RIG-I and MDA5 and downstream signaling (39). IFN- α , however, does not up-regulate in vitro the expression of other transcripts found up-regulated in SLE neutrophils that might be related to NETosis. These include (i) NET integral proteins (that is, histone-encoding transcripts), (ii) DNA-modifying proteins (that is, LL37 and HMGB family members), (iii) neutrophil activators (that is, C5), and (iv) components of the nuclear

pore complex (that is, NUP205), among others. Whether other cytokines than IFN- α are involved in predisposing SLE neutrophils to NETosis remains to be elucidated. TNF- α -activated neutrophils have been recently described to undergo NETosis upon incubation with anti-MPO (myeloperoxidase) antibodies from patients with ANCA-related vasculitis, and NET-related material could be visualized in kidney sections from these patients (40). Whether, in addition to anti-RNP, other SLE-specific specificities, including the most prevalent anti-dsDNA (double-stranded DNA) antibodies, induce NETosis of SLE neutrophils remains to be determined.

NETosis may play a pathogenic role in SLE by inducing and/or amplifying IFN production by pDCs. NET-induced activation of pDCs is likely to take place during normal immune responses to infectious agents. The complex genetic background of SLE patients (41), however, could provide multiple amplification steps for the perpetuation and subsequent pathogenicity of neutrophil-pDC interactions. Indeed, polymorphisms in genes expressed by neutrophils, such as ITGAM/CD11b, rank among the highest in the scale of SLE susceptibility. Polymorphisms in genes along the IFN and TLR signaling pathways (that is, IRF5, TLR7, IRAK1, STAT4, etc.) could amplify the response of SLE neutrophils to TLR7 triggering. Polymorphisms affecting thresholds of B cell activation (that is, BLK, BANK1, LYN, etc.) and/or deficient removal of ICs might contribute to prolonged neutrophil exposure to activating ICs. Polymorphisms in Fc γ RIIa could affect the internalization and/or endosomal trafficking of SLE-specific ICs in neutrophils and pDCs. Finally, polymorphisms in genes involved in DNA degradation (that is, TREX1) might alter SLE NET DNA content and the subsequent activation of pDCs.

Whereas the above-described mechanisms apply to mature neutrophils, immature neutrophils do not respond to type I IFN because they fail to phosphorylate STAT1 (signal transducer and activator of transcription 1) in response to receptor binding (15). Thus, immature neutrophils might not be susceptible to anti-RNP-induced NETosis. This would explain the survival and expansion of these cells in the SLE microenvironment, giving rise to the SLE “granulopoiesis signature” that we have previously reported (9). NETs from about two-thirds of pediatric SLE patients tested in our studies are potent inducers of IFN- α production by pDCs. A similar proportion of pediatric SLE patients display a blood granulopoiesis signature and go on to develop nephritis at some point during the course of disease. Indeed, neutrophil-specific proteins measured in the urine of SLE patients have been described to be among the best surrogate markers of kidney disease (20). Finally, impairment of DNase I function and failure to dismantle NETs have been recently reported to correlate with kidney involvement in SLE (42). Longitudinal studies will be necessary to assess whether NETosis can be used as a biomarker or a predictor of tissue damage in SLE patients.

Our results reconcile the presence of two transcriptional signatures in SLE PBMCs—type I IFN and granulopoiesis—that, especially when combined, can be used as surrogate measures of disease activity (9, 43). Overall, they highlight the role of neutrophils as a fundamental piece in the puzzle of lupus pathogenesis. Furthermore, they explain how anti-RNP antibodies might contribute to the production of IFN via induction of a peculiar form of neutrophil death that represents source of highly immunogenic DNA. There, all the necessary ingredients converge to trigger the adaptive immune system through a self-amplifying pathogenic loop. This loop might also be set up in response to exogenous agents, such as bacterial and/or fungal infections, that may trigger and/or exacerbate NET formation and remain the main cause of mortality in SLE patients (44).

MATERIALS AND METHODS

Patient information

This study was approved by the Institutional Review Boards of University of Texas Southwestern Medical Center, Texas Scottish Rite Hospital for Children, and Baylor Health Care Systems in Dallas, Texas. Informed consent was obtained from all patients or their parent/guardian. Blood samples were obtained from patients fulfilling the diagnosis of SLE according to the criteria established by the American College of Rheumatology (45). Disease activity was assessed by the SLEDAI (46) as measured on the day of blood collection. Healthy pediatric controls were children visiting the clinic either for reasons not related to autoimmunity or infectious diseases or for surgery not associated with any inflammatory diseases. The clinical data and demographic characteristics of these patients are summarized in tables S2 and S3.

Human neutrophil isolation

Blood from healthy donors and pediatric SLE patients was collected using ACD tubes (BD Vacutainer). Neutrophils were isolated by Ficoll-Paque and Dextran T-500 gradients (Fluka Biochemika). The preparation contained greater than 98% neutrophils as confirmed by flow cytometry using CD16 (R&D Systems), CD11b (BD Biosciences), and CD66b (AbD Serotec) antibodies. Trypan blue exclusion showed the viability to be greater than 96% for all preparations.

Cell culture and neutrophil stimulation

SLE and healthy neutrophils were cultured in complete RPMI medium supplemented with 2% AS at a density of 5×10^6 cells/ml in 12-well tissue culture plates. Imiquimod-R837 (10 μ g/ml) (Invivogen), anti-RNP IgG (10 μ g/ml), or IFN- α 2 (1000 IU/ml) (Schering Corp.) was added, and the cells were incubated at 37°C for 6 hours. For microarray or reverse transcription-PCR (RT-PCR) experiments, cells were spun and resuspended in RLT buffer (RNeasy mini kit, Qiagen) for subsequent RNA isolation. All the supernatants were used for later experiments and for Luminex analysis to determine cytokine levels.

RT-PCR

RNA samples were converted to complementary DNA (cDNA) using the High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. RNA integrity was assessed using the 2100 Bioanalyzer (Agilent Technologies). Quantitative PCR was performed on selected targets (TLR2, TLR9, and IL-8) using predeveloped primers and probes (TaqMan Gene Expression Assays, Applied Biosystems) on the ABI Prism 7900 Sequence Detection System. Expression results were calculated as described by the manufacturer. The endogenous β_2 -microglobulin control was used for correcting the results with the comparative threshold cycle method for relative quantification.

Purification of anti-RNP antibodies from SLE patients

Serum samples from SLE patients were obtained from Golden West Biologicals, the Binding Site, and SeraCare. All samples were filtered through a 0.45- μ m polyvinylidene fluoride syringe before purification. Anti-RNP titer levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Corgenix). Samples were compared with a positive control provided by the manufacturer. Positive anti-RNP samples had an activity greater than 22 U/ml. IgG from anti-RNP-positive patients was purified using HiTrap Protein G HP column (GE Healthcare). Once purified, IgG was desalted and then quantified.

Induction of NET formation

SLE and healthy neutrophils (10^5) were stained with calcein green AM (Invitrogen), seeded on poly-L-lysine-covered eight-well chamber slides, and allowed to settle. Cells were incubated in complete RPMI supplemented with 2% AS for 2 hours, treated with either PMA (25 nM) or anti-RNP-IC (10 μ g/ml), or left unstimulated. For labeling of DNA, Sytox Red (1 μ M; Molecular Probes) or 4',6-diamidino-2-phenylindole (DAPI) was used. Slides were mounted in Prolong Gold antifade media (Molecular Probes) and examined with Leica SP2 RS SE confocal microscope.

Quantitation of NETosis

Supernatants from cultured neutrophils were digested with micrococcal nuclease (250 mU/ml) (Worthington Biochemical Corp.) to dismantle the NET scaffold. The free DNA in the supernatants was then quantified by incubating the samples with Sytox Orange (2.5 μ M; Molecular Probes) for 10 min at room temperature. The relative fluorescence was then read with a fluorometer with filter setting of 544 (excitation)/590 (emission) (SpectraMax M2; Molecular Devices). A standard curve was built with Lambda DNA Standard (Invitrogen). As a positive control, NETs were induced by incubating the neutrophils with PMA (25 nm) for 12 hours.

HMGB1 and LL37 quantification

Supernatants from cultured neutrophils were collected, and HMGB1 and LL37 levels were determined by ELISA (HMGB1 ELISA Kit II Central Institute, Shino-Test Corp.; Human LL37, Hycult Biotechnology).

Purification and activation of pDCs

Buffy coats were obtained from Carter Blood Care and fractionated over Ficoll gradients. pDCs were isolated from the PBMCs using a negative selection kit (Dynabeads Human DC Enrichment kit, Invitrogen) and sorted by flow cytometry as CD123⁺HLA-DR⁺CD11c⁻CD3⁻CD14⁻CD15⁻CD16⁻CD19⁻CD56⁻ on a FACSaria (BD Biosciences). Purity was routinely >98%. pDCs were cultured at a density of 5×10^4 cells per well in 96-well round-bottom plates in 200 μ l of complete RPMI supplemented with 10% fetal calf serum (FCS). Oligodeoxynucleotide (ODN) 2216 (CpG A) (Invivogen) was used as a positive control for TLR9 activation. Other conditions included anti-RNP-IC (10 μ g/ml), supernatants from either RNP-IC-stimulated healthy or SLE neutrophils (20% final concentration), or IgG control antibodies (10 μ g/ml).

Immunofluorescence

Neutrophils (10^5 cells) were seeded on poly-L-lysinated glass cover slides and treated with media or purified anti-RNP IgG (10 μ g/ml) in complete RPMI supplemented with 2% AS. After 3 hours, cells were fixed with 4% paraformaldehyde and DNA was stained using Hoechst 33342 (Invitrogen). For NET-protein staining, maximum NET release was triggered by treating neutrophils with PMA (25 nM) for 3 hours. Specimens were then fixed with 4% paraformaldehyde, and unspecific binding sites were blocked with 5% goat serum in phosphate-buffered saline (PBS) for 30 min. For LL37, HMGB1, and nigrosin-eosin (NE) staining, cover slides were incubated with mouse anti-human LL37 (clone D-5, Santa Cruz Biotechnology), rabbit anti-human HMGB1 (Cell Signaling), or mouse anti-human NE (clone SPM205, Santa Cruz Biotechnology) followed by the appropriate fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (Fab fragment, all from Santa Cruz Biotechnology). DNA was counterstained with Hoechst 33342. Cover slides were mounted onto glass slides using Prolong Gold mounting media (Invitrogen) before acquisition.

Mechanistic studies to understand RNP-IgG-induced NET formation

SLE neutrophils were pretreated with mouse anti-human CD32 (20 µg/ml, azide-free, clone 3D3, BD Biosciences), the NADPH oxidase inhibitor DPI (10 µM, Calbiochem), or the TLR7 inhibitor IRS-661 (1 µM, gift from F. Barrat) for 10 min before starting the stimulation with anti-RNP/Sm IgG (10 µg/ml) as described previously. Heat-aggregated IgG was prepared by heating (65°C for 30 min) purified human IgG. Large protein precipitates were then removed by centrifugation at 1000g for 2 min.

NET-induced pDC activation

Highly purified pDCs (25,000 cells) were stimulated with 20% of neutrophil-derived cell-free supernatants (NETs) in the presence of TLR9 inhibitor ODN-TTAGGG (Invivogen). After 18 hours of culture, cells were analyzed by fluorescence-activated cell sorting (FACS) (maturation markers), whereas cell-free supernatants were used for IFN quantification (CBA Flex Set from BD Biosciences).

pDC phenotype and cytokine assays

pDCs were stained with fluorochrome-labeled anti-CD80, anti-CD83, and anti-CD86 antibodies (BD Biosciences) and analyzed on a FACS-Canto (BD Biosciences). Supernatants of stimulated pDCs were collected after overnight culture. Cytokine levels (IFN- α , IL-6, TNF- α , and IP-10) were determined by using bead-based cytokine multiplex analysis (Luminex; Bio-Rad).

Microarray analysis

RNA was isolated using the RNeasy kit (Qiagen) and amplified and labeled with Illumina TotalPrep RNA amplification kit (Applied Biosystems). An Agilent 2100 Analyzer (Agilent Technologies) was used to assess RNA integrity. Biotinylated complementary RNA (cRNA) was hybridized to Illumina Human-6 Beadchip Array version 2 and scanned on Illumina Beadstudio 500. Fluorescent hybridization signals were assessed with Beadstudio software (Illumina), and statistical analysis and hierarchical clustering were performed with GeneSpring 7.3.1 software (Agilent Technologies). Functions and interactions of differentially expressed genes were studied using Ingenuity Pathways Analysis (IPA 8.5).

Statistical analyses

The data were expressed as means. Statistical significance of differences was determined by Student's t test and analysis of variance (ANOVA) (adjusted for Dunn's test) ($P < 0.05$ was considered statistically significant).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

SLE neutrophils are prone to death and display prominent signatures of type I IFN, TLR signaling, and cell death. **(A)** Healthy ($n = 11$) and SLE ($n = 11$) neutrophils were cultured with 2% autologous serum (AS). Cell viability was evaluated at different time points using trypan blue. *** $P < 0.0001$, ANOVA (adjusted for Dunn's test). **(B)** Microarray analysis of purified blood neutrophils from healthy children ($n = 12$) and pediatric SLE patients ($n = 21$). One thousand five hundred and sixty-four transcripts were differentially expressed in SLE neutrophils ($P < 0.01$, nonparametric t test). Genes representative of the three most prominent pathways according to Ingenuity Pathways Analysis algorithms are shown. **(C)** Microarray analysis of neutrophils from two healthy donors (HD1 and HD2) incubated for 6 hours with either 20% AS or sera from 10 pediatric SLE patients. Data were normalized to autologous sera cultures. Genes from the three most representative networks according to Ingenuity Pathways Analysis algorithm are depicted. **(D)** Healthy neutrophils were cultured with 20% AS or sera from four pediatric SLE patients (SLES) with active disease (SLEDAI > 4). TLR7, TLR8, and TLR9 expression was analyzed using real-time PCR. Fold up-regulation was calculated as the difference in cycle threshold after 1- and 6-hour in vitro incubation relative to the expression at time 0. **(E)** Neutrophils from healthy children (HN, $n = 6$) and SLE patients (SLEN, $n = 7$) were cultured overnight in the presence or absence of a TLR7 agonist (R837, 10 $\mu\text{g/ml}$). IL-8 production was analyzed by Luminex assay. *** $P < 0.0001$, paired t test. **(F)** SLE neutrophils ($n = 4$) were incubated with media, anti-RNP antibody (10 $\mu\text{g/ml}$), or LPS (25 nM) for 6 hours. Left: IL-8 production was detected by Luminex. Right: cells were stained for CD66b surface expression. Results are displayed as quantification of mean fluorescence intensity (MFI). ** $P = 0.001$; *** $P < 0.0001$, ANOVA (adjusted for Dunn's test).

Fig. 2.

Anti-RNP antibodies induce NETosis of SLE neutrophils. (**A** and **B**) Neutrophils from healthy children (**A**) and SLE patients (**B**) were stained with calcein green. Cells were incubated in 2% AS for 3 hours (**A** and **B**, **a** and **b**) and treated with anti-RNP IgG (10 μ g/ml) (**A** and **B**, **d** to **f**) or with PMA (25 nm) (**A** and **B**, **g** to **i**). Sytox Red was used to stain DNA and detect cell death. Neutrophils from healthy children display intact membranes after 3 hours of culture with medium (**A**, **a** to **c**). Under the same conditions, SLE neutrophils undergo significant apoptosis (**B**, **a** to **c**). Anti-RNP antibodies induce apoptosis of healthy neutrophils (**A**, **d** to **f**) and NETosis of SLE neutrophils (**B**, **d** to **f**). PMA induces the release of extracellular DNA in both healthy (**A**, **g** to **i**) and SLE (**B**, **g** to **i**) neutrophils. These data are representative of at least six independent experiments. (**C**) NET DNA quantification. Supernatants from healthy and SLE neutrophils with and without previous activation with anti-RNP antibodies were digested with micrococcal nuclease to dismantle the NET scaffold. Free DNA in the supernatants was quantified by incubation with Sytox Orange followed by relative fluorescence measurement after building a standard curve using λ DNA. As a positive control, NETs were induced by incubating neutrophils with PMA (25 nm) for 12 hours. * $P = 0.01$, one-way ANOVA with post test Dunn's correction.

Fig. 3. SLE NETs are loaded with LL37 and HMGB1. **(A)** Activation with anti-RNP antibodies leads SLE, but not healthy neutrophils, to secrete high levels of LL37 (left) and HMGB1 (right) as quantified by ELISA on culture supernatants (P values were obtained by paired t test). **(B)** LL37 and HMGB1 colocalize following a globular pattern along SLE NETs. NET release was triggered by treating healthy and SLE neutrophils with PMA (25 nM) for 3 hours. Specimens were then fixed and incubated with antibodies against LL37 and HMGB1 followed by the addition of FITC- or TRITC-conjugated secondary Fab fragments, respectively. DNA was counterstained with Hoechst 33342. Magnification, $\times 40$. This experiment is representative of four independent experiments.

Fig. 4.

SLE NETs induced by anti-RNP antibodies are potent activators of pDCs. **(A)** pDCs from healthy donors were cultured with medium (a), CpG A (10 µg/ml) (b), anti-RNP IgG (10 µg/ml) (c), supernatant (20%) from healthy neutrophils without (d) or with (e) activation with anti-RNP IgG, and supernatant (20%) from SLE neutrophils without (f) or with (g) activation with anti-RNP IgG (10 µg/ml). Top panel depicts the light microscopy images after overnight culture under the above-described conditions. Expression of HLA-DR, CD80, CD86, and CD83 was assessed by flow cytometry in the same cultures (gated on live cells). **(B)** These experiments were repeated using 3 independent healthy pDC donors and 17 SLE sera for induction of CD80 and CD86 expression and 13 sera for induction of CD83 expression. *** $P < 0.0001$, one-way ANOVA with post test Dunn's correction. **(C)** Cytokine measurement in the supernatants of pDCs activated as described in (A). *** $P < 0.0001$, one-way ANOVA with post test Dunn's correction.

Fig. 5.

Mechanism underlying the generation of anti-RNP antibody-induced SLE NETs and their downstream effects on pDCs. **(A)** Neutrophils from SLE patients ($n = 4$) were pretreated for 10 min with Fc γ RIIa blocker (anti-human CD32), the NADPH oxidase inhibitor diphenylene iodonium (DPI) (left and middle panels), or the TLR7 inhibitor IRS-661 (right panel) before incubation with anti-RNP antibodies. Neutrophil supernatants were then added to pDC cultures. IFN- α secretion (left) and expression of the maturation marker CD83 (middle and right panels) were assessed by Luminex and flow cytometry, respectively. HG IgG, heat-aggregated Ig control. * $P = 0.01$; *** $P < 0.0001$, one-way ANOVA with post test Dunn's correction. **(B)** Addition of TLR9 inhibitors (ODN-TTAGGG) or DNase I inhibits

the capacity of SLE NETs to induce pDC activation as measured by IFN- α secretion (left) and CD83 expression (right), respectively; ODN-TTAGGG did not inhibit NET-induced pDC maturation (middle). ** $P = 0.001$, paired t test.

Fig. 6.

Treatment of healthy neutrophils with IFN- α renders them susceptible to NETosis upon subsequent activation with anti-RNP antibodies. pDCs were cultured in the presence of medium, CpG A (10 μ g/ml), anti-RNP IgG (10 μ g/ml), supernatants (20%) from healthy neutrophils (HN/ctrl), healthy neutrophils treated with anti-RNP IgG (HN/RNP), or IFN- α (1000 IU, HN-IFN- α) or from healthy neutrophils treated for 6 hours with IFN- α ; washed; and incubated with anti-RNP IgG (HN-IFN- α /RNP). **(A)** Flow cytometry was used to assess induction of expression of activation markers (CD80, CD86, CD83) after overnight cultures. **(B)** Luminex assay was used to assess secretion of IFN- α , TNF- α , and IL-6 in the same cultures. * P = 0.01; *** P < 0.0001, one-way ANOVA with post test Dunn's correction.