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NETs mitochondrial DNA and its autoantibody in Systemic Lupus Erythematosus and a proof-of-concept trial of metformin

Haiting Wang, Ting Li, Sheng Chen, Yueying Gu, Shuang Ye

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Haiting Wang, MM, Ting Li, MD, Sheng Chen, MD, Yueying Gu, MD, Shuang Ye, MD: Department of Rheumatology, Renji Hospital South Campus, School of Medicine, Shanghai JiaoTong University, Shanghai 200001 China

Address correspondence to Shuang Ye, MD, Department of Rheumatology, Renji Hospital South Campus, School of Medicine, Shanghai JiaoTong University, 145 Shandong C Rd, Shanghai 200001, China. E-mail:ye_shuang2000@163.com

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Abstract

Objective. Neutrophil extracellular traps (NETs) are found to be important in systemic lupus erythematosus (SLE) pathogenesis by activating plasmacytoid dendritic cells (pDCs) and type I interferon (IFN) pathway. NETs composed of self-DNA are considered of nuclear origin, and are a major source for anti-DNA autoantibody (Ab) generation. This study evaluated whether mitochondrial DNA (mtDNA) reside in NETs, and together with anti-mtDNA Abs cause dysregulation of pDC-IFN-α pathway and its clinical implication in SLE.

Methods. Patients with SLE (n=102), rheumatoid arthritis (n=30) and healthy donors (n=40) were enrolled in our in vitro studies. NETs were generated from phorbol 12-myristate 13-acetate (PMA)-stimulated peripheral neutrophils. Immunofluorescent staining determined NETs formation ex vivo and in lupus nephritis (LN) renal biopsy samples. mtDNA levels and type I IFN-inducible gene scores (IFN scores) were measured by quantitative PCR. Anti-mtDNA Abs, anti-double strands (ds)-DNA Abs and IFN-α were detected by enzyme-linked immunosorbent assay. Isolated NETs, mtDNA or dsDNA, combined with anti-mtDNA or dsDNA IgG, and other culture conditions were applied to stimulate purified pDCs. In the proof-of-concept trial, another 113 SLE patients were enrolled. We evaluated the efficacy and safety of metformin on a background of corticosteroids and conventional immunosuppressive agents in patients with mild/moderate lupus. The primary endpoint was to measure the efficacy of metformin on reducing disease flare.

Results. We first identified mtDNA in NETs and anti-mtDNA Abs was elevated in

SLE patients compared with controls and significantly correlated with IFN scores and disease activity index. We next detected the presence of anti-mtDNA Abs was disproportionately associated with lupus nephritis (LN), and correlated better than anti-dsDNA Abs to LN activity index. mtDNA was deposited in NETs in LN renal biopsies. In addition, mtDNA/anti-mtDNA was greater inducers of pDC IFN-α production via TLR9 engagement than dsDNA/anti-dsDNA. We then assessed the effect of an old drug, metformin, on down-regulating NET-mtDNA-pDC-IFNα pathway. Metformin indeed decreased PMA-induced NET formation and CpG-stimulated pDCs IFN-α generation. Ultimately, a proof-of-concept trial that treat mild/moderate SLE patients with metformin add-on resulted in decreased clinical flare-up, prednisone exposure and body weight.

Conclusion. Our data establish a link between mtDNA in NETs, anti-mtDNA Abs, and pDC-IFN-α pathogenesis in SLE, and highlight that specific strategies attempting to down-regulate this pathway, like metformin, may turn out to be new approaches to treat SLE.

Key words: systemic lupus erythematosus (SLE), lupus nephritis (LN), neutrophil extracellular traps (NETs), mitochondrial DNA (mtDNA), plasmacytoid dendritic cells (pDCs), type I interferon (IFN), metformin

The type I interferon (IFN) pathway plays a pivotal role in the pathogenesis of systemic lupus erythematosus (SLE). It is well known that self-DNA and other nuclear antigens, along with their autoantibodies (Abs), are potent stimulators of natural IFN-α producing cells such as plasmacytoid dendritic cells (pDCs)(1). Recently, neutrophil extracellular traps (NETs) were found to be important in SLE, as they promote pDCs differentiation and activation (2,3). When NETs components, including self-DNA and antimicrobial peptides, engage innate immunity pattern recognition receptors, such as Toll-like receptor 9 (TLR9), they mediate pDC-IFN-α pathway activation (4,5). NETs-DNA is thought to be of nuclear genomic origin. Our first question was to determine whether NETs from SLE patients contained mitochondrial DNA (mtDNA). Indeed, it was shown that mtDNA reside in NETs from healthy donors (6). However, whether there is increased mtDNA residing in NETs in lupus and the possible mechanistic relevance to the pDC-IFN-α pathway requires further investigation.

Human mtDNA is a 16,569-nucleotide pair, closed circular molecule present in the mitochondrial inner membrane where reactive oxygen species (ROS) are produced. Compared with nuclear DNA, protected by histones, mtDNA is more vulnerable to injury. mtDNA evolved from bacterial genomes and contains unmethylated cytosine-phosphate-guanine (CpG) DNA repeats (7), which are highly potent TLR9 ligands (8,9). In addition, circulating mtDNA induces systemic inflammatory syndrome that triggers profound tissue injury (10). Thus, the hypothesis that NETs-mtDNA contributes to pDC-IFN-α pathway activation in SLE is plausible.

Furthermore, since an immune complex is likely required to facilitate the DNA component accessing intracellular TLR-9, we investigated whether anti-mtDNA Abs are present in SLE. This study investigated the clinical relevance of anti-mtDNA Abs to the lupus phenotype and its possible synergistic effect with NETs-mtDNA.

Given the mechanistic relevance of NETs-mtDNA/Abs and pDC-IFN-α pathway in SLE holds true, the next important question is to ask what would be our approach to down-regulate this process? Metformin is the mainstay treatment for type 2 diabetes mellitus that has diverse mechanism of actions. Its anti-oxidative properties (11) and anti-inflammatory effects (12), as well as excellent safety profile, make metformin a convenient candidate in this regard. The formation of NET is ROS-dependent (13, 14), while metformin can selectivity inhibit mitochondrial respiratory chain complex I and decrease the NADPH oxidase activity, thus leading to a decrease in ROS production (11). After in vitro experiments to observe the effect of metformin on down-regulating NET-mtDNA-pDC-IFN-α pathway, we take one step forward to design a randomized, proof-of-concept trial to evaluate the efficacy and safety of metformin on a background of corticosteroids and conventional immunosuppressive agents in patients with mild/moderate lupus.

MATERIALS AND METHODS

Clinical Study design

Patients

We enrolled age and sex matched SLE patients (n=102) , rheumatoid arthritis patients (n=30) and healthy donors (n=40) in our in vitro studies. Another 113 SLE

patients were enrolled in the clinical trial. All SLE patients were at least 18 years old and recruited from the Department of Rheumatology, Shanghai Renji Hospital, fulfilled the American College of Rheumatology (ACR) classification criteria for SLE (15). All patients' clinical data were recorded including the assessment of SLE Disease Activity Index (SLEDAI) (16). Other inclusion criteria in clinical trial were a mild/moderate disease activity with no British Isles Lupus Assessment Group (BILAG) A or no more than two B (17) and a stable treatment regimen with fixed doses of prednisone (0-30 mg/day), antimalarial, or immunosuppressive drugs for at least 30 days (cyclophosphamide for at least 6 months as an exception). The main exclusion criteria in clinical trial were fulfill the diagnosis of diabetes (18); previous treatment with any biological agents within 6 months; previous exposure of metformin within 30 days before screening or previous history of intolerant to metformin; pregnancy; current or recent infection. Of the 102 SLE patients in *in vitro* experiment, 76 had LN, and renal biopsies were obtained in 38 patients. Subtypes of LN and active/chronic index were documented according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification criteria (19).The research protocol was approved by the institutional review board of Renji Hospital. All clinical investigations were performed according to the Declaration of Helsinki.

Clinical trial design

Eligible patients for clinical trial were randomized in a 1:1 ratio to metformin add-on or just conventional treatment by using random number table method. Subjects

randomized to metformin will receive a target dose of 500 mg three times daily. Investigators were allowed to adjust the dose according to the patients' tolerance. At 12 months, the average dose of metformin in the experimental group is 1078mg (500mg to 1500mg). The regimen for conventional treatment was based on the patient's disease manifestations and was in accordance with standard of care. Investigators tapered the prednisone dose on the basis of their clinical judgment. The modified SELENA-SLEDAI Flare Index (SFI) (20, 21) and body weight were assessed every 4 weeks with prescribed laboratory tests. Adverse events were recorded at every study visit.

Endpoints

The primary endpoint was to evaluate the effect of metformin on reduction in disease flare for patients with mild or moderate SLE with a common background of corticosteroids and conventional immunosuppressive agents. The major secondary endpoints were the influence of metformin on corticosteroid sparing effect and the influence of metformin on patients' body mass index (BMI).

This trial is registered with Chinese Clinical Trial Registry (ChiCTR-TRC-12002419).

Neutrophil isolation and stimulation

Five ml of whole-blood was obtained from SLE patients and HDs using ACD tubes (BD Vacutainer). Neutrophils were isolated immediately by a one-step gradient centrifugation method using polymorphoprep (Axis-Shield). The mononuclear cell layer was aspirated for total RNA extraction. The neutrophil layer was isolated, and

resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS) at a density of 5×10^6 cells/ml (2). The preparation contained greater than 98% neutrophils as confirmed by flow cytometry (FACS Calibur) using anti-CD15 antibody staining (BD Bioscience). Trypan blue exclusion showed the viability was greater than 96% for all preparations. Neutrophils were cultured in 24-well/96-well tissue culture plates. Neutrophils were incubated with inhibitors 30 min before stimulation as indicated. The NADPH oxidase inhibitor DPI (Calbiochem) was used at 5-10 μM and metformin (Sigma) at 1-1000 μM. Phorbol 12-myristate 13-acetate (PMA, Sigma) (10ng/ml) was added to form NETs, and the cells were incubated at 37 °C. After 3 hours of stimulation, cells were spun down and cell-free supernatant was collected. Supernatants were stored at −20 °C until use.

Quantification of NETs

NETs generated by activated neutrophils were digested with 500 mU/ml micrococcal nuclease (MNase; Worthington Biochemical Corp.). The nuclease activity was stopped with 5 mM EDTA and the culture supernatants were collected and stored at 4°C until further use. Picogreen (Invitrogen), a fluorescent DNA dye, was added, and the DNA content was quantified by fluorescence spectrometry. The relative fluorescence was then read with a fluorometer with filter setting of 480 (excitation)/520(emission). A standard curve was built with Lambda DNA Standard (2, 14).

NETs Triple/Double Immunofluorescence Staining

SLE and HD neutrophils were seeded on 0.001% poly-L-lysine covered slides,

and stimulated with PMA (20ng/ml) as described above. Cell membranes were stained with calcein green AM (Invitrogen). For labeling of DNA, 4′,6-diamidino-2-phenylindole (DAPI) was used. mtDNA was stained using MitoSox Red (5 μM, Invitrogen). Slides were mounted in Prolong Gold antifade media (Molecular Probes) and examined with a Leica or Carl Zeiss confocal microscope. Frozen sections from renal biopsy specimens were also stained accordingly.

DNA/RNA Isolation and quantitative PCR (qPCR)

DNA from supernatants of PMA-stimulated neutrophils was purified using QIAamp® DNA Mini Kit (Qiagen). Nuclear DNA is undetectable in the supernatant, as indicated by the Ct values (>30) of two genomic house-keeping genes (RPL13A and GAPDH) (Supplementary Figure 1). A qPCR assay for measuring mtDNA copy numbers was then set up. A specific human mitochondrial cytochrome oxidase b (Cyto B) sequence was cloned and constructed into a PGM-T plasmid in order to generate a standard curve (Supplementary Figure 2). Total RNA extracted from peripheral blood mononuclear cells (PBMC) using Trizol Reagent (Invitrogen) was reverse transcribed into cDNA with the Superscript II Reverse Transcriptase kit (Takara, Shiga, Japan). The concentration of mtDNA and the transcriptional levels of type I IFN-inducible genes (IFN-induced protein with tetratricopeptide repeats [IFIT]1, IFIT3, myxovirus resistance 1 [Mx1], oligoadenylatesynthetase [OAS]1, and lymphocyte antigen 6 complex, locus E [Ly6e]) were measured by SYBR Green qPCR using Premix Ex TaqTM (Takara) via the ABI PRISM 7900 system (22). Primer sequences are shown in Supplementary Table 1.

Anti-mtDNA Abs enzyme-linked immunosorbent assay (ELISA) and IgG purification

Mitochondria were isolated from HD peripheral blood leukocytes using Mitochondria Isolation Kit (Thermo). mtDNA was extracted from isolated mitochondria using DNeasy Blood & Tissue kit (Qiagen). DNA Coating Solution (Pierce) was used to immobilize mtDNA(4 μg/ml) on an ELISA plate. The coating plate was incubated with 1/100 dilution of serum for 2 h at 37 °C. The secondary antibody, anti-human IgG coupled with horseradish peroxidase, was added and incubated for 1 h. Substrate solution was added thereafter. A microplate reader determined the absorbance at 450 nm within 5 minutes after the addition of the Stop Solution.

Anti-dsDNA Abs titers in serum were measured in parallel using an ELISA kit (EUROIMMUN). Anti-mtDNA Abs predominant samples were arbitrarily defined as having anti-mtDNA titers greater than 1.5 optical density (OD) value and anti-dsDNA titers less than 0.4 OD value, while anti-dsDNA Abs predominant samples had anti-dsDNA titers greater than 1.2 OD value and anti-mtDNA titers less than 1 OD value. IgG from these samples or from HD sera were purified using a HiTrap protein G HP column (GE Healthcare). Once purified, IgG was quantified using BCA Protein Assay Kit (Pierce) and then stored at −80 °C (2).

Purification and activation of pDCs

HD Blood leukocytes (buffy coat) were obtained from Shanghai Veteran Blood Station and fractionated over Ficoll gradients. pDCs were isolated from PBMCs using

a negative selection kit (Diamond Plasmacytoid Dendritic Cell Isolation Kit, Miltenyi Biotec). The preparation contained greater than 98% pDCs as confirmed by flow cytometry using anti-CD123 and CD304 antibody (Miltenyi Biotec) staining. pDCs were cultured at a density of 5×104 cells per well in 96-well plates in 200 μl of complete RPMI supplemented with 10% FCS and stimulated overnight before IFN-α was measured. The TLR9 agonist CpG-2216 (1 μg/ml) and TLR7 agonist R837 (10 μg/ml) were added (both from InvivoGen). Metformin (1-100 μM) was added 30 min before stimulating with CpG-2216 as indicated. Other conditions included mtDNA, dsDNA (human peripheral blood leukocytes genome DNA), anti-mtDNA-IgG (10 μg/ml), anti-dsDNA-IgG (10 μg/ml), control IgG (10 μg/ml), supernatants from PMA-stimulated SLE neutrophils with or without DNase I (Sigma) (6 U/ml). IFN-α was measured by an ELISA kit (PBL Biomedical Laboratories).

Statistical analysis

An intent-to-treat approach was used for the analysis of primary and secondary outcomes in the clinical trial. On the basis of previous reports and the clinical opinion of the principal investigator, it was estimated that 50 percent of the patients in conventional treatment group would have an increase in objective manifestations of disease within 12 months (23). A 50 percent reduction in the rate of clinical flare-ups was considered of clinically significance. For an alpha error of 0.05(two-side) and a beta error of 0.20, the estimated sample needed was 57 subjects per group.

Two-tailed Student's t-test test, chi-square analysis and Spearman correlation were performed where indicated. For flare-free survival data, the Mantel-Cox test was

conducted. For these tests, $P \leq 0.05$ was deemed significant.

RESULTS

The presence of mtDNA in NETs and anti-mtDNA Abs in sera are greatly enhanced in active SLE patients and correlate with disease activity. Neutrophils isolated from the blood of SLE patients and HDs were stimulated with PMA to induce NETs formation. Combined two-color DNA (Sytox Red) and cell membrane (calcine green) staining suggested SLE neutrophils underwent more extensive NETosis (Figure 1A), as evidenced by significant web-like structure formation and higher quantity of NETs measured by DNA fluorescence, all in line with previous reports (3, 24). Then qPCR was used to measure whether NETs DNA release contained mtDNA sequences. Significantly increased mtDNA copies were observed in the supernatants of PMA-stimulated neutrophils from SLE patients (n=63) versus HDs (n=24) (Figure 1B). It is noteworthy that the circulating mtDNA in plasma was significantly higher in lupus patients, about one order of magnitude, than in HDs (Figure 1C), albeit there is no statistically significant correlation between NETs-mtDNA and plasma mtDNA ($p=0.0799$; $r=0.2018$). More importantly, NETosis released mtDNA levels were positively correlated with SLE disease activity, as determined by SLEDAI and IFN scores (Figure 1D).

Since abundant NETs comprise a major source of autoantigens in SLE, we measured anti-mtDNA Abs in the sera of SLE patients (n=102) and controls. Forty-one percent of SLE patients were positive for anti-mtDNA Abs (determined by mean plus two times SD from 40 HDs with a cutoff OD value = 1.311). In

comparison, none of the RA (n=30) controls displayed a positive serology response to mtDNA (Figure 2A). Additionally, the titers of anti-mtDNA Abs were also associated with SLE patient PBMC IFN scores and SLEDAI (Figure 2B). Interestingly, the levels of anti-mtDNA Abs significantly correlated with classic anti-dsDNA Abs titers measured by ELISA (Figure 2B). Not surprisingly, anti-mtDNA Abs was associated with LN phenotype similar to anti-dsDNA Abs (Supplementary Table 2). To further discriminate the function of anti-mtDNA and anti-dsDNA Abs, we obtained the samples according to arbitrarily defined different specificities (Figure 2B, boxed area) and purified their IgG for further *in vitro* study.

NETs mtDNA and anti-mtDNA Abs are associated with active lupus nephritis. As the major phenotype of SLE, the impairment of NETs degradation was implicated in LN patients (24). We analyzed renal biopsy samples from LN patients by *in situ* immunofluorescent staining, to determine the presence of NETs-mtDNA in the renal biopsy tissue. We detected NETs by staining with a DNA dye (DAPI), a specific mtDNA dye (MitoSOX Red) and an anti-myeloperoxidase (MPO) antibody. NETs components, especially mtDNA and MPO, were colocalized in close proximity to neutrophil infiltrates in affected glomeruli (Figure 2C) compared with normal kidney tissues which were obtained from a nephrectomy sample distant to tumor tissues (Figure 2C). In addition, the presence of anti-mtDNA Abs was disproportionately associated with active disease rather than chronic lesions among proliferative LN (class III or IV with/without V) (Figure 2D).Moreover, anti-mtDNA Abs was more frequently present in the serum of patients with significant manifestation of

proteinuria (Figure 2E). Although anti-dsDNA Abs had a similar correlation, anti-mtDNA Abs had a better performance in terms of correlation with LN activity index and the quantity of proteinuria (Figure 2F and Supplementary Figure 3). These results indicate that mtDNA in NETs and anti-mtDNA autoantibodies are probably involved in LN.

mtDNA and anti-mtDNA Abs are stronger inducers of pDC-IFN-α production than dsDNA and anti-dsDNA Abs. NETs components, such as antimicrobial peptide (LL37, HNPs) coated DNA, are potent pDC-IFN-α inducers (3, 25). We evaluated the specific role of mtDNA and anti-mtDNA in this process. First, free human mtDNA(40 μg/ml) or dsDNA (40 μg/ml) alone was unable to activate pDCs. However, pDCs were activated when human mtDNA or dsDNA was complexed with their antibody IgG (10 μg/ml) purified from SLE serum. These data are consistent with previous reports (4, 5) that demonstrated the Fc portion of IgG from SLE patients serum interacts with CD32 (FcγRII) on pDCs and delivers DNA to intracellular pools of TLR9. It is noteworthy that mtDNA complexed with SLE-derived anti-mtDNA IgG (10 μg/ml) promotes pDCs to secrete more IFN-α than dsDNA/anti-dsDNA complexes (Figure 3A). pDC-IFN-α production induced by DNA/anti-DNA complexes occurs in a DNA dose-dependent manner, and mtDNA/anti-mtDNA are more potent IFN-α inducers compared with dsDNA/anti-dsDNA (Figure 3B). We then used NETs, i.e., cell-free supernatants of PMA-activated neutrophils, isolated from SLE patients to stimulate pDCs. These supernatants induced substantial levels of IFN-α in pDCs (Figure 3C) (3). As

predicted, IFN-α production was greatly enhanced by the addition of anti-DNA IgG purified from SLE patients serum, but not by IgG from HDs. IFN-α levels were doubled when induced by NETs with anti-mtDNA IgG versus anti-dsDNA IgG (Figure 3C). We then depleted DNA (mtDNA or nuclear DNA) using DNase I (6 U/ml), which abrogated over 90% of NETs/anti-mtDNA-stimulated pDCs IFN-α producing activity. DNase I eliminated the IFN-α inducing effect of CpG-2216, a synthetic TLR9 agonist, as opposed to the lack of effect on TLR7-induced IFN-α production by a TLR7 agonist (R837) (Figure 3D). This further demonstrates that the DNA component of NET is required for pDC-IFN-α activation in a TLR-9 dependent manner, and that mtDNA is more efficient than dsDNA in the immune complex that promotes this process.

Metformin down-regulates NETs/mtDNA-pDC-IFN-α pathway. We examined the effect of metformin on PMA-induced NET production in purified peripheral blood neutrophils from heathy donors. Pretreatment of neutrophils with metformin before addition of PMA inhibited NET formation in a concentration-dependent manner (Figure 4A). The viability of neutrophils is not changed by metformin and there is no apoptosis as stained with FITC-conjugated Annexin V and propidium iodide (PI) (Supplementary Figure 4). NETosis is dependent on ROS production and the NADPH oxidase inhibitor diphenyleneiodonium (DPI) prevented NET formation upon activation with PMA, which was used as a positive control. NET-DNA release by metformin pretreated neutrophils showed a significant decrease after 40 min of PMA activation (Figure 4B). Metformin decreased mtDNA copies in NETs by 57% at 40

minutes after PMA stimulation as compared with neutrophils treated with PMA alone(Figure 4C). In addition, metformin can inhibits CpG or mtDNA/anti-mtDNA Abs stimulated IFN-α generation by pDCs and the effect is in a dose dependent manner (Figure 4D-E); Within the pharmacologic meaningful range (5~50μM), the IFN-α production is reduced by 50~80%. Therefore, we carried on a proof-of-concept clinical trial to evaluate the efficacy and safety of metformin add-on to a background of corticosteroids and conventional immunosuppressive agents in mild/moderate lupus patients.

Metformin can reduce clinical flare, prednisone exposure and body weight in SLE patients. From Sept 1, 2012, to Jul 1, 2014, 113 patients with systemic lupus erythematosus were randomly assigned to add-on metformin (n=56) or just conventional treatment (n=57). Supplementary table 3 shows the trial profile. The two groups did not differ in any of the main baseline characteristics including the exposure of immunosuppressive agents (table 1). The rate of disease flares was reduced during 12 months with metformin group, when assessed with SLEDAI and SFI (figure 5A). The relative risk of SFI in patients receiving metformin was 0.49 (95% CI: 0.26, 0.96; P=0.04), in other words, add-on metformin reduced the risk of disease flares by 51% over conventional treatment. In the meantime, the prednisone exposure in the metformin add-on group is lower than conventional treatment group. The proportions of patients with at least a 50% reduction in prednisone dose were significantly greater with metformin at every visit from weeks 16 to 24 and week 32 (figure 5B).The tapering of prednisone from the baseline was significantly greater in

the metformin add-on group than in the conventional treatment group at week 24, 32 and 48 (figure 5C), however the overall exposure of prednisone (area under the curve) is lower, the statistical significance cannot be appreciated .

The mean change in body weight index (BMI) between metformin add-on group and conventional treatment group reached statistical significance from the 8th week to the completion of the study (figure 5D). There is an increase of body weight of conventional treatment group during the first 24 weeks and go plateau thereafter, while the BMI of metformin add-on group continued to decline.

In the metformin add-on group, three patients discontinued the study because of side effects. Two patients complained of nausea and diarrhea and one experienced slightly alanine transaminase elevated (less than two times the upper limit of normal) during the dose escalation. After stopping metformin, these symptoms disappeared. Metformin administration caused minimal side effects in five of 56 subjects finishing the follow up. These patients complained of mild GI discomfort, which resolved with reduction of the dose. No hypoglycemia, lacticacidosis and other serious adverse reaction were reported.

DISCUSSION

In the current study, we are trying to draw lines to connect several established or unestablished dots, i.e.,NETosis, mtDNA along with its autoantibody, and pDC-IFN-α pathway, for a better understanding of part of the pathogenesis of SLE.

NETosis is a neutrophil-specific form of cell death. Unlike apoptosis or necrosis, NETosis is characterized by the formation of NETs. Composed of DNA, histones and

antimicrobial peptides, NETs amplify pDC-IFN-α pathway activation in SLE, and act as a major source of lupus auto antigens(2,3). A recent study constructed an in vivo model［Cl-amidine treated New Zealand mixed 2328 (NZM) mice］, to test this widely accepted concept. NZM mice is a model of lupus relevant to type I IFNs and replicates a number of the features of aberrant of NET formation in human lupus. Treatment of NZM mice with a chemical inhibitor of NET improved the lupus phenotype (26). These previous observations highlight the role of NETosis in the pathogenesis of SLE.

Mitochondria are endosymbionts that originated from purple bacteria approximately 1.5×10^9 years ago and are the only organelle of the mammalian cell that contains its own DNA except for the nucleus. It is hardly a surprise that mtDNA is present in NETs. Indeed, mtDNA, but not nuclear DNA, first joins NETs formation when neutrophils are still viable (6). It is possible that mitochondria and mtDNA are actively involved in the process of NETosis. As an example, pharmacological or genetic approaches to block ROS production suggested that NET formation is ROS dependent (6), while mitochondria are both the source and target of ROS (27). The precise mechanism of mitochondria and mtDNA involvement in NETosis is still undetermined. Nonetheless, our data, for the first time, provide direct evidence that mtDNA release by netting neutrophils is greatly enhanced in SLE patients. mtDNA contains a higher frequency of unmethylated CpG dinucleotides, similar to bacterial DNA, which can promote innate immune responses through TLR9 (8, 9). Our data further confirmed that NETs-mtDNA, in the presence of its autoantibodies, are

two-fold more potent compared with nuclear (ds) DNA in terms of inducing pDCs IFN-α production. One of the technical limitations of this study is that the possibility of cross-reaction and difference in affinity between the anti-mtDNA and anti-dsDNA antibodies. To develop monoclonal antibody against mtDNA will be our next step to solve this problem.

Anti-mtDNA Abs was detected in over 40% SLE patients in this study. Both NETs mtDNA and serum anti-mtDNA Abs correlated with disease activity and the presence of a type I IFN response. Anti-mtDNA Abs, but not NETs-mtDNA or circulating mtDNA (data not shown), were associated with active LN. Our data suggested that anti-mtDNA Abs might have biomarker value, along with the classic anti-dsDNA Abs, as a panel of anti-DNA Abs in SLE, which could provide useful information both relevant to renal involvement and IFN-signature responses. To validate the performance of anti-mtDNA Abs, large-scale clinical studies are warranted. Our *in vitro* study was consistent with a previous report that NETs DNA-containing immune complexes (with anti-DNA Abs) stimulated pDCs to produce type I IFN via a FcγR and TLR9 dependent pathway (5). Our in situ data, i.e., co-localization of mtDNA in NETs structures in LN samples, in line with previous studies in LN (24, 28) indicated that NETs/mtDNA could be actively involved in immune-mediated renal injury.

Various studies bas highlighted the role of metformin in autoimmune inflammatory disease (29-32). In our in vitro study, we showed that metformin decreased NET-DNA release and NET-mtDNA copies in cultured neutrophils.

Moreover, CpG-stimulated pDCs IFN-α generation were inhibited by 50~80% by metformin within the pharmacologic meaningful range (5~50μM). Therefore, our hypothesis is that metformin may serve as an adjunct treatment to reduce SLE disease activity.

In our clinical trial, the frequency of SFI flares of patients in metformin add-on group was 51% less compared to those patients in conventional treatment group. Metformin displayed steroid-sparing effects, although no statistical difference was observed in the overall exposure of prednisone between the two groups. Without any directed caloric restriction or increase in habitual exercise, metformin also effectively reduced body weight in SLE patients which has positive impact both on patients' well-being and cardiovascular risk. Whether metformin could reduce the cardiovascular events in SLE patients deserves further long term investigation. One of the major limitations of the current study is the open-label, non-blinded design without a placebo arm, which introduces certain bias; nevertheless, metformin can reduce SLE flares without compensating by more prednisone exposure, self-sufficiently suggested its therapeutic effect. Another limitation is that whether metformin could down-regulate NETosis, mtDNA release, and IFN-α signature *in vivo* was not addressed in the proof-of-concept trial. In fact, the effect of metformin is probably multi-facet and other types of immune cells maybe affected. As an example, Yin, et al., recently reported that metformin could restore lupus CD4+ T cell function in vitro and reverse lupus phenotype in animal models through tuning T cell metabolism (33).

Taken together, we believe that specific strategies interrupting the NETs-mtDNA orchestrated type I IFN pathway might be a novel approach to treat SLE and metformin is a promising choice that deserve further multicenter, randomized placebo controlled trial.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising itcritically for important intellectual content, and all authors approvedthe final version to be published. Dr. Ye had full access to all of thedata in the study and takes responsibility for the integrity of the dataand the accuracy of the data analysis.

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Figure 2. NETs mtDNA and anti-mtDNA Abs are associated with active lupus nephritis. (A) Autoantibodies against mtDNA in sera from patients with SLE, RA, or HDs. (B) The titers of anti-mtDNA Abs correlated with the SLEDAI,IFN scores and anti-dsDNA Abs titers in SLE patients (n=50). (C) In situ immunofluorescence analysis of frozen sections of renal biopsies from individuals with lupus nephritis and normal kidney tissues. Colocalization of DNA (blue), mtDNA (red) and neutrophil granule markers, MPO (green) indicates intra-glomerular NET formation. Increased magnification of boxed area shows NET deposition inside glomerular capsule. Scale bars, 50 μm. (D) Patients with active renal damage [A] had a higher percentage of anti-mtDNA Abs than patients with chronic renal damage [C] among proliferative LN (class III or IV with/without V). A/C group was defined as mixed active and chronic lesions. (E) Percentages of LN patients with significant anti-mtDNA antibody titers (cutoff value OD index, 1.311) among LN patients with proteinuria great than 0.5 g/d and patients with proteinuria less than 0.5 g/d by chi-square test. (F) Correlation between LN activity index and anti-mtDNA or anti-dsDNA. Each symbol represents an independent patient, and horizontal bars represent the mean.*p<0.05, ***p<0.001, ns=not significant.

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Figure 4. Metformin down-regulates NETs/mtDNA-pDC-IFNα pathway. (A) Neutrophils were pre-incubated with various concentrations of metformin for 0.5 h and then stimulated with PMA (10 μg/ml). Three hours after the stimulation, the supernatants were collected and NET-DNA was quantified. (B) Neutrophils were activated with PMA in the presence of 100 μM metformin (white bars), 10 μM of the NADPH oxidase inhibitor (DPI, gray bars) or in the absence of these

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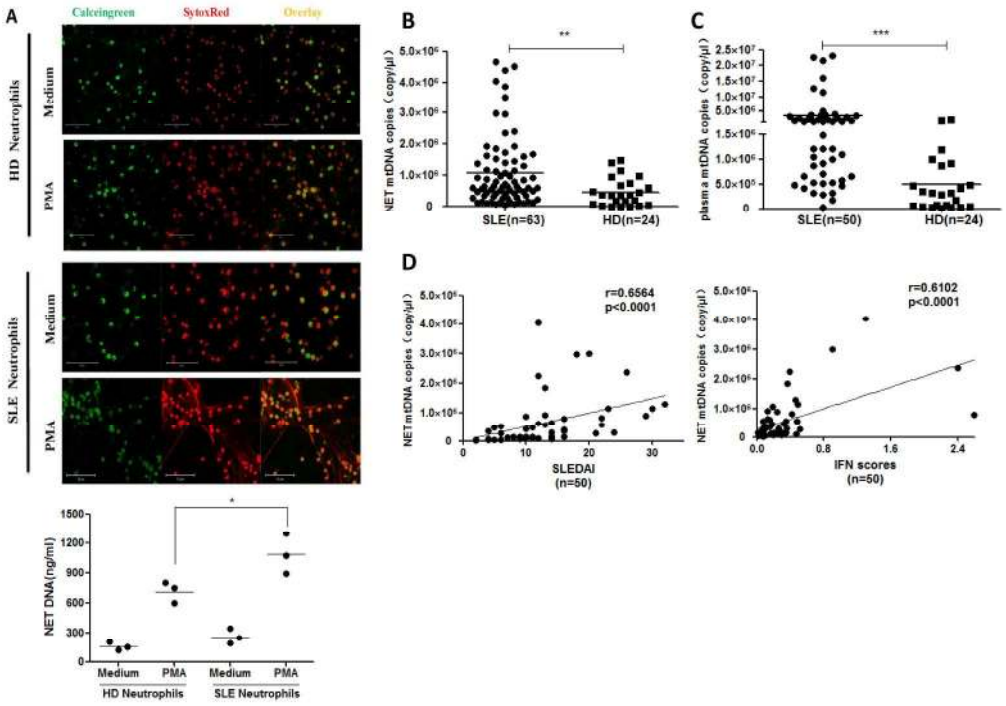

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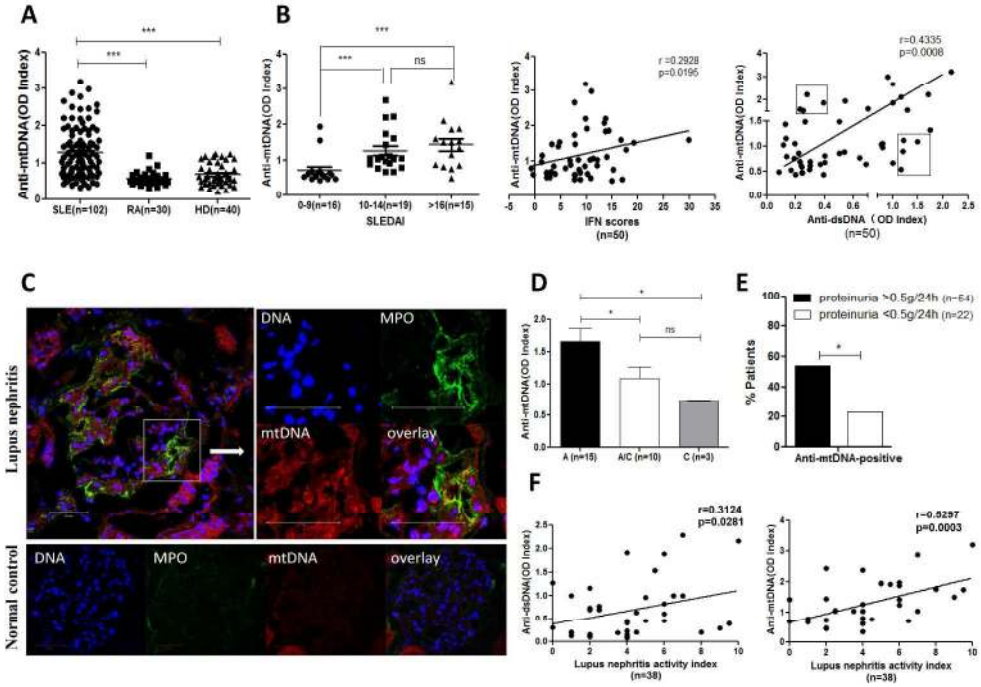

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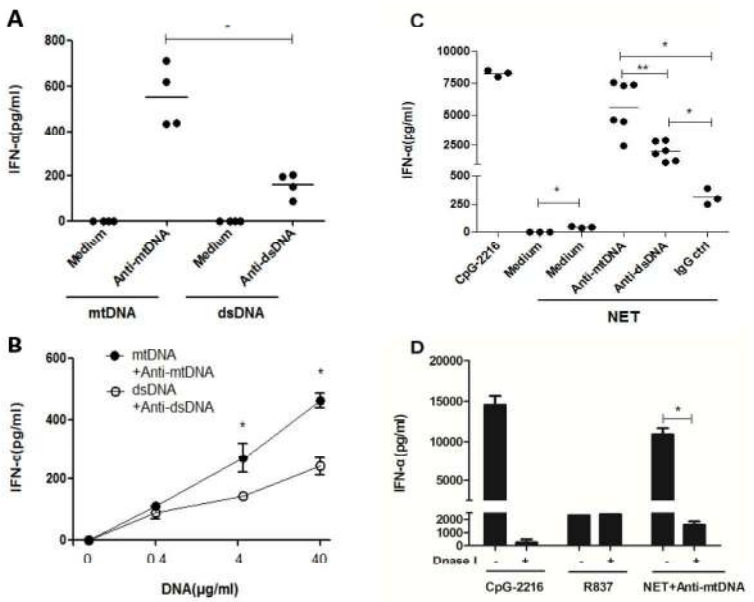

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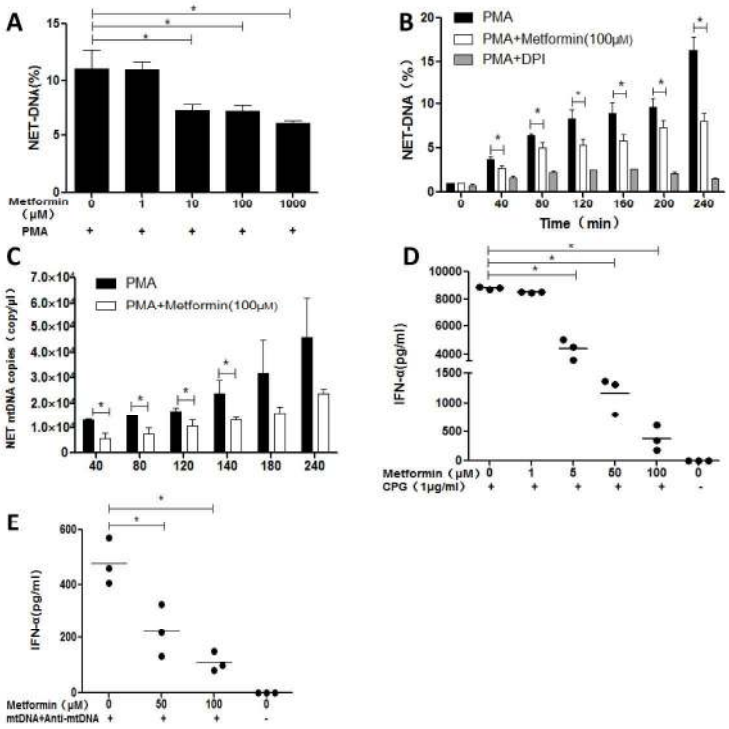

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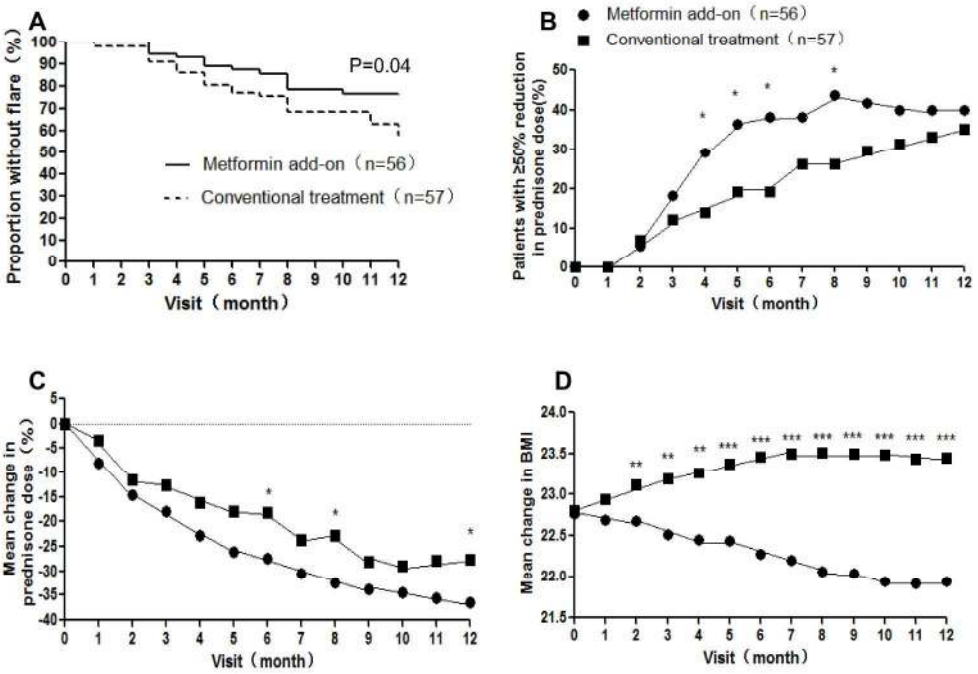

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	Metformin (n=56)	Conventional treatment (n=57)
Age (years)	30.7(13.2)	32.7((14.0)
Women	51 (94%)	53 (95%)
Disease duration (years)	3.5(3.8)	4.1(4.8)
SLEDAI	3.0(3.7)	3.4(3.5)
SLEDAI score≥10	5(9%)	5(9%)
Baseline SELENA-SLEDAI organ involvement		
CNS	0(0%)	0(0%)
Serosal	0(0%)	0(0%)
Haematological	6(11%)	3(5%)
Musculoskeletal	1(2%)	2(4%)
Dermal	5(9%)	3(5%)
Renal	7(13%)	8(14%)
Proteinuria≥1.5 (g/24h)	4(7%)	5(9%)
Prednisone		
Dose(mg/day)	13.3(8.8)	12.7(7.3)
＞7.5mg/day at baseline	41(73%)	45(79%)
Immunosuppressive drug		
Mycophenolate	13(23%)	15(27%)
Azathioprine	7(13%)	7(13%)
Methotrexate	12(21%)	6(11%)
Leflunomide	1(2%)	0(0%)
Ciclosporin	0(0%)	5(9%)
Thalidomide	4(7%)	3(5%)
Cyclophosphamide	3(6%)	4(7%)
Antimalarial drug	**52(92%)**	**51(89%)**
Biomarkers		
Anti-dsDNA(IU/ml)	30.3(32.6)	37.9(37.89)
C3 concentration (g/L)	0.84(0.25)	0.76(0.25)
C3 concentration less than 0.9g/L	32(59%)	35(64%)
C4 concentration(g/L)	0.17(0.08)	0.15(0.08)
C4 concentration less than 0.1g/L	13(24%)	18(32%)
IgG (g/L)	14.2(3.5)	14.0(6.1)
IgA (g/L)	2.7(1.5)	2.4(1.0)
IgM (g/L)	1.3(0.8)	1.1(0.8)

Table 1: Baseline clinical characteristics of patients

Data are number (%) or mean (SD).SLEDAI=Systemic Lupus Erythematosus Disease Activity Index.C3=complement C3. C4=complement C4.Ig=immunoglobulin. Anti-dsDNA was measured using radioimmunoassay (Farr assay) with a detectable range of 7-100 IU/ml. Patients who were positive at baseline with an anti-dsDNA assay greater than 7 IU/ml。