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Original Research

The Role of Plasma Cell-Free Mitochondrial DNA and Nuclear DNA in Systemic Lupus Erythematosus

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Abstract

Background: The roles of plasma cell-free (pcf) mitochondrial DNA (mtDNA^{pcf}) and nuclear DNA (nDNA^{pcf}) in the pathogenesis of systemic lupus erythematosus (SLE) remain unclear. We analyzed the relative copies of mtDNA^{pcf} and nDNA^{pcf} and investigated their association with the levels of plasma 8-hydroxy-2'-deoxyguanosine (8-OHdG), plasma malondialdehyde (MDA) and mRNA of leukocyte C-type lectin domain family 5 member A (CLEC5A) in SLE patients. **Methods**: A total of 80 SLE patients and 43 healthy controls (HCs) were enrolled. Their plasma samples were subjected to the measurements of mtDNA^{pcf} copies, nDNA^{pcf} copies, 8-OHdG and MDA, respectively. Their leukocytes were analyzed for CLEC5A mRNA expression. **Results**: SLE patients had higher nDNA^{pcf} copies (2.84 ± 1.99 *vs.* 2.00 ± 0.88, $p = 0.002$), lower mtDNA^{pcf} copies (4.81 ± 6.33 *vs.* 9.83 ± 14.20, $p = 0.032$), higher plasma 8-OHdG (0.227 ± 0.085 *vs.* 0.199 ± 0.041 ng/mL, $p = 0.016$), lower plasma MDA (3.02 ± 2.20 *vs.* 4.37 ± 2.16 μM, $p = 0.001$) and similar leukocyte CLEC5A mRNA expression levels (1.21 ± 1.17 *vs.* 1.26 ± 1.05, $p = 0.870$), as compared with those of HCs. Among the HCs, SLE patients with SLE Disease Activity Index (SLEDAI) $\leq$8, and SLE patients with SLEDAI >8, their respective mtDNA^{pcf} copies decreased stepwisely (9.83 ± 14.20 *vs.* 6.28 ± 7.91 *vs.* 3.19 ± 3.35, $p = 0.054$). The nDNA^{pcf} copies of HCs, SLE patients without nephritis, and SLE patients with nephritis were increased stepwisely (2.00 ± 0.88 *vs.* 2.63 ± 1.74 *vs.* 3.16 ± 2.34, $p = 0.043$). Among SLE patients, higher nDNA^{pcf} copies were associated with higher levels of plasma 8-OHdG ($p < 0.001$) but lower plasma MDA ($p = 0.019$). Among HCs but not SLE patients, higher nDNA^{pcf} copies ($p = 0.013$) or lower mtDNA^{pcf} copies ($p < 0.001$) were related to higher levels of leukocyte CLEC5A mRNA expression. **Conclusions**: Higher nDNA^{pcf}, lower mtDNA^{pcf}, increased ROS-elicited oxidative DNA damage and dysregulated leukocyte CLEC5A expression might be implicated in the pathogenesis of SLE.

Keywords: plasma cell-free mitochondrial DNA (mtDNA^{pcf}); plasma cell-free nuclear DNA (nDNA^{pcf}); malondialdehyde (MDA); 8-hydroxy-2'-deoxyguanosine (8-OHdG); C-type lectin domain family 5 member A (CLEC5A); systemic lupus erythematosus (SLE)

1. Introduction

Systemic lupus erythematosus (SLE) is characterized by the dysregulation of immunocompetent cells that can generate pathogenic autoantibodies after self-antigen exposure to attack systemic organs [1]. The pathogenesis of SLE are multifactorial [2,3]. However, few studies have discussed the potential roles of plasma cell-free DNA (DNA^{pcf}) [4].

Under normal circumstances, DNA molecules are located intracellularly and embedded compactly in the nucleus as nuclear DNA (nDNA) or in the mitochondrial nucleoid as mitochondrial DNA (mtDNA) [5]. On the contrary, cell-free DNA (DNA^{cf}) are the DNA molecules that are released into the tissue fluid from cells all over the whole body [6]. They have been detected in cerebrospinal fluid [7], pleural fluid [8], saliva [9], urine [10] and plasma [11] for different purposes. The DNA^{cf} present in blood circulation is called "plasma cell-free DNA (DNA^{pcf})", and most of them are originated from circulating hematopoietic cells [12]. Analysis of DNA^{pcf} has been employed for the diagnosis of acute myeloid leukemia as a non-invasive tool, which is as accurate as the invasive bone marrow aspiration or biopsy [13]. Human DNA^{pcf} was first described by Mandel and Metais in 1948 [11]. From then on, the

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clinical usefulness of DNA^{pcf} has been emphasized in predicting the outcomes of critical patients with myocardial infarction [14], sepsis [15] or stroke [16]. High levels of DNA^{pcf} were first found in SLE patients in 1966 [17]. Later on, some studies suggested that DNA^{pcf} might serve as an exposed self-antigen to trigger the pathogenesis of SLE [18]. Because the DNA^{pcf} is mainly derived from intracellular mtDNA or nDNA, it is of great interest and clinical relevance to explore the roles of mitochondrial DNA^{pcf} ($mtDNA^{pcf}$) and nuclear DNA^{pcf} ($nDNA^{pcf}$) simultaneously in SLE patients.

Dysregulated antioxidant capacity with imbalanced production of reactive oxygen species (ROS) has been speculated in the development of SLE [19,20]. Abnormal ROS levels would cause oxidative damages or oxidative modifications in the intracellular or extracellular components of cells *in situ*, nearby, or remotely. These components may include DNA, lipid, proteins, and phospholipids. The events would trigger subsequent cellular dysfunction or autoimmune reactions in SLE patients [19–21]. Elevated plasma 8-hydroxy-2'-deoxyguanosine (8-OHdG) representing oxidative DNA damage [21–23] and plasma malondialdehyde (MDA) representing lipid peroxidation [21,22,24] have been reported in SLE patients with different clinical implications. The levels of accumulated 8-OHdG and/or MDA in the plasma could be effective surrogates to represent the oxidative stress in SLE patients [25].

Viral infection causing incidental self-antigen presentation has been proposed as a possible mechanism underlying the autoimmune reaction in SLE. Similar to the immune interaction implicating high levels of interferon (INF) observed between invading virus and host pattern/pathogen recognition receptors (PRRs), a phenomenon of type I INF signature has also been recognized as a prominent feature of SLE [26]. A clinical trial revealed that the treatment of anti-INF-alpha monoclonal antibody, sifalimumab, could achieve a significant improvement of symptoms/signs in moderate to severe SLE patients [27]. Recently, the importance of human C-type lectin domain family 5 member A (CLEC5A, a member of PRRs) in interacting with the invading virus was reported [28]. Teng *et al.* [29] reported that through toll-like receptor 3 (TLR, a subtype of PRRs), influenza virus infection in bone marrow-derived macrophages from $CLEC5A^{-/-}$ mice would result in an increase of IFN-alpha. Based on these findings, we have speculated that decreased or dysregulated leukocyte CLEC5A expression might be involved in SLE pathogenesis.

In this study, we focused on the measurement of copies of $mtDNA^{pcf}$ and $nDNA^{pcf}$ to explore their association with the levels of plasma 8-OHdG and plasma MDA as well as the leukocyte CLEC5A mRNA transcripts in SLE patients. We aimed to elucidate the role of DNA^{pcf} in the pathogenesis of SLE.

2. Materials and Methods

2.1 Recruitment of SLE Patients and the Healthy Controls (HCs)

According to the classification criteria for SLE and scored by the SLE Disease Activity Index (SLEDAI)-2000 [30–32], a total of 80 non-acute SLE patients (female/male = 67/13, mean age 45.4 $\pm$ 12.7 years) under regular follow-up and treatment in the outpatient department (OPD) of the Division of Allergy, Immunology and Rheumatology in Taipei Veterans General Hospital were recruited in this sudy. Their medications consisted of steroids, hydroxychloroquine, azathioprine, mycophenolate mofetil or NSAID according to clinical conditions. Another 43 healthy individuals, who had no evidence of systemic diseases, e.g., autoimmune diseases, diabetes, infections, cardiovascular diseases or malignancy and matched for age (within 10 years, 40.2 $\pm$ 10.4 years) and sex (female/male = 35/8), were recruited as the healthy controls (HCs) for comparative study. The demographic data of SLE patients regarding organ involvement and laboratory profiles were recorded in detail. Regarding the lupus nephritis, it was defined by a protein-to-creatinine ratio (or 24-hour urine protein) exceeding 500 mg protein/24 hours or by red blood cell casts in the urine [30]. Disease activity/severity was evaluated by using the SLEDAI-2000 scoring system [32,33].

Besides, another 3 active SLE patients with severe clinical presentations were enrolled. These included Case A with nephritis, pleuritis and pericarditis, Case B with acute lupus nephritis and rapidly progressive glomerulonephritis who was undergoing dialysis, and Case C with severe pulmonary arterial hypertension. Their therapeutic regimens consisted of intravenous rituximab (Mabthera™, 500 mg) on days 1 and 14 preceded by a pre-medication with 100 mg of intravenous methylprednisolone. Blood samples were drawn before rituximab treatments on days 1 and 14.

2.2 Purification of Plasma Cell-Free DNA (DNA^{pcf}) and Extraction of the mRNA & DNA from Leukocytes

Approximately 10 mL of peripheral venous blood was drawn into a tube containing EDTA (VACUETTE® with EDTA, Greiner Bio-One). After centrifugation at 3000 g for 10 minutes at 4 °C, the supernatant plasma and leukocyte-enriched buffy coat were collected, respectively. An aliquot of 200 μL plasma was subjected to the purification of plasma cell-free DNA (DNA^{pcf}) using QuickcfDNA™ Serum and Plasma Miniprepkit (Epigenetics) according to the manufacturer's instructions [34]. The eluted DNA^{pcf} was kept at –20 °C until use.

The leukocytes from 42 SLE patients and 24 HCs were isolated for RNA extraction using the TRI™ reagent (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). With oligo-dT primers in a 50-μL reaction buffer, an aliquot of 2 μg of purified RNA was reverse-transcribed to cDNA (1X) by the Ready-To-Go reverse transcription – polymerase

chain reaction (RT-PCR) kit (GE Healthcare, Amersham, UK). The cDNA thus obtained was kept at –20 °C until use [20].

Genomic DNA of the leukocytes from a healthy control (HC-No.42) was purified through the standard phenol-chloroform extraction and isopropanol precipitation procedure as described previously [35]. The DNA pellet was dissolved in nuclease-free distilled water and kept at –20 °C until use.

2.3 Standard Curves for DNA and cDNA Quantification

Quantitative real-time PCR (Q-PCR) using SensiFAST SYBR® Hi-ROX Kit (BIOLINE, London, UK) was applied to determine the DNA copies and cDNA copies through a standard curve and their threshold cycle (Ct) values. To establish standard curves for calculations, genomic DNA and cDNA from the leukocytes of HC-No.42 were serially diluted by 4-fold, from 30 to 0.0018310546875 ng/μL for DNA and from 0.25 (4× dilution) to 0.00390625 (256× dilution) for cDNA, respectively, and were then subjected to Q-PCR for the determination of their Ct values.

The oligonucleotide sequences of the primers used for amplification of mtDNA (the *tRNA*$^{Leu(UUR)}$ gene region) and nDNA (the *18S rRNA* gene region) were mtF3212: 5′-CACCCAAGAACAGGGTTTGT-3′/mtR3319: 5′-TGGCCATGGGATTGTTGTTAA-3′ and 18SF1546: 5′-TAGAGGGACAAGTGGCGTTC-3′/18SR1650: 5′-CGCTGAGCCAGTCAGTGT-3′, respectively [36]. The equations for mtDNA and nDNA quantifications were established as follows:

(Ct value of analyzed sample mtDNA) = (–3.6972) × log (mtDNA copies of analyzed sample/mtDNA copies of reference sample) + 20.6390 (R^2 = 0.9967) and (Ct value of analyzed sample nDNA) = (–3.8037) × log (nDNA copies of analyzed sample/nDNA copies of reference sample) + 21.014 (R^2 = 0.9980), respectively.

Using these 2 equations, the mtDNA copies and nDNA copies of the analyzed sample relative to mtDNA copies and nDNA copies of the reference sample were calculated.

The primer sequences for CLEC5A cDNA amplification and 18S rRNA cDNA amplification were CLEC5AF: 5′-GTTTCACCACCACCAGGAGC-3′/CLEC5AR: 5′-GGCATTCTTCTCACAGATCC-3′ and 18S rRNAF: 5′-CTCAACACGGGAAACCTCAC-3′/18S rRNAR: 5′-CGCTCCACCAACTAAGAACG-3′, respectively [20,37]. The equations for CLEC5A and 18S rRNA cDNA quantifications were set as follows:

(Ct value of analyzed sample CLEC5A cDNA) = (–3.3563) × log (CLEC5A cDNA copies of analyzed sample/CLEC5A cDNA copies of reference sample) + 23.418 (R^2 = 0.9922) and (Ct value of analyzed sample 18S rRNA cDNA) = (–3.2571) × log (18S rRNA cDNA copies of analyzed sample/18S rRNA cDNA copies of reference sample) + 18.824 (R^2 = 0.9973), respectively.

Using these 2 equations, the CLEC5A cDNA copies and 18S rRNA cDNA copies of the analyzed sample relative to CLEC5A cDNA copies and 18S rRNA cDNA copies of the reference sample were calculated, respectively.

2.4 Determination of mtDNA^{pcf} Copies, nDNA^{pcf} Copies and the Leukocyte CLEC5A mRNA Expression Levels

Q-PCR was used for the quantification of mtDNA^{pcf}, nDNA^{pcf}, leukocyte CLEC5A cDNA and leukocyte 18S rRNA cDNA copies, respectively. For each Q-PCR tube, 1 μL of DNA^{pcf}/or 1 μL of cDNA (16× dilution) of analyzed sample was amplified in a 10-μL reaction mixture that contained 0.25 μL of each primer (20 μM), 5 μL of SensiFAST SYBR® Hi-ROX premix, and 3.5 μL of PCR-grade water. Simultaneously, 1 μL of HC-No.42 leukocyte DNA (1 ng/μL)/or cDNA (16× dilution) and PCR-grade water were used as the reference (positive) and negative controls, respectively. The Q-PCR protocol contained a hot start at 95 °C for 10 minutes followed by 45 cycles of amplifications at 95 °C for 15 seconds and 60 °C for 60 seconds. By equations established above, the mtDNA^{pcf} copies, nDNA^{pcf} copies, leukocyte CLEC5A cDNA copies and leukocyte 18S rRNA cDNA copies of analyzed samples relative to those of HC-No.42 leukocyte, were calculated, respectively. We defined the CLEC5A mRNA expression level as total CLEC5A cDNA copies divided by total 18S rRNA cDNA copies in the present study. Finally, relative mtDNA^{pcf} copies, nDNA^{pcf} copies and CLEC5A mRNA expression levels of analyzed samples were calculated after adjusting with the mtDNA^{pcf} copies, nDNA^{pcf} copies and leukocyte CLEC5A mRNA expression level of HC-No.42 as 1.000. Each reaction was done in duplicate to get the average of data.

2.5 Determination of Plasma 8-OHdG

Part of the results of plasma 8-OHdG in the present analyses had been reported in other studies for different purposes [20,38]. An aliquot of 200 μL of plasma, filtered by an ultra-filter (CENTRICON®, Ultracel YM-10 membrane, Millipore, Amicon, USA) with a cut-off molecular weight of 10 kDa, was subjected to centrifugation at 10,000 g at 4 °C for 2 hours to get rid of the interfering substances. Then, an aliquot of 50 μL of filtered plasma was employed for the measurement of 8-OHdG content by using the highly sensitive 8-OHdG Check ELISA kit (Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd. Haruoka, Fukuroi, Shizuoka, Japan) according to the manufacturer's instructions. Each reaction was done in duplicate to get the average for data presentation [20,38].

2.6 Determination of Plasma MDA

In the present study, we measured the plasma MDA, without the hydrolysis of plasma sample, by a spectrophotometric assay kit (MDA-586, OxisResearch, Inc. Portland, OR, USA) according to the procedure recommended by

the manufacturer, which involved the reaction with a chromogenic reagent N-methyl-2-phenylindole (NMPI) to form an intensely colored carbocyanine dye with a maximum absorption at 586 nm. A standard curve was established by using the referenced MDA samples at the concentration range of 0–4 μM and the plasma MDA levels in clinical samples were then calculated [39].

2.7 Statistical Analysis

The results are presented as the mean ± standard deviation (mean ± S.D.). The continuous variables between HCs and SLE patients were compared using the *t*-test or Mann-Whitney *U* test when appropriate. The continuous variables among HCs, SLE patients with SLEDAI ≤8 and SLE patients with SLEDAI >8 or among HCs, SLE patients without clinical manifestations and SLE patients with clinical manifestations were compared using Jonckheere-Terpstra trend test to demonstrate their trends of distributions. Alterations of continuous variables before and after the treatment of rituximab were compared by Wilcoxon signed-ranks test. Relationships between two continuous variables were evaluated by using Pearson's or Spearman's *rho* correlation and are presented with the correlation coefficient (CC). Differences and associations were considered significant when *p*-values were less than 0.05.

3. Results

3.1 Demographic Data of the 80 SLE Patients

A total of 80 SLE patients (13 men) with a mean age of 45.4 years were recruited. Their demographic data are listed in Table 1. Their mean and median SLEDAI were 9.4 and 8, respectively. Their mean and median anti-dsDNA antibody titers, serum complement 3 (C3) levels and serum complement 4 (C4) levels were 122.4 and 80 IU/ mL, 83.0 and 76.9 mg/dL, and 16.5 and 15.3 mg/dL, respectively. Using the cutoff values of >15 IU/mL, <90 mg/dL and <10 mg/dL, 66 (82.5%), 52 (65.0%) and 9 (11.3%) SLE patients were classified as harboring positive anti-dsDNA antibody, hypo C3 and hypo C4, respectively. Regarding the clinical manifestations, 39 (48.8%) suffered from CNS involvement, 31 (38.8%) nephritis, 18 (22.5%) skin rash, 24 (30.0%) alopecia, 12 (15.0%) oral ulcer and 52 (65.0%) complement decrease, respectively.

Table 1. Demographic data of the 80 SLE patients recruited for this study.

Demographic data	Mean ± S.D./n (%)
Gender	
Female/Male	67 (83.8)/13 (16.3)
Age (yrs)	45.4 ± 12.7
SLEDAI	9.4 ± 6.0
Median	8
Anti-dsDNA antibody titers (IU/mL)	122.4 ± 127.4
Median	80.0
Positive anti-dsDNA antibody (>15 IU/mL)[a]	66 (82.5)
Serum complement 3 (C3) levels (mg/dL)	83.0 ± 25.8
Median	76.9
Hypo C3 (<90 mg/dL)[b]	52 (65.0)
Serum complement 4 (C4) levels (mg/dL)	16.5 ± 7.4
Median	15.3
Hypo C4 (<10 mg/dL)[c]	9 (11.3)
CNS involvement	39 (48.8%)
Nephritis	31 (38.8%)
Skin rash	18 (22.5%)
Alopecia	24 (30.0%)
Ulcer	12 (15.0%)
Complement decrease (Hypo C3, hypo C4 or both)[d]	52 (65.0%)

SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; CNS, central nervous system; [a], >15 IU/mL was classified as positive anti-dsDNA antibody; [b], <90 mg/dL was classified as hypo C3; [c], <10 mg/dL was classified as hypo C4; [d], hypo C3, hypo C4 or both were classified as complement decrease.

3.2 Distributions of mtDNA^{pcf} Copies, nDNA^{pcf} Copies, Plasma MDA, Plasma 8-OHdG and Leukocyte CLEC5A mRNA Expression Levels among 43 HCs and 80 SLE Patients

SLE patients had lower mtDNA^{pcf} copies than did the HCs (median, 2.28 *vs.* 2.76; mean ± S.D., 4.81 ± 6.33 *vs.* 9.83 ± 14.20, $p = 0.032$, Table 2). On the contrary, SLE patients had higher nDNA^{pcf} copies than the HCs (median, 2.12 *vs.* 1.77; mean ± S.D., 2.84 ± 1.99 *vs.* 2.00 ± 0.88, $p = 0.002$, Table 2). Regarding the plasma markers reflecting oxidative damages, SLE patients had higher levels of plasma 8-OHdG than HCs (median, 0.207 *vs.* 0.187; mean ± S.D., 0.227 ± 0.085 *vs.* 0.199 ± 0.041, $p = 0.016$); but they had lower levels of plasma MDA than HCs (median, 1.82 *vs.* 4.06; mean ± S.D., 3.02 ± 2.20 *vs.* 4.37 ± 2.16, $p = 0.001$, Table 2). The leukocyte CLEC5A mRNA expression levels did not differ significantly between SLE patients and HCs (median, 0.781 *vs.* 0.965; mean ± S.D., 1.21 ± 1.17 *vs.* 1.26 ± 1.05, $p = 0.870$, Table 2).

3.3 Distributional Changes in mtDNA^{pcf} Copies and nDNA^{pcf} Copies among 43 HCs and 80 SLE Patients without or with the Presence of Different Clinical Manifestations

Among HCs, SLE patients with less activity (SLEDAI ≤8) and SLE patients with higher activity (SLEDAI >8), they exhibited a trend of decreasing mtDNA^{pcf} copies in order (median, 2.76, 2.73 and 2.14; mean ± S.D., 9.83 ± 14.20, 6.28 ± 7.91 and 3.19 ± 3.35, $p = 0.054$; Table 3, Left). Although not significant, such a trend of decrease was also observed among HCs, SLE patients without complement decrease and SLE patients with complement decrease (median, 2.76, 2.62 and 2.03; mean ± S.D., 9.83 ± 14.20, 5.02 ± 5.49 and 4.70 ± 6.79, $p = 0.062$; Table 3, Left) and among HCs, SLE patients without nephritis and

Table 2. Distributions of mtDNA^{pcf} copies, nDNA^{pcf} copies, plasma MDA, plasma 8-OHdG and leukocyte CLEC5A mRNA among 43 HCs and 80 SLE patients.

Parameters	HCs (n = 43)	SLE (n = 80)	*p*-value[a]
Plasma cell-free DNA (DNA^{pcf})			
mtDNA^{pcf} copies			
Mean ± S.D.	9.83 ± 14.20	4.81 ± 6.33	0.032
Median	2.76	2.28	
nDNA^{pcf} copies			
Mean ± S.D.	2.00 ± 0.88	2.84 ± 1.99	0.002
Median	1.77	2.12	
Plasma oxidative damage markers			
8-OHdG (ng/mL)			
Mean ± S.D.	0.199 ± 0.041	0.227 ± 0.085	0.016
Median	0.187	0.207	
MDA (μM)			
Mean ± S.D.	4.37 ± 2.16	3.02 ± 2.20	0.001
Median	4.06	1.82	
Parameter	HCs (n = 24)	SLE (n = 42)	*p*-value[a]
Leukocyte CLEC5A mRNA level			
Mean ± S.D.	1.26 ± 1.05	1.21 ± 1.17	0.870
Median	0.965	0.781	

mtDNA, mitochondrial DNA; mtDNA^{pcf} copies, plasma cell-free mtDNA copies; nDNA, nuclear DNA; nDNA^{pcf} copies, plasma cell-free nDNA copies; MDA, malondialdehyde; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; CLEC5A, C-type lectin domain family 5 member A; HC, healthy control; SLE, systemic lupus erythematosus; [a] comparison between HC and SLE patients, using *t*-test or Mann-Whitey *U* test when appropriate.

SLE patients with nephritis (median, 2.76, 2.27 and 2.28; mean ± S.D., 9.83 ± 14.20, 5.38 ± 6.95 and 3.90 ± 5.18, $p = 0.082$; Table 3, Left). However, such a trend in mtDNA copies were not conspicuous regarding the abnormal anti-dsDNA antibody ($p = 0.360$), CNS involvement ($p = 0.133$), skin rash ($p = 0.254$), alopecia ($p = 0.273$) nor oral ulcer ($p = 0.343$), respectively (Table 3, left).

Regarding the distributions of nDNA^{pcf} copies among HCs, SLE patients without nephritis and SLE patients with nephritis, they exhibited a tendency of increase (median, 1.77, 2.01 and 2.53; mean ± S.D., 2.00 ± 0.88, 2.63 ± 1.71 and 3.24 ± 2.40, $p = 0.043$; Table 3, Right). Although there seemed to have an inclination, such a trend was present regarding the individual domain of involvement including skin rash ($p = 0.069$) and alopecia ($p = 0.073$) (Table 3, Right). Otherwise, the distributions of nDNA^{pcf} copies were irrelevant to the score of SLEDAI ($p = 0.333$), abnormal anti-dsDNA antibody ($p = 0.475$), CNS involvement ($p = 0.696$), oral ulcer ($p = 0.188$) nor complement decrease ($p = 0.411$) (Table 3, Right).

3.4 The Distributions of mtDNA^{pcf} and nDNA^{pcf} Copies and Their Association with Plasma 8-OHdG, Plasma MDA and Leukocyte CLEC5A mRNA Expression Levels in 43 HCs and 80 SLE Patients

As shown in Table 4, among 80 SLE patients, their distributions of mtDNA^{pcf} copies were not related to the distribution of plasma 8-OHdG ($p = 0.471$) nor plasma MDA ($p = 0.132$). However, it is interesting that their distributions of nDNA^{pcf} copies were positively correlated with plasma 8-OHdG ($p < 0.001$; CC = 0.457) and negatively correlated with plasma MDA ($p = 0.019$; CC = –0.262), respectively.

Among the 43 HCs, their distributions of mtDNA^{pcf} copies ($p = 0.009$, CC = 0.392) and nDNA^{pcf} copies ($p = 0.016$, CC = 0.366) were positively correlated with the levels of plasma 8-OHdG, significantly. On the contrary, their distributions of mtDNA^{pcf} copies ($p = 0.167$) and nDNA^{pcf} copies ($p = 0.849$) were not related to the levels of plasma MDA (Table 4).

Significantly, the leukocyte CLEC5A mRNA levels were related inversely to mtDNA^{pcf} copies ($p < 0.001$, CC = –0.677) and positively to nDNA^{pcf} copies ($p = 0.013$, CC = 0.501) among the analyzed 24 HCs. However, leukocyte CLEC5A mRNA levels were not related to mtDNA^{pcf} copies ($p = 0.205$) and nDNA^{pcf} copies ($p = 0.207$), respectively, among the 42 analyzed SLE patients.

3.5 Alterations of mtDNA^{pcf} Copies, nDNA^{pcf} Copies and Plasma 8-OHdG, Plasma MDA and Leukocyte CLEC5A mRNA Expression Levels in 3 SLE Patients Undergoing Rituximab Treatment

In Table 5, changes of mtDNA^{pcf} copies, nDNA^{pcf} copies and plasma 8-OHdG, plasma MDA and leukocyte CLEC5A mRNA expression levels in 3 SLE patients un-

Table 3. Fluctuation of the distributions in mtDNA^{pcf} copies and nDNA^{pcf} copies from 43 HCs to 80 SLE patients with the absence or presence of different clinical manifestations.

	mtDNA^{pcf} copies			nDNA^{pcf} copies		
	Mean ± S.D.	Median	p-value[a]	Mean ± S.D.	Median	p-value[a]
Disease activity index (DAI)			0.054			0.333
HCs (n = 43)	9.83 ± 14.20	2.76		2.00 ± 0.88	1.77	
SLEDAI ≤8 (n = 42)	6.28 ± 7.91	2.73		3.14 ± 2.22	2.21	
SLEDAI >8 (n = 38)	3.19 ± 3.35	2.14		2.50 ± 1.67	1.96	
Anti-dsDNA antibody status			0.360			0.475
HCs (n = 43)	9.83 ± 14.20	2.76		2.00 ± 0.88	1.77	
SLE with anti-dsDNA antibody ≤15 IU/mL (n = 14)	3.72 ± 5.87	1.75		4.43 ± 2.77	4.62	
SLE with anti-dsDNA antibody >15 IU/mL (n = 66)	5.04 ± 6.44	2.43		2.50 ± 1.62	2.00	
CNS status			0.133			0.696
HCs (n = 43)	9.83 ± 14.20	2.76		2.00 ± 0.88	1.77	
SLE without CNS involvement (n = 41)	6.17 ± 8.04	2.02		3.23 ± 2.03	2.42	
SLE with CNS involvement (n = 39)	3.38 ± 3.36	2.28		2.42 ± 1.89	1.80	
Renal status			0.082			0.043
HCs (n = 43)	9.83 ± 14.20	2.76		2.00 ± 0.88	1.77	
SLE without nephritis (n = 49)	5.38 ± 6.95	2.27		2.63 ± 1.74	2.01	
SLE with nephritis (n = 31)	3.90 ± 5.18	2.28		3.16 ± 2.34	2.53	
Skin status			0.254			0.069
HCs (n = 43)	9.83 ± 14.20	2.76		2.00 ± 0.88	1.77	
SLE without skin rash (n = 62)	4.41 ± 5.22	2.16		2.83 ± 2.06	2.03	
SLE with skin rash (n = 18)	6.17 ± 9.28	2.30		2.87 ± 1.80	2.29	
Scalp status			0.273			0.073
HCs (n = 43)	9.83 ± 14.20	2.76		2.00 ± 0.88	1.77	
SLE without alopecia (n = 56)	4.49 ± 5.41	2.27		2.78 ± 2.03	2.12	
SLE with alopecia (n = 24)	5.55 ± 8.18	2.3		2.96 ± 1.95	2.22	
Oral mucosa			0.343			0.188
HCs (n = 43)	9.83 ± 14.20	2.76		2.00 ± 0.88	1.77	
SLE without ulcer (n = 68)	4.56 ± 6.03	2.03		2.90 ± 2.01	2.21	
SLE with ulcer (n = 12)	6.23 ± 7.98	3.12		2.45 ± 1.94	1.87	
Complement status			0.062			0.411
HCs (n = 43)	9.83 ± 14.20	2.76		2.00 ± 0.88	1.77	
SLE without complement decrease (n = 28)	5.02 ± 5.49	2.62		3.22 ± 1.96	2.40	
SLE with complement decrease (n = 52)	4.70 ± 6.79	2.03		2.63 ± 2.00	1.98	

mtDNA, mitochondrial DNA; mtDNA^{pcf} copies, plasma cell-free mtDNA copies; nDNA, nuclear DNA; nDNA^{pcf} copies, plasma cell-free nDNA copies; HC, healthy control; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; CNS, central nervous system; [a]Comparison among HCs, SLE patients with SLEDAI ≤8 and SLE patients with SLEDAI >8 or among HCs, SLE patients without clinical manifestations and SLE patients with clinical manifestations (e.g., abnormal anti-dsDNA antibody, CNS involvement, nephritis, skin rash, alopecia, oral ulcer or complement decrease), using Jonckheere-Terpstra trend test to demonstrate their trends of distributions.

dergoing rituximab treatment are listed. Because only the data of 3 patients were analyzed, the clinical relevance was limited and insufficient to draw a conclusion. Nevertheless, regarding the alterations of mtDNA^{pcf} copies and leukocyte CLEC5A mRNA expression levels, the changes were universally increased although not statistically significant (p = 0.109).

4. Discussion

In summary, we have demonstrated that: (1) SLE patients tended to have lower mtDNA^{pcf} copies, higher nDNA^{pcf} copies, higher levels of plasma 8-OHdG, and lower levels of plasma MDA than did HCs, but they had similar leukocyte CLEC5A mRNA levels (Table 2). (2) SLE patients with higher SLEDAI tended to have lower mtDNA^{pcf} copies, and SLE patients with nephritis had higher nDNA^{pcf} copies but lower mtDNA^{pcf} copies (Table 3). (3) In SLE patients, higher nDNA^{pcf} copies were correlated with higher levels of plasma 8-OHdG but lower levels of plasma MDA (Table 4). (4) In HCs, higher nDNA^{pcf} or lower mtDNA^{pcf} copies were associated with higher leukocyte CLEC5A mRNA expression levels, which showed no associations in SLE patients (Table 4) and (5) In active SLE patients, increases in mtDNA^{pcf} copies and

Table 4. The distribution of mtDNA^{pcf} and nDNA^{pcf} copies along with their association to plasma 8-OHdG, MDA and leukocyte CLEC5A mRNA in 43 HCs and 80 SLE patients.

	mtDNA^{pcf} copies			nDNA^{pcf} copies		
Association of	Overall (n = 123)	HCs (n = 43)	SLE (n = 80)	Overall (n = 123)	HCs (n = 43)	SLE (n = 80)
Plasma 8-OHdG						
CC[a]	0.100	0.392	0.082	0.460	0.366	0.457
p-value	0.273	0.009	0.471	<0.001	0.016	<0.001
Plasma MDA						
CC[a]	–0.142	–0.214	–0.170	–0.251	0.030	–0.262
p-value	0.117	0.167	0.132	0.005	0.849	0.019
Association of	Overall (n = 66)	HCs (n = 24)	SLE (n = 42)	Overall (n = 66)	HCs (n = 24)	SLE (n = 42)
Leukocyte CLEC5A mRNA level						
CC[a]	–0.344	–0.677	–0.200	0.253	0.501	0.199
p-value	0.005	<0.001	0.205	0.040	0.013	0.207

mtDNA, mitochondrial DNA; mtDNA^{pcf} copies, plasma cell-free mtDNA copies; nDNA, nuclear DNA; nDNA^{pcf} copies, plasma cell-free nDNA copies; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; CLEC5A, C-type lectin domain family 5 member A; HC, healthy control; SLE, systemic lupus erythematosus; CC, correlation coefficient; [a], Pearson or Spearman's *rho* correlation coefficient (CC) if appropriate.

Table 5. Alterations of mtDNA^{pcf} copies, nDNA^{pcf} copies and plasma 8-OHdG, plasma MDA and leukocyte CLEC5A mRNA expression levels in 3 SLE patients undergoing rituximab treatment.

	Case	Pre-rituximab	Post-rituximab	Alterations	*p*-value[a]
Plasma cell-free DNA (DNA^{pcf})					
mtDNA^{pcf}					0.109
	A	0.37	5.96	Increase	
	B	1.11	2.02	Increase	
	C	3.35	142.67	Increase	
nDNA^{pcf}					0.285
	A	0.14	0.30	Increase	
	B	1.94	1.01	Decrease	
	C	8.05	3.68	Decrease	
Plasma oxidative damage markers					
8-OHdG (ng/mL)					0.593
	A	0.532	1.251	Increase	
	B	0.949	0.706	Decrease	
	C	0.572	0.687	Increase	
MDA (μM)					0.593
	A	2.25	2.76	Increase	
	B	5.03	3.84	Decrease	
	C	1.06	1.03	Decrease	
Leukocyte CLEC5A mRNA level					0.109
	A	0.60	3.16	Increase	
	B	2.54	3.25	Increase	
	C	0.74	3.24	Increase	

mtDNA, mitochondrial DNA; mtDNA^{pcf} copies, plasma cell-free mtDNA copies; nDNA, nuclear DNA^{pcf}; nDNApcf copies, plasma cell-free nDNA copies; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; CLEC5A, C-type lectin domain family 5 member A; [a], Wilcoxon signed-rank test.
Case A, nephritis, pleuritis and pericarditis; Case B, acute lupus nephritis and rapidly progressive glomerulonephritis undergoing dialysis; Case C, severe pulmonary arterial hypertension.

leukocyte CLEC5A mRNA levels were found after rituximab treatment (Table 5). We have thus speculated that lower mtDNA^{pcf} copies, higher nDNA^{pcf} copies, oxidative damages/stress as well as dysregulated leukocyte CLEC5A expression might be implicated in the pathogenesis of SLE. A proposed mechanism is illustrated in Fig. 1.

Higher levels of DNA^{pcf} in SLE patients was first described by Tan *et al.* in 1966 [17]. Up to now, DNA^{pcf}

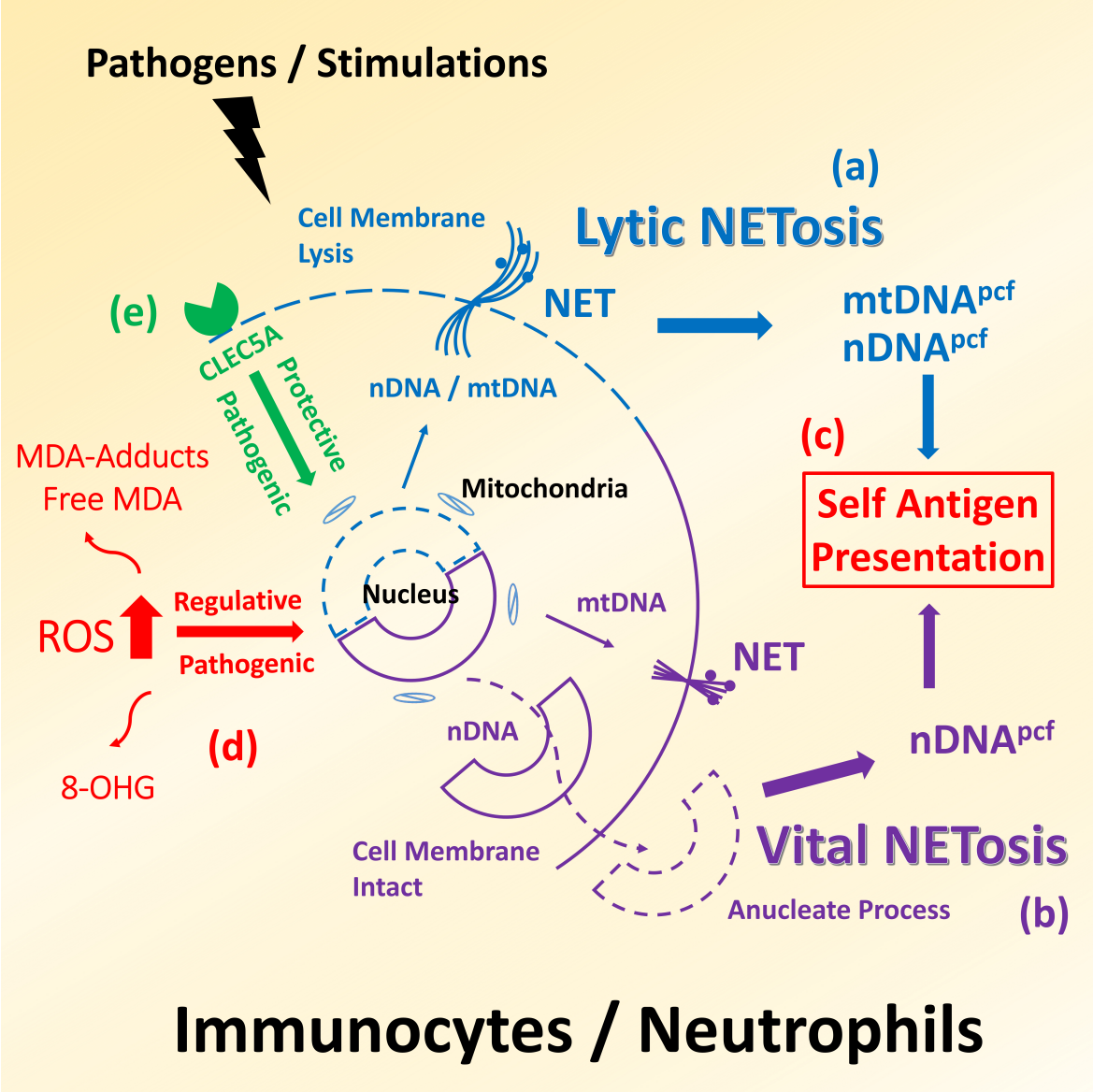

Fig. 1. Illustration is a proposed mechanism of DNA^{pcf} released from immunocytes/neutrophils under pathogen invasion or stress stimulations, which is involved in the self-antigen presentation in SLE pathogenesis. (a) Lytic NETosis, DNA molecules, including nDNA and mtDNA, as well as other proteins in extruded NET can entangle the pathogens and trigger immune reactions to result in a subsequent lysis of their own cell membrane and the death of invading microbes. (b) Vital NETosis, different from the cell death during lytic NETosis, some immune cells secrete only mtDNA, harboring high immunogenicity, into NET, which enable them to maintain alive even if they become anucleate. (c) Both lytic and vital NETosis could cause the release of DNA into blood circulation and involve the presentation of self-antigen. (d) ROS plays an important regulator in adjusting NETois, and the degrees of ROS-elicited modifications could be reflected by the abundance of 8-OHdG and MDA. (e) CLEC5A can drive human immune response to defend viral infections and is a critical receptor in innate immune system causing NETosis.

has been regarded as an important biomarker in autoimmune rheumatic diseases [40]. Nevertheless, DNA^{pcf} including mtDNA^{pcf} and nDNA^{pcf} and their potential implications in disease development were less explored in SLE. Giaglis *et al.* [41] reported that a high level of DNA^{pcf} is resulted from mtDNA^{pcf} rather than from nDNA^{pcf} in SLE. However, Truszewska *et al.* [42] found that high DNA^{pcf} is due to high nDNA^{pcf} in SLE. They also failed to find differences in mtDNA^{pcf} copies between SLE patients and HCs. The present investigation has revealed higher nDNA^{pcf} but lower mtDNA^{pcf} copies in SLE patients compared with those of HCs (Table 2). Similar to the Q-PCR protocol described by Giaglis and Truszewska and those reported in the literature [41–43], we measured the mtDNA^{pcf} and nDNA^{pcf} copies in a given volume through the established standard curves. There are small differences between

the absolute copies reported by Giaglis and Truszewska and the relative copies detected in the present investigation. Although the data by Giaglis and Truszewska were generated in more precise manner, the results of the present study could still provide useful information. So to speak, our patients exhibited higher SLEDAI of 8 in median than the cohort in Truszewska's of 7.42 in average or in Giaglis' of 2 in median, indicating more severe clinical manifestations in our patients. These similarities and disparities suggest that the mechanism underlying the release of intracellular DNA into plasma to become nDNA^{pcf} or mtDNA^{pcf} in SLE patients needs re-appraisal. Through cell death, degradation or damage and extrusion of intracellular molecules, partial or complete genomes from *in situ* or nearby tissue cells that may or may not contain invading viruses or microbes, would be continuously shed into human body fluids to form DNA^{cf} [44]. Different from the other types of DNA^{cf}, most DNA^{pcf} are originated from hemopoietic cells [12]. As a result, necrosis, apoptosis, degradation or damages of dysregulated SLE-immunocytes might be the major contributors to the release of SLE-DNA^{pcf} [40,45]. Among the different types of immunocytes, neutrophil is the most abundant one with plenty of DNA molecules and has been implicated in the trigger and perpetuation of SLE [46]. DNA^{pcf} from dysregulated neutrophils might be involved in the pathogenesis of SLE.

Similar to necrosis, apoptosis or exocytosis of general cells, neutrophils are able to undergo a unique mechanism of metamorphosis resulting in neutrophil extracellular trap (NET), which is accordingly called "NETosis", to deal with the invading pathogens or sterile inflammation (Fig. 1). Stimulated by ROS and extruding web-like NET, which is composed of a nucleus-derived decondensed DNA coated with histones, granular proteins, and cytoplasmic proteins, into the extracellular space, some neutrophils can entangle the pathogens and trigger immune reactions to result in a lysis of their own cell membrane and the death of invading microbes, termed lytic NETosis (Fig. 1a) [47]. Different from the lytic NETosis, some "immortal neutrophils" may undergo vital NETosis to keep intact cell membranes, in which they only extrude a small amount of mtDNA into NET, allowing themselves to remain alive and continue to exert antimicrobial activity, even if they become anucleated (Fig. 1b) [48,49]. Through the release of neutrophil nDNA^{pcf} and mtDNA^{pcf}, NETosis might lead to a self-antigen exposure and participate in the pathogenesis of SLE (Fig. 1c) [40,50,51].

As shown in Fig. 1, both lytic and vital NTEosis in neutrophils could contribute to the release of nDNA molecules, which could account for our results of higher nDNA^{pcf} in SLE patients than in HCs (Table 2). In our cohort, SLE patients tended to present lower levels of mtDNA^{pcf} (Table 2) and such a decrease was highly correlated with the high SLEDAI (Table 3). This implies that mtDNA are captured in the NET without releasing into the plasma, which is a possible result of vital NETosis. The mtDNA-enriched NET may triggered a vicious cycle of immune response because the high CpG motif in mtDNA that would propagate immunogenicity cascade in innate immunity [52]. Therefore, based on the findings of low mtDNA^{pcf} copies and higher median SLEDAI of 8 as demonstrated in our cohort, similar mtDNA^{pcf} copies and less higher mean SLEDAI of 7.42 in Truszewska's cohort [42] or high mtDNA^{pcf} copies but low median SLEDAI of 2 in Giaglis' cohort [41], we speculated that the different results of mtDNA^{pcf} or nDNA^{pcf} might be hybrid results from diverse proportions of lethal (lytic) and vital NETosis (Fig. 1a–c) regulated by oxidative stress (Fig. 1d). To validate this speculation, we need again to evaluate the NETosis activity and the differences among intracellular DNA, cell membrane-bound DNA and DNA^{pcf} of SLE patients in the future.

Lupus nephritis is an important clinical manifestation in SLE, and the role of oxidative DNA damage has been evaluated [53]. However, the role of DNA^{pcf} remained unclear. Because 8-OHdG is a stabilized product after oxidative DNA damage, it is an optimal marker to reflect the ROS levels. Our preliminary results showed that SLE patients harbored higher levels of plasma 8-OHdG and higher nDNA^{pcf} copies than did HCs' (Table 2), and the high plasma 8-OHdG levels were related to high nDNA^{pcf} copies among SLE patients (Table 4). It is suggested that high oxidative stress might cause an abundant release of nDNA from impaired leukocytes during NETosis. We also found that SLE patients with nephritis had higher nDNA^{pcf} copies (Table 3), suggesting that damaged kidney might be related to impaied clearance of NET remnant or nDNA^{pcf}. As a result, higher nDNA^{pcf} copies were detected and the vicious cycle sustained. On the contrary, we did not find any association between the levels of plasma 8-OHdG and mtDNA^{pcf} copies in SLE patients, but SLE patients with nephritis tended to have a lower mtDNA^{pcf} copies (p = 0.082) (Table 3). However, Fernandez *et al.* [54], reported that an elevation in mtDNA^{pcf} would define a subgroup of SLE patients with membranous lupus nephritis. Due to the dynamic changes of intracellular and extracellular mtDNA molecules and the inconsistency in reported literature, Truszewska normalized their ratios between intra to extra cellular mtDNA copy number to correlate the occurrence of nephritis, and it showed good association [42]. Besides the mtDNA^{pcf}, the high levels of urine mtDNA contents from damaged kidney (i.e., a kind of mtDNA^{cf} in urine) have been advocated to correlate severity of nephritis [55,56]. The precise role of mtDNA either intracellular or extracellular to reflect lupus nephritis needs further investigation.

On the other hand, we failed to observe a higher level of plasma MDA in SLE patients (Table 2) and the levels of plasma MDA were inversely correlated with that of nDNA^{pcf} (Table 4). Unlike the stabilized 8-OHdG, MDA

is the peroxidized product of lipid with highly reactive activity, the unstable free form could conjugate with proteins through covalent alterations of amide and amine groups of peptides to form MAD adducts at a post-translational level, giving rise to neo-epitopes that can elicit autoantibody responses [52]. The literature has demonstrated that high levels of MDA adducts would trigger obvious autoimmune reactivity in SLE patients [57]. Similar to oxidized low density lipoprotein, MDA adducts could induce the formation of NET and propagate immune reaction [58]. More precisely speaking, MDA could exist in free and conjugated (adduct) forms, and a free form MDA could positively extrapolate total MDA to predict oxidative stress in exhaled breath condensate as well as urine. But the relevant role of free MDA in plasma/serum, nasal fluid, or saliva to predict total MDA remained controversial and dismal. Different proportions of free MDA and conjugated MDA (MDA-adducts) in different clinical samples might account for these discrepancies [59,60]. In the present study, what we measured was the free plasma MDA rather than MDA adducts and lower levels of plasma MDA were inversely correlated with high $nDNA^{pcf}$ copies in SLE patients (Table 4). It is suggested that large amounts of MDA were underestimated because of the presence of high proportion of MDA-protein adducts in SLE patients.

CLEC5A can drive human immune response to defense viral infections [29,37]. Recent studies showed that CLEC5A is a critical receptor in innate immune system, causing NETosis to deal with Dangue virus [28,37] or Japanese encephalitis virus [61]. Among the 24 HCs, we found that higher levels of leukocyte CLEC5A mRNA level were associated with higher $nDNA^{pcf}$ copies or a lower $mtDNA^{pcf}$ copies, suggesting a possible role of DNA^{pcf} in CLEC5A related immune response (Table 4). High nDNA in the NET is required for neutrophils to trigger suicidal NETosis and to defend against invading pathogens [62]. The present results have indicated that the leukocytes in healthy subjects hold functional CLEC5A to trigger proper immune reaction. In contrast, such function may vanish or be dampened in SLE patients, because the above association becomes absent among SLE leukocyte CLEC5A mRNA transcripts and $nDNA^{pcf}$ copies, suggesting an impaired leukocytes/neutrophils in SLE [46]. Influenza infection in bone marrow-derived macrophages of $CLEC5A^{-/-}$ mice (dampened CLEC5A) would induce an increase in the secretion of IFN-α [29], which is similar to the IFN signature in SLE [26]. Of note, although the added cohort of 3 SLE patients was small in number, the present preliminary results showed an increase of leukocyte CLEC5A mRNA level after the treatment with rituximab (Table 5). Taken together, we propose that an aberrant expression and impaired function of CLEC5A may be implicated in the pathogenesis of SLE.

Similar to CLEC5A, cyclic guanosine monophosphate (GMP) - adenosine monophosphate (AMP) synthase (cGAS) is also a member of PRR to act as an additional first-line host immune defense that can induce type I IFN response through its interaction with downstream stimulator of interferon genes (STING), i.e., the cGAS-STING pathway [63]. Recently, many immunological studies have focused on the cGAS-STING pathway as another player in the pathogenesis of autoimmune diseases, including SLE [64]. It is speculated that cGAS could sense and interact with free plasma intrinsic or extrinsic DNA fragments derived from engulfed NETs to trigger immune cascade [65]. This process would be more potent if the cGAS is stimulated by free mtDNA fragments [66,67]. Intriguingly, in the present investigation, we observed that SLE patients with higher SLEDAI tended to have lower $mtDNA^{pcf}$ copies extracellularly. But we have also demonstrated a lower mtDNA copy number intracellularly in our previous report [35]. In addition, the $mtDNA^{pcf}$ copies was increased after rituximab treatment in severe SLE patients (Table 5). These dynamic alterations of mtDNA strengthen the possibility of the interaction among $mtDNA^{pcf}$ molecules and consumption of mtDNA fragments through PRR, cGAS or CLEC5A, which might contribute to the pathogenesis of SLE.

Viral infection has been proposed to play a role in the SLE pathogenesis. A meta-analysis demonstrated that SLE patients express higher IgG titers to EBV viral capsid antigen (VCA) and early antigen (EA), and exhibit higher positive rates for EBV DNA [68]. Furthermore, thanks to the new computational methods to account for the genetics of human diseases, it has been found that nearly half of SLE risk loci are occupied by the Epstein-Barr nuclear antigen 2 (EBNA2) protein and many co-cluster with human transcription factors [69]. As to the lupus nephritis, some studies have revealed that a higher serum EBV load is associated with renal damage, and the involved kidneys can express higher EBV-latent membrane protein-1 (EBV-LMP1) and EBV-encoded RNA 1 (EBER-1) [70,71]. Notably, higher EBV loads were correlated with higher DNA^{cf} levels in SLE patients [70]. The measurement of DNA^{pcf} copies to dissect the interrelations between EBV infection and self-antigen presentation in SLE requires further investigations.

5. Conclusions

In conclusion, we have demonstrated that higher $nDNA^{pcf}$ copies, lower $mtDNA^{pcf}$ copies, increased ROS-elicited oxidative DNA damages and dysregulated leukocyte CLEC5A expression might participate in the pathogenesis of SLE. Further evaluation of the underlying mechanisms in this regard is necessary in the future.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

HTL, CSL, CYT and YHW designed the study. HTL, CSL and SCP carried out the experiments. HTL, CSL, SCP and WSC wrote the original draft. CYT and YHW reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of Taipei Veterans General Hospital (IRB No: 2013-04-043B).

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Conflict of Interest

The authors declare no conflict of interest. Given his role as Guest Editor and Editorial Board Member, Yau-Huei Wei had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Pietro Gentile.

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