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Microparticles in the blood of patients with SLE: Size, content of mitochondria and role in circulating immune complexes

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ABSTRACT

Objective: Microparticles (MPs) are small extracellular vesicles released from apoptotic or activated cells through a blebbing process. MPs express surface molecules from their parental cells and they bind IgG to form circulating immune complexes (MP-ICs) in patients with systemic lupus erythematosus (SLE). Through investigation of MP size, IgG expression, content of nucleic acids and mitochondrial molecules, we hypothesized that unrecognized particle populations can be identified in SLE.

Methods: We investigated 327 well-characterized SLE patients and 304 controls divided into two sets (280/280 and 47/24). We measured MPs by flow cytometry using a gating strategy to encompass small (0.2–0.7 μm) and large (0.7–3.0 μm) MPs. Nucleic acids were labeled with SYTO 13 and mitochondria with MitoTracker. Expression of mitochondria markers TOM-20 and Hexokinase 1 and the presence of IgG was investigated.

Results: MPs staining with SYTO 13 were more frequent in 280 SLE patients compared to 280 controls. In 47 SLE patients, levels of large MPs were elevated compared to 24 controls. The majority of large MPs contained mitochondria (mitoMPs). The number of mitoMPs associated positively with high disease activity, anti-dsDNA antibodies and pro-inflammatory cytokines. Patients with active lupus nephritis had higher levels of mitoMPs and IgG-positive mitoMPs.

Conclusion: Blood of patients with SLE contain a previously unrecognized population of circulating large MPs with bound IgG and mitochondrial proteins. Levels of these particles are related to several measures of active SLE, suggesting that these structures may have a role in disease pathogenesis.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease that primarily affects young women and leads to inflammation and injury of multiple organs especially the kidneys [1]. A key element in disease pathogenesis is the expression of antibodies to nuclear molecules (antinuclear antibodies or ANAs), including DNA, RNA and proteins that associate with nucleic acids [2,3]. In SLE, the load of circulating autoantigens is likely increased because of either enhanced cell death or impaired clearance of dead and dying cells [4]. During active disease, ANAs can bind to their target autoantigens to form immune complexes (ICs) which bind complement. When these complexes are deposited in tissues, they can induce organ manifestations e.g. nephritis and skin lesions and damage [5–7]. Furthermore, ICs can be internalized by innate immune cells where they stimulate the production of type 1 interferons through activation of nucleic acid receptors [8].

In addition to increases in cell death and impaired clearance of dead and dying cells, the course of SLE is marked by the activation of lymphoid and myeloid cells as well as platelets [9,10]. Importantly, during apoptosis and activation of these cells, downstream signaling events lead to the extracellular release of microparticles (MPs) by a blebbing process [11]. MPs are small extracellular vesicles that can be defined in terms of size, with usual dimensions considered to be 0.1–1.0 μm in diameter [12,13]. In terms of composition, MPs carry a wide variety of cytoplasmic and nuclear components including DNA and RNA [14]. MPs expose phosphatidylserine (PS) on the outer surface but, in contrast to MPs from control subjects, the majority of MPs in SLE do not

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expose PS [15,16]. Consistent with their content of nuclear autoantigens, MPs in the blood of SLE can bind to immunoglobulins and form ICs [17–19].

Among other subcellular structures that appear in the blood, mitochondria are immunologically active and represent novel players in the pathogenesis of immune-mediated disease [20–22]. Like MPs, mitochondria can be released from cells during cell activation and cell death and display properties similar to those of MPs [20]. In addition to acting as particles, mitochondria can be components of MPs [23]. In view of their presumed origin as endosymbiotic bacteria to control energy metabolism, mitochondria share many similarities with prokaryotes and express immunostimulatory molecules. Among these molecules, mitochondrial DNA (mtDNA) can induce immune responses because of its content of CpG motifs; mtDNA may be particularly prone to oxidation because of its proximity to the electron transport chain, thereby affecting its immunological properties [22,24].

In the present study, we have characterized MPs in the blood of SLE patients and their content of nucleic acids and mitochondrial molecules using flow cytometry. Because of evidence that extracellular vesicles are heterogeneous in size as well as composition, we modified the gates to also include measurement of larger particles. Using a novel gating strategy to capture particles as large as approximately 3 μm, our results demonstrate that large MPs express more mitochondrial molecules (mitoMPs) than smaller particles and that large MPs are more frequent in ICs in SLE. We observed positive associations between levels of mitoMPs and major SLE features, suggesting that mitoMPs play a role in disease pathogenesis.

2. Patients and Methods

2.1. Study population

We studied the expression of MPs in the blood of patients in a large cohort of consecutive SLE patients, who received care at the Department of Rheumatology, Karolinska University Hospital between 2004 and 2011. All patients fulfilled at least four of the 1982 revised classification criteria for SLE according to the American College of Rheumatology [25]. SLE disease activity was assessed by SLAM [26]. Renal activity was assessed by the British Isles lupus assessment group (BILAG) index [27]. Analysis of nuclear content in MPs was performed in 280 SLE patients and 280 “SLE-free” population controls, individually matched to the SLE patients for age, sex and region of living. Detailed description of patient and control characteristics is found elsewhere [15].

Analyses of mitochondrial molecules were performed in a separate, not overlapping set of 47 SLE patients from the same cohort. For these experiments, we included 24 healthy control subjects matched to the SLE patients for age and sex. As a disease control, we included 20 patients with acute coronary syndrome (ACS), referred to as cardiovascular disease (CVD) controls. Samples were drawn 3–5 days after the ACS. Blood from all participants were collected using the same standardized protocol. Blood samples were drawn into citrated tubes and centrifuged within 1 h at 2 570 g for 20 min in room temperature (RT) and subsequently stored at −80° as platelet-poor plasma (PPP). The local ethic committee at Karolinska Institutet approved the study and all experiments were performed in compliance with good clinical practice and laboratory practice. All study subjects gave written informed consent to participate.

2.2. Laboratory tests

High-sensitivity (hs) CRP, fibrinogen and creatinine were measured with BN ProSpec System (Dade Behring, Deerfield, IL, USA). Cystatin C was analyzed on Architect Ci8200 analyzer with cystatin C reagents from Gentian (Moss, Norway). Estimated (e) glomerular filtration rate (GFR) was calculated from serum cystatin C (eGFRcystatin C) results in mg/L as previously described [28]. TNF-α, interleukin (IL)-6, IL-8, Interferon-γ-induced protein 10 (IP-10), Monocyte chemotactic protein 1 (MCP-1) were analyzed in EDTA plasma using a multiplex assay (K15054D; Mesoscale Discovery, Gaithersburg, MD). Antinuclear antibodies (ANA) were analyzed by indirect immunofluorescence (IFL) on HEp-2 cells (ImmunoConcepts, Sacramento, CA, USA). Antibodies to specific nuclear antigens (dsDNA, SSA-Ro52, SSA-Ro60 and SSB) were analyzed by multiplexed bead technology (Luminex) using BioPlex 2200 system (Bio-Rad, Hercules, CA, USA).

3. Flow cytometric analysis of MPs and mitoMPs

3.1. Detection of nuclear content in MPs

Frozen PPP from 280 SLE patients and 280 matching controls was thawed in a water bath at 37 °C for 5 min and centrifuged at 2 000*g* for 20min at RT. The supernatant was re-centrifuged at 13 000*g* for 2 min at RT. Subsequently, 20 μl of the supernatant was incubated for 20 min in the dark with 5 μl of SYTO 13 (Invitrogen, Paisley, UK) together with anti-IgG-PE (Abcam, Cambridge, UK), in order to determine nuclear content of MPs. MPs were identified on the basis of size and considered to be between 0.3 and 1.0 μm in diameter and positive for SYTO 13. The MP gate was determined using Megamix-plus beads (BioCytex, Marseille, France), which is a mix of beads with diameters of 0.1 μm, 0.3 μm, 0.5 μm and 0.9 μm.

3.2. Detection of small and large MPs and mitoMPs, in 47 SLE patients

Frozen PPP from 47 SLE patients, 24 healthy controls and 20 CVD controls, were thawed and first centrifuged at 2 000*g* for 20 min at RT. The upper part of the supernatant was transferred to new tubes and centrifuged for 20 800*g* for 45 min, at RT. The supernatant was discarded, and the particle enriched pellet was used for analysis. Subsequently, 20 μL of the pellet was incubated 10 μl MitoTracker deep red FM (500 nM, Invitrogen, Paisley, UK) to assess mitochondrial content. Staining for mitochondrial protein was performed with 5 μl anti-TOM20-FITC, 5 μl anti-hexokinase 1 (HK1) Dylite 755 (Abcam, Cambridge, UK). Anti-IgG-FITC (5 μl) was also added to detect immune complexes (Abcam, Cambridge, UK).

The flow cytometric protocol was modified in order to include larger MPs compared to conventional definition on MPs. As seen is Fig. 1A, MPs were gated in two separate gates, based on forward scatter (i.e., size); small MPs (~0.3–0.7 μm) and large MPs (~0.7–3.0 μm). The gates were determined using Megamix-plus beads as described above, together with a 3.0 μm bead (Spherotech Inc, Lake Forest, IL, USA). Later, each subpopulation was investigated for 1) mitochondria content (MitoTracker positive) and 2) expression of TOM20 and/or HK1 as well as IgG. Representative dot-plots are shown in Fig. 1B–C. All samples were measured on a Beckman Gallios instrument (Brea, CA, USA). Conjugate isotype-matched immunoglobulin with no reactivity against human antigens was used as a negative control to define the background noise of the cytometric analysis. The intra- and inter-assay coefficients of the flow cytometric analysis were less than 9.0% respectively.

3.3. Phenotyping mitoMPs

In order to investigate the origin of mitoMPs we labeled mitoMPs against a platelet marker and T-Cell marker. Briefly, an MP-enrich pellet was obtained as described above. 20 μL of the pellet was incubated with 10 μl of MitoTracker deep red FM together with 5 μl CD42a-FITC (GPIX) and CD3-Dylite 755 (Both Beckman Coulter, CA, USA). After 20 min incubation in the dark, the samples were fixed using BD Cellfix and measured with the same panel as described above. Conjugate isotype-matched immunoglobulin with no reactivity against human antigens was used as a negative control to define the

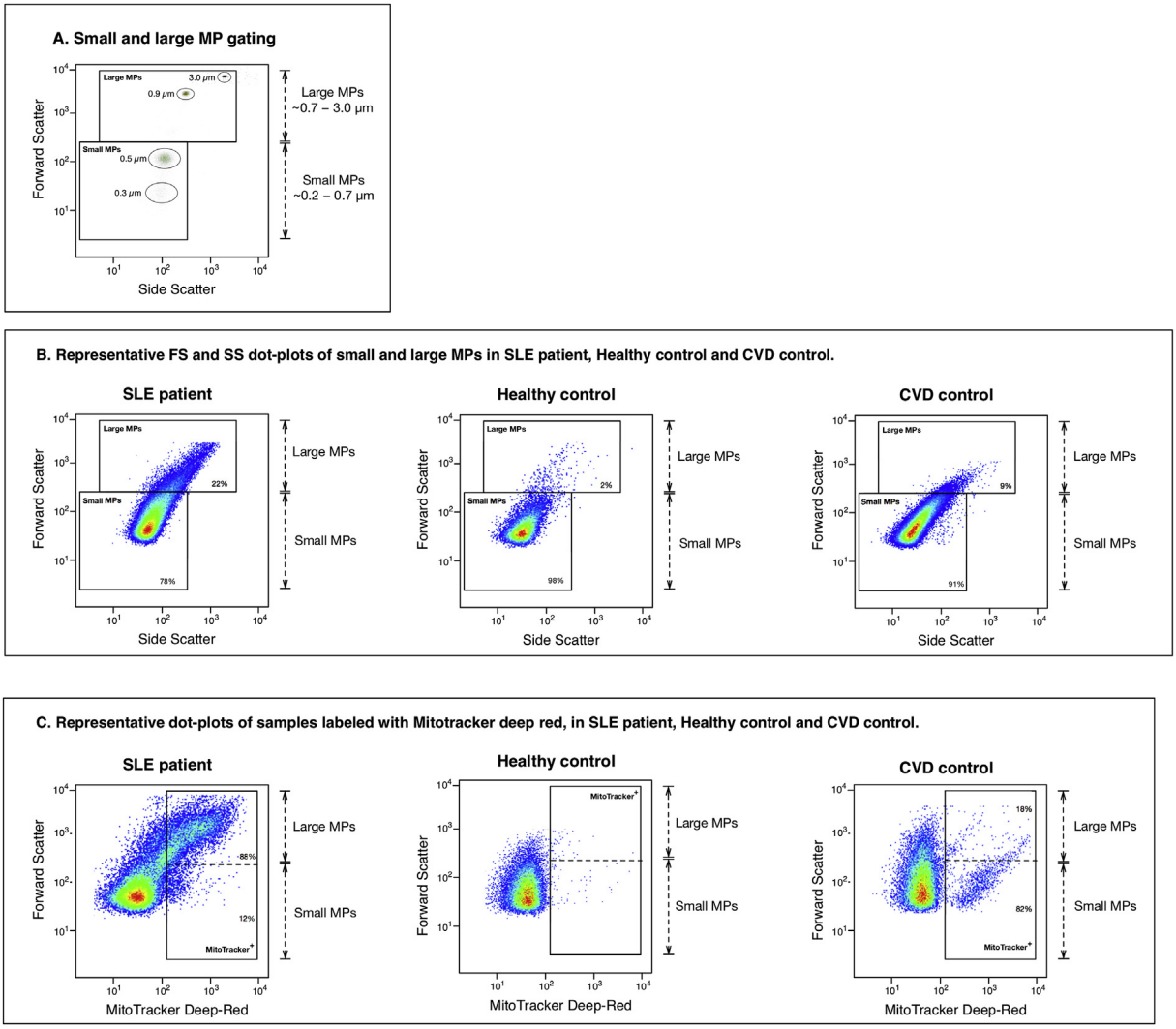

Fig. 1. Flow cytometric analysis of small and large MPs and mitoMPs. Representative dot plots demonstrating: A) Gating strategy for small and large MPs based on beads calibration. B) Representative samples demonstrating concentration of small and large MPs in SLE patients, healthy controls and CVD controls. C) Representative dot plots of SLE patients, healthy controls and CVD controls labeled with MitoTracker.

background noise of the cytometric analysis.

3.4. Statistical analysis

Prior to statistical analysis, data were log transformed, if necessary, to obtain a normal distribution. Patients and controls were compared with t-test. Within the patient group, associations between MP subtype and clinical/laboratory characteristics were investigated with linear regression or t-test, and results are reported as direction of association and p-value. The Bonferroni correction was used to adjust for multiple comparisons. A p-value ≤ 0.05 was considered significant. Statistical analysis was performed using JMP software (SAS Institute, v12.0, Cary, North Carolina, USA).

4. Results

4.1. Nucleic acids and IgG in MPs from SLE patients and matched controls

Supplement Table 1 presents the basic clinical characteristics of the SLE patients and controls. Using plasma from these subjects, we determined the number of MPs containing nucleic acids (SYTO 13^+) and expression of IgG on the surface of MPs. As these results indicate, MPs with nucleic acid and IgG are more frequent in the blood of SLE patients compared matched controls ($p < 0.0001$, Fig. 2).

4.2. Large MPs and mitoMPs in SLE patients

Having established information on the MP content of nucleic acids and expression of IgG in SLE patients and controls, we analyzed blood from 47 additional patients to investigate the mitochondrial content of particles and the relationship to IgG expression. We also analyzed blood from 20 healthy and 24 CVD controls.

In these experiments, we modified the gating strategy in view of studies indicating that MPs in immune complexes may be larger than the usual upper size limit for MPs of 1.0 μm [23,29]. Initially, we investigated the distribution of small (~0.3–0.7 μm) and large MPs (0.7–3.0 μm) (Fig. 3). Blood from SLE patients had higher levels of large MPs than both healthy and CVD controls ($p < 0.0001$ and $p < 0.001$, respectively Fig. 3A). The levels of small MPs were significantly higher in SLE and CVD controls compared to healthy controls. ($p < 0.0001$, Fig. 3A).

4.3. Distribution of particle types

In SLE patients, the majority of the mitochondria positive particles (MitoTracker positive), referred to as *mitoMPs*, were observed in the

Table 1
Associations between various subtypes of microparticles; large and small microparticles and mitoMP phenotype, and SLE features in 47 patients.

	MPs^{LARGE}	MPs^{SMALL}	mitoMPs	$mitoMPs^{TOM20+HK1}$	$mitoMPs^{IgG}$
Clinical variables					
Age	ns	ns	ns	ns	ns
Gender female	ns	ns	ns	(+) p = 0.007	ns
Disease duration	ns	ns	ns	ns	ns
Smoking (Current)	ns	ns	ns	ns	ns
SLAM ≥6	ns	ns	ns	(+) p < 0.03	ns
Autoantibodies					
Anti-dsDNA	p = 0.0065	p = 0.010	(+) p = 0.0001	(+) p = 0.005	(+) p = 0.0001
Anti-Nucleosome	ns	ns	(+) p = 0.0001	(+) p = 0.008	(+) p = 0.0001
Anti-SSA	ns	ns	ns	ns	ns
Anti-SSB	ns	ns	ns	ns	ns
Inflammation markers					
Signs of ongoing renal lupus: < !–Soft-enter Run-on– > activity (BILAG A-C)	ns	ns	(+) p = 0.014	ns	(+) p = 0.010
TNFα (pg/mL)	(+) p = 0.0003	(+) p = 0.007	(+) p = 0.002	(+) p = 0.0003	(+) p = 0.01
IL-6 (pg/mL)	(+) p = 0.02	ns	(+) p = 0.0012	ns	(+) p = 0.006
IL-8 (pg/mL)	ns	ns	(+) p = 0.037	(+) p = 0.01	ns
IP-10 (pg/mL)	(+) p = 0.03	ns	(+) p = 0.032	(+) p = 0.003	ns
MCP-1 (pg/mL)	(+) p = 0.01	ns	(+) p = 0.008	(+) p = 0.0001	(+) p = 0.025
MPO (pg/mL)	(+) p = 0.01	ns	(+) p = 0.004	(+) p = 0.03	(+) p = 0.015

Values are given as direction [+/−] and MP phenotype. p-values (t-test) represent adjustment according to Bonferroni test. ns = non significant. HK1 = Hexikinase 1; BILAG = British Isles lupus assessment group; TNFa = Tumor necrosis factor alpha; IL = Interlukin; IP10 = Interferon gamma-induced protein 10; MCP-1 = Monocyte Chemoattractant Protein-1; MPO = Myeloperoxidase.

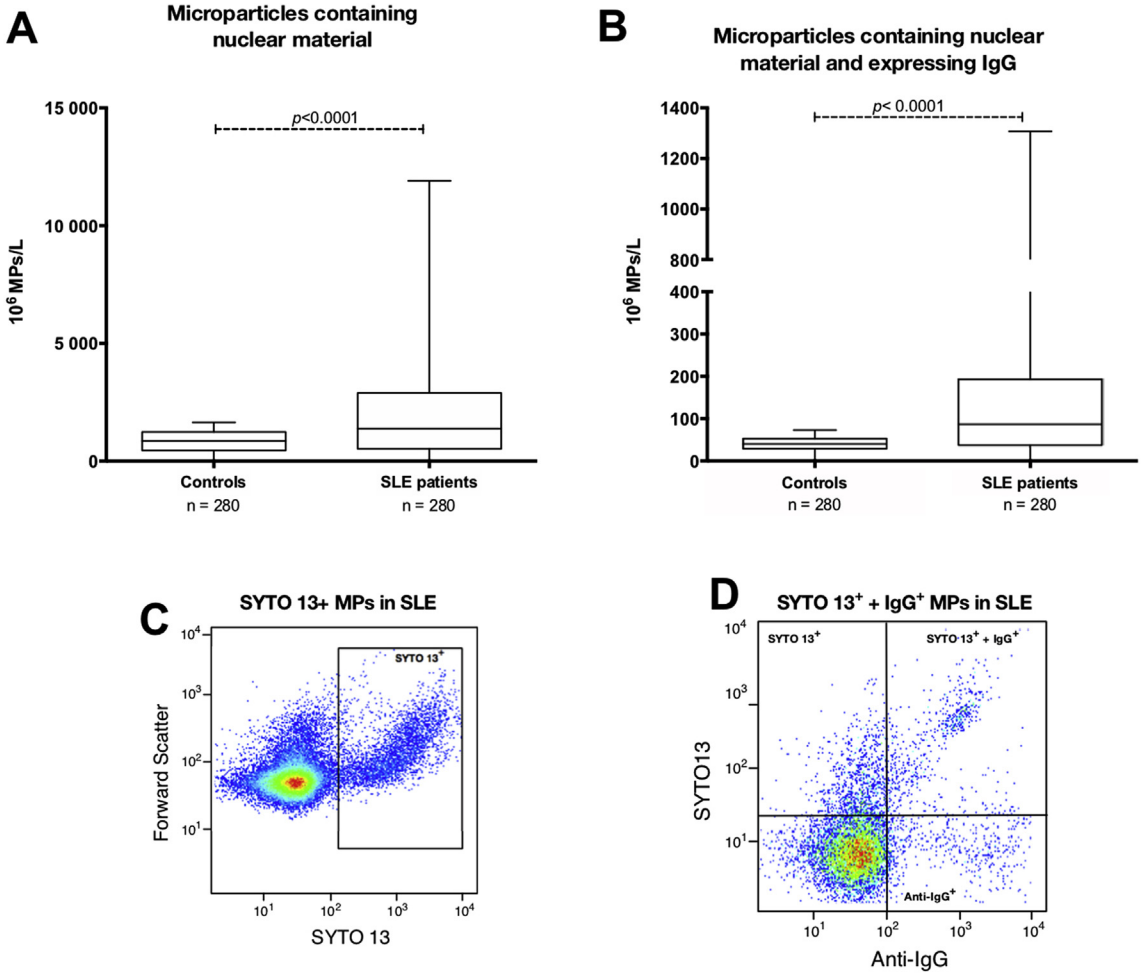

Fig. 2. Microparticles (MPs) containing nuclear material (DNA and/or mRNA) and expressing IgG in 280 patients with systemic lupus erythematosus and 280 controls. (A) MPs defined by size and SYTO 13 binding by flow cytometry. (B) MPs defined by size, SYTO 13 binding and binding of anti-IgG by flow cytometry. C-D) Representative flow cytometric plots demonstrating SYTO 13^+ MPs and SYTO 13^+ MPs co-expressing IgG. Bars represent median values and line indicates min and max values.

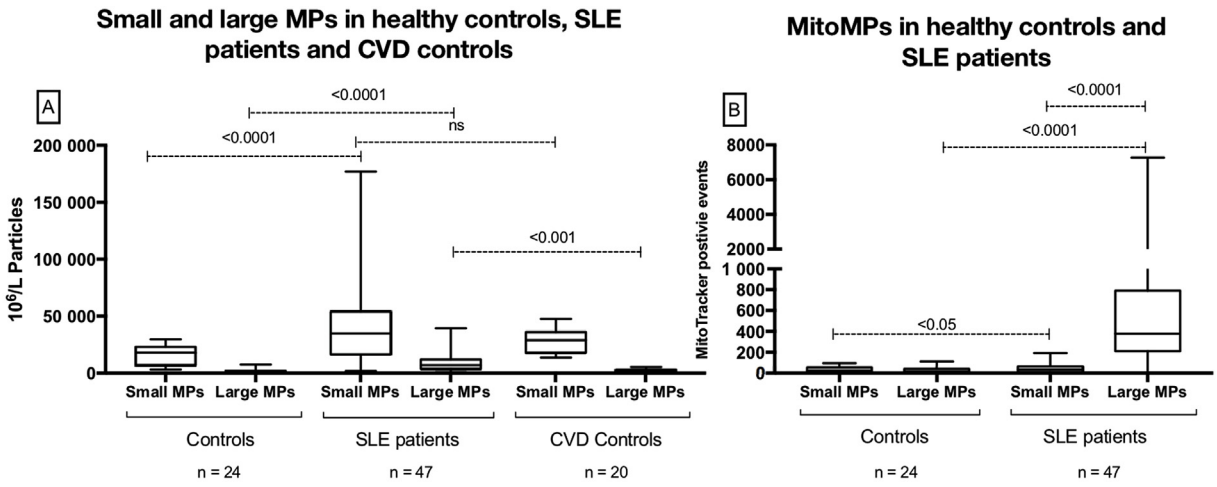

Fig. 3. Small and large MPs and mitoMPs in patients with systemic lupus erythematosus, healthy controls and disease controls. (A) Number of small and large MPs defined by size, analyzed by flow cytometry (B) Number of mitoMPs i.e. large MPs containing mitochondria, defined by size and MitoTracker positivity. Bars represent median values and line indicates min and max values.

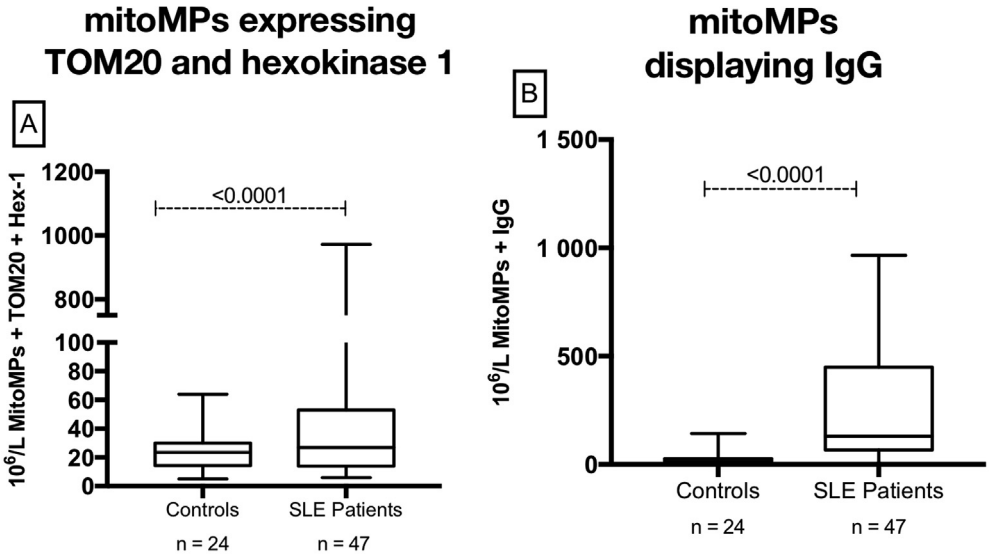

Fig. 4. mitoMPs in 47 patients with systemic lupus erythematosus and 24 healthy controls. (A) Number of mitoMPs expressing outer mitochondria markers TOM20 and hexokinase 1. (B) Number of mitoMPs displaying IgG. Bars represent median values and line indicates min and max values.

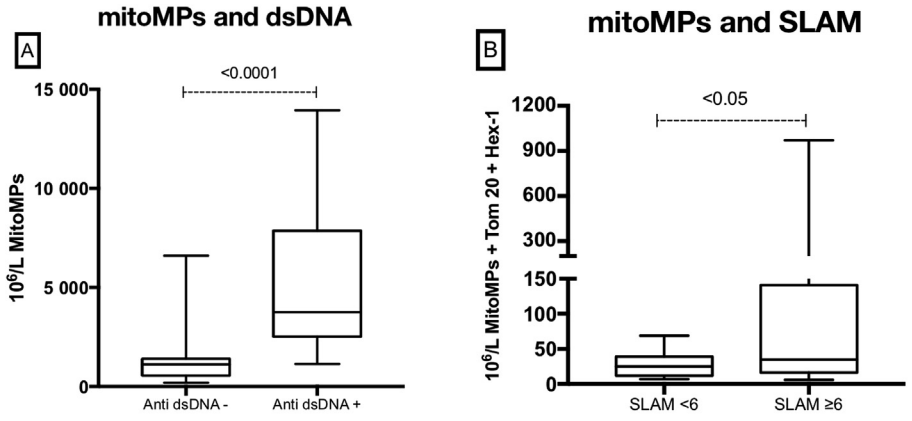

Fig. 5. mitoMPs and clinical features. (A) Number of mitoMPs in 47 SLE patients negative or positive for dsDNA antibodies. (B) Number of mitoMPs expressing TOM20 and hexokinase-1 in 47 SLE patients with and without active disease, as measured by SLAM (equal or above 6). Bars represent median values and line indicates min and max values.

large MP gate (See Fig. 1B for plots and Fig. 3B for levels). mitoMPs also exposed outer mitochondria markers TOM20 and HK1 more frequently compared to smaller MPs (See supplement Fig 1 for dot plots and Fig. 4A for concentrations). Moreover, the majority of the IgG-positive particles were located in the larger MP gate, although smaller MPs were also positive for IgG (Supplement Fig 2). The levels of mitoMPs displaying IgG were significantly higher in SLE patients compared to healthy controls (Fig. 4B). Approximately, one third of the mitoMPs

were IgG positive.

4.4. Phenotyping mitoMPs

mitoMPs were phenotyped based on platelet or T-cell origin. Results demonstrated that the majority of the mitoMPs were of platelet origin ($CD42a^+$; 829; 197; 5171) compared to T-cell origin ($CD3^+$; 276; 64; 1551).

4.5. Associations between mitoMPs and SLE features

Next, we analyzed the relationship between different MP populations and clinical features of SLE. The number of large MPs associated more strongly with typical SLE features than did the number of small MPs (Table 1). In addition, levels of mitoMPs correlated significantly with the presence of anti-dsDNA ($p < 0.0001$, Fig. 5A) and anti-nucleosome ($p < 0.0001$, Table 1). No association with anti-SSA and/or anti-SSB was observed (Table 1). Patients with higher disease activity as defined as a SLAM ≥6 had higher numbers of mitoMPs displaying outer mitochondria markers TOM20 and HK1 ($p < 0.05$, Fig. 5B). Also, female patients had significantly higher concentrations of mitoMPs expressing outer mitochondria markers ($p < 0.05$, Table 1). Moreover, various phenotypes of mitoMPs were associated with levels of pro-inflammatory cytokines (Table 1). Patients with signs of ongoing renal lupus activity (BILAG Index A-C) had significantly higher levels of mitoMPs and IgG-coated mitoMPs (Table 1). No correlations.

5. Discussion

These studies provide new insights into the expression of MPs in SLE by employing a gating strategy to assess a wider size range of particles than usual flow cytometric approaches. By this means, we identified a population of large MPs that appeared in much greater abundance in the blood of patients with SLE compared to controls. We also demonstrated that the population of large MPs contained mitochondria as shown by MitoTracker staining as well as the hexokinase 1 and TOM20 mitochondrial proteins; such MPs can be termed mitoMPs. Since levels of mitoMPs were associated with higher disease activity, positivity for anti-dsDNA and levels of pro-inflammatory cytokines, these studies provide evidence for a role of MPs and mitochondria in clinically important SLE events.

In these studies, we first used staining with SYTO 13 to demonstrate a significant increase in the number of MPs containing nucleic acids in SLE. SYTO 13 binds both DNA and RNA, although it has preference for double-stranded DNA [30]. As previously shown [31], the binding of SYTO 13 by MPs released from apoptotic cells *in vitro* reflects the presence of both DNA and RNA, likely resulting from translocation of nuclear molecules into particles as cell death proceeds. Since apoptotic cells can produce MPs containing nucleic acids, our findings suggest that the MPs in lupus blood result from apoptosis; this possibility is consistent with previous studies showing enhanced apoptosis and impaired clearance of apoptotic cells in SLE [4,32]. In addition, SYTO 13^+ MPs present in patient blood displayed IgG, indicating that, similar to particles generated *in vitro*, MPs *in vivo* are antigenically active.

In studies to delineate the relationship of MPs to disease activity, we divided the MPs by size into two sub-populations: small and large MPs. This strategy is based on recent reports that MPs can appear as larger particles by flow cytometry and be components of immune complexes [17,29]. In addition, MP subpopulations that express IgG have been shown to associate with clinical SLE characteristics such as higher disease activity and more vascular damage [17]. Our studies on the properties of large MPs also relates to the presence of mitochondria in these structures. As shown in recent studies, MPs released during platelet activation can contain mitochondria as internal components, but cells may also release free mitochondria [23,33].

In our experiments, we demonstrated the presence of mitochondria in MP populations by labeling with MitoTracker deep red FM, a cell-permeable dye that accumulates in active mitochondria. Our findings indicate that the majority of the MitoTracker positive events (~80–90%) were present in the large MP gate, suggesting that mitochondria are more frequent in larger particles compared to smaller MPs. The diameter of mitochondria is approximately 200–500 μm [23,33] as such, MPs that contain mitochondria may be larger than MPs not containing mitochondria.

While our study has focused on blood from patients with SLE, the presence of large MPs may not be specific for this disease. As shown in a study by Cloutier and colleagues on synovial fluid of patients with rheumatoid arthritis (RA), MPs can be heterogeneous in size and include a subpopulation of the size range of approximately 700–3000 nm [29]. Furthermore, in this study, the larger particles were associated with IgG, thus representing ICs. In contrast, the small particles in synovial fluid were not associated with IgG. Interestingly, in the study of Cloutier and colleagues, synovial fluid from patients with psoriatic arthritis lacked large particles and contained few MP-ICs. That study, however, did not assess the properties of MPs in blood although other studies have indicated that, in contrast to synovial fluid, RA blood does not contain MP-ICs.

Other evidence that particles may be larger in size comes from studies on the properties of particles in the context of malignancy. These studies have identified a population of extracellular vesicles that have been termed large oncosomes [34]. Large oncosomes range in size from greater than 1 to greater than 10 μm. While the origin of these structures and their relationship to malignancy have not yet been elucidated, the findings on large oncosomes nevertheless suggest that the size range of particles may be greater than usually considered. As a result, studies using gating strategies with 1 μm as an upper limit in size may miss detection of large particles. Together, these findings suggest that the appearance of large particles may not be specific to SLE although appearance of MP-ICs in either the blood or other fluid (e.g., synovial fluid) may depend on the source of MPs as well as the amount and specificity of autoantibodies.

As shown in studies on patients as well as in animal models, extracellular mitochondria occur in the blood in a variety of clinical settings and can stimulate inflammation [23,24,35]. Because of molecular similarities with bacterial PAMPs (pathogen associated molecular properties), mitochondrial components can act as DAMPs (damage associated molecular patterns) and stimulate innate immunity. In addition to labeling samples with MitoTracker, we used antibodies to TOM20 and HK1 to determine if mitochondrial proteins are present in an immunologically accessible form in mitoMPs. Such accessibility could result from permeability of the mitochondria or MPs to allow antibody binding; accessibility could also result from the presence of the enzymes on the surface of mitochondria or MPs, perhaps related to translocation events that occurs *in vivo*. Interestingly, the majority (~61%) of the TOM20 and HK1 positive events were present in the mitoMP population. Since we can also detect IgG in the mitoMP population, mitoMPs may be a preferential source of ICs. Alternatively, once bound by IgG, these structures may become larger than usual MPs.

When using gates that detect larger MPs, the number of particles is strongly associated with important features of SLE such as levels of anti-dsDNA, disease activity and levels of pro-inflammatory cytokines (Table 1). This association is even greater with mitoMPs and its phenotype (TOM20/HK 1 and IgG positive). Importantly, levels of mitoMPs revealed a strong association with levels of anti-dsDNA and anti-nucleosome antibodies; these findings contrast to those of a previous study which failed to demonstrate major associations between levels of MPs of "conventional" size (0.1–1.0 μm) and SLE features [15]. Other investigators have shown that the levels of MPs in immune complexes correlate with clinical features in both rheumatoid arthritis and SLE and our results are consistent with these reports [17,29].

In the course of SLE, mitoMPs containing mitochondria could increase the accessibility of extracellular mitochondria, with the intrinsic

immunological properties of mitochondria driving inflammation. Indeed, the positive association of levels of mitoMPs with levels of pro-inflammatory cytokines suggests that these mitoMPs could promote immune cell activation and inflammation [36–38]. In particular, patients with signs of ongoing renal lupus activity (BILAG, A-C) had significantly higher levels of mitoMPs and IgG-coated mitoMPs in our study, suggesting that IgG-coated mitoMPs may also deposit in renal tissue during active nephritis. In this regard, Nielsen et al. have provided evidence that MP components are present in the kidney in SLE, supporting this possibility [39].

This study has a number of limitations. Thus, while we have demonstrated the presence of larger mitoMPs, we have not defined a mechanism to account for the larger size. Larger mitoMPs could be the results of aggregation of several smaller particles by IgG, which could bind or cross-link particles together. Alternately, the larger mitoMPs may represent an apoptotic body or like structure. Similarly, we have not shown a mechanism by which mitochondria or mitochondrial molecules appear in the larger particles. While a larger particle may be better able to accommodate an intact mitochondrion, a smaller particle could contain either a fragmented mitochondria or mitochondrial proteins that have translocated into the cytoplasm. Finally, while our data suggest correlations between levels of mitoMPs with disease activity in cross-sectional studies, the utility of this marker in longitudinal studies has not been established.

At present, the analysis of extracellular vesicles is undergoing important changes related to improvement in instrumentation, advances in analytic strategies and elucidation on different particle subtypes or subpopulations. Thus, while, MPs are often phenotyped according to size (between 0.1 and 1.0 μm) and expression of PS [40,41], we and others have shown that the majority of MPs in SLE are negative for PS as measured by annexin V or lactadherin staining; this situation could occur because of the shielding of PS by autoantibodies possibly through intermediate binding of antigenic scavenger molecules like β2-glycoprotein-I [16,42]. In the present study, we have gone beyond the classical definition of MPs, incorporating a gating strategy that allows assessment of particles in the size range of 0.7–3.0 μm. Ordinarily, in MP analysis, these larger particles would not be measured since their size exceeds the usual upper limit of 1.0 μm. It is important to note that the refractive index of beads and MPs differs [43]. Thus, the cut-off presented in the present study are based on the bead size. Taken together, our findings suggest that events in SLE pathogenesis may lead to the occurrence of larger MPs differing in composition and phenotypic properties from those encountered in other settings. Future studies will investigate the basis of these differences and the utility of different MP phenotypes as biomarkers of disease activity in SLE.

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Declaration of conflicting interests

SE is an employee of AstraZeneca. All other authors declare that they have no conflicting interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.05.003.

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