

Increased IgG on Cell-Derived Plasma Microparticles in Systemic Lupus Erythematosus Is Associated With Autoantibodies and Complement Activation

Christoffer T. Nielsen,¹ Ole Østergaard,¹ Line Stener,¹ Line V. Iversen,² Lennart Truedsson,³ Birgitta Gullstrand,³ Søren Jacobsen,⁴ and Niels H. H. Heegaard¹

Objective. To quantify immunoglobulin and C1q on circulating cell-derived microparticles (MPs) in patients with systemic lupus erythematosus (SLE) and to determine whether immunoglobulin and C1q levels are correlated with clinical and serologic parameters.

Methods. Sixty-eight clinically well-characterized SLE patients, 38 healthy controls, 6 patients with systemic sclerosis (SSc), and 6 patients with rheumatoid arthritis (RA) were included. The numbers of annexin V-binding MPs displaying IgG, IgM, or C1q were enumerated by flow cytometry. MP protein levels were determined by mass spectrometry in clinically defined subsets of SLE patients and controls. The MP IgG load was determined by flow cytometric analysis of all samples from SLE patients and healthy controls.

Results. SLE patients had significantly increased total and relative numbers of IgG-positive MPs ($P = 0.0004$), with a much higher average IgG load per MP ($P < 0.0001$) than healthy controls. Quantitative mass spectrometry of purified MPs verified significantly in-

creased IgG, IgM, and C1q levels in SLE patients. In RA and SSc patients, the average IgG load per MP was significantly lower than in SLE patients ($P = 0.006$ and $P = 0.05$, respectively). Also, the IgM load and C1q load per MP were significantly higher in SLE patients than in the control groups ($P < 0.05$), except for IgM in the RA group. IgG-positive MPs were significantly associated with the presence of anti-double-stranded DNA, anti-extractable nuclear antigen, and antihistone antibodies, with total IgG, and with decreased leukocyte counts. Average IgG load per MP was associated with lower concentrations of MPs, the presence of anti-C1q antibodies, and complement consumption.

Conclusion. Our findings indicate that circulating cell-derived MPs in SLE patients carry increased loads of IgG, IgM, and C1q and that IgG MPs are associated with autoantibodies and complement activation. The findings link immunologic reactions on MPs with the etiology of SLE.

Systemic lupus erythematosus (SLE) is an autoimmune disease with a wide range of clinical manifestations (1). One of the serologic hallmarks of SLE is the presence of circulating, high-affinity autoantibodies against nuclear constituents (2). These autoantibodies may form circulating proinflammatory immune complexes (ICs) that directly trigger plasmacytoid dendritic cells (PDCs) and the complement system, e.g., in the kidneys (3). Also, IC and autoantibody activation of SLE neutrophils prone to NETosis, i.e., specialized neutrophil cell death, contributes to the sustained PDC activation that is typical of SLE (4,5). The etiology of antinuclear autoimmunity in SLE remains unclear, but persistent, poorly cleared circulating cellular remnants, including microparticles (MPs) and neutrophil extracellular traps, are likely sources of immunogenic autoantigens (6–9).

Supported by the Danish Rheumatism Association (grant 233-527-17.10.05), the Lundbeck Foundation (grant 39/06), the Lægeforeningens Forskningsfond, the Fonden til Lægevidenskabens Fremme, the Aage Bang Foundation, and the Novo Nordisk Foundation (grant 5990 to Dr. Jacobsen).

¹Christoffer T. Nielsen, MD, Ole Østergaard, MSc, PhD, Line Stener, MSc, Niels H. H. Heegaard, MD, DSc(Med): Statens Serum Institut, Copenhagen, Denmark; ²Line V. Iversen, MD: Statens Serum Institut and Bispebjerg Hospital, Copenhagen, Denmark; ³Lennart Truedsson, MD, PhD, Birgitta Gullstrand, PhD: Lund University, Lund, Sweden; ⁴Søren Jacobsen, MD, DSc: Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark.

Drs. Nielsen and Østergaard contributed equally to this work. Drs. Jacobsen and Heegaard contributed equally to this work.

Address correspondence to Niels H. H. Heegaard, MD, DSc(Med), Department of Clinical Biochemistry and Immunology, Statens Serum Institut, Building 85, Room 240, Artillerivej 5, DK-2300 Copenhagen S, Denmark. E-mail: nhe@ssi.dk.

Submitted for publication July 1, 2011; accepted in revised form January 5, 2012.

Circulating cell-derived MPs are heterogeneous submicrometer-sized vesicles that are shed from the surface of cells, constitutively or during activation or apoptosis (10,11). Apoptotic MPs formed *in vitro* contain nuclear autoantigens that are accessible to autoantibodies (6,12,13). IgG from SLE patients and patients with other systemic autoimmune diseases associated with antinuclear antibodies (ANAs) binds late apoptotic cells, inhibiting their uptake by monocyte-derived macrophages (14). Extrapolated to *in vivo* conditions, this suggests an increased load of apoptotic material that cannot be cleared in SLE patients, consistent with murine models of SLE (15).

Other studies have shown that SLE sera may increase phagocytosis of necrotic cells in an autoantibody- and complement-dependent process (16–18). Only a few studies of SLE have addressed the presence of circulating MPs and their endogenous surface cargo. In a study published in 2002, no difference was found in the number of C1q-positive MPs in SLE patients and healthy controls (19). Recently, however, it was shown that anti-DNA autoantibodies from SLE patients may bind to MPs from apoptotic cell lines, suggesting that this could take place *in vivo*. Accordingly, ICs with particle properties were found with higher frequency in SLE patients than in controls (13). We have recently shown that circulating MPs are heterogeneous and represent both cell-derived MPs and material of unknown origin in the same size range (20). The cell-derived MPs can be defined by their binding of annexin V.

The aim of the present study was to evaluate the putative role of MPs in SLE as circulating antigenic targets and carriers of ICs. We quantitated the IgG, IgM, and C1q associated with annexin V-binding (*i.e.*, cell-derived) MPs from healthy controls, disease controls, and clinically well-characterized SLE patients, using the independent but complementary techniques of flow cytometry and mass spectrometry.

PATIENTS AND METHODS

Patients. Sixty-eight SLE patients (62 women and 6 men) who fulfilled the American College of Rheumatology (ACR) criteria for SLE as updated in 1997 (21) were included in the study. Their median age was 39 years (range 21–76 years), and the median disease duration was 10 years (range 0–37 years). At inclusion, 46 patients were taking disease-modifying antirheumatic drugs. All patients were white, except for 2, who were Asian. The healthy control group consisted of 33 women and 5 men (all white) who were not receiving any treatment. Their median age was 45 years (range 24–62 years).

For the analysis of MP-associated IgG, IgM, and C1q by both flow cytometry and mass spectrometry, a subset of 12 SLE patients was selected based on clinical presentation and matched with 12 healthy controls, 6 patients with rheumatoid arthritis (RA), and 6 patients with systemic sclerosis (SSc). The 12 SLE patients were divided into 4 groups of 3 patients each. Patients in group 1 had biopsy-proven active nephritis and high disease activity according to the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) version of the SLE Disease Activity Index (SLEDAI) (22) (SLEDAI >12), and patients in group 2 had previous biopsy-proven nephritis, current quiescent disease, and a low SLEDAI (of 0). Patients in group 3 had no history of nephritis, current active disease other than nephritis, and a high SLEDAI (of >10). Patients in group 4 had no history of nephritis, current quiescent disease, and a low SLEDAI (of 0). The 6 patients with RA fulfilled the ACR 1987 revised classification criteria (23). The 6 patients with SSc fulfilled the ACR classification criteria (24) and included 3 patients with diffuse cutaneous SSc and 3 patients with limited cutaneous SSc.

The study was approved by the local ethics committee (approval number H-B-2007-130) and carried out according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to inclusion in the study.

Clinical and paraclinical assessment. The clinical characteristics of the SLE patients are summarized in Table 1. Disease activity was scored using the Safety of Estrogens in Lupus Erythematosus National Assessment version of the SLEDAI. Cumulative organ damage was assessed using the Systemic Lupus International Collaborating Clinics/ACR Damage Index (25). Baseline clinical manifestations were recorded according to the definitions of the SLEDAI.

Routine clinical biochemical parameters were obtained for all patient samples. Additionally, ANAs were detected by indirect immunofluorescence on HEp-2 cells in the SLE and SSc patient samples. Antibodies against double-stranded DNA (dsDNA) and histones were determined by enzyme-linked immunosorbent assay (ELISA) in all SLE patient samples, which were also screened for anti-extractable nuclear antigen (anti-ENA) using the Diastat ENA single-well screen ELISA from Euro-Diagnostica. In SLE patient samples, antiphospholipid antibodies (IgG/IgM anticardiolipin and IgG/IgM β_2 -glycoprotein I) and antibodies against C1q, cyclic citrullinated peptide (CCP), Ro 60, Ro 52, and La were measured by ELISA. Anti-CCP antibodies were also measured in RA patient samples. C1q was determined by rocket immunoelectrophoresis. Anti-Scl-70 antibodies were detected by line immunoassay in the SSc patient samples. Complements C3 and C4 were measured by immunonephelometry on an Immage System (Beckman Coulter) in the SLE patient samples.

Blood sampling and isolation of platelet-poor plasma (PPP). Venipuncture was performed with a 21-gauge needle. The first tube was used for autoantibody and complement analyses (20). Blood for MP preparation was then collected into 9-ml citrate tubes (Vacuette sodium citrate 3.8%; Greiner). Immediately after collection, blood cells were removed by centrifugation (at 1,800g for 10 minutes at 21°C) followed by a second centrifugation step (at 3,000g for 10 minutes at 21°C) to obtain PPP. The PPP was divided into

Table 1. Clinical characteristics of the 68 patients with SLE*

Disease manifestations†	
Renal disease	11 (16)
Vasculitis	4 (6)
Arthritis	7 (10)
Rash	3 (4)
Alopecia	5 (7)
Mucosal ulcers	6 (9)
Serositis	2 (3)
Leukopenia	8 (11)
Thrombocytopenia	3 (4)
SLEDAI, mean ± SD (range)†	5 ± 5 (0–21)
Autoantibodies and complements‡	
Anti-dsDNA	30 (44)
Antihistone	48 (71)
Anti-ENA	14 (21)
Anti-C1q	17 (25)
Low C3‡	33 (49)
Low C4‡	46 (68)
Low C1q‡	17 (25)
Treatment†	
Prednisolone ≤7.5 mg daily	15 (22)
Prednisolone >7.5 mg daily	14 (21)
Antimalarials	13 (19)
Azathioprine, MTX, or mycophenolate mofetil	30 (44)
SLICC/ACR Damage Index, mean ± SD (range)†	1 ± 1 (0–8)
Antiphospholipid syndrome	15 (22)

* Except where indicated otherwise, values are number (%) of patients. Clinical manifestations were defined and scored as in the Safety of Estrogens in Lupus Erythematosus National Assessment version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). Anti-dsDNA = anti-double-stranded DNA; anti-ENA = anti-extractable nuclear antigen; MTX = methotrexate; SLICC/ACR = Systemic Lupus International Collaborating Clinics/American College of Rheumatology.

† At study inclusion.

‡ Values below the reference interval of the assay.

250- μ l aliquots, snap-frozen in liquid nitrogen, and stored at -80°C .

Isolation, labeling, and flow cytometric analysis of MPs. MPs were isolated from PPP as previously described (20). The MPs were double-labeled with annexin V and a panel of titrated monoclonal antibodies against either IgG1, IgM, or C1q. Allophycocyanin (APC)-conjugated annexin V was used in all tubes (final dilution 0.0052 $\mu\text{g}/\text{ml}$; Becton Dickinson). For flow cytometric analysis of C1q-positive MPs, 5 μl of the MP suspension was diluted in 45 μl calcium-containing phosphate buffered saline (PBS; 2.5 mM CaCl_2) followed by addition of 5 μl APC-conjugated annexin V and 5 μl fluorescein isothiocyanate (FITC)-conjugated anti-C1q (IgG1; final dilution 0.130 $\mu\text{g}/\text{ml}$) (clone MhC5B9; Cedarlane) or isotype-matched control antibodies (FITC-conjugated IgG1) (clone MOPC-21; Becton Dickinson). For the detection of IgG1 and IgM the following method was used. A 5- μl suspension of MPs was diluted in 35 μl of calcium-containing PBS and then incubated with 5 μl of APC-conjugated annexin V and 5 μl of the prediluted specific antibody or isotype-matched control antibody followed by 10 μl of R-phycoerythrin anti-mouse IgG. The antibodies and concentrations used were as follows: anti-human IgG1 (IgG2b, 1.75 $\mu\text{g}/\text{ml}$) (M1325; Sanquin),

anti-human IgM (IgG1, 0.052 $\mu\text{g}/\text{ml}$) (clone MH15-1; Sanquin), R-phycoerythrin anti-mouse IgG (rabbit F[ab']₂, 0.44 $\mu\text{g}/\text{ml}$; Dako), and isotype-matched control antibodies (IgG2b, clone HYB 100-01; Statens Serum Institut and IgG1, clone MOPC-21).

The MPs, APC-conjugated annexin V, and antibodies were incubated in the dark for 60 minutes. After incubation, 900 μl of calcium-containing PBS was added. For the annexin V control experiments, samples were prepared with annexin V and isotype-matched control antibodies using PBS-citrate instead of calcium-containing PBS. The samples were analyzed within 1 hour using a FACSCalibur flow cytometer (Becton Dickinson) controlled by CellQuest software, version 5.1.1, in the “high” flow rate mode. Flow rate was measured before or after each experiment. All channels were recorded with logarithmic gain. Acquisition time was 60 seconds. All samples were processed batch-wise in random order.

MP gating was accomplished using 1- μm beads (20). The gated MPs were then plotted based on their binding of annexin V (fluorescence channel 4) and the antibody (fluorescence channel 1 or fluorescence channel 2). This enabled us to separate cell-derived MPs (annexin V positive) with surface-bound IgG1 (hereinafter called IgG), IgM, or C1q from soluble ICs with particle properties (26). The fluorescence threshold of the negative controls (isotype controls and annexin V in PBS-citrate) was used for gating. Annexin V-positive MPs that were either antibody positive or negative were enumerated, and the geometric mean fluorescence intensity (MFI) of the antibody staining of the double-positive MPs was recorded. Data were analyzed using FlowJo software, version 7.6.1 (Tree Star). Plasma concentrations (numbers of MPs/ml) were calculated on the basis of MP counts per unit time, flow rate, and net dilution during sample preparation.

Characterization of MPs by liquid chromatography tandem mass spectrometry (LC-MS/MS). Aliquots (1 ml) of PPP from each of the 3 individuals in each of 12 groups (4 SLE groups, 4 healthy control groups, 2 RA groups, and 2 SSC groups, n = 36 total) were thawed on ice and washed in PBS-citrate (at 19,000g for 30 minutes at 22°C), leaving 50 μl fluid, followed by resuspension of the MP pellet to a total volume of 1,000 μl before repeating centrifugation a total of 5 times. The final pellet was resuspended in the remaining 50 μl PBS-citrate and frozen.

Purified and concentrated MP preparations (50 μl) were thawed and precipitated with trichloroacetic acid/acetone by applying the first steps from a 2-dimensional cleanup kit (Bio-Rad). After precipitation, the proteins were resolubilized in 8M urea, 50 mM NH_4HCO_3 and digested for 3 hours using endoproteinase Lys-C (Wako) before dilution of the samples to 2M urea and continued digestion overnight by porcine trypsin (V5111; Promega) at room temperature. Samples were then frozen until analysis by LC-MS/MS.

Digested samples were whirlimixed and centrifuged to remove any insoluble material. Pools of the 3 samples from each of the 12 groups were made by mixing 15- μl aliquots of the relevant digests followed by 2-fold dilution in solvent A (2% [volume/volume] acetonitrile, 0.1% [v/v] formic acid) to ~ 200 ng/ μl . In order to compare peptide spectral counts with the plasma MP concentrations measured by flow cytometry, samples were loaded based on the initial plasma volume and not on the protein concentration. Thus, 5 μl of diluted sample

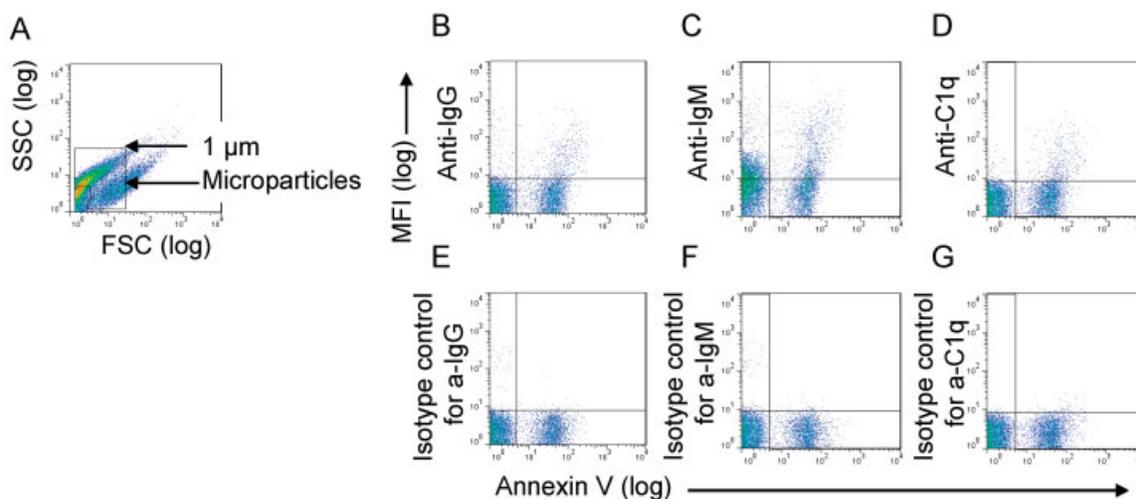


Figure 1. Representative flow cytometry density plots of microparticles (MPs) double-stained with annexin V and monoclonal antibodies against IgG, IgM, and C1q. **A**, Identification of MPs by size, as particles smaller than $1\ \mu\text{m}$ in a forward scatter (FSC) and side scatter (SSC) plot. **B–D**, Plots of MPs based on binding of annexin V and antibodies, allowing identification of the binding of the antibodies to bona fide cell-derived MPs (i.e., annexin V binding). Soluble immune complexes may also be classified as MPs when gated only by size. Annexin V-binding MPs that were either positive or negative for anti-IgG (**B**), anti-IgM (**C**), or anti-C1q (**D**) were enumerated, and the geometric mean fluorescence intensity (MFI) of the antibody staining of the double-positive MPs was obtained. **E–G**, Fluorescence threshold of the isotype controls.

was loaded onto an Acclaim PepMap C18 precolumn (300 μm inner diameter, 5 mm long, 5 μm particles; Dionex), desalted in-line, and separated on an Acclaim PepMap C18 analytical column (75 μm inner diameter, 150 mm long, 3 μm particles; Dionex) with a 70-minute gradient controlled by a Dionex Ultimate 3000 system connected to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray source (Proxeon). The flow rate was 200 nl/minute; the mobile phases consisted of solvent A and solvent B (95% [v/v] acetonitrile and 0.1% [v/v] formic acid). The gradient went from 0% to 35% solvent B in 60 minutes, followed by 10 minutes with 100% solvent B; then data acquisition was stopped and the column was re-equilibrated with solvent A. Orbitrap MS data were acquired as full scan spectra (300–1,800 mass/charge [m/z]) with 60,000 resolution at 400 m/z. MS/MS data were recorded in parallel in a data-dependent mode by fragmenting the 5 most abundant ions (charge state +2 or higher) by collision-induced dissociation in the LTQ ion trap at 35% collision energy using dynamic exclusion (40 seconds) to minimize repeated fragmentation of the same peptides.

Protein identification and relative quantitation using spectral counts. Recorded raw files were initially converted into Mascot Generic Files (mgf) using DTASuperCharge, version 2.0a7, which is part of MSQuant (27). The mgf files were subjected to database searching (Mascot version 2.2) with the following settings: for the database, SwissProt 56.0 (518,415 sequences and 182,829,264 residues); for taxonomy, *Homo sapiens*; for enzyme, trypsin, 1 missed cleavage site allowed. There were no fixed modifications, and variable modifications were oxidation (M) and deamidation (NQ). The peptide tolerance was 10 parts per million, and the MS/MS tolerance was 0.6 daltons. The resulting DAT files from the

Mascot search were further analyzed and combined in Scaffold, version 3.00.07, filtering the data set to contain only high-confidence peptide spectral matches resulting in a 0.2% protein false discovery rate for the entire data set.

The number of peptide spectral matches associated with each identified protein is termed the spectral count for that protein. This number is conveniently accessed since it is calculated as part of the protein identification process. It correlates with protein abundance and can therefore be used for relative quantitation between samples (28,29). Thus, spectral counts were used in the present study to quantitate proteins in selected samples relative to their presence in other samples.

Statistical analysis. MP concentrations, MFI, and spectral counts were not normally distributed. Data are presented as dot plots in the figures, with lines showing the median. Differences between 2 groups were determined by Mann-Whitney U test. Differences between more than 2 groups were tested by one-way Kruskal-Wallis analysis of variance followed by Mann-Whitney U test to compare the SLE samples with the other groups individually. Univariate correlation analyses were performed by calculating Spearman's rank correlation coefficients (r). P values less than 0.05 were considered significant. GraphPad Prism version 5 was used for the statistical calculations and plots.

RESULTS

Detection of IgG-, IgM-, and C1q-positive MPs by flow cytometry. MPs were gated first by size (Figure 1A) and then by antibody and annexin V binding

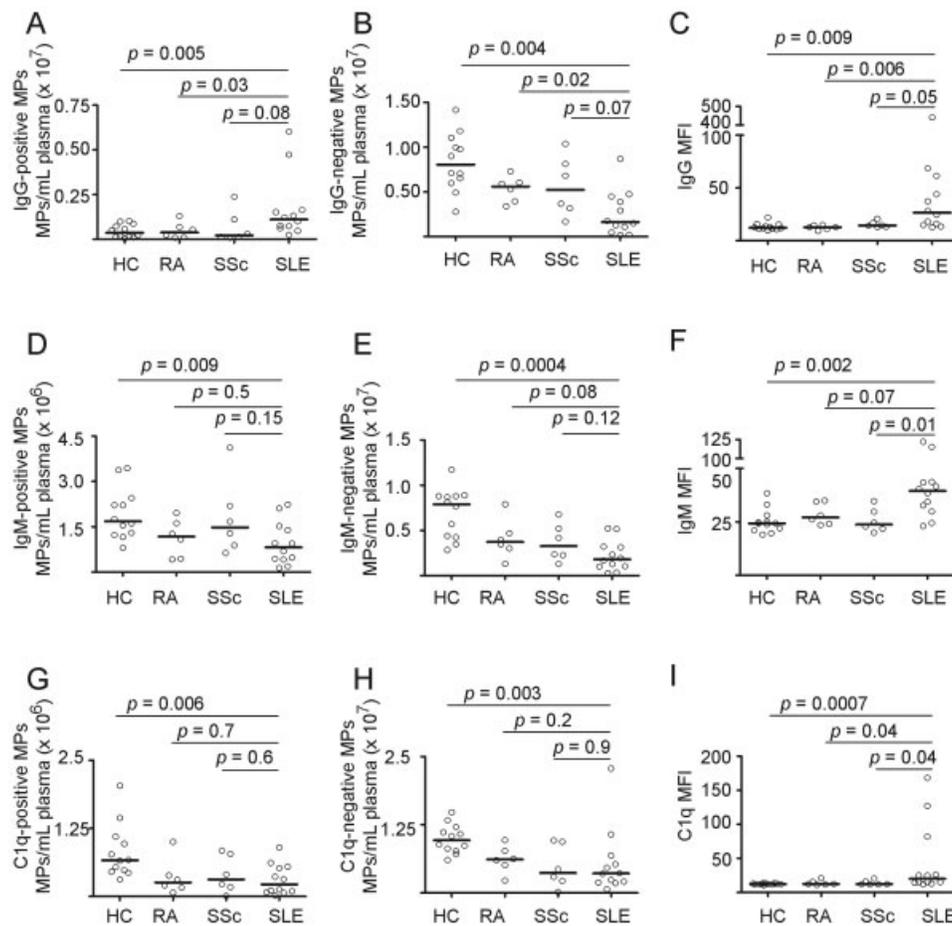


Figure 2. Plasma concentrations of annexin V-binding MPs and MFIs of antibody staining on the double-positive MPs in healthy controls (HC; $n = 12$), patients with rheumatoid arthritis (RA; $n = 6$), patients with systemic sclerosis (SSc; $n = 6$), and patients with systemic lupus erythematosus (SLE; $n = 12$). **A–C**, Plasma concentrations of annexin V-binding IgG-positive MPs (**A**), plasma concentrations of annexin V-binding IgG-negative MPs (**B**), and IgG MFI (**C**). The IgG-positive MP concentration and IgG MFI were higher in the SLE patients than in all other groups. **D–F**, Plasma concentrations of annexin V-binding IgM-positive MPs (**D**), plasma concentrations of annexin V-binding IgM-negative MPs (**E**), and IgM MFI (**F**). **G–I**, Plasma concentrations of annexin V-binding C1q-positive MPs (**G**), plasma concentrations of annexin V-binding C1q-negative MPs (**H**), and C1q MFI (**I**). IgM-positive and C1q-positive MPs were decreased in all of the disease groups compared with healthy controls, whereas the MFI for both IgM and C1q was increased in the SLE patients. Circles represent individual subjects; horizontal lines represent the median. Data for each antibody were obtained from the same subjects. P values were determined by Mann-Whitney U test. See Figure 1 for other definitions.

(Figures 1B–G). In some SLE and RA patients, we detected events classified as MPs by size that only bound the antibody and not annexin V (Figures 1B–D). This was most pronounced for anti-IgM. These events most likely represent a mixture of signals from both soluble ICs and cell-derived MPs that do not bind annexin V. Only annexin V-binding MPs were further analyzed, i.e., all flow cytometric results in this study were obtained using cell-derived MPs.

Increased MP-bound IgG, IgM, and C1q levels in patients with SLE, detected by flow cytometry. We characterized IgG, IgM, and C1q on MPs from a subset

of the SLE patients ($n = 12$) and from both healthy controls ($n = 12$) and 2 autoimmune disease control groups (RA patients [$n = 6$] and SSc patients [$n = 6$]) (Figure 2).

Overall, IgG-positive MPs were significantly increased in the SLE patients compared with both the healthy controls and the RA patients ($P = 0.005$ and $P = 0.03$, respectively), while there was no significant difference between the SLE patients and the SSc patients ($P = 0.08$) (Figure 2A). As a measure of the average load of IgG per annexin V-positive MP, the MFI was calculated. The IgG MFI in the samples from SLE

patients was significantly higher than in all of the other groups ($P \leq 0.05$ for all comparisons) (Figure 2C).

In contrast to the increased IgG-positive MP concentration, the concentrations of IgM-positive and C1q-positive MPs were significantly decreased in the SLE patients compared with healthy controls ($P = 0.009$ and $P = 0.006$, respectively), whereas the levels did not differ significantly from those in the disease controls (Figures 2D and G). Both the IgM-positive and C1q-positive MP counts correlated directly with the total count of annexin V-positive MPs in the sample. However, despite lower numbers of MPs, the MFI values for both IgM and C1q were significantly increased in the SLE patients compared with the control groups ($P < 0.05$), except for IgM MFI in the RA group ($P = 0.07$) (Figures 2F and I).

Thus, MPs from SLE patients had higher MFIs for IgM, C1q, and IgG. The C1q MFI measurements correlated with the IgG MFI and IgM MFI measurements ($r = 0.45$, $P = 0.006$ and $r = 0.61$, $P < 0.0001$, respectively), suggesting that the higher MFI values in SLE may represent MPs with IgG, IgM, and C1q present in fixed ratios compatible with the presence of ICs on the MPs.

Verification of increased MP-associated IgG, IgM, and C1q levels in SLE patients by proteome profiling using tandem mass spectrometry. The 36 individual samples analyzed for MP-bound IgG, IgM, and C1q using flow cytometry (Figure 2) were pooled into 12 groups of 3 samples each, based on clinical presentation, as described in Patients and Methods. Purified MPs from each pool were analyzed using LC-MS/MS after trypsin treatment. The use of sample pools reduced the workload of this multistep and time-consuming technique. Based on tryptic fragment masses (m/z values), peptide fragment patterns (yielding amino acid sequences), and the spectral counts of the individual peptides, their parent proteins can be identified and quantitated. LC-MS/MS thus allows unequivocal antibody-independent identification of the whole compartment of specific proteins (the proteome) in a sample above a given threshold of abundance and typically spanning 4–5 decades of protein concentrations.

In all cases the mean concentrations of annexin V-positive MPs derived by flow cytometry correlated linearly with the spectral counts of the cytoskeletal protein myosin 9 ($r = 0.72$, $P = 0.008$) and the integral platelet membrane protein integrin- α IIb (CD41) ($r = 0.72$, $P = 0.008$) (Figures 3A and B). We thus used the myosin 9 spectral count as a measure of the MP numbers

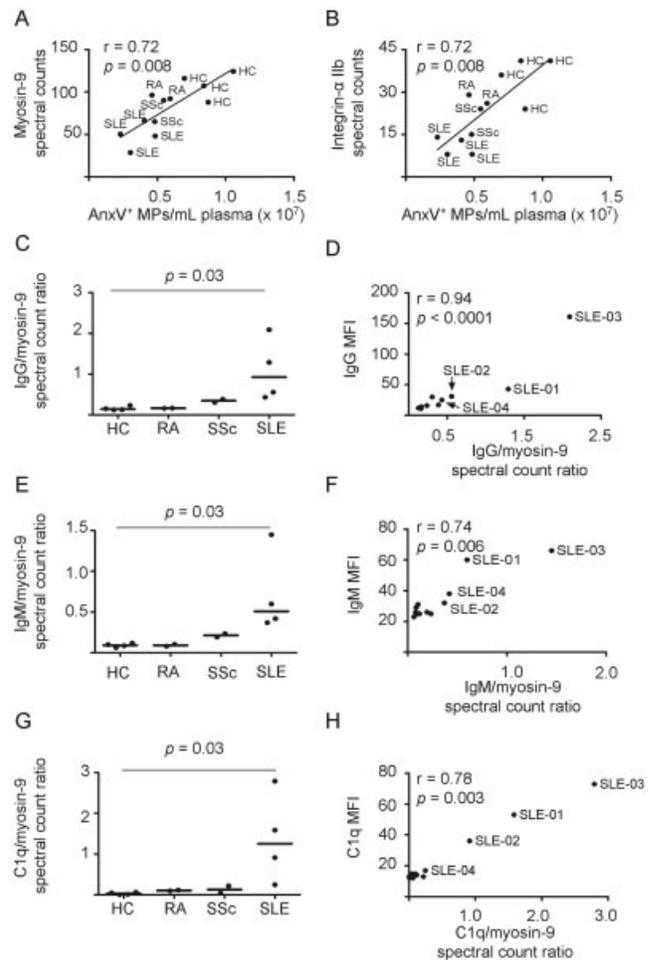


Figure 3. Correlations of spectral counts of myosin 9, integrin- α IIb, and MP-associated IgG, IgM, and C1q with concentrations of annexin V (AnxV)-positive MPs and IgG, IgM, and C1q MFIs. Subjects were grouped into 4 pools of healthy controls (HC), 2 pools of patients with rheumatoid arthritis (RA), 2 pools of patients with systemic sclerosis (SSc), and 4 pools of patients with systemic lupus erythematosus (SLE). Each pool contained 3 subjects ($n = 36$ subjects total). **A** and **B**, Correlation of myosin 9 (**A**) and integrin- α IIb (**B**) spectral counts, determined by liquid chromatography tandem mass spectrometry (LC-MS/MS), with mean annexin V-positive MP concentrations, determined by flow cytometry. Myosin 9 was used as a marker of the numbers of MPs in each sample pool analyzed by LC-MS/MS. **C**, **E**, and **G**, Ratio of myosin 9 to IgG (**C**), IgM (**E**), and C1q (**G**). The levels of IgG, IgM, and C1q relative to myosin 9 were higher in the SLE group than in the control groups. **D**, **F**, and **H**, Correlation of the MP-associated IgG (**D**), IgM (**F**), and C1q (**H**) spectral counts with the MFIs for all 3 molecules detected by flow cytometry. SLE-01, SLE-02, SLE-03, and SLE-04 represent the 4 SLE groups described in Patients and Methods. Patients in groups 1 and 3 had active disease. Low values were found for all controls by both LC-MS/MS and flow cytometry. Circles represent sample pools; horizontal lines represent the median. P values were determined by Kruskal-Wallis one-way analysis of variance. See Figure 1 for other definitions.

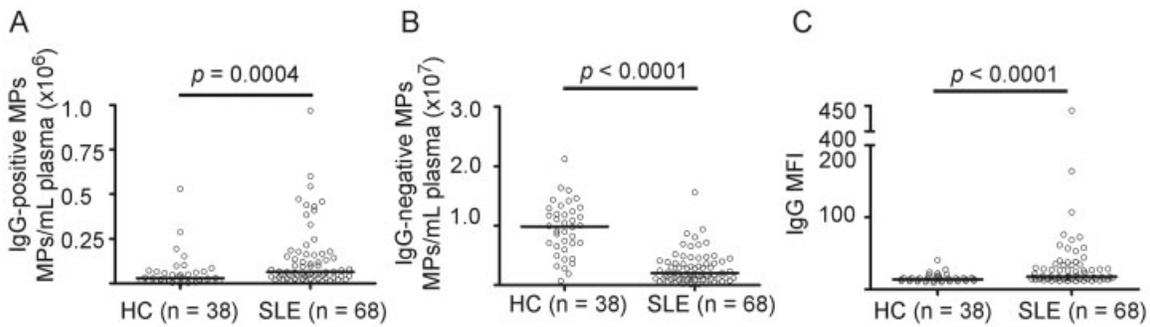


Figure 4. Plasma concentrations of annexin V-binding MPs with and those without detectable surface-bound IgG and MFI of IgG staining, determined by flow cytometry in healthy controls (HC) and patients with systemic lupus erythematosus (SLE). **A** and **B**, Plasma concentrations of IgG-positive (**A**) and IgG-negative (**B**) MPs. Compared with the healthy controls, the SLE patients had significantly higher levels of IgG-positive cell-derived MPs (annexin V-positive MPs with surface-bound IgG) and decreased levels of IgG-negative cell-derived MPs (annexin V-positive MPs with no surface-bound IgG). **C**, IgG MFI. The MFI was significantly higher in the SLE patients than in the healthy controls. Circles represent individual subjects; horizontal lines represent the median. *P* values were determined by Mann-Whitney U test. See Figure 1 for other definitions.

in the samples. There were higher spectral counts of IgG, IgM, and C1q relative to the myosin 9 spectral counts in the pooled MP samples from SLE patients compared with the control groups (Figures 3C, E, and G). The 2 pools of samples from SSc patients had slightly higher levels of IgG and IgM than both the healthy control and RA groups. In this respect, the SSc sample pools were intermediate between the SLE pools and the other 2 control pools. The increased MP-associated IgG, IgM, and C1q levels found by LC-MS/MS analysis of the SLE samples correlated well with the flow cytometry data, i.e., the IgG, IgM, and C1q MFI values ($r = 0.94$, $P < 0.0001$; $r = 0.74$, $P = 0.006$; and $r = 0.78$, $P = 0.003$, respectively) (Figure 3D, F, and H). We also found that the SLE pools representing active disease (SLE group 1 and SLE group 3) had the highest levels of all 3 of these molecules (Figures 3D, F, and H).

Increased numbers of IgG-positive MPs with a higher IgG load in SLE patients, detected by flow cytometry. The 68 SLE samples were individually evaluated for annexin V, IgG double-positive MPs and compared with samples from the 38 healthy controls (Figure 4). Consistent with the observations described above, the numbers of cell-derived MPs with surface-bound IgG were significantly increased in the group of all SLE patients ($P = 0.0004$). The IgG MFI was also highly significantly increased in the SLE patient group ($P < 0.0001$) (Figure 4C). The IgG-negative MPs were significantly decreased ($P < 0.0001$) (Figure 4B) as was the total concentration of annexin V-positive MPs, as previously shown in this patient cohort (20). Consequently, the proportion of circulating cell-derived MPs with surface-bound IgG was significantly increased in

the SLE group (median [5th–95th percentile] 3.5% [0.7–22.3%] in the SLE patient group versus 0.4% [0.02–4.3%] in the healthy control group; $P < 0.0001$).

Association of MP-bound IgG with complement consumption and presence of autoantibodies. Univariate analyses of the correlation of the concentrations of IgG-positive MPs and the IgG MFI with clinical and serologic parameters were performed for all samples (Table 2). The concentrations of IgG-positive MPs were positively associated with higher concentrations of annexin V-positive MPs, plasma IgG levels, anti-dsDNA,

Table 2. Correlation analysis of the clinical and MP parameters of the 68 patients with SLE at study inclusion, by Spearman’s rank correlation*

	IgG-positive MPs		IgG MFI	
	r	P	r	P
Total annexin V-positive MPs	0.32	0.009	-0.26	0.03
Active nephritis	-0.12	0.3	-0.0059	0.96
SLEDAI	0.11	0.4	0.11	0.4
Platelets	0.05	0.73	-0.11	0.4
Leukocytes	-0.25	0.04	-0.11	0.4
Plasma IgG	0.30	0.01	0.16	0.2
Anti-dsDNA†	0.31	0.01	0.23	0.06
Anti-ENA	0.38	0.001	0.042	0.74
Antihistone	0.29	0.02	0.08	0.50
Anti-C1q	0.19	0.1	0.39	0.001
C4	-0.17	0.2	-0.50	<0.0001
C3	-0.21	0.09	-0.40	0.0007
C1q†	-0.032	0.8	-0.25	0.04

* MP = microparticle; SLE = systemic lupus erythematosus; MFI = mean fluorescence intensity; SLEDAI = SLE Disease Activity Index; anti-ENA = anti-extractable nuclear antigen.

† Anti-double-stranded DNA (anti-dsDNA) and C1q were measured in 67 of the patients.

anti-ENA, and antihistone levels, and lower leukocyte levels. Interestingly, the IgG MFI was highly correlated with lower levels of complements C4, C3, and C1q and with the presence of anti-C1q (but not with the other autoantibodies) and with lower levels of total numbers of annexin V-positive MPs. IgG-positive MPs and IgG MFI were not associated with other clinical manifestations, SLEDAI, anti-SSA/SSB, or antiphospholipid antibodies.

DISCUSSION

It has been proposed that MPs carry autoimmunogenic material and ICs in SLE patients, and this is the first study to verify highly significantly increased concentrations of IgG-positive cell-derived MPs and a higher average IgG load on MPs from SLE patients compared with both disease controls and healthy controls (6,13,30,31). Exposure of autoantigens on circulating MPs in SLE patients has several implications. First, autoantigen-presenting MPs may be highly autoimmunogenic due to the adjuvancy of liposome-like MPs, and IgG associated with MPs further enhances immunostimulation as observed with DNA- and RNA-containing ICs (32–34). MPs may also be important activators of the interferon- α pathway, which is believed to be crucial for the proinflammatory state in SLE (3). Exploration of the immunostimulatory properties of MPs alone and of IC-carrying MPs compared to soluble ICs containing nucleic acids would thus be of interest. Second, bound immunoglobulins may initiate the classical pathway of complement and contribute to the systemic complement activation observed in SLE (14,16,18,19,35,36). Third, MPs provide adhesion and costimulatory molecules that result in IC deposition when MPs bind to various cells, e.g., to endothelial cells in kidney glomeruli. This may explain the presence of autoantibodies at sites where specific epitopes are absent (37). Under normal circumstances, most plasma MPs derive from platelets and erythrocytes (38–40). Under inflammatory conditions, MPs from nucleated cells such as lymphocytes, granulocytes, and endothelial cells become more prominent (41–43).

In the present study, the numbers of IgG-positive MPs were significantly associated with the presence of circulating anti-dsDNA, anti-ENA, and antihistone autoantibodies, with plasma IgG levels, and with total annexin V-positive MP concentrations. Interestingly, the IgG MFI was associated with anti-C1q and activation of the classical pathway of complement. These data suggest that the complement system is activated by cell-derived

MPs carrying ICs in SLE patients. Also, while no simple correlation between IgG load and disease activity was found, the mass spectrometry data obtained with a set of homogeneous pools of SLE patient samples showed the highest levels of MP IgG, IgM, and C1q in the 2 pools from patients with active disease.

Few studies of endogenous surface-bound molecules of MPs from patient samples have been published. In RA patients, the number of circulating MPs displaying C-reactive protein correlated with the numbers of C1q-bearing MPs (44), and in early RA the numbers of MPs positive for C1q, serum amyloid P component, and C-reactive protein were elevated (45). We are unaware of previous studies enumerating and quantitating IgG on cell-derived circulating MPs from SLE patients.

We were able to detect IgG associated with bona fide MPs by counting only particles that bind annexin V and by gating based on subcellular particle size (20). The mode of binding of the MP-associated IgG was not determined in our experiments. The IgG may be bound to its specific antigens and/or may be bound through complement receptors or Fc receptors and may already be present on the parent cells generating the MPs (46). Further studies, including continued mining of the proteomic data that represent >500 individual proteins, may answer some of these questions, including the provenance of the MPs. Interestingly, in the SSc samples we found an intermediate increase in MP IgG and IgM content by mass spectrometry. These findings could suggest binding of antibodies to SSc autoantigens such as Scl-70 or centromeres on MPs in SSc patients.

With regard to the consequences of IgG interacting with MPs, a few studies suggest that SLE-associated autoantibodies inhibit Fc receptor binding and the uptake of late apoptotic cells by monocyte-derived macrophages. This would lead to an increased half-life of MPs and thus an accumulation of circulating cellular remnants (14). It was recently proposed that a similar autoantibody-mediated mechanism may interfere with the degradation of neutrophil extracellular traps in subsets of SLE patients (8). However, recent studies have not shown increased levels of MPs in SLE patients (13). Indeed, in our previous study we found decreased numbers of MPs in SLE patients (20). Also, in the case of dead and dying cells, autoantibodies appear to promote complement-dependent phagocytosis (16,18,47,48). In the present study, we found that the concentration of annexin V-positive MPs was inversely correlated with the IgG MFI, supporting the notion that MP opsonization may enhance MP clearance.

Analysis of MPs from fresh samples would be

preferable since it is possible both that MPs may be lost and that they may be induced by sample handling (49). However, it is difficult to avoid the need to freeze samples when working with larger numbers of patients. Also, for studying immunoglobulins on MPs, the samples have to be washed, and this inevitably leads to losses. In the present study, we therefore used standardized conditions for sample collection, prepared PPP immediately using relatively high-speed centrifugation, stored frozen samples for comparable lengths of time, analyzed the samples in a random order, and labeled samples with annexin V to ensure detection of true cellular MPs, as was recently recommended (49). Also, we have evaluated the effect of freezing in samples from both healthy controls and SLE patients and consistently found that freezing of the samples resulted in lower numbers of total MPs, whereas the concentrations of annexin V-positive MPs were almost completely unaffected (20).

Taken together, our data indicate that plasma MPs carry antigens accessible to autoantibodies and that complement-activating ICs may form on MPs in SLE patients. These data are consistent with recent observations by Ullal and coworkers (13), who showed that anti-dsDNA antibodies bind to MPs from plasma and that some SLE patients had increased numbers of ICs with particle properties correlating with anti-dsDNA levels. However, our study is the first to measure and document significant differences between controls (both healthy and disease) and SLE patients in numbers and intensities of double-labeled circulating MPs. Important findings in our study also include the observations that the number of IgG-positive MPs correlated with IgG and anti-dsDNA, antihistone, and anti-Sm/RNP (anti-ENA) levels in serum. IgG MFI levels were also correlated with serum anti-C1q levels and consumption of complement components.

Circulating cell-derived MPs may be a source of immunogenic autoantigens accessible for autoantibody binding in SLE, and our results support the idea that this binding may result in IC formation on MPs in SLE patients, activating the complement system. MPs may thus take center stage both in the pathogenesis and in the perpetuation and modulation of disease activity in SLE.

ACKNOWLEDGMENTS

We thank Lærke Kjeldsen and Martin Lademann for assistance with the preliminary experiments. We also thank

laboratory technician Kirsten Beth Hansen for invaluable help with the preparation of the samples.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Heegaard had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Nielsen, Østergaard, Iversen, Jacobsen, Heegaard.

Acquisition of data. Nielsen, Østergaard, Stener, Iversen, Truedsson, Gullstrand.

Analysis and interpretation of data. Nielsen, Østergaard, Jacobsen, Heegaard.

REFERENCES

- Jacobsen S, Petersen J, Ullman S, Junker P, Voss A, Rasmussen JM, et al. A multicentre study of 513 Danish patients with systemic lupus erythematosus. I. Disease manifestations and analyses of clinical subsets. *Clin Rheumatol* 1998;17:468–77.
- Rahman A, Isenberg DA. Systemic lupus erythematosus. *N Engl J Med* 2008;358:929–39.
- Obermoser G, Pascual V. The interferon- α signature of systemic lupus erythematosus. *Lupus* 2010;19:1012–9.
- Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med* 2011;3:73ra19.
- Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med* 2011;3:73ra20.
- Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med* 1994;179:1317–30.
- Cline AM, Radic MZ. Apoptosis, subcellular particles, and autoimmunity. *Clin Immunol* 2004;112:175–82.
- Hakim A, Furnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A* 2010;107:9813–8.
- Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nat Rev Rheumatol* 2010;6:280–9.
- Beyer C, Pisetsky DS. The role of microparticles in the pathogenesis of rheumatic diseases. *Nat Rev Rheumatol* 2010;6:21–9.
- Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* 2009;19:43–51.
- Cline AM, Radic MZ. Murine lupus autoantibodies identify distinct subsets of apoptotic bodies. *Autoimmunity* 2004;37:85–93.
- Ullal AJ, Reich CF III, Clowse M, Criscione-Schreiber LG, Tochacek M, Monestier M, et al. Microparticles as antigenic targets of antibodies to DNA and nucleosomes in systemic lupus erythematosus. *J Autoimmun* 2011;36:173–80.
- Reefman E, Horst G, Nijk MT, Limburg PC, Kallenberg CG, Bijl M. Opsonization of late apoptotic cells by systemic lupus erythematosus autoantibodies inhibits their uptake via an Fc γ receptor-dependent mechanism. *Arthritis Rheum* 2007;56:3399–411.
- Botto M, Dell'Agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F, et al. Homozygous C1q deficiency causes glomerulone-

- phritis associated with multiple apoptotic bodies. *Nat Genet* 1998;19:56–9.
16. Grossmayer GE, Munoz LE, Weber CK, Franz S, Voll RE, Kern PM, et al. IgG autoantibodies bound to surfaces of necrotic cells and complement C4 comprise the phagocytosis promoting activity for necrotic cells of systemic lupus erythematosis sera. *Ann Rheum Dis* 2008;67:1626–32.
 17. Munoz LE, Janko C, Chaurio RA, Schett G, Gaipal US, Herrmann M. IgG opsonized nuclear remnants from dead cells cause systemic inflammation in SLE. *Autoimmunity* 2010;43:232–5.
 18. Munoz LE, Janko C, Grossmayer GE, Frey B, Voll RE, Kern P, et al. Remnants of secondarily necrotic cells fuel inflammation in systemic lupus erythematosis. *Arthritis Rheum* 2009;60:1733–42.
 19. Nauta AJ, Trouw LA, Daha MR, Tijsma O, Nieuwland R, Schwaeble WJ, et al. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *Eur J Immunol* 2002;32:1726–36.
 20. Nielsen CT, Ostergaard O, Johnsen C, Jacobsen S, Heegaard NH. Distinct features of circulating microparticles and their relationship to clinical manifestations in systemic lupus erythematosis. *Arthritis Rheum* 2011;63:3067–77.
 21. Hochberg MC, for the Diagnostic and Therapeutic Criteria Committee of the American College of Rheumatology. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosis [letter]. *Arthritis Rheum* 1997;40:1725.
 22. Petri M, Kim MY, Kalunian KC, Grossman J, Hahn BH, Sammaritano LR, et al, for the OC-SELENA Trial. Combined oral contraceptives in women with systemic lupus erythematosis. *N Engl J Med* 2005;353:2550–8.
 23. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315–24.
 24. Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980; 23:581–90.
 25. Gladman D, Ginzler E, Goldsmith C, Fortin P, Liang M, Urowitz M, et al. The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index for systemic lupus erythematosis. *Arthritis Rheum* 1996;39:363–9.
 26. Gyorgy B, Modos K, Pallinger E, Paloczi K, Pasztoi M, Misjak P, et al. Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. *Blood* 2011;117:e39–48.
 27. Schulze WX, Mann M. A novel proteomic screen for peptide-protein interactions. *J Biol Chem* 2004;279:10756–64.
 28. Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevinsky JR, et al. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol Cell Proteomics* 2005;4:1487–502.
 29. Zhang B, VerBerkmoes NC, Langston MA, Uberbacher E, Hettich RL, Samatova NF. Detecting differential and correlated protein expression in label-free shotgun proteomics. *J Proteome Res* 2006;5:2909–18.
 30. Schiller M, Bekeredian-Ding I, Heyder P, Blank N, Ho AD, Lorenz HM. Autoantigens are translocated into small apoptotic bodies during early stages of apoptosis. *Cell Death Differ* 2008; 15:183–91.
 31. Pisetsky DS, Gauley J, Ullal AJ. Microparticles as a source of extracellular DNA. *Immunol Res* 2011;49:227–34.
 32. Pisetsky DS, Lipsky PE. Microparticles as adjuvants in the pathogenesis of SLE. *Nat Rev Rheumatol* 2010;6:368–72.
 33. Lovgren T, Eloranta ML, Bave U, Alm GV, Ronnblom L. Induction of interferon- α production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. *Arthritis Rheum* 2004;50:1861–72.
 34. Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J Clin Invest* 2005;115:407–17.
 35. Gasser O, Schifferli JA. Microparticles released by human neutrophils adhere to erythrocytes in the presence of complement. *Exp Cell Res* 2005;307:381–7.
 36. Walport MJ. Complement and systemic lupus erythematosis. *Arthritis Res* 2002;4 Suppl 3:S279–93.
 37. Van Bavel CC, Fenton KA, Rekvig OP, van der Vlag J, Berden JH. Glomerular targets of nephritogenic autoantibodies in systemic lupus erythematosis [review]. *Arthritis Rheum* 2008;58:1892–9.
 38. Orozco AF, Lewis DE. Flow cytometric analysis of circulating microparticles in plasma. *Cytometry A* 2010;77:502–14.
 39. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. *Blood Rev* 2007;21:157–71.
 40. Ullal AJ, Pisetsky DS, Reich CF III. Use of SYTO 13, a fluorescent dye binding nucleic acids, for the detection of microparticles in vitro systems. *Cytometry A* 2010;77:294–301.
 41. Erdbruegger U, Grossheim M, Hertel B, Wyss K, Kirsch T, Woywodt A, et al. Diagnostic role of endothelial microparticles in vasculitis. *Rheumatology (Oxford)* 2008;47:1820–5.
 42. Guiducci S, Distler JH, Jungel A, Huscher D, Huber LC, Michel BA, et al. The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis. *Arthritis Rheum* 2008;58:2845–53.
 43. Sellam J, Proulle V, Jungel A, Ittah M, Miceli Richard C, Gottenberg JE, et al. Increased levels of circulating microparticles in primary Sjögren's syndrome, systemic lupus erythematosis and rheumatoid arthritis and relation with disease activity. *Arthritis Res Ther* 2009;11:R156.
 44. Biro E, Nieuwland R, Tak PP, Pronk LM, Schaap MC, Sturk A, et al. Activated complement components and complement activator molecules on the surface of cell-derived microparticles in patients with rheumatoid arthritis and healthy individuals. *Ann Rheum Dis* 2007;66:1085–92.
 45. Van Eijk IC, Tushuizen ME, Sturk A, Dijkmans BA, Boers M, Voskuyl AE, et al. Circulating microparticles remain associated with complement activation despite intensive anti-inflammatory therapy in early rheumatoid arthritis. *Ann Rheum Dis* 2010;69:1378–82.
 46. Duffau P, Seneschal J, Nicco C, Richez C, Lazaro E, Douchet I, et al. Platelet CD154 potentiates interferon- α secretion by plasmacytoid dendritic cells in systemic lupus erythematosis. *Sci Transl Med* 2010;2:47ra63.
 47. Gullstrand B, Martensson U, Sturfelt G, Bengtsson AA, Truedsson L. Complement classical pathway components are all important in clearance of apoptotic and secondary necrotic cells. *Clin Exp Immunol* 2009;156:303–11.
 48. Sarmiento LF, Munoz LE, Chirinos P, Bianco NE, Zabaleta-Lanz ME. Opsonization by anti-dsDNA antibodies of apoptotic cells in systemic lupus erythematosis. *Autoimmunity* 2007;40:337–9.
 49. Dey-Hazra E, Hertel B, Kirsch T, Woywodt A, Lovric S, Haller H, et al. Detection of circulating microparticles by flow cytometry: influence of centrifugation, filtration of buffer, and freezing. *Vasc Health Risk Manag* 2010;6:1125–33.