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Circulating Apoptotic Microparticles in Systemic Lupus Erythematosus Patients Drive the Activation of Dendritic Cell Subsets and Prime Neutrophils for NETosis

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Objective. **Circulating chromatin-containing apoptotic material and/or neutrophil extracellular traps (NETs) have been proposed to be an important driving force for the antichromatin autoimmune response in patients with systemic lupus erythematosus (SLE). The aim of this study was to determine the exact nature of microparticles in the circulation of SLE patients and to assess the effects of the microparticles on the immune system.**

Methods. **We analyzed microparticles isolated from the plasma of patients with SLE, rheumatoid arthritis (RA), and systemic sclerosis (SSc), as well as from healthy subjects. The effects of the microparticles on blood-derived dendritic cells (DCs) and neutrophils were assessed by flow cytometry, enzyme-linked immunosorbent assay, and immunofluorescence microscopy.**

Results. **In SLE patients, we identified microparticles that were highly positive for annexin V and apoptosis-modified chromatin that were not present in healthy subjects or in RA or SSc patients. These microparticles were mostly CD31+/CD45– (endothelial), partly CD45+/CD66b+ (granulocyte), and negative for B and T cell markers. Microparticles isolated from the plasma of SLE patients increased the expression of the costimulatory surface molecules CD40, CD80, CD83, and CD86 and the production of proinflammatory cytokines interleukin-6, tumor necrosis factor, and interferon-α by blood-derived plasmacytoid DCs (PDCs) and myeloid DCs (MDCs). SLE microparticles also primed blood-derived neutrophils for NETosis. Microparticles from healthy subjects and from RA or SSc patients exhibited no significant effects on MDCs, PDCs, and NETosis.**

Conclusion. **Circulating microparticles in SLE patients include a population of apoptotic cell–derived microparticles that has proinflammatory effects on PDCs and MDCs and enhances NETosis. These results underline the important role of apoptotic microparticles in driving the autoimmune response in SLE patients.**

Microparticles are generally defined as small vesicles with a size between 100 nm and 1 μm that are formed by budding of the cell membrane upon stimulation or during the late stages of apoptosis (1). Circulating microparticles are ubiquitous in the blood of healthy individuals, where they are mostly derived from (activated) platelets or erythrocytes and to a lesser extent from (activated) endothelial and immune cells. These microparticles play an active role in coagulation and intercellular communication and assist in activation or suppression of the immune system, depending on their parental cell origin (2). Changes in the concentration and/or composition of circulating microparticles have been described in various autoimmune diseases, including rheumatoid arthritis (RA) (3), systemic sclerosis (SSc) (4), polymyositis/

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dermatomyositis (5), and systemic lupus erythematosus (SLE) (6–10). For SLE, the reported microparticle-related changes remain somewhat inconclusive. In particular, the identification and characterization of circulating microparticles derived from apoptotic cells, which have been proposed to be an important trigger for the antichromatin autoimmune response that typically occurs in SLE patients (11,12), have not been assessed.

Processing of potential damage-associated molecular patterns (DAMPs) during apoptosis largely determines whether the immune system will react to dying cells (13). In general, DAMPs remain hidden inside the cell during early apoptosis, where they can later be released. The role of apoptotic microparticles in the processing of DAMPs is still unclear, although they contain potential DAMPs, such as high mobility group box chromosomal protein 1 (HMGB-1) (14) and interleukin-1α (IL-1α) (15). The basic components of chromatin, DNA and histones, have also been identified as ligands for pattern-recognition receptors Toll-like receptor 2 (TLR-2), TLR-4, and TLR-9 (16–18). Interestingly, mice lacking caspase-activated DNase are unable to form apoptotic microparticles and were found to be protected against a pristane-induced autoimmune response to chromatin (19).

We have previously shown that apoptotic microparticles stimulated murine bone marrow–derived dendritic cells (DCs), whereas apoptotic cell bodies that remained after the release of microparticles did not (20,21). In addition, we have observed that specific apoptosis-associated histone modifications, which are targeted by lupus autoantibodies, are incorporated in in vitro–generated apoptotic microparticles (22–24). These histone modifications include acetylation of H4K8,12,16, acetylation of H2BK12, and trimethylation of H3K27. Importantly, hyperacetylated nucleosomes, and not non–apoptotic-derived nucleosomes, were found to induce maturation of murine bone marrow–derived DCs (22). Injection of an H4K8,12,16Ac peptide into lupus mice prior to disease manifestations aggravated the subsequent disease symptoms, while injection of the unmodified peptide equivalent exhibited no effect on the disease. Other investigators have observed that apoptotic microparticles have the capacity to activate murine and human DCs; however, studies of the contents and immunologic effects of apoptotic microparticles conducted thus far have focused on in vitro–generated microparticles (25–29).

In addition to apoptotic microparticles, neutrophil extracellular traps (NETs) have recently been proposed as a potential source for circulating chromatin in SLE patients (30–34). In the present study, we investigated the presence, contents, and origins of apoptotic microparticles in the plasma of SLE patients and evaluated their potential role in the activation of blood-derived dendritic cells and neutrophils.

PATIENTS AND METHODS

Patients. Blood samples from patients with biopsy-proven proliferative lupus nephritis (World Health Organization class III, IV, Vc, or Vd), RA, or SSc, as well as from healthy control subjects were collected at the Radboud University Medical Center. All patients with SLE met ≥4 American College of Rheumatology criteria for the disease (35). Disease activity was measured with the SLE Disease Activity Index (SLEDAI) (36). The use of human blood was approved by the local ethics committee, and written consent was obtained from the patients and healthy controls.

Isolation and characterization of microparticles. Plasma was centrifuged at 500*g* for 5 minutes at 4°C, and the supernatant was removed and centrifuged at 20,800*g* for 10 minutes at 4°C. The pellet containing the microparticles was washed with phosphate buffered saline containing 0.1% bovine serum albumin, and the microparticles were incubated for 30 minutes at 4°C with lupus-prone mouse–derived monoclonal antibodies (mAb) or with mouse IgG2a (UPC-10; Sigma). For staining of chromatin in microparticles, we used a panel of lupus-prone mouse–derived monoclonal antibodies, consisting of #34 (anti-H3 [37]), KM-2 (anti-H4K8,12,16Ac [22]), BT164 (anti-H3K27me3 [23]), and LG11-2 (anti-H2BK12Ac [24]). Purification of these monoclonal antibodies was performed as described previously (22–24,37). The specificity of each batch of purified monoclonal antibody was validated by comparing the reactivity in enzyme-linked immunosorbent assay (ELISA) with the respective modified histone peptide, H3(18–37) for #34, H4(1–22)K8,12,16Ac for KM-2, H2B(1–18)K12Ac for LG11-2, and H3(21–34)K27me3 for BT164 and its unmodified equivalent. After washing, microparticles were incubated for 20 minutes at 4°C with Alexa Fluor 488–conjugated goat anti-human Ig (heavy and light chain; Life Technologies). After washing, samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). All buffers were filtered through a 0.2-μm filter, and buffers without plasma-derived microparticles exhibited a negligible number of counted events.

Where indicated, plasma-isolated microparticles were stained with phycoerythrin (PE)–conjugated anti-CD3 (UCHT-1; Beckman Coulter), anti-CD19 (J3-119; Beckman Coulter), anti-CD31 (WM59; BD Biosciences), or mouse IgG1 (MOPC-21; BD Biosciences), and/or fluorescein isothiocyanate (FITC)–conjugated anti-CD45 (J33; Beckman Coulter), anti-CD66b (80H3; Beckman Coulter), or mouse IgG1 (MOPC-21; BD Biosciences). Additionally, microparticles were taken up in annexin V buffer and stained with FITC-conjugated annexin V according to the manufacturer's protocol (BioVision). For determining the concentration of microparticles, AccuCheck calibrated beads were added according to the manufacturer's protocol (Life Technologies).

Isolation of plasmacytoid DCs (PDCs) and myeloid DCs (MDCs). Buffy coats were obtained from volunteer donors according to institutional guidelines and with their written consent. CD1c+ MDCs and PDCs were purified by positive selection using anti-CD1c–conjugated and anti–blood dendritic cell antigen 4 (anti–BDCA-4)–conjugated magnetic

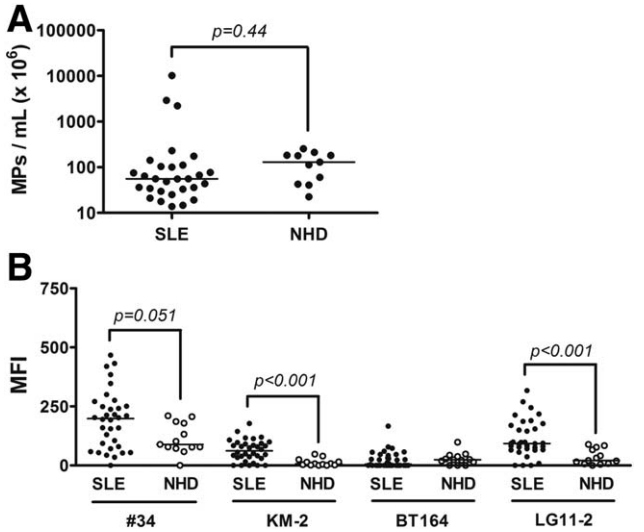

Figure 1. Microparticle (MP) concentrations in the plasma of systemic lupus erythematosus (SLE) patients and normal healthy donors (NHD), with more apoptosis-modified chromatin present in SLE patients. **A,** The concentration of microparticles in the plasma of SLE patients (n = 27) and healthy controls (n = 11) was determined by flow cytometry using 6.4-μm counting beads. **B,** Microparticles isolated from the plasma of SLE patients and healthy controls were stained with monoclonal antibodies #34 (anti-H3), KM-2 (anti-H4K8,12,16Ac), BT164 (anti-H3K27me3), or LG11-2 (anti-H2BK12Ac) and analyzed by flow cytometry. The background of the respective microparticles determined by staining with an isotype control (mouse IgG2A) was subtracted. Each symbol represents an individual subject; horizontal lines show the mean. MFI = mean fluorescence intensity.

microbeads, respectively (Miltenyi Biotec), as previously described (38). Purity was routinely as much as 95%, as assessed by double staining with BDCA-2+/CD123+ for PDCs and with CD11c+/CD1c+ for MDCs (Miltenyi Biotec). PDCs (with 10 ng/ml of recombinant human IL-3; CellGenix) and MDCs were cultured overnight in X-Vivo 15 medium (Lonza) supplemented with 2% human serum, at a concentration of 10^5 cells/100 μl/well in a 96-well round-bottomed plate. To determine the effect of microparticles on PDCs and MDCs, microparticles were isolated from plasma as described above and added for 16 hours at a concentration of 20% (volume/volume). As a positive control for activation, PDCs and MDCs were activated overnight in the presence of 5 mg/ml of R848 (M362; Axxora) or 1 μg/ml of lipopolysaccharide (LPS) (catalog code tlrl-3pelps; InvivoGen), respectively.

Flow cytometry and ELISA. For experiments with DCs, microparticles were isolated according to the method described above, the microparticle pellet was subsequently resuspended in the same volume of medium without serum, and the protein concentration was determined using the bicinchoninic acid assay (Sigma-Aldrich). Thereafter, serum was added directly to the microparticle preparation and adjusted to acquire equal amounts in microparticle-derived protein equivalents, and 20 μl was added to DCs in a 96-well plate containing 200 μl of medium per well.

The phenotype of PDCs and MDCs was determined using PE-conjugated anti-CD80, anti-CD83, and mouse IgG1, allophycocyanin-conjugated CD86 and mouse IgG1 (all BD Bioscience), and PE-conjugated anti-CD40 (Beckman Coulter). For determination of cytokine production, supernatants were collected from PDC/MDC cultures after 16 hours of incubation. Production of interferon-α (IFNα) and IL-6 was analyzed according to standard ELISA techniques using murine monoclonal capture and horseradish peroxidase–conjugated anti-IFNα antibodies (Bender MedSystems) or anti–IL-6 antibodies (Sanquin). Tumor necrosis factor (TNF) production was measured using a human TNF ELISA kit according to the manufacturer's protocol (BD Biosciences).

Induction and analysis of NETosis. Polymorphonuclear neutrophils (PMNs) were isolated from EDTA-anticoagulated whole blood using Lymphoprep (StemCell Technologies) as previously described (39). Isolated PMNs were then stimulated for 3 hours with 10 μg/ml of LPS (*Escherichia coli* O55:B5; Sigma-Aldrich) either alone or in combination with 20% (volume/volume) plasma-derived microparticles. Subsequently, NETs were harvested with 5 units/ml of micrococcal nuclease (Worthington) and quantified by measuring NET-derived DNA content on a Tecan Infinite 200 Pro multimode reader with Sytox Orange (Life Technologies). All measurements were confirmed by immunofluorescence microscopy on a Zeiss Axio Imager M1 fluorescence microscope.

Statistical analysis. Data are reported as the mean ± SEM of at least 3 experiments. Significance was determined by Student's *t*-test, Mann-Whitney U test, or one-way analysis of variance followed by Bonferroni correction. GraphPad Prism software was used for the analyses. *P* values less than 0.05 were considered significant.

RESULTS

Presence of apoptotic microparticles in the plasma of SLE patients. We measured the total concentration of microparticles in plasma samples from SLE patients with biopsy-proven proliferative lupus nephritis and active disease as well as from healthy individuals. As shown in Figure 1A, there was no significant difference in the concentration of microparticles between SLE patients and healthy individuals. Counted events represented membrane-containing structures, since treatment with 1% Triton X-100, which destroys membranous particles, completely abolished the population (data not shown). Subsequently, we evaluated the presence of apoptosis-modified histones by staining isolated microparticles with a panel of monoclonal antihistone antibodies (22–24,37). While apoptosis-modified histones were largely absent in samples from healthy controls, plasma-derived microparticles from the majority of SLE patients contained apoptosis-associated modifications as probed with mAb KM-2 and LG11-2, which recognize histone H4K8,12,16Ac and H2BK12Ac, respectively (Figure 1B). This resulted in significant differences in the mean fluorescence intensity signal obtained with these mAb in samples from SLE patients and healthy controls. A similar tendency was observed for histone H3 as probed with

mAb #34 (anti-H3), though the difference did not reach significance. There was no difference in the staining with mAb BT164 (anti-H3K27me3).

Interestingly, in plasma samples from SLE patients, we observed a particular population of microparticles characterized by a relatively high forward scatter (FSC) and low side scatter (SSC) profile, which was absent in plasma samples from healthy subjects (Figure 2A). Using calibrated beads in flow cytometry and electron microscopy, we determined that the size of SLE microparticles was distributed between 300 nm and 1.5 μm (data available upon request from the corresponding author). In addition, SLE plasma–derived microparticles contained a population with high expression of annexin V (Figure 2A), which corresponded to the SLE-associated population observed in the FSC/SSC plot. Microparticles positive for mAb LG11-2 (anti-H2BK12Ac) were also predominantly present in this same population (Figure 2B). Similar results were observed for microparticles positive for mAb KM-2 and #34 (data not shown). Microparticles in the plasma of healthy controls were weakly positive for annexin V and antihistone mAb, and no association with a specific FSC/SSC population was found. Thus, the plasma of SLE patients contains a specific population of microparticles that is absent from the plasma of healthy subjects. The strong staining for annexin V and the presence of apoptosis-modified histones clearly indicate that these microparticles must originate from apoptotic cells.

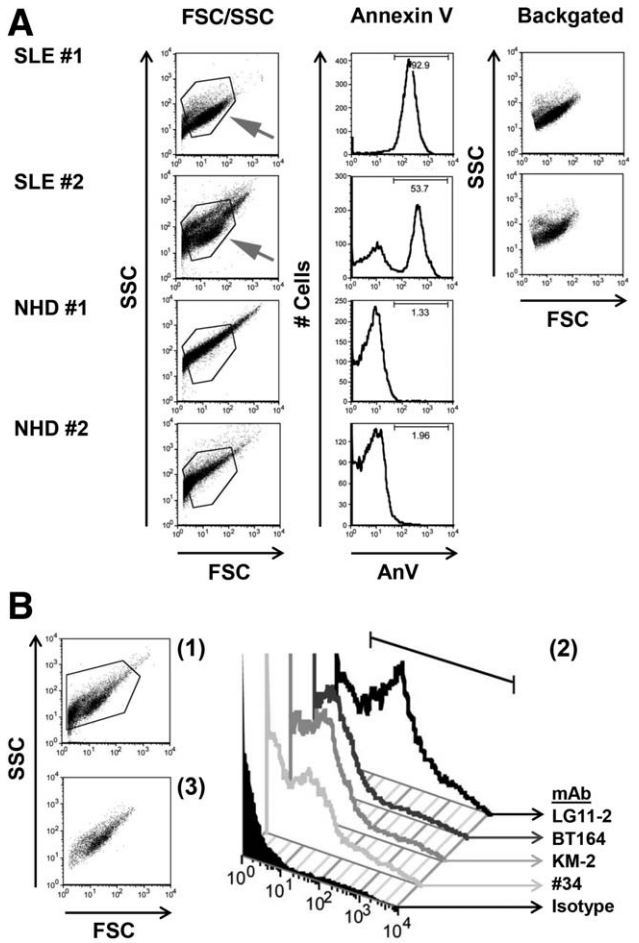

Figure 2. Presence of a specific population that represents apoptotic cell–derived microparticles in circulating microparticles from systemic lupus erythematosus (SLE) patients. **A,** Representative histograms of plasma-derived microparticles from 2 SLE patients with different amounts of the SLE-associated population and from 2 normal healthy donors (NHD). **Arrows** in the FSC/SSC plots (left) indicate a population of microparticles with a relatively high FSC and low SSC profile. Microparticles were then stained with annexin (AnV)–fluorescein isothiocyanate (middle), and the population highly positive for annexin V was backgated into the FSC/SSC plot (right) for the 2 SLE patients. **B,** Representative histograms of SLE-derived microparticles (panel 1) that were stained with antihistone monoclonal antibodies (mAb) LG11-2 (anti-H2BK12Ac), BT164 (anti-H3K27me3), KM-2 (anti-H4K8,12,16Ac), #34 (anti-H3), or mouse IgG2A (isotype) (panel 2). Microparticles that were positive for LG11-2 were backgated into the FSC/SSC plot (panel 3).

SLE-derived apoptotic microparticles from endothelial cells. We next determined the cellular origin of the apoptotic cell–derived microparticles in SLE patients. Since our population of microparticles was positive for chromatin, we could exclude nonnucleated platelets and erythrocytes as a potential source. The majority of the apoptotic cell–derived microparticles stained positive for CD31 (Figure 3A), ranging from 65 to 95% between patients (Figure 3D), which is commonly used as a marker for endothelial cells but is also present on certain hematopoietic cells. Subsequently, double staining with CD45, a marker for all hematopoietic cells, demonstrated that ~15% of the CD31+ apoptotic microparticles were also CD45+. We then analyzed markers for T cells (CD3), B cells (CD19), and granulocytes (CD66b) to determine which hematopoietic cell type could be the origin of the CD45+ microparticles. A total of 5–15% of the CD31+ apoptotic cell–derived microparticles were positive for CD66b (Figure 3B), while apoptotic microparticles were completely negative for CD3 and CD19 (Figure 3C). Thus, circulating apoptotic cell–derived microparticles in SLE patients are mainly derived from endothelial cells (CD31+/CD45–; ~60%) and to a lesser extent from granulocytes (CD31+/CD66b+; ~15%) and other as-yet-unidentified sources (~25%).

SLE-derived microparticles and activation of blood-derived PDCs and MDCs. We isolated microparticles from the plasma of SLE patients and healthy controls and evaluated their effect on CD1c+ MDCs and BDCA-4+ PDCs isolated from the peripheral blood of healthy donors. Incubation of PDCs with micro-

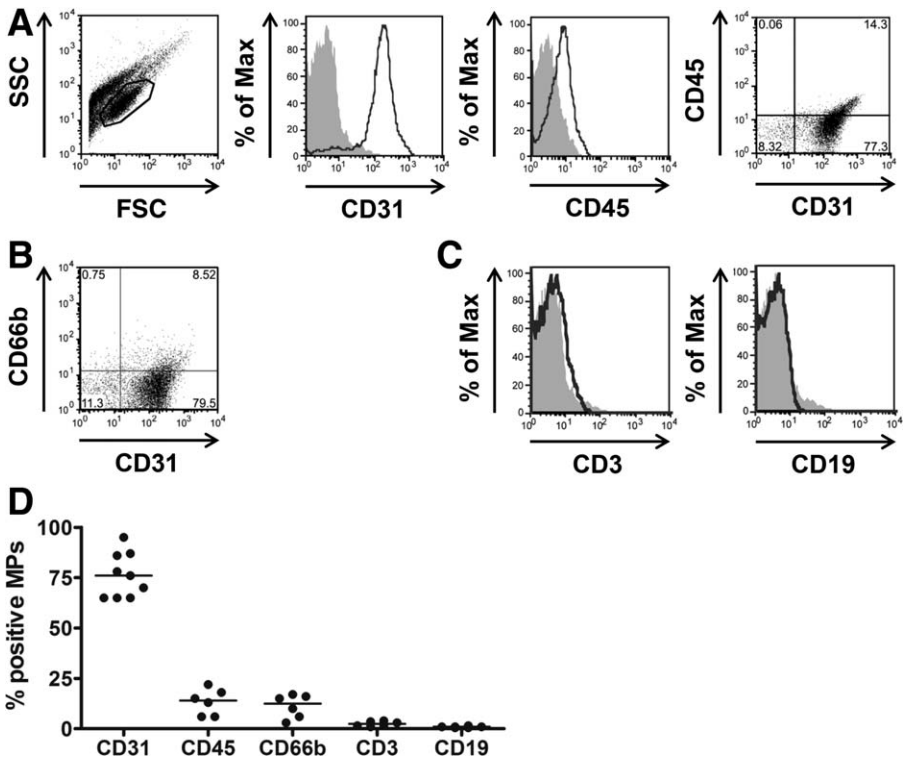

Figure 3. Endothelial cells as the primary source of apoptotic microparticles (MPs) in systemic lupus erythematosus (SLE) patients. **A,** Microparticles isolated from the plasma of SLE patients were stained with phycoerythrin (PE)–conjugated anti-CD31 and fluorescein isothiocyanate (FITC)–conjugated anti-CD45. The SLE-specific (apoptotic cell–derived) population, based on the FSC/SSC profile, was gated for further analysis (left). Representative histograms of the single and double staining for CD31 and CD45 are also shown (middle and right). Shaded histograms show the respective isotype antibody that was used as a control for background staining. **B** and **C,** SLE-derived apoptotic microparticles were analyzed using FITC-conjugated anti-CD66b in combination with PE-conjugated anti-CD31 (**B**) or with PE-conjugated anti-CD3 or anti-CD19 (**C**). **D,** The percentage of apoptotic microparticles positive for the indicated markers is shown for samples from 6–9 SLE patients. Each symbol represents an individual subject; horizontal lines show the median.

particles from SLE patients resulted in a significant increase in the expression of the costimulatory molecules CD80, CD83, and CD40, while incubation with those from healthy controls exhibited no significant effect (Figure 4A). Expression of CD86 was not appreciably affected by the addition of microparticles from SLE patients or healthy controls. Microparticles from SLE patients also induced a more pronounced production of the proinflammatory cytokines IFNα, IFNγ, and TNF in PDCs, while microparticles from healthy controls had no effect on the production of these cytokines (Figure 4A).

When MDCs were incubated with plasma-derived microparticles from SLE patients or healthy controls, we observed a small increase in the expression of CD86 (Figure 4B). The expression of CD80 and CD83 was increased only in the presence of microparticles from SLE patients, while no significant effect of microparticles on the expression of CD40 was found. Furthermore, SLE microparticles induced the production of both IL-6 and TNF (Figure 4B). Thus, in contrast to microparticles derived from healthy controls, microparticles isolated from the plasma of SLE patients are able to activate both PDCs and MDCs, as indicated by a significant up-regulation of several costimulatory molecules and a more pronounced production of proinflammatory cytokines.

Absence of chromatin in microparticles from RA and SSc patients and lack of induction of DC activation. To determine if the above-described characteristics of plasma-derived microparticles were specific for SLE, we also tested microparticles from the plasma of patients with rheumatoid arthritis (RA) and systemic sclerosis (SSc). We found that plasma from RA and SSc patients also contained an annexin V+/CD31+ microparticle population (Figure 5A). However, these microparticles exhibited a low signal for our panel of antichromatin mAb (Figure 5B), which was comparable to that for microparticles from healthy controls (Figure 1B).

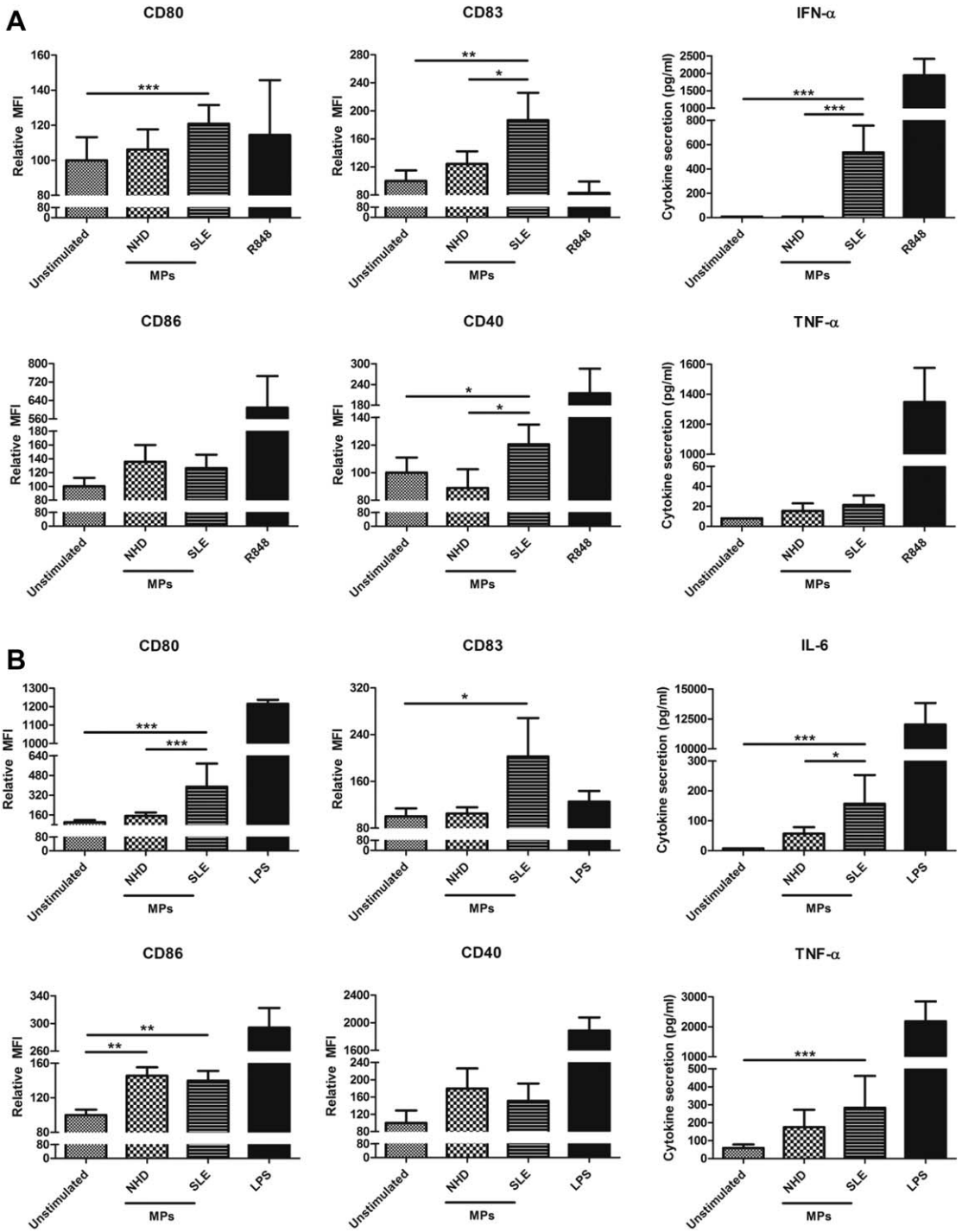

Figure 4. Capacity of systemic lupus erythematosus (SLE)–derived microparticles (MPs) to stimulate plasmacytoid dendritic cells (PDCs) and myeloid dendritic cells (MDCs). Freshly isolated PDCs (**A**) or MDCs (**B**) from normal healthy donors (NHD) were left unstimulated (with PDCs in the presence of interleukin-3 [IL-3]) or were incubated overnight with microparticles isolated from the plasma of healthy donors or SLE patients. Surface expression of costimulatory molecules CD80, CD83, CD86, and CD40 was then determined by flow cytometry. Supernatants from microparticle-stimulated PDC and MDC cultures were analyzed for proinflammatory cytokines interferon-α (IFNα), tumor necrosis factor (TNF), and IL-6. R848 and lipopolysaccharide were used as a positive control for PDC and MDC activation, respectively. Values are the mean ± SEM of 4 independent experiments using microparticles from at least 8 SLE patients and 8 healthy donors. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$. MFI = mean fluorescence intensity.

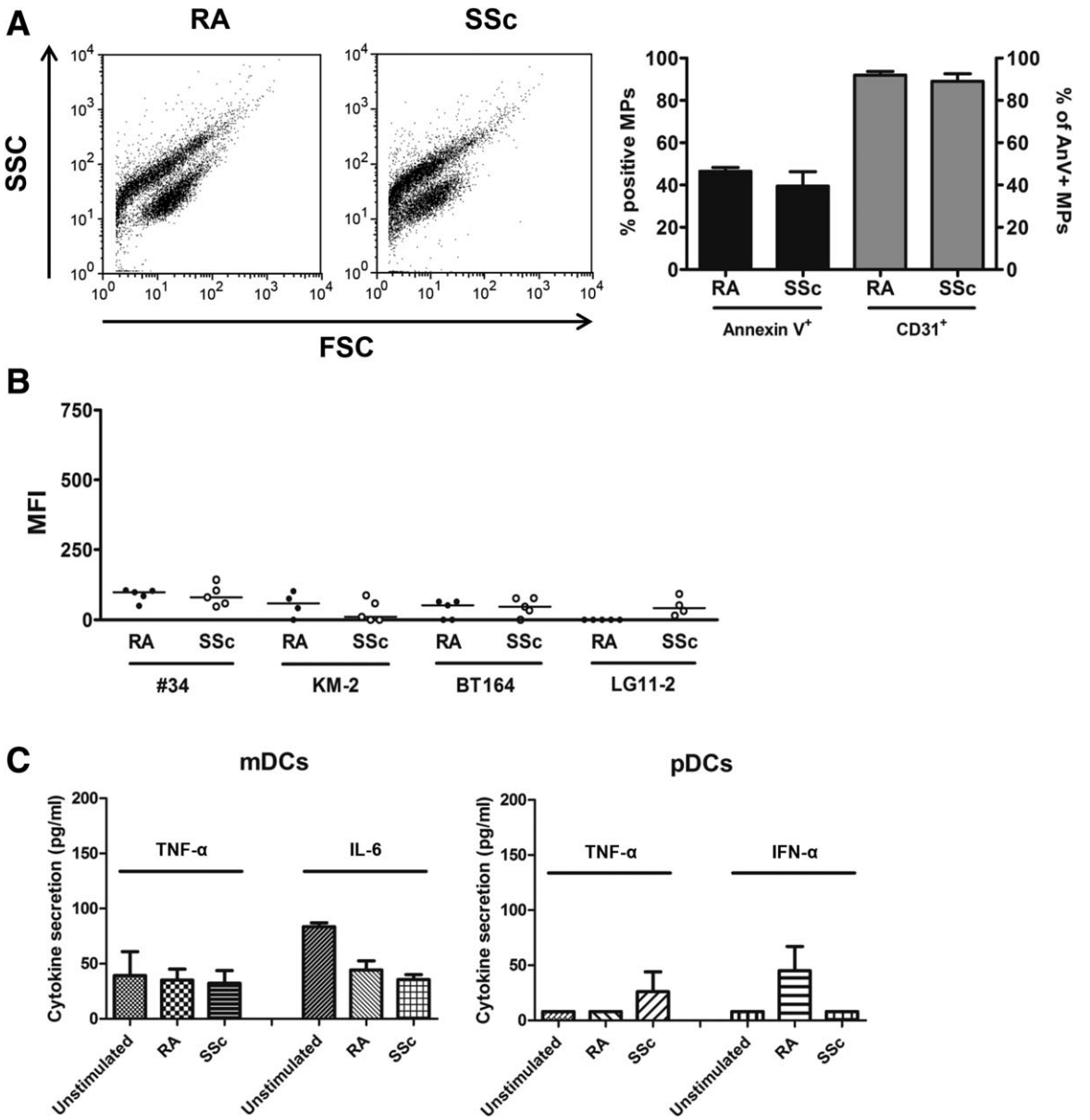

Figure 5. Chromatin levels in microparticles (MPs) derived from rheumatoid arthritis (RA) and systemic sclerosis (SSc) patients and lack of dendritic cell (DC) stimulation. **A,** Representative FSC/SSC plot of plasma-derived microparticles from an RA patient and an SSc patient (left). Microparticles from RA and SSc patients were stained with fluorescein isothiocyanate (FITC)–conjugated annexin V (AnV) or CD31 (right). Values are the mean ± SEM of 5 subjects per group. **B,** Mean fluorescence intensity (MFI) of plasma-derived microparticles from RA and SSc patients stained with monoclonal antibodies #34 (anti-H3), KM-2 (anti-H4K8,12,16Ac), BT164 (anti-H3K27me3), or LG11-2 (anti-H2BK12Ac), as determined by flow cytometry. Each symbol represents an individual subject; horizontal lines show the mean. **C,** Levels of the proinflammatory cytokines tumor necrosis factor (TNF), interleukin-6 (IL-6), and interferon-α (IFNα) in supernatants from microparticle-stimulated plasmacytoid DC (PDC) and myeloid DC (MDC) cultures. Values are the mean ± SEM of at least 5 subjects per group.

In addition, plasma-derived microparticles from RA and SSc patients could not induce the production of proinflammatory cytokines in PDCs and MDCs (Figure 5C). Therefore, the chromatin content and proinflammatory capacity of annexin V+/CD31+ microparticles in SLE is distinct from that in RA and SSc.

Priming of PMNs for NETosis by SLE-derived microparticles. NETs have recently been proposed as an additional source of modified chromatin and have been associated with propagation of the antichromatin response in SLE patients (30–34,39). We hypothesized that a change in the composition of circulating micropar-

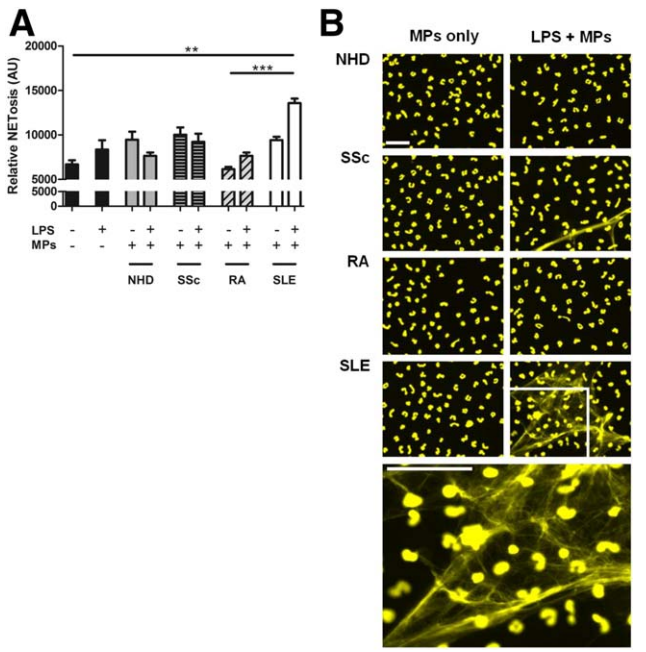

Figure 6. Systemic lupus erythematosus (SLE)–derived microparticle (MP) priming of neutrophils for lipopolysaccharide (LPS)–induced NETosis. **A,** Freshly isolated polymorphonuclear neutrophils from healthy donors were incubated for 3 hours with 10 μg/ml of LPS (*Escherichia coli* serotype O55:B5), with 20% (volume/volume) microparticles derived from normal healthy donors (NHD) or SLE, systemic sclerosis (SSc), or rheumatold arthritis (RA) patients, or with a combination of both LPS and microparticles. Neutrophil extracellular trap (NET)–derived DNA was quantified with Sytox orange, and fluorescence intensities are shown. Values are the mean $\pm$ SEM of 5 subjects per group. ** = $P < 0.01$; *** = $P < 0.001$. **B,** NET release in response to microparticles either alone or in combination with LPS was confirmed by immunofluorescence microscopy following the staining of NET-derived DNA with Sytox orange. Bottom image is a higher-magnification view of the boxed area in the right panel of the SLE sample above. Bars = 50 μm.

ticles in SLE patients might also influence the formation of NETs by neutrophils. We therefore evaluated whether plasma-derived microparticles could either directly induce NETosis or affect LPS-induced NETosis in blood-derived polymorphonuclear neutrophils (PMNs). PMNs isolated from healthy controls and incubated with microparticles alone (from the plasma of healthy donors or SLE, RA, or SSc patients) did not result in a significant release of NETs (Figures 6A and B). Incubation of PMNs with a combination of SLE microparticles and 10 μg/ml of LPS (*E coli* serotype O55:B5) resulted in robust NET formation, whereas microparticles from healthy donors or RA and SSc patients did not influence NETosis (Figures 6A and B). Interestingly, LPS alone failed to induce NET formation, which is consistent with work from others who show that LPS alone may not be sufficient to induce NETosis but that additional "priming" stimuli (e.g., granulocyte–macrophage colony-stimulating factor, IL-5/IFNγ) are required (40). Therefore, circulating microparticles derived from SLE patients specifically prime neutrophils for LPS-induced NETosis in blood-derived neutrophils.

DISCUSSION

In this study, we showed that microparticles present in the circulation of SLE patients have different characteristics from those in healthy controls. In particular, we found that plasma from SLE patients possessed a specific microparticle population with a different size and granularity that was highly positive for annexin V and contained apoptosis-modified chromatin. Although an increase in the concentration of circulating microparticles has been observed in SLE patients (9,10,28), we and other investigators have found a change in the composition of microparticles without an increase in the concentration (6,7). Importantly, we determined the total concentration of microparticles without using specific cell markers, whereas other investigators sometimes used specific cell markers. Plasma from RA and SSc patients also contained annexin V+/CD31+ microparticles but did not contain (apoptotic) chromatin as in the SLE-derived microparticles. These data are consistent with the existing literature (3,4), which describes disease-related changes in endothelial microparticles in these autoimmune diseases. Moreover, the absence of chromatin-containing microparticles in RA and SSc is consistent with the notion that circulating apoptotic chromatin is a specific hallmark of SLE.

There are several arguments to conclude that the SLE-associated microparticle population examined in our study was derived from apoptotic cells. First, the presence of modified histones in the microparticles indicates that apoptosis is involved, since the translocation of chromatin into microparticles occurs exclusively during cell death. Second, the increase in forward scatter of the SLE-specific microparticle population observed with flow cytometry fits with previous observations of an increased size of apoptotic microparticles as compared to microparticles released by living cells (1). Furthermore, the SLE-specific microparticle population expresses phosphatidylserine, which is massively externalized during apoptosis. We also demonstrated that our flow cytometry results should indeed be attributed to microparticles and not to protein complexes as described by György et al (41,42), since treatment with 1% Triton X-100 completely abolished the population (data not shown).

Based on the expressed surface molecules, we showed that SLE-associated microparticles originate

mostly from endothelial cells and partially from granulocytes. To our knowledge, this is the first clue to the origin of circulating apoptotic microparticles in SLE patients. These findings also support a recent study that demonstrated an increase in endothelial-derived microparticles in SLE patients with active disease, which was reduced after immunosuppressive therapy (43). Interestingly, injection of microparticles derived from apoptotic endothelial cells induces an inflammatory response in mice (15). Although circulating endothelial microparticles are sometimes used as a marker of endothelial damage in patients (44), including those with renal disease (45), these studies did not specifically examine well-defined apoptotic microparticle populations. They instead included microparticles derived from activated, nonapoptotic endothelial cells. Nonetheless, we cannot exclude the possibility that vascular damage contributes to the generation of apoptotic endothelial microparticles in our cohort of SLE patients. Since all of our patients had active (renal) disease at the time of plasma retrieval, apoptotic microparticles from other sources could play a more prominent role during the initial phase of disease development.

Importantly, we demonstrated that microparticles isolated from the plasma of SLE patients can activate blood-derived PDCs and MDCs, while microparticles from the plasma of healthy controls or RA and SSc patients did not exhibit such an effect. This is consistent with previous experiments using apoptotic microparticles generated in vitro after induction of apoptosis in either cell lines or blood-derived cells (20,21,25–27). We particularly observed an increase in the production of proinflammatory cytokines, while the increased expression of costimulatory molecules was less pronounced. IFNα production is independent of NF-κB activation in PDCs, and it is therefore possible to dissociate CD80 and CD86 induction from IFNα. However, one would expect that TNF and IL-6 production would coincide with CD80, CD83, and CD86 expression. Other investigators have also found this discordance between cytokine production and the expression of costimulatory molecules (26,33), which could be explained by the difference in the type and sensitivity of the assay used (flow cytometry versus ELISA).

The mechanisms involved in microparticle-induced activation of DCs are not completely clear. While activation of MDCs by apoptotic microparticles was found to be independent of cytosolic TLRs (20), TLR-9 was implicated in the sensing of microparticle-derived DNA (27). Importantly, the latter occurred independently of Fcγ receptor IIA, in contrast to activation by nucleic acid–containing immune complexes (46). We suggest that apoptosis-modified histones in microparticles can interact with cell surface receptors, such as TLR-2 and TLR-4 (16), which have been implicated in the pathogenesis of SLE (47–49). Further experiments are needed to unravel the exact receptors/pathways used by microparticles to activate MDCs and PDCs. Finally, we found that SLE-derived microparticles, but not RA- or SSc-derived microparticles, primed neutrophils for LPS-induced NETosis. The formation of NETs in SLE patients has been associated with activation of neutrophils by nucleic acid–containing immune complexes (33,34). With regard to microparticles, only an effect of placental microparticles on the formation of NETs has been demonstrated (50). Our findings imply that the presence of a high concentration of apoptotic microparticles in the circulation of SLE patients can enhance the formation of NETs, which amplifies the autoimmune response and chronicity of the disease.

In conclusion, apoptotic microparticles have been proposed as an important driver of the chromatin-directed immune response in SLE patients. We show that SLE patients have distinct apoptotic cell–derived microparticles in their circulation and that these have a proinflammatory effect on PDCs and MDCs and enhance NETosis.

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. Van der Vlag 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. Dieker, Tel, Pieterse, van der Vlag.

Acquisition of data. Dieker, Tel, Pieterse, Thielen, Rother, Bakker, Fransen, Dijkman.

Analysis and interpretation of data. Dieker, Tel, Pieterse, Berden, de Vries, Hilbrands, van der Vlag.

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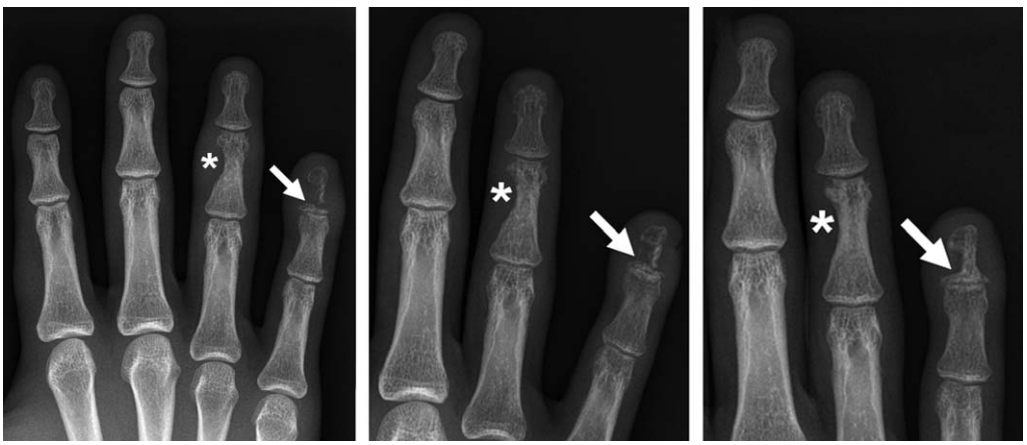
Clinical Images: Osseous sarcoidosis revealed by a pathologic fracture and successfully treated with methotrexate and prednisone



The patient, a 31-year-old man, presented with painful swelling of the distal part of the little finger of the right hand after minor trauma. The only significant medical history was pulmonary sarcoidosis diagnosed 2 years earlier, which had not been treated. The only other abnormality on physical examination was painless swelling of the second, third, and fourth distal phalanges of the right hand. Laboratory tests showed an elevated erythrocyte sedimentation rate, a normal serum calcium level, and a low vitamin D level. The angiotensin-converting enzyme level was moderately elevated. Radiographs revealed a pathologic comminuted intraarticular fracture of the distal phalanx of the little finger (**arrow**), with multiple osteolytic lesions as well as soft tissue masses (**asterisk**) (**left**). Chest computed tomography subsequently revealed multiple bilateral micronodular opacities with bilateral hilar and mediastinal node enlargement. A bone scan revealed increased uptake in the hands, feet, and right knee. The diagnosis of skeletal sarcoidosis was confirmed by surgical biopsy of the fifth distal phalanx, which showed typical nonnecrotizing granulomatous inflammation. After 2 days of treatment with prednisone and methotrexate (15 mg/week), the patient experienced a significant reduction in pain, with progressive recorticalization of the distal (**arrows**) and middle (**asterisks**) phalanges within 3 months (**middle**), which persisted after 1 year (**right**). Sarcoidosis is characterized by noncaseating granulomas infiltrating affected organs; more than 90% of patients have a primary pulmonary manifestation. Bone involvement is typically associated with pulmonary involvement and skin lesions (1). Pathologic fractures caused by osseous sarcoidosis are rare (2), but this diagnosis should be considered in cases of lytic bone lesions.

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