



Research article

Induction of NF- κ B inflammatory pathway in monocytes by microparticles from patients with systemic lupus erythematosusKaren Álvarez^a, Juan Villar-Vesga^b, Blanca Ortiz-Reyes^a, Adriana Vanegas-García^{c,d}, Diana Castaño^a, Mauricio Rojas^{a,e}, Gloria Vásquez^{a,c,*}^a Grupo de Inmunología Celular e Inmunogenética (GICIG), Sede de Investigación Universitaria (SIU), Facultad de Medicina, Universidad de Antioquia (UDEA), Carrera 53 No.61-30, Medellín, Colombia^b Grupo de Neurociencias de Antioquia, Área de Neurobiología Celular y Molecular, Facultad de Medicina. Sede de Investigación Universitaria (SIU), Universidad de Antioquia (UDEA), Calle 70 No.52-21, Medellín, Colombia^c Grupo de Reumatología, Facultad de Medicina, Universidad de Antioquia (UDEA), Carrera 53 No.61-30, Medellín, Colombia^d Sección de Reumatología, Hospital Universitario San Vicente Fundación, Calle 64 No.51D-154, Medellín, Colombia^e Unidad de Citometría de Flujo, Sede de Investigación Universitaria (SIU), Universidad de Antioquia (UDEA), Carrera 53 No.61-30, Medellín, Colombia

ARTICLE INFO

Keywords:

Autoimmunity
Inflammation
NF- κ B pathway
Lupus

ABSTRACT

Background: Elevated levels of circulating microparticles (MPs) and molecules of the complement system have been reported in patients with systemic lupus erythematosus (SLE). Moreover, microparticles isolated from patients with SLE (SLE-MPs) contain higher levels of damage-associated molecular patterns (DAMPs) than MPs from healthy controls (CMPs). We hypothesize that the uptake of MPs by monocytes could contribute to the chronic inflammatory processes observed in patients with SLE. Therefore, the aim of this study was to evaluate the expression of activation markers, production of proinflammatory mediators, and activation of the NF- κ B signaling pathway in monocytes treated with CMPs and SLE-MPs.

Methodology: Monocytes isolated from healthy individuals were pretreated or not with pyrrolidine dithiocarbamate (PDTC) and cultured with CMPs and SLE-MPs. The cell surface expression of CD69 and HLA-DR were evaluated by flow cytometry; cytokine and eicosanoid levels were quantified in culture supernatants by Cytokine Bead Array and ELISA, respectively; and the NF- κ B activation was evaluated by Western blot and epifluorescence microscopy.

Results: The cell surface expression of HLA-DR and CD69, and the supernatant levels of IL-6, IL-1 β , PGE2, and LTB4 were higher in cultures of monocytes treated with SLE-MPs than CMPs. These responses were blocked in the presence of PDTC, a pharmacological inhibitor of the NF- κ B pathway, with concomitant reduction of I κ B α and cytoplasmic p65, and increased nuclear translocation of p65.

Conclusions: The present findings indicate that significant uptake of SLE-MPs by monocytes results in activation, production of inflammatory mediators, and triggering of the NF- κ B signaling pathway.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder of unknown etiology with a broad spectrum of clinical manifestations, mainly characterized by the presence of multiple circulating antibodies against a variety of self-antigens [1]. Several studies have shown that an increased rate of apoptosis and diminished removal of apoptotic cells favor the development of autoimmunity [2, 3]. During apoptosis, cells release diverse subcellular vesicles, including apoptotic bodies and microparticles (MPs), which differ in size, origin, and composition [4, 5].

These vesicles act as modulators of the immune response and represent a primary source of autoantigens in patients with SLE [6].

Microparticles are a heterogeneous population of membrane vesicles (0.1–1 μ m diameter) primarily derived from the plasma membrane of different cells during processes of cell activation and death. These vesicles contain different cellular components and play essential roles in the intercellular communication involved in processes, such as inflammation, coagulation, angiogenesis, and apoptosis [7, 8]. Microparticles can act through different mechanisms, including the release of molecules into the extracellular environment, the triggering of enzymatic cascades by

* Corresponding author.

E-mail address: gloria.vasquez@udea.edu.co (G. Vásquez).<https://doi.org/10.1016/j.heliyon.2020.e05815>

Received 15 June 2020; Received in revised form 21 July 2020; Accepted 18 December 2020

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molecules exposed on the surface, direct interactions with target cells, and the transfer of intracellular molecules and surface receptors to acceptor cells by membrane fusion or internalization [8]. Also, MPs can bind to circulating antibodies to form immune complexes, which, in turn, interact with target cells through complement and Fc receptors [9].

High levels of circulating MPs have been reported in patients with SLE [10, 11, 12]; moreover, these MPs have a higher content of immunoglobulins, complement components, and damage-associated molecular patterns (DAMPs), in comparison with MPs from healthy controls [13, 14, 15]. Thus, the recognition of these extracellular vesicles by cells of the immune system could contribute to the chronic inflammatory processes observed in the course of SLE. However, this hypothesis has not been extensively studied, and many proinflammatory mechanisms induced by MPs in patients with SLE remain to be elucidated.

Monocytes are considered critical players in the development of SLE. Patients with SLE have monocytes with different phenotypic and functional alterations [16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26] that produce inflammatory mediators and perpetuate the autoimmune response through antigenic presentation and co-stimulation of self-reactive T- and B-cells. When compared to monocytes from healthy controls, monocytes from patients with SLE overexpress different molecules: Fc gamma receptor I (FcγRI, CD64) [16–18], adhesion, and costimulatory molecules such as CD40L [19], and the Intercellular Adhesion Molecule-1 (ICAM-1, CD154) [18]. These molecules could reflect the activation state of monocytes, and suggest they might be more efficient antigen-presenting cells in patients with SLE. Additionally, in *in vitro* experiments, monocytes from patients with SLE overproduce inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) [21, 22], interleukin 6 (IL-6) [22, 23], IL-1β [22], and BAFF [26]. Moreover, the accumulation of CD16⁺ monocytes in renal tissues correlate with impaired renal function in lupus nephritis [27, 28]. Monocytes could also play a key role in vesicle removal as expected from their potent phagocytic activity that allows them to clear circulating apoptotic bodies, microorganisms, and immune complexes.

The NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells) proteins comprise a family of transcription factors that regulate the expression of different genes, including those coding for pro-inflammatory cytokines, chemokines, and adhesion molecules [29]. This family of transcription factors play an essential role in the survival of lymphocytes, activation of innate immune cells, and therefore, the initiation of a normal immune response. The canonical NF-κB pathway involves various steps, including the phosphorylation, ubiquitination, and degradation of the NF-κB Inhibitor alpha (IκBα) that leads to the nuclear translocation of the NF-κB p50 and p65 subunits. Constitutive activation of the NF-κB pathway is frequently associated with inflammatory diseases, such as rheumatoid arthritis and SLE [30]; also, in response to TNF-α, the monocytes of patients with SLE show greater NF-κB nuclear translocation and lower p-IκBα levels than monocytes from healthy individuals [31]. The variety of molecules contained in MPs of patients with SLE could contribute to greater NF-κB activation in cells interacting with the vesicles.

Considering the role of monocytes in the development of SLE and their ability to clear dead cells components [32], as well as the constitutive activation of the NF-κB pathway observed in inflammatory diseases [30] and the higher nuclear translocation of NF-κB in TNF-α treated-monocytes from patients with SLE [31], this study aimed to evaluate the activation, production of inflammatory mediators, and the triggering of the NF-κB signaling pathway in monocytes exposed to microparticles isolated from the plasma of patients with SLE.

2. Materials and methods

2.1. Patients and controls

Thirty patients diagnosed with SLE, according to the American College of Rheumatology criteria [33, 34], were enrolled in the present

study. They were evaluated by the Grupo de Reumatología (Rheumatology Group) de la Universidad de Antioquia (GRUA, by its Spanish acronym) at Hospital Universitario San Vicente Fundación (HUSVF, by its Spanish acronym), in Medellín, Colombia. Also, 15 gender-matched healthy individuals who were similar to patients in age participated in the study as healthy controls (HCs). Additionally, blood samples were drawn from 10 healthy individuals (age range = 24–31 years) for isolating peripheral mononuclear cells (PBMCs) and monocytes. All HCs declared not to be taking any medication or having any active infectious disease. All individuals agreed to participate in the study voluntarily by signing an informed consent approved by the Ethics Committee of the Institute of Medical Research in the Faculty of Medicine at Universidad de Antioquia.

2.2. Isolation and characterization of microparticles

Microparticles from patients with SLE (SLE-MPs) and controls (CMPs) were isolated from 4 mL of citrated venous blood (BD-Vacutainer® 3.8% sodium citrate tubes; Becton Dickinson, San Jose, CA) as previously described [35]. Briefly, the blood sample was centrifuged at 1,800 x g for 10 min at 21 °C to obtain total plasma. This plasma was then centrifuged twice at 3,000 x g for 20 min at 21 °C to remove the platelets and obtain platelet-poor plasma (PPP). Finally, 1 mL of PPP was centrifuged at 16,900 x g for 1 h to allow the sedimentation of MPs. These vesicles were suspended in filtered Dulbecco's Phosphate-buffered saline (1X DPBS; Gibco, Grand Island, NY), taking care not to disturb them, and kept at –70 °C until use.

The isolated vesicles were confirmed to be MPs (0.1 μm–1 μm in diameter) by flow cytometry analysis using reference latex microspheres with diameters of 0.1 μm, 0.5 μm, 1.0 μm, and 2.0 μm (Polysciences, Inc. Warrington, PA). The acquisition threshold for size (FSC-A) and granularity (SSC-A) parameters were set with filtered sheath fluid. Samples were acquired on a FACSCanto™ II flow cytometer (Becton Dickinson) with FACSDiva™ Software version 6.0 (Becton Dickinson).

The protein content of the MPs was quantified by the bicinchoninic acid (BCA) method (Thermo Scientific, Waltham, MA) following the manufacturer's instructions. The absorbance of samples was read at 562 nm using a Universal Microplate Reader BiotekELx800NB spectrophotometer (Biotek Instruments, Winooski, VT). Protein concentrations were calculated using a standard curve made with known concentrations of bovine serum albumin (BSA).

2.3. Isolation of peripheral blood mononuclear cells

PBMCs were isolated from EDTA-anticoagulated venous blood (BD-Vacutainer® tubes; Becton Dickinson) after centrifugation at 900 x g for 30 min at 21 °C on a density gradient (1.077 g/mL) of Histopaque (Sigma Aldrich, St. Louis, MO). The cells were washed twice with PBS at 300 x g for 10 min and suspended in RPMI (Gibco, Grand Island, NY) supplemented with penicillin (100 U/mL)/streptomycin (100 μg/mL) (Cambrex-Bioscience, Walkersville, MD), and 10% inactivated fetal bovine serum (iFBS). Cell viability was evaluated by trypan blue (Sigma Aldrich, St. Louis, MO) dye exclusion in Neubauer chamber: percentages ≥95% were observed.

2.4. Labeling of microparticles

Microparticles (1000 μL) obtained from blood samples of HCs were mixed with 1 μM carboxyfluorescein succinimidyl ester (CFSE; Thermo scientific, Waltham, MA) and incubated at 37 °C for 10 min; then 100 μL of iFBS were added, and vesicles were incubated at room temperature for 5 additional minutes. Finally, the vesicles were washed twice with RPMI-1640 supplemented with 10% iFBS at 16,900 x g for 1 h. The supernatant was removed, the pellet was suspended in 1 mL filtered PBS and the CFSE-labeled CMPs were acquired on a FACSCanto™ II flow cytometer.

2.5. Binding and uptake of microparticles

PBMCs (1×10^5) from healthy controls were cultured for 1 h with or without CFSE-labeled CMPs (30, 50, and 100 $\mu\text{g}/\text{mL}$ of protein). The cells were then stained with anti-CD14-MY4-RD1 (clone 322A-1; Beckman Coulter, Brea, CA), anti-CD3-V510 (clone OKT3; Biolegend, San Diego, CA), and anti-CD19-V450 (clone HIB19; BD Pharmingen, San Diego, CA) mAbs, incubated in darkness at room temperature for 20 min, washed with PBS, and acquired on the flow cytometer. An initial acquisition was run in the FACSCanto™ II flow cytometer to evaluate the percentage of binding/uptake of MPs by the PBMCs. Subsequently, 0.01% v/v trypan blue was added to the acquisition tubes to quench the fluorescence of the MPs bound to cell surfaces, and a second acquisition was run on the cytometer to evaluate the percentage of uptake of MPs by the cells. The percentages of both, binding/uptake and uptake of MPs by different leukocyte populations were determined with the Overton subtraction algorithm (FlowJo™ Software version X; Ashland, OR: Dickinson and Company 2019).

2.6. Enrichment of monocytes and effect of microparticles on the expression of cell surface markers

Monocytes were enriched from the peripheral blood samples of healthy individuals by negative selection using the RosetteSep™ Human Monocyte Enrichment Cocktail (StemCell Technologies, Vancouver, Canada) according to the manufacturer's instructions. The purity of monocytes was $\geq 90\%$. Approximately 1×10^5 monocytes were pretreated with different doses (0, 1, 5, and 25 μM) of pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF- κ B activation [36, 37]. Monocytes were then stimulated for 6 h, under adherent conditions, with CMPs or 5 $\mu\text{g}/\text{mL}$ Lipopolysaccharide (LPS from *Escherichia coli*, serotype O111:B4; InvivoGen, San Diego, CA) (positive control). All cultures were performed at 37 °C and 5% CO₂ in 96-well flat-bottom plates in a final volume of 200 μL of RPMI-1640 supplemented with penicillin (100 U/mL), streptomycin (100 $\mu\text{g}/\text{mL}$), and 10% iFBS previously filtered through a 0.20 μm -Acrodisc® syringe filter. After incubation, the cell surface expression of activation markers (HLA-DR and CD69) was evaluated by flow cytometry.

The viability of PDTC-treated cells was assessed with propidium iodide (PI) and DIOC₆ staining. Additionally, to rule out any effect of 25 μM PDTC on the cellular ability to bind and uptake MPs, PBMCs treated or not with PDTC were incubated with CFSE-labeled CMPs for 1 h to evaluate the percentages of binding and uptake of MPs by cells.

2.7. Effect of microparticles on the production of cytokines and eicosanoids by monocytes

Monocytes (1×10^5) isolated from healthy individuals and pretreated with or without 25 μM PDTC for 1 h were stimulated with 30 $\mu\text{g}/\text{mL}$ vesicles (SLE-MPs or CMPs). Culture supernatants were collected after 6 and 24 h incubation and stored at 20 °C to evaluate the concentrations of cytokines and eicosanoids.

The concentration of TNF- α , IL-1 β , and IL-6 was evaluated in 6 h-supernatants by CBA (Human Inflammatory Cytokine kit; BD Biosciences, San Diego, CA) following the manufacturer's instructions. Additionally, the levels of PGE₂ (6-hour supernatants) and BAFF and LTB₄ (24-hour supernatants) were quantified by ELISA following the manufacturers' instructions (PGE₂ and LTB₄ kits; Abcam, Cambridge, United Kingdom. BAFF kit; R&D Systems, Minneapolis, MN). The PDTC-mediated inhibition of the production of these inflammatory mediators was expressed in terms of percentage of inhibition in relation to the PDTC-unexposed control.

2.8. Effect of microparticles on the activation of the NF- κ B signaling pathway

PBMCs (2×10^6) from healthy individuals pretreated or not with PDTC for 1 h were cultured with 30 $\mu\text{g}/\text{mL}$ vesicles (CMPs or SLE-MPs) or

5 $\mu\text{g}/\text{mL}$ LPS for 30 min. Then, cells were collected and washed with cold PBS for preparing cell lysates, as described below, to evaluate the levels of p65 and I κ B α .

2.8.1. Nuclear and cytoplasmic fractionation

Microparticles-treated PBMCs (2×10^6) were lysate to obtain cytoplasmic and nuclear fractions as described elsewhere [38]. Briefly, cells were centrifuged at 1,000 $\times g$ for 5 min at 4 °C, and the cell pellet was suspended in 200 μL of buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5% (v/v) IGEPAL, 0.5 mM DTT, and protease inhibitor), incubated at 4 °C for 20 min, and centrifuged at 6,000 $\times g$ for 10 min at 4 °C to collect the supernatant (cytoplasmic fraction). The cell pellet was then washed twice with buffer A, suspended in 200 μL of buffer C (20 mM HEPES pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF, and protease inhibitor), incubated at 4 °C for 30 min, and centrifuged at 14,500 $\times g$ for 10 min at 4 °C to collect the supernatant (nuclear fraction). The cytoplasmic and nuclear lysates were stored at -70 °C. Immediately before use, 20 μL of buffer D (20 mM HEPES pH 7.9, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF, and protease inhibitor) were added to cell lysates and their protein content were quantified by the BCA method.

2.8.2. Total cell lysate

Microparticles-treated PBMCs (2×10^6) were suspended in 100 μL of lysis buffer (25 mM Tris-HCl, 120 mM NaCl, 0.5% IGEPAL, 50 mM NaF, 1 mM Na₃VO₄, and protease inhibitor), incubated at 4 °C for 20 min, and centrifuged at 14,500 $\times g$ for 10 min at 4 °C. The cell lysate supernatant was collected and stored at -70 °C until use. Before use, the protein content of the total cell lysates were quantified by the BCA method.

2.8.3. Protein electrophoresis and western blotting

Proteins in cell lysates were separated by electrophoresis (7 μg protein per lane) in 15% polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) under reducing conditions at 140 V for 1 h. A wet protein transfer onto polyvinylidene fluoride (PVDF) membranes was performed at 40 V, 4 °C, for one hour using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). After transfer, membranes were covered with blocking buffer (5% skim milk, 0.05% Tween-20 in 1X PBS) for 1 h at room temperature. The membranes were then incubated at 4 °C overnight with mouse anti-human p65, I κ B α , and β -actin antibodies (IgG1 isotypes, Santa Cruz Biotechnology, Dallas, TX) diluted 1:200, 1:1000, and 1:8000 in blocking buffer, respectively. After, the membranes were incubated with RDye®800CW-conjugated goat anti-mouse IgG polyclonal antibody (1:7000 in blocking buffer, LI-COR Biosciences, Lincoln, NE) at room temperature for 1 h. Finally, fluorescent bands were evaluated using the Li-Cor Odyssey®9120 Infrared Imaging System with the ODYSSEY® application software (LI-COR Biosciences; Lincoln, NE). The relative protein band expression was calculated after densitometry analysis and normalization to the β -actin signal that was used as a loading control.

2.9. Epifluorescence microscopy

Monocytes (300×10^5) isolated from peripheral blood of healthy individuals were cultured on 12 mm glass coverslips (Marienfeld, Lauda-Königshofen, Germany), previously conditioned with a poly-L-lysine solution (Sigma Aldrich) for 2 h at 37 °C. These cells were then treated for 30 min with 30 $\mu\text{g}/\text{mL}$ of vesicles (CMPs or SLE-MPs) or 5 $\mu\text{g}/\text{mL}$ LPS (positive control), fixed with 4% paraformaldehyde for 20 min at 37 °C, incubated with 50 mM NH₄Cl for 10 min, permeated with 0.25% Triton X-100 for 5 min, and blocked with 2% FBS for 1 h at room temperature. After, the cells were incubated for 1 h at room temperature with a primary anti-p65 mAb (isotype IgG1, 1:150; Santa Cruz biotechnology, Dallas, TX), washed with PBS, and then incubated for 1 h with a secondary goat anti-mouse IgG (1:2500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) polyclonal antibody, Alexa fluor 488™

Phalloidin (1:1000; Thermo Scientific), and Hoechst 33342 (1:5000; Invitrogen, Carlsbad, CA). Finally, the cells were preserved on the glass coverslips with FluorSave™ solution (Calbiochem, San Diego, CA). Slides were analyzed with an inverted epifluorescence microscope (IX 81 Olympus, Tokyo, Japan) using Image-Pro Plus 2D Image Analysis software. At least six different fields were evaluated for each experimental condition. The images were analyzed using ImageJ software (WS Rasband, National Institute of Health, Bethesda, MD, USA, <http://imagej.nih.gov/ij>). Cell localization of p65 was analyzed by merging nucleus/p65 and cytoplasmic/p65 images and calculating the Pearson's correlation coefficients.

2.10. Statistical analysis

The expression per cell of HLA-DR and CD69 induced by CMPs and SLE-MPs in monocytes was compared with the Kruskal-Wallis test. The expression of the surface activation markers, the levels of cytokines and eicosanoids, the $\text{I}\kappa\text{B}\alpha$ degradation, and the p65 cytoplasmic in monocytes pretreated or not with PDTC and stimulated or not with MPs, were compared using the two-way Analysis of Variance (ANOVA) with Bonferroni post hoc test. In the case of microscopy assays, the cell localization of p65 was assessed with Pearson's correlation coefficient. Then, the correlation coefficients derived from different treatments were compared with one-way ANOVA, with Bonferroni post hoc test for contrasting means. We use ranks including the minimal and maximal value as the criterion to show the variability to make evident the most relevant and real differences and to avoid bordering differences. A residual normality test was performed to validate the statistical model. In all cases, a p-value ≤ 0.05 was considered statistically significant. The statistical analysis was run in GraphPad Prism version 6 (GraphPad Software; La Jolla, CA).

3. Results

3.1. Microparticles are mainly ingested by monocytes

Because different types of cells can interact with vesicles, through several mechanisms including receptor-mediated interactions and even membrane fusion, the ability to bind and uptake microparticles was compared between monocytes, T- and B-cells. To this purpose, PBMCs from 10 healthy individuals were incubated for 1 h with CFSE-labeled microparticles (CMPs) at different concentrations (30, 50, and 100 $\mu\text{g}/\text{mL}$ protein). The size and characteristics of these MPs have been previously reported by our group [35]. The CFSE labeling of the MPs used in the present study was highly efficient and reached $86.3 \pm 2.6\%$ (Figure 1a). The comparative analysis of flow cytometry histograms showed that MPs were mainly bound and ingested by monocytes, followed by B-cells, and to a lesser degree, by T-cells (Figures 1b and 1c).

3.2. SLE-MPs induce increased expression of HLA-DR and CD69 in monocytes through a mechanism inhibited by PDTC

The highest capacity of monocytes to bind and uptake MPs could induce cellular activation; therefore, the expression of cell activation antigens (HLA-DR and CD69) was evaluated in monocytes of healthy individuals exposed to CMPs and SLE-MPs. The expression (Mean Fluorescence Intensity/MFI) of both markers increased in monocytes stimulated with SLE-MPs ($p \leq 0.01$) in comparison with unstimulated monocytes (Figures 2a and b). Similarly, the CD69 MIF also increased in monocytes exposed to CMPs ($p \leq 0.05$) in comparison with unstimulated monocytes (Figure 2b); CMPs also induced an increase in the expression of HLA-DR, but it was not statistically significant (Figure 2a).

Then, to establish whether the MP-induced activation of monocytes depended on the activation of the NF- κB pathway, monocytes were pretreated with different concentrations of PDTC (1, 5, and 25 μM). This molecule inhibited the MP-induced increase of HLA-DR and CD69 in monocytes in a dose-dependent manner (data not shown), suggesting that at least partially, the activation of NF- κB pathway was involved. As PDTC (25 μM) induced the highest percentages of inhibition for HLA-DR and CD69 (55.8% and 32.0%, respectively) (Figures 3a, 3b, and 3c), it was the concentration of the inhibitor used in the subsequent experiments.

An additional experiment was carried out to discard any deleterious effect of PDTC on cell viability. To this purpose, monocytes were treated with PDTC for 6 h; then, cells were stained with DIOC₆ and PI to evaluate the mitochondrial membrane potential and the plasma membrane integrity, respectively. The PDTC did not significantly affect any of those parameters (data not shown). Additionally, the inhibitor did not affect the cellular ability to bind and uptake the microparticles.

3.3. SLE-MPs induce increased levels of cytokines and eicosanoids through a mechanism inhibited by PDTC

Monocytes from healthy individuals were pretreated or not with PDTC and cultured in the presence of CMPs or SLE-MPs; as positive controls, PDTC-treated monocytes were exposed to IFN- γ or LPS. The levels of cytokines and eicosanoids were measured in culture supernatants. Compared to unstimulated monocytes, cultures treated with SLE-MPs showed a significant increase of IL-6 ($p \leq 0.01$) (Figure 4a), IL-1 β ($p \leq 0.05$) (Figure 4b), PGE₂ ($p \leq 0.01$) (Figure 4e), and LTB₄ ($p \leq 0.05$) (Figure 4f), while CMPs induced non-significant increases of those inflammatory mediators. The exposure of monocytes to SLE-MPs and CMPs caused a non-significant increase in the TNF- α levels (Figure 4c) and did not increase the levels of BAFF (Figure 4d).

The cultures of PDTC-treated monocytes exposed to SLE-MPs showed decreased concentrations (percentage respect to the PDTC-unexposed monocytes) of IL-6 (86.5 %; $p \leq 0.001$), IL-1 β (77.3%; $p \leq 0.001$), PGE₂ (57.4%; $p \leq 0.001$), and LTB₄ (50.7%; $p \leq 0.05$) (Figures 4a, 4b, 4e, 4f).

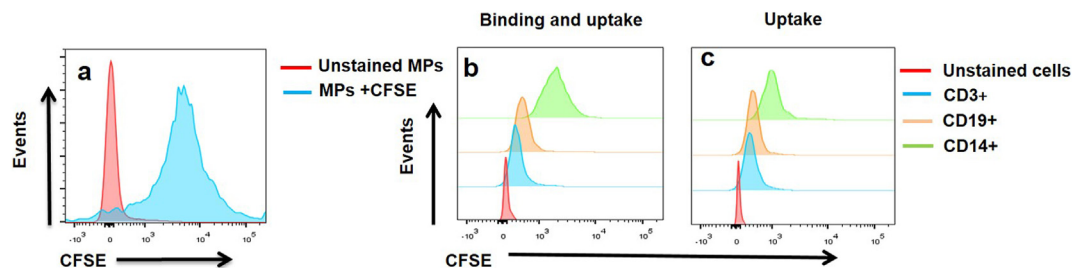


Figure 1. Microparticles from healthy controls are mainly bound and ingested by monocytes. Representative histogram of CMPs (MPs obtained from blood cells of healthy controls) stained with CFSE (a). Representative histograms of binding and uptake of CMPs (100 $\mu\text{g}/\text{mL}$) by CD14+, CD19+, and CD3+ cells from a patient with SLE (b). Representative histograms of uptake of CMPs (100 $\mu\text{g}/\text{mL}$) by CD14+, CD19+, and CD3+ cells from a patient with SLE. Similar results were observed in additional experiments ($n = 10$) and with different concentrations of MPs.

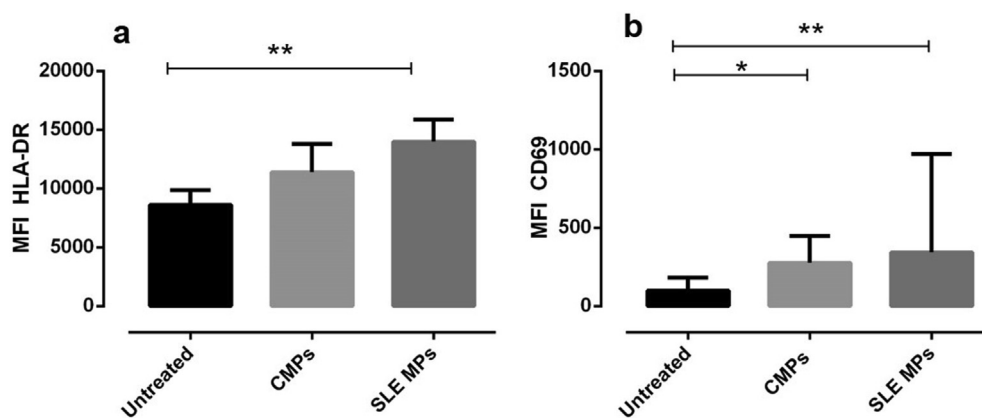


Figure 2. Microparticles from patients with SLE increase HLA-DR and CD69 expression on monocytes. MFI of HLA-DR (a) and CD69 (b) on monocytes treated with or without 30 $\mu\text{g}/\text{mL}$ of CMPs (MPs from controls) and SLE MPs (MPs from patients with SLE). Comparisons among groups were performed using the Kruskal–Wallis test; * $p \leq 0.05$; ** $p \leq 0.01$. Results are shown as mean \pm range, $n = 7$. The asterisks indicate statistically significant differences.

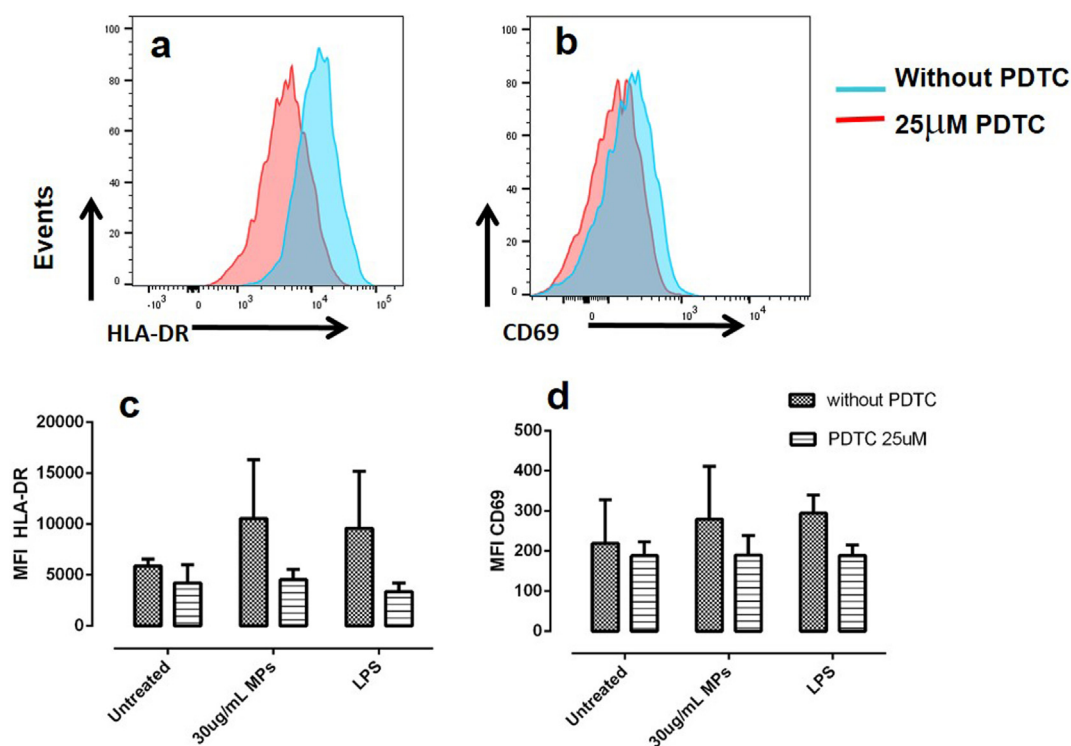


Figure 3. PDTC antagonizes the expression of HLA-DR and CD69 induced by MPs on monocytes. Representative histograms of HLA-DR and CD69 expression in monocytes stimulated with MPs in the presence (red histogram) and absence (blue histogram) of PDTC (a). MFI of HLA-DR (b) and CD69 (c) on monocytes treated with or without CMPs (MPs from controls) in the presence and absence of PDTC. Results are shown as media \pm range. Data were compared using two-way ANOVA; $n = 3$. The asterisks indicate statistically significant differences.

and 4f). PDTC induced a non-significant reduction in the levels of IL-6 and IL-1 β in cultures with CMPs (Figures 4a and 4b).

3.4. Microparticles induced decreased levels of I κ B α and cytoplasmic p65 in PBMCs

As PDTC inhibited the proinflammatory effects of SLE-MPs on monocytes, the effect of microparticles on the activation of the NF- κ B pathway was evaluated. PBMCs from healthy individuals were treated with or without PDTC for 1 h and stimulated with CMPs, SLE-MPs, or LPS (positive control) for 30 min. Supplementary Figure in comparison with unstimulated cells, the total lysate from PBMCs treated with CMPs an SLE-MPs showed reduced levels of the I κ B α protein (Figure 5a: lanes 2

and 3), although there was no significant difference between exposure to SLE-MPs and CMPs. The pretreatment of cells with PDTC inhibited the degradation of I κ B α induced by MPs (Figure 5a: lanes 5, 6, and 7). The consolidated densitometry analysis for the I κ B α levels, expressed as the fold change with respect to unstimulated cells, is shown in Figure 5c.

On the other hand, in comparison with untreated cells, PBMCs cultured with CMPs and SLE-MPs showed a reduced cytoplasmic level of p65 (Figure 5b: lanes 2 and 3); moreover, this change was prevented in the presence of PDTC (Figure 5b: lanes 5 and 6). There were no significant differences between treatments with CMPs and SLE-MPs. The consolidated densitometry analysis for the cytoplasmic p65 protein, expressed in terms of fold change with respect to unstimulated cells, is shown in Figure 5d.

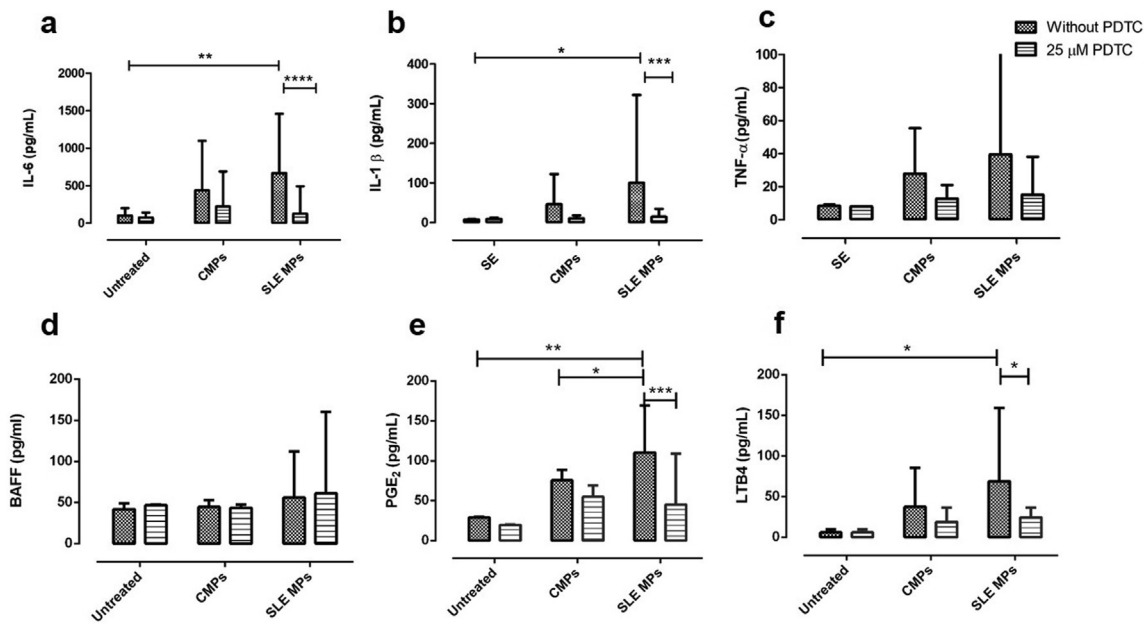


Figure 4. SLE-MPs increase the levels of IL-6, IL-1 β , PGE₂, and LTB₄ in cultures of monocytes, and PDTC inhibits this effect. Levels of IL-6, IL-1 β , TNF- α , and BAFF (a–d) and eicosanoids PGE₂ (e) and LTB₄ (f) in culture supernatants of PDTC-treated monocytes exposed to CMPs (MPs from controls) and SLE-MPs (MPs from patients with SLE). Results are shown as media \pm range. Data were compared using two-way ANOVA * p \leq 0.05, * p \leq 0.01, *** p \leq 0.00; n = 10. The asterisks indicate statistically significant differences.

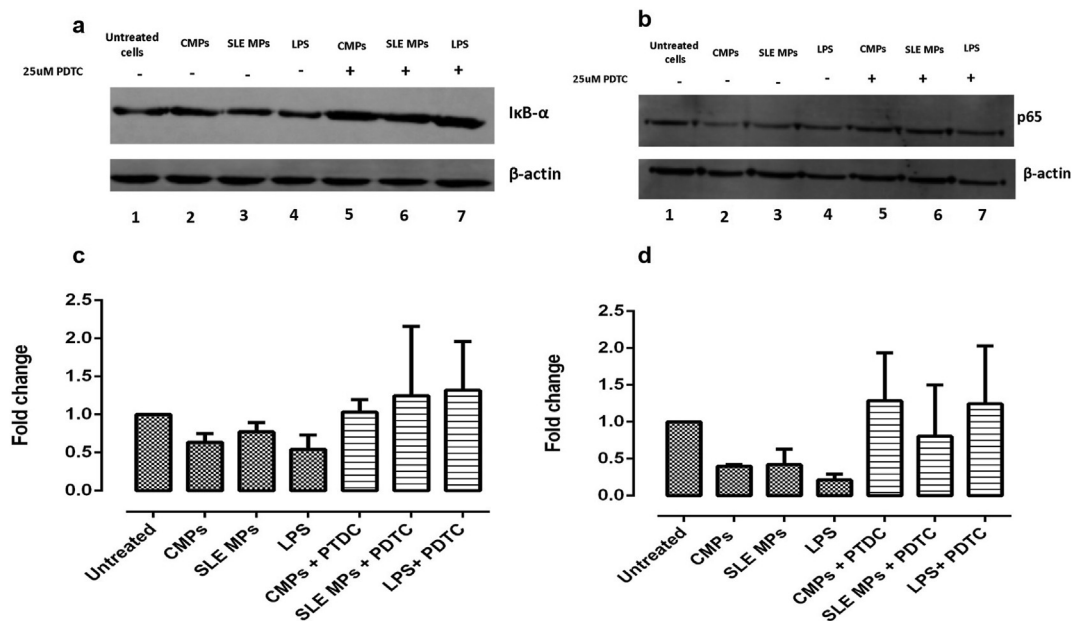


Figure 5. PBMCs treated with SLE-MPs and CMPs show reduced levels of I κ B α and cytoplasmic p65, and PDTC inhibits this reduction. Representative immunoblots are shown for I κ B α (a) and p65 (b). Densitometry analysis of the levels of the I κ B α (c) and the cytoplasmic p65 protein (d) expressed as the fold change with respect to unstimulated cells. β -actin protein immunoblot was performed to ensure equal sample loading. Results are shown as media \pm range. Data were compared using two-way ANOVA; n = 5.

3.5. Microparticles induced the nuclear translocation of p65 in monocytes

After, to confirm the findings observed on western blotting, monocytes from peripheral blood of healthy individuals were treated with CMPs, SLE-MPs, or LPS (positive control) to evaluate the localization of p65 in the nucleus and cytoplasm by epifluorescence microscopy (Figure 6a). The analysis of individual and merged fluorescent images of the nucleus, cytoskeleton and p65 showed that p65 was located mainly in the cytoplasm of unstimulated monocytes while it increased in the

nucleus after treatment with CMPs (p < 0.05), SLE-MPs (p < 0.01), and LPS (p < 0.001) (Figure 6b). There was no significant difference in the nuclear translocation of p65 between treatments with CMPs and SLE-MPs.

4. Discussion

This study showed that binding or uptake of SLE-MPs by monocytes induced cell activation as evidenced by the increased expression of the

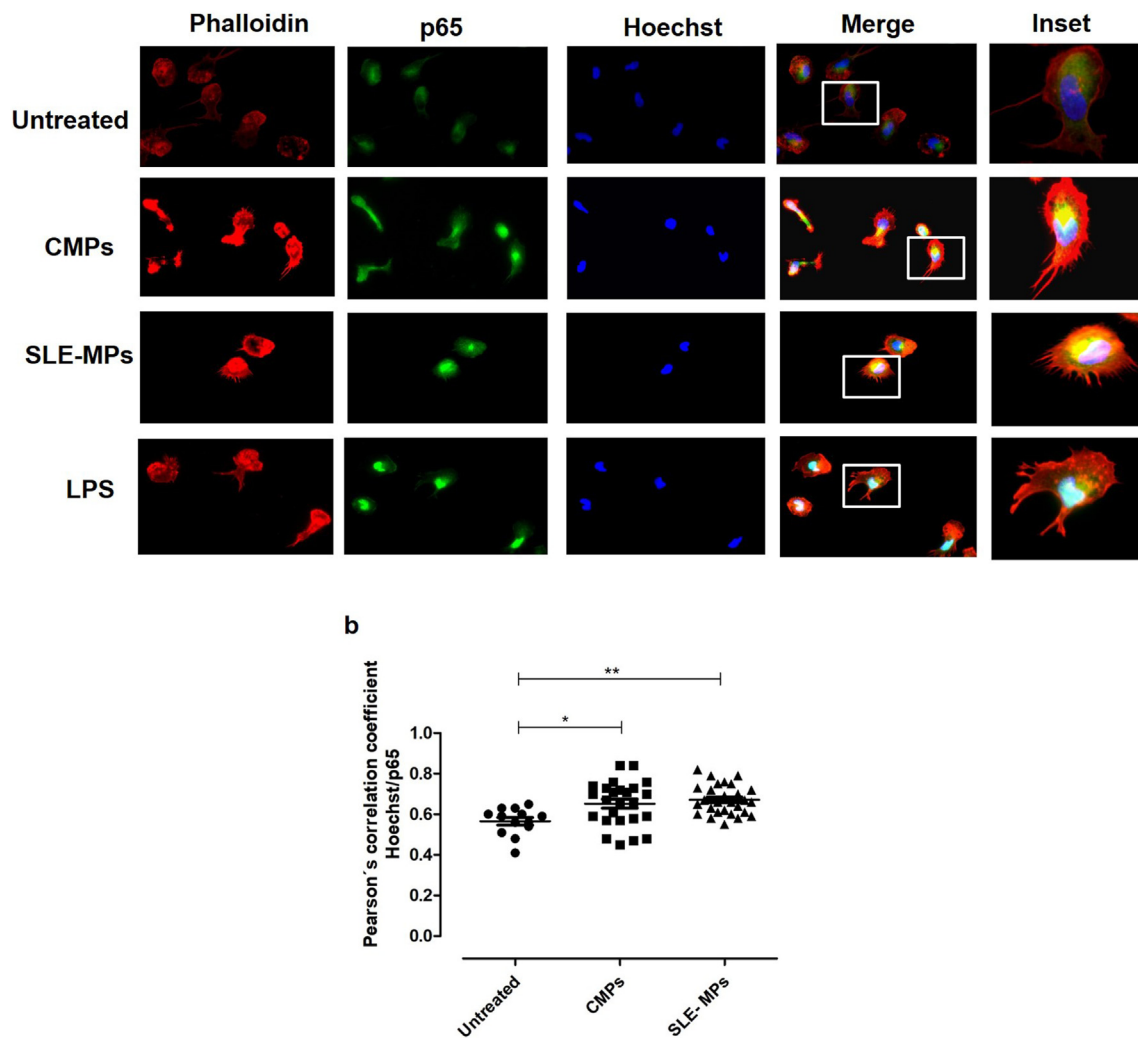


Figure 6. Microparticles induce the nuclear translocation of p65 in monocytes. The nuclear translocation of the NF- κ B subunit p65 was analyzed through epifluorescence microscopy. Individual images from the nucleus (blue), phalloidin (red), and p65 (green), and image overlapping (merge) (a). Colocalization of Green and blue fluorescent signals was evaluated in all cells found in six random fields using the Pearson's correlation coefficient (b); $n = 5$. The asterisks indicate statistically significant differences.

surface activation markers, HLA-DR and CD69, and the higher production of inflammatory mediators (IL-6, IL-1 β , PGE₂, and LTB₄). Additionally, the treatment of monocytes with MPs induced the nuclear translocation of p65, suggesting the activation of the NF- κ B signaling pathway. Therefore, we proposed that in patients with SLE, the binding or uptake of MPs by monocytes could trigger signaling pathways that drive the chronic inflammatory process underlying the pathogenesis of the disease. In the present study, MPs were mainly bound and internalized by monocytes, which could be explained by their potent phagocytic activity and critical role in the removal of material released from dying cells [28]. The inefficient uptake of MPs by T-cells suggests the process is mainly mediated by endocytic mechanisms and not merely by membrane fusion.

Previous studies have shown that MPs have several effects on the immune response; for example, MPs induce increased surface expression of CD80, CD86, CD83, and HLA-DR in plasmacytoid dendritic cells (pDCs) [39]; and augment the synthesis of inflammatory mediators, such as cytokines (IL-6, IL-1 β , IL-8, TNF- α) and eicosanoids (PGE₂, prostacyclin/PGI₂, thromboxane B₂/TXB₂) by different types of cells [40, 41, 42, 43, 44]. Additionally, circulating MPs isolated from patients with SLE have known inflammatory effects: they induce the production of IFN- α , IFN- γ , and TNF- α in pDCs; and IL-6 and TNF- α in myeloid dendritic cells

[45]. That is to say, the uptake of MPs by monocytes could alter their cell phenotype and function, as previously described in patients with SLE.

The uptake of CMPs by monocytes increased the CD69 expression while the uptake of SLE-MPs induced an increase even higher of CD69; and moreover, of HLA-DR. The higher expression of those molecules reflect the activated state of monocytes and point their higher cell ability for antigen presentation, an event that could be critical in autoimmunity.

Also, SLE-MPs increased the levels of IL-6, IL-1 β , PGE₂, and LTB₄ (Figure 4), inflammatory mediators that play an important role in the pathogenesis of SLE. IL-6 is found in high concentration in the plasma of patients with SLE in whom promotes the production of IgG and the differentiation of plasma cells to antibody-secreting cells [46, 47, 48]. Similarly, elevated levels of IL-1 β have been reported in the serum and urine of patients with SLE [49]. Additionally, PGE₂ modulates many immune processes at inflamed tissues, including edema, fever, and the production of inflammatory cytokines [50].

Chae and colleagues demonstrated that PGE₂ induces a higher synthesis of IL-6, IL-10, IFN- γ , and NO in the murine model of pristane-induced lupus [51]. Moreover, the treatment of human monocytes with LTB₄ raised the production of IL-1 and IL-6 [52,53]. Curiously, various cytokines promote the production of proinflammatory eicosanoids, e.g., IL-1 and TNF- α induce the synthesis of PGE₂ [54]; meaning that the

production of proinflammatory eicosanoids and cytokines is interrelated. Therefore, it is possible to suggest that PGE₂ and LTB₄, induced by MPs, might contribute to the deregulated production of IL-6 and IL-1 β in patients with SLE; and in turn, cytokines would induce the synthesis of eicosanoids, contributing this way to the amplification of the inflammatory response in patients with SLE. Furthermore, the pretreatment of macrophages with PGE₂ induces a reduced removal of apoptotic cells [54], pointing that the high levels of PGE₂ induced by SLE-MPs could also contribute to the decreased ability of monocytes to remove microvesicles and apoptotic cells. The persistence of MPs in the extracellular space exposes the immune system to their self-antigen content and also favor the appearance of neoantigens as a result of post-translational modifications [55]. PDTC is a potent NF- κ B inhibitor that blocks the I κ B α release from NF- κ B in the cytoplasm. The pretreatment of monocytes with PDTC inhibited the effect of microparticles, as evidenced by the lower levels of IL-6, IL-1 β , PGE₂, and LTB₄, and suggested the NF- κ B pathway was involved in the production of those inflammatory mediators (Figure 4). The effect of PDTC was more significant in monocytes exposed to SLE-MPs than to CMPs; that means that the synthesis of inflammatory mediators could be activated through different signaling pathways, depending on the source of MPs (patients vs. controls).

Therefore, it is plausible to hypothesize that compared to CMPs, SLE-MPs contain more components for the activation of different signaling pathways, including the NF- κ B one. Previous evidence obtained from our Group [35, 56] and other authors [14] shown that SLE-MPs contain higher levels of HMGB1 and CD40L [11] than their normal counterparts. Our previous observations also support that circulating MPs from SLE patients are not only found in a higher proportion [10, 11, 12], but also vary in composition with respect to the MPs of healthy individuals. Phenotypic analyzes of these vesicles reveal that different molecules, especially immunoglobulins and complement components [15], are found in a higher proportion in the MPs of patients with SLE. Additionally, an increase in the frequency of C1q + MPs and immune complexes with IgM and IgG has been described in patients with SLE; the latter were positively correlated with disease activity and IgM + negatively [35]. DAMPs such as HMGB1 [56] and other molecules such as tissue factor, CD40L, vascular cell adhesion molecule 1 (VCAM-1) [14], glycolytic enzymes and apoptogenic proteins [13], are found in a higher proportion in the MPs of patients with SLE. Therefore, these vesicles could induce a more pronounced effect on monocytes compared to the MPs of healthy individuals, helping to perpetuate the inflammatory process in SLE.

HMGB1 and CD40L could interact with TLR2/4 and CD40 in the target cells and trigger the NF- κ B signaling pathway [57, 58]. Depending on the activation state of the cell they come from, microparticles can have different contents of mRNA, proteins, receptors, and enzymes which will define the effect of vesicles on the recipient cells [59]. In this study, the inhibitory effect of PDTC on the synthesis of cytokines and eicosanoids induced by MPs on monocytes showed that the proinflammatory effect was related to an NF- κ B mediated mechanism. PBMCs treated with MPs showed lower levels of cytoplasmic p65 and I κ B α in the total cellular lysate; and, in addition, that monocytes treated with the microvesicles exhibited a higher nuclear translocation of p65, especially after treatment with SLE-MPs. Consistent with the present results, previous studies showed that following treatment with MPs, the nuclear translocation of NF- κ B increased in neutrophils, monocytes, and macrophages, as well as synovial fibroblasts from patients with rheumatoid arthritis; what is more, the inhibition of the NF- κ B pathway reduced the production of cytokines [43, 60]. Although previous studies had suggested the role of NF- κ B in the proinflammatory effect induced by MPs, the present study provides the first evidence that SLE-MPs induce the activation of monocytes by a mechanism dependent at least partially on the triggering of the NF- κ B signaling pathway. This route probably contribute to the constitutive activation of the NF- κ B pathway observed in patients with SLE.

Previous studies have proposed that in addition to having a role in the inflammatory process, the NF- κ B pathway can modulate the phenotype of

extracellular vesicles. For example, the inhibition of the NF- κ B signaling pathway reduces the levels of tissue factor in MPs derived from endothelial cells after stimulation with *Chlamydia pneumoniae* [55]. Additionally, exosomes isolated from the plasma of NF κ B-knockout mice have an altered protein composition when compared to exosomes from wild type mice [56]. Then, it is possible to consider that an activated NF- κ B pathway could contribute to the higher number and altered composition of circulating MPs in patients with SLE.

The findings in the present study provide evidence that MPs could contribute to the inflammation observed in patients with SLE. When circulating monocytes bind and uptake the microparticles, the cell surface expression of HLA-DR and CD69 increase, as well as the production of IL-6, IL-1 β , PGE₂, and LTB₄. The MP-induced activation of monocytes, at least partially mediated by the NF- κ B pathway, could favor the cell migration to peripheral tissues, enhance the antigen presentation to autoreactive T-cell in an immunogenic environment, and promote the activation of autoreactive B-cells, favoring this way, the perpetuation of the inflammatory state in patients with SLE.

Declarations

Author Contribution Statement

K. Álvarez, M. Rojas and G. Vásquez: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

J. Villar-Vesga and B. Ortiz-Reyes: Performed the experiments; Analyzed and interpreted the data.

A. Vanegas-García: Contributed reagents, materials, analysis tools or data.

D. Castaño: Analyzed and interpreted the data; Wrote the paper.

Funding Statement

G. Vásquez was supported by Fondo Colombiano de Investigaciones Científicas y Proyectos Especiales “Francisco José de Caldas” (1115- 569-33389). K. Álvarez was supported by Fondo Colombiano de Investigaciones Científicas y Proyectos Especiales “Francisco José de Caldas” (Joven Investigador 2015). This work was supported by Universidad de Antioquia (Sostenibilidad).

Data Availability Statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2020.e05815>.

Acknowledgements

We thank Marta Mesa for her English Language Wording review and correction.

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