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Research paper

# Platelet-derived microparticles generated *in vitro* resemble circulating vesicles of patients with rheumatoid arthritis and activate monocytes

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ABSTRACT

Patients with rheumatoid arthritis (RA) have increased amount of platelet-derived microparticles (PMPs) positive for citrullinated peptides (CPs) that form immune complexes (PMPs-ICs). Monocytes are important inflammatory mediators that play a role in the clearance of PMPs-ICs. We aimed to generate PMPs-ICs *in vitro* and determine its effect on monocytes from patients with RA and healthy individuals (HI). PMPs from patients showed platelet markers, mitochondria content, and phosphatidylserine exposure similar to PMPs from HI. However, patients had a higher frequency of IgG+ and CPs+ vesicles than HI. PMPs-ICs generated *in vitro* were similar to the circulating vesicles of patients with respect to IgG- and CPs-positivity. PMPs-ICs induced proinflammatory cytokines and CX3CR1 expression in monocytes from HI, and IL-10 and CD36 upregulation in monocytes from patients. These results suggest that PMPs-ICs induce activation of monocytes, with a pro-inflammatory response in HI and a more tolerant response in cells of patients with RA.

## 1. Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease characterized by the presence of IgG autoantibodies directed against citrullinated peptides (ACPA) [1,2], which can form immune complexes (ICs) [2,3] and contribute to tissue damage and inflammation in synovial joints [3]. High amounts of ICs formed with citrullinated fibrinogen have been found in serum and synovial fluid of patients with RA [4,5]. In addition, these ICs have a proinflammatory effect on macrophages by inducing the production of tumor necrosis factor (TNF)-α through the binding of citrullinated peptides (CPs) by Toll-like receptor (TLR)-4 and IgG by Fc gamma receptors (FcγR) [6]. This response could be directly related to joint and systemic inflammation in patients with RA.

TNF-α is an important inflammatory mediator in RA immunopathology; monocytes and macrophages are the main cellular sources of this cytokine [7]. In patients with RA, these phagocytes are increased in the joints [8]. In murine models of RA, circulating monocytes were shown to infiltrate the joints and contribute to tissue damage at the disease onset as well as during the persistent disease states [9]. In addition, these cells can differentiate into synovial macrophages, promote adhesion and migration of other leukocytes to the affected site, and mediate the degradation of cartilage extracellular matrix and angiogenesis [10]; besides, they are also involved in the resolution of inflammation and tissue repair [9].

Extracellular vesicles (EVs) are a collection of heterogeneous membrane-bound carriers with complex cargoes, which can be released by a variety of cells during physiological and pathological conditions [11,12]. EVs can be divided by size, content, and biogenesis in apoptotic bodies (> 1000 nm), microparticles (or microvesicles, 100–1000 nm), and exosomes (< 100 nm) [12]. Microparticles (MPs) are the heterogeneous population of EVs with phosphatidylserine (PS) exposure containing organelles such as mitochondria [13] and various macromolecules (lipids, proteins, nucleic acids), which are suitable for post-translational modifications [14–17]. In addition, MPs contain CPs and form ICs in the blood and synovial fluid of patients with RA [18]. Higher counts of platelet-derived MPs (PMPs, CD41a+) have been found in synovial fluid and in circulation in patients with RA [18,19]. Furthermore, PMPs isolated from patients with RA were shown to exert a proinflammatory effect on human synovial fibroblasts of healthy

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individuals (HI) and to induce the production of cytokines such as IL-6 and IL-8 [20]. On the other hand, PMPs generated *in vitro* forming ICs (PMPs-ICs or PMPs-IgG+) induced the secretion of leukotriene B4 by human neutrophils [18]. In addition, monocytes and macrophages were found to internalize MPs of different sources [21–23], which induced the production of reactive oxygen species; release of cytokines such as IL-6, IL-8, and TNF-α; and activation of NF-κB [24,25].

Taking in consideration these findings, we proposed that PMPs-ICs generated in the context of RA can activate monocytes and induce a functional response and migration pattern related with joint and systemic inflammation. Therefore, we evaluated the effect of these vesicles generated *in vitro* on the response of freshly-isolated monocytes from HI and patients with RA.

## 2. Materials and methods

### 2.1. Patients with RA and HI

Eighteen patients with RA diagnosed according to the American College of Rheumatology (ACR)/European League Against Rheumatism criteria 2010 were enrolled [26]; all patients were positive for anti-cyclic citrullinated peptides (anti-CCP) antibodies and/or rheumatoid factor (seropositive); no patients received biological therapy. Patients were recruited at the Rheumatology Service of Hospital Universitario San Vicente Fundación (HUSVF Medellín, Colombia). The patients were 17 females and 1 male (age range, 40–75 years). According to the Disease Activity Score based on the evaluation of 28 joints (DAS-28), half of the patients had active disease (≥2.6) [26]. In addition, 41 HI (40 females and 1 male) were enrolled in this study from the general population at the Universidad de Antioquia (Medellín, Colombia). Each participant voluntarily signed an informed consent form. The study was approved by the Universidad de Antioquia's Medical Research Institute and the HUSVF Ethics Committees.

### 2.2. In vitro generation of PMPs

Venous whole blood samples of patients with RA and HI were anti-coagulated with citrate (Improve Medical, Guangzhou, China) and total plasma was separated by centrifugation at 1800*g* for 10 min at room temperature; platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were separated at 3000*g* for 20 min at room temperature. Next, $20–40 \times 10^6$ platelets from PRP were stimulated with different amounts of phorbol 12-myristate 13-acetate (PMA), Ionomycin, collagen type IV and adenosine diphosphate (ADP), all from Sigma-Aldrich (St Louis, MO), in 200 µL of filtered Dulbecco's Phosphate-buffered saline 1× (DPBS, Gibco, Grand Island, NY) supplemented with 1 mM calcium chloride (Merck Millipore, Darmstadt, Germany) for 30 min. Platelets were precipitated at 3,000*g* for 20 min. The supernatant was collected and centrifuged at 16,900*g* for 60 min to obtain PMPs. These vesicles were washed and preserved at − 70 °C in fresh filtered DPBS until further use. Supplementary Fig. 1A (Fig. S1A) shows the amount of platelets remaining in these PMPs preparations (< 8%).

### 2.3. Characterization of circulating PMPs and PMPs generated in vitro

MPs were isolated and characterized as described in our previous report [27]. Briefly, circulating MPs were isolated from PPP of patients with RA and HI at 16,900*g* for 60 min. PMPs were generated *in vitro* as already explained. Filtered sheath fluid and DPBS were used to set the threshold in the flow cytometer BD LSR Fortessa with FACS DIVA software (BD Biosciences, San Diego, CA). Size characterization of vesicles from both sources was performed using polystyrene spheres of known sizes (Fluoresbrite Calibration Grade Size Range Kit, including spheres with diameters of 0.5, 1.0, 2.0, 3.0, and 6.0 µm; and Fluoresbrite YG Microspheres with diameter of 0.1 µm. Polysciences, Inc. Warrington, PA). The vesicles were quantified by flow cytometry using reference beads (Beckman Coulter, Brea, CA) and by acquisition of a constant volume of vesicle suspension at a constant flow for 60 s. The vesicles were labeled with anti-human CD41a (platelet marker, clone HIP8 with different fluorochromes), CD62P-PECy5 (Clone AK-4), CD154-PE (Clone 24–31) and CD63-FITC (Clone H5C6) antibodies, all from BD Biosciences, for 20 min in dark at room temperature.

The percentage of extracellular vesicles bearing mitochondria was determined by DIOC6 (Dihexyloxacarbocyanine Iodide, Invitrogen, Carlsbad, CA) staining for 20 min and treatment with rotenone (100 nM) for another 20 min. The DIOC6+ vesicles were expressed as the difference between the frequency of DIOC6+ events before and after treatment with rotenone (Fig. S1B). In addition, PMPs were stained with Annexin-V (BD Biosciences) using its union buffer 1× (BD Biosciences) for 20 min and with a specific primary rabbit polyclonal anti-human CPs antibody (clone ab100932, Abcam, San Francisco, CA) and the respective secondary antibody (anti-rabbit IgG H&L-Alexa Fluor 647, clone ab150079, Abcam) for 30 min. These vesicles were washed and immediately acquired in the flow cytometer BD LSR Fortessa with FACS DIVA software and analyzed with FlowJo v10 software (Ashland, OR). The percentage of MPs CD41a+ (PMPs) was calculated inside the region of total circulating MPs; the frequency of the other markers (Annexin V, DIOC6, CD62P and CD154) was calculated inside the region of MPs CD41a+ (PMPs); therefore, in this case, total CD41a+ events were assumed to be 100%. The fluorescence minus one (FMO) method was performed for each fluorochrome to determine the positive and negative events; CPs were an exception, and the average of the negative controls (frequency of PMPs and platelets stained with only the secondary antibody and alternative with non-relevant primary antibody of the same isotype plus secondary antibody) was used.

Some PMPs were evaluated by scanning transmission electron microscopy (STEM). For this, vesicles were fixed with 2.5% glutaraldehyde and deposited on copper-coated carbon STEM grids. Next, the samples were dried in the grid at 40 °C and contrasted with uranyl acetate and lead citrate. Samples were then dried at room temperature and evaluated using a Tecnai G2 F20 (FEI company, Hillsboro, OR) microscope. Images were recorded with an Ultra Scan 1000XP-P camera (Gatan, Inc, Pleasanton, CA).

### 2.4. Formation of ICs with PMPs (opsonization)

The formation of ICs with PMPs was performed as described in our previous report [27]. Briefly, several PMPs batches were generated by mixing PMPs from 3 to 4 donors each. Each batch was thawed and quantified using flow cytometry, as described previously. For opsonization, $8 \times 10^5$ PMPs were incubated with 7.5–15 µg/mL purified IgG for 60 min at 37 °C. For IgG isolation, a mixture of plasma from 16 patients with seropositive RA (anti-CCPs > 250 Units according to QUANTA Lite CCP3 IgG ELISA, Inova Diagnostics, San Diego, CA) and affinity chromatography of the NAb™ Protein G Spin kit (Thermo Fisher Scientific Inc. Pittsburgh, PA) were used following the manufacturer's instructions. The unbound fractions and those joined to the column were collected. Subsequently, dialysis of the fractions using a dialysis membrane in DPBS was performed for 20 min at 4 °C; the IgG obtained was frozen at − 70 °C. Protein concentration was quantified by bicinchoninic acid assay (BCA, Thermo Fisher Scientific Inc). Enrichment of IgG was verified by protein electrophoresis with silver staining and western blot (data not shown). The final anti-CCP concentration in IgG preparations was 286.3 Units.

The unbound antibodies were washed away with fresh filtered DPBS at 16,900*g* for 60 min. The formation of PMPs-IgG+ complex was assessed using flow cytometry after staining with a $F(ab')_2$ anti-IgG fragment conjugated with Alexa Fluor 488 (Jackson ImmunoResearch, West Grove, PA) for 30 min at 4 °C (Fig. S1C).

### 2.5. Western blotting

Purified fibrinogen (1 μg/mL) and protein extracts of PMPs (1 μg/mL), with and without citrullination with PAD4 enzyme (Sigma Aldrich, St. Louis, MO), and 1–2 × $10^6$ platelets (10 μg/mL) were separated by electrophoresis in 10% SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, BIO-RAD, Hercules, CA) gel. Proteins were transferred to PVDF membranes (Thermo Fisher Scientific Inc) and were incubated with one of the following primary antibodies for 2 h at room temperature: anti-human citrulline peptides IgM (1:2500, clone F95, Merck Millipore), fibrinogen IgG (1:250, polyclonal antibody, Abcam), PAD IgG (1:100, polyclonal antibody, Acris, Herford, Germany), or β-actin (1:8000, clone C4, Santa Cruz Biotechnology, Dallas, TX). Next, membranes were washed thrice and incubated with the respective secondary antibody for 1 h at room temperature: anti-mouse IgM conjugated to HRP (1:5000, SouthernBiotech, Birmingham, AL) and anti-IgG polyclonal secondary antibodies labeled with IRDye 680RD and IRDye 800CW (1:5000–10,000, LI-COR Biosciences, Lincoln, NE). The antibody with HRP was revealed using SuperSignal™ West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific Inc.) and CL XPosure™ films (Thermo Fisher Scientific Inc) and SRX-101A Tabletop Processor from Konica Minolta (Tokyo, Japan). Anti-IgG were revealed by fluorescence with LI-COR Odyssey Fluorescent Imager.

### 2.6. Isolation and culture of monocytes

Monocytes from HI and patients with inactive RA were enriched with defibrinated peripheral venous blood using Rosette Sep Human Monocyte Enrichment Cocktail (STEMCELL Technologies British Columbia, Vancouver, Canada), according to the manufacturer's instructions and as described in our previous report [27]. Thereafter, 2.5 × $10^5$ monocytes were cultured alone or with a batch of PMPs and PMPs-ICs at a monocyte:vesicle ratio of 1:1 or 10 μg/mL LPS (positive control, data not shown; lipopolysaccharide from *Escherichia coli*, serotype 0111:B4, Sigma-Aldrich, MO). All cultures were performed in 250 μL of RPMI-1640 Glutamax (GIbco) supplemented with 5% autologous serum that was previously inactivated and depleted of MPs, in sterile polystyrene 12 × 75 mm tubes (Fisher scientific, Hampton, NA) for 6 h at 37 °C, 5% $CO_2$ and non-adherent conditions [27]. Monocytes were harvested for further flow cytometric analysis, and the supernatant was collected and stored at −70 °C until measurement of cytokine levels.

To investigate whether a second stimulus with PMPs-ICs affects the monocyte response to these vesicles, monocytes from three HI were stimulated with PMPs-ICs for 6 h in the same conditions as described above. Next, the whole culture medium was replaced with fresh medium and cells were left resting overnight. After 12 h, monocytes were restimulated with PMPs-ICs for 6 h and their supernatant was collected for cytokine measurements.

### 2.7. Immunofluorescence and viability staining of monocytes

Monocytes were washed with DPBS and incubated with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (L/D, Thermo Fisher Scientific Inc) reagent at 4 °C in dark for 15 min. Next, monocytes were washed twice with DPBS and incubated with blocking buffer (0.01% sodium azide, 10% fetal bovine serum (FBS, Gibco), and 1% bovine serum albumin (BSA IgG free, Jackson ImmunoResearch)) for 15 min. Cells were stained with different panels of antibodies at 4 °C in dark for 20 min. The monocyte purity was > 90% for all samples (Fig. S1D). All surface markers assessed in this study were analyzed in CD14++ (high) monocytes (Fig. S1D). Monocyte activation was studied using anti-human CD69-PE (Clone FN50), CD64-APC (Clone 10.1), CD36-APC (Clone CB38, also known as NL07), CD32-PE (Clone 3214509), and CCR2-APC (Clone 48607) antibodies, all these from BD Bioscience; anti-human CX3CR1-PE (Clone 2A9-1), and CCR5-PECy5 (Clone J418F1) antibodies, both from Biolegend. Finally, cells were washed and immediately acquired in the flow cytometer BD LSR Fortessa with the FACS DIVA software and analyzed in FlowJo v10 software. The expression of these molecules was normalized as fold change, calculated by dividing the mean fluorescence intensity (MFI) of each molecule in the stimulated cells (PMPs or PMPs-ICs) by the MFI of the unstimulated cells.

### 2.8. Cytometric bead array (CBA)

The cytokines IL-1β, TNF-α, IL-8, IL-6, and IL-10 were evaluated using CBA Multiplexed Bead-Based Immunoassays (BD Bioscience, CA) according to the manufacturer's guidelines. Samples were immediately acquired in the flow cytometer BD LSR Fortessa with the FACS DIVA software and analyzed using FlowJo v10 software.

### 2.9. Statistical analysis

Comparison between two unpaired groups was performed with the non-parametric Mann–Whitney test. Comparison among three or more groups was performed with the non-parametric Kruskal–Wallis test and Dunn's *post hoc* test. In addition, data with two sources of variation were analyzed using two-way Analysis of Variance (ANOVA) with Bonferroni *post hoc* test. For all analyses, p value $\leq 0.05$ was considered statistically significant. Analyses were performed with Graph Pad Prism 6 of Graph Pad Software Inc. (La Jolla, CA). Although the statistical test is shown in every figure legend, p values are shown only in figures where statistically significant differences were found.

## 3. Results

### 3.1. Patients with RA have a high percentage of circulating PMPs that contain CPs and form ICs

The gating strategy to analyze circulating PMPs and their membrane markers in patients with RA and HI is shown in Fig. 1A. The results showed that platelets are a frequent source of circulating MPs in patients with RA and HI (approximately 50% and 40%, respectively) (Fig. 1A-B). These structures contained platelet activation markers P-selectin (CD62P+) and CD40L (CD154+) as well as exposed PS (Annexin V+), and were stained with DIOC6+ (Fig. 1A-B). PMPs from patients with RA and HI exhibited similar size range and similar expression patterns for the mentioned labeling agents (Fig. 1B); however, patients with RA showed a tendency for more circulating PMPs that contained mitochondria (Fig. 1B). Notably, the percentages of PMPs CPs+ and IgG+ in patients with RA were significantly higher than those in HI (Fig. 1C), indicating that increase in PMPs that contain CPs and form ICs is a remarkable feature in patients with RA.

### 3.2. PMPs generated in vitro from platelets from HI contain CPs and form ICs

For the *in vitro* studies, PMPs were generated from platelets from HI using various treatments (PMA, ionomycin, collagen type IV, and ADP) (Fig. S2 A-B). Platelets cultured under control conditions (without treatment or stimulus) released PMPs [28] (Fig. S2 A-B); however, collagen type IV induced a higher number of PMPs that shared phenotypical characteristics with the circulating PMPs from patients with RA compared with other treatments (Fig. S2 B-C). According to these results and based on the knowledge that collagen seems to induce the release of PMPs in the context of RA [20,29], we selected this treatment for the *in vitro* generation of PMPs. The strategy used to define the size of PMPs and the characterization of these structures as extracellular vesicles [30] are shown in Fig. 2.

PMPs generated *in vitro* and platelets stimulated with collagen type

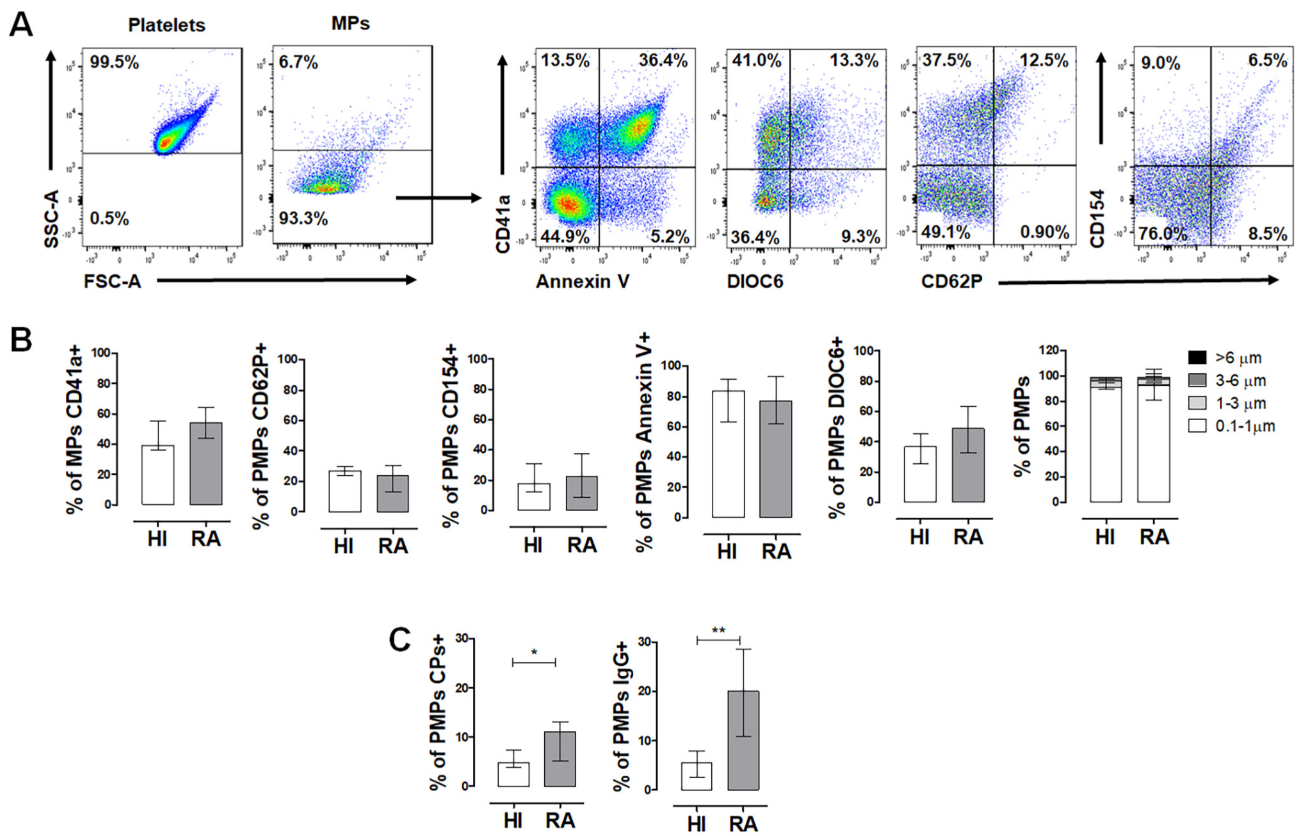


**Fig. 1.** Platelets were a frequent source of circulating MPs. A. Representative pseudo color plots of platelets and MPs (left) are shown with SSC-A (Side Scatter) and FSC-A (Forward Scatter) parameters. Representative analysis of circulating MPs from healthy individuals (HI, right) is shown according to positivity for CD41a (platelets marker) and the concomitant expression of phosphatidylserine (Annexin-V+), the presence of mitochondria (DIOC6+), and the expression of activation markers (CD62P and CD154). B. Frequencies of circulating MPs CD41a+ (PMPs) calculated inside the region of total circulating MPs are shown; the frequency of the other markers (Annexin V, DIOC6, CD62P and CD154) shown in A is also presented and was calculated inside the region of MPs CD41a+. Data are shown as median and interquartile range. Mann–Whitney test. Frequencies of MPs of different sizes (right) were evaluated inside CD41a+ region with use of commercial reference beads. Data are shown as mean and standard error of the mean (SEM). Two-way ANOVA test and Bonferroni *post hoc* test were performed. C. Frequency of circulating MPs from patients with RA and HI with positive expression of CD41a together with CPs and IgG. Results are shown as median and interquartile range. Mann–Whitney test, $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$. B-C HI, n = 5; RA, n = 10.

IV were CPs+ (Fig. 3A). The percentage of CPs+ events in PMPs and platelets and the MFI of CPs in these structures were similar between HI and patients with RA (Fig. 3B-C). However, the MFI of CPs in PMPs showed a tendency to be higher than that in platelets in both study groups, suggesting enrichment of CPs on the membrane of PMPs (Fig. 3A-C).

Total proteins from PMPs and platelets from HI and patients with RA were assessed by Western blot; the results supported the presence of CPs in these structures (band around 75 kDa) in both study groups (Fig. 3D). Although this band exhibited a similar size than the α chain of fibrinogen, a protein that is highly citrullinated in patients with RA [5,31–33], and PMPs and platelets contain this protein, fibrinogen does not seem to be citrullinated in these structures (Fig. S3A-C). It is important to note that CPs signal increased in platelets with collagen type IV treatment compared with unstimulated structures (Fig. S3D). Moreover, PAD enzyme was detected in platelets from HI and patients with RA but not in PMPs; it is interesting to note that all patients with RA evaluated seem to have reduced levels of PAD (Fig. 3D and data not shown). To enrich CP content in PMPs, *in vitro* citrullination of these vesicles was performed. Although PAD4 citrullinated the fibrinogen chains (Fig. S3B), it was not successful with PMPs, because the buffer solution alone used for this reaction (without the enzyme) altered the phenotype of these vesicles (diminished the expression of PMPs markers CD41a, Annexin V, and CPs) (Fig. S3E).

To obtain PMPs-ICs, PMPs generated *in vitro* from HI and patients with RA were opsonized after their incubation with IgG from anti-CCP + patients (Fig. 3E). PMPs from both study groups bound IgG in similar proportions (Fig. 3E). Therefore, PMPs and PMPs-ICs+ generated from HI were used to investigate the *in vitro* response of monocytes.

### 3.3. PMPs-ICs increase CD36 on monocytes from patients with RA and CX3CR1 on HI cells

Binding and uptake assays of PMPs and PMPs-ICs showed that almost all monocytes from patients with RA and HI internalized these vesicles, mainly those forming ICs (Fig. S4). To determine the monocyte activation and migration pattern after PMPs-ICs treatment, the membrane expressions of CD69, HLA-DR, CD64, CD32, CD36, CX3CR1, CCR2, and CCR5 were evaluated (Figs. 4 and S5). There was a significant increase in HLA-DR and the chemokine receptor CCR5 on monocytes from patients with RA and HI in response to PMPs-ICs. In addition, the scavenger receptor CD36 was found upregulated only on monocytes from patients with RA, whereas the chemokine receptor CX3CR1 and CD32 were upregulated only on monocytes from HI in response to PMPs-ICs (Figs. 4 and S5). There was no significant induction in the expressions of CD69, CD64, and CCR2 in both patients with RA and HI (Fig. S5). Interestingly, CD36 expression in monocytes from patients with RA was significantly higher than that in monocytes from HI, whereas the CX3CR1 expression in monocytes from HI was higher than that in monocytes from patients with RA (Fig. 4).

These results showed that monocytes from patients with RA respond to PMPs-ICs in a different manner than monocytes from HI, which

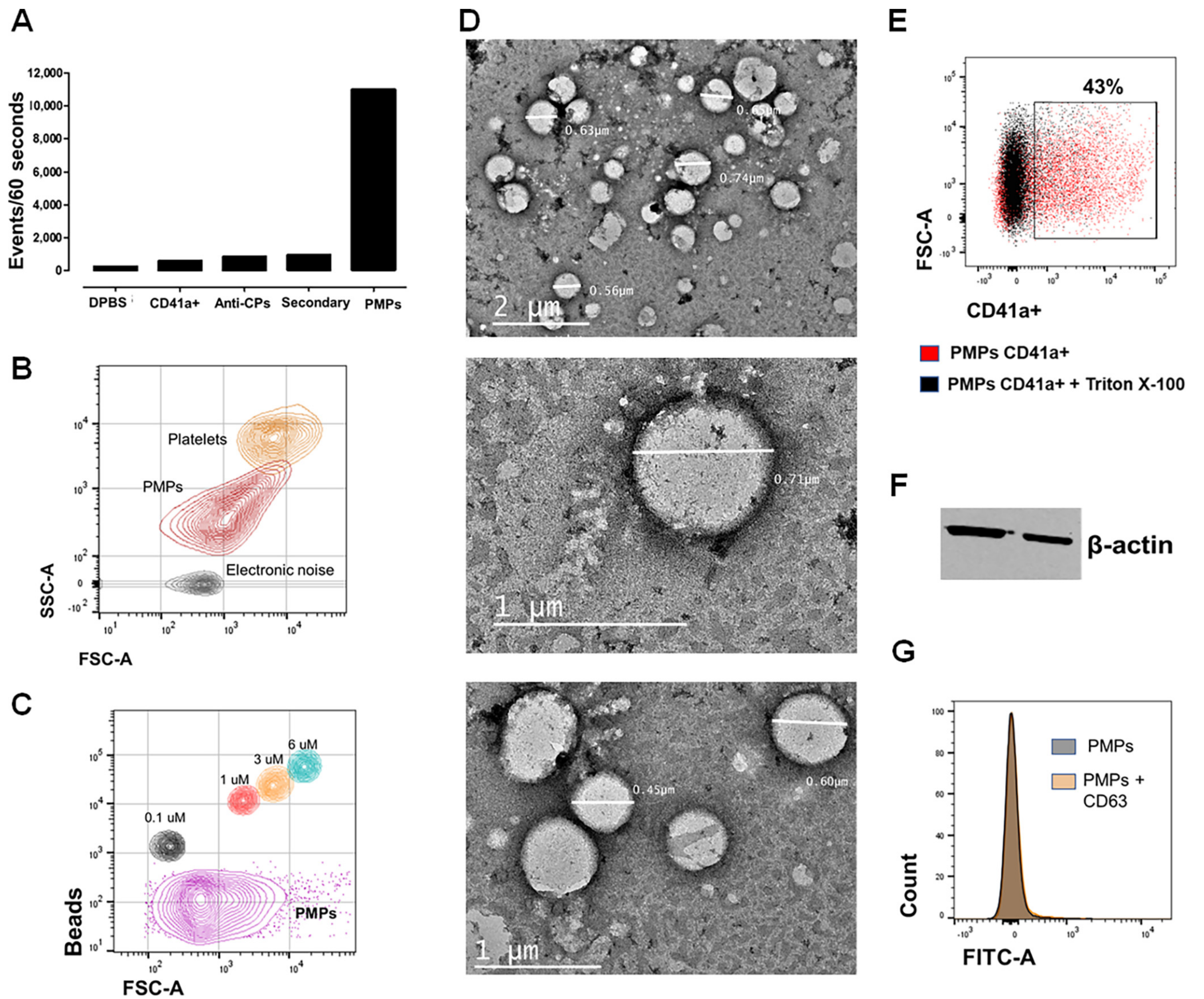


**Fig. 2.** Characterization of extracellular vesicles as PMPs. A. Negative controls for buffer and antibodies aggregation. Number of events observed in DPBS and antibodies (anti-CD41a, anti-CPs, and secondary antibody) acquired alone in the flow cytometer using the MPs parameters in 1 min. Results of at least two independent experiments are shown. There was only minimal noise or non-specific signal of the buffer and antibodies compared with the number of PMPs detected (diluted in DPBS). B. Size determination of PMPs. The gate defining PMPs generated *in vitro* was located above the electronic noise and below the platelets. C. Representative contour plots of the size analysis of PMPs performed by flow cytometry using standard sized beads based on the FSC-A parameter and bead fluorescence. D. Vesicular nature and size of PMPs. Representative microphotographs of PMPs using STEM show the vesicular shape and sizes expected of MPs. E. PMPs are sensitive to detergent treatment. Representative FSC-A vs CD41a dot plots of PMPs untreated (black) and 0.05% Triton X-100 treated (red). Results of at least two independent experiments are shown. FSC-A parameters and CD41a positivity were promptly altered after Triton X-100 treatment. F. Representative western blot showing the presence of β-actin in two different samples of PMPs. G. Representative histogram of CD63 expression on PMPs. This molecule was not detected in three independent experiments, suggesting the vesicles are not exosomes."

suggests a difference in the mechanism of activation.

### 3.4. PMPs and PMPs-ICs induce high levels of proinflammatory cytokines in monocytes from HI and IL-10 in monocytes from patients with RA

To determine monocyte activation, cytokine levels were measured in the supernatant of monocytes cultured with PMPs and PMPs-ICs. PMPs induced a significant increase in the levels of proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α in the supernatant of monocytes from HI (Fig. 5) as compared to that in unstimulated cells. With respect to PMPs-ICs response, there was even greater amount of these detected cytokines compared with PMPs (Fig. 5); no significant changes in IL-10 levels were observed in the supernatant of monocytes from HI after treatment with PMPs and PMPs-ICs. No cytokine accumulation was detected when monocytes were cultured with IgG alone (data not shown). Monocytes from patients with RA and those from HI responded to PMPs and PMPs-ICs in a similar manner with respect to IL-1β production; however, there were no significant changes for monocytes from patients with RA (Fig. 5). Moreover, monocytes from patients with RA seemed to produce TNF-α, IL-6, and IL-8 after treatment with PMPs and PMPs-ICs; however, this was to a lesser extent than that observed with monocytes from HI (Fig. 5). Monocytes from patients with RA incubated with these vesicles accumulated higher levels of IL-10 than those under control conditions (Fig. 5). Furthermore, significant differences were found between the response of monocytes from patients with RA and HI with respect to TNF-α and IL-6 levels after treatment with PMPs and with respect to IL-1 β, TNF-α, IL-6, and IL-10 levels in response to PMPs-ICs.

These findings showed that patients with RA produced lower levels of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α and

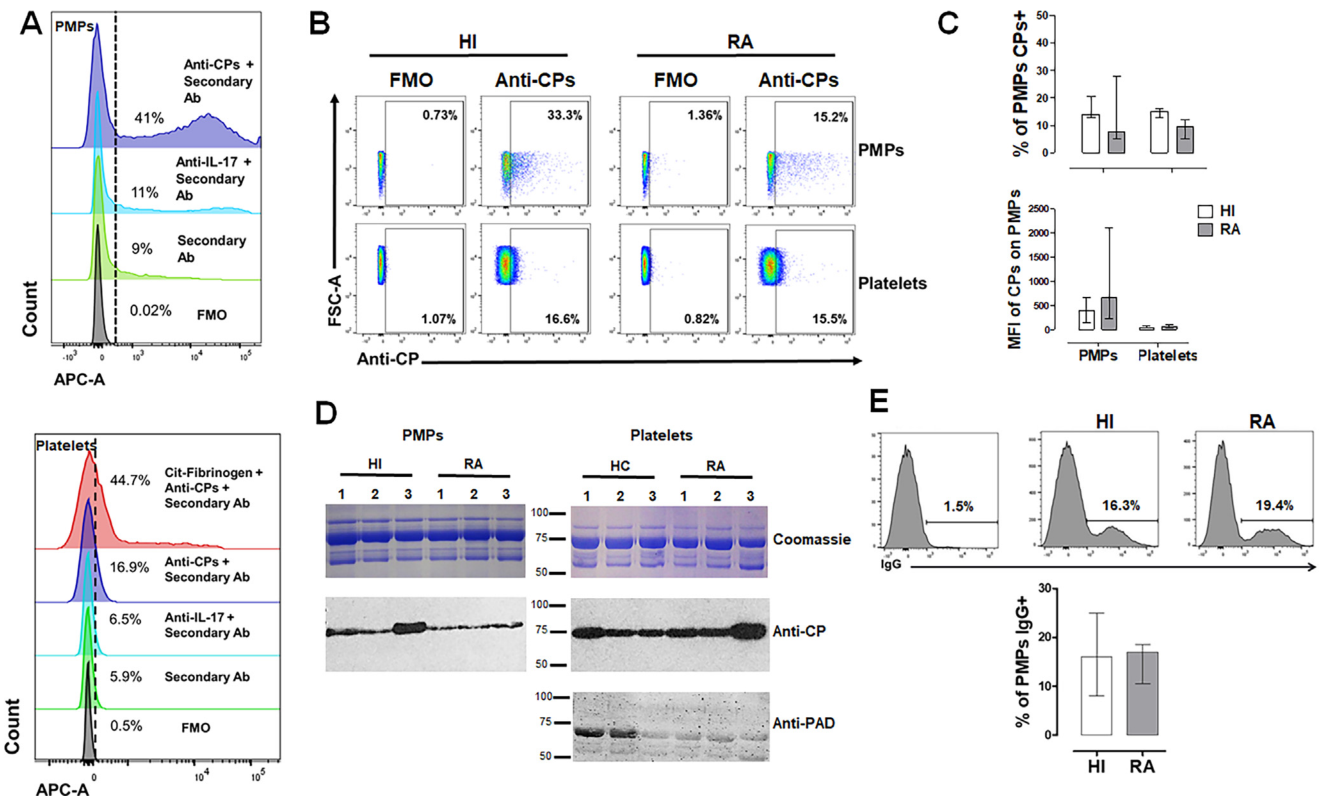


**Fig. 3.** PMPs generated *in vitro* and platelets from patients with RA and HI that were stimulated with collagen type IV expressed CPs and form ICs. A. Representative histograms of CPs staining by flow cytometry in PMPs and platelets of a HI, showing their respective negative controls [FMO in gray, secondary antibody alone in green, and an irrelevant primary antibody (anti-IL-7, same isotype of anti-CPs antibody) in light blue] and positive control (platelets incubated with 10 mg/mL citrullinated fibrinogen for 30 min at 37 °C in orange, Cit-Fibrinogen). B. Representative pseudo color plots and C. frequency of CPs+ in PMPs generated *in vitro* and platelets stimulated with 10 ng/mL collagen type IV from patients with RA and HI. Results are shown as mean and standard error of the mean (SEM). Two-way ANOVA test and Bonferroni *post hoc* test (HI, n = 6 and RA, n = 4). D. SDS-PAGE gel and Western blot of PMPs generated *in vitro* and platelets stimulated with 10 ng/mL collagen type IV. In the upper part the gel bands between 50 and 100 kDa are shown by Coomassie blue staining. Below, the positivity for citrullinated peptides and PAD is shown by Western blotting of three different HI and patients with RA. These results are representative of at least three independent experiments. E. In the upper part, representative histograms of PMPs generated with 10 ng/mL collagen type IV from a HI and patient with RA that were IgG+ after opsonization with 15 µg/mL IgG from patients with RA. Below, frequency of PMPs-IgG+ generated with 10 ng/mL collagen type IV and after opsonization with 7.5 µg/mL IgG from patients with RA. Results are shown as median and interquartile range. Mann–Whitney test (HI, n = 7; RA, n = 6).

notably higher levels of the anti-inflammatory IL-10 than HI after incubation with PMPs-ICs.

### *3.5. Previous exposure of monocytes to PMPs-ICs induces a tolerogenic response of these cells*

The lower levels of cytokines produced by monocytes from patients with RA after treatment with PMP-ICs may be explained by the

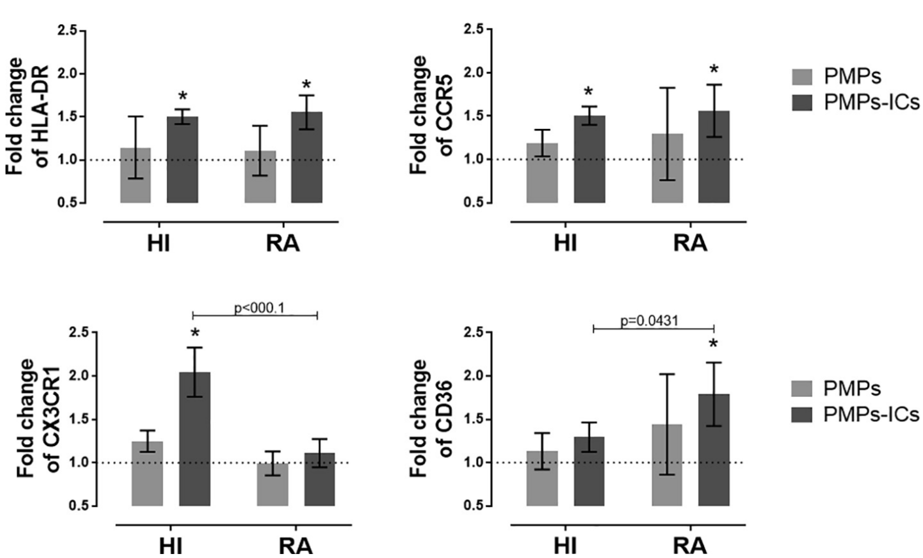


**Fig. 4.** PMPs-ICs upregulated CX3CR1 in monocytes from HI, CD36 in patients with RA, and HLA-DR and CCR5 expression in both study groups. Normalized fold change of the membrane expression of HLA-DR, CCR5, CX3CR1 and CD36 for monocytes from HI or patients with RA in response to PMPs or PMPs-ICs (1:1 ratio) (HI, n = 3; RA, n = 4). Two-way ANOVA test and Bonferroni *post hoc* test, *p ≤ 0.05. Symbols alone indicate comparison with control conditions (vehicle, dotted line) and p values over bars indicate intergroup comparisons. Results are shown as mean and standard error of the mean (SEM).

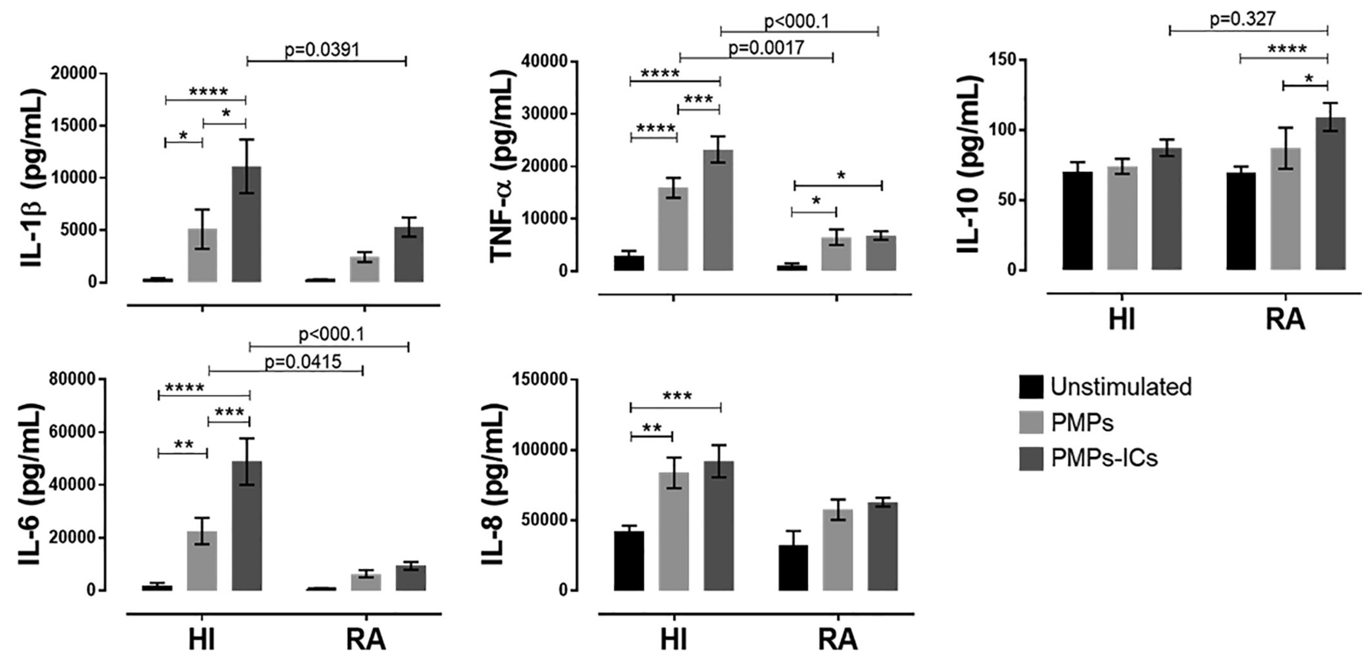


**Fig. 5.** Monocytes from patients with RA and HI produced pro-inflammatory cytokines after treatment with PMPs and PMPs-ICs. Levels of IL-1β, IL-6, TNF-α, IL-8, and IL-10 accumulated in the supernatant of monocytes from HI and patients with RA cultured with or without PMPs or PMPs-ICs (1:1 ratio) (HI, n = 5; RA, n = 4). Two-way ANOVA test and Bonferroni *post hoc* test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001. Symbols indicate comparison with control conditions (vehicle) and the exact p value is reported for intergroup comparisons. Results are shown as mean and standard error of the mean (SEM).

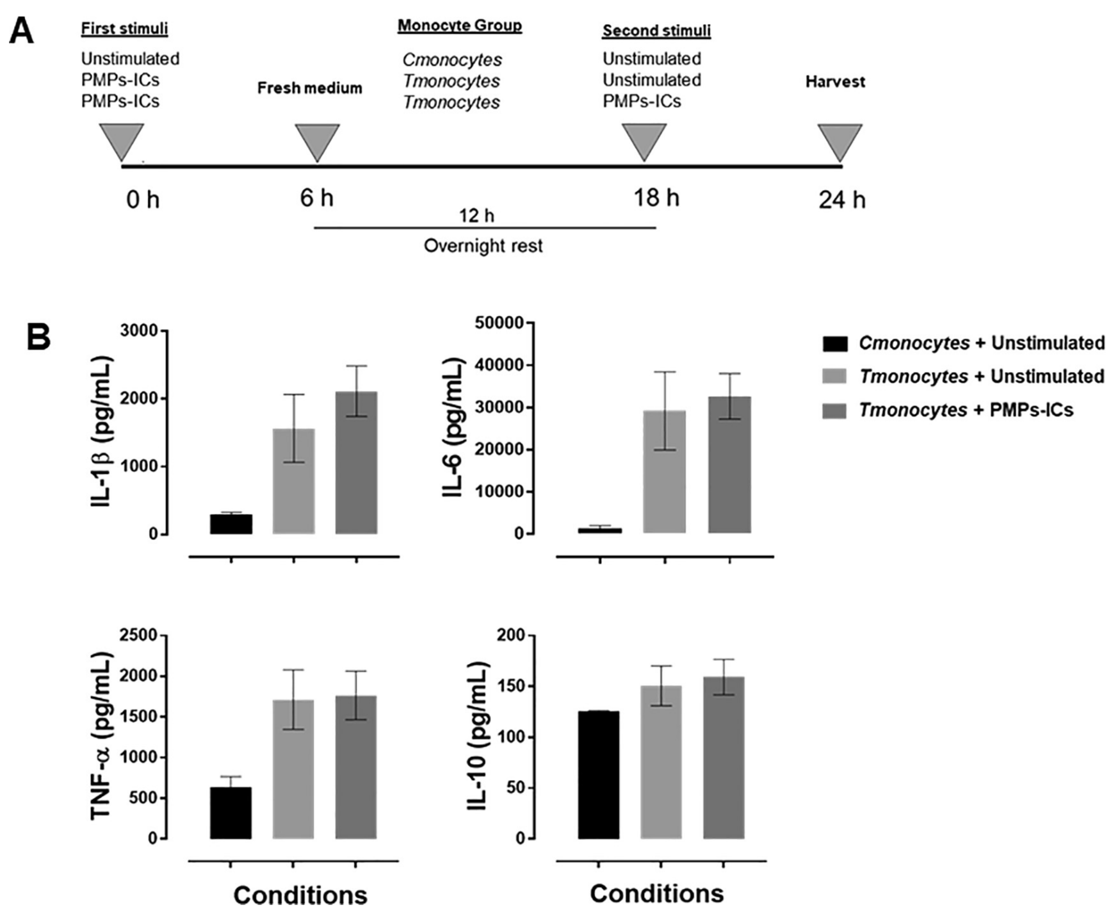


**Fig. 6.** Monocytes from HI did not respond to a second stimuli with PMPs-ICs. A. Enriched human monocytes from HI were stimulated with PMPs-ICs for 6 h (first stimuli), washed and left resting overnight; cells were restimulated for another 6 h. B. The levels of IL-1β, IL-6, TNF-α, and IL-10 were measured in the supernatants of these cultures with CBA. Kruskal–Wallis test and Dunn's *post hoc* test; results are presented as median and interquartile range, n = 3. Cmonocytes: control monocytes; Tmonocytes: tolerant monocytes.

induction of innate memory, specifically the induction of tolerance; therefore, we evaluated whether a second stimulus with PMPs-ICs affects the response of monocytes from HI to these vesicles. Monocytes exposed to PMPs-ICs accumulated levels of IL-1β, IL-6, TNF-α, and IL-10 at 24 h, despite complete replacement of culture media after 6 h of stimulation (Fig. 6). Interestingly, repeated exposure of these cells to PMPs-ICs after 12 h of rest did not induce any further accumulation of the measured cytokines. This suggests that the monocytes from HI that were previously exposed to PMP-ICs acquired tolerance to a second round of exposure to the same vesicles.

## 4. Discussion

According to our findings and in agreement with previous studies [34,35], platelets are a frequent source of MPs in patients with RA and HI. In addition, we found that PMPs from patients with RA and HI share several common characteristics, namely, expression of the activation markers CD62P and CD154, presence of viable mitochondria (sensible to rotenone treatment Fig. S1B) [13], and exposure of PS. The notable differences found between these vesicles were the higher percentage of circulating PMPs-CPs+ and PMPs-IgG+ (PMPs-ICs) in patients with RA as compared to that in HI. These findings highlight that these structures are an important source of autoantigens in RA and a source of ICs, as previously suggested [19,20]. Previous studies have mainly focused on the characterization of the total circulating MPs and PMPs in the synovial joints of patients with RA [19]; however, there is little information pertaining to the phenotype of circulating PMPs in these patients. Therefore, our results contribute to a better knowledge of the composition of circulating PMPs in patients with RA.

Platelets cultured under control conditions (without treatment or stimulus) released PMPs that could be related to the tendency to self-fragmentation of these structures [36]; however, a significant increase was observed in response to different stimuli such as ionomycin and collagen type IV. Collagen type IV was shown to induce the release of a high count of PMPs that shared some characteristics with the circulating PMPs found in patients with RA, suggesting this process plays an important role in the pathogenesis of RA [18]. The PMPs generated *in vitro* contained CPs and formed ICs upon incubation with IgG from patients with RA. *In vitro* induction of PMPs with collagen has been performed in many studies [20,29,37,38]; however, these structures have not been directly compared with the circulating PMPs of patients with RA. In addition, although *in vitro* PMPs-ICs have been previously generated [20], these structures were opsonized with antibodies against fibrinogen and not with IgG from patients with RA, as was done in the present study. These results suggest that PMPs generated *in vitro* with collagen type IV can be used to evaluate the response of leukocytes in a context similar to that of RA. In addition, our data justify the use of platelets from HI as a source for PMPs and PMPs-ICs with a similar phenotype as that of vesicles found in patients with RA.

Collagen exposure due to articular damage can induce the binding and activation of circulating platelets through the recognition of collagen by the Glycoprotein VI (GPVI) receptor, which leads to release of PMPs [20]. In a murine model of RA based on K/BxN serum transfer, a mice strain with deficient GPVI receptor (*Gp6-/-*) exhibited a decrease in the generation of MPs *in vivo*, mild ankle thickness, lower inflammation, and lesser bone and cartilage erosion as compared to that in wild type mice [20]. Platelets contain vimentin and fibrinogen [39,40], which are target proteins for citrullination and relevant autoantigens in RA [4,41]; however, we did not find any previous report regarding CPs and PAD expression in platelets. Thus, to the best of our knowledge, this is the first study that demonstrates the presence of these molecules in platelets. In addition, it is important to note that although CPs were detected in platelets, their signal did not correspond to the α chain of fibrinogen (Fig. S3C) and their expression increased with collagen type IV activation (Fig. S3D). In addition, we found that platelets (but not PMPs) contain PAD enzyme, which suggests that citrullination could be performed in platelets. Nevertheless, this hypothesis needs to be further probed; in addition, how collagen type IV increase CPs, what proteins are citrullinated in platelets, the identification of the specific PAD isoform in platelets and whether megakaryocytes also contribute to citrullination is yet to be studied. Moreover, the finding that platelets from patients with RA seem to exhibit lower levels of PAD than HI is counterintuitive; further studies are required to confirm this assumption and to understand its implication in RA immunopathology.

Regarding PMPs-ICs generation *in vitro*, it is important to consider that IgG used in this study was enriched only with protein G and not with citrullinated proteins. Thus, although CPs were present in PMPs and there were high titers of anti-CCPs in IgG preparations from patients with RA, our evidence cannot dismiss the participation of other molecules or direct components of PMPs, such as carbamylated antigens and anti-carbamylated antibodies, in the formation of ICs. Further studies are required to establish the contribution of different autoantigens and autoantibodies in PMP opsonization.

Platelets can interact and adhere to leukocytes in synovial joints [41] and in circulation [42]. In a similar manner, we proposed that PMPs may interact with different cells, and mediate similar responses as platelets [43]. On the other hand, monocytes have been implicated in the clearance of circulatory MPs, PMPs, and the PMPs-ICs [22,27,44] and are important mediators of the systemic inflammatory response observed in RA [9,10,45,46]. Because platelets are a frequent source of circulating MPs, we investigated the effect of PMPs and PMPs-ICs on monocyte activation. Our findings showed that monocytes from HI and patients with RA release IL-1β, TNF-α, IL-6, and IL-8 after incubation with PMPs; therefore, although PMPs-ICs induced a more significant response, PMPs alone also induced some response from monocytes, which suggests that these structures contain immune stimulatory components that are not related with the formation of ICs. Macrophages were shown to recognize CPs via TLR4 [5], which led to the activation of the NF-κB pathway and the synthesis of proinflammatory cytokines like TNF-α. Indeed, CPs have been proposed as alarmins or DAMPS (danger associate molecular patterns) [47], and therefore PMPs seem to be a source of these inflammatory signals.

P-selectin (CD62P), that is also expressed by PMPs, was shown to play an important role in platelet-monocyte binding, and to increase the COX-2 (cyclooxygenase 2) pathway in monocytes [43]. PMPs that contain functional mitochondria may also contribute to monocyte activation [48,49], which, upon internalization, could alter the bioenergetics of the cell and calcium concentration. In addition, some components of these vesicles, such as mitochondrial DNA, can behave as DAMPS and contribute to the mentioned response [49]. Thus, CPs, P-selectin, and mitochondria contained in PMPs may at least partly explain the observed response of monocytes to PMPs. Furthermore, other components not evaluated in this study, such as micro RNA and functional enzymes, may also modulate the monocyte responses.

Interestingly, PMPs-ICs induced a higher accumulation of the proinflammatory cytokines IL-1β, TNF-α, IL-6, and IL-8 as compared to that induced by PMPs, which can be partially explained by the observed greater tendency of monocytes from HI to internalize PMPs-ICs as compared to PMPs. Moreover, FcγR that are widely expressed on monocytes, may also contribute to the differences in internalization between these structures. FcγR receptors induce the translocation of NF-κβ and calcium influx [50], which may also contribute to the more prominent proinflammatory response of monocytes to PMPs-ICs as compared with PMPs. These results are consistent with the findings in murine and human macrophages using ICs formed with citrullinated fibrinogen [5]. Therefore, the described proinflammatory profile of monocytes from HI together with the increase in the markers HLA-DR, CD32, CX3CR1, and CCR5 may be associated with polarization of these cells to classical activation in response to PMPs-ICs [51].

In addition, the higher expression of CX3CR1 established in response to PMPs-ICs by monocytes from HI suggests that these vesicles regulate the expression of molecules associated with endothelial interaction. In fact, CX3CR1 is the only chemokine receptor involved in rolling and firm adhesion of leukocytes to the endothelium [52,53]. This receptor participates in the patrolling function of monocytes over the endothelium, to maintain its integrity through the removal of apoptotic cells, MPs, and other debris [44]. Therefore, PMPs-ICs may induce the production of molecules in HI that are associated with the protective function of monocytes against endothelial injury. However, for some reason, this phenomenon was not observed with monocytes from patients with RA, despite the high levels of CX3CR1 ligands found in the synovial joints of these patients [54]. This may contribute to the endothelial damage observed in these patients; however, further studies are required to probe this assumption.

Interestingly, monocytes from patients with RA responded to PMPs and PMPs-ICs in a differential manner compared with HI, accumulating the anti-inflammatory cytokine IL-10 and increasing the expression of CD36. Scavenger receptor CD36 induces phagocytosis of extracellular material that contain PS, among other ligands [55]. Upon recognition of PS in apoptotic bodies, this receptor may internalize these in a silent or steady manner [56,57]. This phenomenon may have happened in our study because a considerable proportion of our PMPs externalized PS, and this pathway is believed to effect the clearing of MPs [12]. CD36 priming induces IL-10 [57] and an autocrine mechanism is involved in the up regulation of this receptor. Release of IL-10 together with CD36 upregulation was shown to be associated with polarization of monocytes, which may reflect an alternative mechanism of activation [58]. However, monocytes from patients with RA also enhanced the release of TNF-α in response to PMPs-ICs, which is associated with classical activation of these cells [51].

In addition, monocytes from patients with RA exhibited lower accumulation of the proinflammatory cytokines IL-1β, TNF-α, and IL-6 as compared to monocytes from HI. This response may be related to lower production or a higher autocrine consumption of these molecules. Also, these responses could be affected by patients' treatment strategies. Biological therapies, such as anti-TNF-α and anti-IL-6, may in part be useful because they target monocyte proinflammatory responses [59,60]; therefore, patients receiving these treatments were excluded from this study. However, effects of other pharmacological interventions [such as nonsteroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs)], cannot be ruled out. Finally, the lower production may be associated with previous monocyte activation. Interestingly, freshly isolated monocytes from patients with RA exhibited a higher MFI of CD69 as compared to those from HI (Fig. S6), which suggests that these cells were previously primed in circulation. Therefore, the *in vitro* responses of monocytes from patients with RA could be related to a refractory state induced by PMPs-ICs. This state of tolerance is induced after a second encounter with a given stimuli (for example lipopolysaccharide), which leads to a lower proinflammatory response of NF-κB-associated cytokines, together with induction of IL-10 as compared to the first response [61,62]. Hence, to understand whether a second stimulus with PMPs-ICs was related with tolerance as hypothesized for monocytes from RA, monocytes from HI were stimulated for a second time with PMPs-ICs; there was no further accumulation of the measured cytokines compared with control cultures, which suggests that monocytes from HI previously exposed to PMPs-ICs become tolerant to a second round of exposure to the same vesicles. These results allow us to propose that monocytes from patients with RA could have had a previous exposure to PMPs-IC *in vivo,* which induced a refractory or tolerant state; therefore, these cells showed attenuated response *in vitro* as compared to that of monocytes from HI. However, further studies are required to evaluate this hypothesis with use of diverse tools such as epigenetic and transcriptional approaches [63].

### 4.1. Conclusion

The results of this study suggest that PMPs and particularly PMPs-ICs are potent activators of monocytes that induce a proinflammatory response. Therefore, these vesicles may contribute to the RA immunopathology, for instance by inducing the secretion of systemic inflammatory mediators IL-1β and TNF-α. This response profile differed when cells from patients with RA were evaluated, possibly because of tolerant state of monocytes related to a previous activation by PMPs-ICs; however, these cells continued to release cytokines at a certain level, which may perpetuate the proinflammatory response in these patients. Therefore, interventions to modulate platelet activation and generation of these PMPs and PMPs-ICs, as well as blockade of the interaction of these vesicles with monocytes would be promising targets for treatment of patients with RA.

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## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.12.002.

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