Rheumatology International (2019) 39:1377–1387
https://doi.org/10.1007/s00296-019-04337-1

Rheumatology
INTERNATIONAL

OBSERVATIONAL RESEARCH

Characterization of cell-derived microparticles in synovial fluid and plasma of patients with rheumatoid arthritis

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Received: 26 February 2019 / Accepted: 25 May 2019 / Published online: 14 June 2019

Abstract

Microparticles (MP) are proposed to play a role in the pathogenesis of rheumatoid arthritis (RA). This study aimed to profile cell lineage-specific MP in patients with RA, osteoarthritis (OA), and healthy controls (HC) in synovial fluid and circulation. Patients with RA ($n=40$), OA ($n=30$) and HC ($n=33$) were included. Cell-free synovial fluid (SF) and platelet-poor plasma samples were stained with annexin V APC and antibodies against CD45, CD20, CD14, CD4, CD8, CD66b, and CD61 for multicolor flow cytometry. Mann–Whitney *U* test/unpaired *T* test was used to assess intergroup differences among RA and OA SF and clinical, serological phenotypes of RA based on normality distribution; Kruskal–Wallis test with Dunn's multiple comparisons for comparing plasma MPs among RA, OA, and HC. Correlation between MP proportions and disease parameters was assessed by Spearman's correlation. The proportion of annexin V^+ MP in SF of patients with RA [5 (6.35)] [median (IQR)] was higher compared to OA [1.8 (1.35), $p<0.001$] and plasma of patients with RA [3.45 (5.63)] compared to OA [1.85 (1.4)] and HC [0.9 (1.1), $p<0.001$]. Leukocyte-derived [0.85 (1.17)], granulocyte-derived [0.4 (2.05)], monocyte-derived [0.4 (0.4)], and T cell-derived MP [$CD4^+$ – 0.1 (0.1); $CD8^+$ – 0.1(0.1)] were higher in RA SF ($p<0.001$). Platelet-derived MP (PMP) were the major fraction [1.5 (4.23), $p<0.001$] in RA plasma. Leukocyte-derived MP were higher in RA plasma [0.1 (0.2); $p<0.001$) than OA and HC. Annexin V^+ MP and PMP were higher in the SF of RA with extra-articular manifestations ($n=15$), as compared to those without ($n=25$) ($p=0.02$; $p<0.01$, respectively). High SF granulocyte-derived MP were observed in patients with established RA ($n=24$), ACPA-positive RA ($n=32$) compared to their negative counterparts ($p=0.03$; $p=0.02$, respectively). Our observations of higher proportions of cell-derived MP in the plasma and synovial fluid of DMARD-naïve RA patients, their clinical and serological phenotypes suggest their role in dynamic cross talk between the joint and systemic circulation, disease pathology, and progression.

Keywords Rheumatoid arthritis · Osteoarthritis · Microparticles · Synovial fluid · Plasma · Flow cytometry · Autoimmunity

Introduction

Rheumatoid arthritis (RA), characterized by joint inflammation and articular cartilage damage, is the most commonly encountered autoimmune inflammatory rheumatic disease in rheumatology practice. Although significant progress has been made in the understanding of the disease, elucidating pathophysiological pathways of RA and identifying reliable biomarkers for follow-up is an ongoing area of research. Communications between various immune cells via direct interaction or released mediators are identified as key drivers of inflammation in RA [1]. Microparticles (MP) are one of such released mediators that participate in the intercellular communication [2].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00296-019-04337-1) contains supplementary material, which is available to authorized users.

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MP are a heterogeneous group of bioactive membrane-bound extracellular vesicles. They are 0.1–1 μm in size and express phosphatidylserine (PS) and other phospholipids on their surface [2]. They are released from various immune cells during cellular activation and apoptosis. Depending on the cell of origin, MP carry remnants of cells (cytoplasmic and nuclear components) and express cellular antigens on their surface [3]. Hence, MP may be regarded as liquid biopsies of the dying and activated cells in a given pathogenic condition [4]. In a pathogenic environment of RA, these vesicles may function as damage-associated molecular patterns (DAMPs), source of autoantigens and the site for immune complex formation and antigen presentation, or may be involved in horizontal transfer of miRNA, inflammatory cytokines, proteases that alters the phenotype of the recipient cells, thus enhancing ongoing inflammation [5, 6]. Identifying the origin of the MP is vital as they indicate the predominant cell type involved in a pathological condition [7].

Circulating microparticles are reported to be elevated in various autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) [8–10]. Studies in RA have shown a higher concentration of MP in patients independent of the cell count [11] as compared to healthy individuals. However, there is significant variation in the proportions of MP reported in RA versus osteoarthritis (OA) [12]. The difference in the MP proportions reported seems to be a result of heterogeneity in the duration and treatment of RA. Though the proportions of platelet-derived microparticles (PMP) correlate with disease activity and vasculitis in RA, the MP derived from leucocytes are proposed to be more important in RA synovium [13].

Delineating the precise secretome which includes MP in RA can pave the way for identifying newer biomarkers for disease activity and damage, noncell based therapeutics and in determining the right kind of vector for targeted drug delivery [14].

In our previous study, we noted overall elevated proportions of MP in the plasma and synovial fluid of treatment-naïve patients with RA [15] as compared to healthy controls and OA. But still, there is inconsistency in the current literature concerning the proportions of MP and their correlation with RA phenotype and disease activity. The goal of the present study was to identify the distribution of various cell-derived MP in circulation and the synovial fluid in a homogenous group of treatment-naive RA and to compare their proportions with OA and healthy controls (HC) and further to compare in clinical and serological subtypes of RA.

Materials and methods

Study participants

The study was conducted at the Department of Clinical Immunology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry. Samples were collected between March 2015 and December 2017. Consecutive patients satisfying American College of Rheumatology [ACR] criteria, 2010 for RA [16], with synovitis who were not on any conventional or biologic disease-modifying antirheumatic drugs (DMARDs) including glucocorticoids were recruited for the study. Forty patients (35 females, 5 males, mean age 43.6 ± 10.9 years.) with RA were recruited in the study. Individuals with mechanical knee pain and joint swelling were assessed for crystal arthropathies and chondrocalcinosis by ultrasonography and X-rays of knee joints, in the absence of which they were classified as OA. The OA controls were clinically screened for co-morbidities, with focused history and examination. Patients with a clinical suspicion of an alternate diagnosis for swollen joint, including infection, trauma, and hemarthrosis were excluded from the study based on clinical and radiological evidence. Thirty patients (27 females, 3 males, Mean age 50.6 ± 6.6 years.) diagnosed with knee osteoarthritis (OA) without calcification of the articular cartilage were included as disease controls and 33 age and sex similar healthy individuals (26 females, 7 males, mean age 43.4 ± 7.4 years.) without family history of autoimmune disorders as healthy controls (HC).

Patients with RA with at least one joint deformity defined clinically were classified as having deforming disease. Patients were classified to have extra-articular manifestations if they presented with features of secondary Sjogren's syndrome, vasculitis, interstitial lung disease, etc. Those with an age of disease onset ≤ 55 years were classified as young-onset RA (YORA) and the others as late-onset rheumatoid arthritis (LORA), as well as early RA (disease duration of ≤ 6 months) vs. established RA (disease duration > 6 months). RA patients with diabetes mellitus, hypertension, hypothyroidism, and malignancy were not included in the study. The study was approved by the institute ethics committee and conducted as per the Declaration of Helsinki (JIPMER Protocol No. JIP/IEC/2013/1/107 dated 15.03.2013) [17].

Sample collection and processing

Five ml of synovial fluid was collected in ethylenediamine tetraacetic acid (EDTA) tubes from the inflamed knee joint

of the study participants from the RA and OA groups using a 21-gauge needle. Five ml of peripheral venous blood was collected from the RA, OA, and HC groups in an EDTA tube. Cell-free synovial fluid was separated by centrifugation at 1550*g* for 20 min at 21 °C [13]. Platelet-poor plasma [18] was separated from collected samples by double centrifugation (1800*g* for 10 min, 21 °C followed by 3000*g* for 10 min at 21 °C), snap-frozen in liquid nitrogen and stored at − 80 °C until used for the experiments [19].

Flow cytometry

The frozen plasma and synovial fluid samples were thawed on ice, diluted 1:10 with annexin V binding buffer (BD Biosciences, USA), and labeled with annexin V APC and cell lineage markers such as anti-human CD45 APC H-7 (BD Biosciences, USA), anti-human CD20 BV421 (BD Biosciences, USA), anti-human CD14 PE-Cy7 (BD Biosciences, USA), anti-human CD4 BB515 (BD Biosciences, USA), anti-human CD8 PE-CF594 (BD Biosciences, USA), anti-human CD66b PE (BD Biosciences, USA), and anti-human CD61 BV510 (BD Biosciences, USA) for 15 min in dark, resuspended in 300 µl annexin V binding buffer, and analyzed on BD FACSAria III sorter with FACSDIVA software (BD Biosciences, USA). A low threshold was set at 200 on forward scatter channel (FSC) and allophycocyanin (APC) channel. As an internal size control, MP gating was accomplished using 0.2 µm, 0.5 µm, and 1 µm beads of flow cytometry Sub-micron Particle Size Reference kit (Thermofisher Scientific, USA) (Supplementary Fig. 1). Annexin V only stained samples were run as a control. Only annexin V^+ events in the MP gate region were further subgrouped based on CD45, CD20, CD14, CD4, CD8, CD66b, and CD61 expression. All PS-exposing MP are identified by annexin V positivity. Annexin V^+ MP and their cell lineage-specific subpopulations were defined based on cell lineage-specific markers, and gating strategy is represented in Supplementary Fig. 2. Results were expressed as a percentage of total events.

Serological assays

Serum samples obtained from all the study subjects were analyzed by nephelometry (BN ProSpec System, Siemens, Germany) for rheumatoid factor (RF) and high sensitivity C-reactive (hsCRP) levels. Test values > 15.9 IU/ml were considered positive for RF and values > 3.02 g/l were considered as high hsCRP. Anti-cyclic citrullinated peptide antibodies (ACPA) titer was estimated by ELISA (AESKULISA CCP kit, AESKU Diagnostics GmbH & Co. KG, Germany), and a level above 25 U/ml was considered positive.

Data analysis

Categorical variables were expressed as *n* [percentage (%)] and continuous variables with a normal distribution as mean ± SD. Data on MP proportions are represented as median % [interquartile range (IQR)] unless otherwise stated. Mann–Whitney *U* test or unpaired T test was used to assess variation between synovial fluid MP of RA and OA and intergroup differences among clinical and serological phenotypes of RA. Proportions of plasma MP among RA, OA, and HC groups were compared by Kruskal–Wallis test with Dunn's multiple comparisons. Normality of continuous data was assessed by the Kolmogorov–Smirnov test. Correlation between MP proportions and disease parameters (DAS28 ESR, ACPA titers) was measured by Spearman correlation. GraphPad Prism Version 8 was used for the statistical analysis.

Results

Demographical, clinical, and serological assessment of study participants

Clinical characteristics (age at onset, disease duration, the presence of deformities, extra-articular manifestations), disease activity assessment based on disease activity score using 28 joint counts modified by erythrocyte sedimentation rate (DAS28 ESR) [20], seropositivity for RF and ACPA and hsCRP levels of RA patients are summarized in Table 1.

MP profiling for cell lineage markers

Synovial fluid MP profile in RA and OA

Synovial fluid of RA patients [5% (6.35)] had significantly elevated proportion of annexin V^+ MP compared to OA [1.8% (1.35); $p < 0.001$], and leukocyte-derived MP (annexin V^+ $CD45^+$ MP) were also increased significantly in RA [0.85% (1.175)], compared to OA [0.1% (0.125)], ($p < 0.001$). The B cell-derived MP (annexin V^+ $CD45^+$ $CD20^+$) were not detectable in both the groups. T_h cell-derived MP (annexin V^+ $CD45^+$ $CD20^-$ $CD14^-$ $CD4^+$) [0.1% (0.1)] and T_c cell-derived MP (annexin V^+ $CD45^+$ $CD20^-$ $CD14^-$ $CD8^+$) [0.1% (0.1)] were significantly higher in RA compared to OA [T_h cell-derived MP, 0% (0), $p < 0.001$ and T_c cell-derived MP, 0% (0)], $p < 0.001$, respectively). Monocyte-derived MP (annexin V^+ $CD45^+$ $CD20^-$ $CD14^+$) were significantly higher in RA [0.4% (0.4)] compared to OA [0.1% (0.1)], ($p < 0.001$).

Table 1 Clinical and serological features of patients with rheumatoid arthritis ($n=40$)

Characteristics	RA ($n=40$)
Clinical characteristics	
Age at onset (years) (Mean ± SD)	41.9 ± 11.4
Disease onset (*n*, %)	
Young-onset RA (YORA) (≤ 55 years)	35 (87.5%)
Late-onset RA (LORA) (> 55 years)	5 (12.5%)
Disease duration (*n*, %)	
Early RA (≤ 6 months)	16 (40%)
Established RA (> 6 months)	24 (60%)
Deformities (*n*, %)	
Deforming disease	22 (55%)
Non-deforming disease	18 (45%)
Extra-articular manifestations (*n*, %)	
Present	15 (37.5%)
Absent	25 (62.5%)
SICCA	10 (25%)
Rheumatoid nodules	3 (7.5%)
Interstitial lung disease	2 (5%)
Vasculitis	0 (0%)
Secondary Sjogren's syndrome	2 (5%)
Disease activity (*n*, %) (based on DAS28 ESR score)	
Low (DAS28 ESR < 2.6)	0 (0%)
Moderate active (DAS28 ESR 3.2–5.1)	8 (17.5%)
High disease activity (DAS28 ESR > 5.1)	35 (82.5%)
DAS28 ESR (Mean ± SD)	6.0 ± 0.8
Serological characteristics	
Positive	33 (82.51%)
Negative	7 (17.5%)
Rheumatoid Factor (RF) (*n*, %)	
Positive	28 (70%)
Negative	12 (30%)
Anti-CCP (ACPA) (*n*, %)	
Positive	32 (80%)
Negative	8 (20%)
Inflammatory markers (*n*, %)	
hsCRP	
High	40 (100%)
Normal	0
Erythrocyte Sedimentation rate (ESR) (mm/h) (Mean ± SD)	58.3 ± 21.4

SD standard deviation, *RA* rheumatoid arthritis, *hsCRP* high sensitivity C-reactive protein, *n* number, % percentage

Granulocyte-derived MP (annexin V^+CD66b^+) were significantly higher in RA [0.4% (2.05)] compared to OA [0% (0)]), ($p<0.001$). Synovial fluid proportions of annexin V^+ MP and subpopulations in RA and OA are represented in Fig. 1a–f and Table 2 and Supplementary Figs. 3–9.

Synovial fluid MP profile (cell lineage markers) among clinical phenotypes of RA

Synovial fluid MP profile was further compared between clinical subtypes of RA, i.e., young-onset RA (YORA) vs. late-onset RA (LORA), early vs. established RA, deforming vs. non-deforming disease, and RA with and without extra-articular manifestations. Granulocyte-derived MP were elevated in established RA [0.65% (3.08)] than early RA [0.15% (1.35)], ($p=0.03$). Annexin V^+ MP [6.6% (9.4)] and platelet-derived MP [0.1% (0)] were significantly increased in the synovial fluid of patients with extra-articular manifestations than those without annexin V^+ MP, 3.9% (5.85), ($p=0.02$) and platelet-derived MP, 0% (0.1), ($p<0.01$) respectively, (Fig. 1g–i, Supplementary Table 1.3 and 1.5). No correlation was observed with levels of various MP and disease activity measured by DAS28 ESR.

Synovial fluid MP profile (cell lineage markers) among serological phenotypes of RA

Synovial fluid MP profile was compared among serological phenotypes of RA, i.e., ACPA-positive RA vs. ACPA-negative RA. Synovial fluid of patients with ACPA-positive RA [0.65% (2.15)] contained more annexin V^+ $CD66b^+$ granulocyte-derived MP than patients with ACPA-negative RA [0.05% (0.33)], ($p=0.02$) (Fig. 1j, Supplementary Table 1.6).

A weak positive significant correlation was observed between ACPA titers (U/ml) and T_h cell-derived MP (annexin V^+CD45^+ $CD20^-CD14^-$ $CD4^+$) (Spearman $r=0.323$) as well as with granulocyte-derived MP (annexin V^+CD66b^+) (Spearman $r=0.375$) (Supplementary Table 1.8).

Plasma MP profile (cell lineage markers) in RA, OA and HC groups

The percentage of plasma annexin V^+ MP significantly varied between the groups; RA, OA, and HC ($p<0.001$). Further, RA [3.45% (5.63)] had significantly higher plasma proportions of annexin V^+ MP compared to OA [1.85% (1.4)], ($p<0.01$) and healthy controls [0.9% (1.1)], ($p<0.001$). Plasma annexin V^+ MP were also found at significantly higher proportions in patients with OA than healthy controls ($p=0.02$). The proportions of leukocyte-derived MP (annexin V^+ $CD45^+$) were significantly different across the three groups, ($p<0.001$). RA plasma [0.1% (0.2)] had significantly higher leukocyte-derived MP (annexin V^+ $CD45^+$) compared to OA [0%

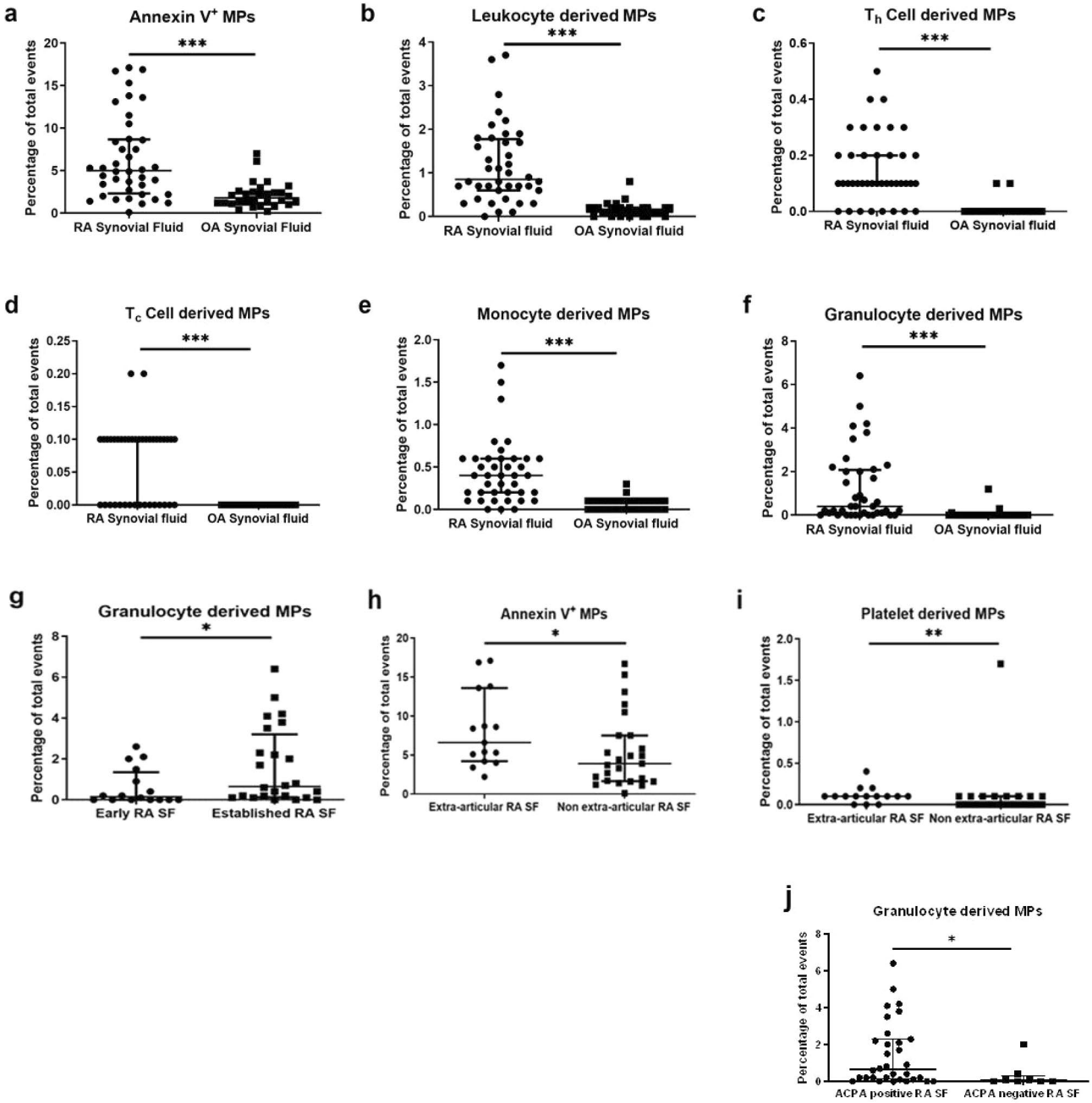

Fig. 1 Synovial fluid Annexin-V$^+$ MP (**a**) and subpopulations (**b**–**f**) in Rheumatoid Arthritis ($n=40$) vs. osteoarthritis ($n=30$); **g**) synovial fluid Granulocyte derived MPs in Early RA ($n=16$) vs. Established RA ($n=24$); **h**, **i** synovial fluid Annexin V$^+$ MPs and Platelet-derived MPs in Rheumatoid Arthritis (RA) patients with Extra-articular manifestations ($n=15$) vs. RA patients without extra-articular manifestations ($n=25$); **j** Synovial fluid Granulocyte derived microparticles in ACPA positive RA ($n=32$) vs. ACPA negative RA ($n=8$); Intergroup differences were evaluated by Mann–Whitney *U* test or unpaired *T* test. Horizontal lines indicate median with interquartile range (IQR); ***$p<0.001$; *SF* synovial fluid, *RA* rheumatoid arthritis, *OA* osteoarthritis; Early RA, Early Rheumatoid Arthritis (disease duration $\leq$ 6 months); Established RA, Established Rheumatoid arthritis (disease duration > 6 months); Extra-articular RA, Rheumatoid arthritis with extra articular manifestations; Non- extra articular RA, Rheumatoid arthritis without extra articular manifestations; ACPA positive RA, Anti-citrullinated protein antibodies positive RA; ACPA negative RA, Anti-citrullinated protein antibodies negative RA

(0.1)], ($p<0.01$) as well as HC [0% (0)], ($p<0.001$). Platelet-derived MP varied significantly higher among RA as compared to the OA and HC group ($p<0.001$). Annexin V$^+$ CD45$^-$ CD61$^+$ platelet-derived MP were higher in the plasma of RA patients [1.5% (4.23)] compared to OA [0.65% (0.85)], ($p=0.02$) and HC [0.2%

Table 2 Synovial fluid MP and cell-specific subtypes in RA ($n=40$) and OA ($n=30$) (expressed as a percentage of total events); Intergroup differences were evaluated by Mann–Whitney *U* test or unpaired *T* test

S. no.	Parameter	RA ($n=40$) Median (IQR)	OA ($n=30$) Median (IQR)	*p* value (Two tailed)
1	Annexin V^+ MPs	5 (6.35)	1.8 (1.35)	< 0.001
2	Leukocyte derived MPs (Annexin V^+ $CD45^+$)	0.85 (1.175)	0.1 (0.125)	< 0.001
3	B Cell derived MPs (Annexin V^+ $CD45^+$ $CD20^+$)	0 (0)	0 (0)	NA
4	T_h Cell derived MPs (Annexin V^+ $CD45^+$ $CD20^-$ $CD14^-$ $CD4^+$)	0.1 (0.1)	0 (0)	< 0.001
5	T_c Cell derived MPs (Annexin V^+ $CD45^+$ $CD20^-$ $CD14^-$ $CD8^{+)}$	0.1 (0.1)	0 (0)	< 0.001
6	Monocyte derived MPs (Annexin V^+ $CD45^+$ $CD14^+$)	0.4 (0.4)	0.1 (0.1)	< 0.001
7	Granulocyte derived MPs (Annexin V^+ $CD66b^+$)	0.4 (2.05)	0 (0)	< 0.001
8	Platelet derived MPs (Annexin V^+ $CD45^-$ $CD61^+$)	0.1 (0.1)	0 (0.1)	NS

IQR interquartile range, *NS* not significant, *NA* not applicable, *RA* rheumatoid arthritis, *OA* osteoarthritis
$^{***}p<0.0001$

(0.35)], ($p<0.001$). Plasma annexin $V^+CD45^-CD61^+$ platelet-derived MP were also found at higher proportions in patients with OA than healthy controls ($p=0.01$). Leukocyte-derived MP subpopulations like annexin V^+ $CD45^+$ $CD20^+$ B cell-derived MP, annexin V^+ $CD45^+$ $CD20^-$ $CD14^-$ $CD4^+$ T_h cell-derived MP, annexin V^+ $CD45^+$ $CD20^-$ $CD14^-$ $CD8^+$ T_c cell-derived MP, annexin V^+ $CD45^+$ $CD20^-$ $CD14^+$ monocyte-derived MP, and annexin V^+CD66b^+granulocyte-derived MP were not detectable in the plasma (Fig. 2 and Table 3).

Plasma MP profile (cell lineage markers) among clinical phenotypes of RA

No significant differences were found in any of the cell-derived MP among various clinical phenotypes of RA, i.e, YORA vs. LORA, early RA vs. established RA, deforming RA vs. non-deforming RA, RA with extra-articular manifestations vs. without extra-articular manifestations.

Plasma MP profile (cell lineage markers) among serological phenotypes of RA

No significant differences were found in any of the cell-derived MP among various serological phenotypes of RA, i.e., seropositive RA vs. seronegative RA, i.e., ACPA-positive RA vs. ACPA-negative RA.

Discussion

We undertook this study to identify the distribution of various cell-derived MP in circulation and the synovial fluid in a group of treatment-naive RA patients. For comparison, the proportions of MP in patients with knee OA and healthy controls were analyzed. Further, we have compared the various cell-derived MP in clinical and serological phenotypes of RA.

The results of our study demonstrated elevated microparticle levels in synovial fluid as well as plasma of patients with RA compared to OA and healthy controls. Higher levels

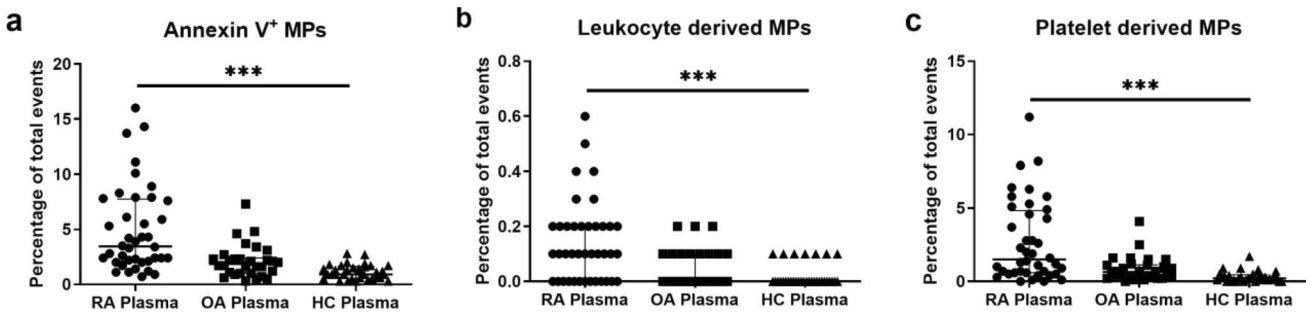

Fig. 2 Plasma Annexin-V^+ MP and subpopulations in Rheumatoid Arthritis ($n=40$) vs. osteoarthritis ($n=30$) vs. healthy controls ($n=33$); Intergroup differences were evaluated by Kruskal–Wallis test. Horizontal lines indicate median with IQR; *IQR* interquartile range, $^{***}p<0.001$; *RA* rheumatoid arthritis, *OA* osteoarthritis, *HC* healthy controls

Table 3 Plasma MP and cell-specific subtypes in RA, OA, and HC (expressed as a percentage of total events); Intergroup differences were evaluated by Kruskal–Wallis test with Dunn's Multiple Comparisons

S. no.	Parameter	RA ($n=40$) Median (IQR)	OA ($n=30$) Median (IQR)	HC ($n=33$) Median (IQR)	Kruskal–Wallis test, p value	Dunn's multiple comparison Test	Multiplicity adjusted p value
1	Annexin V^+ MPs	3.45 (5.63)	1.85 (1.4)	0.9 (1.1)	< 0.001	RA Plasma vs. OA Plasma	< 0.01
						RA Plasma vs. HC Plasma	< 0.001
						OA Plasma vs. HC Plasma	0.02
2	Leukocyte derived MPs (Annexin V^+ $CD45^+$)	0.1 (0.2)	0 (0.1)	0 (0)	< 0.001	RA Plasma vs. OA Plasma	<0.01
						RA Plasma vs. HC Plasma	< 0.001
						OA Plasma vs. HC Plasma	NS
3	B Cell derived MPs (Annexin V^+ $CD45^+$ $CD20^+$)	0 (0)	0 (0)	0 (0)	NA		
4	T_h Cell derived MPs (Annexin V^+ $CD45^+$ $CD20^-$ $CD14^-$ $CD4^+$)	0 (0)	0 (0)	0 (0)	NA		
5	T_c Cell derived MPs (Annexin V^+ $CD45^+$ $CD20^-$ $CD14^-$ $CD8^{+)}$	0 (0)	0 (0)	0 (0)	NA		
6	Monocyte derived MPs (Annexin V^+ $CD45^+$ $CD14^+$)	0 (0)	0 (0)	0 (0)	NA		
7	Granulocyte derived MPs (Annexin V^+ $CD66b^+$	0 (0)	0 (0)	0 (0)	NA		
8	Platelet derived MPs (Annexin V^+ $CD45^-$ $CD61^+$)	1.5 (4.23)	0.65 (0.85)	0.2 (0.35)	< 0.001	RA Plasma vs. OA Plasma	0.02
						RA Plasma vs. HC Plasma	< 0.001
						OA Plasma vs. HC Plasma	0.01

IQR Interquartile range, *NS* not significant, *NA* not applicable, *RA* rheumatoid arthritis, *OA* osteoarthritis, *HC* healthy controls

*$p<0.05$; **$p<0.01$; ***$p<0.001$

of leukocyte-derived MP, and their subpopulations were detected in the synovial fluid of RA patients. RA patients with extra-articular manifestations had higher synovial fluid platelet-derived MP than those without extra-articular manifestations. Synovial fluid granulocyte-derived MP were more elevated in RA patients with established RA and those with positive ACPA. Platelet-derived and leucocyte-derived MP in the circulation were higher in RA compared to OA and HC. It is also evident that low amounts of MP are present in degenerative joint disease (OA) as well as in healthy individuals. The important results from various previous reported studies on MP in RA are depicted in Table 4.

Leukocyte-derived MP, especially granulocyte, monocyte, and T cell-derived MP, were higher in the synovial fluid and have been demonstrated to have pathophysiological and prognostic significance in RA across various studies [21–24]. Inflammation, neovascularization, synovial hypertrophy, and bone erosion are the predominant changes that occur in a rheumatoid joint. Leukocyte-derived MP from arthritic joints as demonstrated in vitro stimulates RA fibroblast-like synoviocytes (FLS) and releases chemokines, pro-inflammatory cytokines, MMPs, B cell activation factors and activates classical complement pathway [25–28]. In vitro experiments using the MP generated from Jurkat T cells, and U937 monocytes have shown to induce pro-angiogenic (glutamic acid-leucine-arginine rich or ELR^+) chemokines in RA FLSs [29]. Besides this, monocytes and T cell-derived MP upregulates the production of proinflammatory factors like prostaglandin E2 by RA FLSs [30]. Leukocyte-derived MP may serve a potential source of molecules involved in intercellular communication, joint destruction, in an autoimmune inflammatory milieu.

We observed the significantly higher synovial fluid proportion of annexin V^+ MP as well as platelet-derived MP in RA patients with extra-articular manifestations suggesting a heightened state of local inflammation. There is evidence to show extracellular vesicles (EVs) which comprise MP and exosomes contribute to the progression of the disease beyond joints in RA by aiding transport of information related to T cell exhaustion, especially PD-1 [31]. Likewise, EVs in autoimmune diseases may contribute to epitope spreading as well [32]. Synovial fluid PMP promoted adhesion and motility of RA-FLSs by increasing MMP1 via activating

Table 4 Various reported studies on MPs (especially synovial fluid MPs) in Rheumatoid Arthritis

References	Sample size	MP phenotype	Technique	Inference
Knijff-Dutmer et al. [53]	RA ($n=19$), HC ($n=10$)	Annexin V; Platelet-derived MP (PMP) (CD61)	Flow cytometry	Elevated numbers of PMP in RA plasma; PMP levels correlated with disease activity
Berckmans et al. [54]	RA ($n=10$), Non-RA ($n=10$), HC ($n=20$)	PMP: CD61; LMP: CD4, CD8, CD14, CD20, CD66b, CD66e	Flow cytometry	Elevated numbers of Procoagulant TF positive Leukocyte derived MP in RA synovial fluid
Berckmans et al. [26]	RA ($n=8$), Undifferentiated arthritis control ($n=3$)	PMP: CD61; LMP: CD4, CD8, CD14, CD20, CD66e	Flow cytometry	Elevated numbers of Leukocyte derived MP in RA synovial fluid
Messer et al. [55]	Microcristalline arthritis (MC) ($n=3$), Reactive arthritis (AR) ($n=5$), RA ($n=7$), OA ($n=5$),	Annexin V	Solid-phase capture assay on immobilized annexin V	Increased numbers of synovial MP in RA and MC
Umekita et al. [56]	RA ($n=20$)	PMP: CD61, CD42a; LMP: CD66b, CD16	Flow cytometry	High levels of PMP in RA and correlated with disease activity
Boilard et al. [42]	Juvenile idiopathic arthritis (JIA) ($n=6$), Psoriatic arthritis (PSA) $n=19$), Gout ($n=14$), RA ($n=20$), OA ($n=20$),	PMP (CD41); LMP (CD15, CD14, CD3)	Flow cytometry	High numbers of PMP in RA SF
Gyorgy et al. [57]	RA ($n=10$),OA ($n=10$), and JIA ($n=8$)	MP; Anx V, LMP; CD3, CD4, CD8, CD14, CD19	Nanoparticle tracking analysis, Flow cytometry, mass spectrometry	Elevated B-cell, T-cells derived MP ($CD3^+$ and $CD8^+$) in synovial fluid of RA;Elevated levels of CD41a + vesicles in RA compared to OA and JIA
Cloutier et al. [38]	RA ($n=23$) and PA ($n=18$)	Annexin V, CD41a	High Sensitive Flow cytoetry	High levels of Annexin V^+ MP and $CD41a^+$ MP containing Immune complexes (size ~ 700–3200 nm) in RA compared to PA.
Rodríguez-Carrio et al. [58]	Individuals with cardiovascular risk without autoimmune manifestations (CVR) ($n=72$), RA ($n=113$), HC ($n=33$)	Violet Proliferation Dye (VPD), CD41, CD146, CD66, CD14, Tang-(CD3+CD31+)	Flow cytometry	Altered profile of Plasma MP associated with disease-specific features and traditional CVR factors
Viñuela-Berni et al. [59]	RA ($n=55$; 24 untreated; 31 under conventional therapy), HC ($n=20$)	Annexin V, CD3, CD14, CD19,CD41a and CD62E	Flow cytometry	High proportions of Plasma $CD3^+$, $CD14^+$, $CD19^+$, $CD41^+$ and $CD62E^+$ MP and urine ($CD14^+$, $CD3^+$ and $CD19^+$ MP from RA patients with high disease activity)
Fan et al. [60]	RA ($n=34$), OA ($n=33$), HC ($n=42$)	Annexin V, CD3, CD4, CD161, CD39, CD73	Flow cytometry	$CD4^+CD161^+CD39^+$ and $CD4^+CD39^+CD73^+$ MP were abundantly present in RA patients synovial fluid
Burbano et al. [39]	Seropositive RA ($n=10$) Seronegative RA ($n=5$); HC ($n=5$)	CD41a, CD105, CD45, CD14	Flow cytometry	Elevated proportions of $CD41a^+$ MP, $CD45^+$ MP in seropositive patients, $CD105^+$ MP in anti-CCP^-RF^- group

PMPs platelet-derived MPs, *LMPs* Leukocyte derived MPs, *JIA* Juvenile idiopathic arthritis, *PSA/PA* psoriatic arthritis, *OA* osteoarthritis, *CCP* cyclic citrullinated protein, *HC* healthy controls, *CVR* cardiovascular risk

Erk-mediated NF-κB pathway indicating their possible role in the RA synovium [33].

Granulocyte-derived MP in the synovial fluid of patients with established RA, seropositive RA, particularly ACPA-positive RA, were higher compared to their negative counterparts. Granulocyte derived MP also showed a weak positive correlation with ACPA levels. Protein arginine deiminases (PAD) isoforms 2 and 4 are functional, and citrullination is upregulated in the synovium [34]. The surface proteins of MP in the synovial fluid may get citrullinated to form neoantigens, which may lead to an ACPA-positive state. Neutrophils are the most abundant cells in the synovial fluid and also observed in pannus/cartilage interface in patients with active RA [35]. A large number of dying neutrophils in the articular compartment in RA maintain a constant release of active PADs for extracellular citrullination [36]. These granulocyte derived MP may also reflect a heightened state of NETosis, which causes prolonged exposure of autoantigens to immune cells, ultimately resulting in autoantibody generation [37]. Microparticles can associate with autoantibodies to form MP-associated immune complexes (MP-ICs) and further express autoantigens, including citrulline and vimentin [38, 39]. MP in synovial fluid may thus behave as a source of autoantigens and autoadjuvants in an autoimmune milieu, perpetuating the immune response [40]. Further, granulocyte-derived MP may serve as a biomarker for the long-standing disease.

Elevated platelet-derived MP (PMP) were found in the circulation of patients with RA reflecting a state of systemic platelet activation [12, 13, 41, 42]. Platelet-derived microparticles are known to transport arachidonic acid to RA synovial fibroblasts [43–45], cause leukocyte aggregation [45, 46], and provide binding sites for the assembly of coagulation factors [47]. While few studies have reported a direct correlation of PMP with disease activity in RA [8, 48], others have observed a higher level of P-selectin, which is a marker of platelet activation on platelets of patients with active RA [49]. We failed to demonstrate such a correlation between disease activity and PMP. Although systemic activation of platelets can be easily detected by elevated levels of platelets in circulation, cargo of PMP differs based on their microenvironment [50, 51]. Hence, studying the proteome of circulating PMP cargo in RA may be fruitful to better understand the molecules responsible for local and systemic amplification of inflammation. Also, the observed higher plasma annexin V^+ MP and platelet-derived MP in OA compared to HC might be due to their age.

The strength of our study is that only DMARD and steroid-naive patients with active RA were enrolled, thus avoiding the confounding effect of immunomodulatory therapy. However, the study also suffers from a few limitations. Though the numbers of cases are higher than most reported studies, the results of the subgroup analysis need further validation in a larger cohort. The OA controls were only clinically screened for co-morbidities, with relevant history and examination, but investigations to rule out diabetes and ischemic heart disease were not done.

Technically, the noise and debris detected in the flow cytometry assays were high and the percentages presented here are the percentage of total events collected. Hence, the use of a combination of sophisticated techniques for quantification (i.e., high-sensitive flow cytometry), use of more than one CD marker to describe particular cell-derived MP subsets, and fluorescence triggering technology (flow cytometry) [52] for specific cell-derived MP may provide an accurate in vivo picture of cell-derived vesicles and may further improve the chances of identification of specific cell-derived microvesicles as biomarkers and diagnostic tools. Also in vitro, functional studies on the effect of microparticles on fibroblasts and other immune cells may provide additional clues on their role in RA and OA.

Our observations of higher proportions of cell-derived MP in the plasma and synovial fluid of DMARD-naïve RA patients and their clinical and serological phenotypes suggest their role in dynamic cross talk between the joint and systemic circulation and disease pathology and progression that might result in elevated autoantibody production, immune complex formation, joint damage, and systemic progression of RA. Further delineation of their in vivo functional mechanisms and exploration as biomarker for diseases phenotypes is warranted.

Author contributions VSN, BNRM, CKG, and KV contributed to the conception and design, acquisition of data, analysis, and interpretation of the data and final approval of the version to be published. BNRM and KV drafted the article. VSN critically revised the article for important intellectual content. VSN, BNRM, CKG, and KV agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding The work was supported by Indian Council of Medical Research (ICMR), India (IRIS ID No.: 2012-2279) and JIPMER Intramural Research Fund (JIP/Res/Intra-PhD/01/2014 and JIP/Res/Intra-PhD/02/2015-16).

Compliance with ethical standards

Conflict of interest Ms. Benita NR Michael, Mr. Vallayyachari Kommoju, Dr. Chengappa Kavadichanda Ganapathy, and Dr. Vir Singh Negi declare that they have no conflict of interest.

Ethical approval The study was approved by the JIPMER institute ethics committee and conducted the following Principles of the Declaration of Helsinki (1964) and its later amendments or comparable ethical standards. Protocol No. JIP/IEC/2013/1/107 dated 15.03.2013.

Informed consent Informed consent was obtained from all individual participants included in the study.

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