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Comprehensive characterization of pathogenic synovial fluid extracellular vesicles from knee osteoarthritis

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Abstract

Synovial fluid (SF) extracellular vesicles (EVs) play a pathogenic role in osteoarthritis (OA). However, the surface markers, cell and tissue origins, and effectors of these EVs are largely unknown. We found that SF EVs contained 692 peptides that were positively associated with knee radiographic OA severity; 57.4% of these pathogenic peptides were from 46 proteins of the immune system, predominantly the innate immune system. CSPG4, BGN, NRP1, and CD109 are the major surface markers of pathogenic SF EVs. Genes encoding surface marker *CSPG4* and *CD109* were highly expressed by chondrocytes from damaged cartilage, while *VISG4, MARCO,*

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Patient consent statement

All specimens were acquired with informed consent under IRB approval of Duke University. TKR specimens were acquired at the time of TKR surgery as surgical waste under IRB approval of Duke University.

Ethics approval statement

This study was reviewed and approved by the IRB of Duke University (Pro00011820 pertaining to the Prediction of Osteoarthritis Progression cohort, Pro00011566 pertaining to the Biomarker and Joint Arthroplasty cohort, and Pro00008622 pertaining to surgical waste specimens) and was conducted in compliance with relevant guidelines.

CRediT authorship contribution statement

Xin Zhang: Conceptualization, Methodology, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **Sisi Ma:** Methodology, Formal analysis, Investigation, Validation, Writing – review & editing. **Syeda Iffat Naz:** Formal analysis, Validation. **Vaibhav Jain:** Formal analysis. **Erik J. Soderblom:** Formal analysis, Validation. **Constantin Aliferis:** Supervision, Writing – review & editing. **Virginia Byers Kraus:** Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

None Declared.

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CD163 and *NRP1* were enriched in the synovial immune cells. The frequency of $CSPG4^+$ and $VSIG4^+$ EV subpopulations in OA SF was high. We conclude that pathogenic SF EVs carry knee OA severity-associated proteins and specific surface markers, which could be developed as a new source of diagnostic biomarkers or therapeutic targets in OA.

Keywords

Extracellular vesicles; Knee osteoarthritis; Synovial fluid; Innate immune system; Surface markers; Chondrocytes; Immune cells

1. Introduction

Osteoarthritis (OA), the most common form of joint disorder in the US affecting 1/7 US adults, increases with age, increases age-related morbidity and mortality, and results in annual costs exceeding $65.5 and $71.3 billion due to medical expenses and lost wages, respectively [1–3]. OA involves a complicated pathological remodeling of connective tissues driven by a host of inflammatory mediators within the affected joints [4,5]. To date, there are no approved medicines to prevent or decelerate OA progression. The goal of this work was to identify surface markers of pathogenic synovial fluid (SF) extracellular vesicles (EVs) as targets for diagnostics and therapeutics in OA.

In our previous study, we reported that plasma EVs carry pathogenic cytokines, TNF-α, IL-1β, IL-6 and IFN-γ; the concentrations of TNF-α and IFN-γ within EVs, but not outside EVs, were associated with knee radiographic OA (rOA) progression [6]. Our findings suggest the importance and clinical relevance of evaluating the role of EVs in OA. SF is an ultrafiltrate of plasma with addition of components from cartilage (chondrocytes), synovium (synoviocytes comprised of fibroblasts and immune cells, such as macrophages, T cells, B cells, and neutrophils in OA joints) [7–12]. Previous studies of ours and others suggest a pathogenic role of SF EVs in OA based on several lines of evidence: multiple immune cell-derived EV subpopulations are enriched in OA SF compared with plasma [10]; OA SF EV subsets carry pro-inflammatory cytokines [10]; small EVs from OA SF can induce macrophages to release inflammatory cytokine IL-1β, chemokines (CCL8, CCL15, CCL20 and CXCL1) and MMPs [13]; and IL-1β-stimulated synovial fibroblast-derived small EVs induced expression of OA-related genes in articular chondrocytes [14]. However, the surface markers, cell and tissue origins, and cargo effectors of these EVs are still largely unknown. Herein, we aimed to comprehensively characterize SF EVs, especially pathogenic SF EVs, from knee OA, to identify their biomarkers related to rOA severity. We hypothesize that pathogenic SF EVs carry rOA severity-associated protein cargo and specific surface markers; these surface markers could in future enable development of new OA biomarkers and new therapeutics targeting pathogenic SF EVs.

To test our hypothesis, first, we identified knee rOA severity-associated peptides in SF and plasma EVs (termed EV peptides$^{rOA\text{-}Pos}$); second, we identified peptides that were more abundant in SF EVs than matched plasma EVs (termed EV peptides$^{SF>PL}$); third, we identified EV surface markers from the list of EV peptides$^{SF>PL}$ that were also highly associated with SF EV peptides$^{rOA\text{-}Pos}$; fourth, we validated expression of genes encoding

these surface markers in end-stage knee OA joint tissue cells; last, we validated the presence of EV subpopulations carrying these surface markers in end-stage knee OA SF (Graphical Abstract). To our knowledge, this is the first study reporting a comprehensive non-targeted proteomic analysis of EVs from matched plasma and SF from knee OA patients, thereby identifying an array of surface markers of pathogenic SF EVs with confirmed OA joint tissue origins.

2. Methods

2.1. Study cohorts

The following cohorts were studied: (1) Proteomic cohort: matched plasma and SF specimens collected from study participants with knee OA (n = 16; mean age 69 ± 12 years, range 51–87; mean BMI 33 ± 9 kg/m^2, 50% female) from the Prediction of Osteoarthritis Progression cohort [15], and the Biomarker and Joint Arthroplasty cohort [16] acquired with informed consent under Institutional Review Board (IRB) approval of Duke University. Their knee rOA severity scores (Kellgren Lawrence [K/L], joint space narrowing [JSN] and osteophyte number and size [OST] [6,10,11]) ranged 2–4, 0–4, and 2–10 for index knees (those providing SF), respectively, and 2–8, 0–9, and 4–19 for both knees, respectively (details in Supplementary Table 1). As older adults with OA, these participants were taking generic medicines for OA and other age-related diseases and conditions [10]. (2) Single-cell RNA sequencing (scRNA-seq) cohort: scRNA-seq data as previously published [12], were derived from cartilage and matched synovium of 3 patients with knee OA (mean age 67 ± 2.3 years, 2 female) acquired at the time of total knee replacement (TKR) as anonymized surgical waste specimens. (3) Flow cytometry cohort: SF specimens acquired as anonymized surgical waste specimens at the time of TKR from 23 patients with knee OA (mean age 65 ± 10 years, range 46–88 years; 35% were female). Samples were stored at −80 °C until analysis. All TKR specimens were acquired under IRB approval of Duke University.

2.2. EV separation

As we previously reported [10,17], blood and SF specimens were centrifuged at 3000 rpm for 15 min at 4 °C on the day of collection to remove cells and debris; plasma and SF were aliquoted and frozen at −80 °C. On the day of EV separation, the matched frozen plasma and SF from the ‘proteomic cohort’ were thawed and centrifuged at 2000 g for 10 min at 4 °C to remove debris. SF was digested with hyaluronidase (Streptomyces hyalurolyticus, 10 unit/ml, Sigma-Aldrich) for 1 h at 37 °C. EVs were separated from plasma and SF using polymer-based precipitation (ExoQuick, System Biosciences) following the manufacturer’s instructions and characterized as we previously reported [10,17].

2.3. Mass spectrometry-based proteomics of SF and plasma EVs

The matched SF EVs and plasma EVs were processed for proteomics by the Duke Proteomics and Metabolomics Core Facility as previously described [18], using a nanoAcquity UPLC system (Waters Corp) coupled to an Orbitrap Fusion Lumos high-resolution accurate mass tandem mass spectrometer equipped with a FAIMS Pro system (ThermoFisher Scientific). Detailed information on sample preparation, proteomic analyses, and data normalization are provided in Supplementary Methods. STRING network analysis

[19] was performed to analyze protein interactions and functional enrichment of EV proteomic data.

2.4. Surface marker identification and validation

SurfaceGenie [20] analysis of the EV proteomic data was used to identify putative surface markers based on Surface Protein Consensus (SPC) score. SPC is a sum of the number of predictive datasets in which a protein is deemed to have a surface localization; scores range 0–4. 'Validated' surface proteins were defined as those with SPC ≥ 2, and 'putative' surface proteins were defined as SPC = 1. Membrane localization was validated using UniProt [21](KW-0472), STRING [19] (GO:0031224) and the Human Protein Atlas [22]. The cell origins of surface proteins were identified by Tabula Sapiens [23,24], a cell atlas of 483,152 cells from 24 organs of 15 healthy human individuals.

2.5. ScRNA-seq

Expression of genes encoding SF EV surface markers were evaluated in scRNA-seq data [12] (data set NCBI GEO GSE152805) generated from 36,832 cells from joint tissues of three participants with knee OA undergoing TKR, including 11,579 chondrocytes from damaged sites of the medial tibial cartilage, 14,613 chondrocytes from intact sites of the outer lateral tibial cartilage, and 10,640 synoviocytes from the matched synovium.

2.6. High-resolution multicolor flow cytometry

As previously reported [17], EV pellets were resuspended in double filtered PBS (df-PBS) filtered by 100 nm filters (EMD Millipore) and stained with fluorescence-conjugated antibodies against human CD163, LRP1 (BD Biosciences), CD109, NRP1 (R&D Systems), CSPG4, MARCO (Novus Biologicals), VSIG4 (ThermoFisher Scientific), PTPRS (LifeSpan) and BGN (Bioss). The flow cytometer was configured to ensure that df-PBS acquisition events were below 20 eventss; green fluorescence reference beads of mean size 100, 1000 and 6000 nm (Bangs Laboratories) were used for size estimation (Supplementary Fig. 1A). The fluorescence background was determined using unstained and antibody-stained EVs and UltraComp™ eBeads plus (ThermoFisher Scientific). The percentages and numbers of EVs carrying each marker were determined using a high-resolution Sony MA 900 Multi-Application Sorter (Sony Biotechnology). Flow cytometric data analysis was performed using FCS Express 5 software (De Novo Software).

2.7. Statistical analyses

Analyses performed in this study included: (1) Spearman for assessing correlations between the expression of each peptide in SF EVs or plasma EVs and age, gender, BMI, and knee rOA scores, and correlations between surface markers with other SF EV peptides; since plasma EVs reflect systemic changes influenced by both knees, while SF EVs reflect local changes from the index knee, Spearman correlations were performed between plasma EV peptides and summed knee rOA scores of both knees, as well as SF EV peptides and index knee rOA scores; (2) Comparisons of peptide expression between EVs of matched plasma and SF used a paired *t*-test with FDR adjustment by Benjamini–Hochberg (BH) procedure; (3) To assess the possibility of informative missingness in the proteomic data, all peptides

were evaluated; calculation of missing rate of each peptide (for SF and plasma EV peptides separately) was defined as number of participants with a missing peptide value relative to the total number of participants; comparisons of missing rates between EVs of plasma and SF were performed using McNemar's test with FDR adjustment by BH procedure. Statistical significance was defined as $p < 0.05$ throughout the study with the exception of missing rates that were defined by BH procedure adjusted $p < 0.05$.

3. Results

3.1. EV characterization

Although there are many methods used for EV separation, each method has advantages and disadvantages, and none of the methods is suitable for all downstream analyses [25]. We selected polymer-based precipitation, one of the most frequently used EV separation methods to date, to ensure high yield of EVs from small volumes of human plasma and SF [10,17,26,27]. We have extensively characterized polymer-based precipitation separated EVs using dynamic light scattering, high-resolution multicolor flow cytometry, nanoparticle tracking analysis, transmission electron microscopy, multiplex ELISA and small RNA sequencing, and reported that EVs have various sizes and lipid bilayer structures, and carry an array of surface markers, respiring mitochondrial components, cytokines and miRNAs [10,17,28]. EVs isolated using the same methods as this study, from the same SF and plasma specimens, were previously validated for EV size and number by nanoparticle tracking analysis and flow cytometry, surface markers (CD81, CD9, CD29, CD63, CD4, CD8, CD68, CD14, CD56, CD15, CD19, CD235a, CD41a, CD34, CD31, HLA-ABC, HLA-G, and HLA-DRDPDQ) by flow cytometry, and cytokine cargo (IL-1β, IL-6, TNF-α and IFN-γ) by flow cytometry and ELISA [10]. SF and plasma both carried EVs with a broad range of relative sizes: large (LEVs), 1000–6000 nm; medium (MEVs), 100–1000 nm; and small (SEVs), <100 nm. Notably, we observed that the numbers of EVs of all sizes, and numerous subpopulations ($CD9^+$, $CD81^+$, $CSPG4^+$, and $CD109^+$) were dramatically higher in hyaluronidase treated SF than the untreated SF (Supplementary Fig. 1). We therefore conducted all experiments herein on hyaluronidase treated SF.

3.2. Associations of SF EV peptides with knee rOA severity

Mass spectrometry-based proteomics of SF and plasma EVs identified 8538 peptides corresponding to 670 proteins (Supplementary Table 2) in the knee OA 'proteomics cohort'. The expression of many SF and plasma EV peptides was significantly correlated with demographic variables (age, BMI and gender, Supplementary Fig. 2), which is consistent with previous studies of ours and others reporting age-, BMI- and gender-related SF and plasma EVs in OA [6,29,30]. SF EVs contained a total of 692 peptides significantly positively ($peptides^{rOA\text{-}Pos}$), and 94 peptides significantly negatively ($peptides^{rOA\text{-}Neg}$) correlated (adjusted for age, BMI and gender) with any one of the three knee rOA severity scores: K/L, JSN and OST [6,10,11] (Fig. 1A-C). We also identified 141 $peptides^{rOA\text{-}Pos}$ and 73 $peptides^{rOA\text{-}Neg}$ in plasma EVs (Fig. 1D-F). The higher number of $peptides^{rOA\text{-}Pos}$ in SF EVs compared to plasma EVs (692 vs. 141, Fig. 1C,F), is consistent with SF EVs as more proximal indicators of OA pathology.

3.3. More than half of the 692 SF EV peptides$^{rOA\text{-}Pos}$ were from the immune system, predominantly the innate immune system

Interestingly, based on STRING network analysis [19], 'immune system' was the top reactome pathway of the proteins comprising the SF EV peptides$^{rOA\text{-}Pos}$; a majority (57.4%) of the 692 SF EV peptides$^{rOA\text{-}Pos}$ were from 46 proteins of the immune system (HSA-168256, FDR = 4.49E-11); among the 397 immune system-related SF EV peptides$^{rOA\text{-}Pos}$, 252 were from 36 proteins of the innate immune system (HSA-168249, FDR = 5.92E-13) (Supplementary Fig. 3). The top three groups of immune system-related SF EV peptides$^{rOA\text{-}Pos}$ were from fibronectin (FN1, 19.2%), the complement system (C2, C3, C4A, C4B, C5, C5, C7, C8A, CFH, total 8.2%) and coagulation pathways (TF, F2, F11, F12, F13A, F13B, FGA, FGB, FGG, total 14.6%) (Fig. 1A). These molecules and pathways are known to be enriched in OA tissues and have established pathogenic roles in OA [31–34]. In contrast, among the 141 plasma EV peptides$^{rOA\text{-}Pos}$, only a few were derived from the complement system and coagulation pathways, and none derived from FN1. Our data strongly support the ability of EVs, in particular SF EVs, to reflect OA severity status.

We detected peptides$^{rOA\text{-}Pos}$ from APOA1 and APOB, which is consistent with a previous study reporting that increased levels of APOA1 and APOB are associated with knee OA [35]. It is not clear if these are components of EVs, since EVs carry lipid [36,37], or co-isolated with EVs, since lipoprotein particles cannot be fully separated from EVs by any of the currently available purification techniques [36].

3.4. Peptides enriched in SF EVs compared with plasma EVs

We hypothesize that EV peptides and proteins of a joint tissue origin are likely to be enriched in SF compared to plasma of knee OA participants. A total of 353 peptides$^{SF>PL}$, corresponding to 143 proteins$^{SF>PL}$, were more abundant in SF EVs than plasma EVs (Fig. 2A): 105 peptides$^{SF>PL}$ from 40 proteins$^{SF>PL}$ had a differential quantitative expression (Supplementary Table 3); 106 peptides$^{SF>PL}$ from 54 proteins$^{SF>PL}$ had a differential missing rate (Supplementary Table 4); and 142 peptides$^{SF>PL}$ from 49 proteins$^{SF>PL}$ were exclusive to SF EVs with 0% missing in SF EVs and 100% missing in plasma EVs (Supplementary Table 5). Notably, most often, the exclusivity of expression was at the peptide but not protein level. For instance, we detected 4 EV peptides$^{SF>PL}$ of CRTAC1 (Supplementary Table 5), a systemic biomarker predictor of knee rOA progression identified by our group and others [38,39], that were exclusive to SF EVs; an additional 30 CRTAC1 EV peptides were present in both SF and plasma EVs (Supplementary Table 2). The exception was QRFP that was exclusively present in SF EVs at both the peptide and protein levels.

3.5. SF EV proteins$^{SF>PL}$ are involved in multiple pathways relevant to OA pathogenesis

STRING network analysis [19] identified reports of proteins$^{SF>PL}$ in previous studies on OA (305 publications), EVs (187 publications) and proteomics (474 publications) (Supplementary Table 6). Most SF EV proteins$^{SF>PL}$ were enriched in various vesicles including EVs (Fig. 2B), involved in degradation of extracellular matrix and collagen, and associated with musculoskeletal system disease and inflammatory response (Fig. 2C). A majority (84.9%) of the SF EV proteins$^{SF>PL}$ were functionally and physically linked with

each other in the network (protein-protein interaction enrichment *p* value <1.0e-16, Fig. 2D). The proportion of SF EV proteins$^{SF>PL}$ involved in specific pathways included: skeletal system (50%), connective tissues (45.3%), extracellular matrix proteoglycans (19.7%), collagen degradation (10.4%), response to stimulus (74.4%), musculoskeletal system disease (22.1%), connective tissue disease (17.4%), bone disease (14%), and degenerative disc disease (3.5%) (Fig. 2D). Taken together, these findings support our designation of these peptides as originating from pathogenic SF EVs.

3.6. EV surface proteins$^{SF>PL}$ indicate potential joint tissue origins of SF EVs

Surface markers on EVs can indicate their cellular origins [6,10,17,26] and mediate transfer of EV cargo to recipient cells via ligand–receptor mechanisms [40]. We defined 'validated' surface proteins as those with SPC score ≥ 2; membrane localization was validated using UniProt [21](KW-0472), STRING [19](GO:0031224) and the Human Protein Atlas [22]. We defined 'putative' surface proteins as SPC = 1 (predicted in only one predictive database). We identified 25 peptides$^{SF>PL}$ corresponding to 8 validated surface proteins (CD163, LRP1, NRP1, CD109, CSPG4, MARCO, PTPRS and VSIG4) (Fig. 3A) in SF EVs of the knee OA proteomics cohort. These 25 EV peptides$^{SF>PL}$ were significantly positively correlated with each other (Fig. 3B), suggesting that SF EVs may be generated from limited cell sources. Based on Tabula Sapiens [23,24], a large human cell atlas, the following tissue/cell origins of SF EV surface proteins are suggested: CD163, LRP1, MARCO and VSIG4 from macrophages and various monocytes; NRP1 and CD109 from multiple types of endothelial cells and capillary aerocytes; LRP1 and NRP1 from alveolar fibroblasts and adventitial cells; LRP1 and CSPG4 from various smooth muscle cells; LRP1, NRP1, PTPRS, CD109 and CSPG4 from connective tissue cells (Fig. 3C-D).

We identified an additional 7 peptides$^{SF>PL}$ corresponding to 4 putative surface proteins (ANXA2, ANXA5, BGN and HSP90AB1) on SF EVs (Supplementary Fig. 4A). ANXA2, ANXA5 and HSP90AB1 are some of the main markers identified in EVs from various biological materials in over 300 previous studies in the Vesiclepedia database [41]. BGN and HSP90AB1 peptides$^{SF>PL}$ were significantly positively correlated with most of the surface peptides$^{SF>PL}$ in SF EVs (Supplementary Fig. 4B). As indicated by Tabula Sapiens [23,24], ANXA2, ANXA5 and HSP90AB1 are highly expressed by almost all cell types including connective tissue cells, while BGN is highly expressed by various smooth muscle cells, fibroblasts, and connective tissue cells (Supplementary Fig. 4C).

Each surface peptide was statistically significantly correlated with numerous other SF EV peptides (number range 263–5384), but negatively correlated with few peptides (number range 10–73). Remarkably, the vast majority of SF EV peptides (~5000, approximately 60% of the total identified SF EV peptides) were significantly positively correlated with surface peptides$^{SF>PL}$ of CSPG4, NRP1 (Fig. 3E) and BGN (Supplementary Fig. 4D).

Taken together, these data suggest two major cell origins of SF EVs, synovial connective tissue cells (such as chondrocytes) generating CSPG4^{+}, CD109^{+} and BGN^{+} EVs, and macrophages/monocytes generating CD163^{+}, MARCO^{+} and VSIG4^{+} EVs.

3.7. CSPG4, CD109, BGN and NRP1 are the major surface markers of pathogenic SF EVs in knee OA

Interestingly, among the total 692 SF EV peptides$^{roA\text{-}Pos}$, 643 (92.9%) peptides$^{rOA\text{-}Pos}$ were significantly correlated with at least one of the SF EV surface peptides$^{SF>PL}$, whereas the remaining 49 (7.1%) peptides$^{roA\text{-}Pos}$ were not significantly correlated with any SF EV surface peptides$^{SF>PL}$ (Fig. 4A). In fact, over 60% of SF EV peptides$^{rOA\text{-}Pos}$ were significantly positively correlated with CSPG4, BGN, NRP1 and CD109, and 77% were positively correlated with CSPG4 alone; there were few negative correlations (Fig. 4B).

3.8. Genes encoding SF EV surface markers were highly enriched in knee OA joint tissues

Based on our scRNA-seq data from end-stage OA joint tissue cells [12], *LRP1, ANXA2, ANXA5, BGN, and HSP90AB1* were highly expressed by OA chondrocytes and synoviocytes. Expression of *CSPG4* and *CD109* were 2.4- and 1.7-fold enriched in chondrocytes of damaged cartilage (expression in 56.1% and 60.3% of these chondrocytes, respectively), compared with synoviocytes. *VSIG4, MARCO, CD163, NRP1* and *PTPRS* were 20-, 13-, 16-, 2.0- and 1.8-fold enriched in synoviocytes compared with chondrocytes from damaged cartilage, respectively (Fig. 5A-B). Moreover, as reported in our previous study [12], these genes were significantly enriched in some OA synoviocyte clusters; for instance, *VSIG4, MARCO, CD163 and NRP1* were expressed by large proportions of immune regulatory macrophages (90.3%, 91.2%, 79.1% and 38.5%, respectively) and HLA-DRA^{+} cells (85.9%, 90.8%, 77.3% and 37.1%, respectively), while *PTPRS* was expressed by 46.4% of HLA-DRA^{+} activated pro-inflammatory fibroblasts and 47.4% of synovial intimal fibroblasts. The overall expression (Supplementary Fig. 5), and joint tissue localization patterns (Supplementary Fig. 6) were confirmed by UMAPs of these genes in 36,832 cells from OA joint tissues.

The top 10 genes co-expressed by *CSPG4*$^{+}$ and *CD109*$^{+}$ OA chondrocytes from damaged articular cartilage (Fig. 5C), included genes encoding proteins made up of pathogenic peptides$^{rOA\text{-}Pos}$ (EEF1A1, LUM, FN1) and other OA-associated proteins (CLU, COMP, DCN) that were detected in SF and plasma EVs from knee OA (Supplementary Table 2). Peptides corresponding to these 6 genes were significantly positively correlated with SF EV CSPG4 and CD109 peptides$^{SF>PL}$ (Supplementary Table 7), supporting our determination that CSPG4^{+} and CD109^{+} OA chondrocytes contribute to CSPG4^{+} and CD109^{+} SF EVs.

Similarly, the top 10 genes co-expressed by *VISG4*$^{+}$, *MARCO*$^{+}$, *CD163*$^{+}$, *NRP1*$^{+}$ and *PTPRS*$^{+}$ OA synoviocytes (Fig. 5D), included genes encoding proteins made up of pathogenic peptides$^{rOA\text{-}Pos}$ (EEF1A1, LUM, FN1, PRG4) and other OA-associated proteins (FTL, FTH1. S100A4, B2M, VIM, PLA2G2A, CLU) that were detected in SF and plasma EVs from knee OA (Supplementary Table 2). Peptides corresponding to several of these genes were significantly positively correlated with particular SF EV surface peptides$^{SF>PL}$ including: FTL, FTH1, S100A4 and B2M with VSIG4; FTL and VIM with MARCO; FTL, FTH1 and S100A4 with CD163; VIM, FTL, PLA2G2A, EEF1A1, LUM and FTH1 with NRP1; PLA2G2A, PRG4, CLU, VIM, LUM and FN1 with PTPRS (Supplementary

Table 7). Our data strongly support the conclusion that both chondrocytes and synoviocytes contribute to the pool of pathogenic SF EVs.

3.9. CSPG4$^+$ SF EVs were highly enriched in SF from knee OA participants undergoing TKR surgery

To validate the surface localization of our newly identified SF EV surface markers, we used a surface staining protocol with high-resolution flow cytometry to profile the SF EVs from knee OA participants undergoing TKR surgery. All tested markers (CSPG4, VSIG4, CD109, LRP1, BGN, CD163, MARCO, NRP1 and PTPRS) were detected on the surface of SF EVs of all sizes, with a wide range of frequencies. CSPG4$^+$ and VSIG4$^+$ EVs were the top two enriched EV subpopulations in SF from the TKR cohort (Fig. 6). Interestingly, we also observed SF EV subpopulations that carried multiple surface markers, most notably CSPG4$^+$CD109$^+$, BGN$^+$LRP1$^+$, and VSIG4$^+$MARCO$^+$ EV subpopulations (Fig. 6).

4. Discussion

Our overall strategy was to first cast a relatively wide net with a comprehensive non-targeted EV proteomic analysis in EVs from the matched SF and plasma (proteomic cohort); then, we identified the strongest and most reliable EV markers and biological pathways associated with rOA (KL, JSN and/or OST grades) with validation of these markers in OA joint tissues (scRNA-seq cohort) and SF (flow cytometry cohort). The use of three grading systems for rOA enabled us to evaluate the overall general severity of OA (KL), cartilage degradation (JSN) and bony anabolic response (OST). It is useful to evaluate features of both cartilage degradation and bone anabolism because these are highly dynamic, separate, but coupled processes [42], potentially requiring different therapeutic strategies. The results of this study firmly support EVs, predominantly SF EVs, as potential diagnostic biomarkers or therapeutic targets of OA. Their potential involvement in the pathogenesis of knee OA is based on the demonstrated pathogenic roles of proteins identified as EV peptides$^{rOA\text{-}Pos}$ that were positively associated with knee rOA severity, and more abundant in SF EVs than plasma EVs (SF EV peptides$^{SF>PL}$). We also identified an array of surface markers on SF EVs indicating their cell origins from multiple cell types, chief among them chondrocytes and synoviocytes, and confirmed their gene expression by OA joint tissues. This study adds to the growing body of knowledge that EVs and their cargo play a role in OA [10,13,14].

Among the 692 identified SF EV peptides$^{roA\text{-}Pos}$ in knee OA, 57.4% were from 46 proteins of the immune system, predominantly the innate immune system; this result strongly supported our previous study reporting a pro-inflammatory phenotype of OA SF EVs [10]. Impressively, 133 (19.2%) peptides$^{rOA\text{-}Pos}$ were from one protein–FN1, known to be a key pathogenic driver of OA pathology. FN1 is a component of normal synovial membranes, but FN1 and FN1 fragments are generated during cartilage degeneration and increased in OA SF [43,44]. FN1 fragments function as disease associated molecular patterns (DAMPs) that contribute to OA progression by accelerating production of matrix metalloproteinases (MMPs) and catabolic cytokines that mediate cartilage damage. MMPs (particularly MMPs −1, −3 and – 13) are upregulated in OA and can degrade FN1 to enhance the disease promoting loop [44,45]. FN1 binds to very late antigen-4 (VLA-4), composed of an α

subunit CD49D and a β1 subunit CD29 (also known as Fibronectin Receptor Subunit Beta) [46]. FN1-VLA-4 binding mediates interaction of immune cells with extracellular matrix and the endothelium, and contributes to the transendothelial migration of lymphocytes and the infiltration of lymphocytes into tissues [47]. Our group and others detected CD29 in EVs from humans and mice with various diseases and conditions [10,17,26,48]. We previously reported that the level of CD29 in plasma LEVs and MEVs were statistically significantly associated with knee rOA progression [6]; moreover, SF EVs of all sizes carried even higher levels of CD29 than the matched plasma EVs in knee OA participants [10]. Taken together, these data suggest that SF EVs carrying FN1 peptides and CD29 may contribute to FN1-VLA-4 mediated OA pathology.

In addition, we identified large quantities of SF EV peptides$^{rOA\text{-}Pos}$ originating from complement activation proteins (classical pathway: immunoglobulins; alternative pathway: C3; and Lectin pathway: FCN3 and MASP1) and complement system components (C2, C3, C4A, C4B, C5, C5, C7 and C8A). These results are consistent with previous studies demonstrating that the complement system is activated in OA joints and plays a central role in the pathogenesis of OA [32,49–51]. We also observed that a large proportion of the SF EV peptides$^{rOA\text{-}Pos}$ were from multiple coagulation factors, including components from the intrinsic pathway (F12 and F11), extrinsic pathway (TF), and the common pathway (F2, F13A, F13B, FGA, FGB and FGG) [52], supporting the involvement of the coagulation pathway in the pathogenesis of OA [33,34]. Our data suggest that SF EVs may contribute to the pathogenesis of OA by transporting and delivering immune- and/or proteolysis-activating peptides$^{rOA\text{-}Pos}$ to recipient cells.

Proteins comprised of EV peptides$^{SF>PL}$ in knee OA were highly OA-relevant including extracellular matrix proteoglycan ACAN (constituting ~10% of tissue dry weight in cartilage [55]), and other cartilage extracellular matrix proteins, such as CHAD, FMOD and COMP. We have reported that SF EVs carry proinflammatory cytokines, IL-1β, IL-6 and TNF-α [6], which can induce the release of ACAN, CHAD and FMOD that activate the classical complement pathway, and COMP that activates the alternative complement pathway [49]. SF EV peptides$^{SF>PL}$ from C5, the complement component shared by all complement activation pathways, can stimulate chondrocytes to produce MMPs, such as MMP-3 whose peptides we identified as enriched in SF EVs, and stimulate immune cells to produce proinflammatory cytokines to further promote degradation of cartilage extracellular matrix [32,49].

This study aimed to identify surface markers specific for or highly enriched on SF EVs for development of OA diagnostic biomarkers and therapeutic targets. We identified SF EV peptides$^{SF>PL}$ from 12 proteins with a predicted surface localization (CD109, CD163, CSPG4, LRP1, MARCO, NRP1, PTPRS, VSIG4, ANXA2, ANXA5, BGN and HSP90AB1). These surface proteins were highly associated with each other and co-expressed in OA-related tissues. Amounts of CSPG4, NRP1 and BGN SF EV peptides$^{SF>PL}$ were significantly positively correlated with around 5000 SF EV peptides, supporting their designation as key surface markers of SF EVs. Importantly, amounts of SF EV surface peptides$^{SF>PL}$ from BGN, NRP1, CD109, and predominantly CSPG4, were significantly positively correlated with the majority of SF EV peptides$^{rOA\text{-}Pos}$. Our data suggests that

these surface markers, especially CSPG4, are the major surface markers of pathogenic SF EVs. Thus, these surface markers might be utilized to identify, isolate, suppress, and deplete specific OA pathogenic EV subpopulations. In addition, these surface markers may mediate the transfer of EV cargo to recipient cells expressing the corresponding ligand [40].

Our data demonstrated that SF EVs are more proximal indicators of OA disease severity than plasma EVs. The pathogenic microenvironment of the OA joint may contribute to the prominent pathogenic phenotype of the SF EV proteome. Our scRNA-seq data from end-stage knee OA cartilages and synovium indicated joint tissue expression of genes encoding SF EV surface markers, including: *LRP1, ANXA2, ANXA5, BGN* and *HSP90AB1*, highly expressed by all types of joint tissue cells; *CSPG4* and *CD109*, relatively enriched in chondrocytes from damaged cartilage; and *VISG4, MARCO, CD163, NRP1* and *PTPRS*, relatively enriched in synoviocytes; among OA synoviocyte clusters, *VSIG4, MARCO, CD163 and NRP1* were predominantly expressed by immune cells, whereas *PTPRS* was highly expressed by fibroblasts [12]. The array of genes coexpressed by *CSPG4*$^+$ and *CD109*$^+$ chondrocytes and *VISG4*$^+$, *MARCO*$^+$, *CD163*$^+$, *NRP1*$^+$ and *PTPRS*$^+$ synoviocytes corresponded to proteins abundant in SF EVs further supporting these joint tissue cells as major origins of SF EVs in knee OA. Flow cytometry confirmed that SF from patients with end-stage knee OA was highly enriched in chondrocyte and synoviocyte-associated EVs, such as CSPG4$^+$ and VSIG4$^+$ SF EVs. We also detected SF EV subpopulations that carried multiple SF EV surface markers, including CSPG4$^+$CD109$^+$, BGN$^+$LRP1$^+$, and VSIG4$^+$MARCO$^+$ EV subpopulations of all sizes, further supporting their OA joint origins. Further evaluation of SF EV subpopulations carrying single or multiple pathogenic surface markers could enhance the understanding of the pathogenesis of OA and facilitate future diagnostic test development.

In summary, compared to plasma EVs, we identified SF EVs as more robust indicators for knee rOA severity, as evidenced by dramatically higher quantities of peptides$^{rOA\text{-}Pos}$ in SF EVs. A majority of the 692 SF EV peptides$^{rOA\text{-}Pos}$ were from the immune system, including FN1, the complement system and coagulation pathway that are known to play crucial roles in the pathogenesis of OA [31–34]. We identified CSPG4, CD109, and BGN as the key surface markers of SF EVs, and notably, CSPG4, CD109, BGN, and NRP1, as the major surface markers of pathogenic SF EVs related to rOA severity. *CSPG4* and *CD109* genes were highly expressed by chondrocytes from damaged cartilage, whereas *VISG4, MARCO, CD163*, and *NRP1* genes were relatively enriched in the synovial immune cells in end-stage knee OA joint tissues. These results suggest that both chondrocytes and synoviocytes contribute to the OA SF EV pool. Among all the EV subpopulations in end-stage knee OA SF identified by surface markers in this study, the CSPG4$^+$ subpopulation was the most abundant. These findings suggest that the surface markers identified on these SF EVs could be developed as diagnostic biomarkers and therapeutic targets for OA.

4.1. Study limitations

There were several limitations of this study. Our data suggest that the identified surface markers are highly associated with pathogenic EV peptides in OA; such results necessitate and facilitate future functional studies of specific EV subpopulations that could be isolated

based on their surface markers to explore the underlying mechanism of these EVs in the pathogenesis of OA. Unfortunately, there is still no “gold standard” method of EV isolation that fits all downstream applications. To optimize the use of precious sets of human samples, we selected polymer-based precipitation for isolating EVs from small volumes of biofluids. The major advantages of this method include its simplicity, high yield of EVs, and preservation of EV integrity [10,17,26]. However, a well-known limitation of this method is the potential co-precipitation of non-EV contaminants [53]. We detected <1% $APOA1^+$ particles in polymer-based precipitation isolated EVs [26]. In this study, we also detected peptides from APOA1 and APOB. However, since EVs can also carry lipid [36,37], and all of the currently available methods could not fully separate lipoprotein particles from EVs, it is still hard to determine if these particle associated APOA1 and APOB are of EV origin or are contaminants. Interestingly, a previous study reported a lack of association of APOA1 and high-density lipoprotein in OA SF [54], suggesting that APOA1 in OA SF, at least in part, may be carried by EVs rather than lipoproteins. Another limitation relates to testing a large number of peptides in a relatively small cohort. Nevertheless, using additional human cohorts and methodologies, we validated the presence of genes encoding our newly identified surface markers in the joint tissues and EV subpopulations carrying these surface markers in SF in patients with end-stage OA. Collection of synovial fluid is invasive, requires insertion of a sterile needle into the joint, has potential complications of bleeding and risk for infection, and can be obtained neat (our study) or by lavage. Therefore, continued research to identify comparable EV biomarkers in a ‘patient-friendly’ biofluid, such as plasma, with standardized acquisition and processing protocols, will be needed to advance translation of these findings to the clinic for more ready use as diagnostic tools.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. All proteomic data for this study are available at massive.ucsd.edu.

Abbreviations:

OA	osteoarthritis

SF	synovial fluid
EV	extracellular vesicles
rOA	radiographic osteoarthritis
peptides$^{rOA\text{-}Pos}$	peptides significantly positively correlated with rOA severity scores
peptides$^{rOA\text{-}Neg}$	peptides significantly negatively with rOA severity scores
K/L	Kellgren Lawrence
JSN	joint space narrowing
OST	osteophyte number and size
scRNA-seq	single-cell RNA sequencing
TKR	total knee replacement
SPC	Surface Protein Consensus
df-PBS	double filtered PBS
LEV	large EV
MEV	medium EV
SEV	small EV
FN1	fibronectin
peptides$^{SF>PL}$	peptides more abundant in SF EVs than plasma EVs
proteins$^{SF>PL}$	proteins corresponding to peptides$^{SF>PL}$

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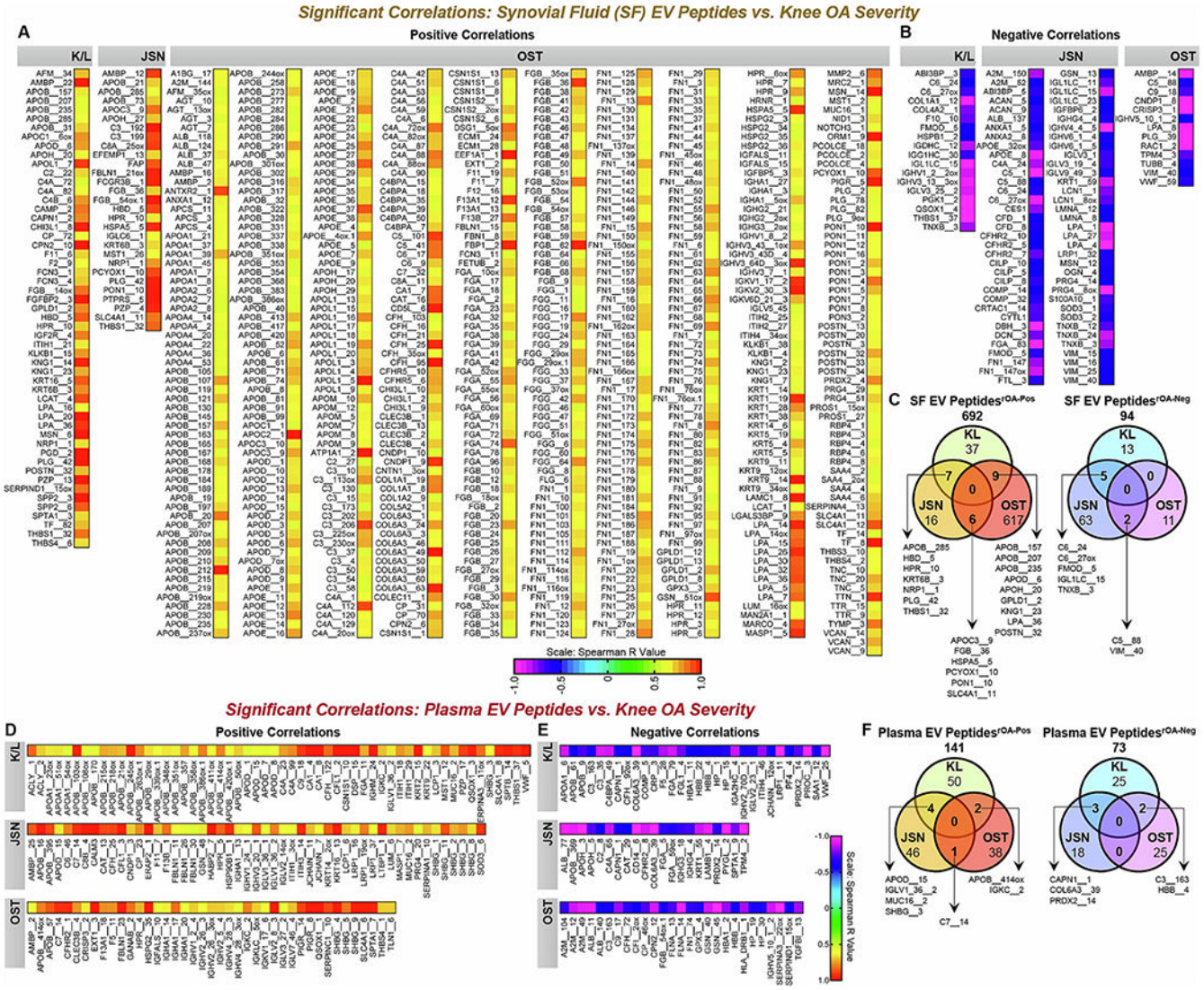


Fig. 1.

Many peptides from SF EVs of knee OA participants were significantly correlated with knee rOA severity. EVs were separated from matched SF and plasma samples from the knee OA 'proteomics cohort' ($n = 16$) and processed for proteomics by high-resolution mass spectrometry coupled with label-free quantification. Spearman correlations, adjusted for age, gender and BMI, were used to assess associations of SF EV peptide (A, B), and plasma EV peptide (D, E) expression, with knee K/L, JSN and OST scores that indicated rOA severity; significant correlations were defined by $p < 0.05$. Heat maps (A, B, D, E) depict the Spearman correlation coefficient r value for statistically significant correlations. Peptide information (gene name, protein name, peptide sequence, confidence of protein identification, accession # and protein position) are provided in Supplementary Table 2. Venn diagrams summarize the number of SF EV (C), and plasma EV (F) peptides that were significantly positively (named as peptides$^{rOA\text{-}Pos}$), and negatively (named as peptides$^{rOA\text{-}Neg}$) correlated with knee rOA severity scores (K/L, JSN and OST).

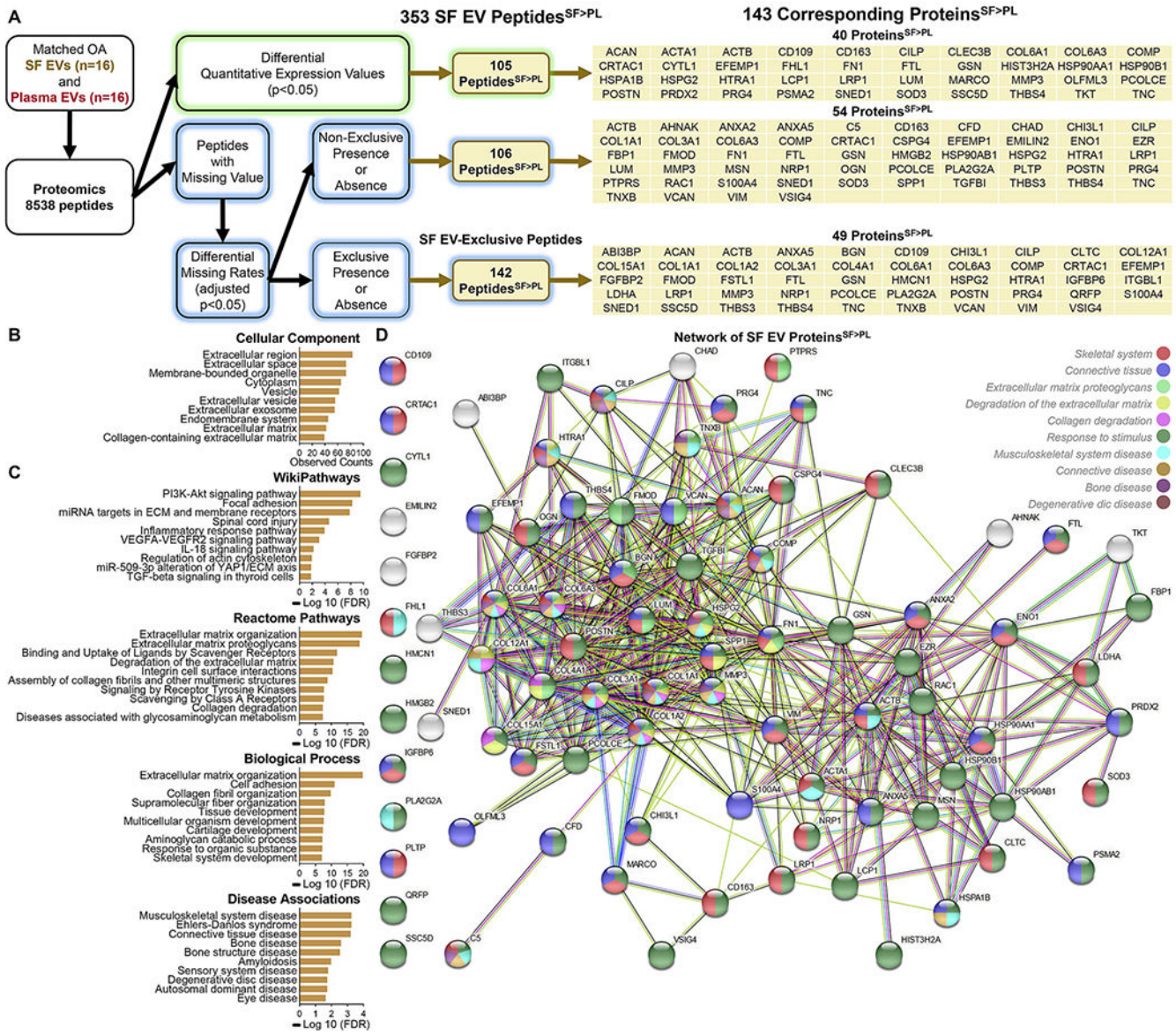
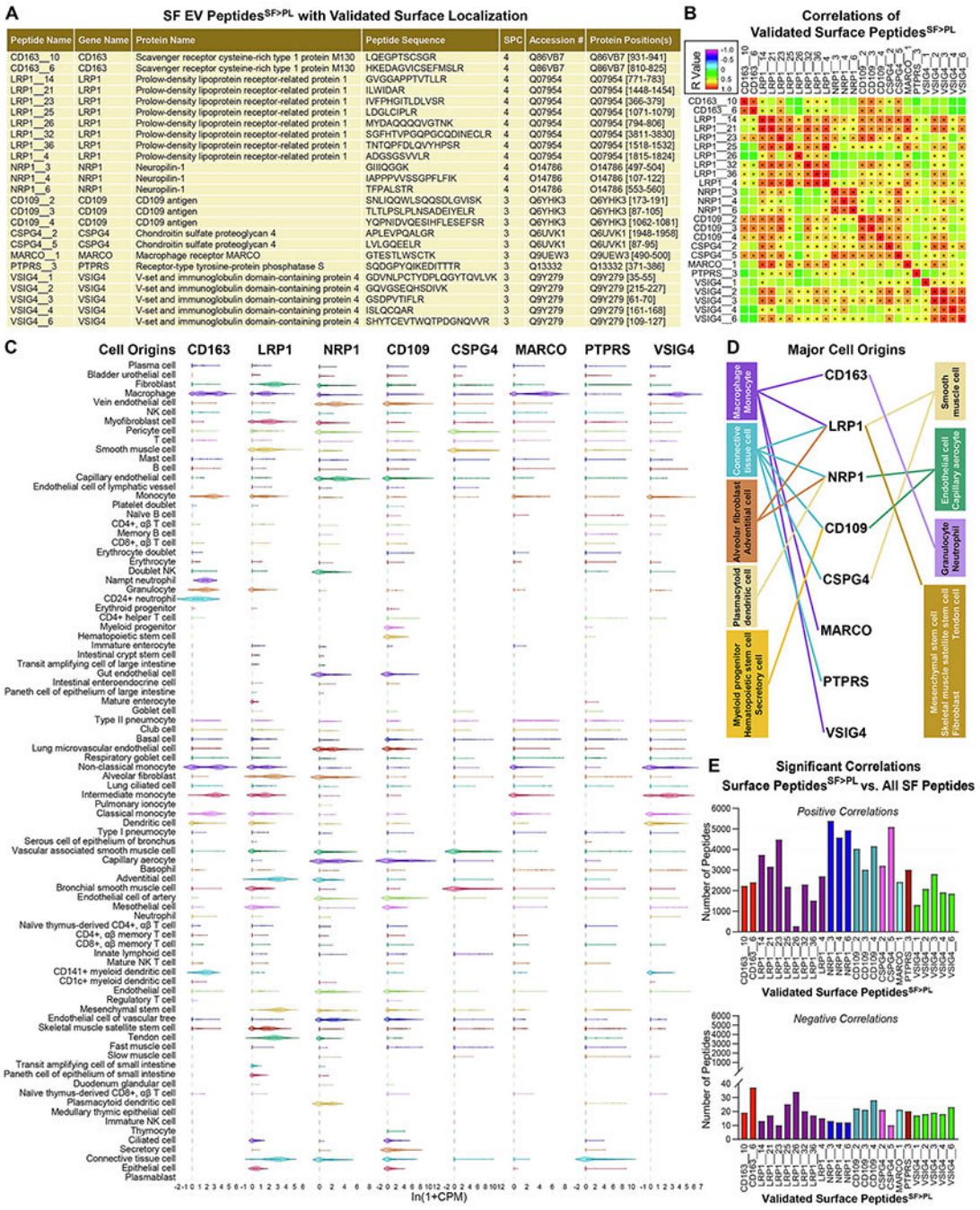


Fig. 2.

Distinct proteomic profiles of SF EVs compared with plasma EVs of knee OA participants. EVs from the matched SF and plasma of the knee OA ‘proteomics cohort’ (n = 16) were processed for proteomics. (A) Sample analysis plan and list of peptides and their corresponding proteins enriched in SF compared with plasma (termed peptides$^{SF>PL}$, proteins$^{SF>PL}$) based on differential missingness (none, some, or compete exclusivity in one group). Comparisons of relative quantitative values were performed using a paired *t*-test with FDR adjustment with the BH procedure; statistical significance was defined as $p < 0.05$. For peptides with some or all values missing in a group, comparisons of missing rates were performed using McNemar’s test with FDR adjustment with BH procedure; statistical significance was defined as adjusted $p < 0.05$. Peptide information is presented in Supplementary Tables 3–5. (B-D) STRING network analysis of the identified proteins$^{SF>PL}$: the bar graphs display the top 10 enriched cellular components based on the observed gene

counts (B), and the top 10 enriched Wikipathways, reactome pathways, biological process and disease associations based on FDR (C); the graph displays the full STRING network (D); the 469 edges indicate the functional and physical protein associations; processes indicated by each node colors of the network are provided in the legend (D).



Peptide Name	Gene Name	Protein Name	Peptide Sequence	SPC	Accession #	Protein Position(s)
CD163__10	CD163	Scavenger receptor cysteine-rich type 1 protein M130	LQEGPTSCSGR	4	Q86VB7	Q86VB7 [931-941]
CD163__6	CD163	Scavenger receptor cysteine-rich type 1 protein M130	HKEDAGVICSEFMSLR	4	Q86VB7	Q86VB7 [810-825]
LRP1__14	LRP1	Prolow-density lipoprotein receptor-related protein 1	GVGIGAPPTVTLLR	4	Q07954	Q07954 [771-783]
LRP1__21	LRP1	Prolow-density lipoprotein receptor-related protein 1	ILWIDAR	4	Q07954	Q07954 [1448-1454]
LRP1__23	LRP1	Prolow-density lipoprotein receptor-related protein 1	IVFPHGITLDLVSR	4	Q07954	Q07954 [366-379]
LRP1__25	LRP1	Prolow-density lipoprotein receptor-related protein 1	LDGLCIPLR	4	Q07954	Q07954 [1071-1079]
LRP1__26	LRP1	Prolow-density lipoprotein receptor-related protein 1	MYDAQQQQVGTNK	4	Q07954	Q07954 [794-806]
LRP1__32	LRP1	Prolow-density lipoprotein receptor-related protein 1	SGFHTVPGQPGCQDINECLR	4	Q07954	Q07954 [3811-3830]
LRP1__36	LRP1	Prolow-density lipoprotein receptor-related protein 1	TNTQPFDLQVYHPSR	4	Q07954	Q07954 [1518-1532]
LRP1__4	LRP1	Prolow-density lipoprotein receptor-related protein 1	ADGSGSVVLR	4	Q07954	Q07954 [1815-1824]
NRP1__3	NRP1	Neuropilin-1	GIIIQGGK	4	O14786	O14786 [497-504]
NRP1__4	NRP1	Neuropilin-1	IAPPPVVSSGPFLFIK	4	O14786	O14786 [107-122]
NRP1__6	NRP1	Neuropilin-1	TFPALSTR	4	O14786	O14786 [553-560]
CD109__2	CD109	CD109 antigen	SNLIQQWLSQQSDLGVISK	3	Q6YHK3	Q6YHK3 [173-191]
CD109__3	CD109	CD109 antigen	TLTLPSLPLNSADEIYELR	3	Q6YHK3	Q6YHK3 [87-105]
CD109__4	CD109	CD109 antigen	YQPNIDVQESIHFLESEFSR	3	Q6YHK3	Q6YHK3 [1062-1081]
CSPG4__2	CSPG4	Chondroitin sulfate proteoglycan 4	APLEVPQALGR	3	Q6UVK1	Q6UVK1 [1948-1958]
CSPG4__5	CSPG4	Chondroitin sulfate proteoglycan 4	LVLGQEELR	3	Q6UVK1	Q6UVK1 [87-95]
MARCO__1	MARCO	Macrophage receptor MARCO	GTESTLWSCTK	3	Q9UEW3	Q9UEW3 [490-500]
PTPRS__3	PTPRS	Receptor-type tyrosine-protein phosphatase S	SQDGPYQIKEDITTTR	3	Q13332	Q13332 [371-386]
VSIG4__1	VSIG4	V-set and immunoglobulin domain-containing protein 4	GDVNLPCTYDPLQGYTQVLVK	3	Q9Y279	Q9Y279 [35-55]
VSIG4__2	VSIG4	V-set and immunoglobulin domain-containing protein 4	GQVGSEQHSDIVK	3	Q9Y279	Q9Y279 [215-227]
VSIG4__3	VSIG4	V-set and immunoglobulin domain-containing protein 4	GSDPVTIFLR	3	Q9Y279	Q9Y279 [61-70]
VSIG4__4	VSIG4	V-set and immunoglobulin domain-containing protein 4	ISLQCQAR	3	Q9Y279	Q9Y279 [161-168]
VSIG4__6	VSIG4	V-set and immunoglobulin domain-containing protein 4	SHYTCEVTWQTPDGNQVVR	3	Q9Y279	Q9Y279 [109-127]

Fig. 3.

Surface peptides$^{SF>PL}$ indicate the putative cell origins of SF EVs in OA. (A) The table displays the SF EV peptides$^{SF>PL}$ with validated surface localization that were defined as SPC ≥ 2 (predicted to be localized to the cell surface in multiple predictive datasets) with confirmed membrane localization by UniProt, STRING and the Human Protein Atlas. (B) Heat map of correlations (Spearman r) of validated surface peptides on peptides$^{SF>PL}$ demonstrating correlation of peptides within a protein and across different proteins; significant results defined by *p < 0.05. (C) Tabula Sapiens were utilized to identify the

cell sources of these validated SF EV surface proteins$^{SF>PL}$ and generated the violin plots. (D) The graph summarizes the co-expression of these validated SF EV surface markers. (E) Spearman analyses were performed to assess correlations between the validated surface peptides$^{SF>PL}$ and other peptides from SF EVs; significant results were defined by $p < 0.05$; the bar graphs display the number of peptides that were significantly positively or negatively correlated with the individual surface peptides$^{SF>PL}$. The lists of significant correlations are presented in Supplementary Table 7.

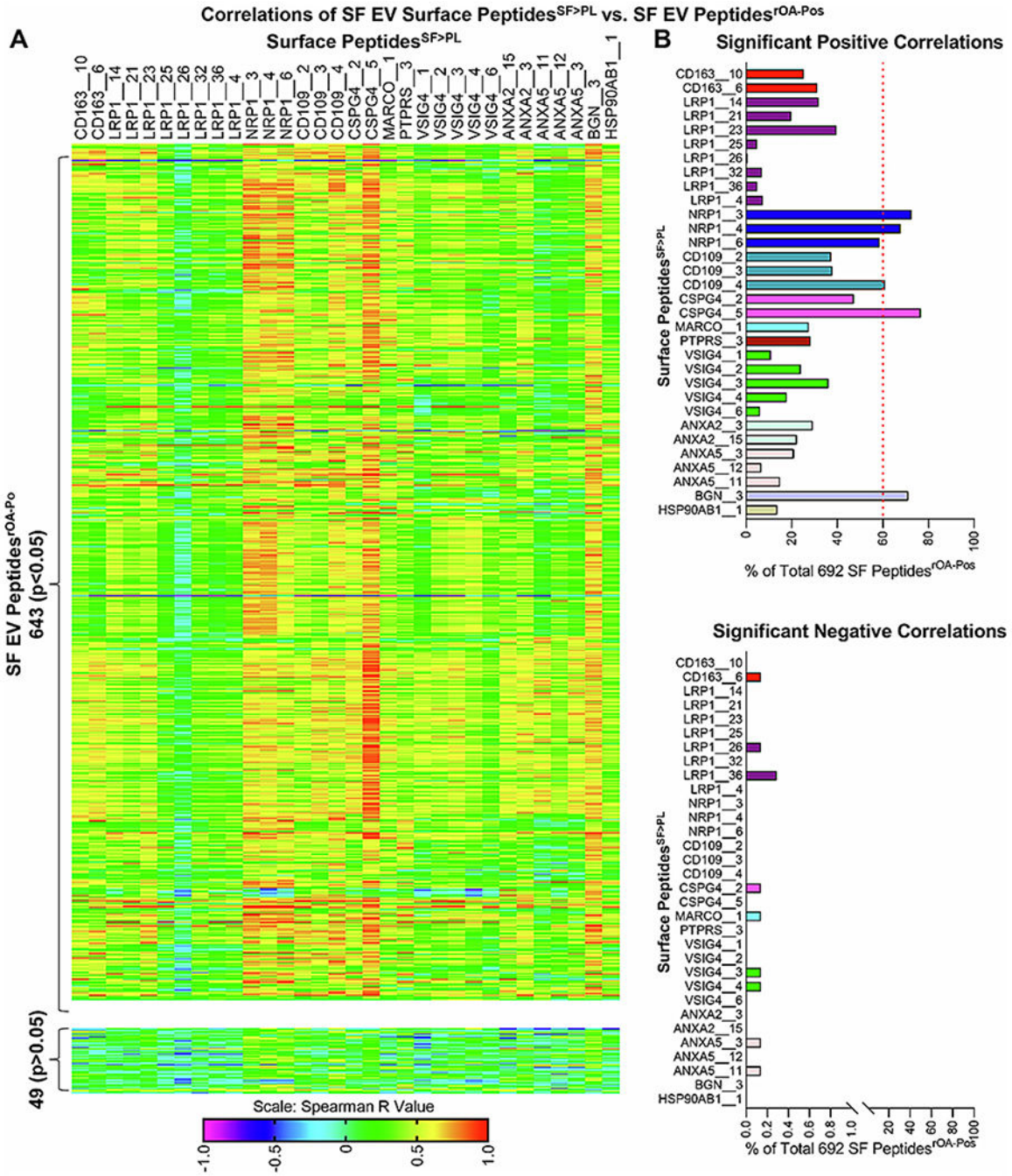


Fig. 4.

CSPG4, CD109, BGN and NRP1 are the major surface markers of pathogenetic SF EVs in knee OA. Spearman analyses were used to assess correlations between the SF EV surface peptides$^{SF>PL}$ and the 692 SF EV peptides$^{rOA-Pos}$. (A) Heat maps depict the Spearman correlation coefficient r values; significant results were defined by $p < 0.05$. The majority (643 of 692, 92.9%) of pathogenic SF EV peptides$^{ROA-Pos}$ were significantly correlated with at least one of the surface peptides$^{SF>PL}$, whereas the rest (49 of 692, 7.1%) of SF EV peptides$^{rOA-Pos}$ were not significantly correlated with any surface peptides$^{SF>PL}$. (B) The

bar graphs display the percentages of the 692 SF EV peptides$^{rOA\text{-}Pos}$ that were significantly ($p < 0.05$) positively or negatively correlated with each of the SF EV surface peptide$^{SF>PL}$.

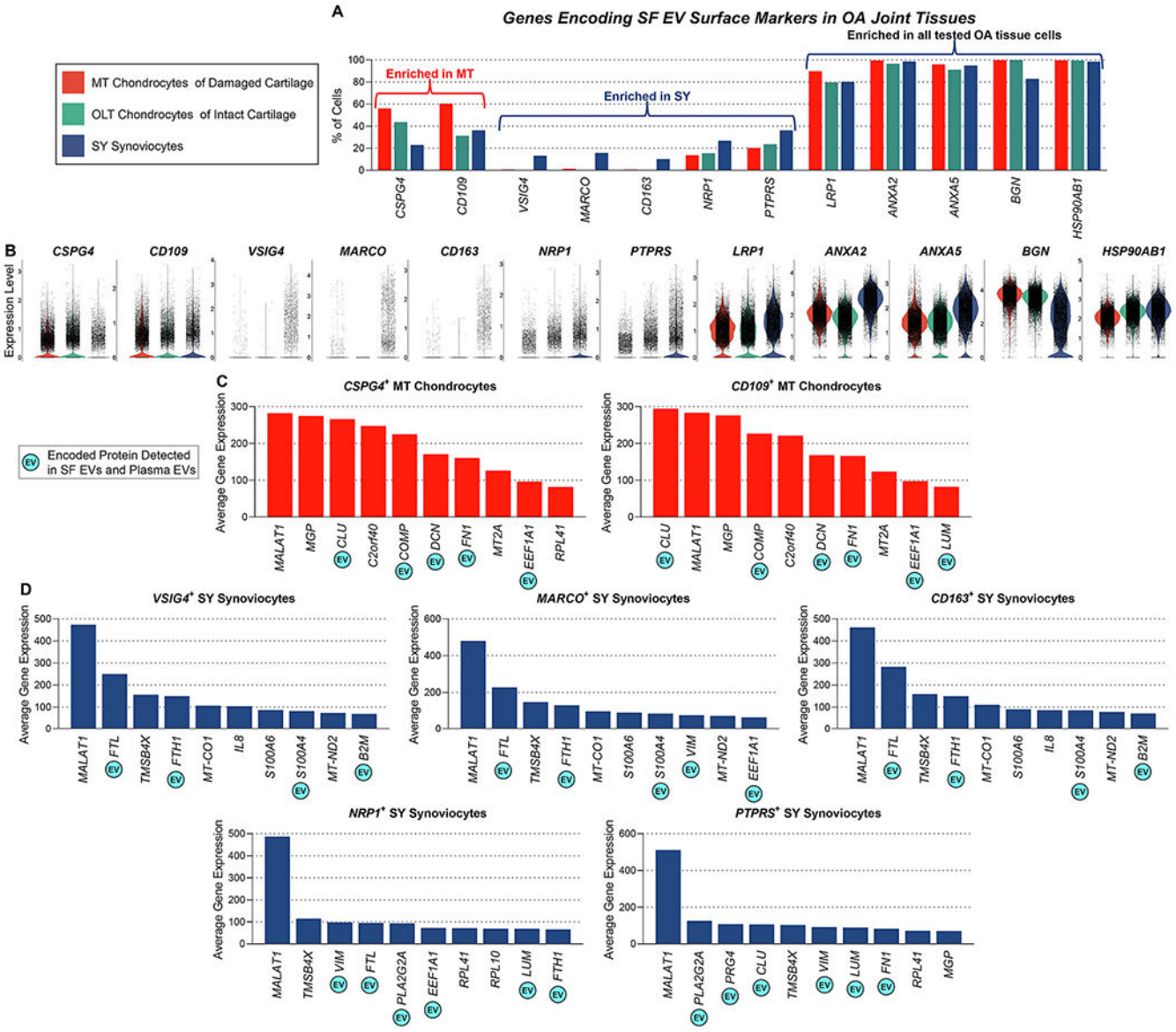


Fig. 5.

Gene expression of SF EV surface markers in knee OA joint tissues. Single cell RNA-seq gene expression data were generated from joint tissues of participants ($n = 3$) with knee OA undergoing joint replacement surgery, including 11,579 chondrocytes from damaged sites of the medial tibial (lesioned) cartilage (MT), 14,613 chondrocytes from intact sites of the outer lateral tibial (non-lesioned) cartilage (OLT), and 10,640 synoviocytes from the matched synovium (SY) as previously described [12]. (A) The bar graph displays the % of cells expressing genes encoding the surface markers in cells derived from human OA joint specimens of the medial tibia (MT, lesioned articular cartilage), outer lateral tibia (OLT, non-lesioned articular cartilage), and synovium (SY). (B) The violin (density) plots display the frequency of cells expressing each gene in the three types of OA joint tissue cells. (C) The bar graphs display the mean gene expression of the top 10 expressed genes in *CSPG4*$^+$ and *CD109*$^+$ MT chondrocytes. (D) The bar graphs display the mean gene expression of the

top 10 expressed genes in *VISG4*$^{+}$, *MARCO*$^{+}$, *CD163*$^{+}$, *NRP1*$^{+}$ and *PTPRS*$^{+}$ synoviocytes. Peptides corresponding to the genes labeled with the “EV” symbol indicate those detected in SF and plasma EVs (Supplementary Table 7), while the peptides corresponding to the genes without the “EV” label were not detected in SF EVs or plasma EVs from knee OA participants (Supplementary Table 2).

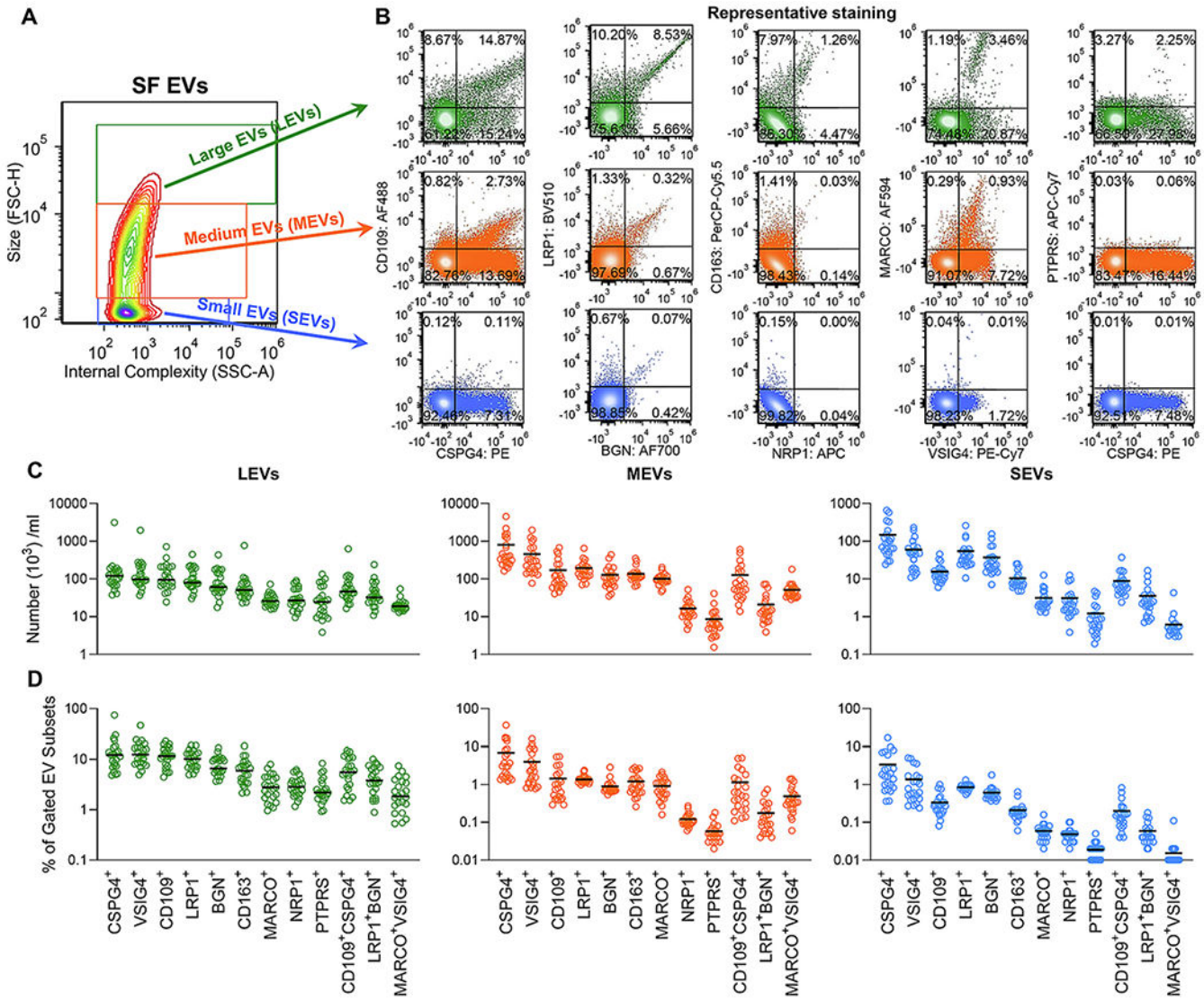


Fig. 6.

Validation of presence and surface localization of validated and putative SF EV surface markers in SF of knee OA participants undergoing TKR surgery. EVs were separated from SF samples from knee OA participants undergoing TKR surgery (n = 23) and profiled for the identified SF EV surface markers by high-resolution flow cytometry. (A) The representative contour plot presents size (FSC-H: Forward Scatter-Height) and internal complexity/granularity (SSC-A: Side Scatter-Area) of LEVs, MEVs and SEVs. (B) The representative color dot plots display all tested surface markers in gated LEVs, MEVs and SEVs from OA SF. (C, D) The scatter plots with mean (black line) present the number (C) and percentages (D) of LEVs, MEVs and SEVs carrying the indicated surface markers.