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Potential Role of Menstrual Fluid-Derived Small Extracellular Vesicle Proteins in Endometriosis Pathogenesiss

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ABSTRACT

Endometriosis, a chronic debilitating disease affects 1 in 7–10 girls and women, who have symptoms of severe chronic pain and subfertility and significantly impacts the overall quality of life. Currently, no effective early diagnostic methods are available for early stages of endometriosis. We used menstrual fluid-derived small extracellular vesicles (MF-sEVs) from women with self-reported endometriosis (laparoscopically diagnosed, $n = 8$) and self-reported without endometriosis and no painful periods ($n = 9$). MF-sEVs were separated using differential ultracentrifugation and characterised using nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), Western Blot, flow cytometry, mass-proteomics analysis and functional assays. Spherical-shaped sEVs were identified with a median diameter of ~120 nm, expressing sEV marker proteins. The MF-sEV proteins were classified as endometrial origin. Over 5000 proteins were identified, ~77% of which were decreased whilst only 22 proteins (largely comprising immunoglobulins) were increased in endometriosis/MF-sEVs compared to control/MF-sEVs. Decreased proteins were involved in nitrogen compound metabolism, immune response, intracellular signal transduction, regulation of programmed cell death, maintenance of cell polarity and actin cytoskeleton organisation. Flow cytometry demonstrated a significant increase in CD86 expression (immune activation marker) in endometriosis/MF-sEVs. Mesothelial cells showed a significant decrease in cellular resistance and junctional protein expression. MF-sEVs are possible contributors to the pathogenesis of endometriosis and may have the potential for early detection of the disease.

1 | Introduction

Endometriosis is the growth of endometrial-like tissue outside the uterus mainly within the peritoneal cavity, affecting ovaries, bowel and bladder (Young et al. 2013), however, rare distal lesions in the lungs, liver, heart and eyes have also been reported (Burney and Giudice 2012, Ceccaroni et al. 2010). It affects up to 10%–14% of reproductive-age women (Giudice and Kao 2004, Kuan et al. 2021, Bulletti et al. 2010, Horne and Missmer 2022). Endometriosis can be a debilitating disease where girls and women suffer severe pelvic pain and it is one of the main causes of infertility (Giudice and Kao 2004, Kuan et al. 2021, Bulletti et al. 2010), severely affecting their quality of life; social activities, education, relationships, career and productivity

(As-Sanie et al. 2019, Soliman et al. 2018, Rowlands et al. 2022).

Sampson's theory posits that retrograde menstruation, where menstrual effluent containing viable endometrial cells/tissue fragments refluxes through patent Fallopian tubes into the peritoneal cavity and establishes lesions on peritoneal surfaces (Sampson 1927). This theory is supported by the presence of a higher volume of refluxed menstrual fluid (MF), endometrial stem/progenitor cells and tissue fragments, and a higher frequency of uterine contractile waves in women with endometriosis than in healthy controls (Halme et al. 1984, Masuda et al. 2021, Salamanca and Beltrán 1995). New research in endometriosis has identified intrinsic endometrial abnormalities (Brosens et al. 2012, Vallvé-Juanico et al. 2019) and the resulting alterations in the endometrial secretome (Llarena et al. 2020) on the pathogenesis of the disease, nevertheless, its aetiology remains elusive (Horne et al. 2017, Irungu et al. 2019). Consequently, there is nearly a decade-long delay in diagnosis, which involves invasive procedures, suboptimal amelioration of pain and a high recurrence rate following surgical excision treatment (Kuan et al. 2021, Singh et al. 2020, Nirgianakis et al. 2020, Guo 2009, Jensen and Coddington 2010).

Cells secrete nano-sized extracellular vesicles (EVs) into the extracellular milieu for intercellular communication (Kim et al. 2015). EVs have a lipid bilayer that protects the cargo: proteins, mRNA, miRNA and metabolites, which provide information on donor cell status. Indeed, cancer cell-derived small EV (sEV) cargoes are donor cell-specific and selectively influence specific recipient cells by upregulating promigratory and proinflammatory signalling, rendering the environment permissive to tumour cell colonisation and metastasis establishment (Urabe et al. 2021, Kogure et al. 2020, Simpson 2017, An et al. 2021). Endometriosis is associated with lesions at local and distal sites, similar to tumour metastases (Young et al. 2013, Gebel et al. 1998, Gilabert-Estellés et al. 2007). We hypothesised that MF-sEV protein cargoes will be different between women with endometriosis compared to controls and that this differential MF-sEV-protein composition in endometriosis will contribute to functional changes in mesothelial cells that may facilitate endometriotic lesion establishment.

2 | Materials and Methods

Our research nurses recruit donors through poster advertisements. They consented to all donor participants, collected samples and the donors were de-identified to prevent bias in donor selection. Menstrual blood was collected from participants (Table 1) recruited from the general community and women undergoing surgery at Monash Health hospitals following informed written consent under the human ethics approvals from Monash Health Human Research Ethics Committee (09394B and RES-20-000-159A). Participants' information was collected at the time of menstrual blood collection that included: menstrual cycle characteristics (average length and duration), bleeding history (period pain and pattern/duration), endometriosis diagnosis (including stage if known), laparoscopic surgery, age, height, fertility status (unknown, normal or infertile, or other) and current medication including hormones and others. Women were divided into two groups; women who self-reported with a previous confirmed laparoscopic diagnosis of endometriosis (endometriosis group, endo) and women who self-reported with no endometriosis, painful periods and did not require laparoscopic investigations (control group without painful periods, ctrl).

2.1 | Menstrual Blood Collection and sEV Isolation

Our experienced research nurses trained the participants to use the menstrual cup. The participants were requested to collect menstrual blood using a menstrual cup for 4–6 h on the second day of menses in the morning (ctrl, $n = 9$ and endo, $n = 8$, Table 1). The menstrual blood was transferred into a 50 mL tube (#339652, ThermoFisher Scientific) and kept in flexible Ice blankets (#IB-80-12×13-1, Icepack Australia) within a lidded jar (#JARW750, The Plastic Man) throughout transport to the laboratory and immediately processed by diluting with ultrafiltered pure PBS (#AM9624, ThermoFisher Scientific) and separating supernatant (MFS) from the mucus, cells and endometrial tissue fragments by centrifugation at 2000 *g* (Eppendorf, 5810R) for 10 min at 4°C. MF supernatant was stored at −80°C for future use. Small extracellular vesicles (sEVs) were isolated by differential ultracentrifugation using our previous methods (Gurung et al. 2020, Gurung et al. 2021). Briefly, the MFS was centrifuged in polypropylene tubes (#337986, Beckman Coulter) at 10,000 *g* (Hitachi Koki VWR V100X, P40ST rotor) for 30 min at 4°C to remove microvesicles and sEVs were pelleted at 100,000 *g* for 70 min at 4°C followed by a wash with PBS twice. Isolated sEVs were resuspended in 100 µL cold PBS, aliquoted into 20 µL volume and stored at −80°C until further use. The analyses on MF-sEVs were performed on independent participant samples.

2.2 | MF-sEV Characterisation

2.2.1 | Western Blotting

The total protein concentrations of the MF-sEVs were quantified using a Micro BCA protein assay kit according to the manufacturer's instructions (#23235, ThermoFisher Scientific) and as utilised before (Gurung et al. 2021, Rai et al. 2021). This assay measures linear absorbance at 562 nm with increasing protein concentrations. 10 µg MF-sEVs were lysed in sodium dodecyl sulphate lysis buffer for 10 min and incubated at 95°C for 5 min. Proteins were electrophoretically separated in a 4%–20% precast gel (#4568094, Bio-Rad) at 110 V for an hour and transferred onto a polyvinylidene fluoride membrane (#1704156, Bio-Rad). The membrane was blocked with 5% milk for an hour at room temperature (RT), then incubated overnight with primary antibodies at 4°C followed by secondary antibodies at RT for an hour with 5 min of washes thrice in between each step (Table 2). The protein bands were developed using Clarity Western ECL Substrate (#1705061, Bio-Rad), visualised using ChemiDoc XRS+system (Bio-Rad) and quantified using Image Lab software (v5.2.2, Bio-Rad). The values of the three conditions were divided by the value of the media alone, setting the media alone to 1 and the MF-sEV treated groups at higher or lower

TABLE 1 | Characteristics of participants.

Donor ID	Age (yrs)	Menses (d)	Length (d)	MF (mL)	Pain, bleeding pattern	BMI	Fertility	Medication	Disease stage
Ctrl-P1	35	5	28	15	<3	24.2	Fertile	None	–
Ctrl-P2	39	5	25	22	None, normal	24.2	Fertile	None	–
Ctrl-P3	22	4	28	12	None, normal	22.9	UnK	None	–
Ctrl-P4	29	5	34	11	None, normal	22.1	Fertile	None	–
Ctrl-P5	23	7	28	15	None, normal	22.3	UnK	None	–
Ctrl-P6	54	4	28	25	None, normal	30	Fertile	None	–
Ctrl-P7	27	5	28	7	None, normal	20	UnK	None	–
Ctrl-P8	22	2	29	2	None, normal	20.9	UnK	None	–
Ctrl-P9	21	5	28	12	None, normal	NA	UnK	None	–
Mean	**30.2**	**4.7**	**28.4**	**13.4**		**23.3**			
Endo-P10	37	3	28	2	5, normal	24.4	Infertile	Prednisone, Panadol, Ibuprofen, Omeprazole	Stage 3 endometriosis & adenomyosis
Endo-P11	37	5	29	7	7/very heavy bleeding	NA	IVF	Progestin-only pill,	Endometriosis, partial removal
Endo-P12	34	8	29	7	9/very heavy bleeding	25.6	UnK	Oral contraceptive pills, Ibuprofen, diazepam	Endometriosis, partial removal
Endo-P13	40	12	NP	25	9/very heavy bleeding	28	UnK	Tramadol, Naproxen	Stage 3 endometriosis & adenomyosis
Endo-P14	34	7	29	5	8/very heavy bleeding	25.4	Miscarriage	None	Stage 2 endometriosis & adenomyosis
Endo-P15	22	6	27	7	9/very heavy bleeding	20.6	UnK	Omeprazole	Endometriosis
Endo-P16	25	5	28	6	4/very heavy bleeding	21.9	UnK	Estelle, Sertraline, Multivitamin	Stage 2 endometriosis
Endo-P17	28	7	27	12	8/very heavy bleeding	20.6	UnK	Escitalopram and Copper IUD	Stage 4 endometriosis
Mean	**32.1**	**6.6**	**28.1**	**8.9**		**23.8**			

Abbreviations: BMI, body mass index; Ctrl, control group without period pain; Endo, diagnosed symptomatic endometriosis; MF, menstrual fluid; NA, not available; Unk, unknown.

TABLE 2 | Antibodies used.

Antibody	Catalogue number/company	Dilution factor	Protein size
Primary antibodies			
Mouse anti-human ALIX (mAb)	# 2171S/ Cell Signaling Technology	1:1000	95 KDa
Rabbit anti-human Syntenin-1 (mAb)	#ab133267/ Abcam	1:1000	32 KDa
Rabbit anti-human β-actin HRP (mAb)	# 12620S/ Cell Signaling Technology	1:1000	45 KDa
Rabbit anti-human Scribble (pAb)	# PA554821/ThermoFisher Sci	1:1000	220 KDa
Rabbit anti-human ZO-1 (pAb)	# 402200/ThermoFisher Sci	1:1000	225 KDa
Rabbit anti-human IgM Recombinant mAb)	#MA5-32549/ThermoFisher Sci	1:1000	75 KDa
Secondary antibodies			
Goat anti-mouse-HRP	#626560/ ThermoFisher Sci	1:2000	–
Goat anti-rabbit-HRP	#626120/ ThermoFisher Sci	1:2000	–

Abbreviations: IF, Immunofluorescence; mAb, monoclonal antibody; pAb, polyclonal antibody; WB, Western Blot.

than the media alone. The new values were multiplied by 100% to present the data in percentage.

2.2.2 | Nanoparticle Tracking Analysis (NTA) and Transmission Electron Microscopy (TEM)

All samples were diluted in 1 mL of ultrafiltered pure PBS (#AM9624, ThermoFisher Sci) to identify the ideal particle concentration per frame value (20–100 particles per frame). The following parameters were set using ZetaView PMX-110 and videos were captured: temperature at 25°C, laser wavelength at 405 nm, filter wavelength: scatter, conductivity: 15,000.00 µS/cm, camera 0.713 mum/px and measurement at 11 positions. Particle Metrix Zetaview Software version 8.05.12 SP1 was used to analyse the diameter, size distribution and particle quantification of MF-sEVs in the captured videos. One millilitre of ultrafiltered pure PBS was loaded into a flow-cell top plate for background before assessing the true MF-sEVs particles. The particle concentration was normalised per microgram (assessed using BCA assay) of MF-sEVs.

The morphology of MF-sEVs was visualised through negative staining and captured using TEM. Carbon-coated copper EM grid (#TEM-FCF300CU50, Sigma-Aldrich) was glow-discharged for a minute (PELCO easiGlow), incubated with MF-sEV droplets for 3 min followed by 1% aqueous uranyl acetate for 30 s at RT then air-dried. The copper grids containing negatively stained MF-sEVs were loaded into a specimen quartet holder and images were taken using JOEL JEM-1400PLUS TEM with an accelerated voltage of 80 kV and a spot size of 2.

2.2.3 | Mesothelial Cell Permeability and Cell Polarity

The MCs (4×10^5) were cultured in a 0.4 µm pore size transwell insert (#3526, Costar) until confluency. 30 µg/mL MF-sEVs ($n = 3$ per group) were added and Trans-Epithelial Electrical Resistance (TEER) was determined by measuring the ohmic resistance (#24072, WPI) every 24 h for the next 48 h. FITC-dextran (40 kDa, 100 µL, #D1845, Life Technologies) was added into the inserts and incubated for 30 min at 37°C in the dark. The FITC-dextran leakage into the lower compartment was measured at 488 nm. MCs in the inserts were lysed and protein extracted using RIPA Lysis and Extraction Buffer (#89900, ThermoFisher Sci) and Halt Protease Inhibitor Cocktail (#87786, ThermoFisher Sci) for 10 min at 4°C and polarity proteins were determined using Western blotting (Table 2). The data were normalised in a similar manner to protein expression under the section "Western Blot."

2.3 | MF-sEV Surface Markers by Flow Cytometry

MF-sEV surface epitope expression was analysed using MACSPlex Exosome Kit (#130122209, Miltenyi Biotec). Twenty microgram of endometriosis or control MF-sEVs were diluted in MACSPlex buffer (MPB) and incubated with MACSPlex Exosome capture beads overnight on a rocker at RT protected from light followed by labelling with detection reagents (APC- anti-CD9, anti-CD63 and anti-CD81) for an hour at RT in the dark. Each step was followed by a wash with 500 µL of wash buffer and centrifugation at 3000 *g* for 5 min. Signals from MPB alone and buffer with positive multiplex beads were plotted using forward and side scatter area (Figure 4A) to set the negative and positive gating respectively followed by visualisation of bead subsets at red (PE,561 nm and APC/640 nm,) and blue (FITC/488 nm) lasers (BD LSR Fortessa flow cytometer) and analysed with FLowJo software (V10.8.1, FlowJo, BD). The APC-median fluorescence intensity (MFI) of the 39 capture bead subsets was quantified and the background was corrected by subtracting respective MFI values from matched buffer technical controls treated exactly as for MF-sEVs samples (buffer+ capture beads+ antibodies) followed by the values of the isotype controls. The MFI for each EV marker was then normalised to mean MFI for the specific EV markers (APC-CD9, -CD63 and -CD81) to obtain normalised MFI (nMFI). GraphPad Prism 9 was used to analyse the data.

2.4 | Tandem Mass Tag (TMT) Proteomic and Data Analysis

MF-sEVs from six control and four endometriosis participants were subjected to TMT proteomic analysis. Samples were lysed in 1% (w/v) Sodium deoxycholate (SDC), 100 mM HEPES pH 8.1 buffer (#D6750-100G, Sigma), and boiled at 95°C for 10 min. Samples were ultra-probe sonicated with 3 × 30-s intervals and cooled between rounds. Protein concentration was measured using a BCA kit according to the kit's manual (#2322, Pierce). Samples were reduced and alkylated with 10 mM TCEP (#77720, Thermo) and 40 mM chloroacetamide (#C0267-100G, Sigma) with incubation at 55°C for 15 min. Trypsin (Promega, #V528X) was added at a 1:50 wt:wt ratio alongside Lys-C at a 1:25 wt:wt ratio (#VA1170, Promega), and samples were incubated at 37°C overnight. Digestion was halted, and SDC was precipitated by the addition of trifluoroacetic acid (TFA) to a final concentration of 1%. Samples were centrifuged at 21,000 *g* for 10 min to ensure SDC precipitation, and the peptide containing supernatant was purified using Stage-tips packed with SDB-RPS (Empore) with a binding capacity of 10 μg for normalisation (Humphrey et al. 2018).

Each sample was labelled with the TMTpro 16plex reagent set (Lot: VC294906, Thermo Scientific) according to the manufacturer's instructions and utilised a singular reference channel containing all samples pooled. Individual labelled samples were then pooled into plexes and high-pH RP-HPLC was used to generate 12 fractions, which have been acquired individually by LC-MS/MS to maximise identifications. The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD042101.

The LC-MS/MS data were acquired using a Dionex UltiMate 3000 RSLCnano for peptide separation and analysed with an Orbitrap Eclipse Tribrid mass spectrometer (Thermo Scientific) with an Acclaim PepMap RSLC analytical column (75 μm × 50 cm, nanoViper, C18, 2 μm, 100Å; Thermo Scientific) and an Acclaim PepMap 100 trap column (100 μm × 2 cm, nanoViper, C18, 5 μm, 100Å; Thermo Scientific) using a 120-min linear gradient with increasing concentrations of 80% acetonitrile (ACN)/0.1% formic acid at a flow rate of 250 nL with three FAIMS compensation voltages (CV) (−40, −55 and −70) with duty cycle times set to 1.3, 1.0 and 0.7 s respectively. The mass spectrometer operated in Data-Dependent Acquisition (DDA) mode with precursors measured in the Orbitrap at a resolution of 120,000 across a scan range of 350–1600 m/z using an RF lens of 40%, an AGC (automatic-gain-control) target of 250%, with injection time set to auto. The mass spectrometer operated in synchronous precursor selection (SPS) whereby fragmentation spectra are generated synchronously alongside tag-based reporter ions at the ms3 level with 10 per duty cycle per CV. Briefly, precursors identified as peptides were isolated using the quadrupole at an isolation width of 0.7 m/z, followed by fragmentation using collision-induced dissociation with a fixed energy of 30%; the fragments were then measured by the ion trap at a turbo scan rate and a maximum injection of time of 35 ms. The real-time search was enabled at a 1% FDR at an Xcorr of 1.2 on doubly charged peptides against the Human SwissProt Proteome (accessed 24th of October 2021) with one missed cleavage, fixed modifications of TMTpro Lysine and N-termini, carbamidomethylation on cysteine and variable oxidation on methionine. Reporter tags were isolated with an isolation window of 0.7 m/z (ms1) and an ms2 isolation of 2 m/z; fragmentation was performed utilising higher collision-induced dissociation using 55% energy and measured in the orbitrap at a resolution of 50,000, an AGC target for 200% and a maximum injection time of 86 ms.

The raw data files were analysed with sequest in Proteome Discoverer version 2.5 (Thermo Scientific) to obtain quantitative ms3 reporter ion intensities, utilising the human SwissProt proteome (Accessed June 2020), which were further analysed using in-house generated R packages. Briefly, before normalisation, proteomic data was filtered for high-confidence protein observations. In addition, contaminants, proteins that have been only identified by a single peptide and proteins not identified/quantified consistently across the experiment were removed. The protein intensity data were converted to a log2 scale, samples grouped by disease conditions and missing values imputed using the "Missing not at Random" (MNAR) assuming the missingness was due to low protein abundance, which was then normalised using the variance-stabilising-normalisation (VSN) method. Both imputations and VSN were conducted by the DEP package (Zhang et al. 2018). Protein-wise linear models combined with empirical Bayes statistics were used for the differential expression analyses. The limma package38 from R Bioconductor was used to generate a list of differentially expressed proteins between endometriosis and control MF-sEVs. A cut-off adjusted *p* value of 0.05 (Benjamini-Hochberg method) along with a fold change of two was applied to determine significantly regulated proteins between the groups. Pathway enrichment map analysis was performed using Gene Ontology (GO) enrichment analysis tool (Ashburner et al. 2000, Carbon et al. 2021, Mi et al. 2019).

2.5 | Statistical Analyses

For statistical analyses (1) a comparison between two groups: an unpaired *t*-test with Welch's correction (not assuming equal standard deviations between groups) was used, (2) a comparison between more than two groups: One-Way ANOVA with the Holm-Sidak multiple comparisons test was used and (3) a comparison between more than two groups over time: Two-Way ANOVA with Sidak multiple comparisons test was used. Significance between groups was considered with the adjusted *p* value cut-off of <0.05.

3 | Results

3.1 | Donor Characteristics

Women with endometriosis had self-reported confirmed surgical endometriosis diagnoses with persistent period pain despite surgeries disease stages ranging from stage 2 to 4 and some associated adenomyosis (endometriosis group, endo). Women without endometriosis were also self-reported, asymptomatic for painful periods and never investigated for endometriosis (control group without painful periods, ctrl). The characteristics of the participants are shown in Table 1. The average age of the control and endometriosis groups were similar, 30.2 years/SD ± 10.9

and 32.1 years/SD ± 6.4, respectively. The duration of menses was slightly longer in endometriosis donors with an average of 6.6 days (SD ± 2.7) versus 4.7 days (SD ± 1.3) in control donors ($p = 0.09$), whilst cycle length was similar in both groups (approximately 28 days). On average 13.4 mL (SD ± 7.0) of menstrual blood was collected from the control women and 8.9 mL (SD ± 7.0) from women with endometriosis in the 4–6 h of collection time ($p = 0.2$). Very heavy menstrual bleeding with a significantly higher pain score of 8–9 during menses was recorded in the endometriosis group compared to the control groups which had no pain and normal bleeding pattern. In addition, verbal reporting of pain and discomfort on using the menstrual cups was reported by women with endometriosis which could have contributed to lesser menstrual blood collected despite reporting very heavy menstrual bleeding patterns. The mean BMI of the participants was similar between the two groups and in the upper normal range, 23.3 (SD ± 3.0) and 23.8 (SD ± 2.8) in the control and endometriosis groups, respectively. Most of the donors had not tried to get pregnant and did not know their fertility status, however, among those that were known, the control group were fertile whilst the women in the endometriosis group had fertility problems such as infertility, required fertility assistance or miscarriage. Furthermore, the most striking finding was that the control group was not taking any medication whilst women in the endometriosis group were taking medications including a wide range of hormones (Prednisone, Progestin-only pill, Oral contraceptive pills and Estelle), analgesics (Panadol, Ibuprofen, Tramadol and Naproxen), anti-depressants (Diazepam, Sertraline and Escitalopram) and others (Omeprazole, multivitamins).

3.2 | sEVs Are Present in MF

We first confirmed the presence of sEVs in the MF samples. MF-sEVs from women with and without endometriosis were enriched in the 100,000 *g* pellets as shown by their round morphology in TEM (Figure 1A) and confirmed by Western Blotting of sEV-marker proteins ALIX/PDCD6IP, β-actin and Syntenin-1 (Figure 1B). Single particle size analysis by NTA demonstrated control-(ctrl) MF-sEVs with a median diameter of 125 nm (Figure 1C/D) and endometriosis-(endo) MF-sEVs of 119 nm ($p = 0.85$). Similarly, the total particle number of MF-sEVs was $5.58 \times 10^{14} \pm$ SD 2.59×10^{14}/μg in the control group and $5.69 \times 10^{14} \pm$ SD 5.65×10^{14}/μg ($p = 0.98$) in the endometriosis group (Figure 1E). Despite the lack of statistical differences between the two groups, there was a wider variation in the particle size and concentration in the endo-MF-sEVs compared to ctrl-MF-sEVs. Overall, MF from control and endometriosis women contains sEVs with characteristics consistent with the minimal information for studies of extracellular vesicle (MISEV) guidelines (Théry et al. 2018).

3.3 | MF-sEVs Are Specific to Endometrial Cells and the Endometrium

To identify differences in women with and without endometriosis, we analysed the MF-sEV protein composition using a TMT-based proteomic analysis. We identified over 5000 proteins in the MF-sEVs from women with and without endometriosis (Table S1). The top 100 abundance sEV proteins such as transmembrane proteins (CD63, CD81 and CD9) and cytosolic proteins (TSG101 and ALIX) indexed in the Exocarta database (Mathivanan et al. 2012) were listed, further validating the presence of sEVs in our MF preparations.

GO analysis of the MF-sEV protein cargo identified critical biological processes represented in cellular communication within the endometrial microenvironment (Table S2). A large proportion of proteins were involved in cellular, metabolic, defence and apoptotic processes, wound healing, tissue regeneration, transport, cell migration, transport and localisation indicating a physiological role of EV proteins at menses in homeostasis and tissue repair. Endocytosis, phagocytosis and cell surface receptor signalling pathways were also identified indicating their mode of communication.

Menstrual blood is composed of endometrial tissue fragments, endometrial secretions and blood from within the endometrium as well as from the peripheral circulation. Given that the sEVs retain the donor cell identity, we interrogated the proteins expressed to validate the endometrial origin of the MF-sEVs. Specific makers for endometrial stem/stromal cells such as SUSD2, CD146, CD140β and CD90 and epithelial progenitor & mature cells such as EPCAM, CDH2, CDH1, CD133 and MUC1 were identified accordingly (Table 3). In addition, MF-sEVs expressing proteins of other endometrial resident cells such as endothelial (CD13, CD29, CD31, CD34, CD36, CD39, CD47, CD54) and various immune cells such as uterine natural killer cells (CD56, CD69), decidual T cells (CD4, CD3D, CD3E, CD3G), neutrophils (CD18/ITGB2), macrophages (CD14, CD163, CD71) and B cells 9 CD20, CD74, HLA-DRA) were also identified further supporting that MF-sEVs mostly originated from the endometrium (Table 3).

Given the recent evidence of a microbial origin of some forms of endometriosis (Muraoka et al. 2023), we examined whether MF-sEVs could provide any clue to this phenomenon. We identified proteins binding to lipopolysaccharide (lipopolysaccharide binding protein/LPB) and lipoteichoic acid (prohibitin-2/PHB2 and histidine-rich glycoprotein/HRG) which were significantly decreased in endo MF-sEVs compared to control MF-sEVs indicative of a decrease response to bacteria in endometriosis participants (Tables S1 and S3). Antimicrobial responses such as Platelet factor 4/PF4, lactoferrin (lactotransferrin)/LTF, cathelicidin antimicrobial peptide/CAMP, S100-A10 and Interferon-induced GTP-binding protein Mx1/MX1 were identified. These were significantly decreased in endo MF-sEVs compared to control MF-sEVs (Tables S1 and S3). In addition to the sEV proteins, MF-sEV preparations consisted of proteins from category 3 such as APOA1, Immunoglobulins, AGO1/2/3, TGFBI, HSP90AA and 4 including lamin A and cytochrome C, as listed in the MISEV2023 guidelines (Welsh et al. 2024).

3.4 | MF-sEV Proteins Reveal Dysfunctional Inter- and Intra-Cellular Communication in Endometriosis

To gain insight into differences in the endometrial sEV-mediated inter-cellular communication between endometriosis and control women, we performed a comparative analysis of the differentially expressed MF-sEVs proteomes. Of the >5000 proteins identi-

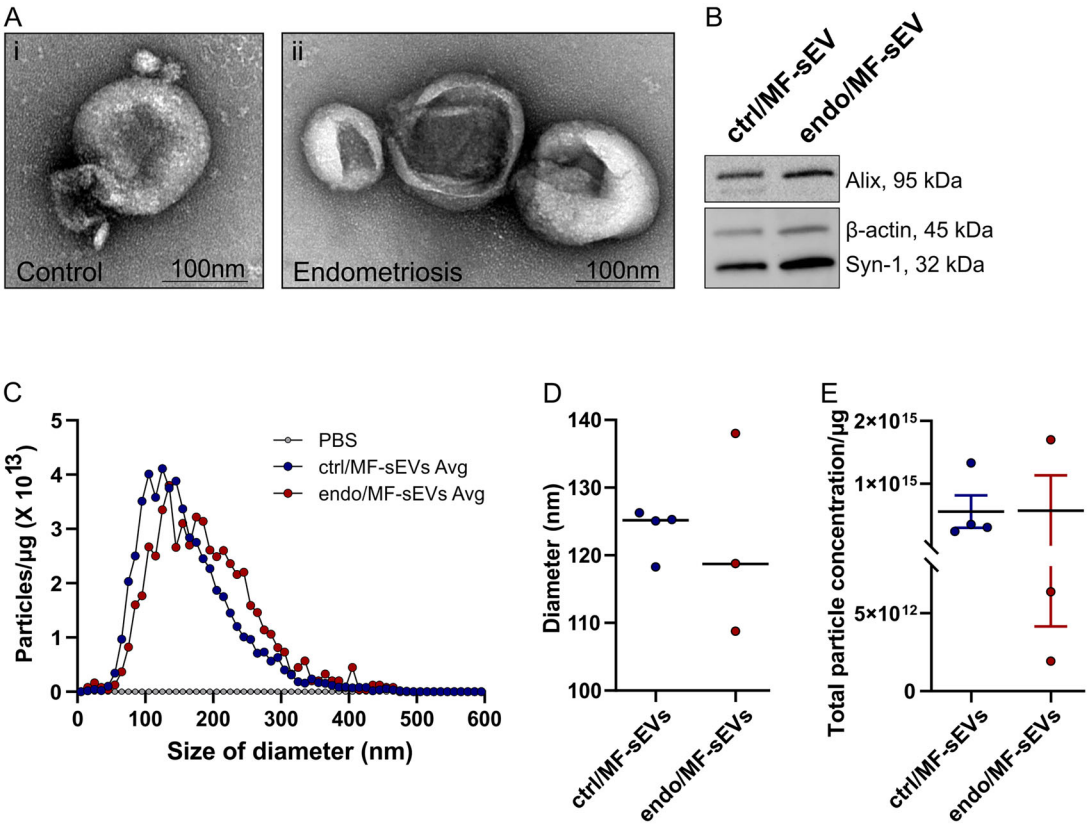

FIGURE 1 | Characteristics of menstrual fluid sEV from endometriosis and control women. (A) Representative TEM images of MF-sEVs from control (i) and endometriosis (ii) women show a characteristic round-shaped morphology ~120 nm diameter. (B) Representative Western blot of EV markers Alix, β-actin and Syntenin-1 on MF-sEVs. (C) Average particle concentration (normalised to per μg MF-sEV proteins) and particular EV size distribution from control and endometriosis groups, $n = 3$/group. (D) The median diameter of control (125 nm) and endometriosis MF-sEVs (119 nm). (E) Total particle concentration normalised to per μg protein in control (5.58×10^{14}) and endometriosis (5.69×10^{14}) MF-sEVs. The two groups were compared using an unpaired *t*-test with Welch's correction.

TABLE 3 | Endometrial cell-specific proteins in MF-sEVs.

Endometrial cell type	Markers
Mesenchymal stem/stromal cells	SUSD2, CD146, CD140β, CD90, CD105, CD73, NOTCH1, CD44
Epithelial progenitor & mature cells	EPCAM, CDH2, CDH1, CD133, CTH, EZR, KRT1, KRT14, KRT18, KRT19, KRT33A, KRT6B, KRT, KRT78, KRT9, MUC1, MUC13, MUC16, MUC4, MUC5AC, MUC5B, MUC6
Endothelial cells	CD13, CD29, CD31, CD34, CD36, CD39, CD47, CD54, CD62, CD102, CD106, CD112, NECTIN2, CD142, CD143, CD144/CDH5, CD147, CD309, TEK, LYVE1, SELP, vWF
Leucocytes:	CD45
Decidual T cells	CD4, CD3D, CD3E, CD3G, CD69, CD8B, HLA-DR
Uterine natural killer cells	CD56, CD69
neutrophils	CD18/ITGB2
Decidual macrophage	CD14, CD163
Macrophages	CD163, CD11b, CD71, CD54, CD86
Dendritic cells	CD11c
B cells	CD20, CD74, HLA-DRA

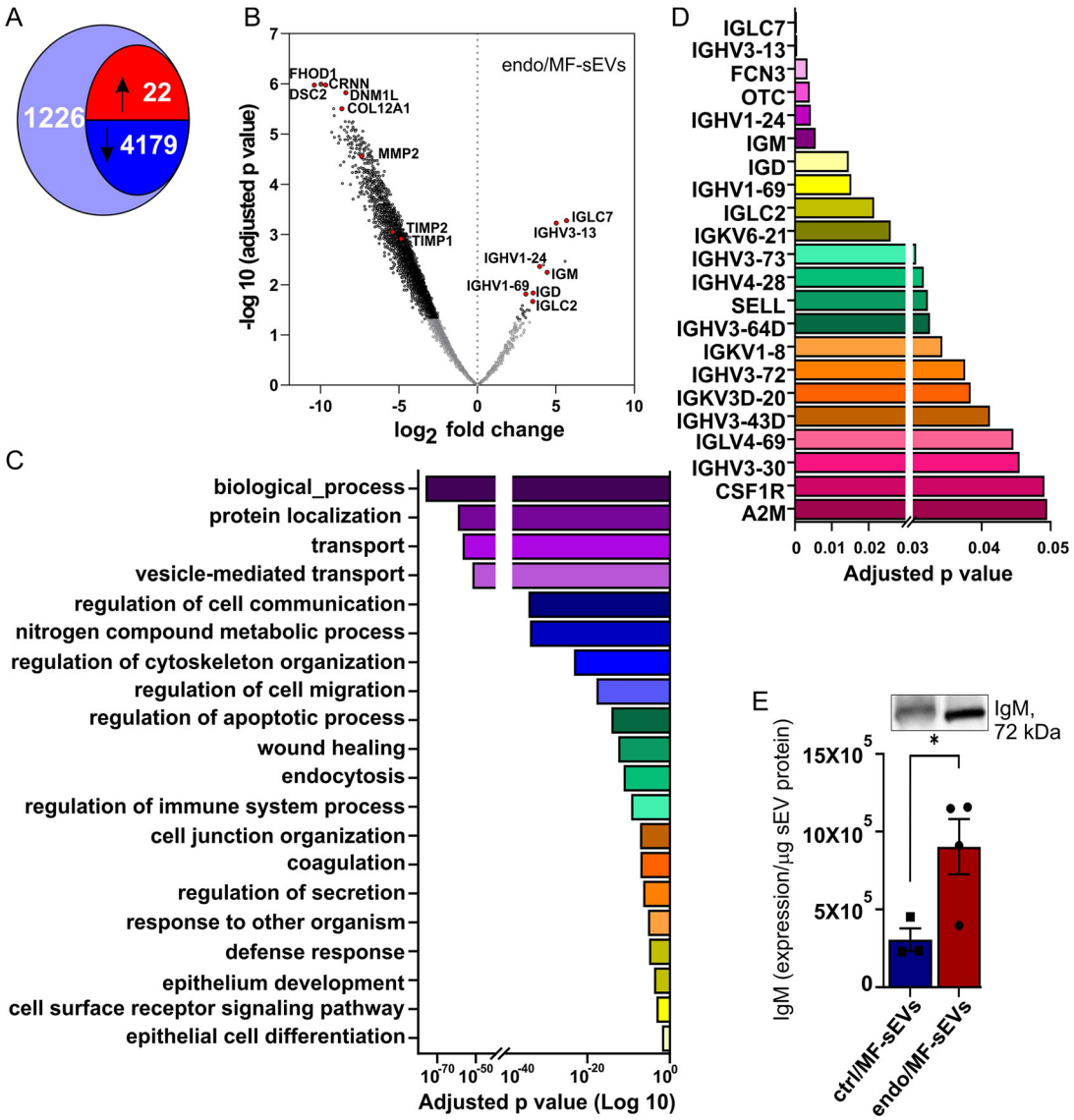

FIGURE 2 | Differential proteins in endometriosis and control MF-sEVs and their relative pathways. (A) Venn diagram showing the common proteins with equal expression in MF-sEVs from control and endometriosis women (1226, purple), and those that were up-regulated (22, red) and down-regulated (4179, blue) in endo/MF-sEVs compared to ctrl/MF-EVs. (B) Volcano plot illustrating significantly differentially abundant proteins (black dots). Functional pathway annotation of significantly regulated pathways in the down-regulated proteins in endo/MF-sEVs (C) and the 22 up-regulated proteins (D). (E) Representative Western blot image of IgM expression in ctrl and endo/MF-sEVs and the graph showing the quantification of three to four independent MF-sEVs from control and endometriosis women. The two groups were compared using an unpaired *t*-test with Welch's correction. $^{*}p = 0.04$.

fied in MF-sEVs, 77.4% were differentially regulated between endometriosis and control women (Figure 2A, B), supporting the notion of intrinsic endometrial abnormalities in women with endometriosis. Surprisingly, ~77% of these proteins were significantly decreased in MF-sEVs from women with endometriosis compared to the controls (Figure 2A, B). Downregulated proteins were the classical sEV cytosolic proteins (TSG101, CHMP, ALIX, FLOT1) indicating the lack of compartmentalisation of proteins by eutopic endometrial cells. Some of the most significantly decreased proteins were DSC2, IL1RN, SDCBP2, SLC1A1, SLC15A1, LGALS7, IL18, IL1R1 and S100P (Table S3). Unsurprisingly, GO Enrichment Analysis showed that these decreased proteins reflected a consequential loss of various vital biological pathways mentioned above together with processes regulating autophagy, response to bacteria, glycolysis and glucogenesis, solute transport, contractile behaviour, cell surface receptor signalling and epithelial cell differentiation and development (Table S4, Figure 2C).

Twenty-two proteins were significantly increased including adhesive molecule A2M, SELL and FCN3 and interestingly, 18 were immunoglobulins (Table S5, Figure 2D); Igλ, IgK and Igδ, IgG and IgM, with heavy chain variable domains that form the antigen binding sites and regulate phagocytosis, classical complement activation and the immunoglobulin mediated-immune response. Western Blot analysis of IgM expression validated the proteomic findings with a significant increase in expression in endo/MF-sEVs compared to the ctrl/MF-sEVs ($p = 0.04$)

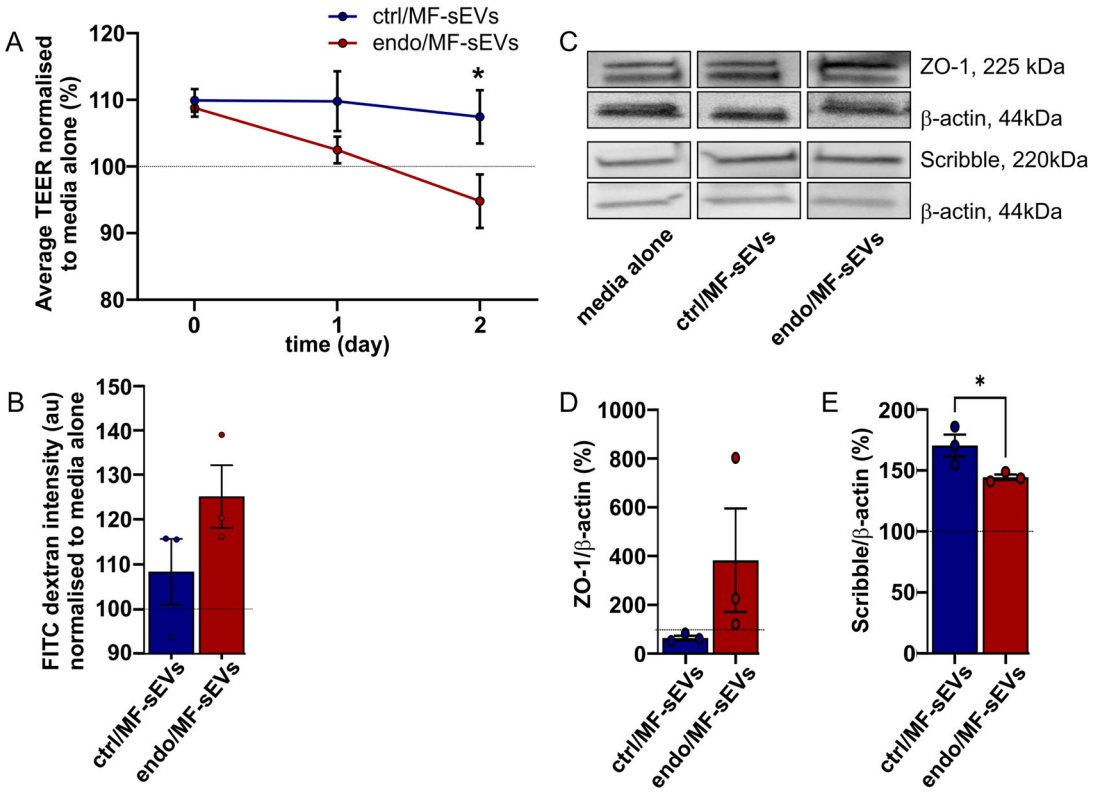

FIGURE 3 | Endo/MF-sEVs modify mesothelial function. (A) Graph showing changes in the transepithelial electrical resistance in mesothelial cells treated with MF-sEVs from women with and without endometriosis. The dotted line represents the readout of MCs treated with media alone. Analysis: Two-way ANOVA with Sidak multiple comparisons test. (B) Graph showing the intensity of FITC-dextran (40 kDa) which has passed through MCs treated with MF-sEVs from women with and without endometriosis. (C) Representative Western blot of ZO-1 and Scribble expression on MCs treated with media alone, ctrl/MF-sEVs, and endo/MF-sEVs and (D) and (E) graph showing their respective quantification. The dotted lines represent the readout of MCs treated with media alone. The graph is presented as the mean ± SEM of three independent MF-sEVs from control and endometriosis women. Analysis: One-Way ANOVA with Holm-Sidak multiple comparisons test, *$p = 014$.

(Figure 2E). Overall, the proteomics data strongly suggest that endometriosis/MF-sEVs contain proteins skewed negatively in abundance and function of endometrial and immune cells providing clues to endometrial health and how they can potentially affect the homeostasis of recipient cells.

3.5 | MF-sEVs Alter Mesothelial Cells Permeability and Barrier Integrity

To assess whether MF-sEVs alter the behaviour of mesothelial cells in areas, where endometriosis lesions are established, we assessed cellular integrity by measuring TEER, permeability and changes in the tight-junctional proteins. The TEER increased slightly after introducing MF-sEVs from both groups compared to the MCs treated with media alone (Figure 3A). The TEER of the ctrl/MF-sEVs treated MCs was maintained throughout 48 h; however, TEER of endo/MF-sEVs treated MCs decreased significantly at 48 h, $p = 0.019$ (Figure 3A). Similarly, there was an increasing trend in FITC-dextran ($p = 0.1$) leakage through the MCs treated with endo/MF-sEVs compared to the control (Figure 3B).

Epithelial cell permeability is maintained by junctional proteins, therefore differential expression of ZO-1 and Scribble were assessed in MCs treated with ctrl/MF-sEVs and endo/MF-sEVs. Western Blot analysis showed a high level of ZO-1 in MCs (Figure 3C), but no statistical differences were found between either MF-EV groups or with media alone (Figure 3D). Similar to the TEER, 48 h post-treatment there was a significant increase in Scribble in the MCs treated with ctrl/MF-sEVs ($p = 0.0003$) and endo/MF-sEVs ($p = 0.002$) compared to media alone, but the increase with endo-MF-sEVs was significantly less compared to the ctrl/MF-sEVs ($p = 0.014$, Figure 3C, E). This change in the expression of junctional proteins indicates that the endo/MF-sEVs cargoes modify the cellular function of MCs in the peritoneal cavity and thus may contribute to the pathogenesis of endometriosis.

3.6 | Surface Markers in MF-sEVs From Women With and Without Endometriosis

To systematically evaluate differences in MF-sEVs surface antigen expression between women with and without endometriosis, a multiplex bead-based flow cytometry assay was utilised (Figure 4A–D). All 37 surface proteins were detected above the detection threshold in all preparations. Both MF-sEVs groups showed a high and similar content of the three surface tetraspanins CD9, CD63 and CD81, supporting the presence of

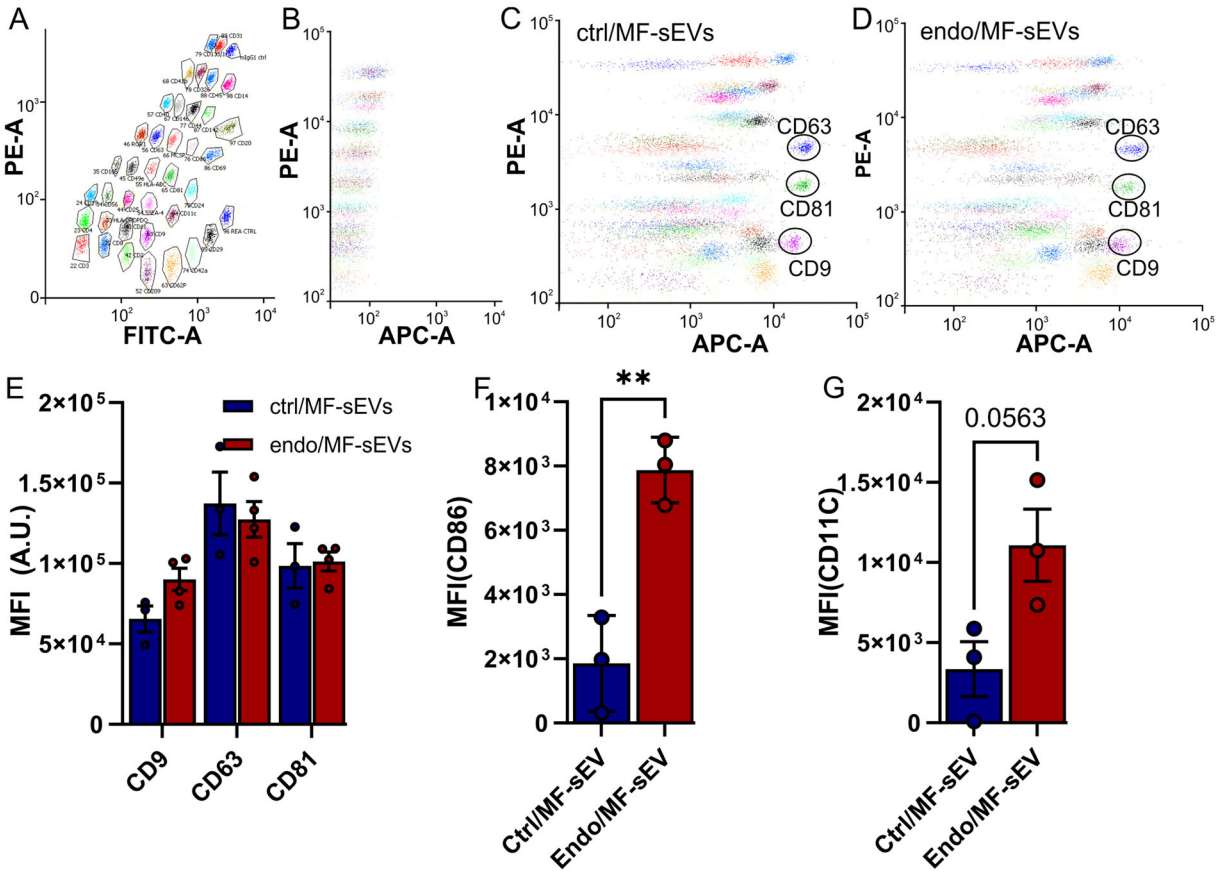

FIGURE 4 | Surface expression of protein markers in MF-sEVs. Representative flow chart of flow cytometry analysis to identify 39 surface markers including two isotype controls using PE and FITC (Beads, A) and APC (beads without detection reagents, (B) channels in MF-sEVs. (C) and (D) representative flow cytometry plots of ctrl/MF-sEVs and endo/MF-sEVs showing the distribution of 39 surface proteins in the APC channel. Graph showing quantification of median fluorescence intensity (MFI) of (E) tetraspanins CD9, CD63, and CD81, (F) CD86 and (G) CD11c in ctrl- and endo-MF-sEVs. The graph is presented as the mean ± SEM of three independent MF-sEVs from control and four endometriosis women. $n = 3$/ctrl and 4/endo. The two groups were compared using an unpaired *t*-test with Welch's correction, $^{**}p = 0.006$.

sEVs (Figure 4C–E). Although there was a trend to increased expression of some surface epitopes in endo-MF-sEVs such as CD2, CD4, CD40 and CD8 (data not shown), only CD86 was expressed significantly higher in the endo/MF-sEVs compared to the ctrl/MF-sEVs ($p < 0.002$, Figure 4F) and CD11c had a trend towards increased expression in endometriosis ($p = 0.052$, Figure 4G). Five markers for dendritic and B cells CD19, CD1C, CD209, MCSP and CD20 (Figure 5A, Table 4) were low in intensity (MFI < 1000) supporting the low cellular frequency of these leukocytes in the endometrium. Thirty-two markers had high expression above 1000 MFI (Figure 5A, Table 4). Stromal/mesenchymal proteins (CD44, CD105, CD146), epithelial markers (SSEA4, CD326), endothelial marker (CD31), decidual monocyte (CD14) and immune cell markers (CD45, CD4, CD40) were detected (Figure 5A, Table 4), denoting that a proportion of the MF-sEVs was of endometrial cellular origin. HLA-DR, HLA-DP, HLA-DQ, and HLA-ABC were also present on sEVs possibly derived from antigen-presenting cells in the endometrium (T cells, macrophages, natural killer and dendritic cells) (Figure 5A, Table 4). Furthermore, the nMFI of canonical protein markers of different immune cells (CD1C, CD2, CD3, CD4, CD8, CD11c, CD19, CD20, CD25, CD56, CD86, CD209) and ROR1, MCSP and CD49e were relatively low, with a mean nMFI below ~5 (Figure 5B). The 16 markers that denote immune cells (CD45, HLA-DRDPDQ, HLA-ABC, CD24, CD40,) stromal/mesenchymal (CD44, CD105, CD142; SSEA4), epithelial (CD29, CD133/1, CD326), endothelial cells (CD31) and platelets (CD41B, CD42a, CD62P) had moderate/high nMFI (nMFI >10) indicating their high expression relative to the three tetraspanin MF-sEV markers (Figure 5B). Cumulatively, our data show that MF-sEVs may make a significant contribution towards the immune system and angiogenic responses that may be important in establishing endometriotic lesions.

4 | Discussion

This study evaluated the proteomes of MF-sEVs from women with self-reported, previously confirmed endometriosis with persistent period pain despite surgery and from women with self-reported no endometriosis and no painful periods and provided clues on their potential role in the pathogenesis of the disease, given that MF is retrogradely shed into the pelvic cavity. Although significant changes in peritoneal fluid-derived EVs in endometriosis have been shown before (Nazri et al. 2020), to our knowledge, this is the first study to date showing a comprehensive list of MF-sEV proteins from women with endometriosis and unaffected women. We identified endometrial-specific proteins in

TABLE 4 | Median fluorescence intensity of surface antigens in MF-sEVs.

	Mean of MFI		Mean of nMFI	
Surface markers	**Control**	**Endometriosis**	**Control**	**Endometriosis**
CD1C	230	338	0.2	0.3
CD2	2809	5210	2.8	4.9
CD3	2034	1105	2.0	1.0
CD4	1579	5158	1.6	4.9
CD8	4648	7294	4.6	6.9
CD9	**65,452**	**90,098**	65.2	84.8
CD11c	3347	8396	3.3	7.9
CD14	5203	7570	5.2	7.1
CD19	349	663	0.3	0.6
CD20	110	538	0.1	0.5
CD24	10,805	19,631	10.8	18.5
CD25	1622	3003	1.6	2.8
CD29	45,825	59,386	45.6	55.9
CD31	25,472	50,024	25.4	47.1
CD40	18,570	36,987	18.5	34.8
CD41B	17,481	27,371	17.4	25.8
CD42a	34,178	50,706	34.0	47.7
CD44	29,039	33,401	28.9	31.4
CD45	21,752	32,935	21.7	31.0
CD49e	5935	7953	5.9	7.5
CD56	4443	3350	4.4	3.2
CD62P	43,401	65,681	43.2	61.8
CD63	**13,„7292**	**127,476**	136.7	119.9
CD69	7411	14,706	7.4	13.8
CD81	**98,503**	**101,248**	98.1	95.3
CD86	1856	6176	1.8	5.8
CD105	34,894	43,663	34.7	41.1
CD133_1	64,544	78,246	64.3	73.6
CD142	13,692	24,353	13.6	22.9
CD146	5206	8459	5.2	8.0
CD209	36	333	0.0	0.3
CD326	37,088	36,161	36.9	34.0
HLA-ABC	18,028	24,189	18.0	22.8
HLA-DRDPDQ	28,464	30,381	28.3	28.6
MCSP	147	215	0.1	0.2
ROR1	713	1730	0.7	1.6
SSEA4	20,601	22,619	20.5	21.3

Note: Bolded are sEVs-specific markers.

Abbreviations: MFI, mean fluorescence intensity; n, normalised.

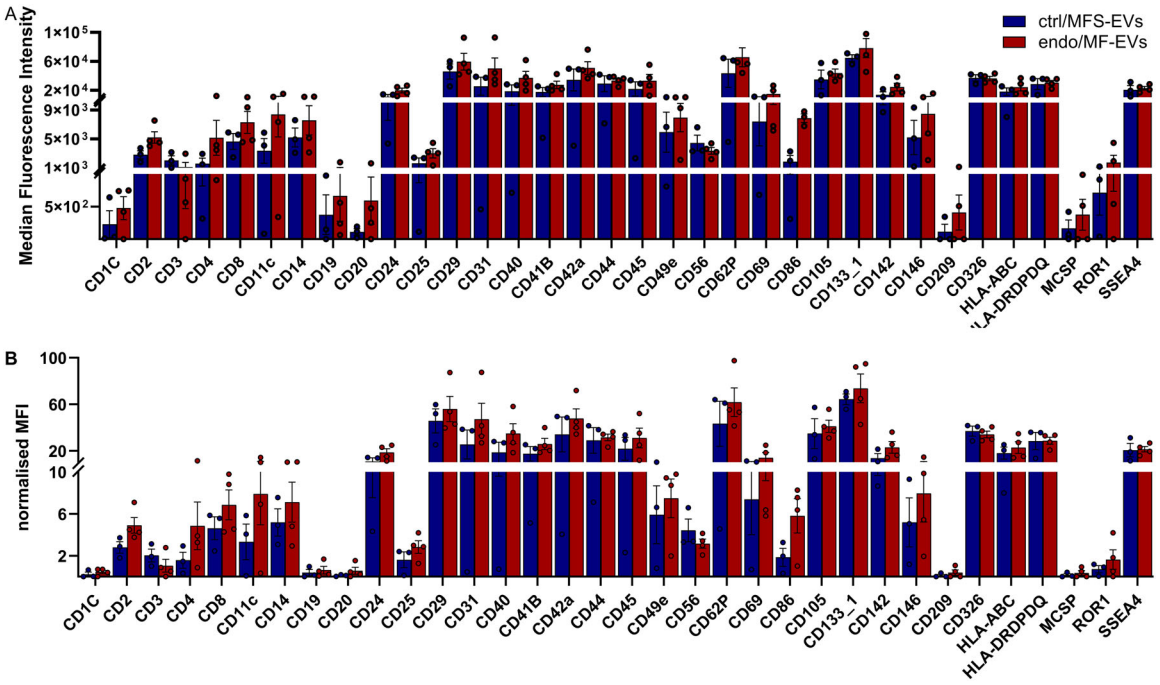

FIGURE 5 | Identification and quantification of surface expression of protein markers in MF-sEVs. Graphs showing (A) median fluorescence intensity of all 37-surface protein expression in ctrl- and endo/MF-sEVs and (B) normalised MFI against tetraspanin CD9, CD63, and CD81 in ctrl- and endo-MF-sEVs. The graph is presented as the mean ± SEM of three independent MF-sEVs from control and endometriosis women. $n = 3$/ctrl and 4/endo. Analysis: An unpaired *t*-test with Welch's correction.

MF-sEVs that regulate key biological processes and identified significant differences between endometriosis and controls. A large proportion of the differentially expressed endo/MF-sEVs proteins were decreased compared to those in controls suggesting defects in protein packaging within the sEVs and dysfunctional intercellular communication to maintain tissue homeostasis in shedding endometrial cells. Dysregulated molecular pathways involving endometrial cell-derived endo/MF-sEVs included cellular process, nitrogen compound metabolism, immune response, intracellular signal transduction, regulation of programmed cell death, establishment or maintenance of cell polarity, regulation of autophagy and actin cytoskeleton organisation. Overall, this study supports the notion that sEV-mediated eutopic endometrial cell communication is deranged indicating potential contribution to endometriotic lesion establishment and progression.

Our study showed that ctrl/MF-sEVs contain cytoskeletal organisation proteins that increased mesothelial cell transepithelial resistance, suggesting a role in preventing peritoneal content leakage into the sub-mesothelial tissues and circulation from retrogradely shed MF into the pelvic cavity. In contrast, endo/MF-sEV content is low in such proteins with limited ability to increase mesothelial barrier resistance, suggesting increased permeation, potentially providing a mechanism allowing endo/MF-sEV to breach this defence barrier normally maintained by tight junctional proteins. The large surface area of the peritoneal mesothelium allows passive dialysis of a large quantity of fluids and substances across the peritoneum (Young et al. 2013, Mutsaers et al. 2016), critical for maintaining peritoneal fluid volume. Women with endometriosis experience abdominal bloating ("endo belly") with a significant increase in lower abdominal girth, associated with increased peritoneal fluid volume causing severe discomfort (Drake et al. 1980, Luscombe et al. 2009) and cyclical local and systemic swelling (Luscombe et al. 2009). Our findings of differential proteins of endo/MF-sEVs and their differential interaction with mesothelial cells support their contribution to endometriotic lesion establishment and symptoms the women experience.

Our findings also showed a decrease in mesothelial cell polarity regulators with high porosity suggesting a potential role in endometrial ectopic lesions establishment in the peritoneum. Inflammation is known to disrupt epithelial barrier integrity such as in the gut in inflammatory bowel diseases leading to epithelial barrier dysfunction (Martini et al. 2017) and immunoglobulins are an integral component of inflammation. Findings from cancer research have demonstrated that cancer cell-derived EVs decrease vascular cell integrity promoting invasion and metastasis. In our study, we identified a significant decrease in MF-sEVs proteins from endometriosis participants that maintain the epithelial tight junctions such as protein kinase C and Rab GTPase, as well as myosin light-chain kinase that decreases tight junctions. Given the increase in TEER in both groups initially and maintained over the experimental time only in the control group but decreased in the endometriosis group, suggests the decrease in junction-maintaining proteins and concomitant increase of immunoglobulin in endo/MF-sEVs may have contributed to the barrier disruption. In addition, a damaged or absent mesothelium following peritoneal surgeries exposes the underlying extracellular matrix (ECM) to endometrial cells present in the menstrual reflux (Dunselman et al. 2001, D'hooghe et al. 1992, Witz et al. 2003). The dynamic process of endometrial cells attaching to the outer perimeter of the MCs allows their invasion through the permissive intercellular space and to the ECM (Witz et al. 2003).

In this regard, we provide evidence for a possible mechanistic role of MF-sEVs in endometriotic lesion establishment.

Our proteomic data demonstrated a significant increase in immunoglobulins in endo/MF-sEVs, including IgM, frequently found in women with endometriosis (Menzhinskaya et al. 2023) which we validated using Western Blotting. This finding supports the concept of endometriosis as an inflammatory disease with an altered immune niche (Vallvé-Juanico et al. 2019). B cells, the primary source of IgM, comprise ~3% of total endometrial leucocytes and are consistently identified mainly in the basalis of human endometrium (Vallvé-Juanico et al. 2019). However, they are also abundant around the luminal and glandular epithelium in the functionalis in an overactivated state often producing antibodies/autoantibodies in women with endometriosis (Hever et al. 2007, Greenbaum et al. 2021, Riccio et al. 2017). IgM plays a vital role in tissue immune homeostasis as well as in clearing apoptotic cells (Sathe and Cusick 2023), but high levels may impair endometrial cellular function involved in embryo receptivity, creating an inhospitable environment for implantation and contributing to infertility (Vallvé-Juanico et al. 2019, Greenbaum et al. 2021, Franco Spegiorin et al. 2010). IgM forms a complex with the polymeric Ig receptor at the basal membrane of epithelial cells and translocates to the apical membrane in vesicles and is secreted into the lumen (Michaud et al. 2020), supporting our findings of IgM in the MF-sEV.

Many groups have observed the presence of plasma proteins in their EV preparations that are categorised as non-vesicular extracellular particles (NVEPs) or contaminants and proteins associated with intracellular compartments that co-isolate with EVs (Welsh et al. 2024, Wu et al. 2024, Tóth et al. 2021). Recent studies have identified these proteins are indeed protein corona of EVs. They are identified as external molecular components of EVs and have functional significance such as maintaining their morphology (Varga et al. 2020), rate of vesicular mobility and migration (Skliar et al. 2018), interaction with other proteins (Tóth et al. 2021) and uptake by and behaviour of recipient cells (Tóth et al. 2021, Willis et al. 2019, Dietz et al. 2023, Wolf et al. 2022, Gomes et al. 2022). Innovative research and advancements in technologies have improved our understanding of EVs and their integral components. Indeed, our current study also identified proteins such as binding proteins to microbial cell membranes and antimicrobial proteins/peptides associated with NVEPs, intracellular components and vesicular corona including immunoglobulins (IgM) in the MF-sEVs preparation, similar to those identified in the blood plasma. Therefore, these proteins may be a part of MF-sEVs identity and integral to their functions.

Our analysis identified increased CD11c and CD86 (inflammatory phenotype) surface antigens in endo/MF-sEVs indicating a pro-inflammatory endometrial environment in women with endometriosis. Pro-inflammatory macrophages predominate in the eutopic endometrium in women with endometriosis which facilitates incomplete clearance and increased survival of the refluxed tissues in the peritoneal cavity (Vallvé-Juanico et al. 2019). Pro-inflammatory macrophages promote angiogenesis, cell migration of Schwan cells, cell proliferation and survival, thus may contribute to the increased immature microvasculature (Kuroda et al. 2009, Van Langendonckt et al. 2004) and nerve fibre infiltration in endometriotic lesions. IL-18 and galectin-7 were decreased in MF-sEVs from women with endometriosis. Decreased levels of IL-18 and NK cell activity have also been reported in the eutopic endometrium of women with endometriosis (Wilson et al. 1994). IL-18 is secreted by endometrial stromal and epithelial cells and is increased in the secretory phase which is critical in inducing cytolytic activity of NK cells but fails to occur in endometriosis (Luo et al. 2006, Jørgensen et al. 2022, Zhang et al. 2004). Galectins, especially galectin-7 is actively accumulated in the decidual stromal cells during the late secretory phase and secreted in the MF, and they play a key role in endometrial repair post-menstruation (Luo et al. 2006, Jørgensen et al. 2022, Evans et al. 2014, Menkhorst et al. 2014). Our data supports previous findings of eutopic endometrial dysfunction, poor decidualisation and the clinical symptoms of infertility that occur in women with endometriosis. Given the differential composition of key endometrial regulators in MF-sEVs in women with endometriosis, MF-sEVs give insight into the disease pathogenesis and may present a potential diagnostic avenue.

5 | Significance

Extensive research has been conducted to understand the pathogenesis of and to identify diagnostic biomarkers for endometriosis. The development of potential diagnostic and treatment interventions necessitates an understanding of the disease pathophysiology. MF (unbiased selection of participants in the current study) is an easily sourced biofluid and our study has demonstrated significant differences in MF-sEV proteins that likely have a pathophysiological role in endometriosis lesion establishment. Although our pilot study is underpowered for sample size, it will enable future larger studies that allow endometriosis subgrouping, to fully establish the value of MF-sEVs towards the development of a minimally invasive diagnostic tool for endometriosis that is so urgently needed.

Author Contributions

Shanti Gurung: conceptualization (lead), data curation (equal), formal analysis (equal), investigation (equal), methodology (equal), software (equal), supervision (equal), validation (equal), writing–original draft (equal), writing–review and editing (equal). **Jacqueline Piskopos**: formal analysis (equal), methodology (equal), validation (equal), writing–original draft (supporting), writing–review and editing(supporting). **Joel Ricky Steele**: data curation (equal), formal analysis (equal), methodology (supporting), software (equal), writing–review and editing (supporting). **Ralf Schittenhelm**: data curation (equal), formal analysis (equal), methodology (supporting), software (equal), writing–review and editing (supporting). **Anup Shah**: data curation (equal), formal analysis (equal), methodology (supporting), software (equal), writing–review and editing (supporting). **Fiona L. Cousins**: methodology (supporting), writing–review and editing (supporting). **Thomas T. Tapmeier**: methodology (supporting), writing–review and editing (supporting). **Caroline E. Gargett**: conceptualization (equal), funding acquisition (equal), resources (equal), supervision (lead), writing–review and editing (equal).

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Ethics Statement

This study was approved by the Monash Health Research Ethics Committee (approval no. RES-20-0000-159A).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD042101.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.