1 **Macrophages alternatively activated by endometriosis-exosomes**
2 **contribute to the development of lesions in mice**

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8 **Running title:** Exosomes promote endometriosis in mice

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18 # Abstract

19 **STUDY QUESTION:** Do exosomes play a role in the pathogenesis of endometriosis
20 in a murine model?

21 **SUMMARY ANSWER:** Exosomes from endometriosis (EMS) can alternatively
22 activate macrophages and thus contribute to the development of lesions in mice.

23 **WHAT IS KNOWN ALREADY:** The pathogenesis of endometriosis, an
24 inflammatory disease, possibly involves peritoneal macrophages. Exosomes are
25 recognized as a new communicator among cells and a key modulator in several
26 inflammatory diseases.

27 **STUDY DESIGN, SIZE, DURATION:** We performed in vitro and in vivo
28 experiments to demonstrate the role of exosomes in modulating macrophages.
29 RAW264.7 cells (macrophages) were used to examine the effects of exosomes on
30 macrophages in vitro. An experiment was also conducted in vivo, as follows. Fifty
31 C57BL/6 female mice were randomly allocated to five control and five experimental
32 groups (n=5/group). The experimental group was injected i.p. with EMS-exosomes
33 derived from eutopic stromal cells, starting on day-7 then every day for 1 week.)The
34 control group received CON-exosomes from mice without endometriosis. Peritoneal
35 macrophages were assessed over the next 6 days. On day 0, all mice were injected i.p.
36 with endometrium to establish the endometriosis model. On day 14, all mice were
37 sacrificed, ectopic lesions were counted and measured.

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38 **PARTICIPANTS/MATERIALS, SETTING, METHODS:** Exosomes were isolated
39 from endometrial stromal cells（ESCs） by ultracentrifugation and characterized
40 through transmission electron microscopy, nanoparticle tracking analysis, and western
41 blot. After treatment with exosomes, the polarization and phagocytic ability of the
42 macrophages were detected by flow cytometry analysis.), immunofluorescent staining,
43 and RT-PCR. C57BL/6 mice were utilized to establish an endometriosis model by i.p.
44 injection of endometrial segments. .

45 **MAIN RESULTS AND THE ROLE OF CHANCE:** After treatment with
46 EMS-exosomes, the macrophages were polarized into an M2-like phenotype and their
47 phagocytic ability decreased（p <0.05 versus treatment with CON-exosomes). The
48 total weight and volume of the lesions in mice treated with EMS-exosomes
49 significantly increased compared with those in mice treated with CON-exosomes
50 (p<0.05). The infiltration of M2-like macrophages was enhanced in the EMS-exosome
51 group (p <0.001 versus treatment with CON-exosomes).

52 **LARGE SCALE DATA: N/A**

53 **LIMITATIONS, REASONS FOR CAUTION:** Detection of endometriosis following
54 exosome treatment was only performed in a murine endometriosis model. Clinical data
55 and additional mechanism studies must be conducted to understand the role of
56 exosomes in the pathogenesis of endometriosis.

57 **WIDER IMPLICATIONS OF THE FINDINGS:** This study emphasizes the

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58 importance of EMS-exosomes in the pathogenesis of endometriosis. Further
59 investigations on the exosome signaling pathways may contribute to the development
60 of effective treatments for endometriosis.

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63 The authors report no conflict of interest.

64 **Keywords:** endometriosis, mice, exosomes, macrophages, polarization, phagocytosis

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67 Introduction

68 Endometriosis is an inflammatory condition characterised by abnormal growth of
69 endometrial-like tissues outside the uterus which affects 6%–10% of reproductive-aged
70 women (Bulun, 2009, Giudice and Kao, 2004). Women with endometriosis typically
71 experience pelvic pain and infertility, which have considerable adverse effects on their
72 quality of life and entail substantial costs. However, the precise pathogenic mechanisms
73 of endometriosis are not well identified, and diagnosis is always delayed, leading to
74 difficulties in medical and surgical treatments (Simoens *et al.*, 2012, Vercellini *et al.*,
75 2014). Although the pathogenesis of this disease remains unknown, patients with
76 endometriosis exhibit immune dysfunction (Paul Dmowski and Braun, 2004).

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77 Emerging evidence suggests that endometriosis is an inflammatory condition that can
78 induce an immune response, which leads to cellular changes (Paul Dmowski and Braun,
79 2004). Peritoneal macrophages play a key role in the establishment and maintenance of
80 endometriosis because they mainly function in the immune regulation of the abdominal
81 cavity and the mediation of homeostasis in the peritoneal environment (Capobianco
82 and Rovere-Querini, 2013). Numerous studies have shown that the density of
83 macrophages is significantly higher in women with ectopic endometrium than that in
84 the control (Berbic *et al.*, 2009, Takebayashi *et al.*, 2015, Tran *et al.*, 2009). In addition,
85 the activation of macrophages and "macrophage-associated cytokines", such as
86 monocyte chemoattractant protein-1, is promoted in women with endometriosis
87 (Akoum *et al.*, 2002). Macrophages are highly plastic cells that can be modified by the
88 tissue microenvironment and categorized into M1 and M2 polarization states (Bronte
89 and Murray, 2015). M1-like macrophages, which are "classically activated", exhibit
90 an inflammatory phenotype, and M2 macrophages, which are "alternatively activated"
91 have an anti-inflammatory phenotype (Wynn and Vannella, 2016). Bacci and his
92 colleagues reported that peritoneal macrophages differentiate into M2-like
93 macrophages in patients with endometriosis and in a murine endometriosis model
94 (Bacci *et al.*, 2009). Hence, the phenotypic switch of macrophages in the peritoneal
95 cavity plays an important role in endometriosis. However, the underlying mechanisms
96 of the crosstalk between macrophages and endometrial cells remain to be unveiled.

97 Exosomes contain various biological molecules, including proteins, nucleic acids, and
98 lipids; these vesicles serve as "communicators" among different types of cells by

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99 transporting cargoes derived from the parent cells and affect physiological and
100 pathological conditions (De Toro *et al.*, 2015, Raposo and Stoorvogel, 2013, van Niel *et*
101 *al.*, 2018). Exosomes from different disease states play a very important role in
102 macrophage polarization (Robbins and Morelli, 2014). Ti et al. demonstrated that
103 exosome-shuttled let-7b derived from lipopolysaccharide-preconditioned
104 mesenchymal stromal cells can modify macrophage polarization (Ti *et al.*, 2015). Wu et
105 al. found that gastric cancer cells secrete exosomes, which promote cancer progression
106 by activating the nuclear factor-kappa B pathway in macrophages (Wu *et al.*, 2016).
107 These studies suggest the important roles of exosomes in macrophage activation and
108 polarization. However, the role of exosomes in endometriosis is poorly understood.
109 Therefore, exploring the potential roles of endometriosis-derived exosomes on
110 macrophage activation and polarization in the process of a disease is of great interest.

111 In this study, we conducted in vitro and in vivo experiments and revealed that the
112 eutopic stromal cells of endometriosis-exosomes polarized macrophages toward the
113 M2-like phenotype and impaired their phagocytic ability, thereby enhancing the
114 development of lesions.

115 Materials and Methods

116

117 Mouse model of endometriosis

118 In this study, all 6-week-old female C57BL/6 mice were obtained from Beijing HFK

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119 Bioscience Company (Beijing, China). Animal care and all procedures were conducted
120 in accordance with the Animal Care and Use Committee of Shandong University
121 (Shandong, China). All mice were caged in groups of five, maintained under
122 controlled conditions, and allowed to have 2 weeks of acclimatization to the
123 environment. The estrous stage was monitored daily by a vaginal smear every morning,
124 and mice with normal estrous cycles were used in subsequent experiments. All animals
125 were placed in environmentally controlled housing with a light/dark cycle of 12/12 h, a
126 stable temperature of 25 ± 1 °C, and a relative humidity of 55% (±10%). All animals
127 were given free access to laboratory food and water. The mouse model of endometriosis
128 was adapted from a model described previously (Bacci *et al.*, 2009, Long *et al.*, 2016,
129 Mariani *et al.*, 2012) and used in our previous study (Yuan *et al.*, 2017, Yuan *et al.*,
130 2018). Donor mice were initially treated with estradiol benzoate (3 µg/mouse, Aladdin,
131 Shanghai, China) and sacrificed after 7 days. The uterus was removed and seeded in a
132 Petri dish containing warm sterile phosphate-buffered saline (PBS). The two uterine
133 horns were isolated and split longitudinally with a pair of scissors. Each uterine horn
134 was identically processed and carefully disrupted into segments smaller than 1 mm by
135 using scissors. The fragments were then injected i.p. into recipient mice using a 1 mL
136 syringe and a 25 guage needle (day 0). Overall, 16 endometrial segments (about 30 mg
137 in total) from one uterine horn were placed in 400 µL of PBS and injected into one
138 receipt mouse. The mice were sacrificed by cervical dislocation 14 days after
139 endometrial tissue injection. In the control group, mice received an injection of PBS i.p,
140 and the method of injection and dosage was same as the endometriosis groups. The

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141 flow diagram of the experimental design is shown in Fig. 1A.

142 Isolation and culture of endometrial stromal cells

143

144 Mice were euthanized 14 days after i.p. injection of endometrial segments or PBS.
145 The abdominal cavity was immediately opened. The uteri were removed, washed with
146 PBS twice, dissected to minute pieces (< 1 mm), and digested with 0.25% (w/v)
147 collagenase II mixed with 0.25% (w/v) collagenase IV (Worthington, Lakewood, NJ,
148 USA) for 60 min at 37 °C. The fragments were filtered through 100 μm aperture
149 sieves to remove debris and through 40 μm aperture sieves to remove epithelial cells.
150 The isolated endometrial stromal cells (ESC) were resuspended in Dulbecco's
151 modified Eagle medium (DMEM)/F12 (Gibco, Beijing, China) containing 10% (v/v)
152 fetal bovine serum (FBS, Gibco), plated onto a 150 cm^2 cell culture flask (Corning,
153 New York, NY, USA), and cultured at 37 °C under 5% CO_2 atmosphere. The purity of
154 ESCs was examined by separate immunostaining for the stromal marker vimentin
155 (EPR3776, Abcam, Cambridge, UK) and through flow cytometry (FCM) analysis.
156 Only cultures that contained more than 95% vimentin-positive cells were included in
157 our study, and cultured cells at 3–5 passages were utilized.

158 RAW264.7 macrophage cells were provided by Chengjiang Gao (Shandong
159 University, School of Medicine, Department of Immunology, Jinan, Shandong, China)
160 and cultured in T25 flasks containing DMEM (Gibco, Beijing, China) supplemented

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161 with 10% (v/v) FBS (Gibco) at 37 °C and 5% CO_2.

162 Isolation of exosomes from ESCs

163 Once 70% confluency was attained, ESCs were washed twice with PBS and cultured in
164 DMEM/F12 (Gibco, Beijing, China) with 10% exosome-free FBS
165 (EXO-FBS-250A-1,System Biosciences, Palo Alto, CA, USA) for 24 h. Exosomes
166 were isolated in accordance with the method reported in previous studies (Lo Cicero *et*
167 *al.*, 2015, Sun *et al.*, 2016, Wu *et al.*, 2018). The culture supernatant was centrifuged at
168 300 g for 10 min to remove cells. The supernatant obtained was centrifuged at 2000 g
169 for 10 min to remove dead cells and the supernatant was then refrigerated at 4 °C
170 overnight. The mixture of these supernatants was centrifuged at 10000 g and 4 °C for
171 30 min to remove cell debris and at 100000 g and 4 °C for 70 min to obtain exosomes.
172 The exosomes were washed by centrifugation at 100000 g and 4 °C for 70 min. The
173 supernatant was poured off, and the pellet was resuspended in 50 μL of PBS. The
174 exosomes were stored at –80 °C. Overall, all exosomes utilized in in vivo and in vitro
175 experiments in our study were isolated from 10 mice with endometriosis and 10 mice
176 without endometriosis. An average of 30 μg of the purified exosomes were obtained
177 from *c.* 3×10^6 ESCs. The isolated exosomes were characterized by transmission
178 electron microscopy, nanoparticle tracking analysis , and western blot.

179 Treatment of macrophages with ESC-derived exosomes

180 ESC-derived exosomes were labeled using a PKH67 Fluorescent Cell Linker Kit

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181 (Sigma-Aldrich, Saint Louis, MO, USA) to detect the direct transfer of the exosomes
182 into macrophages (RAW264.7). The purified exosomes were resuspended in PBS and
183 labeled with PKH67. PBS without exosomes was used as control. Internalization was
184 performed at 37 °C by incubating RAW264.7 cells with EMS-exosomes and
185 CON-exosomes. Fluorescent signals were detected after 24 h.

186 The effects of ESC-derived exosomes on macrophage (RAW264.7 cells) activation
187 and polarization were assessed in vitro. The macrophages were stimulated with
188 EMS-exosomes or CON-exosomes (10 μg/mL) for 24 h. The doses of exosomes were
189 determined according to previous studies (Deng *et al.*, 2009, Singh *et al.*, 2012, Zhao *et*
190 *al.*, 2017). After 24 h of co-culture at 37 °C, the cells were washed and collected for
191 immunofluorescence analysis and RNA harvesting.

192 Treatment of mice with ESC-derived exosomes

193 The mice were injected with EMS-exosomes or CON-exosomes (30 μg/mouse i.p.) on
194 day-7 according to previously reported methods (Deng *et al.*, 2009, Singh *et al.*, 2012,
195 Zhao *et al.*, 2017). The treatment was repeated at 8 p.m. every day **until the**
196 **endometriosis model was built.** Five mice from the EMS-exosome or CON-exosome
197 group were sacrificed for peritoneal macrophage detection on day-6, -4, -2, and 0. On
198 day 0, the mice were treated with exosomes and induced with endometriosis as
199 described above. Five mice from the experimental group or control group were
200 sacrificed after injection of endometrium on day 14. The flow diagram of the
201 experimental design is shown in Fig.1B.

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202 Immunofluorescent staining and hematoxylin-eosin staining

203 Primary ESCs and polarized RAW 264.7 macrophages were identified using
204 immunofluorescent staining. The cells were fixed in ice-cold methanol for 5 min,
205 washed twice with ice-cold PBS, and incubated for 10 min in PBS containing 0.25%
206 Triton X-100. The cells were then washed in PBS twice for 5 min and incubated with
207 1% bovine serum albumin (BSA) in PBS containing Tween-20 (PBST) for 30 min to
208 block nonspecific binding of the antibodies. The ESCs were incubated with
209 anti-vimentin antibody (1:400, EPR3776, Abcam, Cambridge, UK), and RAW 264.7
210 macrophages were incubated with anti-inducible NO synthase (iNOS) antibody
211 (1:100, Abcam, Cambridge, UK) and anti-CD206 antibody (1:200, 15-2, Abcam,
212 Cambridge, UK). The cells were diluted in 1% BSA in PBST in a humidified chamber
213 overnight at 4 °C then washed with PBS twice for 5 min and incubated with the
214 secondary antibody (1;500, Abcam, Cambridge, UK) in 1% BSA for 1 h at room
215 temperature in the dark. The secondary antibody solution was decanted and washed
216 with PBS twice for 5 min in the dark. The slides were mounted in the medium with
217 4′,6-diamidino-2-phenylindole (DAPI, Abcam, Cambridge, UK) for 1 min.

218

219 The 4 μm-thick sections of uterine and ectopic regions were dewaxed and rehydrated
220 in ethanol and water, for hematoxylin-eosin staining, the slides were stained with
221 hematoxylin (CWBIO, Beijing, China) for 5 min and eosin (CWBIO, Beijing, China)
222 for 2 min. For immunofluorescent staining, antigen retrieval was performed in citrate

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223 buffer (pH 6.0) for 15 min. The sections were washed three times in PBS.
224 Non-specific binding was blocked with 1% BSA in PBST for 30 min. The sections
225 were then incubated for 2 h at 37 °C with the primary antibodies, namely, E-cadherin
226 (1:200, R&D Systems, Minneapolis, MN, USA) or vimentin (1:200, EPR3776,
227 Abcam, Cambridge, UK), or IgG control antibodies. The sections were washed with
228 PBS three times and incubated at room temperature with Dylight 488-conjugated
229 donkey anti-rabbit secondary antibody (1:400, Abcam, Cambridge, UK) or Dylight
230 594-conjugated donkey anti-goat secondary antibody (1:400, Abcam, Cambridge, UK)
231 for 1 h. After the sections were washed, the cell nuclei were counterstained with
232 fluorescent mounting medium containing DAPI (Abcam, Cambridge, UK). Images
233 were obtained using a microscope (Olympus BX53, Olympus, Tokyo, Japan) fitted
234 with a digital camera (Olympus cellSens Standard, Olympus).

235 Quantitative real-time PCR

236 The co-cultured macrophages were washed with cold PBS. Total RNA was extracted
237 with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) in
238 accordance with the manual of the product owner. RNA (1 μg) was reverse transcribed
239 into cDNA with ReverTra Ace quantitative PCR RT Master Mix with gDNA Remover
240 (TOYOBO, Osaka, Japan). Each 20 μL of the PCR mixture contained 1× SYBR Green
241 PCR Master Mix (TOYOBO, Osaka, Japan), 30 ng of cDNA, and 300 nM of each
242 specific primer. The primers used for each gene are listed in Table I. Quantitative
243 RT-PCR was performed on an Applied Biosystems 7500RT-PCR System (Applied

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244 Biosystems, USA). The reactions were run in triplicate by using SYBR green gene
245 expression assays. The relative change was normalized to endogenous GAPDH mRNA
246 by using the formula $2^{-\Delta\Delta Ct}$.

247 FCM analysis

248 After treatment with the exosomes at different time points, mouse peritoneal cells
249 were collected on day-6, -4, -2, and 0 and divided equally into two parts for FCM
250 analysis of macrophage polarization and phagocytosis. After euthanizing the animals,
251 peritoneal cells were retrieved by peritoneal lavage with 7 mL (3.5 mL × 2) of ice-cold
252 washing buffer containing Advanced Roswell Park Memorial Institute medium. The
253 peritoneal cavity of each mouse was injected with 1640 medium (Gibco, USA) and 2%
254 heat-inactivated FBS (Gibco, Australia). After shaking the mice, peritoneal cells were
255 collected. In this study, F4/80 and CD11b were used as pan-macrophage markers
256 (Davies *et al.*, 2013). The markers iNOS and CD206 were used to detect M1 and M2
257 macrophages, respectively (Antonios *et al.*, 2013, Moore *et al.*, 2013, Murray *et al.*,
258 2014, Wang and Kubes, 2016). The peritoneal macrophages were incubated with 2%
259 paraformaldehyde for 10 min on ice before antibody staining to avoid adherence to the
260 tube wall. The cells were centrifuged at 400 g for 5 min and washed twice. The cell
261 suspensions were preincubated with anti-mouse CD16/32 monoclonal antibody
262 (1:100,14-016, eBioscience, USA) for 15 min on ice to block Fc receptors. The cells
263 were then incubated with allophycocyanin-conjugated anti-mouse F4/80
264 (1:25,17-4801,eBioscience,USA) and PerCP-Cyanine5.5-conjugated anti-mouse

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265 CD11b (45-0112, eBioscience, USA) at a concentration of 1:100 for 25 min on ice in
266 the dark. After centrifugation at 400 g, the cells were incubated in the
267 fixation/permeabilization buffer (BD Biosciences, USA) for 45 min on ice in the dark.
268 The cells were washed twice with 1×Perm/wash buffer (BD Biosciences, USA) and
269 incubated with Alexa Fluor 488-conjugated anti-mouse iNOS (53-5920, eBioscience,
270 USA) at a concentration of 1:1000 and with
271 phycoerythrin-conjugated anti-mouse CD206 (141706, BioLegend, USA) at a
272 concentration of 1:50 for 45 min in the dark. After washing with 1× Perm/wash buffer,
273 the cells were resuspended in PBS for FCM analysis.

274 A green zymosan phagocytosis assay kit was used (BioVision; Milpitas, CA, USA) to
275 detect macrophage phagocytic ability. Green zymosan was mixed with the
276 macrophages (in a ratio of 1:20) for 30 min under shaking at 37 °C. The unbound
277 zymosan particles were washed with PBS, and the macrophages were centrifuged for
278 FCM analysis.

279 Statistical analysis

280 All data are presented as mean ± SD. Comparisons among multiple groups (three or
281 more) were performed by one-way ANOVA with post hoc test. For comparison
282 between two groups, the two-tailed unpaired Student's t-test was used. Statistical
283 analysis was performed with GraphPad Prism 7.0 (La Jolla, CA, USA). In all
284 comparisons, a P-value less than 0.05 was considered statistically significant.

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285 # Results

286 Exosomes transfer from ESCs to macrophages

287 The morphology of ESCs from mice with endometriosis (EMS-ESCs) and mice
288 without endometriosis (CON-ESCs) were observed by microscopy and
289 immunofluorescent staining (Fig. 2A). EMS-exosomes from EMS-ESCs and
290 CON-exosomes from CON-ESCs showed similar characteristics of 100 nm diameter,
291 biconcave morphological features (Fig. 2B and C), and positive staining for exosomal
292 makers TSG101 and CD63 (Fig. 2D). To elucidate the role of exosomes in the ESC–
293 macrophage crosstalk, we used the RAW264.7 cell line as model of macrophages in
294 vitro. The exosomes were labeled with PKH67 and incubated with the macrophages.
295 ESC-derived exosomes with green fluorescence were observed in the macrophages
296 (Fig.2E). The results suggest the uptake of exosomes by the macrophages.

297 EMS-exosomes convert macrophages to M2-like phenotype and attenuate their
298 phagocytic ability

299 Immunofluorescence analysis was conducted to determine whether EMS-exosomes
300 may affect macrophage polarization. Treatment with EMS-exosomes markedly
301 suppressed M1-like macrophage polarization and enhanced M2-like macrophage
302 polarization, as shown in the immunostaining results with anti-iNOS and anti-CD206
303 (Fig. 3A). To confirm the effect of EMS-exosomes on macrophage polarization, we
304 assessed the transcriptional changes in specific M1 and M2 marker genes by

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305 quantitative RT-PCR. Macrophages treated with EMS-exosomes showed remarkable
306 decreases in the mRNA levels of M1-related iNOS, tumour necrosis factor-α, and
307 interleukin (IL)-12 and increases in the mRNA levels of M2-related arginase-1(Arg-1)
308 and IL-10 compared with those treated with CON-exosomes (Fig. 3B). The FCM
309 analysis showed that the phagocytic ability of the macrophages that absorbed
310 EMS-exosomes decreased compared with that in the control group (Fig. 3C). These
311 data indicate that EMS-exosomes attenuate the phagocytic ability of macrophages and
312 induce M2 macrophage polarization in vitro.

313 EMS-exosomes remodel phenotypes and phagocytic ability of mouse peritoneal
314 macrophages

315 . After treatment with exosomes at different points, the mice were sacrificed to collect
316 peritoneal macrophages for FCM analysis and detection of phenotypes and phagocytic
317 ability. After peritoneal injection of the EMS-exosomes, the total peritoneal
318 macrophages (F4/80+CD11b+ cells) expressed an increased percentage of M2
319 (CD206+) and a decreased percentage of M1 (iNOS). However, in the control group,
320 the percentage of M1 macrophages or M2 macrophages remained unchanged with
321 extended processing time (Fig.4A). Comparison of the percentages of M1 and M2 in
322 both groups showed that after treatment with exosomes for 3 days (Day -4), the
323 proportion of M1 cells showed opposite changes between the two groups. After
324 treatment with exosomes for 5 days (Day -2), the proportion of M2 cells in the
325 EMS-exosome group increased dramatically (Fig.4B and C). These data suggest that

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326 EMS-exosomes reprogramme peritoneal macrophages to the M2 subtype. We then
327 determined the phagocytic ability of the peritoneal macrophages through FCM
328 analysis. Pretreatment with EMS-exosomes attenuated the phagocytic capacity of
329 peritoneal macrophages compared with the control group (Fig.4D and E).

330 Treatment with EMS-exosomes increases the volume and weight of ectopic lesions and
331 promotes M2 macrophage infiltration

332 Following the treatment with exosomes, two uterine horns from a donor normal mouse
333 were fragmented and injected into the peritoneal cavity of two recipient mice (one from
334 the experimental group, and the other one from the control group). Body weight was
335 measured daily, and no significant change was observed. Typical endometriotic tissues
336 were observed 14 days after the peritoneal injection (Fig. 5A). The lesions were
337 collected 14 days after the endometriosis induction (Fig.5B). The total volume of the
338 lesions in EMS exosome-treated mice was higher than that in CON exosome-treated
339 mice (Fig.5C, p=0.012). The total lesion weight also significantly increased (Fig.5D,
340 p=0.014). However, no significant difference was observed in the total number of
341 lesions between EMS exosome-treated mice and CON exosome-treated mice (Fig.5E,
342 p=0.545). To assess whether EMS-exosomes can promote macrophage infiltration in
343 ectopic lesions of the peritoneal cavity, we subjected the lesions to immunofluorescent
344 staining with anti-E-cadherin. The macrophages were examined through
345 immunofluorescent staining with anti-CD206. The results reveal that after
346 pretreatment with EMS-exosomes, more M2 macrophages (CD206+ macrophages)

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347 primarily infiltrated the ectopic glands of the lesions (Fig.5F and G).

348 Discussion

349 In this study, we conducted in vitro and in vivo validation experiments to determine
350 the effects of EMS-exosomes in macrophages. Our current findings provide novel
351 insights for understanding the effects of exosomes on promoting endometriotic lesions
352 in a murine model by modulating macrophage phagocytic ability and phenotype
353 switch.

354 Exosomes are nano-sized membrane vesicles are secreted by most cells. These vesicles
355 contain proteins, nucleic acids, and lipids and exhibit pivotal roles in local and systemic
356 cell-to-cell communication (El-Andaloussi *et al.*, 2012, Piper and Katzmann, 2007,
357 Raposo and Stoorvogel, 2013, Thery *et al.*, 2002). In the research field of endometriosis,
358 exosomes have been rarely investigated. Harp *et al.* reported that exosomes derived
359 from endometriotic stromal cells have enhanced angiogenic effects in vitro (Harp *et al.*,
360 2016). Wu and his colleagues found that exosomes containing miR-214 derived from
361 ESCs inhibited endometriosis fibrosis (Wu *et al.*, 2018). These studies suggested an
362 important role of exosomes in the pathology of endometriosis. In the present study, we
363 focus on the potentially important role of exosomes shed by eutopic ESCs from a
364 murine model of endometriosis in modulating macrophage activation. Through in vitro
365 experiments, we observed that EMS-exosomes were internalized by macrophages,
366 which expressed high levels of CD206 and low levels of iNOS. In addition,
367 macrophage-related cytokine expression was detected by RT-PCR analysis. In our vivo

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368 experiments, administering EMS-exosomes increased the percentages of macrophages
369 expressing high levels of Arg-1 and IL-10 and low levels of iNOS, TNF-α, and IL-12.
370 These data suggest that EMS-exosomes can programme the macrophages to
371 differentiate into M2-like phenotype. The phagocytic ability of macrophages was
372 suppressed after treatment with exosomes derived from endometriosis. These results
373 suggest that ESCs from endometriosis secreted "abnormal" exosomes because they can
374 regulate the phenotype and attenuate the phagocytic ability of macrophages, which may
375 play a crucial role in the early survival of refluent endometrial tissues. These findings
376 led us to hypothesize that exosomes derived from endometriosis may contribute to the
377 development of the disease because they can change the immune condition.

378 Immune dysfunction, particularly the abnormal activation states of peritoneal
379 macrophages, in endometriosis has been detected in the pathogenesis of endometriosis.
380 Numerous immune cells in peritoneal fluid from women with endometriosis are
381 macrophages, which are involved in the pathogenesis and symptoms of the disease
382 (Tran *et al.*, 2009). Haber and his colleagues used liposomal bisphosphonate to deplete
383 peritoneal macrophages in a rat model of endometriosis; the rats showed a reduced
384 implantation rate, implant size, and weight (Haber *et al.*, 2009). Bacci *et al.* reported
385 that macrophages are alternatively activated in patients with endometriosis; in addition,
386 they found that alternately activated M2 macrophages in a mouse model of the disease
387 were required for growth and vascularization of lesions (Bacci *et al.*, 2009). Similar
388 results were also found in rhesus macaques (Smith *et al.*, 2012). Recent studies
389 demonstrated that alternately activated macrophages can infiltrate endometriotic

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390 lesions and promote angiogenesis (Bacci *et al.*, 2009, Capobianco *et al.*, 2011). These
391 data provide evidence that M2 macrophages play a vital role in the pathogenesis of
392 endometriosis. Furthermore, several lines of evidence suggest that
393 endometriosis-relevant macrophages exhibit poor phagocytic ability. Chuang *et al.*
394 found that impairment of the phagocytic ability of macrophages could be due to the
395 down-regulation of CD36 (Chuang *et al.*, 2010, Chuang *et al.*, 2009). However, the
396 mechanisms responsible for the abnormal condition of macrophages in endometriosis
397 remain unknown.

398 Despite more than a century of intensive research, the pathophysiology of
399 endometriosis remains unclear; the most accepted theory is that deciduous endometrial
400 fragments enter the peritoneal cavity and their subsequent proliferation and invasion
401 into the underlying tissues result in endometriotic lesions. Most women have a certain
402 degree of retrograde menstruation but some of them have endometriosis; as such, the
403 endometrium of women with endometriosis is thought to be abnormal and predisposed
404 to successful establishment of ectopic lesions (Carvalho *et al.*, 2011, Lee *et al.*, 2009,
405 Minici *et al.*, 2008). This characteristic change in the eutopic endometrium was also
406 found in experimental murine endometriosis. Ruiz et al. found that the autophagic
407 markers were generally decreased in eutopic endometria from endometriosis-induced
408 mice when compared with eutopic endometria of control mice (Ruiz *et al.*, 2016),
409 which were also found in women with endometriosis (Yang *et al.*, 2017). Lee, et al.
410 demonstrated that normal endometrium, when placed in an ectopic location to create
411 experimental endometriosis in mice, led to characteristic changes in gene expression in

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412 eutopic endometrium (Lee *et al.*, 2009). They found that the expression of Hoxa10,
413 Hoxa11, insulin-like growth factor binding protein-1 and progesterone receptor A
414 decreased in the eutopic endometrium in endometriosis-mice and these important
415 changes were consistent with patients with endometriosis (Gurates and Bulun, 2003,
416 Klemmt *et al.*, 2006, Wu *et al.*, 2005), which were also similarly altered in the eutopic
417 endometrium of baboons with induced endometriosis (Fazleabas *et al.*, 2003, Kim *et al.*,
418 2007). EMS-exosomes derived under a pathological condition are small enough to
419 cross the Fallopian tubes and easily enter the abdominal cavity. Macrophages, as the
420 most abundant cells in the abdominal cavity and the most easily altered by the
421 microenvironment, must respond to exosomes with information from abnormal
422 endometrial cells to remodel the abdominal microenvironment and thus influence
423 disease progression. As our study demonstrated, exosomes derived from endometriosis
424 can remodel the macrophage phenotype to M2 polarization and attenuate the
425 phagocytic ability of macrophages in vivo and in vitro. After treatment with
426 EMS-exosomes, the peritoneal macrophages showed decreased phagocytic ability and
427 a high percentage of M2, thereby contributing to the development of endometriosis and
428 increasing the total volume and weight. At the same time, we found that around the
429 ectopic lesions the number of infiltrating macrophages expressing CD206 (M2-like
430 macrophages) increased after treatment with EMS-exosomes. Hence, treatment with
431 EMS-exosomes significantly promoted macrophage recruitment into the ectopic
432 lesions, consistent with previously reported results (Bacci *et al.*, 2009).

433 This study presents several novel discoveries but still has limitations. Exosomes are a

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434 heterogeneous group of cell-derived membranous structures; as such, biogenesis,
435 including the generation, sorting, releasing, up taking, and fate, of exosomes in
436 recipient cells is very complex (van Niel *et al.*, 2018). The regulatory pathways
437 involved in the biogenesis and secretion of exosomes are not well defined (Thery *et al.*,
438 2002). In the present study, we focus on the effect of EMS-exosomes on macrophages,
439 which are a key regulator of endometriosis. Future studies should determine the
440 mechanisms of the involvement of EMS-exosomes in endometriosis development.
441 Exosomes can be isolated through several methodologies, including ultracentrifugation,
442 gradient ultracentrifugation, commercial kits, size-exclusion chromatography, and
443 field-flow fractionation (Shao *et al.*, 2018). Other new enrichment methods include
444 microfluidic filtering, contact-free sorting, and immunoaffinity enrichment. Given that
445 each isolation method has its own limitations, we chose the most widely used and the
446 gold standard approach according to an online questionnaire in October 2015 drafted
447 by the International Society for Extracellular Vesicles (Gardiner *et al.*, 2016). Existing
448 isolation methods must be further improved for research studies of exosomes.
449 Moreover, emerging technologies, such as microarray-based gene expression analysis
450 and proteomics analysis of exosomes, must be utilized to improve the efficiency and
451 quality of exosome research in endometriosis. In our study, we used the most widely
452 accepted biomarkers for two different populations of macrophages, namely, M1 and
453 M2, which are commonly recognized as the two ends of the spectrum of polarization
454 (Mosser and Edwards, 2008). Subtle subpopulations of plastic macrophages are also
455 present; as such, developing reliable biomarkers for each subpopulation is important to

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456 promote the implementation of "macrophage associated therapeutics.". In the present
457 study, we did not identify the precise molecules, especially the different cargos
458 potentially carried by exosomes that can modulate macrophages. Thus, further studies
459 must be conducted to clarify the exact mechanism and provide additional clinical data.

460 Collectively, our findings highlight a novel mode of communication between ESCs and
461 macrophages. Exosomes from endometriosis play a novel function in regulating
462 macrophage activation and polarization, which could lead to the development of
463 endometriosis.

464

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470 **Authors' roles**

471 Guoyun Wang conceived and designed the study. Huihui Sun analyzed and interpreted
472 the data and drafted the article. Huihui Sun, Ming Yuan, Qiuju Li, Ni li, and Qianwei
473 Zhen performed the experiments. All authors participated in the writing and revision of
474 the article.

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477 ## Conflict of interest

478 The authors report no conflict of interest.

479

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622

623 Figure legends

624

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625 **Figure 1** Flow diagram of the experimental design.

626 **(A).** A murine endometriosis (EMS) model was established by i.p. injection of
627 endometrial segments. On day 14, mice were sacrificed for endometrial stromal cells
628 (ESCs) and exosomes isolation. **(B).** Mice were treated with exosomes from eutopic
629 endometrium of mice with endometriosis (EMS-ESCs) and (for the control) without
630 endometriosis (CON-ESCs) for 7 days, and on day 0 the murine endometriosis model
631 was induced.

632 **Figure 2** Exosomes transfer from ESC to macrophages.

633 **(A).** EMS-ESCs and CON-ESCs observed under phase-contrast microscope and after
634 immunofluorescent staining of vimentin (green) in ESCs (scale bar=200 μm). **(B).**
635 Transmission electron microscopy image of the morphology of exosomes released by
636 ESCs in the culture supernatants (scale bar=200 nm). **(C).** Nanoparticle tracking
637 analysis result on the size distribution of ESC-derived exosomes. **(D).** Western blot of
638 ESC-derived exosomes and immunoblot of CD63 and TSG101. **(E).** Fluorescent
639 microscopy detection of the internalization of PKH-labeled exosomes (green) by
640 macrophages (scale bar=500 nm). DAPI: 4′,6-diamidino-2-phenylindole

641 **Figure 3** EMS-exosomes convert macrophages to M2-like phenotype and attenuate
642 their phagocytic ability.

643 **(A).** Immunofluorescence analysis of macrophage phenotype (iNOS: inducible nitric
644 oxide synthase, green; CD206, red; scale bar=500 nm). **(B).** Expression of M1

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645 macrophage-related markers [iNOS, tumour necrosis factor-α (TNF-α), interleukin
646 (IL)-12] and M2 macrophage markers [arginase-1 (Arg-1)and IL-10] in macrophages
647 after culturing with EMS-exosomes and CON-exosomes. mRNA expression of genes
648 normalized to that of GAPDH and given as relative change. **(C).** FCM detection of
649 macrophage phagocytic ability after treatment with CON-exosomes and
650 EMS-exosomes. n=3 independent experiments, performed in triplicate , *P<0.05,
651 **P<0.01, and ***P<0.001.

652

653 **Figure 4** EMS-exosomes remodel the phenotypes and phagocytic ability of mouse
654 peritoneal macrophages.

655 **(A).** Flow cytometry analysis for peritoneal macrophages of mice after i.p. injection of
656 EMS- exosomes and CON-exosomes at different points. **(B)** and **(C).** Phenotypic
657 switch of macrophages in the peritoneal cavity after treatment with EMS-exosomes and
658 CON- exosomes. **(D).** FCM analysis of the phagocytic ability of the macrophages after
659 treatment with EMS-exosomes and CON-exosomes. **(E).** Treatment with
660 EMS-exosomes attenuate the phagocytic ability of peritoneal macrophages. n=5,
661 *P<0.05, **P<0.01, and ***P<0.001.

662 **Figure 5** Treatment with EMS-exosomes increases the volume and weight of ectopic
663 lesions and promotes M2 macrophage infiltration.

664 **(A).** Validation of the mouse model of experimentally induced endometriosis. HE

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665 staining and immunofluorescent staining of E-cadherin (red) and vimentin (green) in
666 the uterus and ectopic endometrial tissues of mice. **(B).** Assessment of the lesions in the
667 mouse model of endometriosis. **(C)** and **(D).** Total volume and weight of lesions
668 collected on day 14 increased after treatment with EMS-exosomes. **(E).** Total number
669 of the lesions treated with EMS-exosomes and CON-exosomes collected on day 14
670 after disease induction. **(F)** and **(G).** Infiltrating macrophages expressing CD206 (M2
671 macrophage-related markers) increased in number in ectopic tissues after treatment
672 with EMS-exosomes. n=5, *P<0.05, **P<0.01, and ***P<0.001.

673 HE: hematoxylin-eosin staining

674

675

676

677

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Table I Primer sequences for each gene analysed by RT-PCR.

iNOs	Forward (5' to 3')	TCCTGGACATTACGACCCCT
iNOs	Reverse (5' to 3')	CTCTGAGGGCTGACACAAGG
TNF-α	Forward	CCAGACCCTCACACTCAGATCATC
TNF-α	Reverse	GCGTAGACAAGGTACAACCCATCG
IL-12	Forward	TGTGGAATGGCGTCTCTGTC
IL-12	Reverse	GGCGGGTCTGGTTTGATGAT
Arg-1	Forward	GACCACAGTCTGGCAGTTGG
Arg-1	Reverse	TACGTCTCGCAAGCCAATGT
IL-10	Forward	TAGAAGTGATGCCCCAGG
IL-10	Reverse	TCATTCTTCACCTGCTCCACTGC

679 iNOS: inducible nitric oxide synthase, TNF-α: tumour necrosis factor-α, IL: interleukin,
680 Arg-1: arginase-1
681

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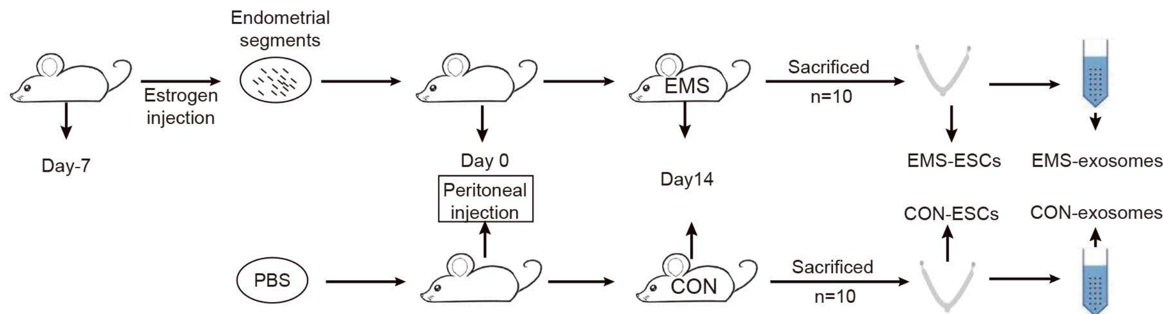

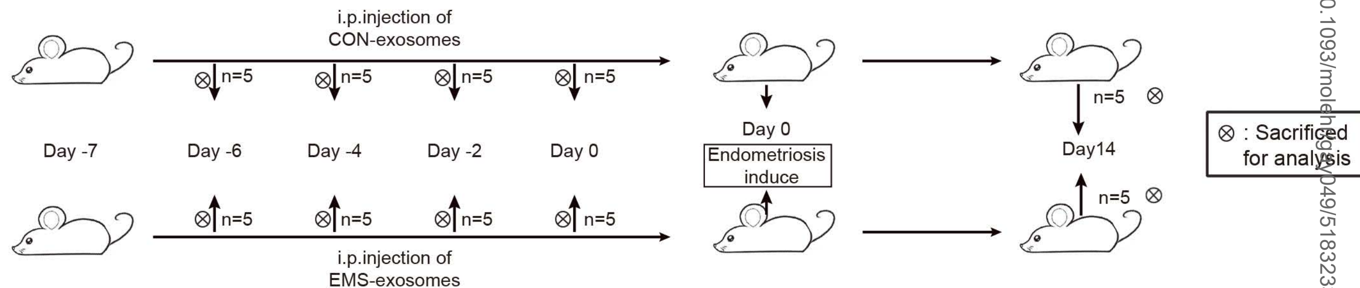

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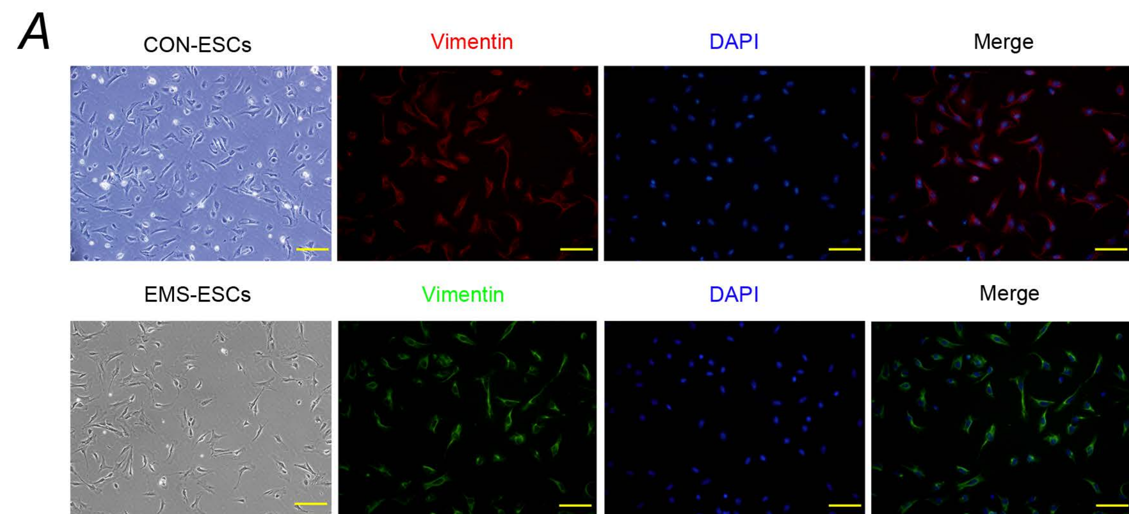

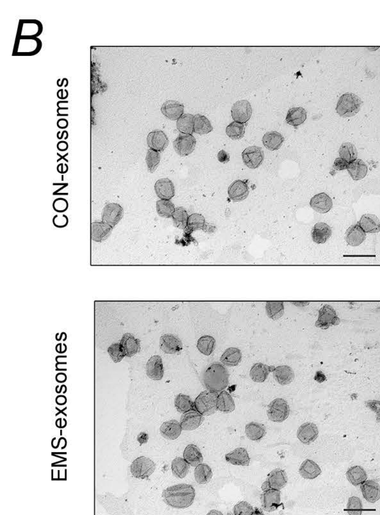

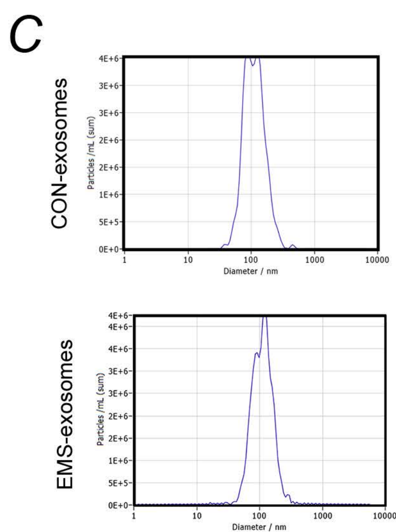

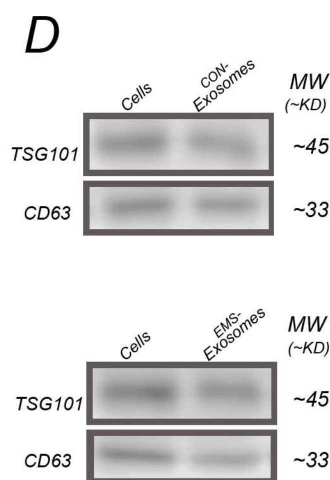

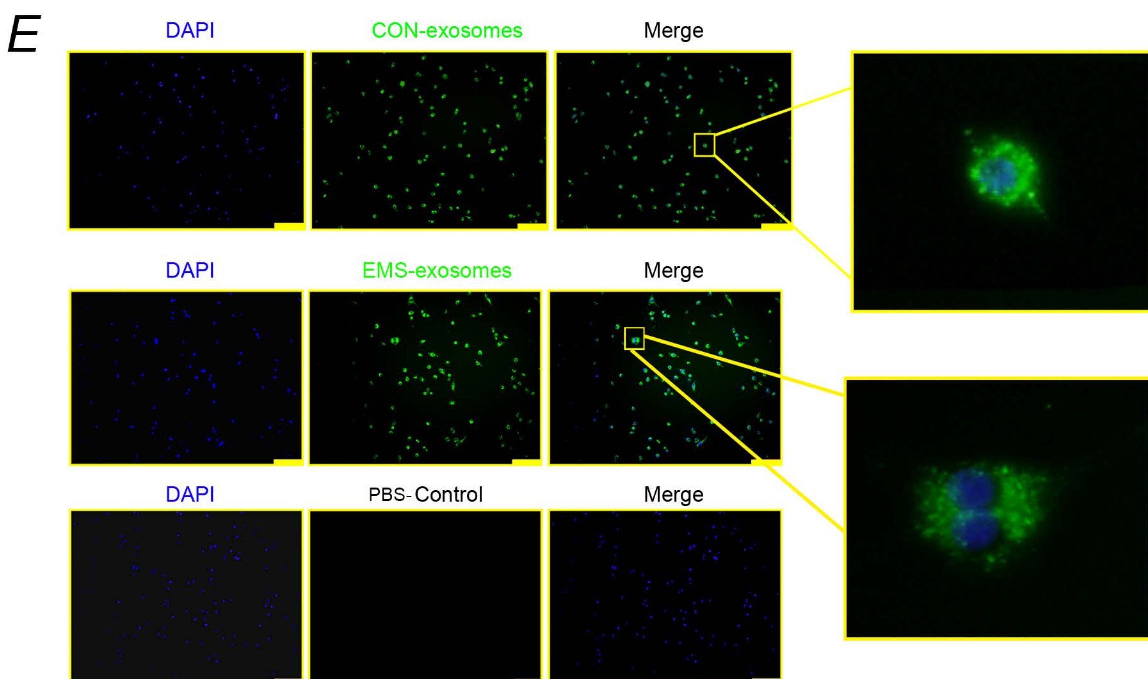

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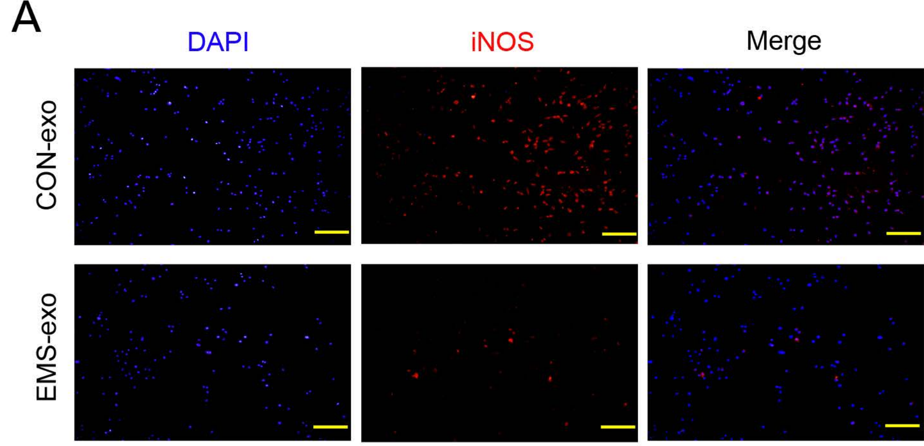

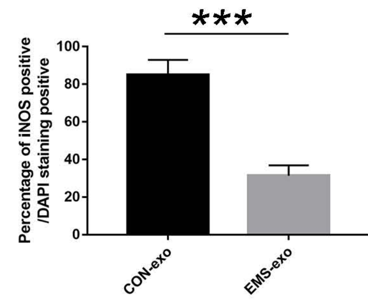

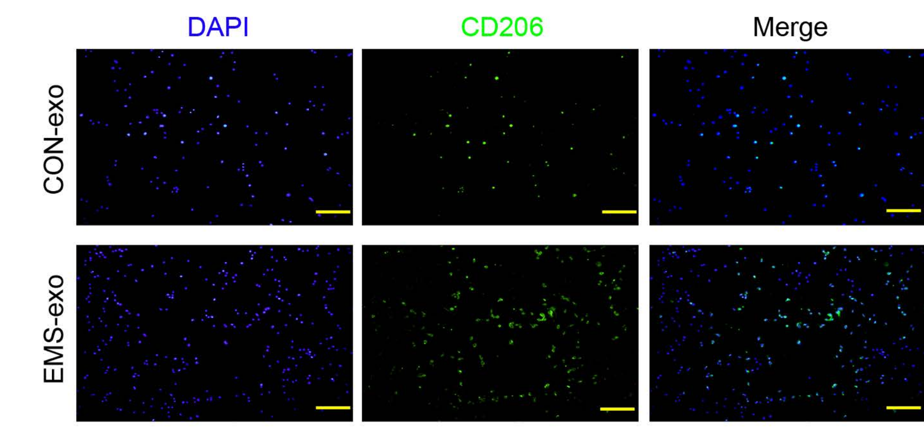

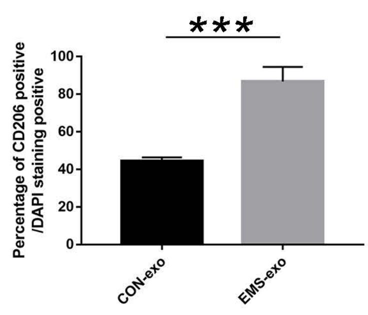

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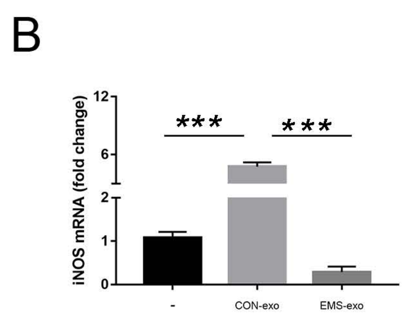

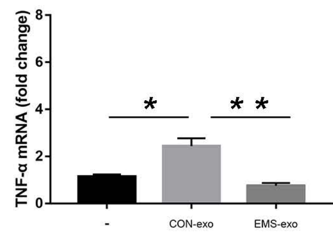

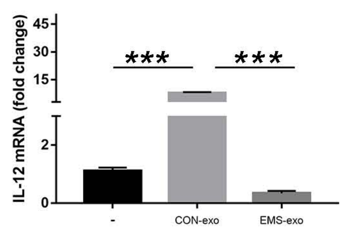

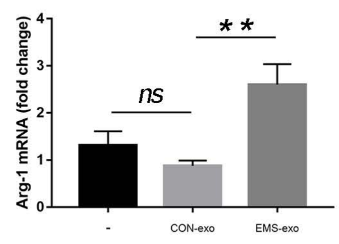

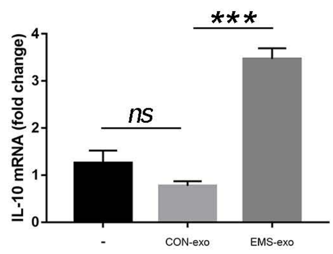

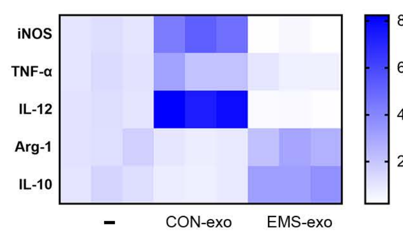

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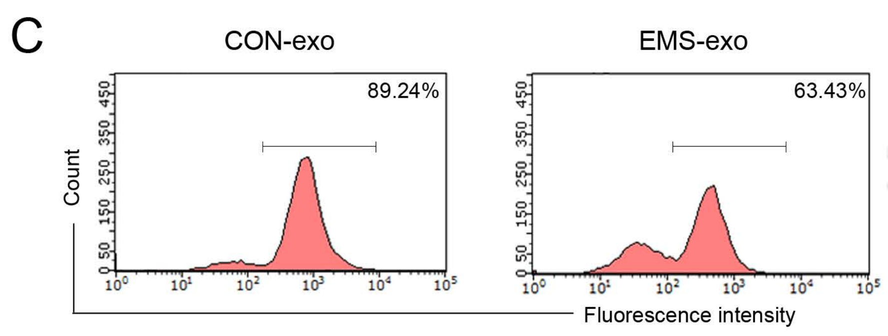

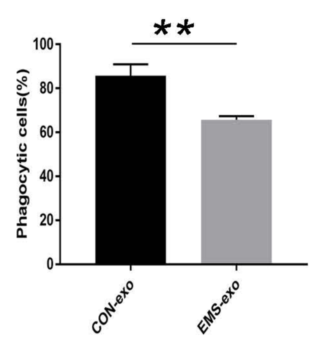

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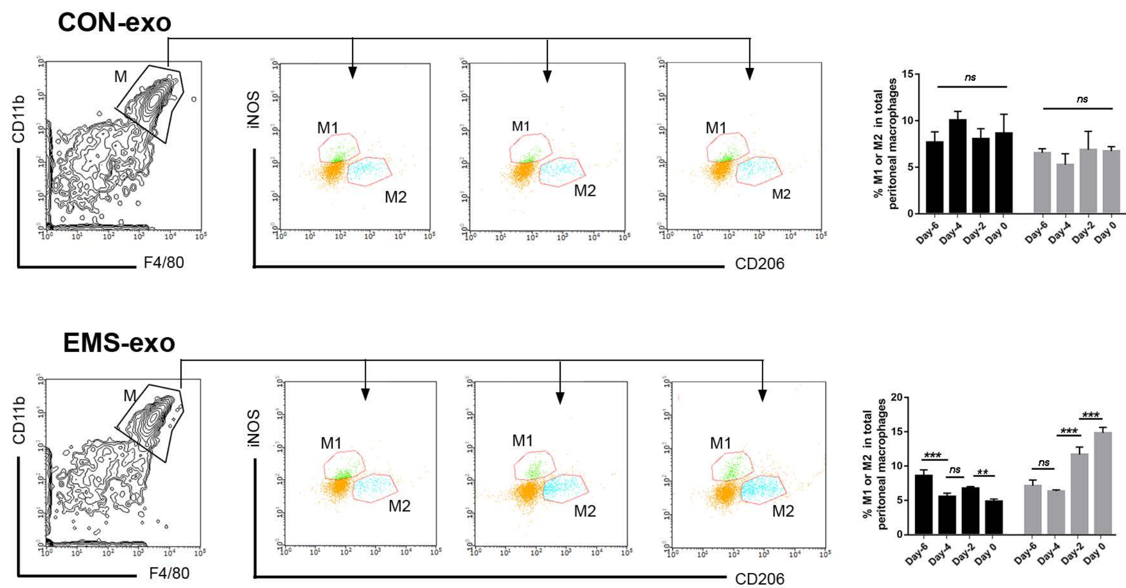

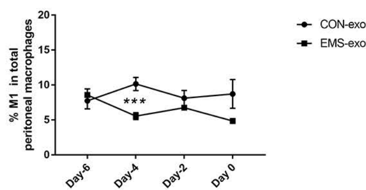

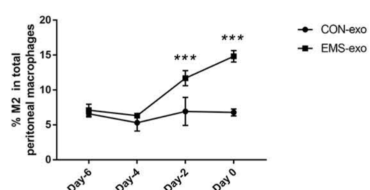

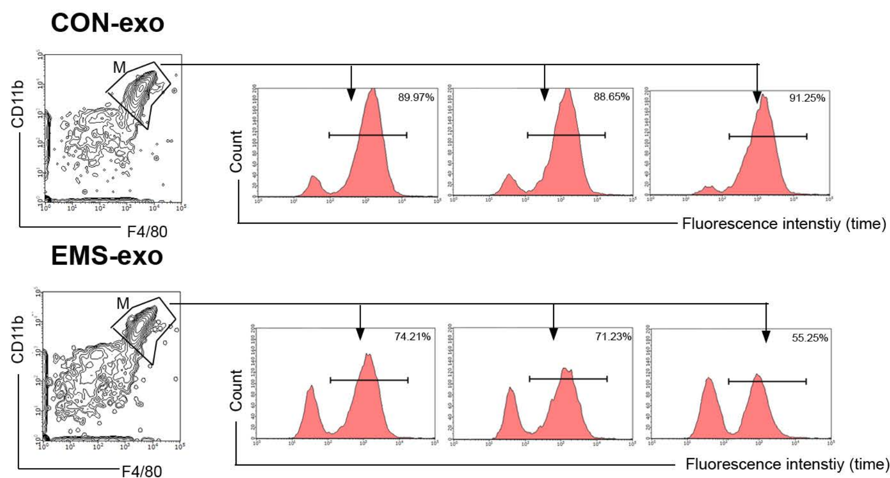

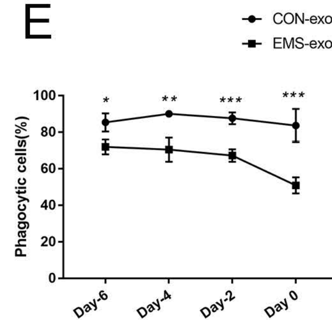

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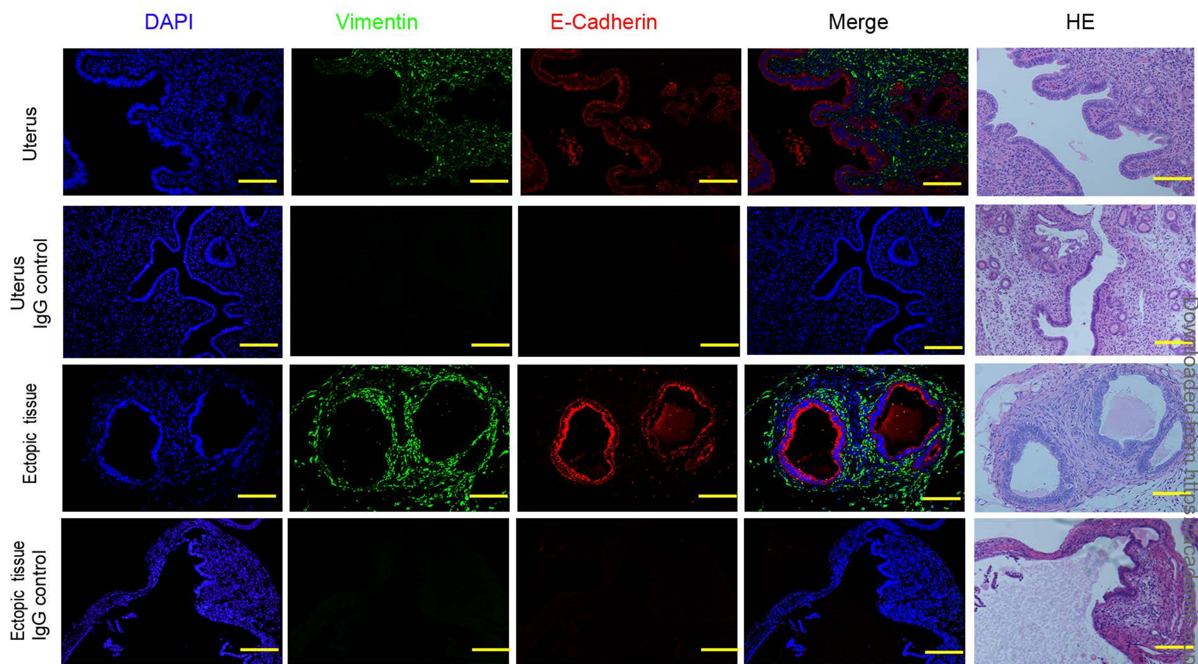

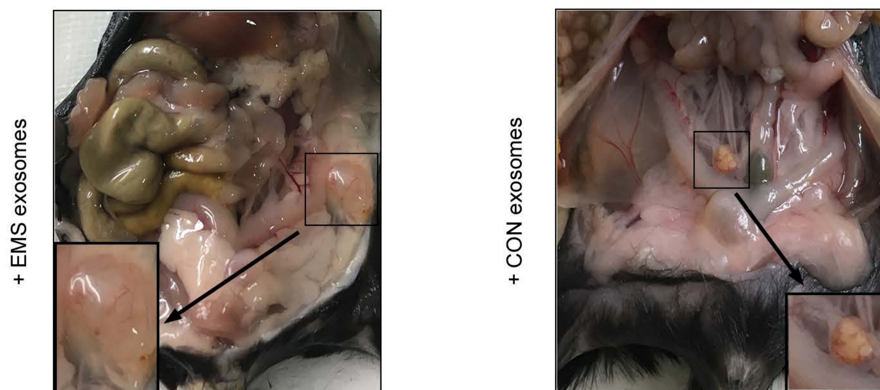

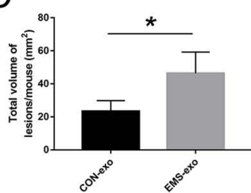

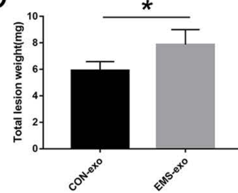

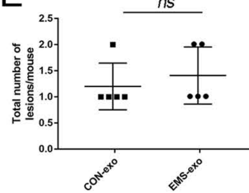

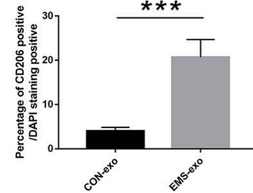

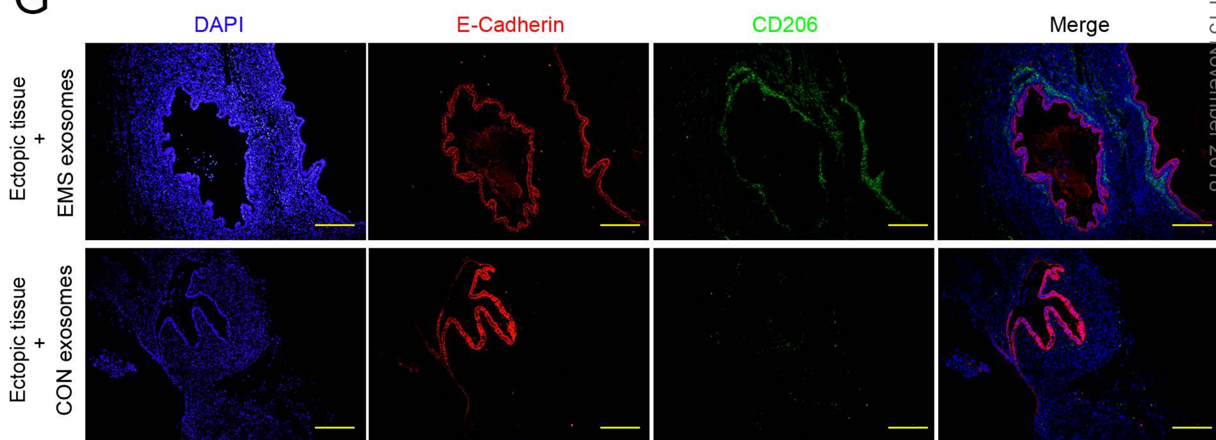

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