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Title

- **Complement Component 3 expressed by the endometrial ectopic tissue is involved in the endometriotic lesion formation through mast cell activation**

Short Title:

- Role of complement component 3 in endometriosis

Authors

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Summary: C3 produced by the endometriotic tissue is involved in the lesion development through mast cell activation

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34 **Abstract**

35 The pathophysiology of endometriosis (EM) is an excellent example of immune
36 dysfunction, reminiscent of tumor microenvironment as well. Here, we report that an interplay
37 between C3 and mast cells (MCs) is involved in the pathogenesis of ectopic EM. C3 is at the
38 epicenter of the regulatory feed forward loop, amplifying the inflammatory microenvironment, in
39 which the MCs are protagonists. Thus, C3 can be considered a marker of EM and its local
40 synthesis can promote the engraftment of the endometriotic cysts. We generated a murine model
41 of EM via injection of minced uterine tissue from a donor mouse, into the peritoneum of the
42 recipient mice. The wild type mice showed greater amount of cyst formation in the peritoneum
43 compared to C3 knock-out mice. This study offers an opportunity for novel therapeutic
44 intervention in EM, a difficult to treat gynecological condition.

45

46 **MAIN TEXT**

47

48 **Introduction**

49 Endometriosis (EM) is a chronic condition that affects about 5-10% of women in
50 reproductive age. EM is characterized by pain and infertility as a consequence of the presence of
51 functional endometrial tissue outside the uterine cavity (*1*). The most common locations for the
52 ectopic implants are the ovaries, peritoneum, and the utero-sacral ligaments. The presence of
53 ectopic tissue in these areas induces a condition of chronic inflammation. Current evidence
54 suggests that immune dysfunction is the most likely causative factor for the EM pathogenesis. In
55 particular, the pathways involved in immune-cell recruitment, cell-adhesion, and inflammatory
56 processes encourage the implantation and survival of endometriotic lesions. Recently, it has been
57 shown that the complement system is one of the most predominant pathways altered in EM (*2*).

58 The complement system is an important part of the innate immunity and acts as a bridge
59 between innate and acquired immune system. It is involved in host defense against infectious
60 agents and altered self. Three different molecules are responsible for the recognition phase of
61 complement: C1q, Mannose-Binding Lectin (MBL), and C3. The binding of the recognition
62 molecules to the target ligands initiate the three different complement pathways: the classical (via
63 C1q), alternative (via C3) and lectin (via MBL) pathway (*3, 4*). The three pathways eventually
64 converge in the formation of the C3 and C5 convertases. This then results in the generation of the
65 main effector molecules of the complement system: the opsonins C3b and C4b, the
66 anaphylatoxins C3a, C4a and C5a, and the Membrane Attack Complex (MAC) that causes the
67 target cell lysis. The small complement fragments, anaphylatoxins, cause local inflammatory
68 responses by acting, for instance, on mast cells (MCs), inducing an increase in blood flow,
69 vascular permeability, and leukocyte recruitment (*5, 6*).

70 The current consensus is that EM involves a local pelvic inflammatory process with
71 altered function of immune-related cells in the peritoneal environment. Recent studies suggest
72 that the peritoneal fluid (PF) of women with EM contains an increased number of activated
73 macrophages that secrete various humoral mediators locally, including growth factors, cytokines
74 and possibly, oxidative products (*7*), leading to the development and progression of EM and EM-
75 associated infertility. Although the contributions of specific immune cell subsets and their
76 mediators to the onset and the course of the inflammatory process in endometrial lesions are still
77 poorly understood, evidence suggests that MCs are crucially involved in the inflammatory process
78 associated with EM. High numbers of degranulated MCs have been found in endometriotic
79 lesions (*8-13*).
80 Complement component C3 is expressed by the epithelial cells found in endometriotic tissue (*14-*
81 *16*). In this study, we examined differential expression of C3 in eutopic and ectopic tissues

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derived from EM lesions and its consequences in the pathogenesis of EM using human EM tissues and C3 gene-deficient mice.

Results

C3 is present in ectopic, but not in eutopic, endometrium and is locally expressed by endometriotic cells

We initially confirmed the presence of C3 in human endometriotic tissue sections by immunofluorescence (IF, fig. S1) and by immunohistochemical assays (Fig. 1), in uterine and ectopic endometrium. IHC, using anti-human C3 polyclonal antibody, showed moderate cytoplasmic expression of C3 by endometrial stromal cells, and rarely in some glandular epithelial cells in proliferative as well as secretory endometrium (Fig.1, A and B). A strong signal was present in adnexal and peritoneal EM (Fig. 1, C to G). C3 was found to be widely distributed in the EM tissue, with variable intensity; it was mostly localized in the glandular-like structures and in the vessels.

In order to investigate the local synthesis of C3, total RNA was isolated from EM cysts and C3 gene expression was analyzed by RT-qPCR. Ectopic EM tissues expressed high levels of C3 transcripts compared to the eutopic normal endometrium (Fig. 2A). To assess the contribution of EM epithelial cells to the local C3 production, we isolated EM epithelial/stromal cells from human EM cysts, which showed the characteristic epithelial phenotype (Fig. 2B); ~98% of the cells were positive for the epithelial marker, Cytokeratin 8/18 (fig. S2). We analyzed their expression of C3 by RT-qPCR using HepG2 (hepatocyte cell line) as a calibration control. AN3CA cells were used to assess the expression of C3 by normal epithelial endometrial cells. Because the IHC analysis revealed a marked positivity in the EM vessels, the expression of C3 was also investigated in endometriotic endothelial cells (EEC) and corresponding eutopic cells (uterine microvascular endothelial cells; UtMEC). We observed a very low expression of C3 transcript by both endothelial cell types (fig. S3). Interestingly, EM epithelial/stromal cells expressed a higher amount of C3 compared to AN3CA and HepG2 cells (Fig. 2C). In addition, these cells secreted the C3 protein in the culture supernatant, as measured via ELISA (Fig. 2D). To establish the specificity and exclusivity of C3 expression, we also measured C5 mRNA level; we could not detect C5 transcripts either in the EM tissue or cultured EM epithelial cells (data not shown).

Pro-inflammatory stimuli induce C3 expression by normal endometrial cells, in an in vitro model of EM

Because the ectopic endometrial microenvironment is characterized by inflammation, we set up an in vitro model of EM, stimulating the endometrial cell line, AN3CA, with pro-inflammatory cytokines. Our results demonstrated that normal endometrial cells under resting conditions were unable to produce C3; however, when stimulated with TNF-α, and to a lesser extent with IL-1β, cells started to express C3 mRNA (Fig. 3A), and secrete an increased level of C3 protein, as measured by ELISA (Fig. 3B) as well as by western blot analysis (Fig. 3, C and D). We demonstrated also that the stimulation of C3 transcription induced by TNF-α was dose-dependent (fig. S4).

$C3^{-/-}$ mice are refractory to developing EM cysts in a syngeneic in vivo model of EM

To further investigate the role of C3 in EM pathogenesis, we set up a syngeneic model of EM in C57BL/6 WT or C3 gene-deficient ($C3^{-/-}$) mice. Estrus was induced in donor animals via

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128 administration of estradiol. Then, the minced uterus of the donor mouse was injected in the
129 peritoneum of 2 recipient mice (Fig. 4A). After 3 weeks, the animals were sacrificed, and the
130 peritoneal cysts were counted. WT mice developed a higher number of cysts compared to C3
131 deficient mice (Fig. 4B), indicating that lack of C3 could prevent the EM cyst formation.

132

133 Peritoneal washing isolated from WT mice with EM present more degranulated MCs 134 compared to $C3^{-/-}$ mice

135 We then analyzed the peritoneal washing of WT and $C3^{-/-}$ mice for the presence of
136 infiltrating leukocytes. The samples were cytocentrifuged and the cells were stained with Giemsa.
137 A high number of leukocytes was observed in the peritoneal washing of both WT and $C3^{-/-}$ treated
138 mice. The number of infiltrating leukocytes was comparable with those observed in WT untreated
139 (CTRL Untr) mice. Surprisingly, the fluids collected from the WT animals contained a lower
140 number of MCs, compared to the $C3^{-/-}$ mice (Fig. 4, C and D). Next, we stained the
141 cytocentrifuged leucocytes with a monoclonal antibody to CD117 (a marker of mature MCs). The
142 peritoneal washing collected from the WT mice contained CD117 positive cells as well (Fig. 4E).
143 We wanted to confirm if the MCs present in WT peritoneal washing were degranulated. We thus
144 measured the levels of MC enzyme, tryptase, in the murine peritoneal washing (Fig. 4F). The
145 ELISA for tryptase demonstrated that WT mice with EM had a higher amount tryptase compared
146 to $C3^{-/-}$ or CTRL mice.

147

148 C3a level is higher in the PF of EM patients, which can act on EM MCs

149 C3a is one of the most important stimuli for MC activation. Thus, we measured the levels
150 of the C3a in the PF of EM patients, and compared them with those obtained from myoma and
151 fibroma patients (women undergoing laparoscopy, without alterations of peritoneal cavity
152 environment). Our results showed that the PF of EM patients had significantly higher level of C3a
153 compared to the control patients (Fig. 5A).

154 We next considered that the C3a present in the peritoneal cavity of EM patients could
155 stimulate the MCs within the EM lesions. We therefore stained human EM tissue sections with
156 toluidine blue to highlight MCs present in the tissue. The histochemical analysis confirmed that
157 EM lesions were rich in MCs (Fig. 5, B to D), compared to normal endometrium (fig. S5); the
158 IHC for C3a on the same sections corroborated the presence of this anaphylatoxin as well (Fig. 5,
159 E and F).

160

161 C3a is involved in an auto-amplifying loop of inflammation between MCs and EM 162 cells

163 In order to reconstruct the cross-talk between EM cells and MCs, we set up a co-culture
164 assay, seeding endometrial cell line (AN3CA) at the bottom of a 24-well plate and placing the
165 HMC-1 (a MC cell line) onto the upper chamber of a transwell system (1 μm diameter pores). To
166 understand the role of C3a in this cross-talk, the co-culture was also performed in the presence of
167 a pool of EM-PF and/or a blocking anti-C3a antibody. AN3CA cells, cultured with HMC-1
168 without any other stimulus, expressed low levels of C3. When HMC-1 cells were stimulated with
169 EM-PF in the co-culture, AN3CA cells began to express higher quantities of C3 (Fig. 6A).
170 Surprisingly, AN3CA cells, stimulated with only EM-PF (in absence of HMC-1), were not able to
171 express C3; they produced C3 only in response to EM-PF stimulated HMC-1, or to TNF-α (used
172 as a positive control). Pre-incubation of EM-PF with a blocking monoclonal anti-C3a antibody
173 completely abrogated the effect of EM-PF stimulation, bringing it to resting values (Fig. 6B).

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174 With a view to gauge a potential novel immunotherapeutic approach for EM, we
175 investigated in our co-culture model the effect of anti-TNF-α antibodies. Infliximab and
176 Adalimumab (both in clinical use) showed a strong effect in blocking C3 expression by AN3CA,
177 whereas Certolizumab did not block significantly the effects induced by the EM-PF on HMC-1
178 cells (Fig. 6C).
179
180

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181 **Discussion**

182 There is sufficient evidence for the expression of complement components and regulators
183 in human endometrium (*17-20*). Dysregulation of the complement system is largely considered to
184 play an important role in the pathogenesis of EM (*2, 21, 22*). The importance of the complement
185 proteins in EM was highlighted recently by high-throughput studies (*2, 21-23*). The complement
186 system appears to dominate the chronic inflammation in EM and remains a precipitating factor in
187 EM-associated ovarian cancer. Complement factors C3, C4A, C7, factor D, factor B, factor H,
188 and mannose-associated serine protease 1 (MASP1), are differentially expressed in EM compared
189 to normal uterine tissues (*2*). Furthermore, single-nucleotide polymorphisms in the C3 gene show
190 association with an increased risk of EM and EM-associated infertility (*15*).

191 The presence of C3 in EM tissue was first highlighted in 1980 (*24*). Subsequent studies
192 confirmed the presence of higher levels of C3 in the EM lesions (*2, 14-16, 21, 23, 25-28*).
193 However, how C3 contributes to the pathogenic mechanisms in EM has not been elucidated yet.
194 We show, in this study, the presence of C3 in eutopic and ectopic endometrium (Fig. 1); a strong
195 signal was observed in EM tissue (Fig 1, C to F). C3 was found to be widely distributed in the
196 EM lesions with variable intensity, being mostly localized in the glandular-like structures and in
197 the vessels.

198 C3 appeared to be produced locally and in higher amounts in the ectopic tissues compared
199 to the normal uterus (Fig. 2A). EM cells, isolated from ovarian cysts of women of fertile age,
200 produced C3 in culture (Fig. 2, C and D) (*22, 26, 27*). We also sought to investigate the factors
201 produced by endometrial implants located in the abdominal cavity, which influenced the C3
202 production, and consequent inflammatory microenvironment in the uterine endometrium (*25*).

203 A potential candidate for the upregulation of C3 expression by endometrial cells is the PF
204 rich in pro-inflammatory factors (*29*). PF TNF-α concentrations are elevated in EM patients; its
205 higher concentration correlates with the severity and stage of the disease (*30-32*). Furthermore,
206 TNF-α and IL-1β are known to be elevated in EM milieu (*29, 33*). The stimulation of endometrial
207 cells with TNF-α and IL-1β induced the production of C3. Surprisingly, the stimulation of
208 endometrial stromal cells with EM-PF did not cause increased C3 gene expression, indicating that
209 the concentration of TNF-α in the PF (around 50 pg/ml) was not sufficient for a direct
210 endometrial cell activation (*31*). We wondered what the source of higher concentration of TNF-α
211 in the endometrial tissue could be. A partial explanation came from the study of $C3^{-/-}$ mice. We
212 observed that WT mice developed a higher number of cysts and presented degranulated MCs in
213 PF, as demonstrated by the presence of a considerably higher amount of tryptase, compared to $C3^{-/-}$
214 mice (Fig. 3). Thus, endometrial cells under pro-inflammatory stimuli express C3; the pro-
215 inflammatory milieu can induce the generation of C3a, which in turn, can activate the MCs.
216 Activated MCs can release histamine, in addition to secreting TNF-α (and a host of inflammatory
217 factors).

218 One of the possible causes of complement activation in the EM microenvironment is the
219 setting off of the coagulation cascade, which is caused by the typical periodic bleeding in the EM
220 tissue. Thrombin cleaves C3 to C3a and C3b; activated platelets are also involved in C3 cleavage
221 (*34*). Another activator of the C3 is heme that is released from hemoglobin during hemolysis;
222 heme induces deposition of C3 fragments on the erythrocytes (*35*). Alternative pathway activation
223 can occur through its up-regulator, properdin, binding to activated platelets promoting C3(H_2O)
224 recruitment and complement activation (*36*). In addition, stimulation of endothelial cells by C3a
225 or other factors promptly induces expression of P-selectin, which by binding to C3b, induces the
226 formation of C3 convertases (*37*).

227 We confirmed the presence of higher levels of C3a in the EM-PF compared to control
228 non-EM healthy women (Fig. 5A). The presence of complement component (C4) and

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229 complement activation products (C3c SC5b-9) in the PF and in the sera of EM women have been
230 previously described (*38*). Significantly higher PF levels of C1q, MBL and C1-INH in women
231 with EM compared to control group has also been reported (*39*).

232 C3a present in the EM-PF can act, through C3aR interaction, which is abundantly
233 expressed on the MCs present in the EM tissue (*9, 25*). The involvement of MCs in the EM lesion
234 formation was previously investigated. A recent study showed that numbers of MCs in total as
235 well as activated MCs were clearly increased in the EM lesions in both animals and humans.
236 Thus, the use of MC stabilizers and inhibitors may prove to be effective in treating EM and its
237 associated pain (*40*). An increased presence of activated and degranulated MCs in deeply
238 infiltrating EM and its close histological relationship with nerves strongly suggests that MCs
239 contribute to the development of pain and hyperalgesia in EM, possibly by a direct effect on the
240 nerve structures (*10*). A diffuse infiltration of numerous MCs was observed throughout the
241 stromal lesions, which exhibited degranulation; scattered granules were also observed. In the
242 eutopic endometrium and normal uterine serosa of the EM patients and the controls, MCs were
243 rarely detected (*11*). We identified MCs in the EM cyst tissues (Fig. 5, B to D). Very few cells
244 were localized in the endometrial stroma, which is characteristic of endometrial gland-like
245 regions. A good proportion of MCs were localized around blood vessels and the interstitium with
246 fibrosis, that is, the fibrotic interstitium of endometrial cysts. MCs thus are likely to be involved
247 in the development and progression of EM. Localization of MCs suggests its particularly close
248 relationship with fibrosis and adhesion (*41, 42*).
249 In conclusion, we demonstrated that the complement component C3 is locally synthesized by
250 ectopic endometrial tissue, while the normal uterine endometrium does not express C3. Normal
251 endometrial cells, under pro-inflammatory stimuli, produce C3. C3 gene-deficient mice present
252 less endometriotic lesions in a syngeneic EM mouse model. C3a seems to recruit massive MCs in
253 the EM lesions, which can have a damaging pathogenic consequence in the ectopic EM. C3 seems
254 central to a regulatory feed forward loop, and is able to amplify the inflammatory
255 microenvironment, in which the MCs are protagonists. This study opens up a new window for the
256 identification of new targets for treating EM.
257

258 **Materials and Methods**

259 **Patients.**

260 Patients and control women were enrolled at the Institute for Maternal and Child Health,
261 IRCCS Burlo Garofolo, Trieste, Italy. The study group (EM) consisted of a total of 7 women,
262 diagnosed with moderate/severe and minimal/mild EM, according to the revised criteria of the
263 American Society for Reproductive Medicine ASRM (*43*).

264 **Primary cell isolation and culture**

265 Endometriotic cells and endothelial microvascular uterine cells (UtMEC) were isolated, as
266 described earlier (*44*).

267

268 **Murine model of EM**

269 $C3^{-/-}$ mice were kindly provided by Prof. Marina Botto, Centre for Complement and
270 Inflammation Research, Department of Medicine, Imperial College, London (UK) and generated
271 as described previously (*45*). The mouse model of EM was adapted from Somigliana and Mariani
272 (*46, 47*).

273 **Gene expression analysis**

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RNA was extracted from cells using the kit supplied by Norgen Biotek Corp. (Aurogene, Rome, Italy) according to the manufacturer's instructions and reverse transcripted to cDNA through SuperMix kit (Bioline). qPCR was carried out on a Rotor-Gene 6000 (Corbett, Qiagen, Milan, Italy) using SYBR™ Green PCR Master Mix (Applied Biosystems, Milan, Italy). Supplementary Table S1 shows the primers used for RT-qPCR. The melting curve was recorded between 55°C and 99°C with a hold every 2s. The relative amount of gene expression in each sample was determined by the Comparative Quantification (CQ) method supplied as a part of the Rotor Gene 1.7 software (Corbett Research) (*48*). The relative amount of each gene was normalized with 18S and expressed as arbitrary units (AU), considering 1 AU obtained from HepG2 cells used as a calibrator.

Measurement of C3a

The levels of C3a in PF from EM patients and C3 in cell culture supernatant were evaluated by ELISA kits purchased from Quidel.

Co-culture and cell stimulation

A confluent 24 well-plate of AN3CA cells was stimulated overnight (ON) with 100ng/mL of TNF-α, or 5ng/mL of IL-1β (both from PeproTech, ListerFish, Milan, Italy), or 10% of pooled EM-PF. For the co-culture study, the cells were seeded at confluence, in the lower part of a 1µm pore TW system (Corning, Milan, Italy), whereas the HMC-1 ($2x10^5$/TW) was present in the upper part, in the presence of 10% of pooled EM-PF, with or without blocking anti-human C3a (MyBiosource, Aurogene, Milan, Italy; 20µg/mL). Subsequently, the cells were lysed for RNA extraction, the supernatant recovered and stored at -80°C.

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Competing interests: The authors declare no competing interests

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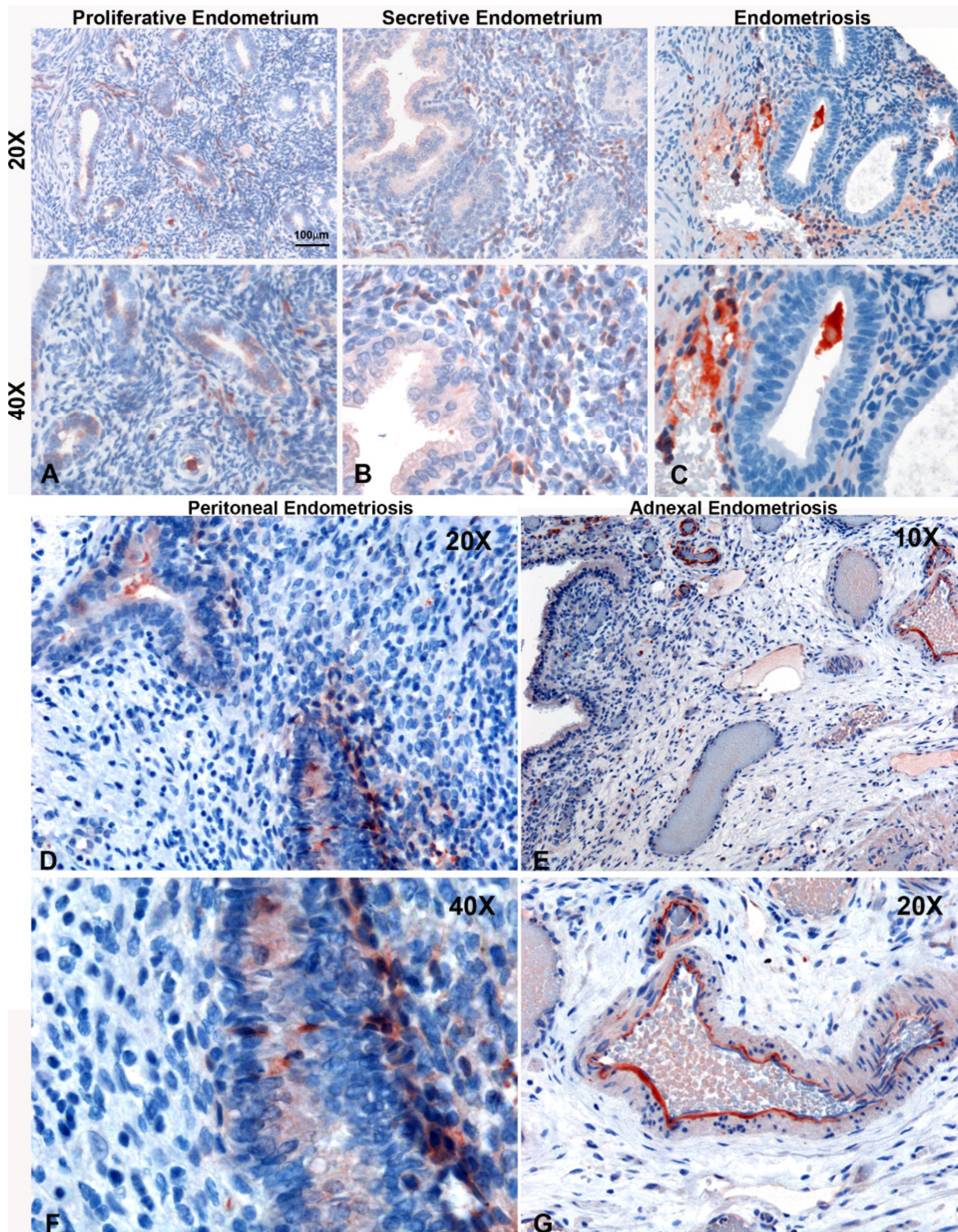
436 ## Figures and Tables



437

438 **Fig. 1. C3 expression by human eutopic and ectopic endometrial tissue.** Representative
439 microphotographs showing expression of C3 by IHC in proliferative normal endometrium (A),

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440 secretive normal endometrium (B), and endometriotic tissue sections (C). In the lower panel: C3
441 IHC on abdominal wall (D and F) and adnexal (E and F) endometriosis (EM). AEC (red)
442 chromogen was used to visualize the binding of anti-human C3 antibodies. Nuclei were
443 counterstained in blue with Harris Hematoxylin; scale bars, 100μm.

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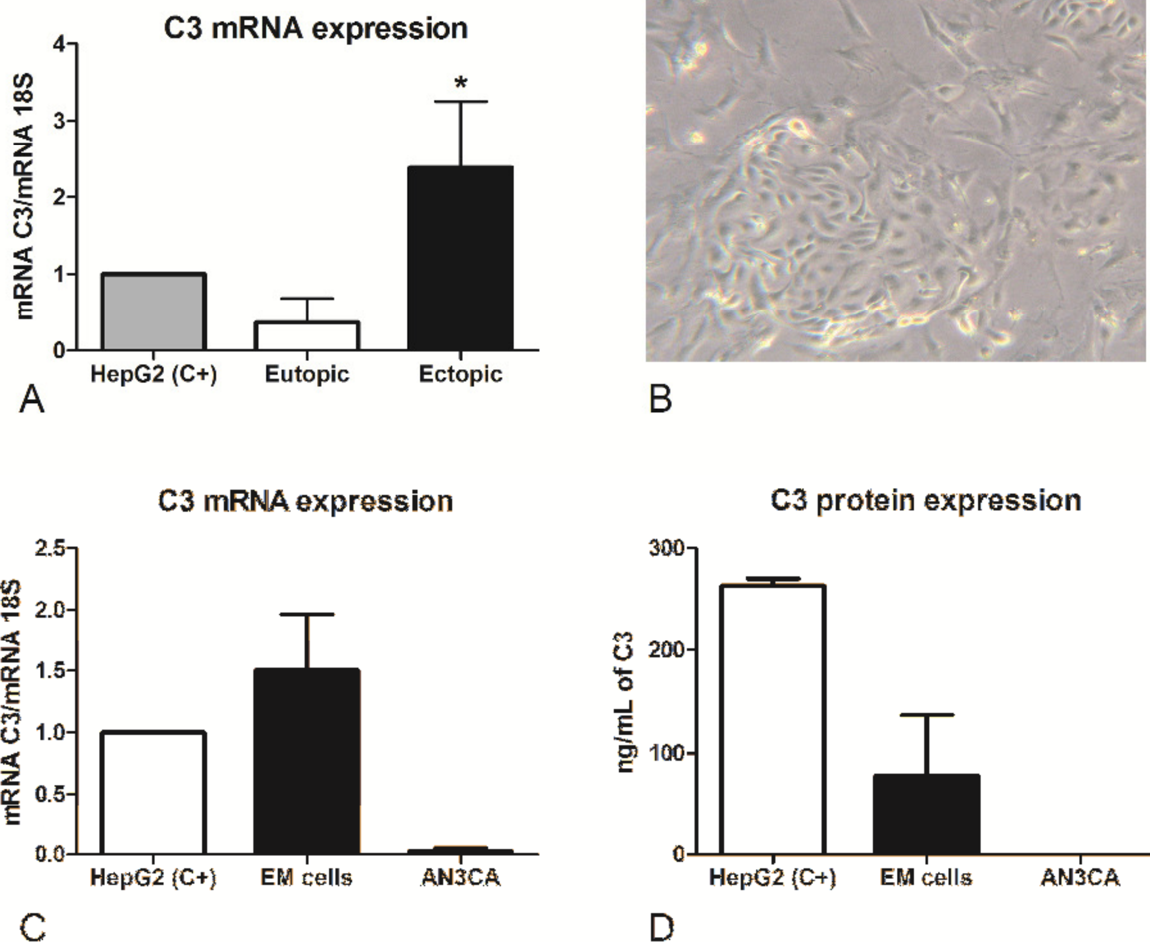

444

445 **Fig. 2. C3 is locally produced in EM tissue.** (A) The gene expression of C3 by endometriotic
446 cysts (n = 4) was investigated by RT-qPCR and compared to normal uterus (n= 3) using HepG2
447 hepatocyte cells as calibrator (AU = 1). 18S was used as the housekeeping gene. Data are
448 expressed as mean ± standard error. * $p<0.05$ (Mann-Whitney U Test). (B) Example of
449 morphological features of endometrial cells isolated from EM cyst biopsies. Images were
450 acquired by phase-contrast microscope, Leica original magnification: 200×. (C) The C3 mRNA
451 expression was measured by RT-qPCR in EM cells (n= 4) and compared to an endometrial cell
452 line, AN3CA. Data are expressed as mean ± standard error. * $p<0.05$ (Mann-Whitney U Test).
453 (D) Protein levels of C3 were assessed by ELISA in the supernatant of cells maintained in culture
454 for 60 h. Data are expressed as mean ± standard error.

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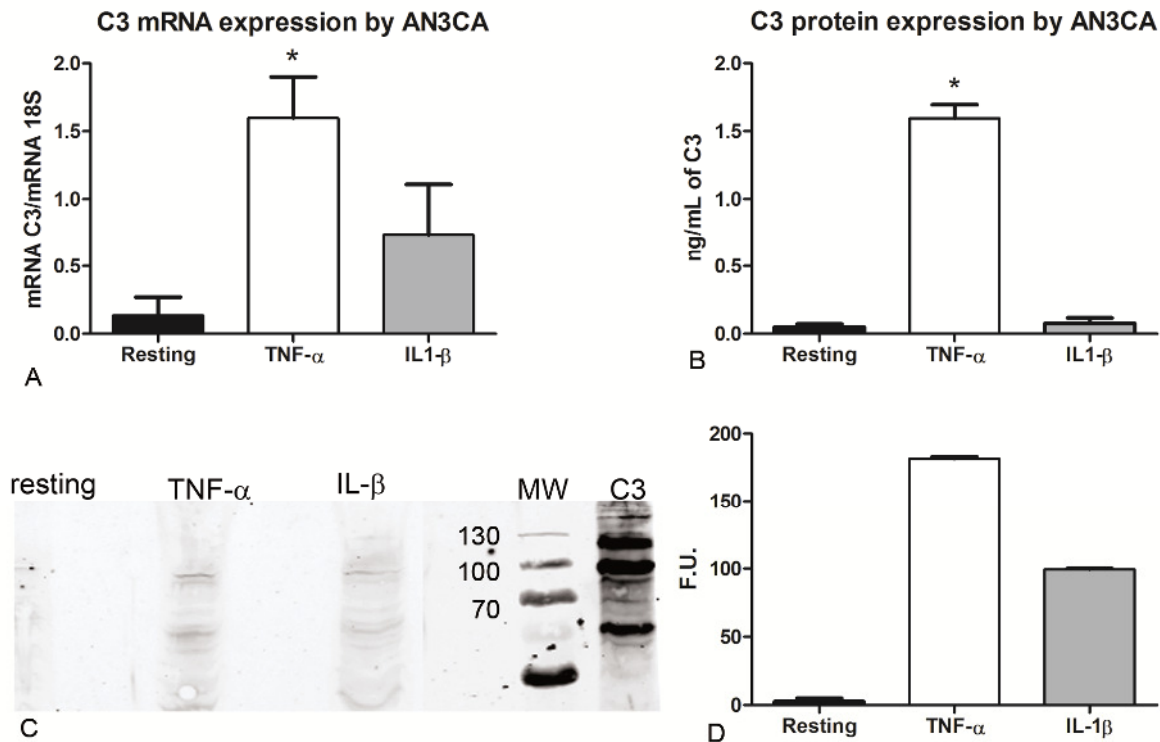

456

457 **Fig. 3. C3 is up-regulated by pro-inflammatory cytokines.** The stimulation of AN3CA cells
458 with pro-inflammatory cytokines induced the up-regulation of C3 expression. (A) AN3CA cells
459 were ON stimulated with TNF-α (100ng/mL) or IL-1β (5ng/mL) and the C3 expression was
460 analyzed by RT-qPCR. Data are expressed as mean of three independent experiments conducted
461 in double ± standard error. * $p<0.05$ (Wilcoxon matched pairs test). (B) Measurement of protein
462 level of C3 by ELISA in AN3CA cell culture supernatant stimulated for 36h with TNF-α
463 (100ng/mL) or IL-1β (5ng/mL). (C and D) The expression of C3 was evaluated in cell lysates by
464 western blot analysis and the intensity of the bands was measured with Odyssey-LICOR scanner.
465 Data are expressed as mean of three independent experiments conducted in double ± standard
466 error. * $p<0.05$.
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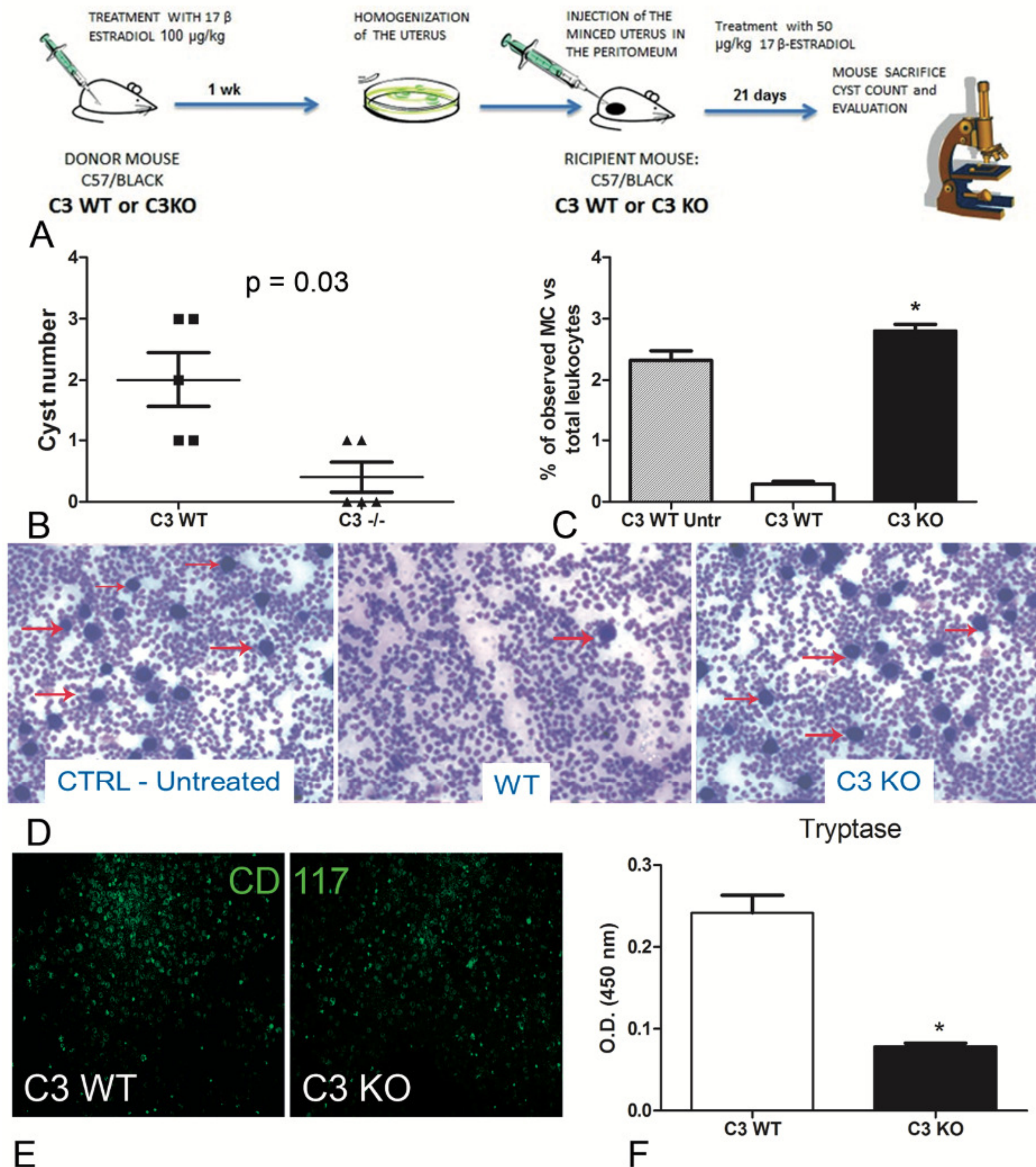

468

469 **Fig. 4. In vivo syngeneic mouse model of EM.** (A) Treatment regimen of C3 WT and gene-
470 deficient mice for generating EM in vivo model. Five C3-/- and WT mice each were injected (i.p.)
471 with minced uterus of a donor mouse; after 3 weeks, the peritoneal cyst formation was evaluated.
472 (B) Number of cysts counted in wild-type (WT, n = 5) or C3-/- (n = 5) mice. Mann-Whitney test
473 P = 0.03. (C and D) Representative images of cytocentrifuged peritoneal washing of untreated
474 WT, EM-induced WT and C3-/- mice (respectively), stained with Giemsa and counted with
475 ImageJ software (Particle Analysis Tool) to obtain relative percentage between total leukocytes
476 and mast cell (MC)/basophil number. MCs/basophils are identified as blue big dots indicated by
477 red arrows. Original magnification 100×. (E) Representative images of cytocentrifuged peritoneal
478 lavage of EM-induced WT mice stained with FITC conjugated anti-mouse CD117. Original
479 magnification 100×. (F) Biochemical characterization of tryptase enzyme present in peritoneal
480 lavage of WT vs C3-/- mice by ELISA. * p<0.05.

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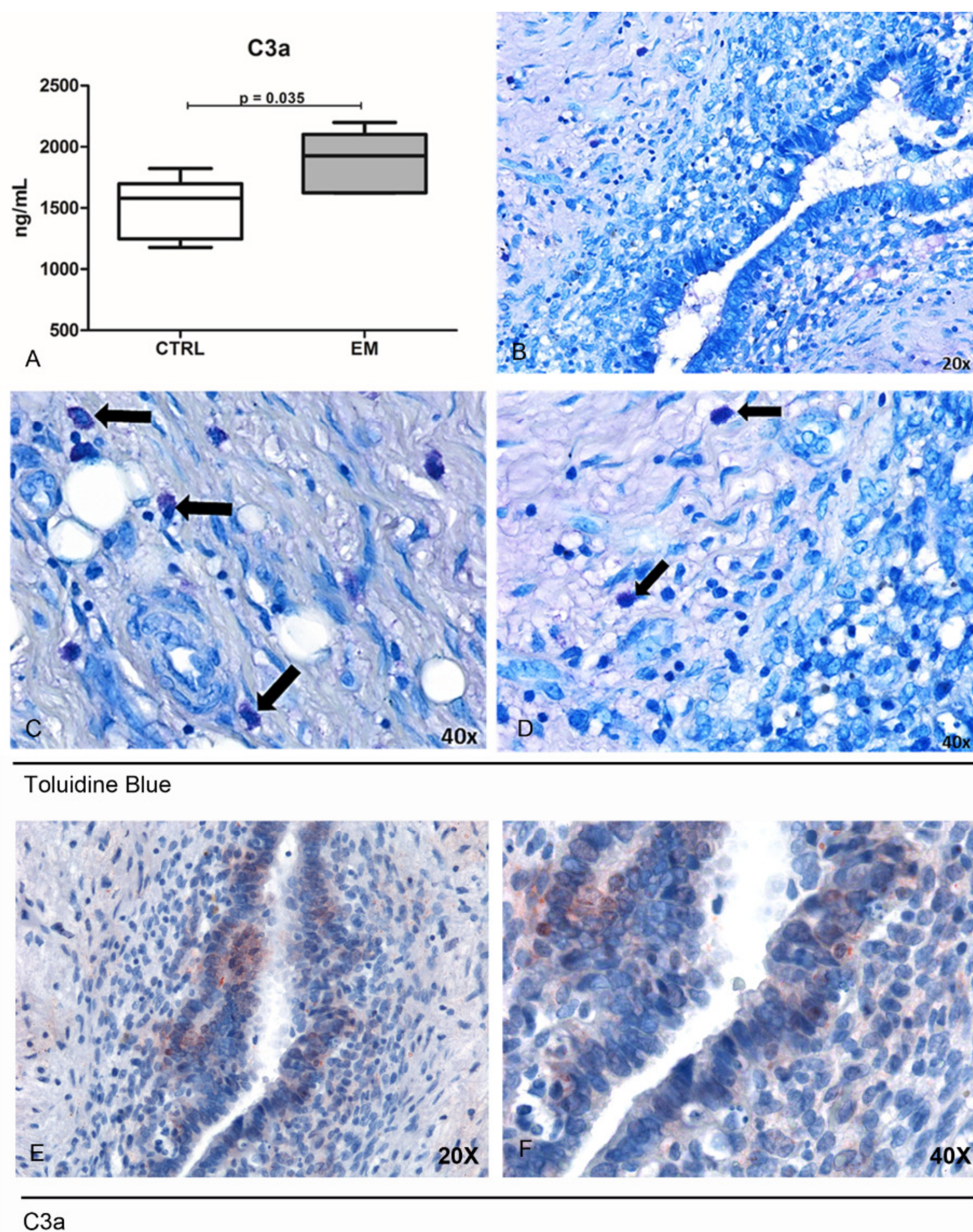

482

483 **Fig. 5. Peritoneal Fluid (PF) derived from EM patients presented elevated levels of C3a that**
484 **likely acts on MCs of the EM tissue.** (A) C3a ELISA evaluation of PF isolated from EM
485 patients (n = 7) compared to control patient group (n = 6). (B to D) Toluidine blue staining of
486 human EM tissue sections for the evaluation of MCs presence. Black arrows indicated MCs. (E
487 and F) Immunohistochemical analysis of C3a in EM tissue sections. AEC (red) chromogen was
488 used to visualize the binding of anti-human C3a antibodies.
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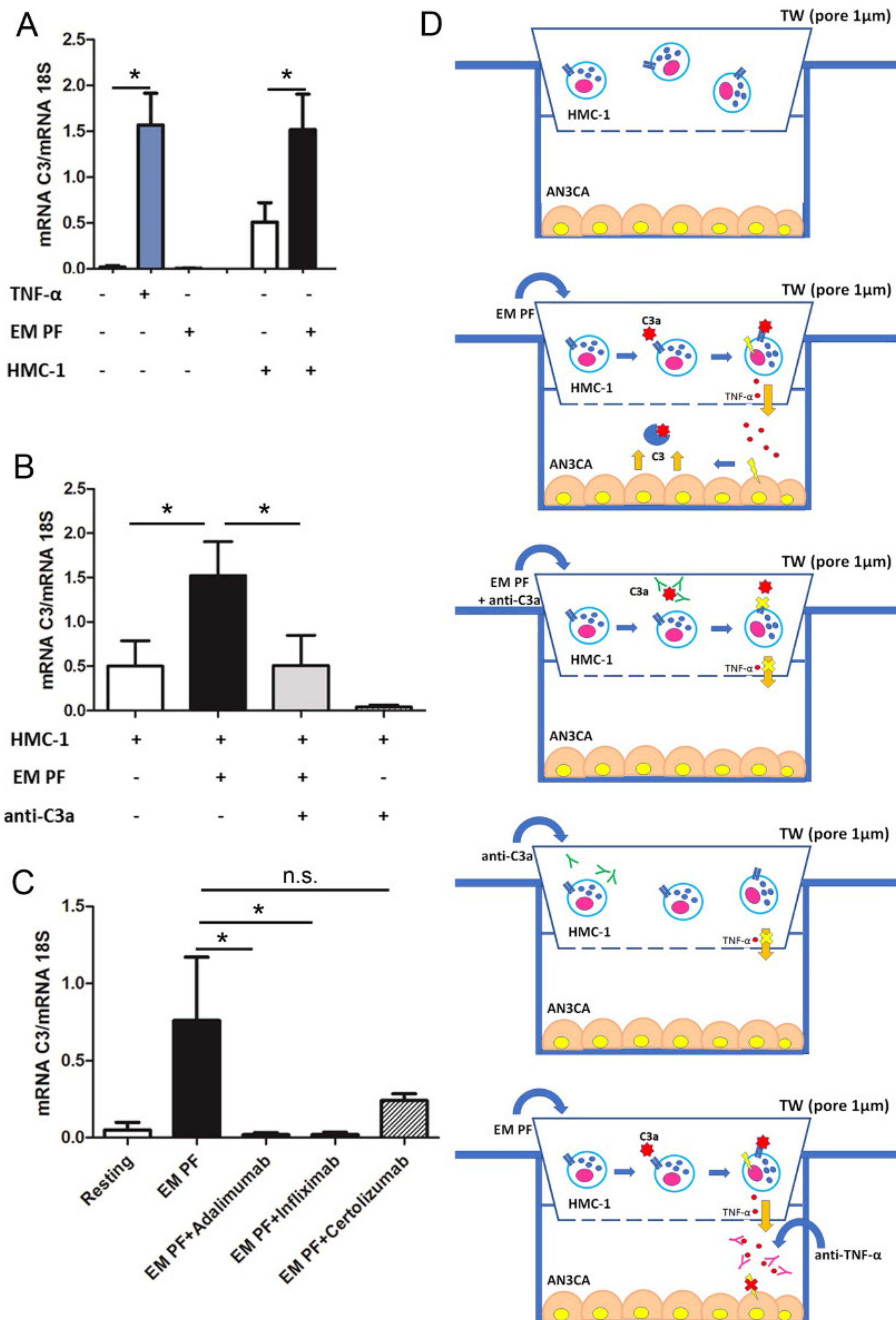

492

493 **Fig. 6. The co-culture of MCs with endometrial cells, in the presence of EM-PF, induced the**
494 **expression of C3, which was inhibited by C3a blocking antibody.** (A) The C3 gene expression
495 was evaluated by RT-qPCR on endometrial AN3CA cells alone (Resting), stimulated with TNF-α

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496 (+TNF-α) or with a pool of EM peritoneal fluid (+EM-PF); or co-cultured with MCs alone
497 (+HMC-1), or stimulated with PFA as positive control (+HMC-1+PFA) or with EM-PF (+HMC-
498 1+ EM PF). Similar experiment was performed in the presence of anti-C3a blocking antibody (B),
499 and the explained by the graphical abstract in (C). (D) Graphical representation of the blocking
500 co-culture experiments. Data are expressed as mean of three independent experiments conducted
501 in double ± standard error. * $p<0.05$.