Co-culture with Macrophages Enhances the Clonogenic and Invasion Activity of Endometriotic Stromal Cells.

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Keywords: Endometrium, endometriosis, macrophages, clonogenicity, endometrial stem cells, colony stimulating factor-1.

ABSTRACT

Objective: To study the effect on endometrial and endometriotic cells after co-culture with macrophages, using clonogenic, invasion and self-renewal assays.

Materials and Methods: Peripheral blood samples, endometrium and endometriotic tissues were collected. Autologous macrophages were co-cultured with endometrial and endometriotic cells. The number of colony forming units (CFU), invasiveness and self-renewal activity after co-culture with macrophages was determined. The cytokine level of colony stimulating factor-1 (CSF-1) from macrophages with and without endometriosis was compared.

Results: Co-culture with macrophages significantly increased the clonogenic and invasion ability of endometriotic stromal cells *in vitro*. Colony stimulating factor-1 (CSF-1) was up-regulated in endometriotic macrophages conditioned medium when compared to those without the disease.

Conclusions: These data suggest that macrophages may increase the proliferation and invasion activity of stromal clonogenic cells in women with endometriosis.

INTRODUCTION

Endometriosis is the presence of endometrial tissue growth outside the uterine cavity and is a benign gynecological disease affecting ~5% of women of reproductive age (1). The sex steroid dependent growth of ectopic endometrial tissues may result in cyclical pelvic pain and infertility. Several proposed theories have implicated the pathogenesis of endometriosis, including retrograde menstruation, peritoneal cell metaplasia, genetic predisposition, and altered immunological surveillance (2). The emerging evidence of somatic stem cells in the human endometrium provides an alternate candidate cell source for the development of endometriosis (3).

The physiological role of stem cells in the endometrium is to maintain the cyclical regeneration of the tissue that occurs after each menstruation. Endometrial epithelial and stromal cells with high clonogenic activity are initiated by stem/progenitor cells (4). The percentage of clonogenic cells in human endometrium does not vary significantly across the menstrual cycle (5). Occasional shedding of endometrial stem cells with colony-forming potential can reach ectopic sites through retrograde menstruation, invading the peritoneum to generate endometriotic lesions (6). Studies examining the eutopic endometrium of women with and without endometriosis revealed striking differences in gene expression that may predispose some women to disease development (7-9). Eutopic endometrial stem cells from women with endometriosis exhibit progesterone resistance which is inherited by their progenies (10). The uncontrolled growth of ectopic endometrial tissue invades the adjacent tissues and is associated with neovascularization and local inflammatory responses. Aberrant production of cytokines and growth, adhesion and angiogenic factors are linked to the occurrence and maintenance of endometriosis (11). How the changes in

59 the inflammatory peritoneal environment influence the behavior of ectopic
60 endometrial stem cells is unknown.

61 Pathogenesis of endometriosis is associated with dysfunctional regulation of the
62 immune system (12), in particular, an increase in macrophages and impairment of
63 their phagocytic activities (13, 14). Hypoxia and tissue stress recruit peripheral
64 macrophages to the endometriotic sites and contribute to the lesion's neovasculature,
65 sustaining the survival of endometrial cells at the ectopic locations. Chemokines
66 produced by stromal cells have a significant role in the infiltration of macrophages
67 into the peritoneal cavity (15, 16). Activation of macrophages is characterized by
68 their secretion of a wide variety of cytokines and growth factors (17). Levels of
69 peritoneal cytokines differ greatly between women with and without endometriosis
70 (18, 19), and higher amounts of cytokines are detected in advanced stages of the
71 disease (20).

72 Little is known about the interactions of macrophages with endometrial colony-
73 forming cells. Here we described the clonal analysis of endometrial and endometriotic
74 cells after co-culture with macrophages and examined how it affects the cell's
75 functional activities.

76

77 **MATERIALS AND METHODS**

78 **Human Tissue Samples**

79 Two types of endometrial tissues were collected: 1) endometrium from women
80 without endometriosis (normal endometrium) and 2) ovarian endometrioma
81 (endometriosis). Endometrial samples (n = 33) were collected from ovulating women

(45.5 ± 0.5 years) undergoing hysterectomy for leiomyoma or adenomyosis. Cyst walls of ovarian endometrioma (n = 32) were collected from women (39.3 ± 1.3 years) undergoing ovarian cystectomy. Only women who had not taken exogenous hormones for three months before surgery were included. Informed written consent was obtained from each patient and ethical approval was obtained from the Cluster Research Ethics Committee/Institutional Review Board of the University of Hong Kong/Hong Kong West Cluster, Hospital Authority, Hong Kong.

The stage of the menstrual cycle was categorized into proliferative (endometrium, n = 19; endometriotic, n = 16) and secretory (endometrium, n = 14; endometriotic, n = 16). The samples were dated based on the reported day of the last menses and histology examination by histopathologists (21). Endometriosis was staged according to the 1996 revised classification of the American Society for Reproductive Medicine (22). Full thickness endometrial tissue samples or ovarian endometriotic cysts were collected in Dulbecco's modified Eagle's medium/Hams F-12 (DMEM/F-12; Life technologies, CA, USA) containing 1% antibiotic (Gibco, MD, USA) and 5% fetal bovine serum (FBS, Gibco). The samples were stored at 4^{o}C and processed within 24 h.

Isolation of Endometrial and Endometriotic Cells

Human endometrial and endometriotic tissues were digested to single-cell suspensions using collagenase type I (300 µg/mL, Worthington Biochemical Corp, NJ, USA) and deoxyribonuclease type I (40 µg/mL, Worthington Biochemical Corp) as described (23). Red blood cells were removed using Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density-gradient centrifugation. Leukocytes were eliminated using anti-CD45 antibody-coated Dynabeads (Life Technologies). Purified epithelial cell

suspensions were separated from stromal cells by using anti-CD368 (EpCAM) antibody-coated microbeads (Miltenyi Biotec Inc. CA, USA).

Macrophage Differentiation and Collection of Conditioned Medium

Peripheral blood mononuclear cells from women with and without endometriosis were isolated with Ficoll-Plaque. Blood samples were collected on the same day as the endometrial or endometriotic tissue. Monocytes were enriched by the Monocyte Isolation Kit II (Miltenyi Biotec Inc., CA, USA) and subsequently differentiated into macrophages *in vitro* according to previous method (24).

Monocytes were stimulated with phorbol-12 myristate 13-acetate (PMA, 50 ng/ml, Sigma-Aldrich, MO, USA) in RPMI 1640 medium (Life Technologies), 10% FBS and 1% penicillin. Differentiation of the monocytes to macrophages was confirmed by morphological changes such as increase in cell size, formation of pseudopodia and adhesion (Supplementary data Fig S1A) and by flow cytometry detection of expression of a macrophage marker CD68 using fluorescein isothiocyanate (FITC) conjugated anti-CD68 antibody (eBioscience, CA, USA) (Supplementary data Fig S1B). To determine the phenotype of macrophages, the cells were co-stained with FITC conjugated anti CD68 (eBioscience) and classical M1 marker allophycocyanin (APC) conjugated anti-CD86 antibody (BD Biosciences) or alternative M2 marker APC conjugated anti-CD206 antibody (eBioscience). Cells were analyzed using a Fortessa flow cytometer (BD Biosciences, CA, USA) in the University of Hong Kong Core Facility. Macrophages were cultured in 6-well transwells (2×10^5 cells/well, EMD Millipore) and 72 h after differentiation, the cells were washed with PBS twice and replaced with RPMI and 1% penicillin. The conditioned media (CM) from macrophages of women with or without endometriosis were collected 48 h later,

centrifuged to remove cellular debris and used for subsequent experiment or stored at -80^{o}C until use.

Co-culture Setup and Colony-Forming Assay

Six different co-cultures were set up: 1) endometrial epithelial cells co-cultured with autologous macrophages and CM (n = 4); 2) endometrial stromal cells co-cultured with autologous macrophages and CM (n = 8); 3) endometriotic epithelial cells co-cultured with autologous macrophages and CM (n=8); 4) endometriotic stromal cells co-cultured with autologous macrophages and their CM (n=13); 5) endometriotic epithelial cells co-cultured with macrophages (without endometriosis) and their CM (n = 3) and 6) endometriotic stromal cells co-cultured with macrophages (without endometriosis) and their CM (n=3). In brief, cells were seeded in duplicate at a clonal density of 500 cells/cm^2 in 6-well plates (BD Bioscience) and were 1) cultured in growth medium only (control), 2) cultured in growth medium supplemented with 50 ng/ml PMA (negative control), 3) co-cultured indirectly with macrophages with 50 ng/ml PMA (2 x 10^5 cells) or, 4) treated with macrophage CM, which was diluted with growth medium at a ratio of 3:7 (v/v). We supplemented PMA to maintain macrophages differentiation in long-term culture. The medium was changed every 7 days, and the colonies formed were regularly monitored using an Eclipse TS100 inverted microscope (Nikon). Endometrial cells were cultured for 15 days (4, 25). Endometriotic cells were cultured for 21 days (23). The colonies formed were fixed with 10% formalin and stained with 1% Toluidine Blue (Sigma-Aldrich) (Supplementary data S2A). Colony-forming units (CFUs) consisting of ≥50 cells were counted to determine the cloning efficiency (CE), which was the percentage of

colonies formed per seeded cell. Large CFUs were defined as colonies with ≥4,000 cells and small CFUs were those with ≤4,000 cells as described (23).

Cell Invasion

Clonally derived endometrial and endometriotic cells were harvested from different conditions, and 2 x 10^5 cells were seeded on Matrigel-coated transwells (24 wells, 8µm pore size, BD Biosciences). After 48 h, cells on the upper surface of the inset membrane were removed with cotton rods, while cells on the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.1% toluidine blue (Supplementary data S2B). The transwells were washed and the invaded cells were lysed with 10% acetic acid. Absorbance of the lysate was measured at 595 nm using a microplate reader (Tecan). Relative invasion was determined by normalization to the control group.

In Vitro Serial Cloning

Individual large epithelial and stromal CFUs from passage 1 (P1) were trypsinized using cloning rings (Sigma-Aldrich) to determine the self-renewal capacity of cells from endometrial and endometriotic cells grown in growth medium and co-cultured with autologous macrophages. A total of three individual large CFUs per patient sample (n = 3) obtained from the clonogenic assays were used. The cell number of each CFU was determined and the cells were re-seeded at a density of 20 cells/cm^2 (26). This process continued until the cells could no longer form CFUs (Supplementary data S2).

Cytokine Array and ELISA

Cytokine Array C3 (RayBiotech Inc., GA, USA) was used to determine the cytokines in the macrophage CM from women with endometriosis (n = 6; proliferative n = 3, secretory n=3) and without endometriosis (n = 6; proliferative n = 3, secretory n=3). The signal intensities of the cytokines were quantified using Quantity One software (Bio-Rad, CA, USA, Supplementary Data S5). The CSF-1 level was determined using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, MN, USA) from women with endometriosis (n = 11; proliferative n = 5, secretory n = 6) and without endometriosis (n = 9; proliferative n = 5, secretory n = 4). Each sample was measured in duplicate. Recombinant CSF-1 (Peprotech, NJ, USA) at 30, 300, and 3000 pg/ml was added to the growth medium of endometrial and endometriotic stromal cells seeded at clonal density (500 cells/cm^2) for 15 days. For neutralization assay, the anti-human colony stimulating factor (CSF-1) monoclonal antibody (10 µg/ml, Peprotech) was added to the endometrial epithelial and stromal cells co-cultured with macrophages and CM without endometriosis.

Flow Cytometry Analysis

The co-expression of CD140b and CD146 on endometrial stromal cells after 15 days of culture in different conditions (n = 5) were analyzed by multicolour flow cytometry as described (26). The cells were incubated with FITC-conjugated anti-CD146 (1 mg/ml, OJ79c clone, mouse IgG1; Thermo Fisher Scientific, MA, USA) and PE-conjugated anti-PDGFRβ (CD140b, 2.5 µg/ml, PR7212 clone, Mouse IgG1, R&D Systems) antibodies in the dark for 45 minutes on ice. Isotype matched controls were included for each antibody. Following the final washing step, the labeled cells were

analyzed by Fortessa flow cytometer (BD Biosciences) in the University of Hong Kong Faculty Core Facility. The cells were selected with electronic gating according to the forward and side scatter profiles (Supplementary Data S3A-D) using the FACSDIVA software (BD Biosciences). Data were analyzed using the FlowJo Software (Tree star Inc.).

Statistical Analysis

Data were analyzed using GraphPad PRISM software (version 5; GraphPad Software Inc., CA, USA). The normal distribution of the data was determined by the D'Agostino-Pearson test. The data were analyzed by a non-parametric one-way ANOVA using Kruskal-Wallis test in multiple groups or using Mann-Whitney test in case of two groups. Differences of $P<0.05$ were considered statistically significant.

RESULTS

Clonogenicity of Human Endometrial and Endometriotic Cells in Co-culture with Autologous Macrophages

Autologous macrophages or their CM were co-cultured with the endometrial and endometriotic cells. Since PMA was used to induce macrophages differentiation, cells treated with PMA alone served as a negative control. To maintain macrophage differentiation in long-term culture, PMA was also supplemented into the co-culture treatment. The total CE (large and small colonies) was $0.33 \pm 0.17\%$ for endometrial epithelial cells (Fig 1A). Treatment with macrophages or their CM did not change the total CE of epithelial cells. There was no difference in the CEs of large endometrial

225 epithelial colonies between groups treated with PMA, macrophages or macrophage
226 CM when compared to the untreated control.

227 For endometrial stromal cells, the total CE (large and small colonies) was 0.31 ±
228 0.10% (Fig 1B). Treatment with macrophages or their CM did not change the total CE
229 of stromal cells. Interestingly, endometrial stromal cells co-cultured with
230 macrophages (0.23 ± 0.08%) produced significantly more large colonies than stromal
231 cells alone (0.06 ± 0.03%, $P<0.05$). Macrophage CM had no effect on the clonogenic
232 growth of the large stromal colonies. The CEs of endometrial stromal small CFUs
233 were similar in all conditions.

234 The overall clonogenicity displayed by endometriotic cells was lower. For
235 endometriotic epithelial cells, there was significant increase in the total CE between
236 the PMA (0.01 ± 0.01%) and the macrophage co-culture (0.14 ± 0.05%, $P<0.05$, Fig
237 2A) group. The proportion of large epithelial clones in the macrophage (0.13 ± 0.05%)
238 and the macrophage CM (0.07 ± 0.03%) treated groups were significantly higher than
239 that of the control (0.003 ± 0.002%, $P<0.05$). No difference was detected for the
240 endometriotic epithelial small CFU in different conditions.

241 For the endometriotic stromal cells, the total CE was 0.01 ± 0.01% and significantly
242 increased after macrophage co-culture (0.19 ± 0.04%, $P<0.001$) and macrophage CM
243 (0.10 ± 0.04, $P<0.05$, Fig 2B). More large endometriotic stromal CFUs formed after
244 co-culture with macrophage (0.14 ± 0.04%, $P<0.001$) and macrophage CM (0.05 ±
245 0.03%, $P<0.05$) when compared with the control (0.0003 ± 0.003%). Endometriotic
246 stromal small colonies also significantly increased when co-cultured with
247 macrophages when compared with the control or the PMA group ($P<0.05$).

Clonogenicity of Human Endometriotic Cells after Co-culture with Macrophages from Patients without Endometriosis

We performed additional co-culture experiments to further investigate the interactions between macrophages and endometriotic cells. Endometriotic epithelial and stromal cells were co-cultured with non endometriotic macrophages and their CM. The CEs for endometriotic epithelial and stromal cells were similar for all the conditions (Fig 3A, B).

Invasion and Self-Renewal Ability of Endometrial and Endometriotic Cells after Co-culture with Autologous Macrophages

There were no changes in the invasiveness of endometrial epithelial cells (Fig 1C). However, co-culture with macrophages or macrophage CM increased the invasion of endometrial stromal cells ($P<0.05$, Fig 1D). For endometriotic samples, the invasiveness of the epithelial cells increased after co-culture with macrophages ($P<0.05$, Fig 2C). This stimulatory effect was also detected on endometriotic stromal cells after co-culture with macrophages and macrophage CM ($P<0.05$, Fig 2D).

The self-renewal ability of cells in the large CFU after co-culture was assessed using a serial cloning strategy. We observed a decline in the number of self-renewal rounds in cells after co-culture when compared to the corresponding control (endometrial epithelial: 1.0 ± 0.1 vs 2.4 ± 0.1, Fig 1E; endometrial stromal: 2.0 ± 0.2 vs 4.0 ± 0.2, Fig 1F; endometriotic epithelial: 0.8 ± 0.3 vs 3.1 ± 0.1, Fig 2E; endometriotic stromal: 1.1 ± 0.4 vs 3.9 ± 0.1; Fig 2F) though the differences were not yet significant.

Since the self-renewal activity of stromal cells declined after macrophage co-culture, we examined the phenotypic expression of the endometrial stromal cells using the

endometrial mesenchymal-like stem cell markers: CD140b and CD146. Flow cytometry analysis of $CD140b^{+}CD146^{+}$ cells on clonally derived stromal cells after co-incubation with macrophages (3.12 ± 2.50%) and their CM (6.28 ± 5.0%) was not significantly different from the control (7.84 ± 3.5%, Supplementary data S3E).

Cytokine Profile of Macrophages from Patients with and without Endometriosis

The macrophage CM from patients with and without endometriosis were compared using a cytokine array for 42 cytokines (Supplementary table S1). Densitometric analysis revealed a 4-fold higher level of CSF-1 in the CM of endometriosis samples (1.11 ± 0.67) than in that of no endometriosis (0.25 ± 0.04, $P<0.05$, Fig 4A, B). Consistently, the amount of CSF-1 released into the CM from endometriotic macrophages was significantly higher (597 ± 140 pg/ml, n = 11) than that from normal endometrial macrophages (159 ± 40 pg/ml, $P<0.05$, Fig 4B) determined by ELISA. However, CSF-1 at concentrations of 30, 300 and 3000 pg/ml did not affect the total CEs of epithelial and stromal cells from endometrial (Fig 5A, C) and endometriotic tissues (Fig 6A, C). The different concentrations of CSF-1 did not affect the invasion ability of endometrial (Fig 5B, D) or endometriotic cells (Fig 6B, D). Although a decline trend in the CEs of endometrial cells were observed after neutralization with CSF-1 antibody, it did not reach statistic significance due to the small sample size (Supplementary data S4A, B).

DISCUSSION

Endometriosis is a multifactorial disease, and its etiology remains uncertain. Among the theories proposed to explain the pathogenesis of endometriosis, Sampson's theory of retrograde menstruation is most widely accepted. In reproductive-age women, a reflux of menstrual tissue enters the peritoneal cavity and embeds into intra-abdominal areas (27). Susceptibility to endometriosis is due to enhanced endometrial cell adhesion to the peritoneum and poor clearance of refluxed endometrial cells by the host immune response (28). Macrophage function is augmented in endometriotic lesions (14). Bacci et al. demonstrated a pro-inflammatory role for macrophages that exacerbates growth and vascularization of endometriotic lesions (29).

In this study, the clonogenicity and invasiveness of endometriotic stromal cells increased significantly after co-cultured with autologous macrophages. Interestingly, the stimulatory effect was not observed when endometriotic stromal cells were co-cultured with macrophages from patients without endometriosis. These observations suggest there may be a two-way communication between macrophages and the endometriotic stromal cells in regulating the proliferation and invasion activity of colony-forming cells. Macrophages can be stimulated by soluble factors derived from endometriotic cells and differentiate in response to the changing microenvironment. Thus, the communication between macrophages and endometriotic cells can facilitate the progression of the disease.

Previously, we demonstrated the existence of colony-forming cells in human endometrium and endometriosis (4, 23). Endometrial and endometriotic cells from large CFUs display properties of somatic stem cells (23, 30). The cells in the large CFUs are heterogeneous, compromising stem cells and their differentiating progenies. Thus, the observed increase of large CFUs may not be due to an expansion of the number of stem cells but rather an expansion of their downstream progenitors or

transit amplifying cells. This notion is supported by our finding that co-culture with autologous macrophages lowered the self-renewal ability of clonally derived endometrial and endometriotic cells in serial cloning assays. Furthermore, clonally derived stromal cells after co-culture with macrophage or CM displayed lower expression of the endometrial mesenchymal stem-like cell surface markers (CD140b and CD146). It is likely that macrophages enhanced the proliferation but readily exhausted the proliferative potential of progenitors/transit amplifying cells of large CFUs.

We also examined the differences of cytokines derived from macrophages from women with and without endometriosis. Since endometrial macrophages have a role in tissue angiogenesis, tissue remodeling and immune defense, a major population of uterine tissue macrophages is alternatively activated (31). Alternatively activated macrophages are more abundant in patients with endometriosis (32) and exacerbate the growth and vascularization of endometriotic lesions (29). In this study, the macrophages from women with and without endometriosis were found to polarized towards the alternatively activated or M2 phenotype and endometriotic macrophages released more CSF-1, which has been associated with the early establishment of endometriotic lesions (33). The level of CSF-1 in the peritoneal fluid of patients with endometriosis is higher than those without (34). CSF-1 can also enhance the proliferation, attachment and invasion of endometrial cells base from *in vitro* and *in vivo* studies (35, 36). However, our results showed that CSF-1 alone did not affect the clonogenicity or invasion activity of endometrial or endometriotic cells. Therefore, the stimulatory activities of macrophages co-culture with endometrial and endometriotic cells could be mediated by one or a cocktail of regulators that were not determined in this study. In addition, it is worth noting that the endometrium would

produce other factors that mediate endometrial macrophage differentiation, and our current *in vitro* model may therefore not fully represent the behavior of these macrophages. A limitation of this study was the source of the macrophage used. Peritoneal macrophages would undoubtedly provide a better insight into the peritoneal phenomenon on endometrial and endometriotic cells. However, to obtain sufficient amount of peritoneal macrophages would be difficult, hence we used peripheral monocyte derived macrophages. Other immune cells such as T cells within the endometrial leukocyte population can also promote the growth and invasion of endometriotic stromal cells (37).

Currently, direct evidence supporting the involvement of endometrial stem/progenitor cells in the etiology of endometriosis is limited. While the existence of endometrial stem/progenitor cells in the endometrial basalis is well documented (38), some evidence supports the presence of endometrial stem/progenitor cells in endometriotic lesions (23, 39). There is also evidence that fragments of the shed endometrial basalis, likely containing endometrial stem/progenitor cells, are more often shed in the menstrual blood of women with endometriosis than in that of healthy control subjects (40, 41). Thus, when exposed to an environment conducive to the formation of endometriosis, such as the presence of dysregulated macrophages, the retrograded endometrial stem/progenitor cells differentiate and their progenies proliferate in ectopic sites, leading to the development of endometriotic lesions. However, whether the altered macrophage changes are primary or secondary occurrences remains uncertain.

In conclusion, the evidence that co-culture of macrophages enhances the clonogenicity and invasion activity of endometriotic stromal cells suggests phagocytic cells and endometriotic cells may contribute to the committed progeny expansion of

retrograde endometrial cells, giving rise to endometriosis. Further work should be undertaken to identify the kinase signals involve in the cell communication between macrophages and endometriotic stromal cells, as these pathways may represent a target for endometriosis treatment.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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FIGURE LEGENDS

Figure 1 – Clonogenicity, invasion ability and self-renewal activity of endometrial epithelial and stromal cells with autologous macrophages. Cloning efficiency (CE) of **(A)** epithelial and **(B)** stromal cells after culture in PMA, co-culture macrophages (co-culture) and macrophage conditioned media (CM) for 15 days. Bars represent total CE (sum of small and large CFUs). White bars indicate large CFU; shaded bars indicate small CFUs. Relative cell invasion capacity of **(C)** epithelial and **(D)** stromal cells after culture in different conditions. Control was set as one. Self-renewal activity of **(E)** epithelial and **(F)** stromal cells co-culture with macrophages. Results reported as means ± SEM; clonogenicity: epithelial n = 4, stromal n = 8; invasion: n = 4, self-renewal n=3. *, [a,b,d,e] $P<0.05$; **, [c] $P<0.01$; *** $P<0.001$. ***; [a-c] are significant differences for large CFUs, [d-e] are significant differences for small CFUs. CFU, colony-forming unit; CM, conditioned medium; PMA, phorbol-12 myristate 13-acetate; SEM, standard error of the mean.

Figure 2 – Clonogenicity, invasion ability and self-renewal activity of endometriotic epithelial and stromal cells with autologous macrophages. Cloning efficiency (CE) of **(A)** epithelial and **(B)** stromal cells after culture in PMA, co-culture macrophages (co-culture) and macrophage conditioned media (CM) for 15 days. Bars represent total CE (sum of small and large CFUs). White bars indicate large CFU; shaded bars indicate small CFUs. Relative cell invasion capacity of **(C)** epithelial and **(D)** stromal cells after culture in different conditions. Control was set as one. Self-renewal activity of **(E)** epithelial and **(F)** stromal cells co-culture with macrophages. Results reported as means ± SEM; clonogenicity: epithelial n = 8, stromal n = 13; invasion: epithelial n = 3, stromal n = 4, self-renewal n = 3. *,[a,c] $P<0.05$; ***,[b] $P<0.001$. [a,b] are significant differences for large CFUs, [c] are significant differences for

small CFUs. CFU, colony-forming unit; SEM, CM, conditioned medium; PMA, phorbol-12 myristate 13-acetate; standard error of the mean.

Figure 3 - The clonogenicity of endometriotic epithelial and stromal cells after co-culture with macrophages from patient without endometriosis.

Cloning efficiency (CE) of **(A)** epithelial and **(B)** stromal cells after culture in PMA, co-culture macrophages (without endometriosis) and macrophage conditioned media (CM) for 15 days. Bars represent total CE (sum of small and large CFUs). White bars indicate large CFU; shaded bars indicate small CFUs. Results reported as means ± SEM; endometriotic epithelial and stromal cells n = 3. CM, conditioned medium; PMA, phorbol-12 myristate 13-acetate.

Figure 4 – Identification of CSF-1 released by macrophages with and without endometriosis. Cytokine arrays of the expression of 42 human cytokines in the macrophage conditioned medium from women with and without endometriosis were evaluated. **(A)** Representative images of the densitometry produced from the cytokine array. **(B)** Arrays were visualized by enhanced luminal-based chemiluminescence and the dot intensities of CSF-1 were quantified by densitometry using Quantity One software. Each bar consists of relative expression (%) for no endometriosis (grey bar) and endometriosis (white bar) of macrophage conditioned medium, n = 6. **(C)** Histogram showing the amounts, in pg/mL, of the CSF-1 as quantified by ELISA, endometrium: n = 9; endometriosis: n = 11. Results reported as means ± SEM; *

434 $P<0.05$. CSF-1, Colony Stimulating Factor-1; ELISA; enzyme-linked immunosorbent
435 assay, SEM, standard error of the mean.

436

437 **Figure 5 – The clonogenicity and invasion activity of endometrial epithelial and**
438 **stromal cells after CSF-1 treatment.**

439 Cloning efficiency (CE) of endometrial **(A)** epithelial and **(C)** stromal cells after
440 treatment with different concentrations of CSF-1: 30, 300 and 3000 pg/ml for 15 days.
441 Bars represent total CE (sum of small and large CFUs). Relative cell invasion
442 capacity of epithelial **(B)** and stromal **(D)** cells after culture in different conditions.
443 Control was set as one. Results reported as means ± SEM; endometrial epithelial and
444 stromal cells n = 3. CFU, colony-forming unit; CSF-1, Colony Stimulating Factor-1;
445 SEM, standard error of the mean.

446 **Figure 6 – The clonogenicity and invasion activity of endometriotic epithelial and**
447 **stromal cells after CSF-1 treatment.**

448 Cloning efficiency (CE) of endometriotic **(A)** epithelial and **(C)** stromal cells after
449 treatment with different concentrations of CSF-1: 30, 300 and 3000 pg/ml for 15 days.
450 Bars represent total CE (sum of small and large CFUs). Relative cell invasion
451 capacity of epithelial **(B)** and stromal **(D)** cells after culture in different conditions.
452 Control was set as one. Results reported as means ± SEM; endometriotic epithelial
453 cells (clonogenicity, n= 7; invasion, n = 3); endometriotic stromal cells (clonogenicity,
454 n = 8; invasion, n=3). CFU, colony-forming unit; CSF-1, Colony Stimulating Factor-1;
455 SEM, standard error of the mean.

456

Supplementary Figure S1 – Induction of macrophage differentiation. (A) Monocytes isolated from peripheral blood mononuclear cells were incubated with phorbol myristate acetate (PMA, 50ng/ml) for 72 h to induce macrophage differentiation. Scale bar: 50 μM. **(B)** Representative histogram for FITC isotype and pan macrophage marker CD68-FITC conjugated. **(C)** The phenotypic expression of macrophages with (n = 3) and without endometriosis (n = 6). Percentage of macrophages expressing M1 marker - CD86 and M2 marker - CD206. Results reported as means ± SEM.

Supplementary Figure S2 –Representative images of the endometrial stromal **(A)** CFUs formed and **(B)** invasion activity under control condition, PMA treatment, co-culture with macrophage and macrophage CM. **(C)** Representative serially passaged images of the CFUs formed by endometrial stromal cells at different passages (P1 to P4).

Supplementary Figure S3 – Gating strategy for co-expression of CD140b and CD146 on human endometrial stromal cells. (**A**) Clonally derived human endometrial stromal cells after 15 days in culture were analyzed by flow cytometry for expression of cell surface markers. Viable cells were selected by their forward scatter (FSC) and side scatter (SSC) profile. (**B**) Cell properties; SSC area (SSC-A) versus SSC height (SSC-H) to gate out cell doublets and aggregates to ensure the signal arises from single cell. (**C**) Single parameter histograms for individual markers CD146-FITC, CD140b-PE. Grey line indicates background fluorescence with isotype matched IgG control. (**D**) Representative dot pot of $CD140b^{+}CD146^{+}$ cells from endometrial stromal cells after co-cultured with macrophage. (**E**) Percentages of $CD140b^{+}CD146^{+}$ cells after culture in different conditions (n = 5). Results are reported as means ± SEM.

Supplementary Figure S4 – The clonogenicity of endometrial epithelial and stromal cells after co-culture with autologous macrophages and treatment with CSF-1 neutralizing antibody.

Cloning efficiency (CE) of **(A)** epithelial and **(B)** stromal cells after culture in PMA, co-culture macrophages (co-culture), co-culture with macrophages together with CSF-1 antibody (co-culture + CSF-1 Ab), macrophage conditioned media (CM) and macrophage conditioned medium together with CSF-1 antibody (CM + CSF-1 Ab) for 15 days. Bars represent total CE (sum of small and large CFUs). White bars indicate large CFU; shaded bars indicate small CFUs. Results reported as means ± SEM; epithelial and stromal cells n = 3. CSF-1, Colony Stimulating Factor-1; CM, conditioned medium; PMA, phorbol-12 myristate 13-acetate.

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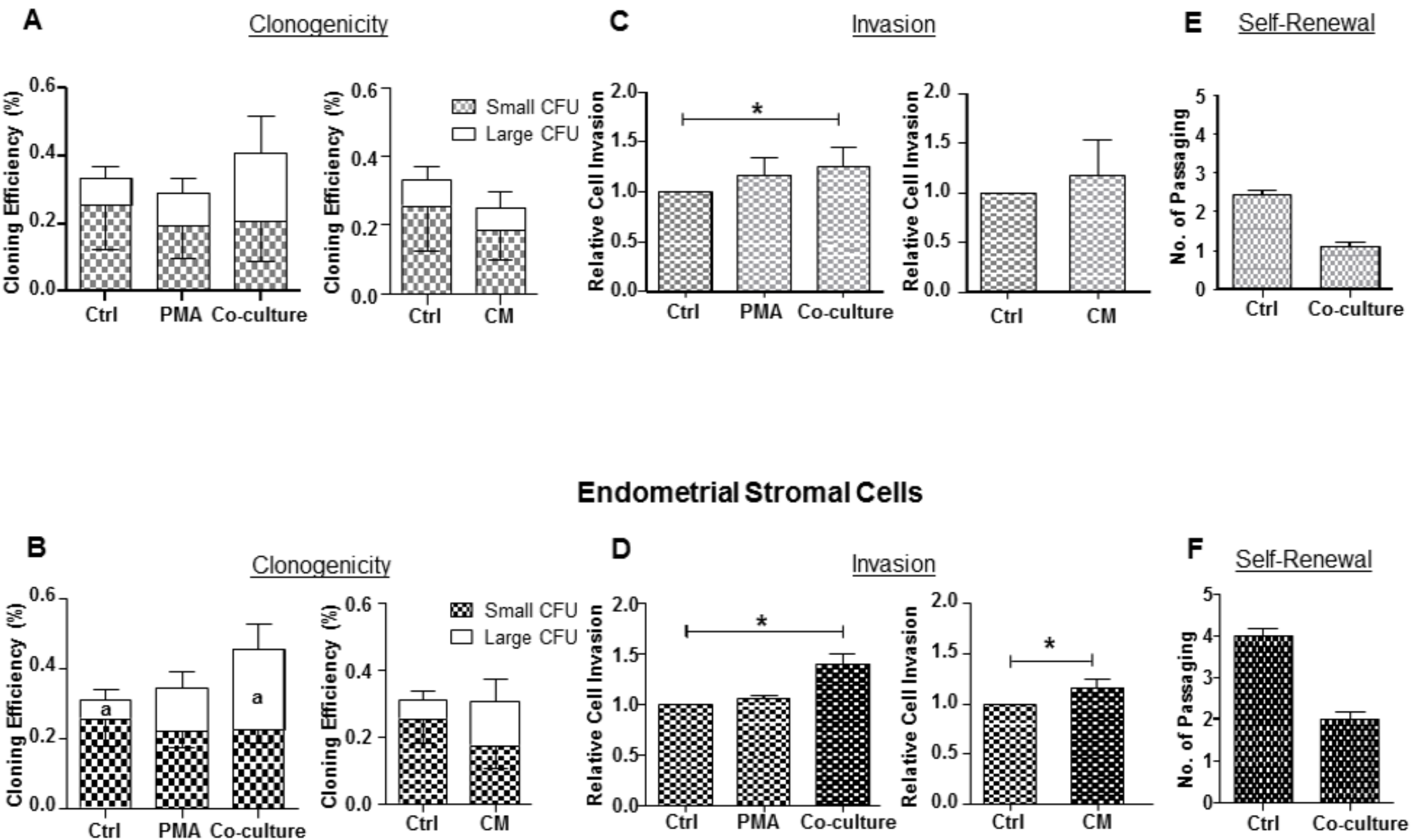
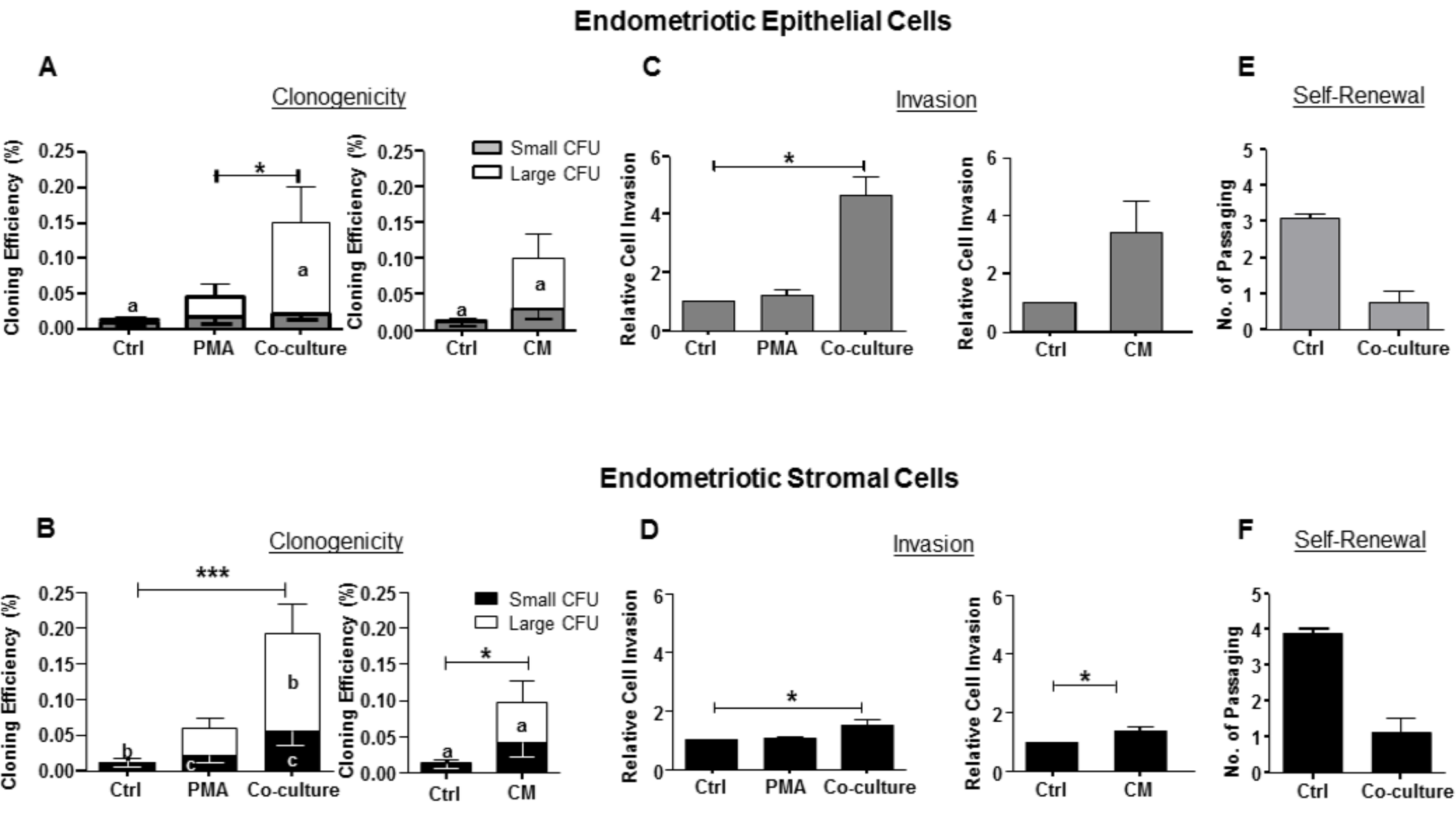
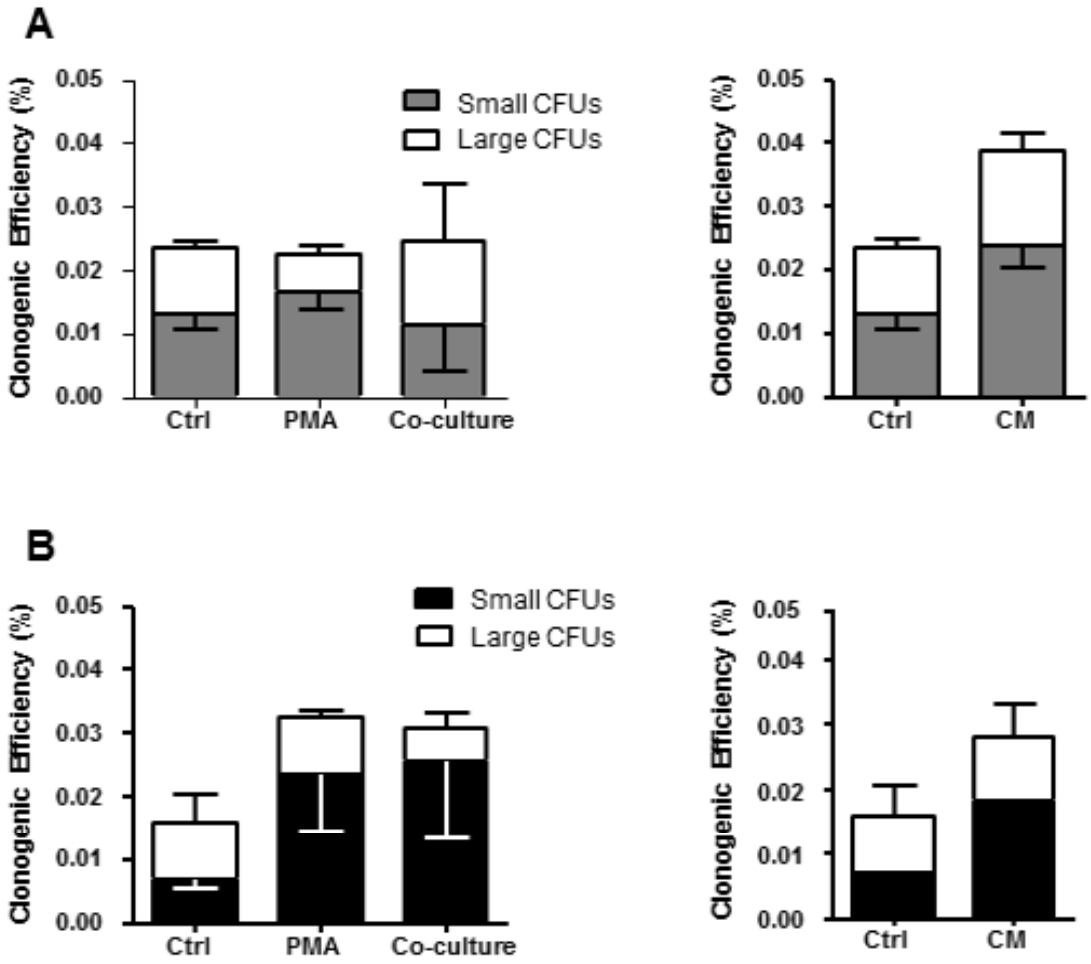

Endometrial Epithelial Cells
A
Clonogenicity
Cloning Efficiency (%)
0.6
0.4
0.2
0.0
Ctrl
PMA
Co-culture
Cloning Efficiency (%)
0.6
0.4
0.2
0.0
Small CFU
Large CFU
Ctrl
CM
C
Invasion
Relative Cell Invasion
2.0
1.5
1.0
0.5
0.0
*
Ctrl
PMA
Co-culture
Relative Cell Invasion
2.0
1.5
1.0
0.5
0.0
Ctrl
CM
E
Self-Renewal
No. of Passaging
5
4
3
2
1
0
Ctrl
Co-culture
Endometrial Stromal Cells
B
Clonogenicity
Cloning Efficiency (%)
0.6
0.4
0.2
0.0
a
a
Ctrl
PMA
Co-culture
Cloning Efficiency (%)
0.6
0.4
0.2
0.0
Small CFU
Large CFU
Ctrl
CM
D
Invasion
Relative Cell Invasion
2.0
1.5
1.0
0.5
0.0
*
Ctrl
PMA
Co-culture
Relative Cell Invasion
2.0
1.5
1.0
0.5
0.0
*
Ctrl
CM
F
Self-Renewal
No. of Passaging
5
4
3
2
1
0
Ctrl
Co-culture

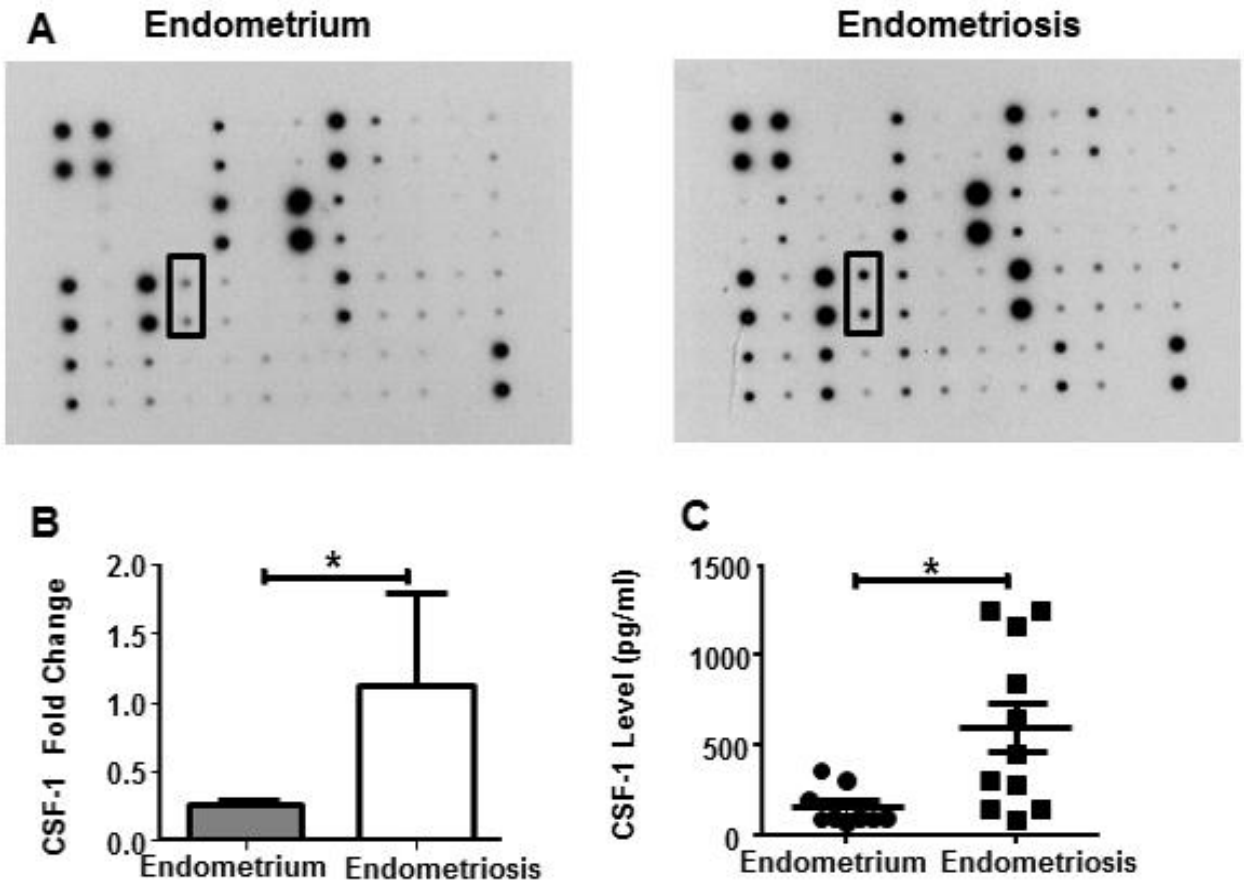
Chan et al.– Figure 2

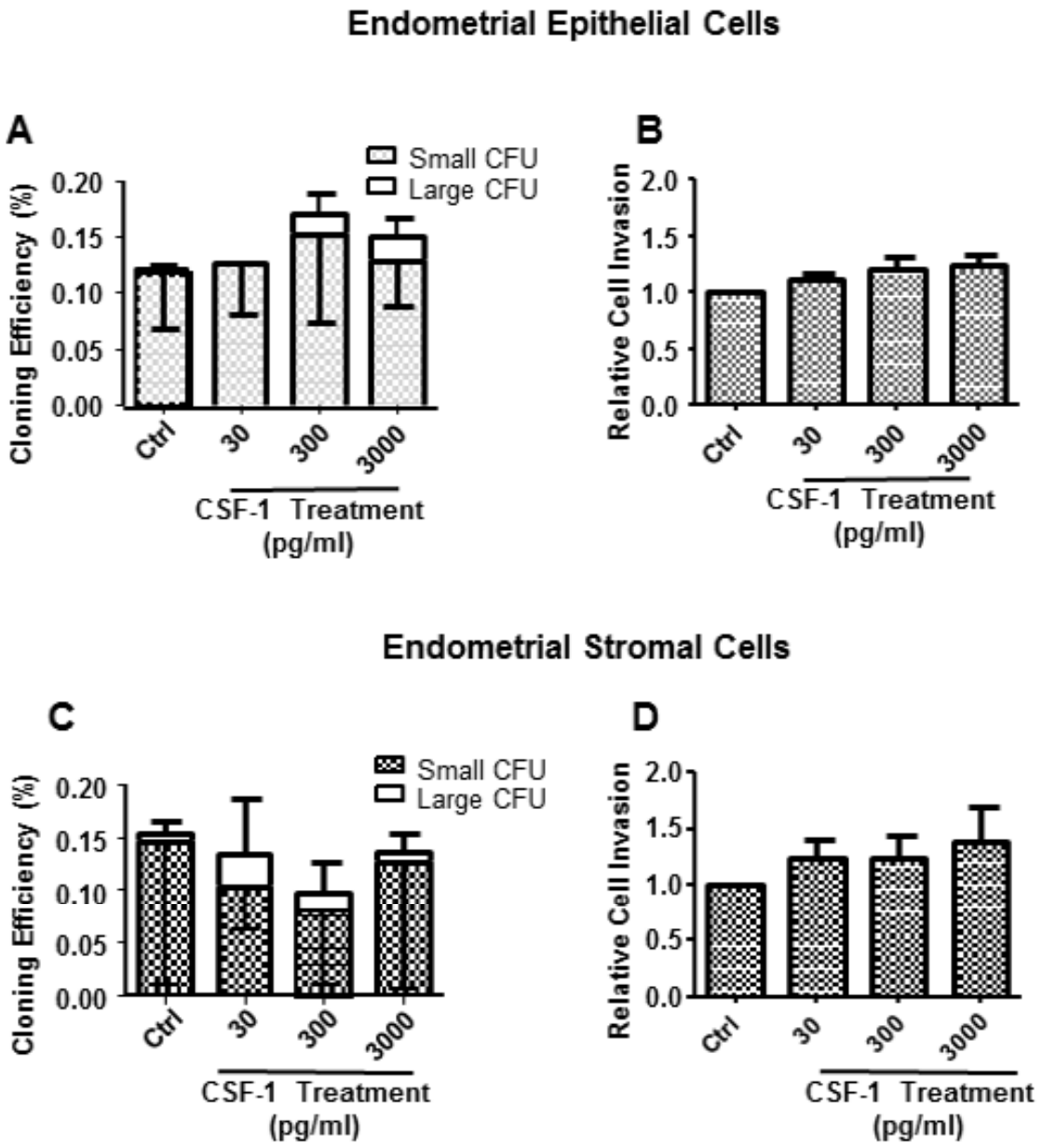


Chan et al. – Figure 3

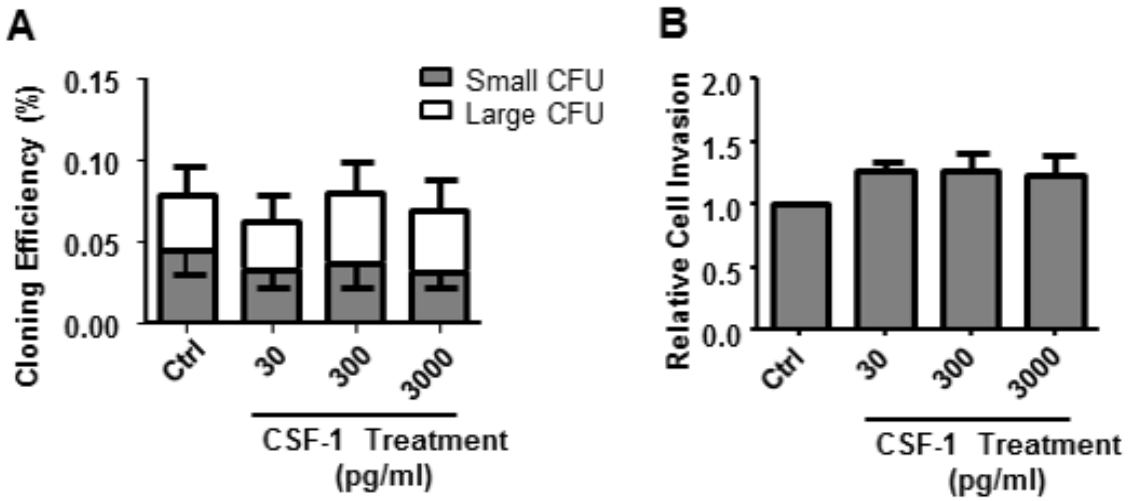


Chan et al – Figure 4

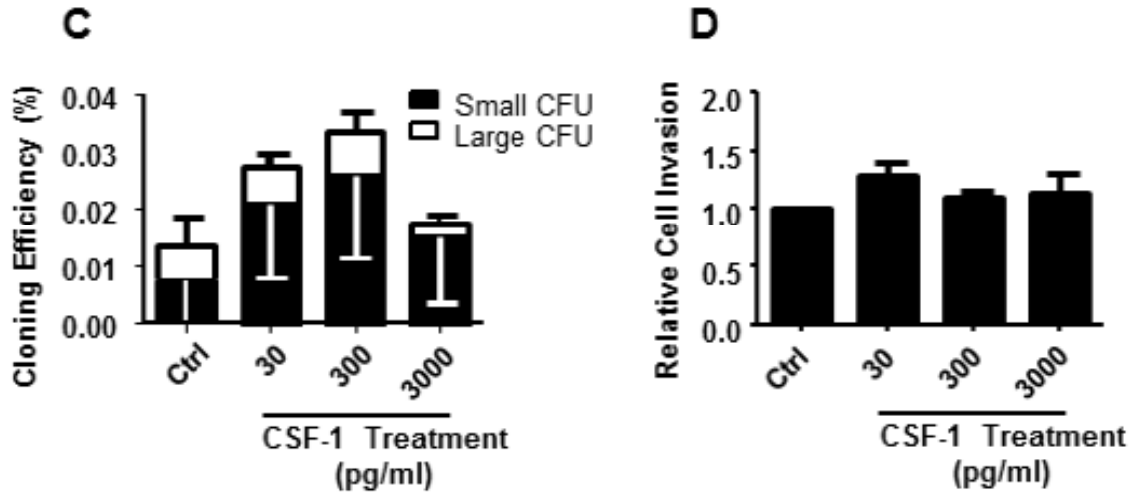


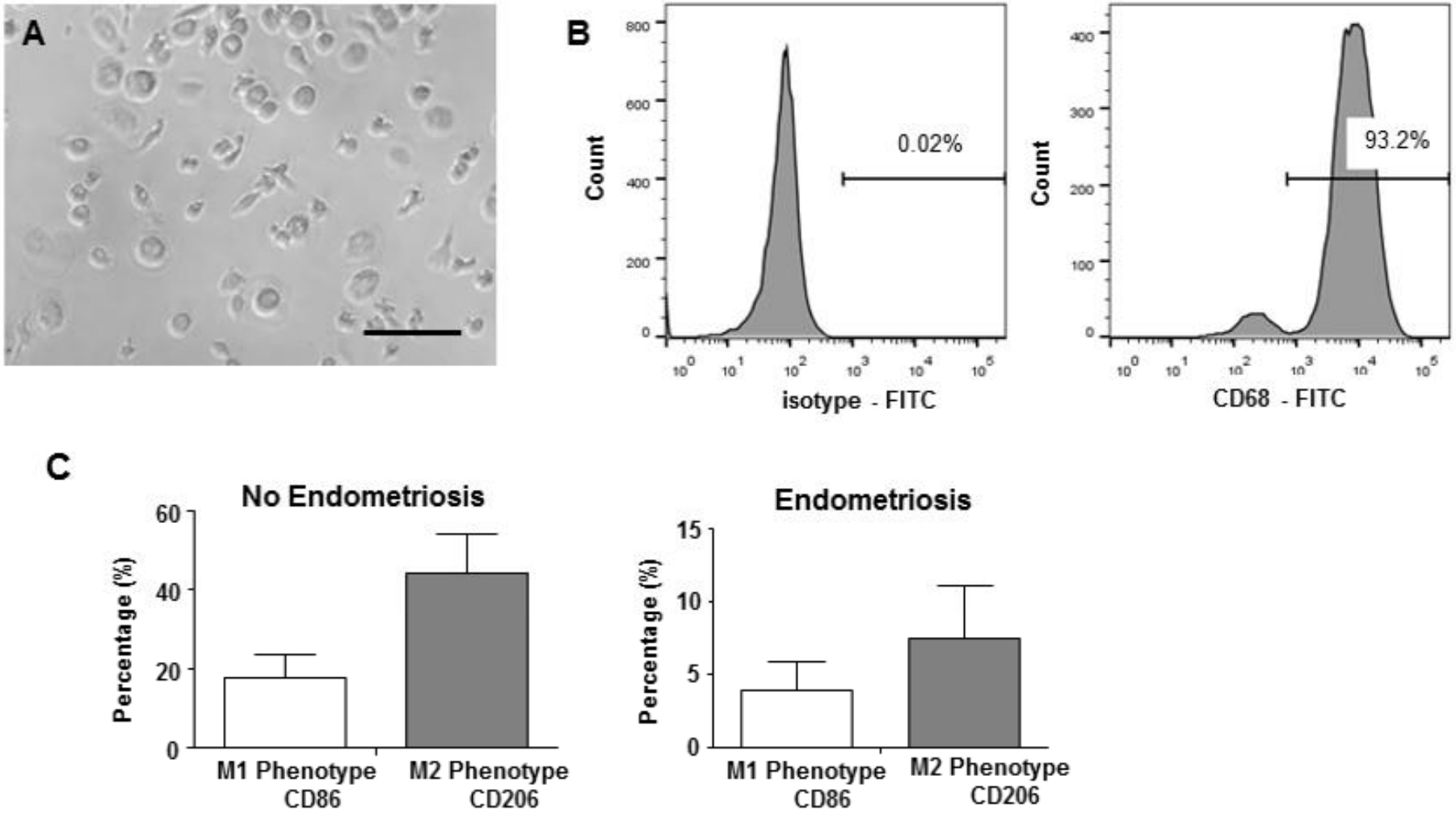

Endometrial Epithelial Cells
A
Small CFU
Large CFU
Cloning Efficiency (%)
0.20
0.15
0.10
0.05
0.00
Ctrl
30
300
3000
CSF-1 Treatment
(pg/ml)
B
Relative Cell Invasion
2.0
1.5
1.0
0.5
0.0
Ctrl
30
300
3000
CSF-1 Treatment
(pg/ml)
Endometrial Stromal Cells
C
Small CFU
Large CFU
Cloning Efficiency (%)
0.20
0.15
0.10
0.05
0.00
Ctrl
30
300
3000
CSF-1 Treatment
(pg/ml)
D
Relative Cell Invasion
2.0
1.5
1.0
0.5
0.0
Ctrl
30
300
3000
CSF-1 Treatment
(pg/ml)

Endometriotic Epithelial Cells

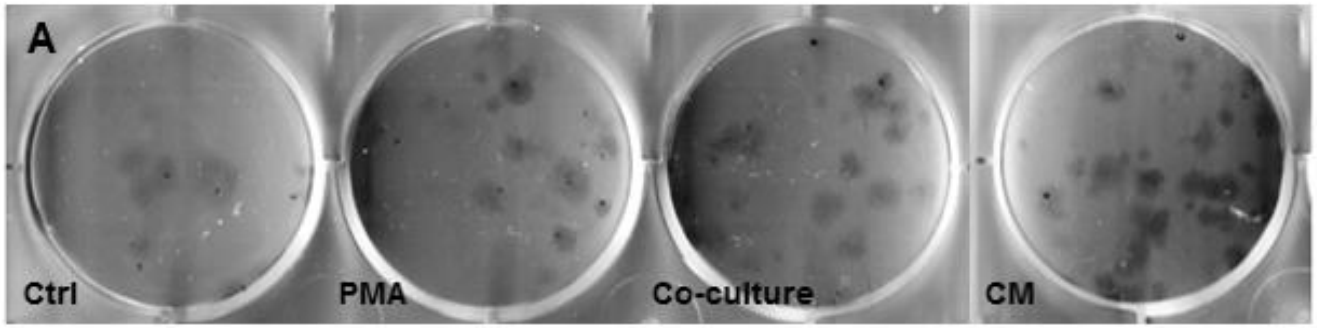


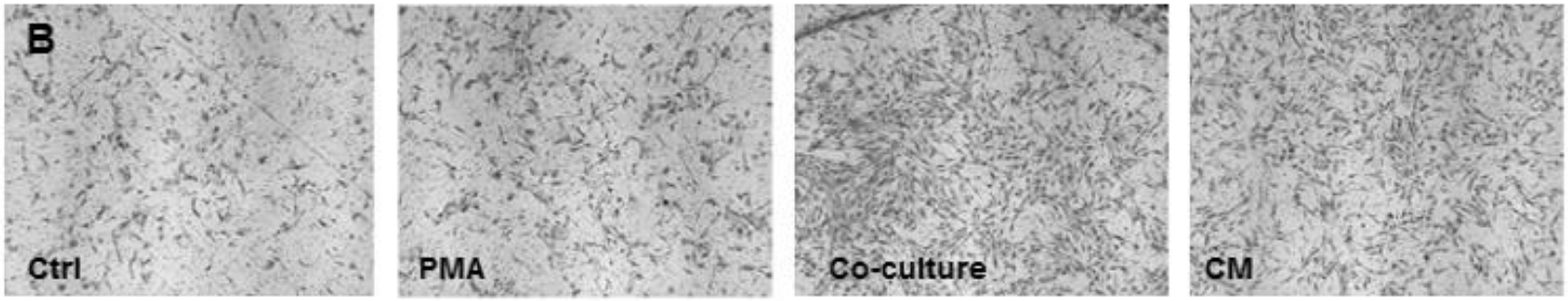
Endometriotic Stromal Cells

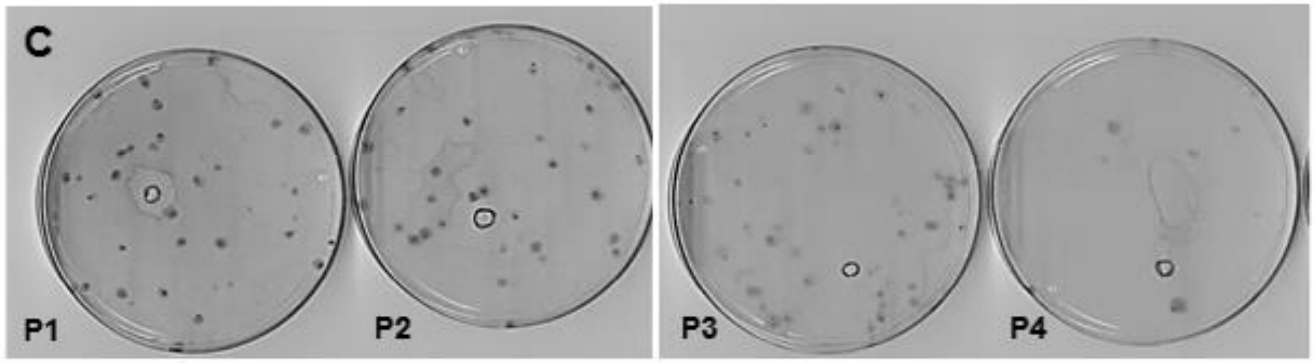



A
B
Count
0.02%
isotype - FITC
Count
93.2%
CD68 - FITC
C
No Endometriosis
Percentage (%)
M1 Phenotype CD86
M2 Phenotype CD206
Endometriosis
Percentage (%)
M1 Phenotype CD86
M2 Phenotype CD206

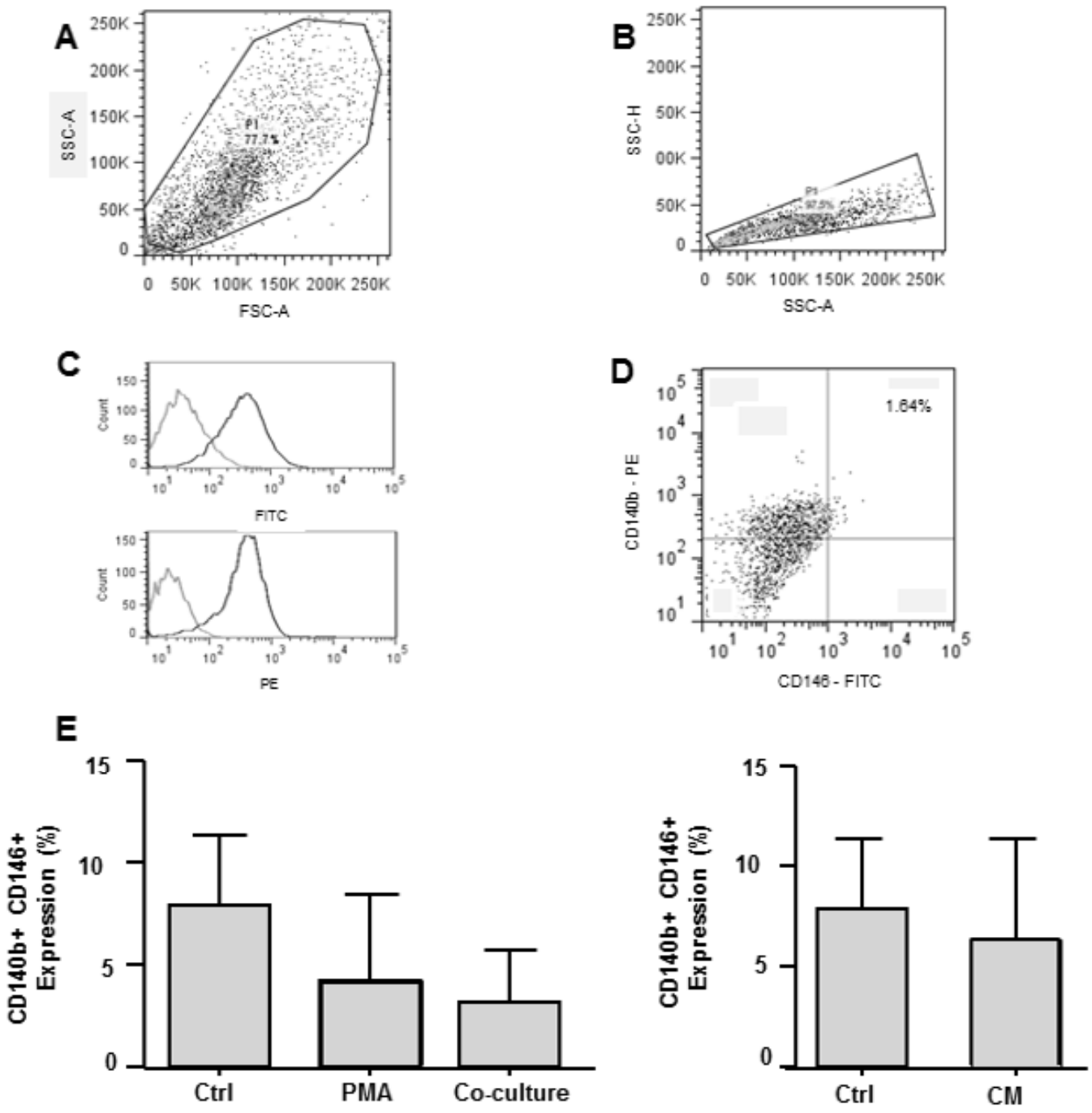
Chan et al. – Supplementary Fig S2



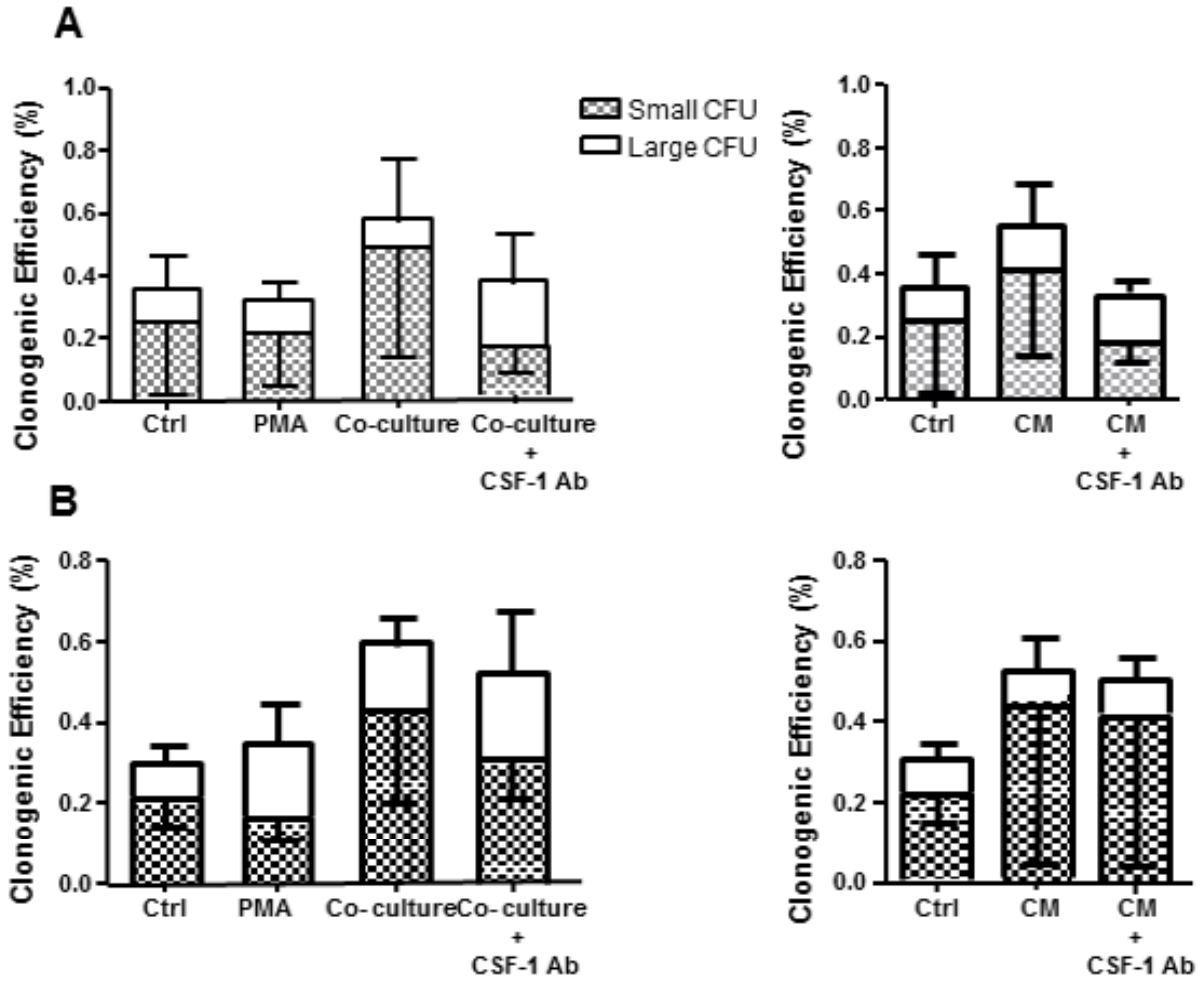




Chan et al. – Supplementary Fig S3



Chan et al. – Supplementary Fig S4



				CXCL5 P=0.09	GCSF P=0.24	GM-CSF P=0.06	GRO a/b/g P=0.31	CXCL1 P=0.13	I-309 P=0.94	IL-1F1 P=0.24	IL-1F2 P=0.18
IL-2 P=0.48	IL-3 P=0.24	IL-4 P=0.33	IL-5 P=0.42	IL-6 P=0.13	IL-7 P=0.24	IL-8 P=0.48	IL-10 P=0.39	IL-12 p40/ p70 P=0.39	IL-13 P=0.70	IL-15 P=0.09	IFN-γ P=0.13
CCL2 P=0.59	CCL8 P=0.39	CCL7 P=0.31	CSF-1 P=0.03	CCL22 P=0.24	CXCL9 P=0.31	MIP-1σ P=0.48	CCL5 P=0.59	SCF P=0.52	SDF-1 P=0.34	CCL17 P=0.39	TGF-β1 P=0.31
TNF-α P=0.39	TNF-β P=0.75	EGF P=0.59	IGF-1 P=0.59	Angiogenin P=0.70	OSM P=0.31	TPO P=0.29	VEGF-A P=0.34	PDGF-BB P=0.82	Leptin P=0.31		

Table 1 – Cytokine array results.