Steroids regulate CXCL4 in the human endometrium during menstruation to enable efficient endometrial repair

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Abstract

Context: Repair of the endometrial surface at menstruation must be efficient to minimize blood loss and optimize reproductive function. The mechanism and regulation of endometrial repair remain undefined.

Objective: To determine the presence/regulation of CXCL4 in the human endometrium, as a putative repair factor at menses.

Patients/Setting: Endometrium was collected throughout the menstrual cycle from healthy women attending the gynecology department. Menstrual blood loss was objectively measured in a subset and heavy menstrual bleeding (HMB) defined as >80ml/cycle. Monocytes were isolated from peripheral blood.

Design: CXCL4 mRNA and protein were identified by RT-qPCR and immunohistochemistry. The function/regulation of endometrial CXCL4 was explored by *in vitro* cell culture.

Results: CXCL4 mRNA concentrations were significantly increased during menstruation. Intense staining for CXCL4 was detected in late secretory and menstrual tissue, localized to stromal, epithelial and endothelial cells. Co-localization identified positive staining in CD68+ macrophages. Treatment of human endometrial stromal (hESC) and endothelial (HEEC) cells with steroids revealed differential regulation of *CXCL4*. Progesterone withdrawal resulted in significant increases in *CXCL4* mRNA and protein in hESCs, whereas cortisol significantly increased CXCL4 in HEECs. In women with HMB, CXCL4 was reduced in endothelial cells during the menstrual phase when compared to women with normal menstrual bleeding. Cortisol exposed macrophages displayed increased chemotaxis towards CXCL4 compared to macrophages incubated with estrogen or progesterone.

Conclusions: Our data implicate CXCL4 in endometrial repair post menses. Reduced cortisol at time of menses may contribute to delayed endometrial repair and HMB, in part by mechanisms involving aberrant expression of CXCL4.

Introduction

The human endometrium displays a remarkable ability to breakdown and fully repair each month in the absence of pregnancy or lactation. Menstruation is triggered by the withdrawal of the ovarian steroid hormones, estrogen and progesterone, as the corpus luteum regresses. This results in a local inflammatory response, including leukocyte influx and edema, which culminates in tissue breakdown by matrix metalloproteases and bleeding (1). Much less is known about the mechanisms and regulation of endometrial repair, but the processes involved appear to be comparable to classic wound healing. These involve temporally overlapping phases of inflammation, resolution of inflammation, tissue formation, tissue remodeling and angiogenesis. In the endometrium this repair process appears to occur in areas of endometrium adjacent to those where breakdown is in progress (2). Delayed repair of the endometrium at menstruation may cause prolonged heavy menstrual bleeding (HMB), which negatively impacts on quality of life for many women.

Macrophages have a well-established role in the repair process at multiple tissue sites (3). They engulf foreign or apoptotic material as part of their phagocytic role and they also secrete a number of proteases, angiogenic factors and growth factors (4). Macrophage depletion has been shown to result in defective repair of skin wounds in the guinea pig (5) and of myocardial injury in mice (6). Endometrial macrophages are present throughout the menstrual cycle, but display a significant increase in number during the perimenstrual phase (7). This increase in the number of tissue resident macrophages is thought to be dependent upon the increase in concentrations of endometrial cytokines that occurs in response to progesterone withdrawal. Cytokines have been implicated in both the recruitment of monocytes into the endometrium and in increased proliferation of macrophages *in situ* (7-9). Recent insights into the phenotype of tissue resident macrophages has revealed that both their plasticity and the prevailing tissue microenvironment influence the ability to adopt pro-wound-healing, pro-resolving and tissue-regenerating phenotypes after injury, reviewed in (10).

CXCL4 (PF4) is a member of the CXC family that has been shown to have a role in chemotaxis of neutrophils and monocytes (11,12). It is currently unknown if CXCL4 is an active chemoattractant within human endometrium but both neutrophils and monocytes are implicated in endometrial repair (13). CXCL4 has been shown to induce differentiation of peripheral blood monocytes, characterized by prevention of spontaneous apoptosis and promotion of differentiation into macrophages in a TNFα and GM-CSF independent fashion (14). CXCL4-stimulated differentiation appears to generate a different macrophage phenotype to the classical M1/M2 subtypes (15). Notably, these macrophages lack expression of the scavenger receptor CD163 (15), are unable to up-regulate heme-oxygenase 1 (15) and do not express the HLA-DR antigen (14) but produce more MMP-7 and MMP-12 protein than other macrophage subtypes (14). In addition, CXCL4 is known to be an angiostatic factor, implicated in inhibition of endothelial cell proliferation (16,17). CXCL4 has been detected at high concentrations at sites of vascular injury (18) and has been found to down-regulate expression of MMP-1 and MMP-3 in human vascular endothelial cells, which may contribute to resolution and repair (19).

As CXCL4 is thought to have a key role in the regulation of angiogenesis, recruitment of monocytes and wound healing, we hypothesized that it has a key role in endometrial repair at the time of menstruation (20). Therefore, we conducted a comprehensive analysis of human endometrial biopsies and utilized *in vitro* cell models to examine the regulation of CXCL4 by steroid hormones including cortisol, as this steroid is thought to play a key role in regulating the local endometrial environment during menstruation. Next, we investigated the impact of CXCL4 on endometrial cells and macrophages. Our results highlight a potential role for this cytokine in the physiological processes of menstruation and endometrial repair.

Methods

Human endometrial tissue collection

Endometrial biopsies (n=61) were collected with a suction curette (Pipelle, Laboratorie CCD, Paris, France) from women (median age 42 years, range 22-50) attending gynecological out-patient departments across NHS Lothian, Scotland. Written consent was obtained from participants and ethical approval granted from Lothian Research Ethics Committee (LREC 07/S1103/29). All women reported regular menstrual cycles (21-35 days) and no exogenous hormone exposure for 2 months prior to biopsy. Women with large fibroids (>3cm) or endometriosis were excluded. Tissue was divided and (i) placed in RNA later, RNA stabilization solution (Ambion (Europe) Ltd., Warrington, UK), (ii) fixed in neutral buffered formalin for wax embedding and (iii) placed in phosphate buffered saline for *in vitro* culture. Cycle stage was determined by (i) histological dating (criteria of Noyes et al. (21)), (ii) reported last menstrual period and (iii) serum progesterone and estradiol concentrations at time of biopsy (Table 1). Samples not consistent for all three criteria were excluded (n=5).

Objective measurement of menstrual blood loss (MBL)

A subset of the participants with biopsies collected in the perimenstrual phase agreed to collect their sanitary ware to allow objective quantification of their menstrual blood loss (MBL) (n=23). Women were provided with the same brand of tampon/pad (Tampax®Always®) and verbal and written instructions on collection. Blood loss was measured using a modified Alkaline-Haematin method as previously described (22,23). A measured MBL of >80ml was classified at heavy menstrual bleeding (HMB) and <80ml as normal (NMB). This method was validated in our laboratory using time expired whole blood applied to the same sanitary products given to participants.

Immunohistochemistry for CXCL4

5µm paraffin sections were dewaxed and rehydrated. Antigen retrieval was by pressure cooker in sodium citrate pH 6 antigen retrieval buffer. Endogenous peroxidase activity was blocked by 3%

hydrogen peroxide. Sections were sequentially incubated in avidin and biotin (Vector Laboratories, Burlingame, CA, USA) and protein block (Dako, Cambridge, UK). Rabbit polyclonal CXCL4 antibody (20µg/ml, Abcam, ab9561, Cambridge, UK) was applied overnight at 4°C. Negative controls were incubated with Rabbit IgG (Dako) at the same concentration as the primary antibody. Biotinylated goat anti-rabbit secondary antibody was used at 1:200 (Vector). Avidin-biotin-peroxidase complex (ABC-Elite; Vector laboratories) was applied for 30 min and liquid diaminobenzidine (DAB) kit (Zymed Laboratories, San Francisco, CA, USA) used for detection. The reaction was stopped with distilled water and sections counterstained with haematoxylin, dehydrated and mounted with Pertex (Cellpath plc, Hemel Hempstead, UK).

Semi-quantitative immunoscoring

Localization and intensity of immunostaining was evaluated in the late secretory and menstrual endometrium of women with objectively measured HMB and NMB by two independent, masked observers. The intensity of staining was graded with a three-point scale (0 = no staining, 1 = mild staining, 2 = strong staining). This was applied to the stromal compartment and endothelial cells. The percentage of tissue in each intensity scale was recorded (24). A value was derived for each of the cellular compartments by using the sum of these percentages after multiplication by the intensity of staining. Average scores are reported unless a discrepancy of >50 points occurred between observers, in these cases the tissue was examined together and a consensus score determined.

Dual immunofluorescence

Endometrial sections were dewaxed, rehydrated, exposed to antigen retrieval and treated with 3% hydrogen peroxidase as above. For CD68/CXCL4 dual immunofluorescence, normal donkey serum was used as a protein block and the sections were incubated with mouse monoclonal CD68 (macrophage marker) antibody (Dako, Glostrup, Denmark) at a 1 in 1000 dilution overnight at 4°C. Donkey anti-mouse peroxidase secondary antibody (Abcam, Cambridge, UK) at a 1:750 dilution was applied for 30 min followed by incubation with TSA™ fluorescein tyramide system (Perkin Elmer, Waltham, MA., USA) for 10 min. The sections were incubated with normal donkey serum for 10 min

followed by 20µg/ml rabbit polyclonal CXCL4 antibody (Abcam) overnight at 4°C. Alexa 546 donkey anti-rabbit secondary antibody (Invitrogen, Paisley, U.K.) was applied at 1:200 for 1h, followed by a 4',6-diamidino-2-phenylindole (DAPI) stain (Sigma, Dorset, UK) for 10 min. CD31/CXCL4 immunofluorescence utilized Novocastra epitope retrieval solution Ph6 (Leica Microsystems, Wetzlar, Germany) and the Leica Bond-Max automated immunostainer (Leica Microsystems). Normal goat serum was used as a protein block prior to incubation with CXCL4 antibody (Abcam) at a 1:2000 dilution for 1h at 37C, omission of primary antibody provided negative controls. Goat anti rabbit secondary antibody (Abcam) was applied before incubation with TSA™ fluorescein tyramide system (Perkin Elmer) for 10 min. Bond wash was followed by Bond epitope retrieval system (Leica), block with normal goat serum and incubation with mouse monoclonal CD31 (Novacastra, Milton Keynes, UK) at a 1 in 600 dilution for 1h. Goat anti-mouse secondary antibody (Abcam) was applied, followed by a 4',6-diamidino-2-phenylindole (DAPI) stain (Sigma, Dorset, UK) for 10 min. All sections were mounted with Permaflour (Thermo Scientific, Waltham, MA, USA) and analyzed on a Zeiss LSM710 confocal microscope system.

Cell culture

Primary human endometrial stromal cells (HESC) were isolated from mid-secretory endometrial tissue (n=3) by enzymatic digestion as previously described (25). HESCs at passage <6 were plated at a density of 10^6 cells per well in 6 well plates in RPMI medium. Cells were serum starved for 24h prior to treatments. Cells were treated with (i) 10nM estradiol for 48h, (ii) 1µM cortisol for 48h, (iii) 1µM progesterone for 6 days or (iv) 1µM progesterone for 6 days followed by serum free media for 48h to mimic progesterone withdrawal.

Human endometrial endothelial cells (HEECs) were a gift from Yale School of Medicine (26). Their isolation (27) and phenotype (28) have been previously described. Serum starved HEECs were treated in an identical manner to HESCs, described above.

Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR)

Concentrations of mRNAs encoded by *CXCL4* were determined by RT-qPCR (Taqman) analysis. Total RNA from cells and endometrial biopsies was extracted using the RNeasy Mini Kit (Qiagen Ltd, Sussex, UK) according to manufacturer's instructions: 100ng RNA samples were reverse transcribed according to standard laboratory protocols (29). A tube with no reverse transcriptase and a further tube with water were included as controls. PCR reaction mixtures were prepared containing Taqman buffer (5.5mM MgCl2, 200µM dATP 200µM dCTP, 200µM dGTP, 400µM deoxyuridine triphosphate), ribosomal 18S primers/probe (Applied Biosystems, Warrington, UK) and specific forward and reverse primers and probes (CXCL4 forward primer agcctggaggtgatcaagg, reverse primer ccattcttcagcgtggcta, Universal probe library number 43, all from Roche Applied Science, Penzberg, Germany) were added for each PCR reaction. Negative controls (water instead of cDNA) were included in each run. PCR was carried out using ABI Prism 7900 (Applied Biosystems, Foster city, CA, USA). Forty cycles were completed (3s 95C, 30s 60C). Samples were analyzed in triplicate using Sequence Detector version 2.3 (PE Biosystems, Foster city, CA, USA), using the comparative threshold method. Expression of target mRNA was normalized to RNA loading for each sample using the 18S ribosomal RNA as a reference.

In cell Western

Following treatments, cells were fixed with 4% NBF for 15 min prior to incubation with blocking buffer (PBS, normal goat serum, water and Triton X-100). Cells were treated with rabbit polyclonal anti-CXCL4 (1:25 Abcam) and mouse monoclonal anti-β-tubulin (1:1000 Sigma) antibodies overnight at 4°C. Cells were washed prior to incubation with goat anti-rabbit IRDye 800CW (Molecular Probes, Eugene, OR, USA) and goat anti mouse Alexa Fluor 680 (Li-Cor Biosciences, Lincoln, NE, USA). The LI-COR Odyssey Infrared Imaging System was used to analyze results.

Macrophage culture

Peripheral blood was obtained from consenting women (LREC 08/S1103/38) on the combined oral contraceptive pill (n=9) to avoid natural hormone fluctuations and monocytes extracted as previously

described (9). Monocytes were cultured into Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, St Louis, MO, USA) with M-CSF (216.21nM) treatment for 5 days to differentiate the cells into macrophages. Macrophages were then treated with 285.71nM GM-CSF (to induce an M0 phenotype), 59.17mM IFNγ (M1 phenotype), 1μM Cortisol (M2 phenotype), Estrogen (10nM) or Progesterone (10nM) for 24h. Cells were washed and re-suspended in serum-free RPMI for 24h, centrifuged, supernatant removed and frozen for use as conditioned media.

Chemotaxis assay

Microslides (Ibidi, Martinsried, Germany) were coated with collagen according to the manufacturer's instructions; collagen was solidified by incubation at 37°C for 30 min. The first well of each capillary had peripheral blood monocyte derived macrophages (PBMC) in RPMI media (Sigma). The connecting well had 20ng/ml CXCL4 (Sigma; the half maximal effective concentration (EC50) for CXCL4 as found by Baltus et al., 2005 (11)) in RPMI media; RPMI alone was used as a negative control. Movement of PBMCs was measured after 24h using an Axiovert 200 microscope (Ziess). Distance measured was converted into percentage movement where complete movement would be 100%, and no movement 0%. The experiment was repeated with PBMCs from 5 different women.

Statistical Analysis

For cell culture, mRNA results are expressed as fold increase, where relative expression of mRNA after treatment was divided by the relative expression after vehicle treatment. For tissue data, results were expressed as a quantity relative to a comparator, a sample of placental cDNA. Data are presented as mean ± SEM and significant differences among raw data (ddCt values) determined using Kruskal–Wallis non-parametric test with Dunn's multiple comparison post-test. Statistical analysis between women with HMB and NMB bleeding at different stages of the cycle was determined using a two-way ANOVA with Bonferroni post-test analysis. Student's t-tests were used for immunoscore data. GraphPad Prism Software was used, version 6 (San Diego, CA, USA). A value of $P < 0.05$ was considered significant.

Results

***CXCL4* mRNA concentrations were increased in menstrual phase human endometrium and CXCL4 localized to epithelial, stromal, endothelial cells and macrophages.**

CXCL4 encoded mRNAs were detected in human endometrial tissue biopsies throughout the cycle an (Figure 1A). *CXCL4* mRNA concentrations were significantly higher in menstrual biopsies compared to those from the proliferative ($P<0.05$), early secretory ($P<0.01$) and mid secretory phases ($P<0.05$).

Immunohistochemistry detected CXCL4 protein in the cytoplasm of epithelial and stromal cells throughout the menstrual cycle, with an increase in staining intensity noted in endometrium collected from women during the secretory and menstrual phases (Figure 1B). Dual immunofluorescence revealed positive CXCL4 staining in CD31+ endometrial endothelial cells during the late secretory/menstrual phase (Figure 1B xi). We observed intense immunostaining of occasional cells within the stromal compartment throughout the cycle. Dual immunohistochemistry revealed CXCL4 was present in the cytoplasm of CD68+ macrophage cells throughout the menstrual cycle (Figure 1C).

Endometrial CXCL4 was regulated by progesterone withdrawal and cortisol

After confirming the presence of CXCL4 in endometrial stromal and endothelial cells, we examined its regulation by steroids using primary endometrial stromal cells (HESCs) and a human endometrial endothelial cell line (HEECs). Treatment with 10nM estradiol mimicked the proliferative phase, 1µM progesterone the secretory phase and sequential progesterone treatment and subsequent removal mimicked the late secretory/menstrual phase. There is mounting evidence that cortisol has an important role in the local endometrial environment at menses (9,20); therefore additional cells were treated with 1µM cortisol. HESCs undergoing progesterone-withdrawal treatments showed a significant increase in concentrations of *CXCL4* mRNA when compared to those treated with vehicle, estradiol or cortisol (Figure 2A). Progesterone withdrawal also significantly increased CXCL4 protein in HESCs when compared to vehicle treated cells (Figure 2B, C).

Interestingly, CXCL4 regulation in HEECs was different to that detected in HESCs. Cortisol treatment of HEECs displayed maximal increases in concentrations of *CXCL4* mRNA (Figure 3A) and protein (Figure 3B, C), which were significantly greater than treatment to mimic progesterone-withdrawal ($P<0.01$).

CXCL4 was significantly decreased in endometrial endothelial cells from women with HMB during the menstrual phase

As *CXCL4* mRNA was maximal in endometrium from the late secretory and menstrual phases of the cycle, we compared mRNA concentrations in endometrial tissue homogenates from these two phases, taken from women with objectively measured menstrual blood loss (MBL). Using a blood loss of >80ml to define HMB, we found no significant differences in mRNA concentrations when comparing women with HMB and NMB (Figure 4A).

As we determined that regulation of CXCL4 varied in stromal and endothelial cells *in vitro*, we hypothesized that cellular levels of CXCL4 may differ in women with HMB and NMB, despite no significant differences in global endometrial *CXCL4* mRNA concentrations. We examined CXCL4 protein by immunohistochemistry in endometrium of the late secretory and menstrual phases from women with NMB and HMB. Semi-quantitative immunoscoring of the stromal compartment and endothelial cells revealed no significant changes during the late secretory phase between women with NMB and HMB (Figure 4B). However, menstrual phase endometrium from women with HMB had significantly decreased CXCL4 staining of endothelial cells versus tissue from women with NMB ($p<0.05$) (Figure 4B, C). In contrast, menstrual stromal compartment staining was not significantly different in endometrium from women with NMB and HMB.

CXCL4 has an augmented chemotactic action on macrophages pre-exposed to cortisol

As CXCL4 increased at menses and co-localized to macrophage cells, we investigated the effect of CXCL4-induced chemotaxis on different macrophage subtypes. Peripheral macrophages were pre-

297 treated to induce different subtypes: M0 (M-CSF pre-treated), M1 (GM-CSF and IFNγ pre-treated)
298 and M2 (cortisol pre-treated) macrophages, or macrophages exposed to a proliferative phase
299 environment (estradiol pre-treatment) or exposed to a secretory phase environment (progesterone pre-
300 treated). These pre-treated macrophages were plated into wells opposite CXCL4 on a multi-
301 channeled microslide. Cortisol-exposed macrophages migrated towards CXCL4 at a significantly
302 higher rate than any of the other macrophage subtypes (Figure 5A, B).
303

Discussion

This manuscript details the presence of CXCL4 in the human endometrium across the menstrual cycle and reveals maximal levels are present during menstruation. Steroid regulation of CXCL4 occurs in human endometrial stromal cells, with significant increases following withdrawal of progesterone. In contrast, endometrial endothelial cells do not display an increase in CXCL4 on progesterone withdrawal, but demonstrate significant increases in response to cortisol treatment. Furthermore, we reveal that women with HMB have significantly reduced CXCL4 in endothelial cells in the menstrual phase, consistent with a defective cortisol response at menses (20). Macrophages pre-treated with cortisol to induce an M2 phenotype migrate significantly faster towards CXCL4 than M0 and M1 subtypes. These data are consistent with CXCL4 having a key role in endometrial breakdown and repair at menstruation.

CXCL4 is present in the human endometrium during menstruation, with both PCR and immunohistochemistry being consistent with maximal detection during the menstrual phase. The functional layer of the endometrial breaks down during menses, with repair occurring simultaneously in adjacent areas (2). Therefore, maximal CXCL4 within the endometrium at this time is consistent with involvement in breakdown and repair of the tissue. Expression of the CXCL4 receptor, CXCR3, has been identified as necessary for efficient wound healing (30). Mice lacking CXCR3 had significantly delayed re-epithelialization and delayed repair of the basement membrane following excisional wounds.

Next, we investigated the regulation of CXCL4 in the human endometrium. Due to the dramatic variations observed across the menstrual cycle, we examined steroid regulation of this cytokine. A series of *in vitro* studies revealed that progesterone withdrawal resulted in a significant increase of CXCL4 expression within endometrial stromal cells, consistent with maximal levels during menstruation. Human endometrial endothelial cells, however, do not express the progesterone

receptor (31), hence it was unsurprising that treatment conditions using progesterone or progesterone withdrawal had no profound effects. However, human endometrial endothelial cells are known to express the glucocorticoid receptor (32) and treatment of these cells with cortisol resulted in a significant increase of CXCL4 expression. We have previously shown that local levels of cortisol regulating enzymes increase in human endometrial tissue during menstruation (33). Therefore, two different steroid hormones have the ability to regulate CXCL4 in endometrial cells to increase concentrations of this putative wound repair factor during menstruation.

As CXCL4 is a putative endometrial repair factor, we examined mRNA concentrations in endometrial tissue sample homogenates from women with HMB and NMB. We hypothesized that women with HMB would have reduced CXCL4 induction during menstruation, leading to inefficient endometrial repair and prolonged, HMB. However, no significant differences in *CXCL4* mRNA concentrations were detected between these two groups of women during the late secretory or menstrual phases. There are two potential explanations for these findings. Firstly, there may be no deregulation of CXCL4 in women with HMB. However, our results suggested that different cell types within the human endometrium have differential regulation of CXCL4 induction with progesterone withdrawal having a significant impact on stromal cells and cortisol regulating CXCL4 in endothelial cells. Examination of homogenized whole endometrial biopsies may mask differential expression of CXCL4 within different cell types in women with heavy versus normal menstrual blood loss. Therefore we examined CXCL4 protein in stromal cells and endothelial cells in women with NMB and HMB during the late secretory and menstrual phases. This revealed that endothelial cell CXCL4 protein was significantly reduced in women with HMB versus NMB during menses, which might be consistent with a defective cortisol microenvironment (33). Our laboratory has previously revealed that the cortisol-inactivating enzyme 11 beta-hydroxysteroid dehydrogenase-2 is significantly increased in endometrium from women with HMB versus NMB, thereby creating a local glucocorticoid deficiency (20). Therefore, we propose that women with HMB have reduced endometrial cortisol leading to decreased CXCL4 in endothelial cells which may contribute to

increased menstrual blood loss. CXCL4 is known to have angiogenic properties (18,19) but its functional role in the endometrium remains to be determined.

CXCL4 is known to be a chemoattractant in a number of tissues, triggering migration of monocytes and macrophages to sites of inflammation (34,35). Herein we show that cortisol treated, M2-like macrophages exhibit increased chemotaxis towards CXCL4 when compared to other steroid treated macrophages. This suggests that the microenvironment created by synthesis of CXCL4 may alter immune cell components. It is also notable that cortisol treated macrophages have been documented to take part in the resolution of inflammation, including removal of apoptotic cells (36). Taken together, these data suggest that CXCL4 may act as a chemoattractant at focal points within the human endometrium that require repair.

In summary, we have identified that CXCL4 is increased in the human endometrium during menstruation, a time consistent with involvement in endometrial repair. Mechanistically, we have revealed that endometrial CXCL4 is regulated by progesterone withdrawal and cortisol. In addition, we reveal that CXCL4 is reduced in endothelial cells of women with HMB at menses. Functionally, CXCL4 appears to have a significant role as a macrophage chemoattractant, particularly for macrophages pre-exposed to cortisol. These data implicate CXCL4 as a key player in the physiological process of endometrial repair post menses.

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Figure Legends

Figure 1. (A) *CXCL4* in whole human endometrial biopsies from across the menstrual cycle reveals maximal expression during the menstrual phase. Each box represents lower quartile, median and upper quartile. Whiskers display minimum and maximum values. *P<0.05, **P<0.01. **(B)** CXCL4 protein in the human endometrium was localized to the cytoplasm of a few stromal cells during the proliferative phase (i and ii). During the early (iii and iv), mid-(v and vi) and late (vii and viii) secretory phases of the menstrual cycle, immunostaining for CXCL4 progressively increased in intensity in both the stroma (St) and secretory glandular epithelium (GE). CXCL4 protein was present throughout the endometrium during the menstrual phase (ix and x) and CXCL4 (green) co-localised to CD31+ve endothelial cells (red) (xi). Insets = negative controls. Arrows indicate CXCL4 positive cells. Scale bar 50µm. **(C)** CXCL4 (green) co-localises to CD68+ve (red) macrophage cells within the endometrium throughout the menstrual cycle. Proliferative (i and ii), late secretory (iii and iv), menstrual (v and vi). Insets show negative controls. Arrows indicate co-localized cells.

Figure 2. Steroid regulation of CXCL4 in human endometrial stromal cells (hESCs). Estrogen, progesterone, progesterone withdrawal or cortisol treatment of hESCs revealed that progesterone withdrawal significantly up-regulated **(A)** CXCL4 mRNA expression (n=5) and **(B)** CXCL4 protein levels detected by in cell Western (n=4) and quantified by densitometry **(C).** Green: CXCL4, Red: β-tubulin; P: progesterone. *p<0.05; **p<0.01.

Figure 3. Steroid regulation of CXCL4 in human endometrial endothelial cells (HEECs). Treating HEECs with estrogen, progesterone, progesterone-withdrawal, and cortisol found that CXCL4 was significantly up-regulated by treatment with cortisol at **(A)** the mRNA level (n=4) and **(B)** protein level (n=4), quantified by densitometry **(C).** Green: CXCL4, Red: β-tubulin; **p<0.01.

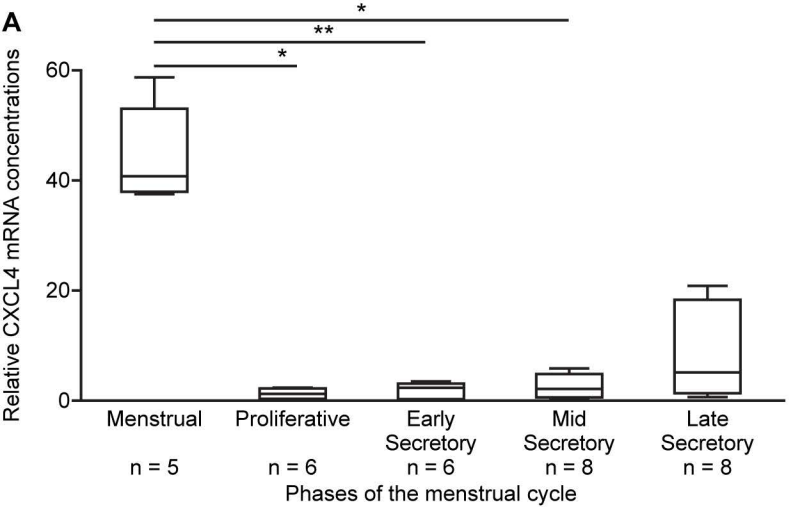
Figure 4. (A) *CXCL4* in late secretory and menstrual endometrial biopsies from women with objectively measured normal (NMB <80ml, white bars) and heavy menstrual bleeding (HMB >80ml, grey bars). **(B)** Immunoscoring of CXCL4 staining of the stromal compartment and endothelial cells in late secretory and menstrual endometrium from women with NMB and HMB. **(C)** Immunohistochemistry staining of CXCL4 in menstrual endometrium from women with NMB and HMB. Inset: IgG matched negative control. Arrow: endothelial cells. LS: late secretory, M: menstrual, St: stromal compartment. NS non-significant, *p<0.05.

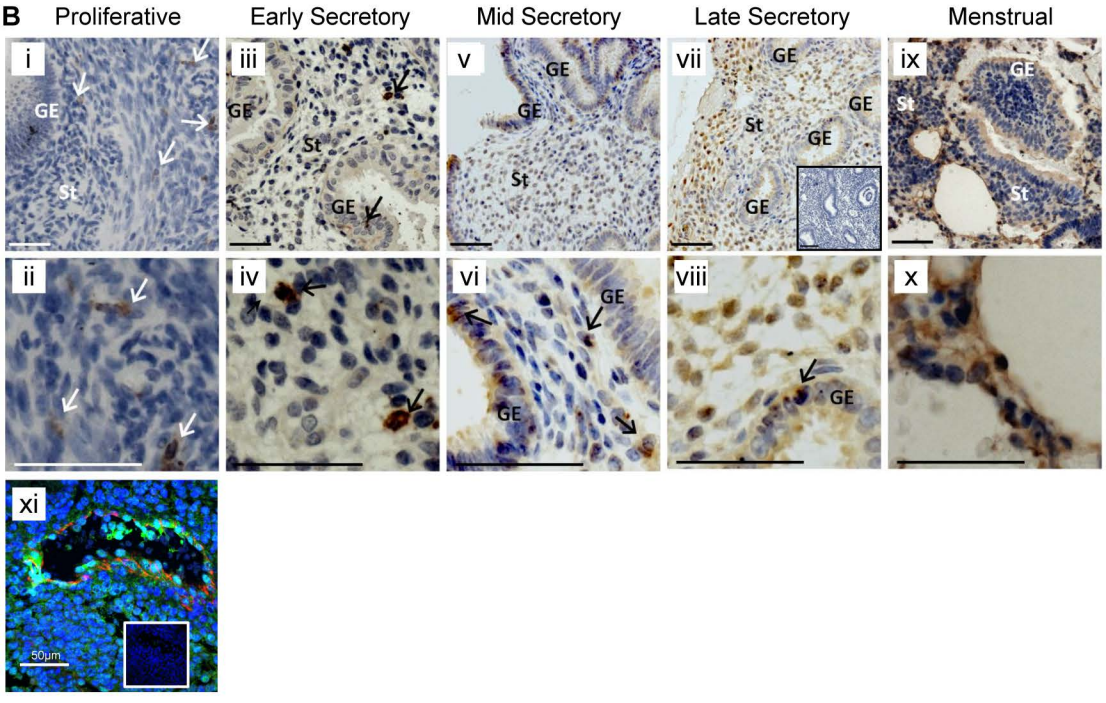
Figure 5. Cortisol-exposed macrophages show increased migration towards CXCL4. **(A)** Pre-treated macrophages (with M-CSF to give an M0 phenotype, GM-CSF and IFNγ to produce an M1 type, cortisol to give an M2 phenotype, estradiol or progesterone) were plated opposite CXCL4 and photographed after 24h. **(B)** Measuring distance travelled (as a percentage of total distance) confirmed macrophages pre-treated with cortisol migrated significantly further than other cells. n=5 separate patient samples; *p<0.05.

Table 1. Classification of endometrial biopsies. NMB normal menstrual bleeding, HMB heavy menstrual bleeding. MBL menstrual blood loss.

Stage of Cycle	**Mean Estradiol** pmol/l (min-max)	**Mean Progesterone** nmol/l (min-max)	**NMB MBL** ml (min-max)	**HMB MBL** ml (min-max)
Proliferative	410 (167-679)	2.8 (1.4-4.6)	-	-
Early Secretory	439 (289-664)	55.4 (26.6-89.9)	-	-
Mid Secretory	585 (301-691)	81.8 (16.1-246.4)	-	-
Late Secretory	275 (59-819)	7.5 (1.1-17.0)	48 (35-62)	200 (85-488)
Menstrual	174 (50-514)	3.4 (1.2-10.6)	40 (26-66)	180 (91-287)

Figure 1


A
Relative CXCL4 mRNA concentrations
60
40
20
0
*
**
*
Menstrual
Proliferative
Early Secretory
Mid Secretory
Late Secretory
n = 5
n = 6
n = 6
n = 8
n = 8
Phases of the menstrual cycle


B
Proliferative
Early Secretory
Mid Secretory
Late Secretory
Menstrual
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
GE
St
50µm

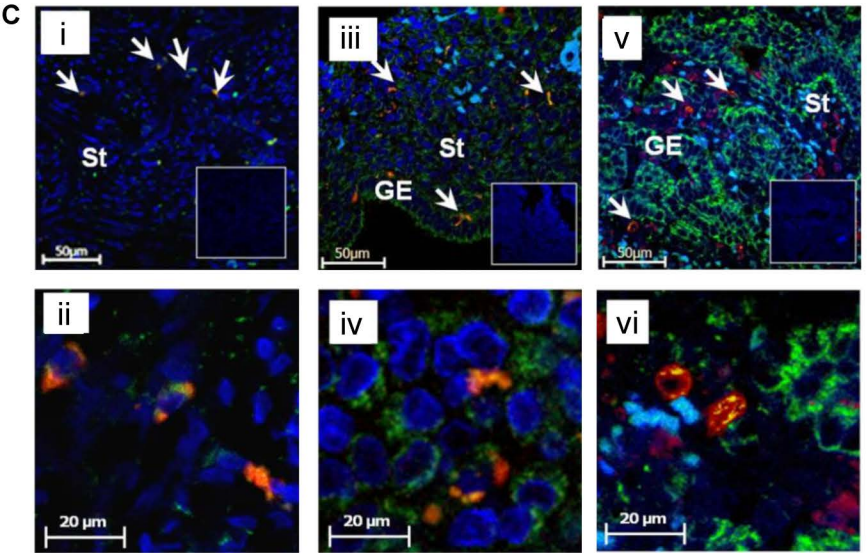

C
i
ii
iii
iv
v
vi
St
GE
50µm
20 µm

Figure 2

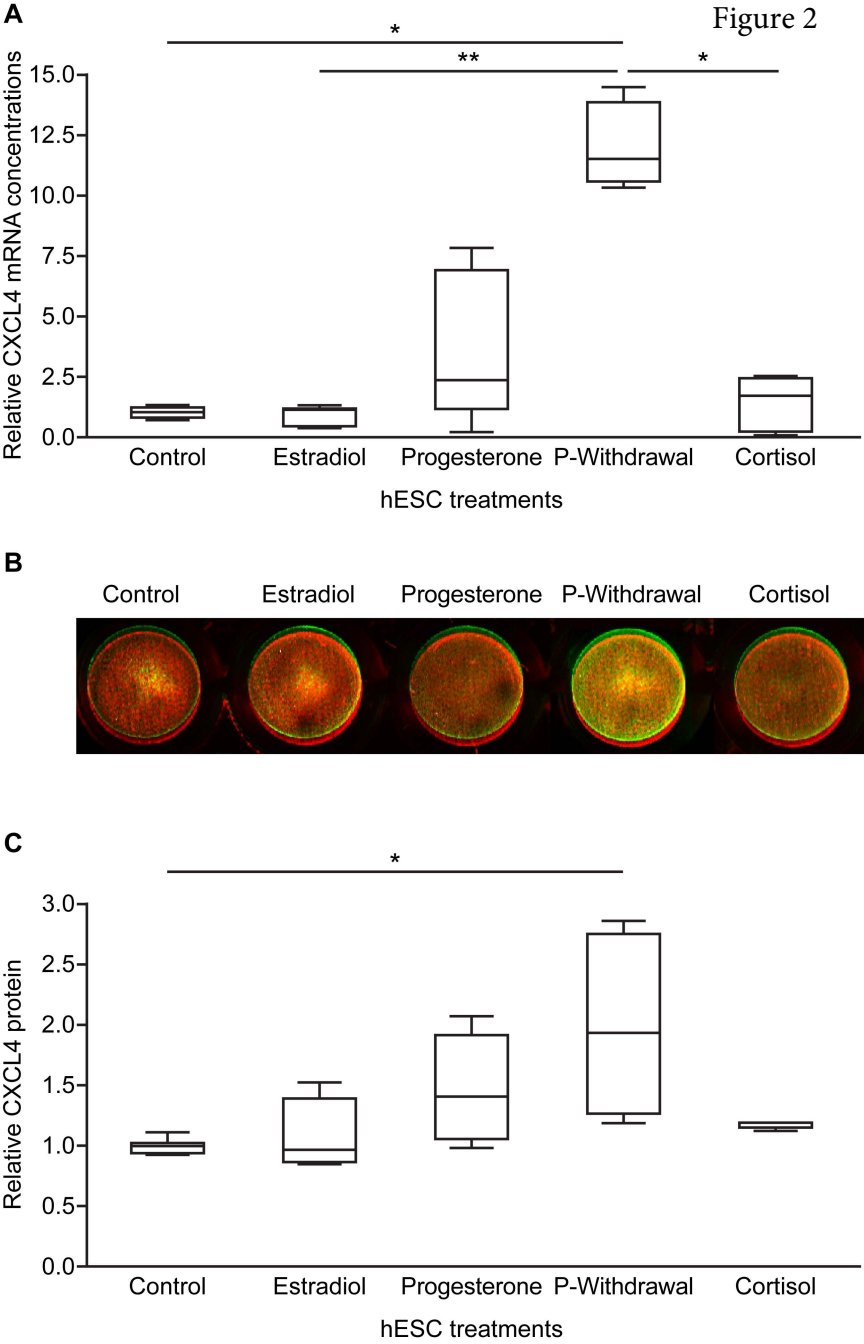

A
*
**
*
Relative CXCL4 mRNA concentrations
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Control
Estradiol
Progesterone
P-Withdrawal
Cortisol
hESC treatments
B
Control
Estradiol
Progesterone
P-Withdrawal
Cortisol
C
*
Relative CXCL4 protein
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Estradiol
Progesterone
P-Withdrawal
Cortisol
hESC treatments

Figure 3

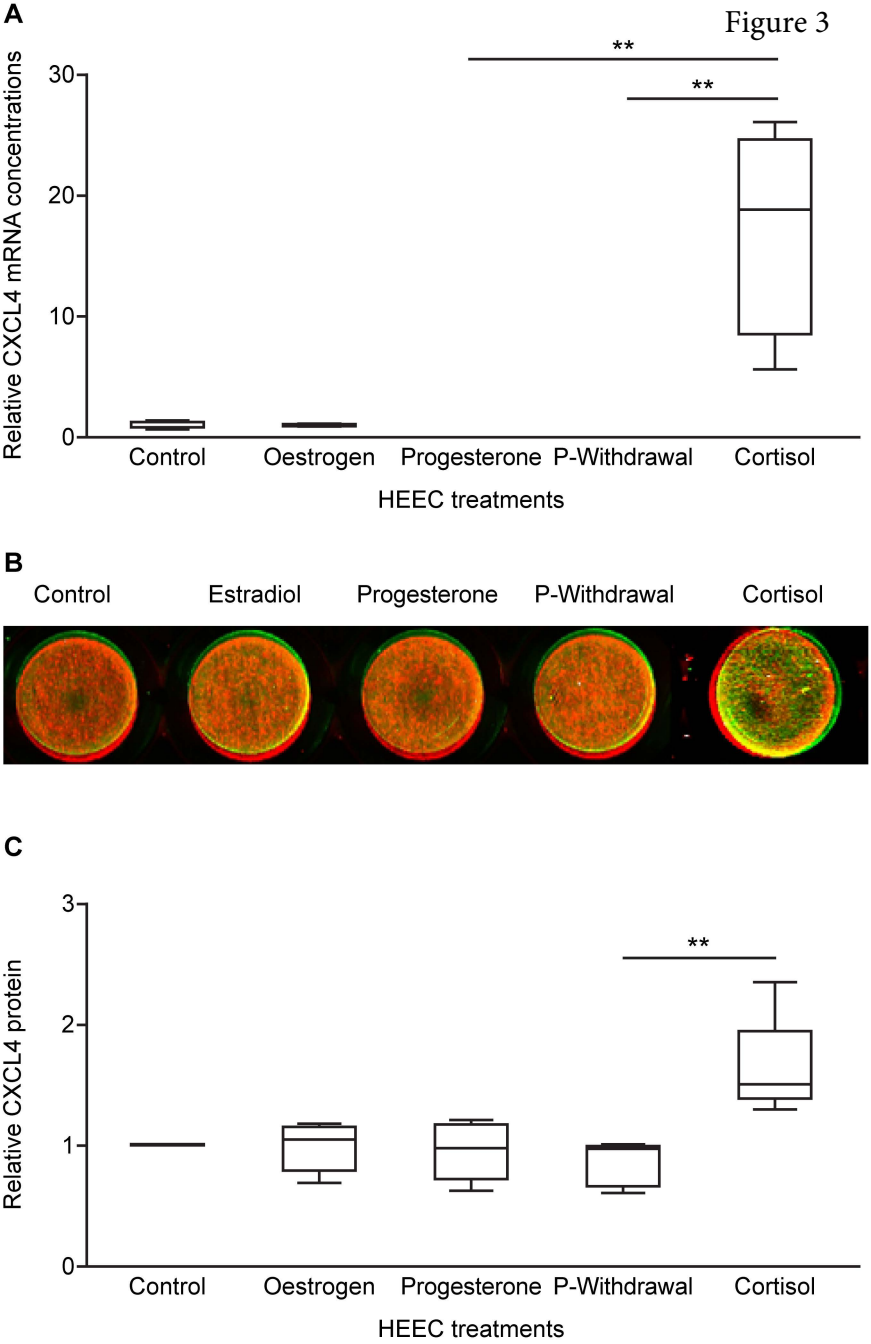
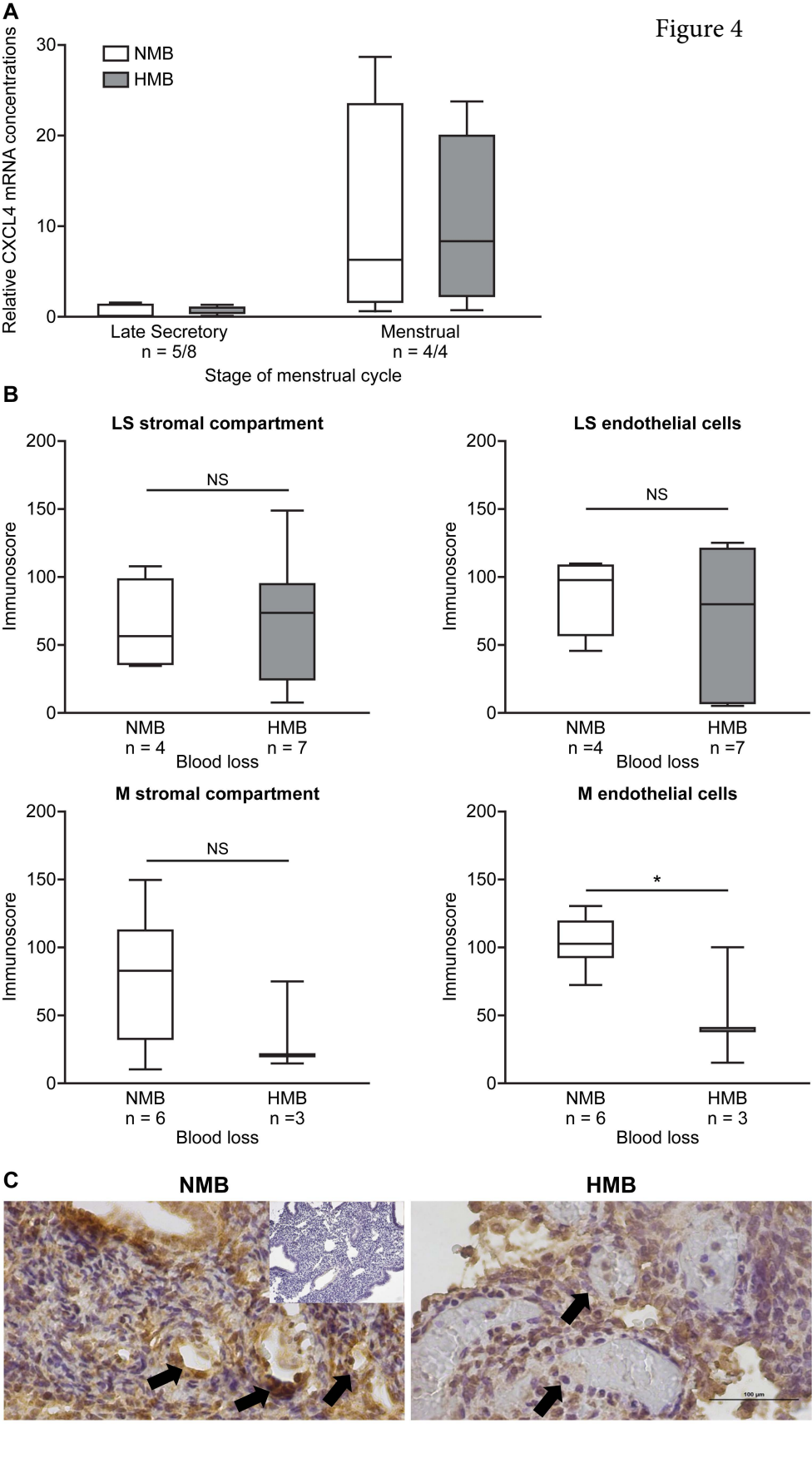
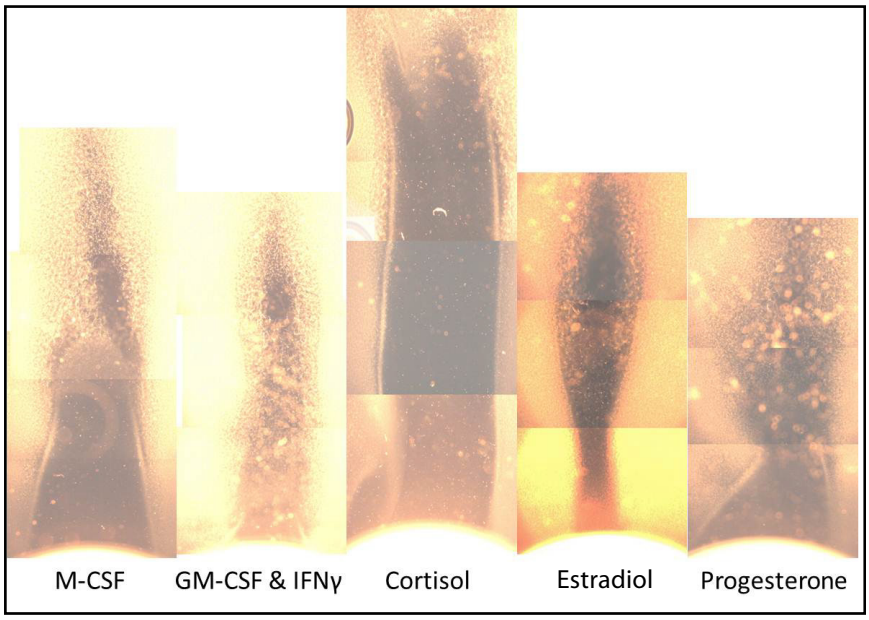

A
Relative CXCL4 mRNA concentrations
30
20
10
0
**
**
Control
Oestrogen
Progesterone
P-Withdrawal
Cortisol
HEEC treatments
B
Control
Estradiol
Progesterone
P-Withdrawal
Cortisol
C
Relative CXCL4 protein
3
2
1
0
**
Control
Oestrogen
Progesterone
P-Withdrawal
Cortisol
HEEC treatments

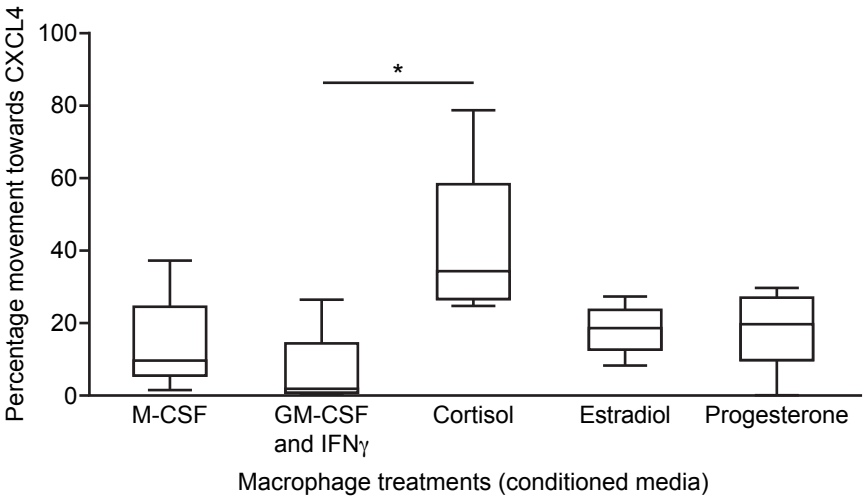
Figure 4


A
Relative CXCL4 mRNA concentrations
30
20
10
0
NMB
HMB
Late Secretory
n = 5/8
Menstrual
n = 4/4
Stage of menstrual cycle
B
LS stromal compartment
Immunoscore
200
150
100
50
0
NS
NMB
n = 4
HMB
n = 7
Blood loss
LS endothelial cells
Immunoscore
200
150
100
50
0
NS
NMB
n =4
HMB
n =7
Blood loss
M stromal compartment
Immunoscore
200
150
100
50
0
NS
NMB
n = 6
HMB
n =3
Blood loss
M endothelial cells
Immunoscore
200
150
100
50
0
*
NMB
n = 6
HMB
n = 3
Blood loss
C
NMB
HMB
100 µm

A


M-CSF
GM-CSF & IFNγ
Cortisol
Estradiol
Progesterone

B


Percentage movement towards CXCL4
100
80
60
40
20
0
*
M-CSF
GM-CSF and IFNγ
Cortisol
Estradiol
Progesterone
Macrophage treatments (conditioned media)