Estradiol Is a Critical Mediator of Macrophage-Nerve Cross Talk in Peritoneal Endometriosis

Estradiol is a critical mediator of macrophage-nerve crosstalk in peritoneal endometriosis

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Running title: The role of estradiol in macrophage-nerve crosstalk in endometriosis

Key words: Endometriosis, neuromodulation, inflammation, estradiol

Number of text pages: 31

Number of figures: 6

Number of tables: 2

Word count: Abstract 220

MS 3916

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The authors have nothing to disclose

These studies were supported by a Society for Endocrinology Early Career Grant awarded to EG, an MRC Programme Grant G1100356/1 to PTKS, a Wellbeing of Women Grant R42533 to AWH and a travel grant from Boehringer Ingelheim Fonds awarded to JT.

Abstract

Endometriosis occurs in approximately 10% of women and is associated with persistent pelvic pain. It is defined by the presence of endometrial tissue (lesions) outside the uterus, most commonly on the peritoneum. Peripheral neuroinflammation, a process characterised by the infiltration of nerve fibres and macrophages into lesions, is believed to play a pivotal role in endometriosis-associated pain. The objective was to determine the role of estradiol in regulating the interaction between macrophages and nerves in peritoneal endometriosis. Using human tissues and a mouse model of endometriosis, we demonstrate that macrophages in lesions recovered from women and mice are immuno-positive for estrogen receptor beta, with up to 20% being estrogen receptor alpha positive. In mice, treatment with estradiol increased the number of macrophages in lesions as well as concentrations of mRNAs encoded by *Csf-1*, *Nt-3* and the tyrosine kinase neurotrophin receptor *TrkB*. Using *in vitro* models we determined that treatment of rat dorsal root ganglia neurons with estradiol increased mRNA concentrations of the chemokine *Ccl-2* that stimulated migration of CSF-1 differentiated macrophages. Conversely, incubation of CSF-1 macrophages with estradiol increased concentrations of brain-derived neurotrophic factor (*BDNF*) and neurotrophin 3 (*NT-3*) that stimulated neurite outgrowth from ganglia explants. In summary, we have demonstrated a key role for estradiol in stimulating macrophage-nerve interactions providing novel evidence that endometriosis is an estrogen-dependent neuroinflammatory disorder.

Introduction

Endometriosis affects 10% of reproductive age women and is associated with persistent pelvic pain [1]. It is defined by the presence of endometrial-like tissue (lesions) found outside the uterus, most commonly on the peritoneum. The mechanisms underlying endometriosis-associated pain are poorly understood but it has been postulated that estrogen-dependent neuroinflammation may be involved [2]. Notably the presence of endometrial tissue fragments on the peritoneum elicits an immune response, including recruitment of macrophages [3], blood vessels and nerve fibres into the resultant lesions [4, 5]. Within the lesions CD68+ macrophages have been detected in close association with nerve fibres [6].

Studies investigating macrophage activation and recruitment have revealed that endometriosis-associated macrophages exhibit a phenotype consistent with the 'alternative' end of the macrophage activation spectrum [7, 8]. In a mouse model of endometriosis that included cell transfer of polarised macrophages, Bacci et al reported that mice injected with pro-inflammatory macrophages (M(IFNγ)) developed microscopic lesions but those injected with alternatively activated macrophages (M(IL-4)) developed larger lesions with a well-developed vasculature [7]. Our studies in a mouse model of endometriosis have revealed that macrophages resident in peritoneal lesions can originate from both the peritoneum and the endometrium [9].

Sensory C, sensory Aδ, cholinergic and adrenergic nerve fibres have been identified within lesions [10, 11] with greater nerve fibre density in areas that exhibit high macrophage density [6]. Studies in zebrafish have shown that macrophages will migrate towards damaged peripheral nerves [12] consistent with a role for neuron-derived factors in immune-nerve cross-talk.

Endometriosis lesions have an estrogen-rich microenvironment associated with enhanced expression of biosynthetic enzymes including aromatase [13]. It is well established

that estrogen action can be mediated by estrogen receptors alpha (ERα) and beta (ERβ), both are widely expressed in the human endometrium [14]. Notably a proportion of the soma of afferent nerve fibres innervating the uterus and peritoneum are reported to express one or both ERs [15]. Although some studies have analysed the expression of ERs in macrophages isolated from the peritoneal fluid of women with endometriosis [16, 17] expression of ERs in lesion resident macrophages has not yet been determined.

The objective of our study was to determine whether estradiol might play a role in the regulation of macrophage-nerve crosstalk in endometriosis both by exploring the expression of ERs in human tissue samples as well as the impact of estradiol (E2) on nerves and macrophages using in vitro and in vivo models.

Material and methods

Human tissues

Eutopic endometrium (n=5) and peritoneal endometriosis lesions (n=10) from patients with endometriosis were collected during laparoscopy (mean age ± SD: 35.1 ± 6.08; range: 26-45 years). Fixed sections of endometriosis lesions were evaluated following staining with H&E and only those that contained both glandular and stromal compartments were used for this study. Dating of eutopic endometrial histology [18] and serum hormone measurements were used to confirm menstrual cycle phase. Endometriosis stage was provided during surgery using the revised American Society of Reproductive Medicine classification (rASRM; I=60%, II= 20%, III=20%). Endometrium (n=5) and peritoneal biopsies (n=8) from women without evidence of clinical endometriosis were also collected during laparoscopy. All patients had regular cycles and had not taken hormones at least 3 months prior to surgery. The study was approved by the Lothian Research Ethics Committee (LREC 11/AL/0376) and the Ethics Committee of Charité – Universitätsmedizin Berlin (EA4/023/05). All patients

provided written, informed consent. Samples were fixed in 4% neutral buffered formalin (NBF) or stored in RNAlater (Applied Biosystems, Warrington, UK).

Mouse model of endometriosis

Endometriosis was induced using wild type and transgenic Cfs1r-EGFP mice (MacGreen) [19] on a C57BL/6 background using a previously validated model of endometriosis as described [9]. In brief, donor endometrial tissue was recovered during induced 'menses' [20] and injected into the peritoneal cavity of recipient mice. Lesions (EL) and peritoneum (EP) were collected from endometriosis mice (n=8) 21days after inoculation of the peritoneum. In an additional group of mice, on day 21 the 17β-estradiol (E2) subcutaneous pellet was either removed (no hormone control; n =5) or retained (E2 treatment; n= 8, mice randomly assigned to groups). After a further 7 days (day 28 of the protocol), mice were culled, lesions and peritoneum removed and either stored in RNAlater or fixed in NBF and processed so that the presence of glands and stroma could be confirmed. Control tissues (uterus, peritoneum) were recovered from naïve control mice (n=6).

Cell culture and estradiol treatments

Dorsal root ganglia (DRG) isolation

Rat DRGs were isolated as previously described [21]. Dissociated DRGs were used for RNA extraction and qRT-PCR. At least 24 hours prior to experiments, the medium was changed to phenol red free media, fetal calf serum (FCS) was stripped with activated charcoal then added to the media at a concentration of 1%. Cells were stimulated with vehicle control (dimethyl sulfoxide (DMSO)), E2 at a final concentration of 10^{-8}M (Sigma, UK), alone or in combination with the anti-estrogen Fulvestrant (ICI 182780, final concentration 10^{-7}M; Tocris, UK). E2 and ICI were dissolved in DMSO to yield stock solutions of 10^{-2}M, all cells were exposed to a final DMSO concentration of 1:1 x 10^4 dilution. DRG-conditioned media

was collected, centrifuged and stored at -80°C and used for the macrophage migration assay. All experiments were performed in triplicates.

Human peripheral blood monocyte isolation and macrophage differentiation

Human venous blood was collected from healthy female volunteers (n=5) with informed consent (LREC 08/S1103/38). To isolate mononuclear leukocytes, blood was centrifuged (350g, 20min) and platelet-rich plasma (PRP) aspirated. Erythrocytes were sedimented with 0.6% (w/v) dextran followed by separation of leukocytes using a Percoll™ (GE Healthcare, UK) gradient and centrifugation at 720g for 20min. Mononuclear leukocytes were aspirated and monocytes isolated by negative selection using a monocyte isolation kit II (Miltenyi Biotech, Surrey, UK). Adherent monocytes were cultured in the presence of colony-stimulating factor 1 (CSF-1; 4ng/ml) to generate monocyte-derived macrophages hereby referred to as M(CSF-1) in accordance with the recently published macrophage nomenclature guidelines [22], for 4 days in Iscove's DMEM (IMDM, Life Technologies, Paisley, UK) containing 10% autologous serum, prepared by re-calcification of PRP (12 well plates, 37 °C in 5% CO2). M(CSF-1) were exposed to DMSO (M(CSF-1+DMSO), 10^{-8}M E2 (M(CSF-1+E2)) or 10^{-8}M E2 plus 10^{-7}M ICI (M(CSF-1+E2+ICI)) for 24h at 37 °C at 5% CO_2 on day 5. Macrophage conditioned media was collected, centrifuged and stored at -80°C until further use for the neurite outgrowth assays. All experiments were performed in triplicates.

RNA extraction and cDNA synthesis

RNA was extracted from DRGs and macrophages using the RNAeasy mini kit (Qiagen, Sussex, UK), concentration and purity was analysed using a Nanodrop (LabTech International, Sussex, UK). cDNA was synthesized using SuperScript® VILO™ (Invitrogen, Paisley, UK) with a starting template of 100ng/µl.

Quantitative real time PCR

Real-time PCR reactions were performed using the Roche Universal ProbeLibrary (Roche Applied Science, West Sussex, UK) and Express qPCR Supermix (Invitrogen) in a 7900 Fast Real-Time PCR with 18S as the endogenous control. Primer sequences (Eurofins MWG operon, Germany) are listed in Table 1. Expression of target genes was related to expression of 18S ribosomal RNA and to an internal control sample using the $2^{-\Delta\Delta Ct}$ method.

Functional assays

Neurite outgrowth assay

Single whole DRG explants from E15.5 rat embryos were incubated with DMEM plus 0.1ng/ml NGF (positive control), IMDM plus autologous serum (negative control) or conditioned medium (CM) from macrophages treated for 24 h with DMSO, E2 or E2+ICI (n=5 female volunteers) for 24h and 48h at 37°C with 5% CO_2 in Poly-D-lysine and Matrigel (BD Biosciences, UK) coated 12 well plates. Neurite outgrowth was analysed as previously described [21]. Neutralisation experiments were performed using anti-BDNF (0.4µg/ml) and anti-NT-3 (0.2µg/ml; R & D systems, Oxford, UK). CM was pre-incubated with neutralising antibodies for 1 hour before experiments were performed. The treatments were performed in duplicate for each of the 5 patients and each duplicate used to incubate 3 DRGs.

Macrophage migration assay

The macrophage migration assay was performed using µ-Slide V1.04 migration slides (Ibidi®, Munich, Germany). CM (80µl) from DRG exposed to DMSO, E2 or E2+ICI (see previous) was aliquoted into one chamber and macrophages (5 X 10^4 cells) were aliquoted into the other chamber and slides incubated for 16h then evaluated using an Axiovert microscope (Carl Zeiss, Germany). A migration score was allocated to each slide based on the distance migrated and the % of macrophages mobilised towards the chamber containing

the DRG media. Neutralisation experiments were performed using an anti-CCL-2 antibody (0.5µg/ml; BioLegend, CA, USA) and an anti-CCL-3 antibody (0.45µg/ml; R and D systems). CM was pre-incubated with neutralising antibodies for 1 hour before experiments were performed. DRG treatments were performed in triplicate in 4 separate experiments and used in migration experiments from 4 macrophage preparations (4 female volunteers).

Immunodetection

Dual immunofluorescence

Dual immunofluorescence was carried out as previously described [21]. In brief, sections were antigen retrieved, blocked for endogenous peroxidase and non-specific epitopes and incubated with primary antibody at 4°C overnight (Table 2). Antibody detection was performed using a secondary F(ab) polyclonal antibody to IgG (HRP) and TSA system kit labelled with Cy3 (red) or fluorescein (green; 1:50 dilution, Perkin Elmer Inc, MA, USA). For detection of the second antigen sections were microwaved in boiling citrate buffer, the second primary antibody applied overnight at 4ºC, and detection as above. The sections were counterstained with DAPI, mounted in Permafluor (Thermo Fisher Scientific, Loughborough, UK) and imaged using a LSM710 confocal microscope and AxioCam camera (Carl Zeiss Inc, UK). Human or mouse uterus was used as a positive control tissue and negative controls had omission of the primary antibody. Macrophage number in lesion and peritoneal sections was quantified by randomly capturing 4 (human) or 3 (mouse) fields of view (FOV) associated with glandular and stromal tissue in endometriosis lesions. A mean was generated and plotted for each biological sample [21].

Immunofluorescence on cultured cells

Cultured DRGs were stained using a NF H chicken anti-neurofilament H antibody (1:1000, Covance, UK) to visualise neurite projections. Cultured macrophages were fixed for 20 mins using ice cold methanol, permeabilised using Triton-X, blocked with Avidin/Biotin

(Vector), and species specific blocking buffer for non-specific epitopes. Macrophages were incubated with primary antibody (CD68, ERα or ERβ) overnight at 4°C. Antibody binding was detected using a biotinylated secondary antibody followed by streptavidin alexafluor 555 and counterstained with DAPI. Images were captured using an Axiovert microscope (Carl Zeiss), Axiovision camera and software.

Statistical analysis

The data were expressed as means ± SEM and were analysed using a one-way ANOVA and Newman Keuls multiple comparison test or a Students T test for two group comparisons. Analysis of QPCR data was performed on transformed values. *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$. Analyses were carried out using GraphPad Prism 6 software.

Results

Macrophages and nerve fibres are found in close association in peritoneal endometriosis lesions

In peritoneal lesions from women with endometriosis, macrophages (immuno-positive for CD68, red) were identified in close association with small diameter nerve fibres, typical of afferent sensory innervation (immunopositive for PGP9.5; green; Fig.1A-B). Clustering of CD68+ macrophages around nerve bundle structures was consistently observed in tissue close to glandular epithelium in peritoneal lesions (Fig.S1). In lesions recovered from transgenic Cfs1r-EGFP (MacGreen) mice, GFP+ macrophages were also detected close to nerve fibres (Fig.1C-D; GFP; red, PGP9.5; green).

Estrogen receptor beta (ERβ) is the predominant estrogen receptor expressed by lesion resident macrophages

Significantly higher numbers of CD68+ macrophages were detected in sections of peritoneal endometriosis lesions compared to sections of unaffected peritoneum ($p<0.001$;

Fig.1E). In women, CD68+ macrophages resident in the eutopic endometrium were immuno-negative for ERα (Fig.S2A; CD68; red, ERα; green) but immuno-positive for ERβ (Fig.S2B, ERβ; green) regardless of whether they had been diagnosed with endometriosis (Fig.S2C-D). In peritoneal biopsies from women without endometriosis ERα was immuno-localised to approximately a tenth of CD68+ macrophages (10.6% ± 3.85) (Fig.1F, Fig.S2E) but all the macrophages were ERβ positive (Fig.S2F). In peritoneal endometriosis lesions approximately a fifth of the CD68+ macrophages (18.3% ± 4.37) were immuno-positive for ERα (Fig.1G) and as in the normal peritoneum they were all immuno-positive for ERβ (Fig.1H). Results obtained in our mouse model of endometriosis mirrored those in women with significantly higher numbers of GFP+ macrophages in lesions ($p<0.05$) compared to the peritoneum of naïve mice (Fig.1I). A tenth of mouse peritoneal macrophages (10.14±6.41) and a quarter of lesion resident macrophages (24.82±5.57) were immuno-positive for ERα (Fig.1J-K) with all GFP+ cells being immuno-positive for ERβ (Fig.1L).

Human peripheral blood monocytes were isolated and differentiated into macrophages that are classified as being at the alternative end of the macrophage activation spectrum [22] by incubating them in the presence of CSF-1 hereafter referred to as M(CSF-1). M(CSF-1) incubated with estradiol are referred to as M(CSF-1+E2). All isolated cells were confirmed as macrophages using CD68 immunocytochemistry (Fig.1M). M(CSF-1+E2) contained mRNAs encoding ERα and ERβ (Fig.S3A-B). Protein localisation revealed a mixed population of ERα positive and negative macrophages; ERα was detected in 66% of cells (Fig.1N) whereas ERβ was detected in all cells (Fig.1O).

Nerve fibres recruit macrophages in an estradiol-dependent manner *in vitro*

We explored the impact of products secreted by DRGs in response to stimulation with E2 on the migration of M(CSF-1) using an *in vitro* macrophage migration assay. Conditioned media (CM) from DRGs stimulated for 24h with DMSO, E2, or E2 plus the anti-estrogen ICI

was placed in one chamber and M(CSF-1) were placed in the other (Fig.2A). M(CSF-1) migrated furthest towards CM from DRG exposed to E2 ($p<0.01$), this effect was not observed using CM from DRG exposed to E2+ICI, indicating an ER specific effect (Fig.2B). Notably, addition of E2 to the medium in the absence of DRG had no effect on the migration of the macrophages (data not shown), verifying a role for E2-dependent DRG derived secretory products in enhancing macrophage migration. QPCR analysis of E2-treated DRGs revealed ER-dependent regulation of a macrophage growth factor and two chemokines. Specifically, mRNA concentrations of colony-stimulating factor 1 (*Csf-1*) were up-regulated by E2 ($p<0.001$; Fig.2C), as were chemokine (C-C motif) ligand 2 (*Ccl2-2*) and 3 (*Ccl-3*) mRNAs ($p<0.05$; Fig.2D and E). Addition of ICI abrogated the effect of E2. Addition of an anti-CCL-2 antibody to DRG CM abolished the E2 induced macrophage chemotactic properties ($p<0.01$) whereas addition of an anti-CCL-3 antibody attenuated E2 induced macrophage chemotactic properties of DRG CM (Fig.2F).

Estradiol induces neurotrophic properties in macrophages

DRGs were cultured in CM from M(CSF-1) and neurite outgrowth was recorded (Fig.3A). CM from E2 activated macrophages M(CSF-1+E2) significantly enhanced neurite outgrowth compared to CM from M(CSF-1+DMSO) or M(CSF-1+E2+ICI) at 24h and 48h (Fig.3B-D; $p<0.001$). Analysis of M(CSF-1) mRNAs revealed that neurotrophins were up-regulated by E2 treatment. Specifically, mRNA concentrations of brain derived neurotrophic factor (*BDNF*) and neurotrophin 3 (*NT-3*) were significantly increased in M(CSF-1+E2) compared to M(CSF-1+DMSO) ($p<0.001$ and $p<0.01$ respectively; Fig.3E and F). This effect was abrogated by the addition of ICI confirming it was receptor-dependent. To verify a role for BDNF and NT-3 in the neurotrophic properties of M(CSF-1+E2), single whole DRG were incubated with CM from M(CSF-1) (Fig.3A) in combination with neutralising antibodies targeted to BDNF or NT-3. The neurotrophic properties of CM from M(CSF-

1+E2) were abolished by anti-BDNF or anti-NT-3 ($p<0.01$; Fig.3G and H). This effect was not observed when the neutralising antibodies were added to DRG cultured in neuronal media in the presence of NGF.

Macrophage infiltration of endometriosis lesions is estradiol-dependent in a mouse model of endometriosis

Lesions recovered from mice exposed to E2 contained significantly more GFP+ cells than mice that had hormonal support withdrawn ($p<0.05$; Fig.4A-C). We have previously reported that *Ccl-2* and *Ccl-5* (*Rantes*) mRNA concentrations were elevated in mouse endometriosis lesions [9] and herein we show that mRNA concentrations of the chemokine *Ccl-3* were also significantly elevated in mouse endometriosis lesions ($p<0.01$) compared to biopsies of naïve uterus and peritoneum (Fig.4D).

***Csf-1, Nt-3* and *TrkB* are estradiol-regulated in a mouse model of endometriosis**

Csf-1 and *Nt-3* mRNA concentrations were significantly higher in lesions than other tissue samples (Fig.5A-B). mRNA concentrations of the tyrosine kinase receptor that binds both Bdnf and Nt-3 (*TrkB*) was also up-regulated in lesions (Fig.5C). Concentrations of mRNAs encoded by *Csf-1, Nt-3* and *TrkB* were significantly higher in the lesions exposed to E2 compared to those recovered from mice in which E2 was withdrawn after lesions were established (Fig.5D-F).

Discussion

Endometriosis is a steroid-dependent disorder; lesions exhibit the capacity for enhanced tissue biosynthesis of estrogens as well as alterations in estrogen receptor protein expression [21, 23]. In the current study, we provide new evidence that estrogens play a key role in the regulation of interactions between macrophages and nerve fibres in endometriosis. Specifically, using *in vitro* model systems we found that DRG neurons produced chemokines

in response to E2 that promoted macrophage recruitment, whilst macrophages stimulated with E2 produced neurotrophins that promoted neuronal outgrowths. Using a mouse model of endometriosis [9] we demonstrated that macrophage infiltration of endometriosis lesions was E2-dependent and that the concentrations of mRNAs encoding *Csf-1*, *Nt-3* and *TrkB* were E2-regulated in mouse lesions.

Estrogen can bind directly to either ERα or ERβ resulting in ligand dependent changes in receptor function and gene expression. We have previously demonstrated that endothelial cells within both normal endometrium [14, 24] and peritoneal lesions [21] are ERβ+/ERα-. Notably, E2 treatment of immortalised human endometrial endothelial cells resulted in changes in gene expression of the axonal guidance factor SLIT3 consistent with the suggestion that neuroangiogenesis can be modulated by estrogen. In this study we used fluorescent immunohistochemistry to explore the ER phenotype of macrophages within eutopic and ectopic endometrium in women and in our mouse model. Notably all CD68+ macrophages in peritoneum and lesions from women, and all GFP+ macrophages in the peritoneum and lesions from mice were immuno-positive for ERβ. In contrast peritoneal and lesion resident macrophages were a mixed population of ERα+ and ERα- cells. There was no clear evidence of either population residing in specific microenvironments, as ERα+ cells were often detected adjacent to ERα- cells. As we have previously reported data from our mouse model of endometriosis showing lesion-resident GFP+ macrophages may originate from both the peritoneum and the 'shed' endometrium [9] we wondered if the heterogeneous expression of ERα might be a reflection of different origins (endometrial macrophages are all ERα-) but since there was no significant difference in proportions of ERα+ cells in lesions and peritoneum this cannot be the case. It therefore remains to be determined what regulates the amount of ERα protein in CD68+ macrophages. One plausible explanation being that the ERα cells have infiltrated from peripheral blood (ERα is expressed by peripheral blood

monocyte-derived macrophages). Macrophages isolated from the peritoneal fluid of women with endometriosis are reported to be immuno-positive for both ERα and ERβ, and that ER immunolocalisation is increased in women with endometriosis compared to women without endometriosis[16, 25]. ER expression in lesion-resident macrophages had not been examined until now. Our findings differed from the previous studies in that ERα was only detected in a subset of macrophages in both peritoneal (from women without endometriosis) and lesion biopsies, we also found no difference in ER immunolocalisation in macrophages from women with and without endometriosis. We suggest that this discrepancy in findings is due to the inherent differences between peritoneal fluid macrophages (naïve and un-stimulated in disease free conditions)[26] and tissue macrophages (activated). Although the phenotype of macrophages present in the eutopic endometrium may alter throughout the menstrual cycle [27], recent reports suggest that they have a phenotype closer to the alternative ('M2-like') end of the macrophage activation spectrum [28], a similar phenotype has been reported for endometriosis-associated macrophages [7]. We postulate that macrophages in the shed endometrium may retain their phenotype and the local 'endometrial' microenvironment of the ectopic endometrial tissue re-programmes the macrophages infiltrating the lesion from the peritoneum.

We and others documented the presence of macrophages and nerves in close association in tissue-sections from lesions [6]. The nuclei of sensory neurons innervating the uterus are known to express estrogen receptors[15], and ER-dependent signaling modulates a range of processes in peripheral nerves[29, 30]. We were therefore interested to explore whether the estrogen dominated microenvironment could play a role in modulating interactions between macrophages and nerves and what regulatory factors may be involved... Using a migration assay to determine whether neurons could release factors influencing macrophages we found evidence that addition of CM from DRGs treated with E2 enhanced macrophage

375 migration and that *Ccl-2* and *Ccl-3* mRNA concentrations in DRGs was ER-regulated.
376 Importantly when an antibody directed against Ccl-2 was added to the CM macrophage
377 migration was abrogated suggesting this was a key E2-dependent factor involved in neuron
378 mediated macrophage migration. Notably elevated CCL-2 concentrations have been detected
379 in peritoneal fluid of women with endometriosis [31]. Moreover, Luk et al demonstrated that
380 CCL-2 concentrations were increased by E2 in endometrial endothelial cells from women
381 with endometriosis [32]. Their study and ours both provide evidence that E2 modulates
382 recruitment of macrophages via CCL-2 to endothelial cells and nerve fibres in endometriosis
383 lesions.

384 CSF-1 is a critical growth factor involved in macrophage survival, proliferation and
385 differentiation [33]. Csf-1 may play a critical role in early development of endometriosis
386 lesions; mice homozygous for a *Csf-1* mutation (*Csf-1* op/op) developed significantly less
387 lesions in a model of endometriosis compared to wild type controls [34]. Elevated CSF-1
388 concentrations have also been reported in the peritoneal fluid of women with endometriosis
389 [35]. In our study, mRNA concentrations of *Csf-1* were increased in lesions recovered from
390 mice and were also increased in DRGs exposed to E2. Based on these results, we suggest that
391 the ER-dependent regulation of CSF-1 in peripheral nerve fibres present in endometriosis
392 lesions may play a role in modulating macrophage survival and phenotype, and is consistent
393 with the hypothesis that neurogenic inflammation is a key process is this disorder.

394 E2 is reported to be neuroprotective [36]; these effects have been linked to E2-
395 dependent expression of BDNF promoting neuron survival, regeneration and synaptogenesis
396 [37]. NT-3 is elevated in the peritoneal fluid of women with endometriosis [38], but the cellular
397 source is uncertain. In this study, E2 increased the neurotrophic properties of macrophages
398 via up-regulation of *BDNF* and *NT-3*, suggesting that this cell type is a key source of these
399 neurotrophins contributing to E2-dependent nerve growth into lesions. Notably, the mRNA

concentrations of *Nt-3* and the neurotrophin receptor *TrkB* were also E2-regulated in the lesions induced in mice complementing the data from the *in vitro* models.

It has been proposed that endometriosis-associated macrophages may identify the ectopic endometrial tissue as a 'wound' and activate pathways supporting cell survival and angiogenesis rather than phagocytosis of ectopic material [39]. Macrophages are vital in the regeneration of damaged nerves following injury to the CNS and PNS and although infiltrating sensory nerves present within endometriosis lesions are not 'damaged' *per se*, they may experience a chemical milieu similar to inflammation in response to trauma [40]. In a mouse model of acute peripheral nerve injury an alternative macrophage response was detected [41], and this phenotype has been associated with a sterile inflammatory environment similar to endometriosis. We suggest that the reciprocal relationship between macrophages and nerves encourages innervation of endometriosis lesions. Moreover, the close proximity of macrophages and nerves within lesions suggests that macrophage-derived cytokines may also contribute to pelvic pain in endometriosis by acting directly on nociceptors generating a pain response and hypersensitivity [40, 42]. We have previously shown that estrogens can also act directly on human sensory neurons to increase the mRNA concentrations of key nociceptive ion channels including *TAC1*, *P2RX3* and *TRPV1* [43], further supporting a role for estrogens in modulating pain response in endometriosis by acting on nerves.

Our data have led us to propose the following model; elevated levels of estrogens present within the lesion microenvironment act to mediate interactions between macrophages and nerve fibres whereby estrogen acts on nerve fibres to enhance the expression of CSF1 and CCL-2, recruiting macrophages to nerve fibres. Reciprocally, estrogens act on macrophages to enhance expression of BDNF and NT-3 which further potentiates neurogenesis into lesions (Fig.6). In conclusion, these new results provide compelling evidence that estrogens produced by the ovaries, as well as within lesions, play a pivotal role

in cross-talk between neurons and macrophages which underpins development of pain symptoms in women with endometriosis. The identification of E2-dependent factors that regulate the process of macrophage-mediated nerve growth into lesions may offer novel targets for inhibition that may be preferred over medically induced hypo-estrogenism.

Acknowledgements

We are grateful to all the women who gave informed consent for the use of their material in this study and to our research nurses Ann Doust and Helen Dewart who organised the tissue collection. We are grateful to Ronnie Grant for preparation of figures. We thank Prof Adriano Rossi for critical feedback on experiments and the manuscript, Dr Uma Thiruchelvam for initial advice on macrophage phenotype and isolation and Dr Maria Luisa Barcena de Arellano for her input.

Author contributions

EG conceived and carried out experiments and analysed data, JT carried out experiments and analysed data, AE carried out experiments, SM and AWH collected patient biopsies, PTKS conceived experiments; EG, AWH and PTKS wrote the manuscript.

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Figure legends

Fig.1. Macrophages are found in close association with nerve fibres in endometriosis lesions and express estrogen receptors. (A-B) Macrophages are found in close proximity to small diameter nerve fibres in human peritoneal lesion biopsies. Dual immunofluorescence carried out using the macrophage marker CD68 (red) and the pan neuronal marker protein gene product 9.5 (PGP9.5; green). Nuclei stained with DAPI (blue). Scale bar = 20 and 50μM, respectively. Inset in (B) is an enlarged image. **(C-D)** Macrophages are also found near small diameter nerve fibres in lesions recovered from a mouse model of endometriosis. To aid in the localisation of macrophages we used the MacGreen mouse; a transgenic mouse with eGFP labelled macrophages for our mouse model of endometriosis. Dual immunofluorescence carried out using an anti-GFP antibody (red) PGP9.5 (green) on lesions recovered from mice. Nuclei stained with DAPI (blue). Scale bar = 50μM. **(E)** Significantly higher numbers of CD68+ macrophages were detected in peritoneal endometriosis lesions (n=10) compared to the healthy peritoneum (n=8). CD68+ cells were counted in 4 randomly selected fields of view (FOV) associated with glandular and stromal tissue in lesions and the mean recorded for each patient sample, statistical analysis was performed using a students unpaired t test, ***:$p<0.001$. **(F-H)** 20% of macrophages in peritoneal lesions are immuno-positive for ERα, 100% are immuno-positive for ERβ. (F) CD68+ ERα+ cells were counted in each FOV (see above) and expressed as a proportion of CD68+ cells, statistical analysis was performed using a one-way ANOVA and Newman Keuls post-test. ***:$p<0.001$. (G) ERα immunolocalisation in endometriosis lesions (CD68; red, ERα; green). Inset shows enlarged area with an ERα+ macrophage (white arrow) adjacent to an ERα- macrophage (yellow arrow). (H) Macrophages in peritoneal lesions are immuno-positive for ERβ (green). Scale bars = 50μM. **(I)** Significantly higher numbers of GFP+ macrophages were detected in peritoneal endometriosis lesions (n=8) compared to the healthy peritoneum (n=6). GFP+ cells

were counted in 4 randomly selected fields of view (FOV) associated with glandular and stromal tissue in lesions and the mean recorded for each sample, statistical analysis was performed using a students unpaired t test, ***:p<0.001. **(J-L)** 25% of macrophages in mouse peritoneal lesions are immuno-positive for ERα, 100% are immuno-positive for ERβ. (J) GFP+ ERα+ cells were counted in each FOV (see above) and expressed as a proportion of GFP+ cells, statistical analysis was performed using a one-way ANOVA and Newman Keuls post-test. ***:p<0.001. (K) ERα immunolocalisation in endometriosis lesions (GFP; red, ERα; green). (L) Macrophages in mouse peritoneal lesions are immuno-positive for ERβ (green). Scale bars = 50μM. **(M-O)** Peripheral blood monocytes were isolated and differentiated into macrophages in the presence of CSF-1. (M) Phenotype of cultured cells was verified using immunocytochemistry for CD68 (red) and expression of (N) ERα and (O) ERβ1confirmed (green). Scale bar = 50μM.

Fig.2. Nerve fibres recruit macrophages in an estradiol-dependent manner via Ccl-2. **(A)** DRGs were stimulated for 24h with vehicle control (DMSO), E2 (10^{-8}M) or E2 plus the anti-estrogen ICI (10^{-7}M). Conditioned media (CM) was retained for use in the macrophage migration assay; macrophages (M(CSF-1); 5 X 10^{-4}M cells per well) were placed in one chamber of the migration slide, DRG CM in the other. **(B)** M(CSF-1) migrated furthest towards CM from DRG exposed to E2 (p<0.01). Migration score was based on % cells mobilised and distance migrated. Mac media: unconditioned macrophage media (-ve control). Macrophages; n=4 patients, embryonic rat DRGs; n = 4 pregnant dams. **(C-E)** Estradiol up-regulates mRNA concentrations of *Csf-1* and chemokines in DRGs. The concentration of mRNAs encoding the macrophage growth factor *Csf-1* and chemokines (*Ccl-2* and *Ccl-3*) was analysed using QPCR in DRG exposed to DMSO, E2, or E2 plus ICI for 24h; (C) colony-stimulating factor (*Csf-1*), (D) chemokine (C-C motif) ligand 2 (*Ccl-2*) / monocyte chemotactic protein 1 (*Mcp-1*) and (E) chemokine (C-C motif) ligand (*Ccl-3*) / macrophage

643 inflammatory protein-1α (*Mip-1α*), n =5 cultures. RQ: Relative quantification. **(F)** The
644 macrophage migration assay was performed as in (B), with the addition of anti-CCL-2
645 (0.5μg/ml) or anti-CCL-3 (0.45μg/ml) antibodies to CM from DRGs exposed to DMSO or
646 E2. Anti-CCL-2 abolished E2 induced chemotactic properties of DRG CM, whereas anti-
647 CCL-3 only attenuated E2 induced chemotactic properties of DRG CM. Statistical analysis
648 was performed using a one-way ANOVA and Newman Keuls post-test. *:$p<0.05$, **:$p<0.01$,
649 ***:$p<0.0001$.

650 **Fig.3. M(CSF-1+E2) exhibit neurotrophic properties via BDNF and NT-3. (A)** M(CSF-1)
651 were exposed to DMSO, E2 or E2 plus ICI, the conditioned media (CM) retained and used to
652 incubate single whole DRG immediately dissected from embryonic rats. **(B-D)** CM from
653 M(CSF-1+E2) enhanced neurite outgrowth compared to CM from M(CSF-1+DMSO). (B-C)
654 Immunocytochemistry performed on DRG using anti-Neurofilament (red) and nuclei
655 counterstained with DAPI (blue). Scale bar = 500μM. (B) DRG grown in CM from M(CSF-
656 1+DMSO) and (C) CM from M(CSF1+E2). (D) Neurite outgrowth was quantified; the score
657 was based on the % coverage of DRG and average length of neurites. DRG media:
658 unconditioned DRG media (NGF present; +ve control), Mac media: unconditioned
659 macrophage growth media (-ve control). **(E-F)** The concentration of mRNAs encoding
660 neurotrophins was analysed using QPCR in M(CSF-1) stimulated with DMSO, E2 or E2 plus
661 ICI for 24h; (E) brain derived neurotrophic factor (*BDNF*) and (F) neurotrophin-3 (*NT-3*),
662 n=5 volunteers. RQ: Relative quantification. **(G-H)** The neurotrophic properties of M(CSF-
663 1+E2) are BDNF and NT-3 dependent. Whole single DRG were cultured for 24h in CM from
664 M(CSF-1+DMSO) or M(CSF-1+E2) for 24h, or CM from M(CSF1+E2) in the presence of an
665 anti-BDNF (0.4μg/ml) or anti-NT-3 (0.2 μg/ml) antibody. (G) Immunocytochemistry
666 performed on DRG using anti-Neurofilament. Scale bar = 500μM. (F) Quantification of
667 neurite outgrowth in DRG incubated with CM from M(CSF-1+DMSO), M(CSF-1+E2) or

M(CSF-1+E2) in combination with anti-BDNF or anti-NT-3. DRG media; unconditioned DRG growth media, Mac media; unconditioned macrophage growth media. Statistical analysis was performed using a one-way ANOVA and Newman Keuls post-test. **:$p<0.01$, ***:$p<0.001$.

Fig.4. Macrophage infiltration of lesions is estradiol-dependent in a mouse model endometriosis. (A) Exposure of endometriosis mice to estradiol (E2; n=6) increased the number of macrophages present in endometriosis lesions compared to lesions from mice that had estradiol support withdrawn 7 days earlier (Control; n=4). The number of macrophages per lesions were counted and normalised to lesion area. **(B-C)** Images show representative fields of view from control mice (B) or mice exposed to E2 (C). Immunofluorescence was carried out using an anti-GFP antibody (green) and nuclei are stained blue with DAPI. Scale bar = 50μM. **(D)** QPCR analysis revealed *Ccl-3* mRNA concentrations were elevated in lesions recovered from a mouse model of endometriosis. mRNA concentrations were measured in the uterus and peritoneum of naïve mice (NU and NP; n=6) and the peritoneum (EP) and lesions (EL) of mice with endometriosis (n=8). Statistical analysis was performed using a Students unpaired t-test or a one-way ANOVA and Newman Keuls post-test. *:$p<0.05$, **:$p<0.01$.

Fig.5. *Csf-1, Nt-3* and *TrkB* mRNA concentrations are elevated in endometriosis lesions and estradiol-regulated in a mouse model of endometriosis. (A-C) *Csf-1, Nt-3* and *TrkB* were elevated in lesions recovered from a mouse model of endometriosis. mRNA concentrations of *Csf-1* (A), *Nt-3* (B) and *TrkB* (C) were measured in the uterus and peritoneum of naïve mice (NU and NP; n=6) and the peritoneum (EP) and lesions (EL) of mice with endometriosis (n=8). **(D-F)** *Csf-1*, *Nt-3*, *TrkB* are regulated by estradiol in mouse endometriosis lesions. In a separate experiment mice were separated into groups that continued exposure to E2 (E2; n=6) or had estradiol treatment withdrawn (control; n=5) for

693 an additional 7 days. Peritoneal (P) and lesion (EL) biopsies were included in the analysis.
694 Peritoneum from naïve mice (naïve P; n=6) was also included. mRNA concentrations of *Csf-*
695 *1* (D), *Nt-3* (E) and *TrkB* (F) were elevated in lesions from mice exposed to E2 compared to
696 control mice. RQ; Relative quantification. Statistical analysis was performed using a one-way
697 ANOVA and a Newman keuls post-test. *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$.

698 **Fig.6. Estradiol enhances the interactions between macrophages and nerve fibres in**
699 **endometriosis.** Schematic representation of macrophage-nerve interactions in endometriosis.
700 Nerve fibres increase the production of CSF-1 and CCL-2 in response to estradiol which
701 enhances macrophage migration. Estradiol also acts on macrophages to increase production
702 of BDNF and NT-3 resulting in increased neurogenesis.

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Table 1: Primers sequences

Gene	Species	Forward	Reverse
Csf-1	Rat	5'CAAGGACTATAAGGAACAGAACGAG3'	5'GAAATTCTTGATTTTCTCCAGCA3'
Ccl-2	Rat	5'AGCATCCACGTGCTGTCTC3'	5'GATCATCTTGCCAGTGAATGAG3'
Ccl-3	Rat	5'GCGCTCTGGAACGAAGTCT3'	5'GAATTTGCCGTCCATAGGAG3'
Csf-1	Mouse	5'GGGGGCCTCCTGTTCTAC3'	5'CCCACAGAAGAATCCAATGTC3'
Ccl-2	Mouse	5'CATCCACGTGTTGGCTCA3'	5'GATCATCTTGCTGGTGAATGAGT3'
Ccl-3	Mouse	5'TGCCCTTGCTGTTCTTCTCT3'	5'GTGGAATCTTCCGGCTGTAG3'
BDNF	Human	5'GTAACGGCGGCAGACAAA3'	5'GACCTTTTCAAGGACTGTGACC3'
NT-3	Human	5'AAAAACGGTTGCAGGGGTAT3'	5'GGTTTGGGATGTTTTGCACT3'
Nt-3	Mouse	5'GGTGGTACCCTCTCCTCACTC3'	5'GAAGAGCCCCTGTCATTCTG3'
TrkB	Mouse	5'TTCTGCCTGCTGGTGATGT3'	5'TCCAGTGGGATCTTATGAAACA3'

726 **Table 2: Antibodies used in immunofluorescence**

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Antibody	Raised in	Tissue	Dilution
ERβ1 (Serotec # MCA1974S)	Mouse	Human	1:500
ERβ (Santa Cruz # SC-8974)	Rabbit	Mouse	1:500
ERα (Vector)	Mouse	Human and Mouse	1:500
PGP9.5 (Dako)	Rabbit	Human and Mouse	1:1000 and 1:3500
CD68 (Clone KPI; Dako)	Mouse	Human	1:1200
GFP (Invitrogen Molecular Probes #A11122)	Rabbit	Mouse	1:1500

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