METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

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

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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 characterized by the infiltration of nerve fibers and macrophages into lesions, plays a pivotal role in endometriosis-associated pain. Our objective was to determine the role of estradiol (E2) in regulating the interaction between macrophages and nerves in peritoneal endometriosis. By using human tissues and a mouse model of endometriosis, we demonstrate that macrophages in lesions recovered from women and mice are immunopositive for estrogen receptor β , with up to 20% being estrogen receptor α positive. In mice, treatment with E2 increased the number of macrophages in lesions as well as concentrations of mRNAs encoded by *Csf1*, *Nt3*, and the tyrosine kinase neurotrophin receptor, *TrkB*. By using *in vitro* models, we determined that the treatment of rat dorsal root ganglia neurons with E2 increased mRNA concentrations of the chemokine C-C motif ligand 2 that stimulated migration of colony-stimulating factor 1–differentiated macrophages. Conversely, incubation of colony-stimulating factor 1 macrophages with E2 increased concentrations of brain-derived neurotrophic factor and neurotrophin 3, which stimulated neurite outgrowth from ganglia explants. In summary, we demonstrate a key role for E2 in stimulating macrophage-nerve interactions, providing novel evidence that endometriosis is an estrogen-dependent neuroinflammatory disorder. (*Am J Pathol* 2015, 185: 2286–2297; <http://dx.doi.org/10.1016/j.ajpath.2015.04.012>)

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 fibers into the resultant lesions.^{4,5} Within the lesions, CD68⁺ macrophages have been detected in close association with nerve fibers.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 polarized macrophages, Bacci et al⁷ reported that mice injected with proinflammatory macrophages [macrophage (interferon γ)] developed microscopic lesions, but those injected with alternatively activated macrophages [macrophage (IL-4)] developed larger lesions with a well-developed vasculature. 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

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Table 1 Primer Sequences

Gene	Species	Forward primer	Reverse primer
<i>Csf1</i>	Rat	5'-CAAGGACTATAAGGAACAGAACGAG-3'	5'-GAAATTCTTGATTTTCTCCAGCA-3'
<i>Ccl2</i>	Rat	5'-AGCATCCACGTGCTGTCTC-3'	5'-GATCATCTTGCCAGTGAATGAG-3'
<i>Ccl3</i>	Rat	5'-GCGCTCTGGAACGAAGTCT-3'	5'-GAATTTGCCGTCCATAGGAG-3'
<i>Csf1</i>	Mouse	5'-GGGGGCTCTCTGTTCTAC-3'	5'-CCACAGAAGAATCCAATGTC-3'
<i>Ccl2</i>	Mouse	5'-CATCCACGTGTGGCTCA-3'	5'-GATCATCTTGCTGGTGAATGAGT-3'
<i>Ccl3</i>	Mouse	5'-TGCCCTTGCTGTCTTCTCT-3'	5'-GTGGAATCTTCCGGCTGTAG-3'
<i>BDNF</i>	Human	5'-GTAACGGCGGCAGACAAA-3'	5'-GACCTTTTCAAGGACTGTGACC-3'
<i>NT3</i>	Human	5'-AAAAACGGTTGCAGGGGTAT-3'	5'-GGTTTGGGATGTTTGCAC-3'
<i>Nt3</i>	Mouse	5'-GGTGGTACCTCTCCTCACTC-3'	5'-GAAGAGCCCCGTGCATTCTG-3'
<i>Ntrk2</i>	Mouse	5'-TTCTGCCTGCTGGTGATGT-3'	5'-TCCAGTGGGATCTTATGAAACA-3'

Sensory C, sensory Aδ, cholinergic, and adrenergic nerve fibers have been identified within lesions,^{10,11} with greater nerve fiber density in areas that exhibit high macrophage density.⁶ Studies in zebrafish have shown that macrophages will migrate toward 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 α (ER α) and β (ER β), both widely expressed in the human endometrium.¹⁴ Notably, a proportion of the soma of afferent nerve fibers innervating the uterus and peritoneum is reported to express one or both ERs.¹⁵ Although some studies have analyzed 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.

Our objective was to determine whether estradiol (E2) plays a role in the regulation of macrophage-nerve cross talk in endometriosis by exploring both the expression of ERs in human tissue samples and the impact of E2 on nerves and macrophages using *in vitro* and *in vivo* models.

Materials and Methods

Human Tissues

Eutopic endometrium ($n = 5$) and peritoneal endometriosis lesions ($n = 10$) from patients with endometriosis were collected during laparoscopy (means $\pm$ SD age, 35.1 $\pm$ 6.08 years; range, 26 to 45 years). Fixed sections of endometriosis lesions were evaluated after staining with hematoxylin and eosin, and only those that contained both glandular and

stromal compartments were used for this study. Dating of eutopic endometrial histological features¹⁸ 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 (I, 60%; II, 20%; III, 20%). Endometrium ($n = 5$) and peritoneal ($n = 8$) biopsy specimens 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 before surgery. The study was approved by the Lothian Research Ethics Committee (number 11/AL/0376) and the Ethics Committee of Charité—University Medicine Berlin (EA4/023/05). All patients provided written, informed consent. Samples were fixed in 4% neutral-buffered formalin or stored in RNAlater (Applied Biosystems, Warrington, UK).

Mouse Model of Endometriosis

Endometriosis was induced using wild-type and transgenic colony stimulating factor 1 receptor—enhanced green fluorescent protein (Csf1r—EGFP) mice (MacGreen; founder stocks were a gift from Bernadette Dutia and Prof. David Hume, The Roslin Institute, The University of Edinburgh, UK)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 menses²⁰ and injected into the peritoneal cavity of recipient mice. Lesions and peritoneum were collected from endometriosis mice ($n = 8$) 21 days after inoculation of the peritoneum. In an additional group of mice, on day 21, the E2 s.c. 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

Table 2 Antibodies Used in Immunofluorescence

Antibody (manufacturer)	Raised in animal	Tissue	Dilution
ER β 1 (no. MCA1974S; Serotec, Kidlington, UK)	Mouse	Human	1:500
ER β (no. SC-8974; Santa Cruz Biotechnology, Dallas, TX)	Rabbit	Mouse	1:500
ER α (Vector Laboratories, Peterborough, UK)	Mouse	Human and mouse	1:500
PGP9.5 (Dako, Cambridge, UK)	Rabbit	Human and mouse	1:1000 and 1:3500
CD68 (clone KPI; Dako)	Mouse	Human	1:1200
GFP (no. A11122; Life Technologies)	Rabbit	Mouse	1:1500

ER, estrogen receptor; GFP, green fluorescent protein; PGP, protein gene product.

the protocol), mice were culled, lesions and peritoneum were removed and either stored in RNAlater or fixed in neutral-buffered formalin, and processed to confirm the presence of glands and stroma. Control tissues (uterus and peritoneum) were recovered from naïve control mice ($n = 6$).

Cell Culture and E2 Treatments

DRG Isolation

Rat dorsal root ganglia (DRG) were isolated as previously described.²¹ Dissociated DRGs were used for RNA extraction and quantitative RT-PCR (RT-qPCR). At least 24 hours before experiments, the medium was changed to phenol red free media, and fetal calf serum 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} mol/L (Sigma, Poole, UK), alone or in combination with the anti-estrogen fulvestrant (ICI 182780; final concentration, 10^{-7} mol/L; Tocris, Birmingham, UK). E2 and ICI were dissolved in DMSO to yield stock solutions of 10^{-2} mol/L, and all cells were exposed to a final DMSO concentration of $1:1 \times 10^4$ dilution. DRG conditioned media (CM) were collected, centrifuged, and stored at -80°C and used for the macrophage migration assay. All experiments were performed in triplicate.

Human Peripheral Blood Monocyte Isolation and Macrophage Differentiation

Human venous blood was collected from healthy female volunteers ($n = 5$) with informed consent (Lothian Research Ethics Committee number 08/S1103/38). To isolate mononuclear leukocytes, blood was centrifuged ($350 \times g$, 20 minutes) and platelet-rich plasma was aspirated. Erythrocytes were sedimented with 0.6% (w/v) dextran, followed by separation of leukocytes using a Percoll (GE Healthcare, Hatfield, UK) gradient and centrifugation at $720 \times g$ for 20 minutes. Mononuclear leukocytes were aspirated and monocytes were isolated

by negative selection using a monocyte isolation kit II (Miltenyi Biotech, Surrey, UK). Adherent monocytes were cultured in the presence of 4 ng/mL colony-stimulating factor 1 (CSF-1) to generate monocyte-derived macrophages, hereby referred to as M(CSF-1) in accordance with the recently published macrophage nomenclature guidelines.²² They were cultured for 4 days in Iscove's Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) containing 10% autologous serum and prepared by recalcification of platelet-rich plasma (12-well plates, 37°C in 5% CO_2). M(CSF-1) were exposed to DMSO [M(CSF-1) + DMSO], 10^{-8} mol/L E2 [M(CSF-1) + E2], or 10^{-8} mol/L E2 plus 10^{-7} mol/L ICI [M(CSF-1) + E2 + ICI] for 24 hours at 37°C using 5% CO_2 on day 5. Macrophage CM were collected, centrifuged, and stored at -80°C until further use for the neurite outgrowth assays. All experiments were performed in triplicate.

RNA Extraction and cDNA Synthesis

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

Quantitative Real-Time PCR

Real-time PCR was 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, Ebersberg, 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 $2^{-\Delta\Delta\text{Ct}}$ method.

Figure 1 Macrophages are found in close association with nerve fibers in endometriosis lesions and express estrogen receptors. **A** and **B**: Macrophages are found in close proximity to small-diameter nerve fibers in human peritoneal lesion biopsy specimens. Dual immunofluorescence was performed using the macrophage marker CD68 (red) and the pan neuronal marker protein gene product 9.5 (PGP9.5; green). Nuclei stained with DAPI (blue). **Inset** in **B** is an enlarged image of boxed area. **C** and **D**: Macrophages are also found near small-diameter nerve fibers in lesions recovered from a mouse model of endometriosis. To aid in the localization of macrophages, we used the MacGreen mouse [a transgenic mouse with enhanced green fluorescent protein (GFP)—labeled macrophages] for our mouse model of endometriosis. Dual immunofluorescence was performed using an anti-GFP antibody (red) PGP9.5 (green) on lesions recovered from mice. Nuclei stained with DAPI (blue). **E**: Significantly more CD68⁺ macrophages were detected in peritoneal endometriosis lesions compared with the healthy peritoneum. CD68⁺ cells were counted in four randomly selected fields of view (FOVs) associated with glandular and stromal tissue in lesions, the mean was recorded for each patient sample, and statistical analysis was performed using a Student's unpaired *t*-test. **F–H**: Of macrophages in peritoneal lesions, 20% are immunopositive for estrogen receptor (ER) α , and 100% are immunopositive for ER β . **F**: CD68⁺ ER α ⁺ cells were counted in each FOV and expressed as a proportion of CD68⁺ cells; statistical analysis was performed using a one-way analysis of variance and Newman Keuls post test. **G**: ER α immunolocalization in endometriosis lesions (CD68, red; ER α , green). **Inset**: Enlarged area of boxed area with an ER α ⁺ macrophage (white arrow) adjacent to an ER α [−] macrophage (yellow arrow). **H**: Macrophages in peritoneal lesions are immunopositive for ER β (green); **inset** is enlarged image of boxed area. **I**: Significantly more GFP⁺ macrophages were detected in peritoneal endometriosis lesions compared with the healthy peritoneum. GFP⁺ cells were counted in four randomly selected FOVs associated with glandular and stromal tissue in lesions, the mean was recorded for each sample, and statistical analysis was performed using a Student's unpaired *t*-test. **J–L**: Of macrophages in mouse peritoneal lesions, 25% are immunopositive for ER α , and 100% are immunopositive for ER β . **J**: GFP⁺ ER α ⁺ cells were counted in each FOV and expressed as a proportion of GFP⁺ cells; statistical analysis was performed using a one-way analysis of variance and Newman Keuls post test. **K**: ER α immunolocalization in endometriosis lesions (GFP, red; ER α , green). **L**: Macrophages in mouse peritoneal lesions are immunopositive for ER β (green). **Insets** in **K** and **L** are enlarged images of boxed area. **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 ER α (**N**) and ER β (**O**) confirmed (green). *** $P < 0.001$. $n = 10$ (**E**, CD68⁺ macrophages in peritoneal endometriosis lesions); $n = 8$ (**F**, CD68⁺ macrophages in healthy peritoneum); $n = 8$ (**I**, GFP⁺ macrophages in peritoneal endometriosis lesions); $n = 6$ (**L**, GFP⁺ macrophages in healthy peritoneum). Scale bars: 20 μm (**A**); 50 μm (**B–D**, **H**, and **L**).

Functional Assays

Neurite Outgrowth Assay

Single whole DRG explants from embryonic day 15.5 rat embryos were incubated with Dulbecco's modified Eagle's medium plus 0.1 ng/mL nerve growth factor (positive control), Iscove's Dulbecco's modified Eagle's medium plus autologous serum (negative control), or CM from macrophages treated for 24 hours with DMSO, E2, or E2 + ICI ($n = 5$ female volunteers). They were incubated for 24 and 48 hours at 37°C with 5% CO₂ in poly-D-lysine and Matrigel (BD Biosciences, Oxford, UK) coated 12-well plates. Neurite outgrowth was analyzed as previously described.²¹ Neutralization experiments were performed using 0.4 µg/mL anti-brain-derived neurotrophic factor (BDNF) and 0.2 µg/mL anti-neurotrophin 3 (NT-3; R&D Systems, Oxford, UK). CM was preincubated with neutralizing antibodies for 1 hour before experiments were performed. The treatments were performed in duplicate for each of the five patients, and each duplicate was used to incubate three DRGs.

Macrophage Migration Assay

The macrophage migration assay was performed using µ-Slide V1.04 migration slides (Ibidi, Munich, Germany). CM (80 µL) from DRG exposed to DMSO, E2, or E2 + ICI was placed in one chamber, macrophages (5×10^4 cells) were placed in the other chamber, and slides were incubated for 16 hours, then evaluated using an Axiovert microscope (Carl Zeiss, Oberkochen, Germany). A migration score was allocated to each slide on the basis of the distance migrated and the percentage of macrophages mobilized toward the chamber containing the DRG media. Neutralization experiments were performed using 0.5 µg/mL anti-chemokine (C-C motif) ligand 2 (CCL-2) antibody (BioLegend, San Diego, CA) and an anti-CCL-3 antibody (0.45 µg/mL; R&D Systems). CM was preincubated with neutralizing antibodies for 1 hour before experiments were performed. DRG treatments were performed in triplicate in four separate experiments and used in migration experiments from four macrophage preparations (four female volunteers).

Immunodetection

Dual Immunofluorescence

Dual immunofluorescence was performed 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 (horseradish peroxidase) and a tyramide signal amplification (TSA) system kit labeled with Cy3 (red) or fluorescein (green; 1:50 dilution; Perkin Elmer Inc., Waltham, MA). For detection of the second antigen, sections were microwaved in boiling citrate buffer, and the second primary antibody was applied overnight at 4°C, and

detected 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). 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 four (human) or three (mouse) fields of view 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; BioLegend, San Diego, CA) to visualize neurite projections. Cultured macrophages were fixed for 20 minutes using ice-cold methanol, permeabilized using Triton X-100 (Sigma), and blocked with Avidin/Biotin (Vector, Peterborough, UK) and species-specific blocking buffer for non-specific epitopes. Macrophages were incubated with primary antibody (CD68, ER α , or ER β) overnight at 4°C. Antibody binding was detected using a biotinylated secondary antibody, followed by streptavidin Alexa Fluor 555 (Life Technologies), 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 $\pm$ SEM and were analyzed using a one-way analysis of variance and a Newman Keuls multiple-comparison test or a *t*-test for two-group comparisons. Analysis of qPCR data was performed on transformed values. Analyses were performed using GraphPad Prism software version 6 (Prism, La Jolla, CA).

Results

Macrophages and Nerve Fibers Are Found in Close Association in Peritoneal Endometriosis Lesions

In peritoneal lesions from women with endometriosis, macrophages (immunopositive for CD68, red) were identified in close association with small-diameter nerve fibers, typical of afferent sensory innervation (Figure 1, A and B). Clustering of CD68⁺ macrophages around nerve bundle structures was consistently observed in tissue close to glandular epithelium in peritoneal lesions (Supplemental Figure S1). In lesions recovered from transgenic Csf1r-EGFP (MacGreen) mice, GFP⁺ macrophages were also detected close to nerve fibers (Figure 1, C and D).

ER β Is the Predominant ER Expressed by Lesion-Resident Macrophages

Significantly more CD68⁺ macrophages were detected in sections of peritoneal endometriosis lesions compared with

Figure 2 Nerve fibers recruit macrophages in an estradiol (E2)-dependent manner via C-C motif ligand (Ccl)-2. **A:** Dorsal root ganglia (DRG) were stimulated for 24 hours with vehicle control [dimethyl sulfoxide (DMSO)], 10^{-8} mol/L E2, or 10^{-7} mol/L E2 plus the anti-estrogen ICI 182780. Conditioned media (CM) were retained for use in the macrophage migration assay; macrophages stimulated with CSF-1 [M(CSF-1)]; 5×10^{-4} mol/L cells per well were placed in one chamber of the migration slide, and DRG CM in the other chamber. **B:** M(CSF-1) migrate furthest toward CM from DRG exposed to E2 ($P < 0.01$). Migration score was based on percentage cells mobilized and distance migrated. Macrophages; $n = 4$ patients, embryonic rat DRGs; $n = 4$ pregnant dams. **C–E:** E2 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 analyzed using quantitative PCR in DRG exposed to DMSO, E2, or E2 plus ICI for 24 hours: Csf-1 (**C**), Ccl-2/macrophage chemotactic protein 1 (**D**), and Ccl-3/macrophage inflammatory protein-1 α (**E**). **F:** The macrophage migration assay was performed as in **B**, with the addition of 0.5 μ g/mL anti-CCL-2 or 0.45 μ g/mL anti-CCL-3 antibodies to CM from DRGs exposed to DMSO or E2. Anti-CCL-2 abolished E2-induced chemotactic properties of DRG CM, whereas anti-CCL-3 only attenuated E2-induced chemotactic properties of DRG CM. Statistical analysis was performed using a one-way analysis of variance and Newman Keuls post test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$. $n = 4$ (**B**, patients for macrophages and pregnant dams for embryonic rat DRGs); $n = 5$ cultures (**C–E**). Mac media, unconditioned macrophage media (negative control); RQ, relative quantification.

sections of unaffected peritoneum ($P < 0.001$) (Figure 1E). In women, CD68⁺ macrophages resident in the eutopic endometrium were immunonegative for ER α (Supplemental Figure S2A) but immunopositive for ER β (Supplemental Figure S2B), regardless of whether they had been diagnosed with endometriosis (Supplemental Figure S2, C and D). In peritoneal biopsy specimens from women without endometriosis, ER α immunolocalized to approximately a tenth of CD68⁺ macrophages ($10.6\% \pm 3.85\%$) (Figure 1F and Supplemental Figure S2E), but all of the macrophages were ER β positive (Supplemental Figure S2F). In peritoneal endometriosis, lesions approximately a fifth of the CD68⁺ macrophages ($18.3\% \pm 4.37\%$) were immunopositive for ER α (Figure 1G) and, as in the normal peritoneum, they

were all immunopositive for ER β (Figure 1H). Results obtained in our mouse model of endometriosis mirrored those in women with significantly more GFP⁺ macrophages in lesions ($P < 0.05$) compared with the peritoneum of naïve mice (Figure 1I). A tenth of mouse peritoneal macrophages (10.14 ± 6.41) and a quarter of lesion resident macrophages (24.82 ± 5.57) were immunopositive for ER α (Figure 1, J and K), with all GFP⁺ cells being immunopositive for ER β (Figure 1L).

Human peripheral blood monocytes were isolated and differentiated into macrophages that are classified as being at the alternative end of the macrophage activation spectrum²² by incubating them in the presence of CSF-1, hereafter referred to as M(CSF-1). M(CSF-1) incubated with E2 are referred to as

Figure 3 Macrophages stimulated with CSF1 and estradiol [M(CSF-1 + E2)] exhibit neurotrophic properties via brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-3. **A:** M(CSF-1) were exposed to dimethyl sulfoxide (DMSO), E2, or E2 plus ICI 182780, the conditioned media (CM) were retained and used to incubate single whole dorsal root ganglia (DRG) immediately dissected from embryonic rats. **B–D:** CM from M(CSF-1 + E2) enhanced neurite outgrowth compared with CM from M(CSF-1 + DMSO). **B** and **C:** Immunocytochemistry performed on DRG using anti-neurofilament (red) and nuclei counterstained with DAPI (blue). **B:** DRG grown in CM from M(CSF-1 + DMSO). **C:** CM from M(CSF-1 + E2). **D:** Neurite outgrowth was quantified; the score was based on the percentage coverage of DRG and average length of neurites. DRG media indicates nerve growth factor present, positive control; Mac media, negative control. **E** and **F:** The concentration of mRNAs encoding neurotrophins was analyzed using quantitative PCR in M(CSF-1) stimulated with DMSO, E2, or E2 plus ICI for 24 hours; BDNF (**E**) and NT-3 (**F**) are shown. **G** and **H:** The neurotrophic properties of M(CSF-1 + E2) are BDNF and NT-3 dependent. Whole single DRGs were cultured for 24 hours in CM from M(CSF-1 + DMSO) or M(CSF-1 + E2) for 24 hours, or CM from M(CSF-1 + E2) in the presence of 0.4 $\mu\text{g}/\text{mL}$ anti-BDNF or 0.2 $\mu\text{g}/\text{mL}$ anti-NT-3 antibody. **G:** Immunocytochemistry performed on DRG using antineurofilament. **F:** Quantification of 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. Statistical analysis was performed using a one-way analysis of variance and Newman Keuls post test. $**P < 0.01$, $***P < 0.001$. $n = 5$ volunteers (**E** and **F**). Scale bar = 500 μm (**B**, **C**, and **G**). DRG media, unconditioned DRG growth media; Mac media, unconditioned macrophage growth media; RQ, relative quantification.

M(CSF-1 + E2). All isolated cells were confirmed as macrophages using CD68 immunocytochemistry (Figure 1M). M(CSF-1 + E2) contained mRNAs encoding ER α and ER β (Supplemental Figure S3, A and B). Protein localization revealed a mixed population of ER α -positive and ER α -negative macrophages; ER α was detected in 66% of cells (Figure 1N), whereas ER β was detected in all cells (Figure 1O).

Nerve Fibers Recruit Macrophages in an E2-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. CM from DRGs stimulated for 24 hours with DMSO, E2, or E2 plus the anti-estrogen ICI were placed in one chamber, and M(CSF-1) were placed in the other chamber (Figure 2A). M(CSF-1) migrated furthest toward 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 (Figure 2B). Notably, the 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 Csf-1 were up-regulated by E2 ($P < 0.001$) (Figure 2C), as were Ccl-2 and Ccl-3 mRNAs ($P < 0.05$) (Figure 2, D and E). The addition of ICI abrogated the effect of E2. The addition of an anti-CCL-2 antibody to DRG CM abolished the E2-induced macrophage chemotactic properties ($P < 0.01$), whereas the addition of an anti-CCL-3 antibody attenuated E2-induced macrophage chemotactic properties of DRG CM (Figure 2F).

E2 Induces Neurotrophic Properties in Macrophages

DRGs were cultured in CM from M(CSF-1), and neurite outgrowth was recorded (Figure 3A). CM from E2-activated macrophages M(CSF-1 + E2) significantly enhanced neurite outgrowth compared with CM from M(CSF-1 + DMSO) or M(CSF-1 + E2 + ICI) at 24 and 48 hours ($P < 0.001$) (Figure 3, B–D). Analysis of M(CSF-1) mRNAs revealed that NTs were up-regulated by E2 treatment. Specifically, mRNA concentrations of BDNF and NT-3 were significantly increased in M(CSF-1 + E2) compared with M(CSF-1 + DMSO) ($P < 0.001$ and $P < 0.01$, respectively) (Figure 3, E 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 DRGs were incubated with CM from M(CSF-1) (Figure 3A) in combination with neutralizing 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 ($P < 0.01$) (Figure 3, G and H). This effect was not observed when the neutralizing antibodies were added to DRG cultured in neuronal media in the presence of NGF.

Macrophage Infiltration of Endometriosis Lesions Is E2 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 ($P < 0.05$) (Figure 4, A–C). We have previously reported that Ccl-2 and Ccl-5 (regulated on activation normal T-cell expressed and secreted) 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 with biopsy specimens of naïve uterus and peritoneum (Figure 4D).

Figure 4 Macrophage infiltration of lesions is estradiol (E2) dependent in a mouse model of endometriosis. **A:** Exposure of endometriosis mice to E2 increases the number of macrophages present in endometriosis lesions compared with lesions from mice that had E2 support withdrawn 7 days earlier (control). The numbers of macrophages per lesion were counted and normalized to lesion area. **B** and **C:** Images show representative fields of view from control mice (**B**) or mice exposed to E2 (**C**). Immunofluorescence was performed using an anti-green fluorescent protein (GFP) antibody (green), and nuclei are stained blue with DAPI. **D:** Quantitative PCR analysis reveals that C-C motif ligand (Ccl)-3 mRNA concentrations are 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, respectively) and the peritoneum (EP) and lesions (EL) of mice with endometriosis. Statistical analysis was performed using a Student's unpaired *t*-test or a one-way analysis of variance and Newman Keuls post test. * $P < 0.05$, ** $P < 0.01$. $n = 6$ (**A**, E2 group); $n = 4$ (**A**, control group); $n = 6$ (**D**, NU and NP groups); $n = 8$ (**D**, EP and EL groups). Scale bar = 50 μm (**B** and **C**). RQ, relative quantification.

Figure 5 Colony-stimulating factor (Csf)-1, neurotrophin (Nt)-3, and tyrosine kinase receptor that binds both Bdnf and Nt-3 (TrkB) mRNA concentrations are elevated in endometriosis lesions and estradiol (E2) regulated in a mouse model of endometriosis. **A–C:** Csf-1, Nt-3, and TrkB are 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, respectively) and the peritoneum (EP) and lesions (EL) of mice with endometriosis. **D–F:** Csf-1, Nt-3, and TrkB are regulated by E2 in mouse endometriosis lesions. In a separate experiment, mice were separated into groups that continued exposure to E2 (E2) or had estradiol treatment withdrawn (control) for an additional 7 days. Peritoneal (P) and lesion (EL) biopsy specimens were included in the analysis. Peritoneum from naïve mice (naïve P) was also included. mRNA concentrations of Csf-1 (**D**), Nt-3 (**E**), and TrkB (**F**) are elevated in lesions from mice exposed to E2 compared with control mice. Statistical analysis was performed using a one-way analysis of variance and a Newman Keuls post test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. $n = 6$ (**A–C**, NU and NP groups); $n = 8$ (**A–C**, EP and EL groups); $n = 6$ (**D–F**, E2 and naïve P groups); $n = 5$ (**D–F**, control). RQ, relative quantification.

Csf-1, Nt-3, and TrkB Are E2 Regulated in a Mouse Model of Endometriosis

Csf-1 and Nt-3 mRNA concentrations were significantly higher in lesions than other tissue samples (Figure 5, A and B). mRNA concentrations of the tyrosine kinase receptor that binds both Bdnf and Nt-3 (TrkB) were also up-regulated in lesions (Figure 5C). Concentrations of mRNAs encoded by *Csf1*, *Nt3*, and *Ntrk2* were significantly higher in the lesions exposed to E2 compared with those recovered from mice in which E2 was withdrawn after lesions were established (Figure 5, D–F).

Discussion

Endometriosis is a steroid-dependent disorder; lesions exhibit the capacity for enhanced tissue biosynthesis of estrogens and alterations in ER protein expression.^{21,23} Herein, we provide new evidence that estrogens play a key role in the regulation of interactions between macrophages and nerve fibers in endometriosis. Specifically, by using *in vitro* model systems, we found that DRG neurons produced chemokines in response to

E2 that promoted macrophage recruitment, whereas macrophages stimulated with E2 produced NTs that promoted neuronal outgrowths. By 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.

ER Expression by Endometriosis-Associated Macrophages Suggests a Heterogeneous Population

Estrogen can bind directly to either ER α or ER β , 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 lesions²¹ are ER β^+ /ER α^- . Notably, E2 treatment of immortalized 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

Figure 6 Estradiol enhances the interactions between macrophages and nerve fibers in endometriosis. Schematic representation of macrophage-nerve interactions in endometriosis. Nerve fibers increase the production of colony-stimulating factor (CSF)-1 and C-C motif ligand (CCL)-2 in response to estradiol, which enhances macrophage migration. Estradiol also acts on macrophages to increase production of brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-3, resulting in increased neurogenesis.

and ectopic endometrium in women and in a mouse model. Notably, all CD68⁺ macrophages in the peritoneum and lesions from women, and all GFP⁺ macrophages in the peritoneum and lesions from mice, were immunopositive for ER β . In contrast, peritoneal and lesion resident macrophages were a mixed population of ER α ⁺ and ER α ⁻ cells. There was no clear evidence of either population residing in specific microenvironments, because ER α ⁺ cells were often detected adjacent to ER α ⁻ cells.

As we have previously reported data from our mouse model of endometriosis showing that the lesion-resident GFP⁺ macrophages may originate from both the peritoneum and the shed endometrium,⁹ we wondered if the heterogeneous expression of ER α might be a reflection of different origins (endometrial macrophages are all ER α ⁻). However, because there was no significant difference in proportions of ER α ⁺ cells in lesions and peritoneum, this cannot be the case. It, therefore, remains to be determined what regulates the amount of ER α protein in CD68⁺ macrophages. One plausible explanation is that the ER α cells have infiltrated from peripheral blood (ER α is expressed by peripheral blood monocyte-derived macrophages).

Macrophages isolated from the peritoneal fluid of women with endometriosis are reported to be immunopositive for both ER α and ER β , and ER immunolocalization increases in women with endometriosis compared with 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 ER α was only detected in a subset of macrophages in both peritoneal (from women without endometriosis) and lesion biopsy specimens^{16,25}; we also found no difference in ER immunolocalization in macrophages from women with and without endometriosis. We suggest that this discrepancy in findings is because of 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 spectrum²⁸; 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 reprograms the macrophages infiltrating the lesion from the peritoneum.

E2 Mediates a Two-Way Dialogue between Macrophages and Nerves

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 ERs,¹⁵ and ER-dependent signaling modulates a range of processes in peripheral nerves.^{29,30} We were, therefore, interested in exploring whether the estrogen-dominated microenvironment could play a role in modulating interactions between macrophages and nerves and what regulatory factors may be involved. By 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 migration and that the mRNA concentrations of Ccl-2 and Ccl-3 in DRGs was ER regulated. More important, when an antibody directed against Ccl-2 was added to the CM, macrophage migration was abrogated, implicating a key E2-dependent factor involved in neuron-mediated macrophage migration. Notably, elevated CCL-2 concentrations have been detected in peritoneal fluid of women with endometriosis.³¹ Moreover, Luk et al³² demonstrated that CCL-2 concentrations are increased by E2 in endometrial endothelial cells from women with endometriosis. Their study and ours both provide evidence that E2 modulates recruitment of macrophages via CCL-2 to endothelial cells and nerve fibers in endometriosis lesions.

CSF-1 is a critical growth factor involved in macrophage survival, proliferation, and differentiation.³³ Csf-1 may play a critical role in early development of endometriosis lesions; mice homozygous for a *Csf1* mutation (*Csf-1* op/op) developed significantly fewer lesions in a model of endometriosis compared with wild-type controls.³⁴ Elevated CSF-1 concentrations have also been reported in the peritoneal fluid of women with endometriosis.³⁵ In our study, mRNA concentrations of *Csf-1* were increased in lesions recovered from mice and were also increased in DRGs exposed to E2. On the basis of these results, we suggest that the ER-dependent regulation of CSF-1 in peripheral nerve fibers present in endometriosis lesions may play a role in modulating macrophage survival and phenotype, and is consistent with the hypothesis that neurogenic inflammation is a key process in this disorder.

E2 is reported to be neuroprotective³⁶; these effects have been linked to E2-dependent expression of BDNF, promoting neuron survival, regeneration, and synaptogenesis.³⁷ NT-3 is elevated in the peritoneal fluid of women with endometriosis,³⁸ but the cellular source is uncertain. In this study, E2 increased the neurotrophic properties of macrophages via up-regulation of BDNF and NT3, suggesting that this cell type is a key source of these NTs contributing to E2-dependent nerve growth into lesions. Notably, the mRNA concentrations of Nt-3 and the NT receptor TrkB were also E2 regulated in the lesions induced in mice, complementing the data from the *in vitro* models.

Endometriosis Is a Neuroinflammatory Disorder

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 after injury to the central nervous system and peripheral nervous system 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 fibers, whereby estrogen acts on nerve fibers to enhance the expression of CSF1 and CCL-2, recruiting macrophages to nerve fibers. Reciprocally, estrogens act on macrophages to enhance expression of BDNF and NT-3, which further potentiates neurogenesis into lesions (Figure 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 hypoestrogenism.

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E.G. conceived and performed experiments and analyzed data, J.T. performed experiments and analyzed data, A.E.-Z. performed experiments, S.M. and A.W.H. collected patient biopsy specimens, P.T.K.S. conceived experiments, and E.G., A.W.H., and P.T.K.S. wrote the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.04.012>.

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