



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Prostaglandin F2alpha-F-prostanoid receptor signaling promotes neutrophil chemotaxis via chemokine (C-X-C motif) ligand 1 in endometrial adenocarcinoma

Citation for published version:

Wallace, AE, Sales, KJ, Catalano, RD, Anderson, RA, Williams, ARW, Wilson, MR, Schwarze, J, Wang, H, Rossi, AG & Jabbour, HN 2009, 'Prostaglandin F2alpha-F-prostanoid receptor signaling promotes neutrophil chemotaxis via chemokine (C-X-C motif) ligand 1 in endometrial adenocarcinoma' *Cancer Research*, vol. 69, no. 14, pp. 5726-33. DOI: 10.1158/0008-5472.CAN-09-0390

Digital Object Identifier (DOI):

[10.1158/0008-5472.CAN-09-0390](https://doi.org/10.1158/0008-5472.CAN-09-0390)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Cancer Research

Publisher Rights Statement:

Europe PMC Funders Group Author Manuscript

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Published in final edited form as:

Cancer Res. 2009 July 15; 69(14): 5726–5733. doi:10.1158/0008-5472.CAN-09-0390.

Prostaglandin F_{2α}-F-Prostanoid Receptor Signalling Promotes Neutrophil Chemotaxis via Chemokine (CXC motif) Ligand-1 in Endometrial Adenocarcinoma

Alison E Wallace¹, Kurt J Sales¹, Roberto D Catalano¹, Richard A Anderson^{1,2}, Alistair RW Williams³, Martin R Wilson¹, Jurgen Schwarze⁴, Hongwei Wang⁴, Adriano G Rossi⁴, and Henry N Jabbour¹

¹MRC Human Reproductive Sciences Unit, The Queen's Medical Research Institute, Edinburgh EH16 4TJ, UK

²Department of Reproductive and Developmental Sciences, The Queen's Medical Research Institute, Edinburgh EH16 4TJ, UK

³Department of Pathology, The Queen's Medical Research Institute, Edinburgh EH16 4TJ, UK

⁴Centre for Inflammation Research, The Queen's Medical Research Institute, Edinburgh EH16 4TJ, UK

Abstract

The prostaglandin F_{2α} (PGF_{2α}) receptor (FP) is elevated in endometrial adenocarcinoma. This study found that PGF_{2α} signalling via FP regulates expression of chemokine (C-X-C motif) ligand 1 (CXCL1) in endometrial adenocarcinoma cells. Expression of CXCL1 and its receptor, CXCR2, are elevated in cancer tissue as compared to normal endometrium and localised to glandular epithelium, endothelium and stroma. Treatment of Ishikawa cells stably transfected with the FP receptor (FPS cells) with 100nM PGF_{2α} increased CXCL1 promoter activity, mRNA and protein expression, and these effects were abolished by co-treatment of cells with FP antagonist or chemical inhibitors of Gq, EGFR and ERK. Similarly, CXCL1 was elevated in response to 100 nM PGF_{2α} in endometrial adenocarcinoma explant tissue. CXCL1 is a potent neutrophil chemoattractant. The expression of CXCR2 colocalised to neutrophils in endometrial adenocarcinoma and increased neutrophils were present in endometrial adenocarcinoma compared with normal endometrium. Conditioned media from PGF_{2α}-treated FPS cells stimulated neutrophil chemotaxis which could be abolished by CXCL1 protein immunoneutralisation of the conditioned media or antagonism of CXCR2. Finally, xenograft tumours in nude mice arising from inoculation with FPS cells showed increased neutrophil infiltration compared to tumours arising from wild-type cells or following treatment of mice bearing FPS tumours with CXCL1-neutralising antibody. In conclusion, our results demonstrate a novel PGF_{2α}-FP pathway that may regulate the inflammatory microenvironment in endometrial adenocarcinoma via neutrophil chemotaxis.

Keywords

Prostaglandin F_{2α}; Endometrial cancer; Neutrophil; CXCL1; CXCR2

Introduction

Endometrial adenocarcinoma is the most common gynaecological malignancy in Western countries, affecting mainly post-menopausal women with a frequency of 15-20 per 100 000 women per year (1). Overexpression of the cyclooxygenase (COX) enzymes and prostaglandins has been demonstrated in endometrial adenocarcinoma as well as a number of other cancer types and gynaecological pathologies (2, 3).

In the reproductive tract, the most commonly synthesised prostaglandins are the E- and F-series prostanoids (4). These are synthesised from arachidonic acid by COX enzymes and prostaglandin synthases, and are then transported out of the cell by a prostaglandin transporter (5) to act in an autocrine/paracrine manner on G-protein coupled receptors (GPCR). The GPCR for PGF_{2α} (FP) is a Gq coupled receptor which upon activation leads to release of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (6). Recently we have demonstrated a role for FP in endometrial adenocarcinoma, with evidence for elevated PGF_{2α}-FP signalling upregulating angiogenic and tumourigenic genes including COX-2 (7), FGF2 (8) and VEGF (9), and increasing proliferation and migration of neoplastic epithelial cells (10-12).

Chemokine (C-X-C motif) ligand 1 (CXCL1, also known as growth-regulated oncogene α) has angiogenic, chemoattractant and inflammatory activities (13). A link between prostaglandins and CXCL1 has been demonstrated, as prostaglandin E₂ signalling induces CXCL1 expression in colorectal cancer cell lines (14). CXCL1 is upregulated in melanoma (15, 16), colorectal (17, 18) and prostate cancer (19) and binds to the CXCR2 receptor (20) to promote the recruitment of neutrophils to sites of inflammation (21). Although the role of neutrophils in cancer is unclear, recent evidence suggests that they may promote tissue remodelling by production of proteases including MMP-9 (22) and angiogenic factors such as VEGF (23), in addition to their classical role as the first line of defence against invading pathogens (24).

In this study, we used a chemokine antibody array to identify CXCL1 as a target gene of PGF_{2α}. We investigated the expression, localisation and regulation of CXCL1 expression mediated via PGF_{2α}-FP signalling in endometrial adenocarcinoma and its downstream regulation of neutrophil influx into endometrial tumours *in vitro* and in endometrial tumour xenografts *in vivo*.

Materials and Methods

Reagents

Indomethacin, PBS, BSA, AL8810, Tri-reagent and PGF_{2α} were purchased from Sigma Chemical Co. (Dorset, UK). PD98059, AG1478, Cyclosporin A and 4C3MQ were purchased from Calbiochem (Nottingham, UK). CXCR2 and Gr-1 (a murine neutrophil marker) antibodies were purchased from R&D systems (Abingdon, UK), and CXCL1 and neutrophil elastase antibodies from Santa Cruz Biotechnology (Autogen Bioclear, UK) and DAKO (Cambridgeshire, UK) respectively. FITC-CD11b, PE-GR-1 and Cy5-CD11c antibodies were obtained from eBioscience (Middlesex, UK). The chemokine antibody array was purchased from RayBiotech (Peterborough, UK).

Patients and tissue collection

Endometrial adenocarcinoma tissue and normal tissue was obtained as detailed in our prior studies (9, 10). Cancer patients were pre-diagnosed to have adenocarcinoma of the uterus, and diagnosis was confirmed histologically in all cases. Normal endometrial tissue was collected from women undergoing surgery for minor gynecological procedures with no

underlying endometrial pathology. Ethical approval was obtained from Lothian Research Ethics Committee and written informed consent was obtained from all subjects before tissue collection.

Cell lines, culture and treatments

Wild-type Ishikawa cells and Ishikawa cells engineered to stably express the full length human FP receptor to the levels observed in endometrial adenocarcinomas, referred to as FPS cells, were cultured as described previously (9). Transient transfections were performed using Superfect (Qiagen, Crawley, UK) as per the manufacturer's protocol. The optimal concentrations of all chemical inhibitors and antibodies were determined empirically by titration using the manufacturer's guidelines as described in our previous studies (25). Cell viability was determined for each inhibitor using the CellTitre 96 Aqueous One Solution assay (Promega, Southampton, UK) as described in our previous studies (10, 25). Cells were treated with vehicle, inhibitor alone, or 100 nM PGF_{2α} alone or in the presence of YM254890 (1 μM), AL8810 (50 μM), 43CMQ (1 μM), AG1478 (200 nM) or Cyclosporine A (1 μM) for the time indicated.

Chemokine antibody array

Conditioned medium (CM) was prepared as described (8). Briefly, FPS cells were stimulated with vehicle or 100 nM PGF_{2α} for 24 hours. The conditioned medium (V-CM or P-CM respectively) was analysed for cytokine expression using the RayBio Human Cytokine Antibody Array 3 kit, following manufacturer's protocol.

CXCL1 luciferase reporter assays

The CXCL1 reporter plasmid consisting of the CXCL1 promoter fused to the firefly luciferase reporter (as described in (26)) was kindly supplied by Professor Ann Richmond (Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee). The CXCL1 promoter firefly luciferase reporter was transfected into FPS cells with pRL-TK (containing the renilla luciferase coding sequence; Promega) as an internal control. FPS cells were cotransfected with control vector (pcDNA3.0) or vector encoding a dominant negative isoform of EGFR, Ras, MEK or NFAT (kindly supplied by Professor Zvi Naor, Tel Aviv University, Israel). The DN constructs have been previously characterised and described (27, 28). Cells were stimulated with vehicle or 100 nM PGF_{2α} for the time indicated in the figure legend. The activity of firefly and renilla was determined using the dual luciferase assay kit (Promega) and total luciferase activity determined relative to the internal renilla control. Data are expressed as fold increase in luciferase activity as compared to vehicle treated cells and are presented as mean ± SEM from at least 3 independent experiments.

Taqman quantitative RT-PCR

CXCL1 and CXCR2 expression in endometrial tissues and FPS cells was measured by quantitative RT-PCR analysis as described previously (1, 2). FPS cells were treated with vehicle, 100 nM PGF_{2α} alone or in the presence of inhibitor, or vehicle and inhibitor alone for 8 hours. RNA samples were then extracted using Tri-reagent following manufacturer's guidelines. RNA samples were reverse transcribed and RT-PCR performed as described previously (1, 2) using sequence specific primers and probes: CXCL1 forward, 5'-GTT TTC AAA TGT TCT CCA GTC ATT ATG-3'; reverse, 5'-CCG CCA GCC TCT ATC ACA GT-3'; probe, 5'-TTC TGA GGA GCC TGC AAC ATG CCA-3'; CXCR2 forward, 5'-TGC TCT TCT GGA GGT GTC CTA CA-3'; reverse, 5'-AGA TCT TCA CCT TTC CAG AAA TCT T-3'; probe, 5'-CCC AGC GAC CCA GTC AGG ATT TAA-3'. Primers and data were analysed and processed using Sequence Detector v1.6.3 (Applied Biosystems).

Expression of analyzed genes was normalised to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Results are expressed as fold increase above cells treated with vehicle and inhibitor. Data are presented as mean \pm SEM from at least 3 independent experiments.

CXCL1 ELISA

CXCL1 protein secretion by FPS cells in the culture media was measured by the Human CXCL1 Quantikine ELISA kit (R&D systems, Abingdon, UK). FPS cells were treated as described above for mRNA, for time indicated in the figure legend. The ELISA was then carried out according to manufacturer's instructions. Optical density of wells was determined by spectrophotometry at 450 nM. Data are presented as mean \pm SEM from at least 3 independent experiments.

Immunohistochemical analysis

Expression of CXCL1, neutrophil elastase, CXCR2 and Gr-1 was localised in endometrial tissue and xenografts by immunohistochemistry using standard techniques as described previously (1, 2). Briefly, following antigen retrieval, sections were blocked in 5% normal rabbit serum (CXCL1 and Gr-1) or normal goat serum (CXCR2 and neutrophil elastase) diluted in TBS with 5% BSA. Subsequently, tissue sections were incubated with goat anti-human CXCL1 polyclonal antibody (2 μ g/ml), mouse anti-human CXCR2 (5 μ g/ml), mouse anti-human neutrophil elastase monoclonal antibody (2 μ g/ml) or rat anti-mouse Gr-1 (5 μ g/ml) overnight at 4°C. Control sections included: no primary antibody, non immune goat, mouse and rat IgG, or CXCL1 antibody pre-absorbed with blocking peptide (20 μ g/ml; Santa Cruz Biotechnology, Autogen Bioclear, UK). Subsequently, sections were incubated with rabbit anti-goat / rat biotinylated or goat anti-mouse biotinylated antibodies (DAKO, UK), followed by streptavidin-horse radish peroxidase complex (DAKO). Colour reaction was developed with 3',3' diaminobenzidine (DAKO). Sections were counterstained in haematoxylin. Images were obtained on a PROVIS microscope at x200 or x400 magnification (Olympus Optical, London, UK) using Canon EOS image capture software (Canon, Woodhatch, Surrey, UK). The number of neutrophils was quantified using neutrophil elastase staining and standard stereological techniques. Briefly, each section was examined using x40 plan apo objective from a BH2 microscope (Olympus, Tokyo, Japan) fitted with an automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK) using a video camera (HV-C20; Hitachi, Tokyo, Japan) and analyzed with Image-ProPlus 4.5.1 software with a Stereology 5.0 plug-in (Media Cybernetics, Wokingham, Berkshire, UK). A total of 40 randomised fields of view were examined and counted (n=7 normal endometrium, n=30 carcinoma) and data are expressed as mean number of cells per mm² of tumour examined.

Immunofluorescence microscopy

CXCR2 expression was colocalised with neutrophil elastase by immunofluorescence microscopy as described previously (8, 29). Briefly, sections were blocked in 5% normal goat serum diluted in PBS with 5% BSA before incubation with mouse anti-neutrophil elastase (1 μ g / ml). Following overnight incubation at 4°C sections were incubated with goat-anti mouse biotinylated Fab, then tyramide signal amplification kit (TSA Fluorescein System, 1:50 dilution, Perkin Elmer, MA). Sections were then microwaved in 0.01M citrate buffer for 30 minutes and endogenous peroxidase blocked using hydrogen peroxide. Non-specific binding was blocked with 5% normal goat serum and sections were incubated with mouse anti-CXCR2 antibody (1 μ g/ml) at 4°C overnight. Sections were again incubated with goat-anti-mouse biotinylated Fab and tyramide signal amplification kit. Nuclei were stained using ToPro (Molecular Probes, UK). Fluorescent images were visualised and

photographed using a Carl Zeiss laser scanning microscope LSM510 (x400 objective; Jena, Germany).

Neutrophil chemotaxis assay

Neutrophil chemotaxis was analysed using transwell inserts (5 μ m pore size, Corning Costar, UK). Neutrophils were purified as previously described (30) and resuspended in serum free media. 750 000 cells were added to the top chamber of the transwell insert and 600 μ l of V-CM or P-CM was added to the bottom chamber. Serum free media alone or with 50 000 pg/ml CXCL1 were added as negative and positive controls respectively. Cells were incubated at 37°C in a 5% CO₂ atmosphere for 1 hour and the plate was gently tapped to dislodge cells adhered to the underside of the membrane. Cells in the bottom chamber were collected and counted at least 6 times using a haemocytometer. Data are expressed as mean \pm SEM from at least 3 independent experiments.

Xenograft tumour model

A suspension of 500 000 Ishikawa wild type (WT) or FPS cells in a total volume of 0.2 ml DMEM was injected subcutaneously into each dorsal flank of CD1-*Foxn1*^{tmu} mice (Charles River, UK). The mice (n=30) were divided into two groups of equal tumour size after engraftment (1 week). The mice were injected twice weekly with 100 μ g IgG (WT and FPS) or CXCL1 neutralising antibody (FPS) via intraperitoneal injection for four weeks. One tumour from each mouse was placed in PBS for flow cytometry analysis and RNA extracted from the second tumour from each mouse. The animals were maintained under sterile conditions in individually vented cages.

Flow cytometry analysis

Xenografts from nude mice were assessed for immune cell infiltrate using flow cytometry (n=15). Briefly, tumours were digested by collagenase treatment at 37°C for 45 minutes. Tissue was then mechanically disrupted into a single cell solution using a syringe and 40 μ m mesh and resuspended in FACS wash (PBS + 1% BSA + 2% formalin). Cells were incubated at 4 °C for 30 minutes in FACS wash containing the following monoclonal antibodies and appropriate isotype controls: FITC-CD11b, PE-Gr-1 and Cy5-CD11c. Red blood cells were lysed using BD FACS lysing solution according to manufacturer's instructions (BD Biosciences, Oxford, UK). Samples were analysed using a FACScalibur cytometer (BD biosystems) using BD CellQuest software. Neutrophils were defined by expression of Gr-1 and CD11b epitope, absence of CD11c and scatter profile.

Statistical analysis

Where appropriate, data were subjected to statistical analysis with ANOVA and Students t-test (GraphPad Prism, San Diego, California, USA).

Results

CXCL1 expression in FPS cells

Changes in cytokine expression in FPS cells in response to PGF_{2 α} -treatment were examined by cytokine antibody array (Figure 1A). A combined upregulation of CXCL1, 2 and 3 as well as CXCL1 alone was observed following 100 nM PGF_{2 α} -treatment of FPS cells for 24 hours compared to vehicle treated cells. To verify this finding, the promoter activity (Figure 1B), mRNA (Figure 1C) and protein (Figure 1D) expression of CXCL1 in response to PGF_{2 α} treatment was examined. All were significantly increased (p<0.01) in response to PGF_{2 α} treatment in a time-dependent manner compared to vehicle treated cells.

Involvement of epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase kinase (MEK) signalling in CXCL1 production

To determine signalling pathways mediating CXCL1 production in FPS cells, we treated cells with vehicle, 100 nM PGF_{2α} alone or with a panel of chemical inhibitors of cell signalling, or inhibitor alone (Figure 2). Treatment of FPS cells with PGF_{2α} for 8 and 24 hours induced a 91.5±8.4 and 22.3±4.7 fold increase in CXCL1 mRNA and protein expression respectively compared to vehicle treated cells (Figure 2A and 2B). This increase was abolished by treatment of cells with a selective inhibitor of Gq (YM254890, p<0.01) and significantly inhibited with the FP receptor antagonist AL8810 (p<0.05) and inhibitors of EGFR (AG1478, p<0.05) and MEK (PD98059, p<0.01). Inhibitors of calcineurin (cyclosporine A, CsA) and protein kinase A (PKA; 43CMQ) did not significantly affect CXCL1 mRNA and protein production.

We confirmed a role for EGFR and extracellular signal-regulated kinase (ERK) in PGF_{2α} mediated CXCL1 production by co-transfecting FPS cells with the CXCL1 promoter and either an empty vector (pcDNA3.0) or dominant negative (DN)-EGFR, DN-Ras, DN-MEK or DN-Nuclear factor of activated T-cells (NFAT; Figure 2C). Treatment of control-vector transfected cells with 100 nM PGF_{2α} showed an elevation of CXCL1 promoter activity of 18.9±3.6 fold which was significantly reduced by co-transfection of cells with DN-EGFR (p<0.05), DN-ras (p<0.01) and DN-MEK (p<0.01), but no significant difference was shown when cells were transfected with DN-NFAT.

CXCL1 and CXCR2 expression in endometrial adenocarcinoma and normal endometrium

Since we had ascertained a role for the FP receptor in regulating CXCL1 in an endometrial adenocarcinoma cell line we next investigated the expression and regulation of CXCL1 in endometrial adenocarcinoma explants by PGF_{2α}. Quantitative RT-PCR analysis showed an increase in the expression of CXCL1 and its receptor CXCR2 mRNA expression in human endometrial adenocarcinoma tissue (n=58) compared to normal endometrium (n=45; 5.9 and 4.2 fold respectively; Figure 3A and 3B; p<0.001). We investigated whether CXCL1 expression in endometrial adenocarcinoma explants was regulated via FP and MEK signalling pathways. Carcinoma tissue was treated with PGF_{2α} in the absence/presence of AL8810 and PD98059 for 24 hours. CXCL1 mRNA was found to be elevated 5.3 ±0.8 fold in response to PGF_{2α} (p<0.05). Co-treatment of tissue with AL8810 or PD98059 significantly reduced this increase in CXCL1 expression (p<0.05).

Localisation of CXCL1 and CXCR2 in endometrial adenocarcinoma

The site of expression of CXCL1 and CXCR2 protein in carcinoma tissue was then determined by immunohistochemistry (Figure 4A). CXCL1 and CXCR2 immunoreactivity was localised to glandular epithelium, vascular endothelial cells and stroma in all well, moderately and poorly differentiated carcinoma sections studied (n=4 each group).

Using serial sectioning, CXCL1 expression could be localised to the same glandular epithelial and vascular endothelial cells as the FP receptor (Figure 4B; highlighted by arrowheads). CXCL1 has been previously described as a potent neutrophil chemoattractant. We next colocalised CXCR2 expression throughout the stroma with expression of neutrophil elastase, a neutrophil marker, in endometrial adenocarcinoma using dual immunofluorescence immunohistochemistry (Figure 4C). No immunoreactivity was observed in sections incubated with non-immune IgG. The number of neutrophils present in endometrial tissue was then quantified using immunohistochemistry for neutrophil elastase (Figure 4D) and was found to be 13.9±2.3 fold higher in cancer compared to sections of normal endometrium (p<0.01).

PGF_{2α}-stimulated CXCL1 induces neutrophil chemotaxis *in vitro* and *in vivo*

We next determined whether the CXCL1 expressed in FPS cells via PGF_{2α}-FP receptor interaction could induce neutrophil chemotaxis. Human neutrophils were purified from peripheral blood and used in a chemotaxis assay. We found a significant increase in neutrophil chemotaxis in response to conditioned media from FPS cells treated with 100 nM PGF_{2α} (P-CM) as compared to vehicle treated cells (V-CM) (Figure 5A). This effect was significantly inhibited with immunoneutralisation of CXCL1 prior to incubation with neutrophils or with the addition of the CXCR2 antagonist SB225002 to P-CM (p<0.001).

To explore whether FP receptor signalling could promote neutrophil migration *in vivo* we injected WT or FPS cells subcutaneously in nude mice. Mice were then regularly injected with control IgG (WT and FPS xenografts) or CXCL1 antibody (FPS xenografts). Tumours formed from FPS cells expressed significantly higher CXCL1 mRNA as compared to WT tumours (Figure 5B) and when analysed by flow cytometry, had increased neutrophil infiltration (Figure 5C, p<0.001). This infiltration was significantly decreased in FPS xenografts injected with CXCL1 neutralising antibody compared to those treated with non-immune IgG (p<0.001). This analysis was confirmed further by immunohistochemistry (Figure 5D), where increased neutrophils were seen distributed throughout FPS xenografts as compared to WT or CXCL immunoneutralised FPS xenografts.

Discussion

The link between inflammation and tumour progression has been demonstrated in a range of studies. For example, elevated expression of inflammatory COX-2 and prostaglandins has been correlated with tumour growth and angiogenesis in prostate, pancreatic and colon cancer (31-33), and the risk of long term inflammation has been demonstrated by studies showing that continued use of specific COX-2 inhibitors (NSAIDs) can significantly reduce cancer occurrence in patients at high risk (34). In the present study we demonstrate that PGF_{2α}-FP signalling can regulate expression of the inflammatory chemokine CXCL1 in endometrial adenocarcinoma cells to modulate neutrophil influx in tumours. To our knowledge, this is the first study to provide a link between inflammatory prostanoids, specifically PGF_{2α}, and neutrophil recruitment in endometrial cancers.

Prostaglandins have been demonstrated to regulate chemokine expression *in vitro* (35, 36). Prostaglandin E₂ (PGE₂) is overexpressed in many cancer types and has been shown to induce CXCL1 production in colon cancer cells which can then promote tube formation and migration of endothelial cells (14). We have previously ascertained a role for the FP receptor and PGF_{2α} signalling in regulating endometrial adenocarcinoma (8-11). In the present study, we investigated a role for the FP receptor in modulating the expression of chemokines using an *in vitro* model system of Ishikawa cells stably expressing the human FP receptor (FPS cells) (9) and a human cytokine antibody array. The array identified CXCL1 as a key cytokine induced by PGF_{2α}-FP signalling. Using FPS cells, which we have previously shown to reproduce the *ex vivo* effects of PGF_{2α} on endometrial adenocarcinoma tissue explants (9), we elaborated the signalling pathways mediating the role of FP on CXCL1 expression using chemical inhibitors and dominant negative mutants of cell signalling pathways. A key effector pathway which has been previously shown to regulate tumourigenic signalling molecules in response to GPCR signalling is the MAPK pathway. The signalling components of this pathway in FPS cells have been identified in our laboratory, where the phosphorylation of the downstream component of the MAPK pathway, ERK1/2, was demonstrated to be mediated by EGFR trans-activation and c-src phosphorylation (9). We found that chemical inhibitors of EGFR and MEK could inhibit CXCL1 production, as did co-transfection of dominant negative EGFR, Ras and MEK. However NFAT, a common regulator of cytokine expression (37), was not involved in

PGF_{2α} mediated CXCL1 production in this cell type. This data is supported by previously published evidence in colorectal adenocarcinoma cell lines where the ERK pathway was also demonstrated to be crucial in the regulation of CXCL1 expression after stimulation with PGE₂ (14).

Overexpression of CXCL1 has previously been demonstrated in a variety of tumour types, including colorectal (18) and melanoma (15), and promotes a variety of cellular functions including cell proliferation in oesophageal cancer (38) and cell invasion in bladder cancer (39). Here we demonstrated elevated expression of CXCL1 and its receptor CXCR2 in endometrial adenocarcinoma compared to normal endometrium. Expression of both was localised to glandular epithelium, stroma and vascular endothelial cells. In addition, treatment of endometrial adenocarcinoma explants with PGF_{2α} caused an increase in CXCL1 expression via FP receptor and ERK1/2 signalling pathways confirming the importance of this signalling cascade in regulating CXCL1 expression *ex vivo*. CXCR2 localisation in neutrophils in endometrial adenocarcinoma suggested that CXCL1 via CXCR2 could play a role in immune cell function. A role for CXCL1 in neutrophil influx has been previously shown in an angiogenic sponge model in the mouse, as endogenous CXCL1 expression increased immediately preceding a neutrophil influx (40).

Here we show by immunohistochemistry that neutrophils are elevated in human endometrial adenocarcinomas. We have also confirmed that CXCL1 is strongly chemotactic to neutrophils as conditioned media from PGF_{2α}-stimulated FPS cells induced chemotaxis of peripheral neutrophils. This chemotaxis was significantly reduced by CXCL1 immunoneutralisation and CXCR2 inhibition using a specific antagonist. In order to determine a role for CXCL1 induced by PGF_{2α}-FP interaction *in vivo* we inoculated nude mice with FPS and WT cells. The increased neutrophils in the resulting FPS tumours as compared to WT were significantly reduced by injection of CXCL1 neutralising antibodies, demonstrating that PGF_{2α} signalling via CXCL1 is influencing neutrophil cell infiltrate in endometrial adenocarcinomas. Neutrophil infiltration into tumours has also been demonstrated to be dependent on CXCL1 chemokine-CXCR2 signalling in a model of melanoma in a CXCR2 null nude mouse (41).

A chemokine-mediated influx of neutrophils is seen in the late secretory phase of the normal endometrium (42). Their role may be dependent on the activating agents and cytokines present, but they are thought to be involved in the breakdown and repair at menstruation by degranulation and the release of proteases which degrade the extracellular matrix (43). They may also be capable of remodelling vasculature, as neutrophils found close to or associated with endothelial microvessels express VEGF during or coincident with angiogenesis in the normal menstrual cycle (44).

The role of neutrophils in endometrial adenocarcinoma is unclear and in our study, similar to other reports (45, 46) neutrophil influx in our xenograft model did not impact on tumour size. However, considering their profound tissue-remodelling capabilities, which have been demonstrated in a number of animal models of other cancer types it is possible that they play a similar role in endometrial cancer. For example, neutrophils have been shown to uniquely produce a TIMP-free MMP-9, a key protease involved in extracellular matrix degradation which may affect the tumour microenvironment by tissue remodelling (24). In addition, the depletion of neutrophils in a mouse model was demonstrated to prevent metastasis of fibrosarcoma cells from the primary tumour (45), suggesting a role for neutrophils in the switch to a metastatic phenotype. In a nude mouse model of breast cancer, overexpression of IL-8, a chemokine related to CXCL1, caused an infiltration of neutrophils which increased invasiveness of the tumour, likely due to an increase in protease production (47). Similarly, the decrease in neutrophil infiltration caused by an inhibition of CXCL1

expression in a nude mouse model of colon cancer significantly decreased metastasis in these animals (48). Furthermore, neutrophils may also promote tumorigenesis through means other than tissue remodelling. In an *in vitro* model of colon cancer, neutrophils promote cellular stress by inducing transient errors in DNA replication in epithelial cells (49), which could ultimately lead to carcinogenesis, whilst neutrophils from ovarian cancer patients released higher levels of reactive oxygen species which could potentially lead to cellular changes which support tumour progression (50).

In conclusion, we provide evidence for a novel PGF_{2α}-FP pathway which can regulate the inflammatory microenvironment in endometrial adenocarcinoma via CXCL1 induced neutrophil chemotaxis.

Acknowledgments

We thank Anne Saunderson for patient recruitment and tissue collection, and Professor S. Howie, Dr S. Battersby, Mrs S. Wright, Ms V. Grant and Miss M. Keightley for helpful discussion and technical assistance.

This study was supported by MRC core funding to HNJ (U.1276.00.004.00002.01)

References

1. Doll A, Abal M, Rigau M, et al. Novel molecular profiles of endometrial cancer-new light through old windows. *The Journal of Steroid Biochemistry and Molecular Biology*. 2008; 108:221–9. [PubMed: 18061438]
2. Jabbour HN, Sales KJ, Smith OP, Battersby S, Boddy SC. Prostaglandin receptors are mediators of vascular function in endometrial pathologies. *Mol Cell Endocrinol*. 2006; 252:191–200. [PubMed: 16701939]
3. Cha YI, DuBois RN. NSAIDs and cancer prevention: targets downstream of COX-2. *Annu Rev Med*. 2007; 58:239–52. [PubMed: 17100552]
4. Hofer G, Bieglmayer C, Kopp B, Janisch H. Measurement of eicosanoids in menstrual fluid by the combined use of high pressure chromatography and radioimmunoassay. *Prostaglandins*. 1993; 45:413–26. [PubMed: 8321911]
5. Reid G, Wielinga P, Zelcer N, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:9244–9. [PubMed: 12835412]
6. Abramovitz M, Boie Y, Nguyen T, et al. Cloning and expression of a cDNA for the human prostanoid FP receptor. *J Biol Chem*. 1994; 269:2632–6. [PubMed: 8300593]
7. Jabbour HN, Sales KJ, Boddy SC, Anderson RA, Williams AR. A positive feedback loop that regulates cyclooxygenase-2 expression and prostaglandin F_{2α} synthesis via the F-series-prostanoid receptor and extracellular signal-regulated kinase 1/2 signaling pathway. *Endocrinology*. 2005; 146:4657–64. [PubMed: 16081631]
8. Sales KJ, Boddy SC, Williams AR, Anderson RA, Jabbour HN. F-prostanoid receptor regulation of fibroblast growth factor 2 signaling in endometrial adenocarcinoma cells. *Endocrinology*. 2007; 148:3635–44. [PubMed: 17478553]
9. Sales KJ, List T, Boddy SC, et al. A novel angiogenic role for prostaglandin F_{2α}-FP receptor interaction in human endometrial adenocarcinomas. *Cancer Res*. 2005; 65:7707–16. [PubMed: 16140938]
10. Sales KJ, Milne SA, Williams AR, Anderson RA, Jabbour HN. Expression, localization, and signaling of prostaglandin F_{2α} receptor in human endometrial adenocarcinoma: regulation of proliferation by activation of the epidermal growth factor receptor and mitogen-activated protein kinase signaling pathways. *J Clin Endocrinol Metab*. 2004; 89:986–93. [PubMed: 14764825]
11. Sales KJ, Boddy SC, Jabbour HN. F-prostanoid receptor alters adhesion, morphology and migration of endometrial adenocarcinoma cells. *Oncogene*. 2008; 27:2466–77. [PubMed: 17968320]

12. Milne SA, Jabbour HN. Prostaglandin (PG) F(2 α) receptor expression and signaling in human endometrium: role of PGF(2 α) in epithelial cell proliferation. *J Clin Endocrinol Metab.* 2003; 88:1825–32. [PubMed: 12679480]
13. Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. *Eur J Cancer.* 2006; 42:768–78. [PubMed: 16510280]
14. Wang D, Wang H, Brown J, et al. CXCL1 induced by prostaglandin E2 promotes angiogenesis in colorectal cancer. *J Exp Med.* 2006; 203:941–51. [PubMed: 16567391]
15. Haghnegahdar H, Du J, Wang D, et al. The tumorigenic and angiogenic effects of MGSA/GRO proteins in melanoma. *J Leukoc Biol.* 2000; 67:53–62. [PubMed: 10647998]
16. Wang D, Yang W, Du J, et al. MGSA/GRO-mediated melanocyte transformation involves induction of Ras expression. *Oncogene.* 2000; 19:4647–59. [PubMed: 11030154]
17. Li A, Varney ML, Singh RK. Constitutive expression of growth regulated oncogene (GRO) in human colon carcinoma cells with different metastatic potential and its role in regulating their metastatic phenotype. *Clin Exp Metastasis.* 2004; 21:571–9. [PubMed: 15787094]
18. Wen Y, Giardina SF, Hamming D, et al. GRO α is highly expressed in adenocarcinoma of the colon and down-regulates fibulin-1. *Clin Cancer Res.* 2006; 12:5951–9. [PubMed: 17062666]
19. Moore BB, Arenberg DA, Stoy K, et al. Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. *Am J Pathol.* 1999; 154:1503–12. [PubMed: 10329603]
20. Addison CL, Daniel TO, Burdick MD, et al. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol.* 2000; 165:5269–77. [PubMed: 11046061]
21. Moser B, Clark-Lewis I, Zwahlen R, Baggiolini M. Neutrophil-activating properties of the melanoma growth-stimulatory activity. *J Exp Med.* 1990; 171:1797–802. [PubMed: 2185333]
22. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell.* 2000; 103:481–90. [PubMed: 11081634]
23. Scapini P, Morini M, Tecchio C, et al. CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. *J Immunol.* 2004; 172:5034–40. [PubMed: 15067085]
24. Ardi VC, Kupriyanova TA, Deryugina EI, Quigley JP. Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America.* 2007; 104:20262–7. [PubMed: 18077379]
25. Sales KJ, Grant V, Jabbour HN. Prostaglandin E2 and F2 α activate the FP receptor and up-regulate cyclooxygenase-2 expression via the cyclic AMP response element. *Mol Cell Endocrinol.* 2008; 285:51–61. [PubMed: 18316157]
26. Nirodi C, NagDas S, Gygi SP, Olson G, Aebersold R, Richmond A. A role for poly(ADP-ribose) polymerase in the transcriptional regulation of the melanoma growth stimulatory activity (CXCL1) gene expression. *J Biol Chem.* 2001; 276:9366–74. [PubMed: 11112786]
27. Levi NL, Hanoch T, Benard O, et al. Stimulation of Jun N-terminal kinase (JNK) by gonadotropin-releasing hormone in pituitary alpha T3-1 cell line is mediated by protein kinase C, c-Src, and CDC42. *Molecular Endocrinology.* 1998; 12:815–24. [PubMed: 9626657]
28. Harris D, Bonfil D, Chuderland D, Kraus S, Seger R, Naor Z. Activation of MAPK cascades by GnRH: ERK and Jun N-terminal kinase are involved in basal and GnRH-stimulated activity of the glycoprotein hormone LH β -subunit promoter. *Endocrinology.* 2002; 143:1018–25. [PubMed: 11861527]
29. Lan HY, Mu W, Nikolic-Paterson DJ, Atkins RC. A novel, simple, reliable, and sensitive method for multiple immunoenzyme staining: use of microwave oven heating to block antibody crossreactivity and retrieve antigens. *J Histochem Cytochem.* 1995; 43:97–102. [PubMed: 7822770]
30. Ward C, Chilvers ER, Lawson MF, et al. NF-kappaB activation is a critical regulator of human granulocyte apoptosis in vitro. *J Biol Chem.* 1999; 274:4309–18. [PubMed: 9933632]
31. Molina MA, Sitja-Arnau M, Lemoine MG, Frazier ML, Sinicrope FA. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. *Cancer Res.* 1999; 59:4356–62. [PubMed: 10485483]

32. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell*. 1998; 93:705–16. [PubMed: 9630216]
33. Jain S, Chakraborty G, Raja R, Kale S, Kundu GC. Prostaglandin E2 regulates tumor angiogenesis in prostate cancer. *Cancer Res*. 2008; 68:7750–9. [PubMed: 18829529]
34. Dannenberg AJ, Subbaramaiah K. Targeting cyclooxygenase-2 in human neoplasia: rationale and promise. *Cancer Cell*. 2003; 4:431–6. [PubMed: 14706335]
35. Pold M, Zhu LX, Sharma S, et al. Cyclooxygenase-2-dependent expression of angiogenic CXC chemokines ENA-78/CXC Ligand (CXCL) 5 and interleukin-8/CXCL8 in human non-small cell lung cancer. *Cancer Res*. 2004; 64:1853–60. [PubMed: 14996749]
36. Takehara H, Iwamoto J, Mizokami Y, et al. Involvement of cyclooxygenase-2--prostaglandin E2 pathway in interleukin-8 production in gastric cancer cells. *Dig Dis Sci*. 2006; 51:2188–97. [PubMed: 17078003]
37. Boyd JH, Divangahi M, Yahiaoui L, Gvozdic D, Qureshi S, Petrof BJ. Toll-like receptors differentially regulate CC and CXC chemokines in skeletal muscle via NF-kappaB and calcineurin. *Infect Immun*. 2006; 74:6829–38. [PubMed: 16982839]
38. Wang B, Hendricks DT, Wamunyokoli F, Parker MI. A growth-related oncogene/CXC chemokine receptor 2 autocrine loop contributes to cellular proliferation in esophageal cancer. *Cancer Res*. 2006; 66:3071–7. [PubMed: 16540656]
39. Kawanishi H, Matsui Y, Ito M, et al. Secreted CXCL1 is a potential mediator and marker of the tumor invasion of bladder cancer. *Clin Cancer Res*. 2008; 14:2579–87. [PubMed: 18451219]
40. Barcelos LS, Talvani A, Teixeira AS, Cassali GD, Andrade SP, Teixeira MM. Production and in vivo effects of chemokines CXCL1-3/KC and CCL2/JE in a model of inflammatory angiogenesis in mice. *Inflamm Res*. 2004; 53:576–84. [PubMed: 15597153]
41. Singh S, Varney M, Singh RK. Host CXCR2-dependent regulation of melanoma growth, angiogenesis, and experimental lung metastasis. *Cancer Res*. 2009; 69:411–5. [PubMed: 19147552]
42. Critchley HO, Kelly RW, Kooy J. Perivascular location of a chemokine interleukin-8 in human endometrium: a preliminary report. *Hum Reprod*. 1994; 9:1406–9. [PubMed: 7989497]
43. Kaitu'u-Lino TJ, Morison NB, Salamonsen LA. Neutrophil depletion retards endometrial repair in a mouse model. *Cell Tissue Res*. 2007; 328:197–206. [PubMed: 17186309]
44. Gargett CE, Lederman F, Heryanto B, Gambino LS, Rogers PA. Focal vascular endothelial growth factor correlates with angiogenesis in human endometrium. Role of intravascular neutrophils. *Hum Reprod*. 2001; 16:1065–75. [PubMed: 11387270]
45. Tazawa H, Okada F, Kobayashi T, et al. Infiltration of neutrophils is required for acquisition of metastatic phenotype of benign murine fibrosarcoma cells: implication of inflammation-associated carcinogenesis and tumor progression. *Am J Pathol*. 2003; 163:2221–32. [PubMed: 14633597]
46. Shojaei F, Singh M, Thompson JD, Ferrara N. Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:2640–5. [PubMed: 18268320]
47. Yao C, Lin Y, Chua MS, et al. Interleukin-8 modulates growth and invasiveness of estrogen receptor-negative breast cancer cells. *International Journal of Cancer*. 2007; 121:1949–57.
48. Yamamoto M, Kikuchi H, Ohta M, et al. TSU68 prevents liver metastasis of colon cancer xenografts by modulating the premetastatic niche. *Cancer Res*. 2008; 68:9754–62. [PubMed: 19047154]
49. Campregher C, Luciani MG, Gasche C. Activated neutrophils induce an hMSH2-dependent G2/M checkpoint arrest and replication errors at a (CA)13-repeat in colon epithelial cells. *Gut*. 2008; 57:780–7. [PubMed: 18272544]
50. Klink M, Jastrzemska K, Nowak M, et al. Ovarian cancer cells modulate human blood neutrophils response to activation in vitro. *Scandinavian Journal of Immunology*. 2008; 68:328–36. [PubMed: 18565119]

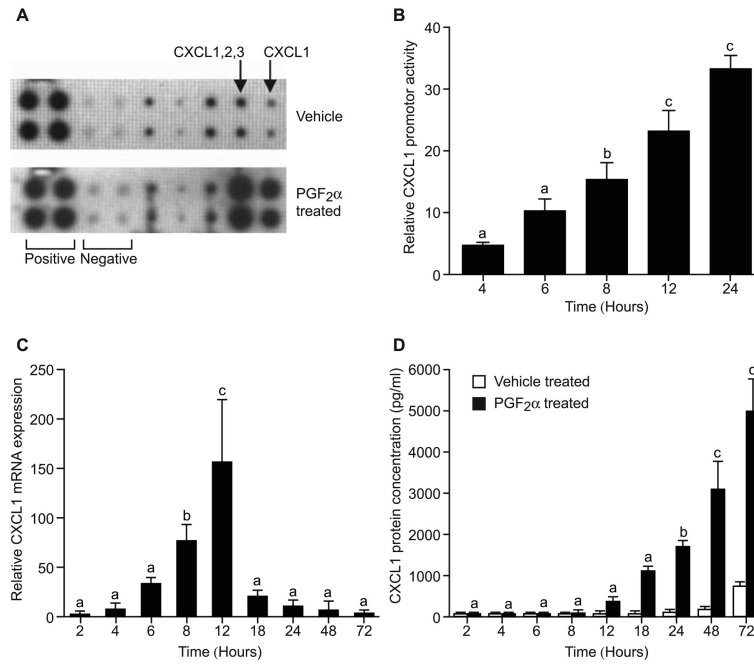
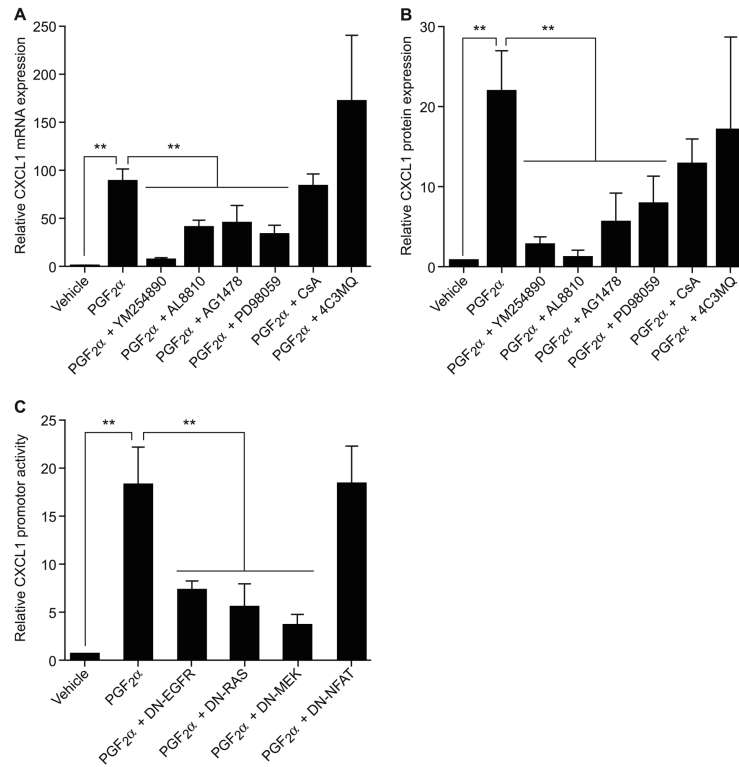


Figure 1. PGF₂α regulates CXCL1 expression in FPS cells. *A:* A human cytokine antibody array was used to determine differences in protein production in the conditioned media of vehicle treated and PGF₂α treated cells. *B:* CXCL1 promoter activity was increased in FPS cells stimulated with 100 nM PGF₂α as compared to vehicle control over a period of 4 to 24 hours. Data are expressed as fold increase in luciferase activity as compared to vehicle treated cells. *C:* FPS cells stimulated with 100 nM PGF₂α show a time dependent increase in CXCL1 mRNA expression as measured by quantitative RT-PCR. Data are expressed as fold over vehicle treated cells. *D:* CXCL1 protein secreted into media was measured by ELISA and was increased after treatment with PGF₂α up to 72 hours. (Columns: mean of at least 3 independent experiments. P<0.05: b is significantly different to a, P<0.01: c is significantly different from a and b)

**Figure 2.**

CXCL1 production in FPS cells is mediated by EGFR, Ras and MEK. **A:** CXCL1 mRNA production, measured by quantitative RT-PCR, was increased after 8 hours PGF_{2α} treatment. This was decreased by co-treatment with YM254890, AL8810, AG1478, and PD98059. No reduction was seen after co-treatment with CsA or 4C3MQ. **B:** CXCL1 protein secretion after 24 hours PGF_{2α} treatment was reduced after co-treatment with YM254890, AL8810, AG1478, and PD98059. No reduction was seen after co-treatment with CsA or 4C3MQ. **C:** CXCL1 promoter activity was measured in cells treated with PGF_{2α} alone or with DN-EGFR, DN-Ras, DN-MEK or DN-NFAT. Significant reduction in activity was seen after 8 hours with all constructs except DN-NFAT. (Columns: mean of at least 3 independent experiments, **p<0.01)

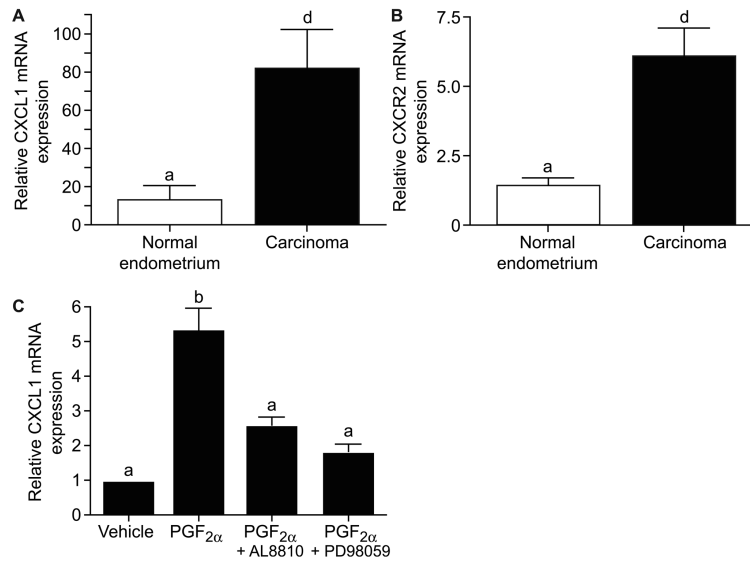


Figure 3.

CXCL1 and CXCR2 expression is increased in endometrial adenocarcinoma. *A*: CXCL1 and *B*: CXCR2 mRNA expression is significantly increased in endometrial adenocarcinoma tissue ($n = 58$) as compared to normal endometrium ($n = 45$). *C*: CXCL1 mRNA is increased in endometrial adenocarcinoma explants ($n=4$) after treatment with PGF_{2α}. Co-treatment with AL8810 or PD98059 significantly decreases this effect. ($P < 0.05$; b is significantly different to a, $P < 0.001$; d is significantly different from a and b)

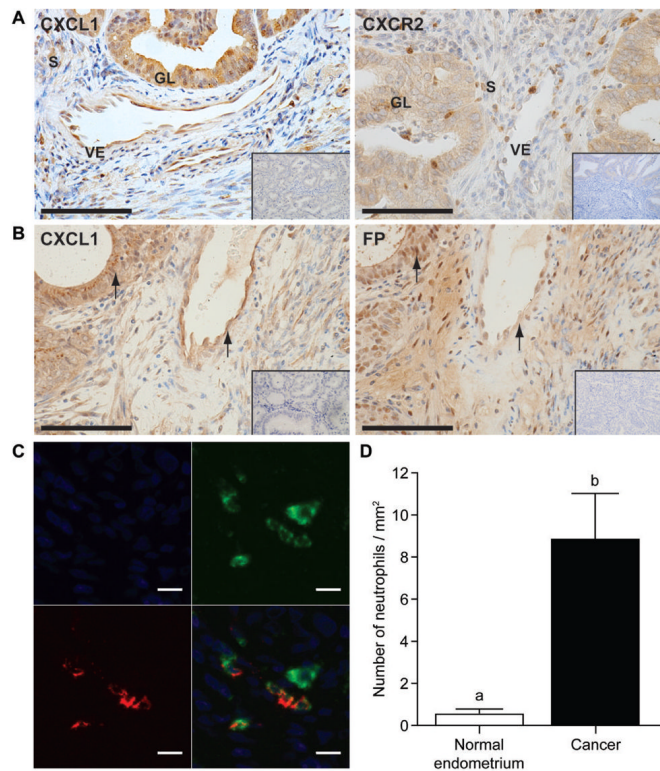


Figure 4.

CXCL1 and CXCR2 are expressed in endometrial adenocarcinoma and colocalise with FP receptor expression and neutrophil elastase respectively. *A*: Immunohistochemical staining of CXCL1 and CXCR2 in moderately and poorly differentiated endometrial adenocarcinoma. GL – glandular, VE = vascular endothelial, S = stromal. *B*: CXCL1 can be colocalised to the same cells as FP receptor (shown by arrowheads) in well differentiated adenocarcinoma by serial sectioning. *C*: Co-localisation of the site of expression of CXCR2 (green) and neutrophil elastase (red). Negative controls are inset. *D*: Carcinoma sections (n=30) and normal proliferative endometrium (n=7) were analysed for expression of neutrophil elastase. Increased expression was seen in cancer (b is significantly different to a; $P < 0.01$). Scale bars in A and B represent 100 μm , in C represent 10 μm .

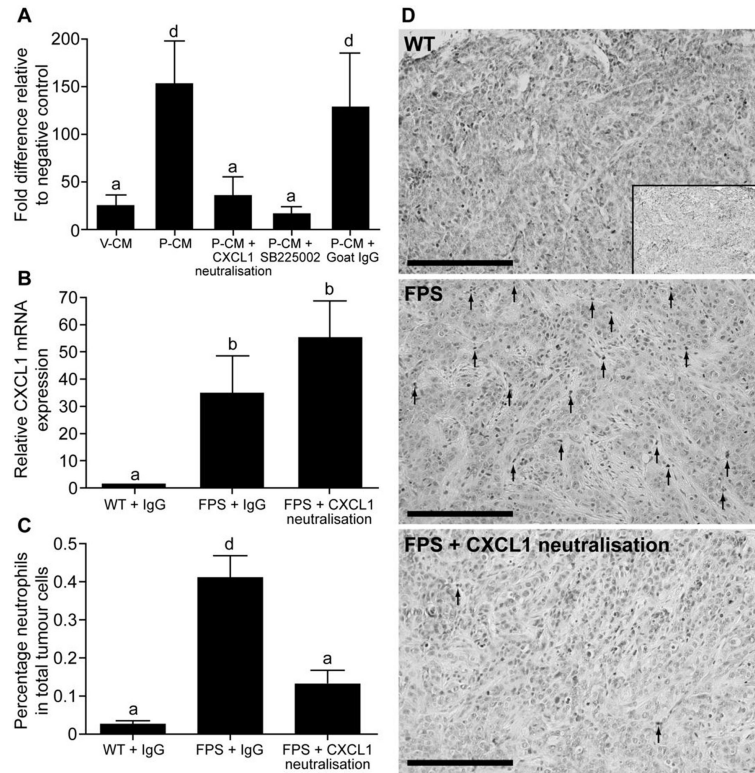


Figure 5.

Neutrophils migrate in response to $\text{PGF}_{2\alpha}$ induced CXCL1 *in vitro* and *in vivo*. **A:** Neutrophil chemotaxis was increased in response to conditioned media from FPS cells treated with $\text{PGF}_{2\alpha}$ for 48 hours (P-CM) as compared to vehicle treated cells (V-CM). CXCL1 immunoneutralisation and the addition of 60 nM SB-225002 significantly decreased chemotaxis. Control goat IgG immunoneutralisation showed no significant effect. Data are expressed as fold over negative control (serum free medium). **B:** CXCL1 mRNA expression is significantly increased in nude mice xenografts formed from FPS cells as compared to WT cell xenografts. Injection of CXCL antibody into xenografts made no significant difference to CXCL1 mRNA expression. **C:** Percentage of neutrophils in tumours from WT and FPS xenografts (n=5 per group), measured by flow cytometry. Increased neutrophil infiltration is seen in FPS xenografts, which is significantly reduced by injection of CXCL1-neutralising antibody. **D:** Localisation of neutrophils in xenografts by Gr-1 staining. Neutrophils are highlighted by arrowheads, scale bars indicate 100 μm (p<0.05: b is significantly different to a, P<0.01: c is significantly different from a and b, P<0.001: d is significantly different from a, b, and c.)