Evidence for the antagonistic form of CXC-motif chemokine CXCL10 in serous epithelial ovarian tumours

Adam Rainczukt*, Jyothsna R. Rao†, Jessica L. Gathercole1, Nicole J. Fainweather1, Simon Chu1, Rina Masadah2, Thomas W. Jobling3, Santanu Deb-Choudhury4, Jolom Dyer5 and Andrew N. Stephens5

1 Ovarian Cancer Biomarker Laboratory, Prince Henry’s Institute of Medical Research, Clayton, VIC, Australia
2 Department of Anatomical Pathology, Hasanudin University, Makassar, Indonesia
3 Department of Obstetrics and Gynaecology, Monash Medical Centre, Clayton, VIC, Australia
4 AgResearch Ltd, Lincoln Research Centre, Christchurch, New Zealand

Patients with high-grade, serous epithelial ovarian carcinoma (HGSOC) are generally diagnosed with extensive peritoneal metastases, and exhibit 5-year survival rates <30%. A subset of these tumours, defined as “immunoreactive,” overexpress mRNA encoding the T-cell-recruiting chemokine CXCL10 (10-kDa interferon gamma-induced protein; C-X-C motif chemokine 10). Tumour-infiltrating CD4+CD8+ T-cells are a well-documented, positive prognostic indicator for HGSOC patients; paradoxically, however, patients diagnosed with HGSOC (overexpressing CXCL10 and therefore theorised to recruit T-cells) typically exhibit poor survival. Recently, an “antagonistic” CXCL10 variant was identified that inhibited leucocyte recruitment to inflamed liver in vivo (Casrouge et al., J Clin Invest 2011;121:308–17). We hypothesised that “immunoreactive” HGSOC might also express antagonistic CXCL10, interfering with leucocyte recruitment and contributing to poor patient prognosis. CXCL10 expression was increased in HGSOC tissues grouped according to pathology, grade and FIGO stage at diagnosis, and its localisation and association with T-cells established by immunohistochemical staining in tissue microarrays. CXCL10 expression was increased in a subset of serous epithelial tumour samples; however, it did not correlate well with CD4+ positive tumour infiltrate. Immunoprecipitation and de novo sequence analysis of CXCL10 identified the N terminally cleaved, “antagonistic” variant of CXCL10 in malignant tumours, and not in benign ovarian disease. The data demonstrate the presence of the antagonistic form of CXCL10 in HGSOC for the first time, and provide a partial explanation for reduced leucocyte infiltration observed in these tumours. We suggest that CXCL10 cleavage and subsequent antagonism of immune cell recruitment may be a feature of the “immunoreactive” HGSOC subtype, leading to early impairment of the immune response and subsequently worsening patient prognosis.

Epithelial ovarian cancers account for between 80 and 95% of ovarian tumours diagnosed, and remain the fourth highest cause of cancer-related mortality for women with an estimated ~22,000 new cases and ~14,000 deaths in the United States in 2010.1 Despite a relatively low population incidence and incremental improvements in surgery and chemotherapy, the 5-year survival rate for patients diagnosed with ovarian neoplasms has improved only marginally over time. Although traditionally classified as serous, mucinous, clear cell or endometroid based on their histological appearance, epithelial ovarian tumours are increasingly considered as different disease subtypes with characteristic molecular genetic alterations (reviewed in Ref. 2). In particular, high- or low-grade serous epithelial tumours are now believed to represent distinct molecular diseases (termed Type I or Type II) with different underlying pathologies. Low-grade (Type I) tumours are believed to progress through benign and borderline stages to malignancy, typically exhibit mutations in KRAS and BRAF (amongst others) and are generally diagnosed at an early stage. By contrast, high-grade (Type II) serous tumours have no well-defined driver point mutations, characteristically overexpress TP53 and metastasise early in their progression. Their aetiology is also unclear, with several hypotheses regarding the location of origin and precursor cell types involved.3

The importance of the host immune response in the development and progression of ovarian tumours has now been firmly established; indeed, as for other cancer types the
presence and type of tumour-infiltrating lymphocytes in ovarian tumours is directly correlated with prognosis. In this context, chemokines play a major role via the recruitment of activated T-lymphocytes and promotion of a Th1-type immune response. The chemokine CXCL10 (10-kDa interferon gamma-induced protein; C-X-C motif chemokine 10) is one of three CXC-type chemokines (CXCL9 or MIG; CXCL10 or IP10 and CXCL11 or ITAC) that bind and signal via their G-coupled protein receptor CXCR3. Expressed in diverse cell types, CXCL10 is a potent chemottractant that recruits activated CD4+ and CD8+ lymphocytes, NK and NKT cells into sites of inflammation (reviewed in Ref. 7). CXCL10 also displays antiproliferative effects on endothelial cells in vitro, and angiostatic and antitumour effects in vivo, and is active against epithelial cells, fibroblasts and other cell and tissue types. These antiproliferative effects may be mediated independently of the main CXCR3 receptor isoform (CXCR3-A) via binding to cell-surface glycosaminoglycans or possibly through one of two other known CXCR3 receptor isoforms (CXCR3-B).

Post-translational processing is an important regulator of CXCL10 function. In particular, the cleavage of a Val-Pro dipeptide from the N-terminus of CXCL10, catalysed by dipeptidyl peptidases (DPPase) DPP4, DPP8 or DPP9, converts it into a powerful antagonist of chemotaxis that retains affinity for the CXCR3 receptor but fails to induce internalisation or receptor-mediated signalling. The importance of this modification in vivo was recently demonstrated by Casrouge et al., who identified an increased abundance of antagonistic, cleaved CXCL10 in circulation in a cohort of patients infected with hepatitis C virus. These patients displayed impaired recruitment of circulating mononuclear cells to the liver, leading to the failure of immune response and an inability to clear the infection after treatment. This in situ generation of antagonistic CXCL10 helped to explain a long-established negative correlation between elevated CXCL10 levels and poor response to therapy in these patients.

Several lines of evidence have suggested key roles for CXCL10 in the pathogenesis of ovarian tumours. Cell lines derived from epithelial ovarian tumours secrete CXCL10 when stimulated with proinflammatory signals including vascular endothelial growth factor (VEGF), prolactin, interferon gamma (IFNγ) or activated NF-κB, all of which have been implicated in ovarian tumour progression. Accordingly, the in vivo secretion of CXCL10 and subsequent recruitment of activated T-lymphocytes into tumour tissue have been associated with improved clinical outcome. However, other studies have suggested that increased expression of CXCL10 does not promote effective tumour suppression, but leads to enhanced lymph node metastasis. Decreased plasma CXCL10 levels in ovarian cancer patients have also been positively correlated with progression-free and overall survival. Although CXCL10 clearly plays an important role in the progression and pathogenesis of ovarian cancers, the relationship between its expression and tumour progression remains undetermined.

The conflict between CXCL10 expression and patient prognosis prompted us to investigate whether cleavage of CXCL10 might occur in ovarian tumours. In this pilot study, we report on the expression of CXCL10 in a cohort of women diagnosed with serous epithelial ovarian tumours, using a combination of tissue expression, immunohistochemical analyses. Immunoprecipitation and mass spectrometry were subsequently used to sequence the N-terminus of CXCL10 and establish the presence of cleaved CXCL10 in ovarian tumours.

Material and Methods
Clinical samples
Protein and mRNA expression studies were carried out using matched samples of tissue, EDTA-chelated plasma and urine, accessed from bio-banked samples collected prospectively from women undergoing surgery for suspected gynaecological malignancies during the period 2007-2010. All samples were obtained from anaesthetised patients who had undergone no prior surgical treatment or neoadjuvant chemotherapy for their tumours. All relevant clinical information, including a thorough histological assessment of tumour type, stage and grade by qualified gynaecological oncologists, CA125 measurement before surgery, age, self-reported menopausal status, pre-existing conditions or medications and any prior history of malignancy were obtained from patient medical records (first deidentified to maintain patient anonymity). Patient details immediately relevant to this study are provided in Table 1. Measurement of serum CA125 and total urine creatinine was performed in the diagnostic pathology laboratory at the Monash Medical Centre, Melbourne, Australia. Ethical approval was obtained from the Southern Health Human Research Ethics Committee (HREC.
Table 1. Summary of patient samples used for mRNA and protein expression studies

<table>
<thead>
<tr>
<th>Group</th>
<th>Pathology</th>
<th>Grade</th>
<th>Stage</th>
<th>Median Age (IQR)</th>
<th>Median CA125 (IQR)</th>
<th>Menopausal status</th>
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<tbody>
<tr>
<td>Control (n = 12)</td>
<td>Serous cystadenoma (n = 4)</td>
<td>n/a</td>
<td>n/a</td>
<td>53 (46–64)</td>
<td>67 (55, 101)</td>
<td>Post</td>
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<tr>
<td></td>
<td>Serous cystadenofibroma (n = 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-ovarian benign (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borderline / low grade (n = 14)</td>
<td>Serous borderline (n = 10)</td>
<td>n/a</td>
<td>n/a</td>
<td>47 (42, 70)</td>
<td>96 (76, 125)</td>
<td>Mixed</td>
</tr>
<tr>
<td></td>
<td>Serous carcinoma (n = 4)</td>
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<td>lc</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Early (n = 5)</td>
<td>Serous adenocarcinoma</td>
<td>3</td>
<td>lb-c</td>
<td>56 (54–57)</td>
<td>111 (48,289)</td>
<td>Post</td>
</tr>
<tr>
<td>Late (n = 28)</td>
<td>Serous adenocarcinoma</td>
<td>3</td>
<td>lllb-c</td>
<td>65 (57,75)</td>
<td>740 (397,2500)</td>
<td>Post</td>
</tr>
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</table>

CXCL10 immunoassay in plasma and urine

Immunassays were performed using a custom MilliPlex™ cytokine assay (MilliPlex MAP Human Cytokine; Millipore, Melbourne, VIC, Australia), as directed by the manufacturer. Individual matched samples of urine and plasma from each patient were assayed in duplicate. Before assay, plasma samples were diluted 1:50 with assay buffer; urine was similarly diluted 1:5. In brief, standards and samples were incubated in the presence of antibody-coupled beads overnight (16–18 hr) at 4°C with mild agitation. Incubation with detection antibodies was performed for 1 hr at room temperature (RT) with mild agitation. Fluorescent intensity measurements were acquired using a Bioplex200 instrument, and concentrations were determined using BioManager analysis software (BioRad, Hercules CA). Urine concentrations of CXCL10 were further normalised against total urine creatinine for each patient. The minimum detectable CXCL10 concentration, as determined by the manufacturer, was 2.2 pg/ml (mean minimum detectable concentration ± 2 standard deviations). Intra-assay variation, as assessed by %CV in median fluorescent intensity across duplicate measurements and standards, was routinely less than 5%.

RNA extraction and analysis by real-time RT-PCR

Total RNA was isolated from tumour lysates using an RNaseasy Mini kit (Qiagen, Melbourne Australia) according to the manufacturer’s instructions. RNA quality was assessed by gel electrophoresis and UV absorbance 260/280 ratios (acceptable range 1.8–2.0). A total of 300 ng RNA was reverse transcribed using SuperScript III (Invitrogen, Melbourne Australia). Quantitative RT-PCR was performed using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Melbourne Australia) with primers for CXCL10 (primer ID: Hs01124251_g1) and human 18S Ribosomal RNA (primer ID: Hs03928985_g1). PCR was carried out as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec and annealing at 60°C for 1 min. The rRNA probe was labelled with VIC™ dye and the probe for CXCL10 was labelled with 6-carboxyfluorescein (FAM). All qPCR was carried out in triplicate. Data values were extracted with the SDS v2.0 software tool (Applied Biosystems), and expression levels were determined using the ΔΔCt (Cycle Threshold) method, with 18S ribosomal RNA as the calibrator.

Preparation of tissue lysates for SDS-PAGE

Tissues used for SDS-PAGE and Western blotting experiments were treated as follows. A 50 mg portion of snap-frozen tumour tissue was washed three times in ice-cold 20 mM Tris (pH 7.4) to remove any visible blood. Each tissue was then homogenised in 400 ml lysis buffer (7 M urea, 20 mM Tris, 2 mM MgCl2, pH 9.0 with HCl) containing 2X EDTA-free protease inhibitor cocktail (Roche, Castle Hill, Australia) and 500 U/ml of benzonase endonuclease (Sigma, Sydney Australia). The homogenate was incubated for 15 min on ice and then diluted with 400 ml of 1 M triethylammonium bicarbonate (TEAB) (Sigma). The solution was vortexed and incubated for a further 15 min on ice, and insoluble material was pelleted by centrifugation at 21,000g for 10 min at 4°C (Eppendorf 5415 rotor). The pellet was rehomogenised in a further 100 ml of 1 M TEAB, and after incubation and centrifugation as above the supernatants were pooled. Samples were then reduced with 5 mM Tris(2-carboxyethyl)phosphine (Sigma) for 1 hr at 60°C and alkylated using 18 mM iodoacetamide (Sigma) for 30 min in the dark. Proteins were then precipitated in 2 vol of acetone as previously described. The precipitated proteins were resuspended in a small volume of urea buffer (7 M urea, 2 M thiourea and 1% C7BzO wt/vol) and assayed according to the method of Bradford. All samples were adjusted to 10 mg/ml in urea buffer, snap-frozen and stored at −80°C before use.

SDS-PAGE and Western blotting

Duplicate protein aliquots for each sample were diluted to 20 ml in 2X LDS sample buffer (Invitrogen, Carlsbad, CA). SDS-PAGE was carried out in a 4–20% criterion polyacrylamide gel (BioRad) at 250 V for 30 min. To avoid any potential for bias during analysis, each sample was assigned a random number from 1 to 1,000 before separation and

loaded in order of random number without any knowledge of sample identity.

Separated proteins were transferred to PVDF membrane using a TransBlot Turbo semidyly transfer device (BioRad). Membranes were dried at 37°C for 1 hr. After re-wetting in 100% methanol, membranes were blocked for 45 min in 5% skim milk powder +0.2% Tween 20 in PBS, and incubated overnight in Tween 20/PBS at 4°C with primary chicken anti-sera against CXCL10 (diluted 1:2,500, AbCam #ab117319) or rabbit anti-sera against β-actin (diluted 1:100,000, Abcam #ab8227). Recombinant CXCL10 (Abcam #ab9810) was used as a control. Secondary antibody was goat anti-rabbit IgG (diluted 1:100,000, Abcam #ab97051) or rabbit anti-chicken IgG (diluted 1:50,000, Abcam #ab97140) conjugated to horseradish peroxidase. Immunostaining was detected using Clarity™ ECL Western Substrate (BioRad) and imaged using a Chemidoc MP (BioRad). Quantitative image analysis was performed using ImageLab 4.1 software (build 16, 2012) using the default settings, and the ratio of CXCL10:β-actin was calculated for every sample (blinded to their identities). Samples were then re-identified and grouped for subsequent statistical analysis.

**Immunohistochemistry**

Immunohistochemical staining of formalin-fixed paraffin sections was carried out using commercially available tissue micro-array slides (US Biomax, Rockville, MD). Arrays contained duplicate 1-mm diameter cores (5-mm thickness) from benign (n = 30) or malignant (n = 94) ovarian tumours, normal cancer-adjacent (n = 5) or histologically normal (n = 5) tissues (see Table 2 and Supporting Information Table S1). Slides were deparaffinised in Histolene (Sigma) and descending grades of ethanol before antigen retrieval. Antigen retrieval was performed as described20 using 50 mM glycine, pH 3.5. Slides were blocked in 10% goat serum, 10% CAS block (Invitrogen) in 5% bovine serum albumin/Tris-buffered saline before overnight incubation with primary antibody against CXCL10 (Abcam ab9807, diluted 1:50), CD45 (Epitomics t69l-1, diluted 1:100), CD45 (Dako, Campbellfield Australia M7254, diluted 1:50) or DPP4 (Abcam ab114033, diluted 1:800). Secondary antibody was biotinylated goat anti-rabbit (Vector Laboratories, Burlingame CA) or anti-mouse (Dako E0433) as appropriate. Slides were incubated with secondary antibody (diluted 1:200) for 30 min, followed by the Vectastain Elite ABC kit according to the manufacturer’s instructions (Vector Laboratories). Antibody binding was detected as a brown precipitate after development with 3,3’-diaminobenzidine tetrahydrochloride (DakoCytomation) and counterstained using Mayer’s haematoxylin (Sigma). Negative controls were performed for all slides using secondary antibody alone, and an immunising CXCL10 peptide used at between 2- and 10-fold excess was employed in blocking experiments (see Supporting Information S2). Slides were dehydrated through ascending grades of ethanol and histology, and mounted with coverslips using dibutyl phthalate xylene (DEPX, BDH; Merck, Melbourne Australia). Slides were imaged using an Aperio ScanScope CS and XT system (Aperio Technologies, Vista CA) at 20× magnification. Representative tumour margins were marked by a qualified pathologist, and serial sections were analysed for staining intensity using Aperio ImageScope (version 11.2.0.780). Staining intensity was scored by the software on a scale of 0 to +3.

**Immunoprecipitation of CXCL10 from tissue lysates**

Anti-CXCL10 antibodies (ab9807) were conjugated to protein A/G agarose at a concentration of 10 mg per 50 ml of protein A/G slurry, using a Pierce Crosslink IP kit (Thermo Scientific, Rockford, IL) as described by the manufacturer. A 20- mg protein aliquot from each tumour lysate was pooled to form four groups (benign, low-grade, high-grade early stage or high-grade late stage). A total of 50 mg protein from each sample pool was diluted 1:10 with lysis/wash buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol, pH 7.4) and pre-clear for 2 hr at room temp by incubation with protein A/G slurry. The supernatant was then harvested and incubated with antibody-conjugated beads overnight at 4°C. Captured antigens were eluted in 0.1 M glycine (pH 2.6) and adjusted to 1 M urea, 100 mM Tris (pH 8.5). A 1/5 aliquot of each sample was assessed by Western

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Table 2. Summary of tissue microarray parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total # patients</td>
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<td></td>
</tr>
<tr>
<td>Age</td>
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<td></td>
</tr>
<tr>
<td>Median</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>IQ range</td>
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</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>46</td>
<td>34</td>
</tr>
<tr>
<td>II</td>
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<td>III</td>
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<td>20</td>
</tr>
<tr>
<td>IV</td>
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<td>4</td>
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<tr>
<td>Unstaged</td>
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<td>1</td>
</tr>
<tr>
<td>Histological Subtype</td>
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</tr>
<tr>
<td>Serous</td>
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<td>50</td>
</tr>
<tr>
<td>Mucinous</td>
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<td>8</td>
</tr>
<tr>
<td>Endometroid</td>
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<td>8</td>
</tr>
<tr>
<td>Clear cell</td>
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<td>3</td>
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<tr>
<td>Benign</td>
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<tr>
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<td>4</td>
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<td>Normal-tumour-adjacent</td>
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<td>4</td>
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<tr>
<td>Grade</td>
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<td>27</td>
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<td>3</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>Ungraded or mixed</td>
<td>13</td>
<td>10</td>
</tr>
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</table>

Full details may be found in Supporting Information S1 and S2.
blotting, whereas the remaining sample was digested with 100 ng of recombinant endoproteinase GluC (Promega, Madison, WI) at 25°C overnight. Peptides were desalted using C18 TopTips (Glygen, Columbia, MD), dried in a vacuum centrifuge and resuspended in 0.05% heptfluoroisobutyric acid (Thermo Fisher Scientific, Melbourne, Australia) for analysis by LC-MS/MS.

**LC-MS/MS**

Desalted peptides were injected into a Dionex Ultimate™ 3000 RSLCnano system (Dionex, Sunnyvale, CA) and loaded onto a Pepswift Monolithic Trap Column (200 nm i.d. x 5 mm) (Dionex) at a flow rate of 10 nl/min. After 7 min the trap column was switched in line with a Pepswift Monolithic Capillary Column (200 nm i.d. x 5 cm) (Dionex) at a flow rate of 1.5 nl/min using mobile phase A (0.05% heptfluoroisobutyric acid in water). Separation was performed in a linear 4–70% gradient of mobile phase B (50% H2O/50% acetonitrile/0.04% trifluoroacetic acid, vol/vol). Peptides were coeluted with HCCA matrix (Bruker Daltonics, Melbourne, Australia) solution at a flow rate of 2 nl/min and spotted directly to 384 spot 800 nm AnchorChip MALDI target plates (Bruker Daltonics), using Proteineer FC II robotic fraction collector (Bruker Daltonics) and allowed to dry.

Mass spectra were acquired using an Ultraflexextreme MALDI-TOF (Bruker Daltonics). WARP-LC 1.2 software (Bruker Daltonics) was used to automate the acquisition of parent ions and selection of up to 20 peptides per target spot for subsequent MS/MS analysis. External calibration was performed automatically on Peptide Calibration Standard II (Bruker Daltonics) spotted onto Anchorchip calibration spots. Spectra were automatically processed in FlexAnalysis 3.3 (Bruker Daltonics). Further processing and identification were performed using ProteinScape 2.1 (Bruker Daltonics). Peptides were searched against the SwissProt database (May16 2012; 536,029 entries) using an in-house version of MASCOT 2.3.01 (Matrix Science, London, UK) with the following parameter settings: no_enzyme (for CXCL10 N-terminal detection) or Glu-C, 1 missed cleavage, Homo sapiens as organism, carbamidomethylation (C) as a fixed modification, oxidation as a variable modification, 80 ppm MS1 tolerance, 1.5 Da MS/MS tolerance, minimum Mascot protein score >30 and peptide score >20. All MS/MS data of CXCL10 peptides and modifications were then also manually evaluated using Biologics 3.2 (Bruker Daltonics).

**MALDI imaging mass spectrometry**

Fresh-frozen tissue sections (12-μm thick) were prepared on indium-tin oxide-coated slides, fixed in 100% ethanol and overlaid with sinnapsin acid matrix solution (10 g/l in 60% acetonitrile and 0.2% trifluoroacetic acid) using a Bruker ImagePrep device (Bruker Daltonics) as recommended by the manufacturer. MALDI images were acquired in the mass/charge range 2,000–20,000 using a Bruker Ultraflex III TOF/TOF (Bruker Daltonics) in linear positive ion mode, with a sampling rate of 0.1 Gs/sec. Acquisition was performed using a Smartbeam laser with a repetition rate of 200 Hz. A spatial resolution of 100 μm was used and each MS spectrum was obtained by accumulation of 400 consecutive laser shots. Images were reconstructed using fleximaging v2.1 (Bruker Daltonics) and normalised using a Ymax/Ymin threshold of 0.5. External calibration was performed using a Protein Calibration Standard (Bruker Daltonics) spotted on one corner of each sample. After image acquisition the matrix was removed from each slide by washing twice in 100% methanol, followed by incubation in 100% acetone for 5 min at RT. Tissue sections were then air dried before H&E staining (as above). H&E-stained sections were scanned at 1,500 dpi resolution and coregistered with their respective ion images. All image analysis was carried out using ClinProTools v2.2 (Bruker Daltonics).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla CA). Immunostaining measurements that returned values below the minimum detectable concentration (MDC) were assigned a value corresponding to half the MDC + 2 SD. Missing values were assigned the median for the particular sample group. All data (immunostaining, protein expression and immunohistochemical staining) were log transformed to approximate normality, and significance was determined using one-way ANOVA and Bonferroni post hoc test. Pairwise comparisons were performed using Student’s t-test; where variances were significantly different between groups, Welch’s correction was applied. Results of p < 0.01 were considered significant.

**Results**

**CXCL10 is overexpressed in serous ovarian tumours**

Tumour-specific CXCL10 expression was assessed by qRT-PCR in a small cohort of patients, matched for age, disease pathology and menopausal status (Table 1). mRNA encoding CXCL10 was significantly higher in both early- and late-stage tumours, relative to a control group comprised of patients with either serous benign ovarian tumours (n = 6) or other nonovarian, benign gynaecological disease (n = 6) (Fig. 1a). Expression was highest in patients with early-stage (Fédération Internationale de Gynécologie et d’Obstétrique (FIGO) stage Ib-c) tumours, with a significant decrease evident in late-stage (FIGO stage IIIC) tumours. Protein expression was then examined in an expanded cohort, comprising additional patients with late-stage tumours as well as a group of borderline/low-grade (Grade 1) tumours (Table 1). CXCL10 was detected in all samples at an apparent molecular mass of ~10 kDa (representative Western blot shown in Fig. 1b). Quantitative analysis of the ratio of total CXCL10: β-actin revealed a significant increase in CXCL10 protein abundance in all high-grade tumour samples relative to controls and also (for early-stage tumours) to samples from borderline/low-grade malignancies (Fig. 1c). Some degradation of β-actin was observed; however, this was an artefact of the high protein load (50 mg per lane) necessary to reliably visualise CXCL10. Highest CXCL10 expression was observed in
patients with early-stage disease, consistent with the elevated mRNA expression observed.

We also evaluated the presence of CXCL10 in urine and plasma samples from the same group of patients. Despite the tumour-specific production of CXCL10 observed, there was no significant increase in secreted levels in either plasma or urine (Supporting Information SI). Indeed, a small but significant decrease was identified in patients with early-stage, high-grade disease, as has previously been reported. The data suggest that despite overexpression of CXCL10 in high-grade ovarian tumours, total circulating/secreted levels of CXCL10 remain relatively unchanged throughout disease progression.

Tissue expression of CXCL10 supports the existence of the “immunoreactive” serous epithelial tumour subtype

Immunohistochemical staining for CXCL10 was performed on tissue microarray slides comprising duplicate tissue cores from (i) histologically normal, (ii) normal cancer-adjacent, (iii) benign or (iv) epithelial tumour tissue sections (see Supporting Information S1 and S2). CXCL10 staining was low in histologically normal, normal-cancer-adjacent (NCAT) and benign stromal tissues (Fig. 2a, panel i; representative images shown in Figs. 3a–3c). Intense CXCL10 staining was observed specifically in association with benign cystic epithelium (Fig. 2a). CXCL10 was present in malignant epithelial tissues in all tumour types (Figs. 3d and 3e and Supporting Information Fig. S2A). Notably, two distinct patterns of CXCL10 reactivity were evident in serous tumours (Fig. 2a). Low-level CXCL10 expression was observed in ~60% of serous tumours, similar to benign and histologically normal ovary (see Fig. 2a and Supporting Information S2). By contrast, ~40% of tumours expressed significantly elevated levels of CXCL10 (Fig. 2a). Total CXCL10 expression in serous tumours was elevated (p < 0.01) compared to normal, NCAT and benign groups (Fig. 2a); other epithelial tumour types showed no significant increase (Fig. 2a), although this may be an artefact of the small numbers examined. The bimodal expression observed in serous tumours was independent of age, stage or tumour grade, suggesting the presence of two different molecular pathological subtypes and consistent with the recently described “immunoreactive” subset of serous ovarian tumours that express elevated levels of CXCL10 mRNA.31
Figure 2. Quantitative analysis of immunohistochemical staining for CXCL10, CD45 and CD3. Tissue microarray slides were stained for CXCL10, CD45 and CD3, representative areas of tumour marked and positive staining scored on an intensity scale of 0 to +3. The % positive cells (total of +2 and +3 scoring cells versus total cell count) were plotted according to sample type. Each circle represents the average % positive cells from duplicate tissue sections per patient. Error bars represent mean ± SEM. (a) % positive cells. Dotted lines represent the mean ± SD for normal tissues. Grey bars on the right-hand side indicate the two populations of serous tumours based on expression levels. (b) Linear regression analysis for CD45 and CD3 vs. CXCL10. Significant correlation coefficients are indicated. (c) Recruitment index for CD45 and CD3 positive cells as a function of CXCL10 expression. Dashed lines represent the mean ± SD for histologically normal tissue. Tissues are divided into "low," "normal" and "high" accordingly. Groups are as follows: normal—histologically normal tissue; NCAT—normal cancer-adjacent tissue; benign—benign tumour; EPI—benign—benign tumour epithelial cell layer; malignant—serous epithelial tumour. **p < 0.01; ***p < 0.001.
CXCL10 expression does not correlate with leucocyte infiltration in serous epithelial tumours

To evaluate T-cell recruitment in response to elevated CXCL10, serial tumour sections were examined for expression of the general leucocyte marker CD45\(^{22}\) and the T-cell marker CD3. CD45-expressing cells were present in low numbers in both normal and cancer-adjacent tissues (Fig. 2a, panel ii; representative images shown in Figs. 3f and 3g), whereas in benign tissues CD45\(^+\) cells were present in elevated numbers particularly at the benign epithelium (Figs. 2a and 3h). By contrast, CD45\(^+\) cells in serous tumours were significantly fewer (\(p < 0.001\)) than in benign tissues, and were not significantly different to histologically normal tissue (Figs. 2a and 3i and 3j). There was a significant positive correlation between CXCL10 and CD45 expression in benign tissues (Fig. 2b, panel i), indicative of the positive influence of CXCL10 expression on leucocyte recruitment. However, in malignant tumour sections there was no apparent correlation between CXCL10 expression and CD45 staining (Fig. 2b).

Immunostaining for CD3 was used to demonstrate the presence of T-cells in serial sections. CD3\(^+\) T-cells were present in low numbers in normal, NCAT and benign stromal tissue, whereas they were significantly elevated in benign epithelium, consistent with CXCL10 and CD45 staining (Figs. 2a, panel iii and 3k—3m). In direct contrast to CXCL10 abundance, CD3\(^+\) cells were not significantly elevated in any tumour type (Figs. 2a, panel iii and 3n and 3o). Linear regression analysis demonstrated a significant positive correlation between CXCL10 and CD3 expression in benign tissues, similar to that observed for CD45; no significant correlation was observed between CD3 and CXCL10 in malignant tissues (Fig. 2b, panel ii).

To further clarify the relationship between CXCL10 and tumour-infiltrating leucocytes, we assessed the ratio of CXCL10:CD45 and CXCL10:CD3 positive cells (recruitment

Figure 3. Immunohistochemical staining of tumour tissue sections for CXCL10 and CD45. Representative tissue sections stained for either CXCL10, CD45 or CD3 are shown. Scale bars are indicated; each 50-\(\mu\)m section is an enlargement of the adjacent 200-\(\mu\)m section. Positive staining appears brown against blue H&E staining. Black arrows indicate isolated CD45-positive cells. Sample types are histologically normal ovarian tissue (a, f, k); normal cancer-adjacent ovarian tissue (b, g, l); benign ovarian tissue (c, h, m) and serous ovarian tumour tissue "type A" (d, i, n) and "type B" (e, j, o). Full TMA slides are provided in Supporting Information S1 and S2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
with an m/z of 2687.4 was also observed specifically in tumour samples, this variant was undetectable in benign tissue lysate (Fig. 4b) or in a sample of recombinant CXCL10 (see Supporting Information S3 and S4). De novo sequence analysis of this peptide identified the amino acid sequence NH$_2$-LSRTVRCTC-ISSNQPVNPRSL- (Fig. 4c, lower panel), corresponding to the same CXCL10 peptide but with the N-terminal Val-Pro dipeptide removed. Importantly, the ratio of intact:cleaved peptide was clearly different in each sample (Fig. 4b) suggesting their differential presence in tumour tissue.

Serial sections were also examined for the presence of DPPase 4 (DPP4), an enzyme known to cleave the N-terminus of CXCL10 both in vitro and vivo. Elevated DPP4 expression was observed in serous and endometroid tumours, whereas benign tissue showed significant decrease in DPP4 expression relative to normal (Supporting Information S2B). Interestingly, DPP4 was also elevated in normal cancer-adjacent tissues. DPP4 and CXCL10 displayed a linear correlation in both benign and malignant tissues, with significantly lower DPP4 expression in benign disease (Supporting Information S2B). The data demonstrate that both N-terminally processed CXCL10—a variant known to directly antagonise leucocyte recruitment—and the enzyme DPP4 are present in serous epithelial ovarian tumours.

**Antagonistic CXCL10 is localised to the tumour epithelium**

There are currently no commercially available antibodies suitable for immunohistochemical studies that can discriminate between intact and cleaved CXCL10—a variant known to directly antagonise leucocyte recruitment—and the enzyme DPP4 are present in serous epithelial ovarian tumours.

**Figure 5. Imaging mass spectrometry of tumour tissue sections for the presence of intact and cleaved CXCL10.** (a) Averaged mass spectra for patient tissue sections from benign, borderline/low-grade, high-grade early-stage and high-grade late-stage tumours. An m/z of 8657.8 or 8461.6 corresponds to the methionine-oxidised form of intact or cleaved CXCL10, respectively. Patient numbers and groups are indicated. (b) Representative IMS images demonstrating the localisation of intact and cleaved CXCL10 in tumour tissue sections for two individual patients. Panels are (i) H&E-stained tissue; (ii) intact CXCL10 (m/z 8657.8); (iii) cleaved CXCL10 (m/z 8461.6) and (iv) overlay of panels (i) and (iii). Scale is indicated. On-tissue peptide digestion and MS/MS analysis confirming the identity of CXCL10 is provided in Supporting Information S5. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
immunohistochemical staining. The identity of CXCL10 in IMS was confirmed by subsequent on-tissue digestion and MS/MS identification of several peptides belonging to CXCL10 (see Supporting Information S5A). The presence of cleaved CXCL10 in these sections also correlated with increased CXCL10 and decreased CD45+ cell infiltrate (see Supporting Information S5B). Together, the data strongly support the hypothesis that proteolytic cleavage of CXCL10 occurs in the tumour epithelium, where it can act to inhibit leucocyte recruitment.

Discussion

Our study provides the first evidence for the presence of N-terminally cleaved CXCL10 (denoted CXCL10(3-77)) in epithelial ovarian tumours and its antagonism of tumour-specific leucocyte recruitment. Inhibition of T-cell recruitment by CXCL10(3-77) was recently established as a prognostically important factor in the pathology of liver disease13; our data suggest that a similar mechanism may also operate in a subset of epithelial ovarian tumours, leading to impairment of the immune response and ultimately influencing prognosis. Ironically, we identified this mechanism in ovarian tumours overexpressing CXCL10, which were previously termed "immunoreactive"21; the presence of N-terminally cleaved CXCL10 in tumours suggests that its role may, in fact, be partially immunosuppressive. The presence of antagonistic CXCL10 in these tumours was not ubiquitous, and represents an additional molecular classifier rather than an absolute. Nevertheless, the hitherto unreported nature of this modification in at least some ovarian tumours is an important biological feature that could partially explain the low leucocyte recruitment observed here and in other studies5,13 and provides a potential new avenue for their treatment.

Chronic inflammation underlies the development, growth and progression of many types of solid tumours; therefore, it is of paramount importance to identify the molecules involved and their protumour or antitumour activities that may influence tumour growth and metastasis. Our mRNA and protein expression data support the recent identification of the "immunoreactive," CXCL10-overexpressing tumour group21 and additionally extend this definition to include patients with high-grade, early-stage (FIGO stage I) disease—representing a subset that was not included in the larger scale analyses reported. Interestingly, we noted higher expression in patients with Stage I disease compared to those diagnosed with more advanced tumours. Decreased CXCL10 expression correlated with increasing stage of cancer has also been reported for both endometrial and cervical cancers22,24 in association with the loss of b-FGF- and/or VEGF-supported induction of CXCL10 expression. Our data suggest that an inverse relationship between CXCL10 expression and disease stage might also occur in serous ovarian tumours, and that CXCL10 expression may be attenuated during disease progression.

Post-translational enzymatic processing regulates the function of a number of chemokines (reviewed in Ref. 25). CXCL10 is an established in vitro substrate for a number of enzymes11,26-30 in particular the DPPases DPP4 and DPP8 (and probably DPP9) that catalyse cleavage of the Val-Pro dipeptide from its N-terminus10,12,21. Recent work established that DPP4-mediated cleavage was responsible for failed leucocyte recruitment in liver13; there has previously been no direct evidence for similar cleavage of CXCL10 in tumours. DPP4 is overexpressed in ovarian tumour tissue10 and cell lines, and has been associated with changes in proliferation, adhesion and migration as well as enhanced sensitivity to paclitaxel.32-34 It is also associated with decreased MMP2 activity and increased levels of tissue inhibitors of metalloproteinases.35 Our data show a significant correlation between DPP4, CXCL10 and reduced leucocyte infiltration in ovarian tumours, suggesting that tumour-expressed DPP4 may be a contributor to the production of antagonistic CXCL10. However, the data do not exclude the possibility that DPP8 and/or DPP9 may also contribute; whilst DPP8 and DPP9 are intracellular enzymes, their activities have also been identified external to cells59, presumably as a result of tissue necrosis. Although both DPP8 and DPP9 are expressed in ovarian cancer cell lines,60 their presence in tumour tissue remains to be determined.

Both the presence and type of infiltrating T-leucocytes present in ovarian tumours are directly correlated with patient prognosis.5,13,37,28 CXCL10 normally functions as a potent chemottractant, recruiting activated CXCR3+ T-cells into sites of inflammation; the CXCL10(3-77), however, acts in a dominant negative manner to bind CXCR3 and prevent signalling or chemotaxis.11,33,34 By contrast to serous benign epithelial tumours, we observed few tumour-infiltrating CD3+ leucocytes (TILs) in malignant tissue despite high CXCL10 expression. Previous reports have also noted low/absent TILs in both ovarian tumour sections5,13 and a mouse xenograft model.39 These findings can now be partially explained by the abundance of antagonistic CXCL10(3-77) in these samples. Benign tumour samples, in which only the intact, chemottractable CXCL10 was present, were able to recruit T-cells; malignant tumours, in which varied levels of CXCL10(3-77) were identified, showed substantially less T-cell recruitment. Recent work has suggested that increased numbers of Treg infiltrate tumours with advancing stage, despite an early Th17 response.60 Therefore, an increased abundance of antagonistic CXCL10(3-77) in these tumours might promote early impairment of activated T-cell recruitment, increasing the effective ratio of infiltration by Treg and ultimately leading to poor prognosis. Therefore, the ratio of intact:cleaved CXCL10 in tumour tissue likely represents an important prognostic feature of the "immunoreactive" subset of serous epithelial ovarian tumours.

Interestingly, there is a long-standing link between type II diabetes (T2D) and prognosis for ovarian cancer patients.61 DPP4 activity has a well-established role in the development of T2D,41 and DPP4-specific inhibitors have become an effective therapy for diabetes management. Although our data do not unambiguously demonstrate which DPPase cleaves CXCL10 in ovarian tumours, it is possible that DPP4 inhibitors may find clinical application in the treatment of patients with ovarian cancers. Indeed, studies...
have suggested that patients prescribed antidiabetes medications have lower risk of hepatocellular carcinoma, and very recently sitagliptin (a DPP4 inhibitor used in the management of T2D) was found to reduce colon cancer in rats. Therefore, the identification of antagonistic CXCL10-77 in epithelial ovarian tumours may represent an important new avenue for targeted therapeutic strategies using existing medications. It is now important to establish which enzyme/s are responsible for CXCL10 processing, the effect of cleaved CXCL10 on the type of immune cell infiltration into tumours and the extent to which this phenomenon might influence epithelial tumour growth and progression.

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References
