The Lymphotactin Receptor Is Expressed in Epithelial Ovarian Carcinoma and Contributes to Cell Migration and Proliferation

Mijung Kim¹, Lisa Rooper², Jia Xie¹, Jamie Rayahin¹, Joanna E. Burdette³, Andre A. Kajdacsy-Balla², and Maria V. Barbolina¹

Abstract

Chemokine receptor-ligand interactions are important to support functioning of both normal and pathologic cells. The expression and function of chemokine receptors in epithelial ovarian carcinoma (EOC) is largely unknown. Here, we report that the lymphotactin receptor (XCR1) was expressed in primary and metastatic human epithelial ovarian carcinoma (EOC) specimens and cell lines. In contrast, expression of XCR1 was not detected in the normal ovary or in human normal ovarian surface epithelial cells. Our data indicate that XCL1 and XCL2 are either present in the malignant ascites or expressed by the ovarian carcinoma cells. The addition of lymphotactin (XCL1 and XCL2) stimulated migration and proliferation of XCR1-positive cells. Reduction of XCR1 expression in ovarian carcinoma cell line SKOV-3 resulted in abrogated diaphragm and peritoneal wall tumor formation and in reduced frequency of colonic, splenetic, and liver nodules in an in vivo xenograft mouse model. Taken together, our data suggest that XCR1 is expressed early during the course of tumorigenic transformation and contributes towards increased cell migration and proliferation, which can facilitate the prometastatic behavior of EOC cells. Mol Cancer Res; 10(11); 1–11. ©2012 AACR.

Introduction

Epithelial ovarian carcinoma (EOC) has a low incidence compared with other common cancers, with approximately 22,000 women diagnosed with EOC annually. However, it is the leading cause of death from gynecologic malignancies and the fifth leading cause of cancer death in women, as over 14,000 patients succumb to this malignancy each year (1). EOC is rarely diagnosed during its early stages, when the chance of survival is 90%. Most patients are diagnosed with EOC when micro- and macrometastases are already present (2). Current treatments are not effective against EOC once it reaches the late metastatic stages. EOC frequently recurs and becomes insensitive to these therapies. Thus, management of the metastatic disease becomes a crucial problem for the treatment of EOC. One possible way to resolve this problem is to target metastasis-specific pathways with novel therapies. Hence, focused identification of novel target pathways and molecules that play a role in metastasis progression could enhance the chances of discovering new and effective therapies. To achieve this goal, we first considered a unique pattern of metastatic dissemination of EOC.

Metastatic EOC colonizes tissues and organs of the peritoneal cavity, whereas the contribution of the hematogenous and lymphagenous spread in forming secondary lesions is minimal (2, 3). Malignant cells attach to the peritoneal linings of the peritoneum and the specialized mesothelial linings of the omental structures called “milky spots,” and develop into metastatic tumors (2, 4–7). This spreading pattern may suggest that the microenvironment of the peritoneal cavity is favorable for homing of EOC metastasis. In fact, peritoneal ascitic fluid is a rich source of growth factors, extracellular matrix proteins, chemokines, and other factors that support and promote peritoneal metastasis (8–10).

Chemokine signaling has been extensively associated with homing of metastasis to distant sites (11). Preliminary quantitative PCR (qPCR) array data in our laboratory suggested that the lymphotactin receptor XCR1, one of the members of the chemokine family of G protein-coupled receptor (GPCRs), was expressed in cell lines of serous EOC. Among chemokine receptors, XCR1 has several unique properties. XCR1 is activated by only 2 ligands, lymphotactin 1 (XCL1) and lymphotactin 2 (XCL2), which are specific only to this receptor (12–15). This high specificity may suggest that the XCL1(2)-XCR1 axis could be a good therapeutic target for processes that depend on these signaling pathways. XCL1 was first reported to attract CD4+ and CD8+ T lymphocytes and natural killer cells (16). Another study showed that XCR1 was expressed by murine CD8+...
dendritic cells and their interaction with XCL1-positive CD8+ T cells regulated cytotoxic immunity (17, 18). Furthermore, XCL1 induced migration of CD4+ and CD8+ T cells (19). As recently shown, XCR1-positive thymic dendritic cells were attracted into medulla by XCL1-positive medullary thymic epithelial cells in vivo (20). The XCL1-XCR1 axis contributes to the progression of various diseases, including rheumatoid arthritis (21), Crohn disease (22), and many others (23). The role of XCL1-XCR1 in cell proliferation, migration, and invasion has been reported in a carcinoma of epithelial origin and an oral squamous carcinoma (24). The current study investigated whether XCR1 was expressed in human specimens of EOC and whether it may have any functional significance in the progression of this disease. XCR1 expression was found in EOC and supports a hypothesis in which the XCL1(2)-XCR1 axes are active in EOC. XCR1 signaling may support tumor cell properties required for progression toward metastasis, such as migration and proliferation, as well as tumor formation. Our data suggest that XCR1 could be a novel drug target for slowing the progression of EOC and perhaps many other cancer types.

Materials and Methods

Reagents and antibodies

Tissue microarrays (TMA) containing specimens of a normal ovary, primary and distant metastatic ovarian carcinomas, and distant metastases from multiple cancer types were obtained from US Biomax (Cat# OV208, OV808, MT2081). The cDNA array prepared from specimens of the normal ovary and primary ovarian carcinoma was obtained from Origene Technologies. XCL1 was obtained from RayBiotech. Human collagen type I and type III and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. The WST-1 proliferation kit was obtained from Takara Bio. XCR1 siRNA, control siRNA (#A), goat anti-human XCR1 antibodies, and rabbit anti-human PCNA antibodies were obtained from Santa Cruz Biotechnology. DharmaFECT was purchased from Dharmacon. Monoclonal anti-β-tubulin antibody was purchased from the Developmental Studies Hybridoma Bank. Matrigel was obtained from BD Biosciences. Transwell cell migration chambers with 8-micron pores were purchased from Corning. Goat anti-human phycoerythrin (PE)-conjugated XCR1 antibodies, anti-goat (PE)-conjugated antibodies, and the XCL1 ELISA DioSet Kit were obtained from R&D Systems. Texas Red-conjugated secondary anti-goat polyclonal IgG antibodies were obtained from Rockland Immunochemicals. Rabbit anti-human XCR1 antibodies were obtained from Novus Biologicals. Anti-rabbit biotin-conjugated antibodies were obtained from Abcam. Vectastain ABC and DAB kits were obtained from Vector Laboratories. The XCL2 Sandwich ELISA Kit and XCL2 protein (a part of the XCL2 ELISA Kit) were obtained from USCN Life Science Inc..

Cell lines

Human ovarian carcinoma cell lines of serous histotype originating from malignant cells in ascites, SKOV-3 and A2780, were obtained from the NCI Tumor Cell Repository. These cell lines were cultured as suggested by the manufacturer for no longer then 20 consecutive passages. The human ovarian carcinoma cell line of serous histotype originating from malignant cells in ascites, Caov-3, was obtained from Dr. M.S. Stack (University of Missouri-Columbia, MO) and propagated in minimal essential media supplemented with 10% FBS for no longer than 15 consecutive passages. The human ovarian carcinoma cell line of serous histotype originating from a primary tumor, IGROV-1, was obtained from the NCI Tumor Cell Repository and cultured as suggested by the manufacturer for no longer then 20 consecutive passages. The borderline EOC cell lines HuIOSBT-1.5 and HuIOSBT-3.3, and the normal immortalized human ovarian surface epithelial cells IOSE29 and IOSE80 were obtained from Dr. N. Auersperg through the Canadian Ovarian Tissue Bank (Vancouver, Canada). The normal immortalized human ovarian surface epithelial cell line HOSE11-12 was obtained from Dr. G.S.W. Tsao (The University of Hong Kong, PR China) and propagated in minimal essential media supplemented with 10% FBS for 5 consecutive passages. The normal immortalized human ovarian surface epithelial cell line T1074 was obtained from Applied Biological Materials and propagated according to the manufacturer’s instructions. The normal (not immortalized) human ovarian surface epithelial cell line HOSEPepC was purchased from SclenCell Research Laboratories and maintained in the conditions suggested by the manufacturer between passages 1 and 2. All cell lines were routinely assessed by cell morphology and their average doubling time.

Mice

Athymic nude – FOXN1NU mice were obtained from Harlan Laboratories and from Charles River Laboratories. All experimental procedures were carried out according to the Institutional Animal Care and Use Committee (IACUC) protocol (#10-060) approved by the Animal Care Committee of UIC.

In vivo tumor formation

For generation of intraperitoneal tumors, 3 × 10⁶ parental SKOV-3 and SKOV-3 stably expressing XCR1shRNA cells were injected intraperitoneally (i.p.) into mice (n = 6). Animals were monitored 3 times weekly for tumor formation, ascites development, and survival up to 9 weeks. Some animals were sacrificed earlier (7.7 weeks the earliest) due to their poor health condition, a large volume of ascites, or large tumors visible through the skin. Animals were sacrificed (on average at 8.5 weeks), dissected, and the abdominal region was examined for tumors. As in most cases it was not possible to determine the margins of the tumors and, in many cases, the tumors presented as large masses that have fused and encased abdominal organs, measurement of the tumor volume associated with a specific organ was not possible. Data analysis was conducted as “yes” in case when tumor were visible and “no” when no nodules were seen regardless of the size found at a specific abdominal organ and tissue and
plotted as a bar graph depicting the percentage of animals bearing metastasis at the indicated tissues and organs. Ascitic fluid was collected and measured. Tumors were collected and paraffin-preserved as described earlier (25).

Procedures for real-time PCR, reverse transcriptase PCR (RT-PCR), immunohistochemistry, flow cytometry, Western blotting, immunofluorescent staining, ELISA, transient transfections, Transwell cell migration, and cell proliferation are described in Supplementary Materials and Methods.

Results
Expression of XCR1 in normal and pathologic ovarian surface epithelia
XCR1 is not expressed in the normal ovary (14). However, the pattern of XCR1 expression during tumorigenic transformation is unknown. Currently, epithelia from both the ovarian surface and the fimbriated edge of the fallopian tube are considered putative precursors of the predominant histotype of serous EOC (26–29). We tested for the expression of XCR1 in human specimens and cells from the normal ovary, normal ovarian surface epithelium (NOSE), normal immortalized ovarian surface epithelium, borderline EOC, primary EOC, and metastatic EOC by real-time PCR, immunohistochemistry, flow cytometry, and Western blot analysis. Our examination of the specimens of a noncancerous ovary and primary ovarian carcinoma by real-time PCR showed that none of the noncancerous ovarian tissues expressed XCR1 RNA, whereas 18 of 41 primary stage I–IV EOC specimens were XCR1-positive (Supplementary Table S2).

Next, the expression patterns of XCR1 protein were correlated with the RNA. By immunohistochemistry, all cases of the normal ovary containing ovarian surface epithelial cells were XCR1-negative (Fig. 1A, Supplementary Table S3). Surprisingly, all immortalized ovarian surface epithelial (IOSE) and borderline EOC cell lines were XCR1-positive (Fig. 1B and C). These data may suggest that XCR1 expression likely occurs early in the process of the tumorigenic transformation for serous EOC that originate from NOSE.

Figure 1. Expression of XCR1 in normal ovarian surface epithelium and the IOSE and borderline EOC cell lines. A, a representative image of XCR1 expression in a specimen from a normal ovary. Black arrow indicates the ovarian surface epithelium in the normal ovary. Brown – XCR1, blue – hemotoxylin. Images were generated using an Aperio ScanScope digital slide scanner. Magnification: ×10. B, expression of XCR1 in the indicated IOSE cell line T1074 was detected by flow cytometry analysis and immunofluorescent staining. Cell surface expression of XCR1 in IOSE was analyzed by flow cytometry analysis. Solid thick line – XCR1 expression, solid thin line – negative control (secondary antibody only). Images [× 40] shown are representative of at least 3 independent experiments. The fluorescent signal from XCR1 staining (red) was overlaid with DAPI staining (blue). C, Cell surface expression of XCR1 in the borderline EOC cell lines was detected with flow cytometry analysis. Solid thick line – XCR1 expression, solid thin line – negative control (secondary antibody only). Representative of at least 3 independent experiments.
To study the functional significance of XCR1 in EOC, XCR1 expression in a panel of EOC cell lines was determined by Western blot analysis, immunofluorescence, and flow cytometry analysis. EOC cell lines except A2780 were XCR1-positive for XCR1 expression and displayed a strong presence of membranous XCR1 (Fig. 2A and B). Western blot analyses confirmed that IOSE and EOC cell lines expressed XCR1, whereas NOSE did not (Fig. 2C). Human specimens of primary and metastatic EOC were immunostained for XCR1 and approximately 50% of primary serous EOC cases were XCR1-positive. XCR1 RNA was detected in 45% of the cases (Supplementary Table S2), and 54% of the human tumor samples were immunopositive for XCR1 (Fig. 3, Supplementary Tables S3 and S4). Moreover, 38% of mucinous EOC cases were positive for the XCR1 protein (Supplementary Table S3), and 50% expressed XCR1 RNA (Supplementary Table S2). Furthermore, 38% \( (n = 8) \) with endometrioid EOC expressed XCR1 RNA (Supplementary Table S2). Finally, the majority (55%) of EOC specimens metastatic to the peritoneal tissues and organs displayed XCR1 protein \( (n = 53) \) (Fig. 2D, Supplementary Tables S3 and S4).

**XCL1 and XCL2 are present in EOC ascites and cell lines**

For XCL1(2)-XCR1–dependent cell signaling to occur, both the ligand(s) and the receptor must interact. Pro-T cells, T cells, and natural killer cells are the major sources of XCL1 (13). XCL1 is expressed in the ovary, spleen, small intestine, and colon, which are common sites of primary and metastatic EOC (30). Secreted XCL1 binds to the glycosaminoglycans (GAG) exposed on cell surfaces (31). Peritoneal ascitic fluid, which commonly accumulates in large volumes in patients with EOC, is a source of a variety of growth factors, metastasis-promoting molecules, and chemokines (8–10). However, whether this fluid contains soluble, not GAG-bound, XCL1 and XCL2 in unknown. Thus, the presence and amount of XCL1 and XCL2 ligands was measured in ascitic fluid. To address this question, ascites specimens of malignant EOC \( (n = 3) \) and borderline gynecologic conditions \( (n = 3) \) were screened using ELISA. Examination of ascitic fluid samples revealed that XCL2 is significantly more abundant with nanomolar quantities than XCL1, which is found in only trace amounts (Table 1). XCL1, which is found in only trace amounts (Table 1). These data could indicate that very little soluble XCL1 is released into the ascitic fluid. Thus, likely sources of XCL1 in the peritoneal cavity include areas in the vicinity of the XCL1-producing cells of the immune system, ovary, and other peritoneal organs as well as cell surface-attached GAGs harboring XCL1 (13, 30, 31). To further investigate the possible source of XCL1 in EOC, we tested the EOC cell lines IGROV-1, SKOV-3, and A2780 and found that XCL1, but not XCL2, RNA is expressed by these EOC cell lines (Fig. 3A). XCL1 protein was also detected in total cell lysates of EOC cell lines A2780 and SKOV-3 using ELISA (Table 1). Interestingly, XCL1 and XCL2 encoding genes are highly homologous and their protein sequences differ by only 2 amino acids; their transcription is dependent on NFAT \( (15, 32) \).

**XCL1 induces proliferation of XCR1-positive cell lines**

Cell proliferation is one of the crucial properties of tumor cells. Metastatic ovarian carcinoma is characterized by a vast intraperitoneal spreading that far exceeds the size of the primary tumor. Chemokine signaling can promote cell proliferation (11, 33). XCL1 has been shown to support the proliferation of XCR1-positive oral epithelial cells (24). Hence, the ability of XCL1 to induce proliferation of XCR1-positive IOSE, borderline EOC, and EOC cell lines was investigated. The \( K_d \) of the XCL1 ligand for the XCR1 is 10 nmol/L (13). Thus, we tested the ability of XCL1 at several concentrations in the low nanomolar range to stimulate cell proliferation. Interestingly, the majority of XCR1-positive cell lines that originated from immortalized NOSE, borderline, and serous EOC were highly proliferative in the presence of XCL1 (Fig. 3B). Generally, cell proliferation increased with increased XCL1 concentration (not shown). XCR1-negative NOSE (HOSEpiC) and EOC (A2780) cells did not proliferate upon XCL1 stimulation (Fig. 3B). Total and nuclear PCNA was positive in cells stimulated with 25 nmol/L XCL1, showing that these cells subsisted in a proliferative phase, in contrast to the serum-starved cells (Fig. 3C). Notably, a few of the XCR1-positive cell lines \( (1074 \text{ and HuOSBT}3,3) \) showed no proliferative response to XCL1 addition. This may be explained by alternative receptor conformations that do not support downstream signaling or quick desensitization of the receptor upon ligand stimulation. Overall, these data suggest that the XCL1/XCR1 axis functions in promoting cell proliferation in most XCR1-positive ovarian cell lines.

**XCL1 and XCL2 induce migration of XCR1-positive cell lines**

Cell migration is essential for metastatic progression. Ovarian carcinoma disseminates locoregionally throughout the peritoneal cavity by shedding malignant cells from the ovary, which is in contrast to the hematogenous or lymphogenous spreading mechanism observed in the majority of solid tumors (3). Moreover, chemokine/receptor interactions play a role in chemotactic cell migration (11, 33). EOC cells disseminate into the peritoneum during the initial steps of metastatic colonization and support of locoregional migration throughout the cavity occurs from cell migration during the late stages of metastatic colonization. Using animal models, another chemokine network, SDF1-CXCR4, has been shown to contribute to homing of EOC cells to the peritoneum up to 30% (34). These data provide a proof-of-principle for the involvement of a chemokine network in the abdominal colonization of EOC. Thus, the ability of EOC cells to engage in XCL1(2)-XCR1 signaling to become more motile was evaluated. To address this question, Transwell cell migration assays were used in which cells were allowed to migrate toward XCL1 and XCL2. The addition of 0.5 and 2 nmol/L XCL2 significantly increased cell migration \( (1.5-\text{ and }2.2\text{-fold, respectively; Fig. 4A}) \). As XCL2 is not currently commercially available as a purified protein, only nontransfected SKOV-3 cells were tested in the migration assay (Fig. 4A). To characterize cell migration in
the presence of XCL1, nontransfected SKOV-3 cells as well as those transiently transfected with either control or XCR1-specific siRNAs were tested. EOC cell migration increased with increasing concentrations of XCL1 and XCL2. The number of cells migrating toward the 10 nmol/L XCL1 solution, chosen based on the $K_d$ of XCL1 binding to XCR1, was double the control medium for both SKOV-3 (Fig. 4A) and Caov-3 (data not shown).

Figure 2. Expression of XCR1 in EOC cell lines. A, cell surface expression of XCR1 in the EOC cell lines using goat anti-human PE-conjugated XCR1 antibodies. Solid thick line – XCR1 expression, solid thin line – negative control (secondary antibody only). Images shown are representative of at least 3 independent experiments. B, expression of XCR1 was tested by immunofluorescent staining of immortalized NOSE and serous EOC cell lines, as indicated. Images were generated using a Zeiss fluorescent microscope and × 40 objective lens. The fluorescent signal from XCR1 staining (red) was overlaid with 4′, 6-diamidino-2-phenylindole (DAPI) staining (blue). A magnified image of XCR1 staining in SKOV-3 converted to a black-and-white image is shown in the lower panel to show membranous immunoreactivity with XCR1 antibodies (white arrows). Intensity of XCR1 staining in cellular protrusions along the dotted line was quantified using the ImageJ (NIH) line scan feature. Bars = 20 μm and 5 μm on the top and bottom, respectively. C, expression of XCR1 was probed in total cell lysates prepared from nonimmortalized NOSE (HOSEpiC), immortalized OSE (T1074), and serous EOC cell lines (IGROV-1 and Caov-3) using goat anti-human XCR1 antibodies. β-Tubulin was used as a loading control. Images were generated using enhanced chemoluminescence application of Chemidoc (Bio-Rad). D, immunohistochemical analysis of XCR1 expression in primary and metastatic specimens of EOC. Representative images of negative and positive cases of primary EOC as well as negative and positive cases of EOC metastasis to the omentum, as indicated. Brown – XCR1, blue – hematoxylin. Images were generated using the Aperio ScanScope digital slide scanner. Magnification: × 10.
Silencing of XCR1 expression by siRNAs resulted in a reduction of cell migration back to the basal level (Fig. 4A). Importantly, the XCR1-negative NOSE cell line, HOSEpiC, did not respond to the addition of exogenous XCL1 (Fig. 4B). Therefore, XCR1 promotes XCL1-dependent migration.

XCR1 expression in the EOC cell lines IGROV-1 and SKOV-3 was silenced and stimulated to migrate in the presence of human ascites fluid (specimen #2, stage IV serous adenocarcinoma, Table 1). Data were compared with cells transfected with a control siRNA. The migration rates were reduced by 60% and 80% in SKOV-3 and IGROV-1,

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**Figure 3.** A, expression of XCL1 and XCL2 RNA in EOC cell lines. Total RNA from IGROV-1, SKOV-3, and A2780 was extracted, cDNA was synthesized, and RT-PCR was conducted as described in the Materials and Methods section. Products of the PCR reaction were loaded in a 2% agarose gel, visualized with ethidium bromide staining, and photographed with the BIO-RAD Chemidoc software. The image has been color inverted for better visualization. M – marker, H – housekeeping gene control RPL-19, 1 – XCL1, 2 – XCL2. The position of the lowest fragment of the marker ladder (500 bp) is indicated. B, XCL1-induced proliferation. NOSE, IOSE, borderline EOC, and serous EOC cell lines, as indicated, were subjected to cell proliferation in the presence and absence of 25 nmol/L XCL1 added to the serum-starved cells (“Starved + 25 nmol/L XCL1,” red bars, “Starved,” blue bars, respectively). Cell proliferation was measured using a WST-1 reagent as described in Methods and plotted. C, SKOV-3 cells were serum-starved (Starved) as well as serum starved and cultured in the presence of 25 nmol/L XCL1 (Starved + 25 nmol/L XCL1) for 24 hours, photographed using a Zeiss microscope with × 40 magnification, collected, and analyzed by Western blot analysis. Expression of PCNA in total cell lysates (in duplicate) was detected by Western blot analysis using rabbit anti-human PCNA antibodies at a 1:200 dilution. β-Tubulin was used as a loading control. Images were generated using the enhanced chemoluminescence application of Chemidoc (Bio-Rad). Immunofluorescent staining of PCNA is shown in the lower panels. SKOV-3 cells were first serum starved and then cultured in the absence (Starved) and presence of 25 nmol/L XCL1 for 24 hours (Starved + XCL1). Nuclear PCNA staining (green fluorescence) was evaluated by immunofluorescent staining. Cell nuclei were stained with DAPI (blue fluorescence). Fluorescent images were generated using the Zeiss AxioObserver fluorescent microscope with × 40 magnification.
XCR1 in Ovarian Carcinoma

Table 1. Concentration of XCL1 and XCL2 in samples of ovarian carcinoma and borderline gynecologic diseases determined by ELISA

<table>
<thead>
<tr>
<th>Sample</th>
<th>XCL1 pg/mL</th>
<th>XCL1 pmol/L</th>
<th>XCL2 ng/mL</th>
<th>XCL2 nmol/L</th>
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<tr>
<td>1 Borderline endometrioid cystadenoma</td>
<td>40</td>
<td>4.0</td>
<td>2.0</td>
<td>0.20</td>
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<tr>
<td>2 Serous adenocarcinoma, stage IV, Grade 3</td>
<td>50</td>
<td>5.0</td>
<td>1.0</td>
<td>0.10</td>
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<tr>
<td>3 Borderline mucinous cystadenoma</td>
<td>50</td>
<td>5.0</td>
<td>1.0</td>
<td>0.10</td>
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<tr>
<td>4 Serous adenocarcinoma, stage IV</td>
<td>48</td>
<td>4.8</td>
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<tr>
<td>5 Serous adenocarcinoma, stage IIIc, Grade 3</td>
<td>37</td>
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<td>6 Serous cyst</td>
<td>50</td>
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<td>7 A2780a</td>
<td>210</td>
<td>21</td>
<td>NDb</td>
<td>NDb</td>
</tr>
<tr>
<td>8 SKOV-3a</td>
<td>83</td>
<td>8.3</td>
<td>NDb</td>
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aTotal protein concentration in the lysates 2.5 mg/mL.
bNot determined.

respectively (Fig. 4C). Notably, the increase in cell migration in the presence of XCL1 was not due to an increase in proliferation because migration assays were conducted within 5 hours of treatment, whereas measurable changes in cell proliferation occurred only after 24 hours. Western blot analyses showed a significant reduction of XCR1 protein expression levels in SKOV-3 and IGROV-1 transfected with XCR1 siRNA (Fig. 4D). Overall, these data suggest that the XCL1(2)-XCR1 axis can promote migration of EOC cells.

XCR1 in ovarian carcinoma cells facilitates dissemination of EOC cells in the peritoneum

To expand the in vitro observations and to validate the role of XCR1 in the formation of metastasis, in vivo xenograft experiments were carried out wherein ovarian carcinoma SKOV-3 cells were injected i.p. into athymic nude mice. XCR1 was silenced using stable transfection of the GFP-tagged plasmid construct containing XCR1. Several stable clones were created and the one with the lowest residual XCR1 expression (50%, Fig. 5A) was chosen (“clone 1”). Terminal metastases were allowed to form. Because of the moribund conditions, experiments were terminated on average 8.5 weeks after injection. Animals injected with the parental SKOV-3 developed gross overt metastasis on omentum, peritoneal wall, colon, diaphragm, liver, mesentery, spleen, stomach, and abdominal lymph nodes (Fig. 5B and D) as well as ascites ranging from 0 to 7 mL (Fig. 5C). In contrast, none of the animals injected with cells deficient in XCR1 expression developed diaphragm nodules and only one mouse in this group had a small nodule on the peritoneal wall. Furthermore, the number of animals per group that developed colonic, liver, splenetic, stomach, and lymph node metastasis was smaller when XCR1 was silenced. (Fig. 5B and D). Animals with reduced XCR1 expression in the SKOV-3 cells generated lower volumes of ascites that ranged between 0 and 4 mL (Fig. 5C). These data indicate that blocking XCR1 can reduce metastatic dissemination of ovarian carcinoma cells in the peritoneum.

XCR1 is expressed in distant metastasis from multiple cancers

Chemokine signaling promotes metastasis likely by supporting homing of malignant cells to their specific niches by facilitating chemotaxis, increasing cell adhesion, and increasing proliferation. Of the tested metastatic EOC specimens, 55% were XCR1-positive. To determine whether XCR1 expression is specific for EOC metastasis, the expression of XCR1 was investigated in other metastatic cancers. Human specimens stained using immunohistochemistry for XCR1 shows that tumors originating in the bladder, breast, cervix, colon, endometrium, esophagus, kidney, larynx, liver, lung, skin, pancreas, prostate, duodenum, rectum, small intestine, stomach, thyroid, and other organs contain XCR1-positive cells (Supplementary Figure S2, Supplementary Table S5). Therefore, XCR1 may have a more general role in metastases of many cancer types.

Discussion

Lymphotactin is a structurally unique stand-alone member of the C class chemokine receptors known to be activated by 2 closely related ligands, XCL1 and XCL2 (13–15, 34). For XCL1(2)-XCR1–dependent signaling to occur, both the ligand(s) and receptor must interact. Our data indicate that XCR1 was not expressed in normal surface epithelial ovarian cells, but its expression increased during the course of malignant transformation. XCL1 was expressed in organs that support the growth and progression of primary and metastatic EOC, namely in the ovary, small intestine, colon, and spleen (30). The cellular sources of XCL1 reported thus far include T cells, natural killer cells, thymocytes, dendritic cells, mast cells, and other leukocytes (13). Information regarding the sources of XCL2 is limited to a few reports that have investigated only a few cellular sources (15). Our examination of the ascitic fluid obtained from patients with EOC and borderline gynecologic diseases indicated that picomolar amounts of XCL1 and nanomolar amounts of XCL2 were present, which were likely secreted into the ascitic fluid by cells that express XCL1(2). Thus, XCR1 is
expressed by transformed ovarian epithelial cells and XCL1 (2) are present in the peritoneal milieu, suggesting that both XCL1-XCR1 and XCL2-XCR1 interactions likely occur during the progression of human EOC and borderline gynecologic diseases.

Cell migration is one of the crucial properties of ovarian cancer cells specifically for tumor expansion, progression, and metastasis. Cell motility in the progression of EOC is required early during the expansion of the primary tumor, while cells are shedding from the primary source and in later stages during expansion of the metastatic tumor. Our data suggest that the XCL1(2)-XCR1 axis could contribute to ovarian cancer cell migration. XCL1 and XCL2 enhanced the chemotactic migration of XCR1-positive EOC cells, thus potentially facilitating movement and tissue specific homing of these metastatic cells to their niche in the peritoneum. Furthermore, metastatic tumor growth is accompanied by cell migration, which may be supported by the XCL1-XCR1 axis. Both XCL1 and XCL2 ligands could stimulate EOC cell migration, particularly after immortalization. Uncontrolled cell proliferation is a hallmark of cancer cells. Interestingly, metastatic ovarian carcinomas reach extremely large sizes, far exceeding those of the primary tumor. Our data suggest that the XCL1-XCR1 axis promotes cellular proliferation, and therefore could potentially contribute to growth of XCR1-positive EOC and XCR1-positive metaplastic cells and borderline tumors. In fact, the results of our in vivo studies have shown the role of XCR1 in facilitation of peritoneal tumor formation. Taken together, the XCL1-XCR1 axis has the propensity to contribute to progression of EOC by enhancing proliferation and facilitating migration.
**Figure 5.** In vivo xenograft study of the role of XCR1 in dissemination of ovarian carcinoma. A, Expression of XCR1 was probed in total cell lysates prepared from SKOV-3 and SKOV-3 stably transfected with scrambled shRNA and XCR1-specific shRNA (clone 1, clone 2, clone 3), as indicated, using goat anti-human XCR1 antibodies (Santa Cruz Biotechnology). β-Tubulin was used as a loading control. Images were generated using enhanced chemoluminescence application of Chemidoc (Bio-Rad). *, P < 0.05. B, photographs of the dissected abdomens of animals injected with parental SKOV-3 cell line (top) and SKOV-3 stably transfected with XCR1shRNA (clone 1; bottom); peritoneal metastasis from the parental SKOV-3, middle. Tumor nodules are outlined. C, average volume of ascites accumulated in each condition, as indicated. D, distribution of metastatic lesions formed on diaphragm, peritoneal wall, colon, liver, spleen, lymph, mesentery, and omentum is shown as the percentage of animals bearing metastases at the site. Statistical significance is calculated using t test. Total number of animals analyzed: 6 for the XCR1shRNA group and 5 for the parental SKOV-3 (one animal was not counted for as the tumors did not take).

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**XCR1 in Ovarian Carcinoma**

A

SKOV-3 Scr sh Clone1 Clone2 Clone3 XCR1

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</tbody>
</table>

B

SKOV-3

SKOV-3 peritoneal wall

XCR1sh

C

Ascites, mL

- SKOV-3
- XCR1sh

D

Animals with metastasis, %

- Diaphragm: *P = 0.002
- Peritoneal wall: *P = 0.008
- Colon
- Liver
- Spleen
- Lymph
- Stomach
- Mesentery
- Omentum
Chemokine axes, including the XCL1-XCR1 axis, have been studied for their ability to induce an immunologic response, which could be exploited to combat cancers by immunotherapy, especially in combination with IL-2 (35). Interestingly, B6C3F1 mouse model studies have shown that XCL1 did not enhance the attraction of immune cells to eliminate EOC (36). These findings may indicate that in certain conditions, such as advanced metastatic cancers, the ability of the lymphotactin axis to attract cells of the immune system to the tumor sites could be weakened. In fact, the accumulation of T-regulatory cells in ovarian tumors is associated with poor prognosis (37). It is possible that even XCR1-positive EOC cells within advanced metastatic tumors fail in attracting antitumor response. On the other hand, our data suggest that XCR1-positive EOC cells may benefit from stimulation of this axis by proliferation and migration. A common feature of metastatic tumors is their ability to escape immune surveillance and use immune cell secretions for tumor progression (38). Hence, it is possible that the XCL1-XCR1 axis in EOC enhances tumor progression as opposed to facilitating antitumor effects exerted by the immune system.

XCL1(2)-XCR1 is a potential drug target due to the high specificity of the interaction between the ligands and the receptor and the lack of cross-reactivity with other members of the chemokine family. Drugs that target chemokine receptors have exhibited some clinical success, supporting the feasibility of targeting this class of receptors (39–41). Currently, pharmacologic agents designed to block XCL1(2)-XCR1 are not available. However, a patented technology based on targeting XCR1-positive cells has been developed (R. Kroczek, Pub. No. US 2010/0310562 A1, "System for delivery into XCR1-positive cells and uses thereof"). These drugs would be most suitable for patients with XCR1-positive EOC. Perhaps anti-lymphotactin therapies used in early-stage EOC patients with no apparent micro- and macrometastasis would aid in the prevention of metastatic disease. Those with established micro- and macrometastasis might also benefit from the reduction of XCL1-induced cell proliferation, by reducing the residual tumor expansion. Further scientific and technological progress is needed to translate the current knowledge on the role of drug targetable proteins, such as XCR1, into therapies that are clinically effective.

Interestingly, previous studies implicated the importance of other chemokine ligand-receptor interactions, such as CXCL12-CXCR4, CXCL1-CXCR1, and CXCL16-CXCR6, in progression and metastasis of ovarian carcinoma (8, 34, 42–44). Current study on the role of XCL1-XCR1 emphasizes the complexity and importance of the chemokine ligand-receptor interactions in the peritoneal milieu of ovarian carcinoma. Together these studies suggest that further examination of other members of the chemokine family for their involvement in pathobiology of ovarian carcinoma may be necessary.

Our data indicate that functional XCR1 is expressed in epithelial ovarian carcinoma. The data suggest that the lymphotactin network in EOC may contribute to the EOC cell migration and proliferation, which are important during metastatic progression. As the lymphotactin receptor is a cell surface receptor of the chemokine family of GPCRs, it represents a potential drug target. Further in vitro and in vivo studies of the role of XCR1 in the progression of ovarian carcinoma and development of pharmacologic small-molecule inhibitors are warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Kim, L. Rooper, J. Xie, J. Rayahin, A. Kajdacsy-Balla, M.V. Barbolina
Writing, review, and/or revision of the manuscript: J. Rayahin, J.E. Burdette, A. Kajdacsy-Balla, M.V. Barbolina
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References


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