Targeting Interleukin-11 Receptor-α Impairs Human Endometrial Cancer Cell Proliferation and Invasion In Vitro and Reduces Tumor Growth and Metastasis In Vivo

Amy L. Winship1,2, Michelle Van Sinderen1,3, Jacqueline Donoghue3,4, Kate Rainczuk1,3, and Evdokia Dimitriadis1,2,3

Abstract

Endometrial cancer contributes to significant morbidity and mortality in women with advanced stage or recurrent disease. IL11 is a cytokine that regulates cell cycle, invasion, and migration, all hallmarks of cancer. IL11 is elevated in endometrial tumors and uterine lavage fluid in women with endometrial cancer, and alters endometrial epithelial cell adhesion and migration in vitro, but its role in endometrial tumorigenesis in vivo is unknown. We injected mice subcutaneously with human-derived Ishikawa or HEC1A endometrial epithelial cancer cells (ectopic), or HEC1A cells into the uterus (orthotopic) to develop endometrial cancer mouse models. Administration of anti-human IL11 receptor (R) α blocking antibody dramatically reduced HEC1A-derived tumor growth in both models and reduced peritoneal metastatic lesion spread in the orthotopic model, compared with IgG. Anti-human IL11Rα retained a well-differentiated, endometrial epithelial phenotype in the HEC1A ectopic mice, suggesting it prevented epithelial-to-mesenchymal transition. Blockade of mouse IL11R with anti-mouse IL11Rα antibody did not alter tumor growth, suggesting that cancer epithelial cell IL11 signaling is required for tumor progression. In vitro, anti-human IL11Rα antibody significantly reduced Ishikawa and HEC1A cell proliferation and invasion and promoted apoptosis. Anti-human, but not anti-mouse, IL11Rα antibody reduced STAT3, but not ERK, activation in HEC1A cells in vitro and in endometrial tumors in xenograft mice. We demonstrated that targeted blockade of endometrial cancer epithelial cell IL11 signaling reduced primary tumor growth and impaired metastasis in ectopic and orthotopic endometrial cancer models in vivo. Our data suggest that therapeutically targeting IL11R could inhibit endometrial cancer growth and dissemination.

Introduction

Endometrial cancer is the most common invasive gynecologic malignancy in developed countries, with more than 280,000 new cases occurring on average annually (1). There is no effective screening test for early detection and there are currently no curative therapies. Alarmingly, the incidence is increasing, particularly in women of reproductive age (2), thought to be attributed to an increase in obesity. Mortality is primarily related to advanced or recurrent disease. Although current radiotherapy or chemotherapy may achieve a transient treatment response, the median survival for these women is less than one year (2).

Type I "endometrioid" carcinoma is the most common type of endometrial cancer, accounting for approximately 85% of cases (3). Type I endometrial tumors are staged according to the guidelines of the International Federation of Gynecology and Obstetrics (FIGO; ref. 4). Increasing tumor grade (G1–G3) is based histologically on the extent to which the cancer differentiates from normal endometrial morphology by loss of the formation of glandular structures and also has increasing metastatic characteristics. Grading therefore includes the extent to which the cancer invades the uterine corpus and the surrounding peritoneum (3). Grade 1 cancers are described as well differentiated in terms of their morphology. These cancers are usually confined to the uterus and have good prognosis following surgical intervention and radiotherapy. Grade 2 endometrial cancers are moderately differentiated and display myometrial invasion within the uterus, as well as spread to nearby pelvic and para-aortic lymph nodes. Grade 3 cancer cells are arranged in a haphazard or disorganized way and do not form glands; hence they are described as poorly differentiated and are highly metastatic (4). Both grade 2 and 3 endometrial cancers have poorer prognosis, compared with grade 1, with metastatic behavior being most closely linked with clinical outcome and cause of death (3).

The cycling endometrium undergoes well-coordinated and regulated processes of proliferation and differentiation in response to estrogen and progesterone, respectively. Subsequently, after menopause, the endometrium becomes atrophic. While it is considered that endometrial cancer most frequently arises when
the quiescent endometrium is affected by hormonal imbalances, molecular or genetic alterations, or a combination of these factors (3), the precise etiology is poorly understood. Once the critical molecular regulators are discovered, targeted, and more effective treatment options may be developed.

Cytokines produced within the tumor microenvironment can promote cancer cell growth, attenuate apoptosis, and facilitate invasion and metastasis, making them attractive therapeutic targets. STAT3 is constitutively active in many human cancers, including endometrial cancer (5) and has the capacity to promote epithelial tumor growth (6). Consequently, STAT3-activating cytokines, such as IL6, are of much interest. In particular, antibodies that neutralize IL6 or block IL6 receptor (R) are in clinical trials for ovarian, prostate, and renal cancers (7,8). In an orthotopic nude endometrial carcinoma model, IL6 promoted tumor growth (9), while targeted inhibition of the IL6 receptor dramatically reduced tumor growth in HEC1A cell–derived subcutaneous xenografts in immunodeficient mice (10).

IL11 is a pleiotropic cytokine that regulates cell cycle, invasion, and migration in numerous cell types (11,12). IL11 is a member of the IL6 family of cytokines, which also includes leukemia-inhibitory factor (LIF), oncostatin M, cardiotoxin-1, ciliary-neurotrophic factor, cardiopin-like cytokine/cytokine-like factor, IL27 and IL31. This family shares a common accessory signaling molecule, glycoprotein (gp) 130, though IL11 signals via its own distinct ligand-specific receptor (R) α subunit. This infers nonredundancy between the IL6 family members that share gp130 signal transducer (13). In the human endometrium, IL11 activates the JAK/STAT3 pathway (14).

The expression of IL11 in the female reproductive tract and reproductive cancers has previously been reviewed (15). In women with endometrial cancer, IL11 levels are elevated in uterine lavage fluid and are positively associated with higher endometrial tumor grades (16). IL11 protein (16) and mRNA (17), along with IL11Rα protein levels are progressively increased with increased endometrial tumor grade (16), which is in line with findings in other tumor types such as ovarian and colorectal (18,19). Studies have reported protumorigenic roles for IL11 in several epithelial cancers, including breast (20) and colorectal cancer (19) and one recent study highlighted a predominant role for IL11 rather than IL6 in mediating gastric cancer (21). We have previously demonstrated that IL11 promotes endometrial cancer cell migration in vitro, via STAT3 (22). However, IL11 regulation of endometrial cancer cell–invasive properties has not been fully evaluated and the specific role of IL11 in endometrial cancer metastasis in vivo remains unknown.

We hypothesized that IL11 contributes to endometrial tumor growth and metastasis in vivo. We aimed to demonstrate this by inhibiting IL11 signaling using mAb specific for the human or mouse IL11Rα subunits and evaluating subcutaneous and orthotopic endometrial tumor growth in female Balb/c athymic nude mice. In vitro, we investigated the effect of IL11 signaling inhibition on Ishikawa and HEC1A endometrial epithelial cancer cell proliferation, invasion, and migration using the xCELLigence real-time monitoring system.

Materials and Methods

Cell line and culture

Ishikawa cells were provided by Dr. M. Nishida (Tsukuba University, Tochigi, Japan, 2014) and cultured in DMEM with 10% fetal calf serum (FCS). HEC1A cells purchased from the ATCC (2013) and cultured in McCoy medium with 10% FCS. Cell line authentication was tested using short tandem repeat analysis (July 2015). To track the cells in vivo bioluminescence imaging, GFP expression was transfected into HEC1A cells using miRiDlAN shMIMIC lentiviral particles (Thermo Scientific) and scrambled negative control. These were transfected at MOI in HEC1A cells (5 × 10³ cells/well), after 72-hour turboGFP expression was confirmed by microscopy and puromycin selection marker added for continued culture.

ELISA

Ishikawa and HEC1A cells were seeded into 12-well culture plates (1 × 10⁵ cells/well; n=3 passages in duplicate) in 1 mL serum-free medium. Media were collected after 24 hours and levels of IL11 secreted by Ishikawa and HEC1A cells were assayed using a human IL11 ELISA (#ELH-IL11; RayBiotech) according to the manufacturer’s instructions.

IL11Rα blocking antibodies

Anti-human (h) IL11Rα, anti-mouse (m) IL11Rα–blocking antibodies and IgG control were provided by CSL Ltd.

xCELLigence real-time functional assays and cell treatments

Experiments were carried out using the RTCA DP xCELLigence instrument (Roche), which was placed in a humidified incubator maintained at 37°C with 95% air/5% CO₂. For proliferation, cells were seeded in E-plate 96 at 10,000 cells/well in 5% FCS medium and the plate was monitored once every 15 minutes for a total of 72 hours. At the time of seeding for xCELLigence real-time proliferation assays, Ishikawa and HEC1A cells were treated with 1 μg/mL, 5 μg/mL, or 10 μg/mL hIL11R α antibody, or IgG control. For subsequent invasion and migration functional assays, HEC1A cells were treated with 5 μg/mL hIL11R α antibody, or IgG. Cell migration and invasion were assessed using CIM-plate 16 (Roche) with 8-mm pores. To measure cell invasion, wells were coated on the top surface of the transwell with Matrigel (BD Biosciences; 1:10 dilution). To measure migration, the same protocol was used on an uncoated plate. Cells were seeded into the top chamber at 10,000 cells/well in serum-free medium and 10% FCS medium added to the bottom chamber for chemotaxis. The CIM-plate 16 was monitored every 15 minutes for 72 hours total. Data was calculated using RTCA software 1.2, supplied with the instrument (ACEA) and exported for statistical analysis.

Flow cytometry and cell-cycle analysis

HEC1A cells were serum starved for 24 hours to synchronize populations into G₀. Medium was replaced with complete media containing 10% FCS, and cells treated with 5 μg/mL hIL11R α antibody, or IgG. Cells were harvested after 8, 24, or 48 hours and fixed overnight in 70% ethanol. Cells were stained with FixCycle PI/RNase staining solution (Molecular Probes) and analyzed on a BD FACSCanto II flow cytometer. Cell cycle and apoptosis analysis and model fitting was performed with FlowJo (FlowJo LLC).

Cytospins and immunocytochemistry

HEC1A cells were cultured with control media only, recombinant human IL11 (100 ng/mL), hIL11R α antibody (5 μg/mL), mIL11R α antibody (5 μg/mL), IgG (5 μg/mL), or IL11 + hIL11R α antibody, IL11 + mIL11R α antibody, or IL11 + IgG for 30 minutes. Cells were trypsinized and cell suspensions collected.
and centrifuged at 438 × g for 10 minutes. The supernatant was discarded and cell pellet resuspended in 1 mL of PBS (200 μL containing 300,000 cells/mL) was applied per well (4-well chamber) by cytocentrifugation at 4,000 U/minute (Hettich Centrifuge, Universal 16A) for 10 minutes. Slides were air-dried overnight at room temperature, then fixed in 70% ethanol for 10 minutes. Immunocytochemistry for phosphorylated (p) STAT3 was carried out as detailed below.

Animals
Animal experiments were conducted in female, 5- to 7-week-old, athymic, BALB/c nude mice purchased from Animal Resources Centre; Western Australia, housed in specific pathogen-free conditions, with food and water available ad libitum and held in a 12-hour light and dark cycle. Use of all animals was in accordance with the guidelines of the Monash Medical Centre Animal Ethics Committee under Ethics Approval number MMCB/2012/07.

Subcutaneous tumor inoculation
Ishikawa and HEC1A were resuspended in serum-free medium at a concentration of 20 × 10^6 cells/mL. Both flanks of each animal were inoculated with 100 μL (2 × 10^6 cells; n = 10 mice/treatment group). Once palpable, tumors were measured with digital calipers (Hare & Forbes Machinery House) and tumor volume calculated using the following formula: (length × width^2)/2 (mm^3; ref. 23).

Orthotopic endometrial tumor inoculation
The orthotopic model was performed as described previously (24) with some modifications. Briefly, mice were anesthetized with ketamine (100 mg/kg) xylazine (5 mg/kg) injected intraperitoneally. Approximately 100 μL of 5% agar was inserted into the mouse vagina using a 1-mL syringe, to form a plug to prevent leakage of injected cells. The dorsal skin was cleaned and sterilized with betadine and a 0.5-cm incision was made in the right lower flank to optimize exposure to the right uterine horn. The distal portion of the horn was identified and externalized. Surgical thread was looped around the top of the uterine horn to prevent leakage of cells out of the oviduct, before a single-cell suspension of 30 μL containing 2 × 10^6 cells was injected into the lumen of the uterine horn. The injection site was closely monitored during and following injection to ensure no spillage into the peritoneal cavity occurred. The incision was then closed with Microl clips and VetBond glue. Mice were monitored and allowed to recover on a heat pad and postoperatively carprofen was administered subcutaneously (5 mg/kg). At the completion of the experiment, in vivo bioluminescence imaging was performed to identify GFP-expressing metastases and micrometastases. Tumor weight was determined as detailed below.

RNA preparation and quantitative real time RT-PCR
Total RNA was isolated from snap-frozen subcutaneous xenograft endometrial tumor tissue using TriReagent (Sigma-Aldrich). Genomic DNA was digested using the DNA free kit (Ambion) according to the manufacturer’s instructions. RNA samples were analyzed by spectrophotometry (Nanodrop) at an absorbance ratio of A260/280 nm to determine RNA concentration, yield, and purity. cDNA was synthesized from total RNA (250 ng) using Superscript III reverse transcriptase (Invitrogen) and analyzed by spectrophotometry at an absorbance ratio of A260/280 nm to determine concentration and purity. Real-time RT-PCR analyses were performed on the ABI 7500HT fast block real-time PCR system (Applied Biosystems) in triplicate (final reaction volume, 10 μL) in 384-well Micro Optical plates (Applied Biosystems). For each sample, 25 ng of cDNA was added to a PCR mix made with the 2× Fast-Start SYBR green master mix containing ROX passive reference dye (Applied Biosystems) and 10 nmol/L primers. The primer sequence details are listed in Supplementary Table S1. The PCR protocol was as follows: 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Relative expression levels were calculated by the comparative cycle threshold method (ΔΔCt) as outlined in the manufacturer’s user manual, with 18s ribosomal RNA serving as the endogenous control for normalization.

IHC and immunofluorescence
Formalin-fixed subcutaneous endometrial tumor sections (4 μm) were dewaxed in histosol (2 × 10 minutes), rehydrated in ethanol, and antigen retrieval performed in 0.01 mol/L sodium citrate (pH 6) before endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 10 minutes. Nonspecific binding was blocked with 10% normal goat serum and 2% normal human serum, in Tris buffered saline (TBS) for 30 minutes. Cleaved caspase-3 (Asp175; Cell Signaling Technology, #9661; 1:500), cytoketatin (Santa Cruz Biotechnology, #sc-h240; 1:200), cyclin D3 (Santa Cruz Biotechnology, #sc-16; 1:1,000), phosphorylated (p)STAT3 (Tyr705; Cell Signaling Technology, #9145s; 1:100), or phosphorylated (p)-p44/42 MAPK (ERK1/2; Thr202/Tyr204; Cell Signaling Technology, #4370; 1:200) antibody was applied overnight at 4°C. Negative control isotype rabbit IgG (Dako) was included for every tissue section. Antibody localization was detected by sequential application of biotinylated goat anti-rabbit IgG (1:200) for 30 minutes followed by Vectastain ABC Elite kit (Vector Laboratories) for 30 minutes. Peroxidase activity was visualized by the application of diamobenzidine substrate (DakoCytomation). Tissues were counterstained with Harris hematoxylin (Sigma-Aldrich) and mounted. Using CellSense software, eight photographs at 20× magnification were taken from each tumor section representing more than 90% of the tumor cross section. A blinded observer counted the numbers of pSTAT3 or cleaved caspase-3–positive cells. The values from each field were averaged and expressed as number of positive cells/field for each tumor. For immunofluorescence co-localization, formalin-fixed sections were treated as described.
above with modifications as follows: nonimmune serum diluted in and washed performed in PBS, primary antibody for vimentin (Santa Cruz Biotechnology #sc-20; 1:1500), cytoketatin (as above), or nonimmune goat IgG (isotype negative control); secondary antibody incubation (donkey α-mouse Alexa Fluor 488 and donkey α-goat Alexa Fluor 594; both 1:200) in nonimmune serum for 2 hours at room temperature; following further washes, NuRed (Life Technologies) was applied for 20 minutes and then sections were mounted using Vectastain (DAKO).

Statistical analysis
Statistical analysis was carried out using GraphPad Prism (GraphPad Software 6.0) and data assessed by Student t test for two groups. Multiple groups were compared using one-way ANOVA, with Tukey post hoc test. Results with P values < 0.05 were considered statistically significant.

Results

Anti-human IL11Rα antibody reduces endometrial cancer cell proliferation and invasion in vitro
HEC1A cells secrete approximate three times higher levels of IL11 protein (597.5 pg/mL± 68.8) compared with Ishikawa cells (226.7 pg/mL± 22.0; Supplementary Fig. S1). Ishikawa and HEC1A endometrial epithelial cancer cells also differentially express the IL11Rα subunit (Supplementary Fig. S1). Endometrial cancer grade 2–derived HEC1A cells express higher levels of IL11Rα mRNA that endometrial cancer grade 1–derived Ishikawa cells, suggesting the potential for different IL11 content with different tumor grade. Ishikawa and HEC1A cells were used for in vitro and in vivo functional studies. Treatment of Ishikawa cells with hIL11Rα antibody significantly reduced cell proliferation after 48 hours (P < 0.05) compared with IgG control (Fig. 1A). There was no concentration–response effect of the antibody at 1.5, or 10 μg/mL, suggesting that the lowest concentration, 1 μg/mL is sufficient to block IL11 signaling and produce a maximal functional effect (Fig. 1A). In HEC1A cells, hIL11Rα antibody treatment significantly reduced cell proliferation after 48 (P < 0.05) and 72 hours (P < 0.01; Fig. 1B). Suppression of IL11Rα activity in the HEC1A cell line by a single dose of the hIL11Rα antibody significantly impaired cell growth by 28% (P < 0.001; Fig. 1C) and increased apoptosis by 22% (P < 0.001; Fig. 1D) relative to the IgG control. In HEC1A cells, hIL11Rα antibody treatment significantly reduced cell invasion (P < 0.05; Fig. 1E) and cell migration at 36 hours (P < 0.05; Fig. 1F) compared with IgG control. In HEC1A cells, STAT3 is phosphorylated under basal conditions and exogenous IL11 further enhanced this (Fig. 1G). Blockade of the IL11Rα using hIL11Rα antibody reduced endogenous STAT3 activation and also impaired IL11-induced STAT3 activation in vitro (Fig. 1G).

Anti-human IL11Rα antibody reduces subcutaneous endometrial tumor growth in vivo
IL11 blockade transiently reduced subcutaneous Ishikawa endometrial xenograft tumor growth between 10 and 15 days after initial administration of the hIL11Rα antibody (P < 0.05; n=10). However, by the study endpoint (21 days treatment), there were no differences in the tumor size between antibody and IgG control treatment groups (Fig. 2A). In the HEC1A endometrial xenograft tumor model, the hIL11Rα antibody significantly reduced tumor volume from 7 days of treatment until the study endpoint at day 25 (226.4 mm³± 38.7 vs. IgG 588.6 mm³± 44.19; P < 0.01; n=10; Fig. 2B). This resulted in a 45% reduction in tumor weight by the study endpoint (hIL11Rα antibody 0.155 g± 0.04 vs. IgG 0.344 g± 0.03; P < 0.01; Fig. 2C and D). IFHC for cyclin D3, a known IL11-regulated cell-cycle target, showed a reduction in cyclin D3 protein levels in HEC1A cell-derived tumors treated with antibody compared with IgG control (Fig. 2E). However, levels were similar between treatment groups in Ishikawa cell-derived tumors (Fig. 2E).

Anti-human IL11Rα antibody reduces intrauterine HEC1A endometrial tumor growth and metastasis in vivo
Intrauterine inoculation of GFP-HEC1A resulted in the formation of primary uterine tumors in the endometrial lining by 7 days and mice became moribund by 4 to 6 weeks. On the basis of this, mice were administered with hIL11Rα antibody or IgG from 7 days postinoculation, for 3 weeks. At the endpoint of the study, in vivo bioluminescence (BLI) imaging revealed an obvious reduction in primary uterine tumor size and also in metastatic lesions (Fig. 3A). This was confirmed by a reduction in endometrial tumor mass (hIL11Rα antibody 0.916 g± 0.17 vs. IgG 0.516 g± 0.09; P < 0.05; n = 10; Fig. 3B) and the number of metastatic lesions in response to hIL11Rα antibody treatment (hIL11Rα antibody 4.55± 0.99 vs. IgG 12.44± 2.39; P < 0.01; Fig. 3C). Endometrial tumor formation was confirmed histologically, revealing large areas of necrosis in hIL11Rα antibody–treated tumors compared with the IgG control group (Fig. 3D).

Targeting epithelial, but not stromal, IL11Rα reduces HEC1A endometrial tumor growth and promotes apoptosis in vivo
To distinguish the effects of IL11 signaling on the tumor epithelial versus stromal compartments, mice with HEC1A subcutaneous tumors were administered with hIL11Rα antibody or IgG control as described previously, or mIL11Rα antibody alone, or in combination with hIL11Rα antibody. Treatment with IL11Rα alone (P < 0.01), or in combination with mIL11Rα antibody (P < 0.05) significantly reduced tumor growth in the HEC1A xenografts by the endpoint of the study at day 31 compared with the mIL11Rα antibody or IgG control groups (Fig. 4A; n=10). Although targeted blockade of the mouse IL11Rα using mIL11Rα antibody transiently reduced tumor growth between 10 and 17 days (P < 0.05), by day 31, there were no differences in the tumor size between mIL11Rα antibody and IgG control treatment groups (Fig. 4A). To determine whether blocking IL11Rα promotes apoptosis in endometrial tumors, cleaved caspase-3 immunostaining was performed on tumor sections (Supplementary Fig. S2A). An increase in the number of apoptotic cells was evident in tumors from mice treated with the hIL11Rα antibody compared with all other treatment groups (hIL11Rα antibody 28± 4 cells/field of view vs. IgG 14± 2 cells/field of view; P < 0.01; Supplementary Fig. S2B).

IL11 inhibition downregulates STAT3 and IL11 signaling components, but does not alter gene expression of other IL6 family cytokine members
In control subcutaneous HEC1A tumors, STAT3 is constitutively active in the tumor epithelium, stroma, and vascular endothelial cells (Fig. 4B–D). Treatment with hIL11Rα antibody significantly reduced endometrial cancer epithelial cell pSTAT3 compared with IgG controls (P < 0.01; Fig. 4B–D). Administration of mIL11Rα antibody alleviated endometrial cancer mouse-
derived, cytokeratin-negative nonepithelial STAT3 activation (likely in stromal and endothelial cells), but had no effect on tumor epithelial pSTAT3 (Fig. 4B–D). Likewise, in vitro the mIL11Rα antibody did not reduce endogenous HEC1A STAT3 activation, or IL11-induced STAT3 activation (Supplementary Fig. S3). In contrast, the hIL11Rα antibody treatment had no effect on ERK1/2 activation compared with control tumors (Supplementary Fig. S4) while reducing IL11 gene expression and significantly...
reducing IL11Rα (P < 0.01) and GP130 (P < 0.05) mRNA in subcutaneous HEC1A tumors (Supplementary Fig. S5). IL6 and LIF mRNA expression were unchanged between groups (Supplementary Fig. S5).

Targeting epithelial IL11Rα retains well-differentiated, epithelial endometrial phenotype

Administration of hIL11Rα antibody, but not mIL11Rα antibody, led to retention of tumor epithelial phenotype as demonstrated by increased cytokeratin immunostaining (Fig. 5A). Cytokeratin staining was localized to the endometrial tumor cell parachyme, with some perinuclear, cytoplasmic, and membranous staining. In contrast, there was a reduction in cytokeratin staining in the IgG control–treated tumors (Fig. 5A). To confirm whether EMT was impaired in response to IL11Rα blockade in the tumor epithelial cells, cytokeratin (epithelial marker) and vimentin (mesenchymal marker) colocalization immunofluorescence was performed. Minimal vimentin, but abundant cytokeratin was found in the hIL11Rα antibody–treated tumor compared with IgG control (Fig. 5B). In response to hIL11Rα antibody treatment, a key EMT regulator, Snail, was significantly downregulated at the mRNA level (P < 0.05), while Twist was downregulated and there was a slight increase in Ecadherin (Ecad) transcript levels (Fig. 5C). Changes in Snail were also confirmed at the protein level by IHC. In the IgG control–treated tumors, Snail localized to the cell cytoplasm and also the nucleus (where it is required to be for active transcription). However, in the hIL11Rα antibody–treated tumor sections, Snail was less abundant with little to no nuclear staining (Fig. 5D).

Discussion

We and others previously demonstrated that IL11 is upregulated in endometrial cancer in women (16,17) and we have shown that IL11 alters endometrial cancer cell migration in vitro, via STAT3 (22). This is the first study to determine a functional role for IL11 in endometrial cancer in vivo and establish that IL11 inhibition impairs endometrial tumor growth and metastasis. Using two xenograft mouse models, subcutaneous and orthotopic, and two antibodies that distinguish between the mouse or human IL11Rα signaling subunit, we demonstrated that targeted blockade of human-derived cancer epithelial cell, but not mouse-derived cell, IL11Rα signaling led to retention of tumor epithelial phenotype as demonstrated by increased cytokeratin immunostaining. The current study provides evidence that IL11 plays an important role in promoting epithelial tumor cell migration and invasion in vitro and in vivo, and the selective targeting of human-derived cancer epithelial cell IL11Rα signaling is a potential approach for the development of targeted therapies for endometrial cancer.
derived stromal or vasculature IL11 signaling is required to reduce tumor growth in vivo (Fig. 6).

Using two endometrial cancer cell lines, IL11Rα blockade reduced IL11 action, but there was a blunted functional response in grade 1 Ishikawa endometrial cancer—derived cells compared with grade 2 HECA1 cells, likely due to differences in IL11 ligand production and/or IL11Rα expression levels between the cell lines. IL11Rα blockade only transiently reduced Ishikawa cell proliferation in vitro and tumor growth in vivo. When protein levels of cyclin D3 were determined, hIL11Rα antibody treatment downregulated this established IL11-regulated cell-cycle target (21,25) in HECA1 tumors compared with IgG control. However, there was no effect of IL11Rα inhibition on cyclin D3 levels in Ishikawa tumors. This finding coincided with a more profound reduction in HECA1 cell proliferation in response to IL11 blockade in vitro, compared with Ishikawa cells. Both Ishikawa and HECA1 cells are responsive to exogenous IL11, as shown previously by STAT3 activation (22). However, as Ishikawa cells endogenously expressed lower IL11Rα mRNA levels than HECA1 cells, this may alter their responsiveness to the blocking antibody. Ishikawa cells also secreted lower levels of IL11 and are innately less invasive than HECA1 cells; they did not invade under control conditions using the xCELLigence system and did not form intrauterine tumors or metastatic lesions in pilot studies in immunodeficient Balb/c mice. This is an important finding and indicates that the reduction in IL11Rα and/or ligand production is crucial to invasive tumorigenic behavior in endometrial cancer cells.

In immunolocalization studies, we have shown IL11 and IL11Rα are not only produced by malignant epithelial tumor cells in human endometrial cancer, but are also highly expressed in tumor-associated vascular cells and stroma (16). Cancer cells rely on the tumor microenvironment, consisting of vasculature, immune cells, and fibroblasts as well as other components for sustained growth and metastasis (26). Targeting the factors that affect the microenvironment improves treatment delivery and patient outcomes (27). We have previously linked IL11 activity with changes in extracellular matrix and vasculature in the endometrium (28). Thus, through the administration of two distinct mouse or human IL11Rα antibodies, we sought to identify the precise effects of targeted IL11 signaling blockade on the malignant tumor epithelial cells versus the tumor microenvironment in xenograft tumors. Interestingly, targeting only tumor epithelial IL11 signaling resulted in reduced tumor growth, despite the fact that the mIL11Rα antibody blocked stromal and vascular endothelial STAT3 activation. The HECA1 cells used in these tumor models express IL11 and IL11Rα mRNA; however, levels were reduced in response to IL11 inhibition in vivo, suggesting that there is the potential for an autocrine signaling loop responsible for tumor establishment. This therefore implies that tumor establishment or progression is not reliant on effects of IL11 on tumor-associated stroma and vascular cells.

In addition to downregulated expression of IL11Rα and gp130, IL11 inhibition resulted in reduced STAT3, with no effect on ERK activation in HECA1 tumors. In addition to the JAK/STAT pathway, IL11 can signal via the MAPK/ERK, or the PI3K pathways (13). We have previously demonstrated that exogenous IL11 phosphorylates STAT3, but not ERK, or AKT in Ishikawa and HECA1 cells (22). This confirms that IL11 is not acting via these pathways. Although it is well established that STAT3 activation prevents tumor cell apoptosis in numerous cancer types (29,30), this is frequently attributed to IL6-mediated STAT3 activation. As IL6 and LIF IL11 family members, are also implicated in the development of numerous solid tumors (31) and endometrial cancer (10), we investigated their expression levels in tumors after hIL11Rα antibody treatment. Targeting IL6 receptor in endometrial cancer has been demonstrated to reduce tumor growth (10); however, in our study, neither IL6 nor LIF mRNA levels were altered, suggesting there was no redundancy between IL11 and these family members.

Blocking IL11 signaling impairs cell proliferation. Furthermore, from immunohistologic analysis of tumors and flow cytometry performed on HECA1 cells, it was evident that IL11 blockade also promoted apoptosis. It is well known that IL11 can play an antiapoptotic role (32) and here we have demonstrated that IL11 blockade induces apoptosis in endometrial tumor xenografts.
Considerable evidence suggests that IL11 is also required for epithelial cell proliferation and survival, leading to the initiation and progression of cancer in the gastric mucosa and colon in humans and mice (21). In addition to cyclin D3 (25), IL11 has been shown to regulate other cell-cycle and cell survival targets, via the STAT3 pathway. While simultaneously suppressing the cell-cycle inhibitor p21, IL11-mediated STAT3 activation also upregulates intrinsic antiapoptotic proteins Bcl-xl, Bcl-2, and survivin (21). Survivin, a protein that promotes proliferation and inhibits apoptosis, is overexpressed in many cancers (33) and is upregulated by trophoblast cells (34). Interestingly, survivin and p21 are regulated in response to IL11 in the uterus in mice (35).

IL11Rα inhibition also impaired the nuclear translocation and mRNA expression of the EMT regulator Snail. This resulted in an epithelial tumor type, characteristic of a less invasive phenotype, likely due to the suppression of epithelial to mesenchymal transition. The transition of an epithelial phenotype towards a more mesenchymal phenotype is a subsequent step, which leads to further progression to invasive disease. Central to this epithelial to mesenchymal transition (EMT) is the activation and induction of Twist and Snail-induced transcription, eventually causing degradation of the basement membrane by induction of matrix metalloproteinases, loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as vimentin (36). In the current study, it was observed that IL11 inhibition resulted in a strong trend in reduced HEC1A cell migration and a significant reduction in invasion in vitro. IL11 blockade downregulated the mesenchymal marker vimentin and led to a retention of cytokeratin-positive epithelial cells in vivo. Most importantly, targeting IL11 impaired peritoneal metastasis in the orthotopic intrauterine xenograft model. Assessment of pathways involved in EMT showed IL11 inhibition significantly downregulated Snail and reduced Twist gene expression. To be transcriptionally active, Snail must be phosphorylated and translocate to the nucleus. We confirmed a dramatic reduction in nuclear phosphorylation of Snail protein following hIL11Rα antibody treatment in orthotopic tumors compared with IgG controls. These findings suggest that IL11 inhibition can prevent cancer cell migration and invasion due to inhibition of EMT (Fig. 6).

The results of the current study support findings in other epithelial-derived tumor types, whereby impaired IL11 signaling has been shown to reduce gastric cancer progression (21) and also prostate cancer metastasis (37) strengthening the rationale for therapeutically targeting IL11 in endometrial cancer. Recently, IL11 was implicated in experimental models of chronic inflammation and associated tumorigenesis, mediated, at least in part, by overactivation of STAT3 (38). In addition, high IL11 and IL11Rα expression are associated with tumor progression, cellular growth, and differentiation and...
poor prognosis in a number of cancers including colorectal (19), gastric (38), hepatocellular (39), and breast (20). IL11 is also highly expressed in metastatic lesions of hepatocellular carcinoma (40), and increases the metastatic potential of breast cancer (41). Furthermore, a recent study using a breast cancer cell line (MDA-MB-468) xenograft model showed that IL11 expressing subclone populations in heterogeneous tumors acted in a non-cell–autonomous manner, causing non IL11-expressing cells to behave in the same manner to drive proliferation and tumor growth (42). Together, these findings highlight an emerging and predominant role for this cytokine in promoting tumorigenesis.

Although little is known of the functional role of IL11 in endometrial cancer, IL11 is essential for normal reproduction in the human and mouse (15). In the cycling human endometrium, IL11 is regulated by prostaglandin E(2) (43). In other epithelial tumor types, IL11 is regulated by hypoxia (44) and oxidative stress (32). Although hypoxia did not alter IL11 levels in endometrial cancer cell lines (unpublished observations). Previously, PGE2 and hypoxia have been shown to synergize to promote cellular proliferation of endometrial adenocarcinoma cells (45). Interestingly, in primary human endometrioid cancer tissue explants and Ishikawa cells, IL11 is regulated by prostaglandin signaling via the calcium-calcineurin nuclear factor of activated T cells (NFAT) pathway (17), suggesting that IL11 expression may be induced in endometrial cancer in response to inflammatory stimuli. While these previous findings provide some insight into what drives IL11 overexpression in endometrial tumors, they do not explain the mechanism(s) of action, which were investigated here.

In conclusion, intact IL11 signaling in endometrial cancer has emerged as an important factor stimulating epithelial tumor cell proliferation and promoting tumor cell transition from an epithelial to a more mesenchymal, more invasive phenotype.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: A.L. Winship, E. Dimitriadis
Development of methodology: A.L. Winship, J. Donoghue, K. Rainczuk

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**Figure 5.** Targeting epithelial, but not stromal, IL11Rx retards well-differentiated, epithelial phenotype and prevents epithelial to mesenchymal transition (EMT) in HECA1 endometrial tumors. A, representative photomicrographs of cytokeratin (epithelial cell marker) immunostained HECA1 subcutaneous tumor sections from mice administered with hIL11Rx antibody, mIL11Rx antibody, hIL11Rx antibody and mIL11Rx antibody combined, or IgG control. B, immunofluorescence colocalization for cytokeratin (epithelial marker; red) and vimentin (mesenchymal marker; green) was performed on hIL11Rx antibody or IgG control intrauterine HECA1 tumor sections. C, quantitative real-time PCR determined mRNA expression levels of key EMT regulators including E-cadherin (Ecad), Twist, and Snail, expressed as ΔΔCt values. Data are mean ± SEM. Student t-test; *, P < 0.05 (n = 10/group). D, representative photomicrographs of Snail immunostained intrauterine tumor sections. Bars, 200 μm (A) and 50 μm (Band D). Insets, negative controls.

**Figure 6.** Proposed mechanism(s) of action of IL11 signaling in endometrial cancer.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.L. Winship, M.V. Sinderen, J. Donoghue. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.L. Winship, M.V. Sinderen, J. Donoghue, K. Rainczuk, E. Dimitriadis Writing, review, and/or revision of the manuscript: A.L. Winship, M.V. Sinderen, J. Donoghue, E. Dimitriadis Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Donoghue, K. Rainczuk Study supervision: E. Dimitriadis

Acknowledgments

The authors thank CSL Limited for providing the anti-human and mouse IL11R blocking antibodies and IgG control. The authors also acknowledge the support of the Victorian Government’s Operational Infrastructure Support Program and the Australian Government NHMRC IRIISS.

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Received August 14, 2015; revised January 13, 2016; accepted January 28, 2016; published OnlineFirst February 4, 2016.

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Grant Support

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Molecular Cancer Therapeutics

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Amy L. Winship, Michelle Van Sinderen, Jacqueline Donoghue, et al.


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