Interleukin-6 Stimulates Defective Angiogenesis

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Abstract

The cytokine IL6 has a number of tumor-promoting activities in human and experimental cancers, but its potential as an angiogenic agent has not been fully investigated. Here, we show that IL6 can directly induce vessel sprouting in the ex vivo aortic ring model, as well as endothelial cell proliferation and migration, with similar potency to VEGF. However, IL6-stimulated aortic ring vessel sprouts had defective pericyte coverage compared with VEGF-stimulated vessels. The mechanism of IL6 action on pericytes involved stimulation of the Notch ligand Jagged1 as well as angiopoietin2 (Ang2). When peritoneal xenografts of ovarian cancer were treated with an anti-IL6 antibody, pericyte coverage of vessels was restored. In addition, in human ovarian cancer biopsies, there was an association between levels of IL6 mRNA, Jagged1, and Ang2. Our findings have implications for the use of cancer therapies that target VEGF or IL6 and for understanding abnormal angiogenesis in cancers, chronic inflammatory disease, and stroke.

Introduction

IL6 is a major tumor-promoting cytokine produced by both malignant and host cells in the tumor microenvironment (1). It is also a downstream product of oncogenic mutations, for example, ras and TP53 (2, 3). Typically via its major downstream signal transducer STAT3, IL6 has both local and systemic protumor actions in experimental and human cancers. In the tumor microenvironment, these include stimulation of malignant cell growth and survival (4), promotion of invasion and metastasis (5), modulation of tumor-promoting T-cell subtypes, involvement in autocrine tumor cell cytokine networks (6), and regulation of the myeloid cell infiltrate (7). Systemic effects of excess IL6 production include induction of acute phase reactants and inflammation (8). There is a complication of many common human cancers.

To add to this catalogue of tumor-promoting actions, there are reports that IL6 stimulates angiogenesis in the tumor microenvironment (9) with evidence that STAT3 signaling induces hypoxia-inducible factor-mediated VEGF-A transcription (10). IL6 is also reported to have direct effects on endothelial cell proliferation and migration (9, 11, 12) and has been implicated in resistance to anti-VEGF antibody treatment in patients (13, 14). In preclinical and clinical studies, we found that a therapeutic neutralizing anti-IL6 antibody reduced systemic VEGF levels in ovarian cancer patients, and that in peritoneal ovarian cancer xenografts, blood vessels were reduced, with a concomitant inhibition of the Notch ligand Jagged1 (7).

This led us to study further the actions of IL6 in normal and cancer angiogenesis. In this article, we present novel evidence that IL6 directly stimulates angiogenesis, but in contrast with VEGF, IL6-stimulated vessels have defective pericyte coverage. We show that this might be due to differential regulation of Notch ligands and Ang2 by these two mediators. Our findings have implications for the use of cancer therapies that target VEGF or IL6.

Materials and Methods

Ethics statement

All animal experiments were approved by the local ethics review process of the Biological Services Unit, Queen Mary University of London (London, United Kingdom) and conducted in accordance with the UKCCCR guidelines for the welfare and use of animals in cancer research.

Aortic ring assay

Angiogenic sprouts were induced from mouse or rat thoracic aortas according to the method of Nicosia and Ottinetti (15). Aortas were dissected from cervical dislocated 8- to 12-week-old male C57BL/6 mice (Charles River) or 180–200 g male Wistar rats (Harlan Laboratories) and sliced into 0.5 mm sections and incubated overnight in serum-free OptiMEM (Invitrogen) at 37°C. Aortic rings were embedded in type I collagen (1 mg/mL) in E4 media (Invitrogen). For mouse aortic rings, the wells were supplemented with OptiMEM with 1% FBS and 30 ng/mL of VEGF (R&D Systems), 50 ng/mL of human IL6 (R&D Systems), or 30 ng/mL of mouse IL6 (R&D Systems) and incubated at 37°C, 10% CO2. Rat aortic ring wells were treated with OptiMEM with 1% FBS and 10 ng/mL VEGF, 10 ng/mL rat IL6, or 10 nmol/L VEGFRI (Cediranib, VEGFR2 inhibitor) and incubated at 37°C.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-15-1227
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Published OnlineFirst June 16, 2015; DOI: 10.1158/0008-5472.CAN-15-1227
10% CO₂. Angiogenic sprouts were counted after 7 days of culture for mouse aortic ring and after 4 days of culture for rat aortic rings. The length of sprouts was quantified using ImageJ software by drawing radial lines from the base of the aortic ring to the tip of the sprouting new vessel. Pericytes were quantified 2.50 microns from the tip of the aortic ring vessel to avoid false-positive quantification of activated fibroblast, which are normally found at the stalk of the vessel. Animals were housed and treated in accordance with UK Home Office Regulations.

Staining of aortic rings

The rat and mouse aortic rings were, respectively, cultured for 1 and 2 weeks before the staining. The rings were washed with PBS, fixed in 4% formaldehyde for 20 minutes. The wells were then washed once in PBS and the rings were permeabilized with 0.5% Triton X-100 in PBS for 30 minutes, before being washed twice in PBS. Of note, 100 μL of BS-1 Lectin FITC (1 mg/ml; Sigma, cat. no. L9381/L5264; 1:200), anti-actin, α-SMA Cy3 (Sigma, cat. no. C6198; 1:500), or anti-NG2 (Millipore, ab5320; 1:200) was added and incubated overnight at 4°C. For IL6Rα staining on aortic rings, 100 μL of the unconjugated (1:200) IL6Rα antibody was left overnight at 4°C. The following day the rings were washed with PBS and incubated with goat anti-rabbit Alexa 488 antibody (Life Technologies, A-11034) for 2 hours at room temperature. The plates were washed twice in PBS and the rings were removed from the 96-well plate, using a syringe needle, placed on a microscope slide, and mounted with Prolong Gold DAPI containing medium (Invitrogen, P36931). The slides were left to dry and imaged using confocal microscopy (Zeiss LSM 510 META).

Tissue culture

Mouse lung endothelial cell (MLEC) was kindly given by Professor Kirbaan Hodivala-Dilke, and was used for most of the in vitro studies. This cell line was isolated and cultured as described previously (16). Human umbilical vein endothelial cells (HUVEC; HPA Laboratories) were grown and cultured in endothelial growth medium (HPA Laboratories) and maintained within 3 to 4 passages.

Ovarian cancer cell lines

The IGROV-1 line was recently characterized as a hypermutated line but unlikely to represent HGSC. The cell line was mycoplasma tested (InvivoGen) and always maintained within 4 to 5 passages before new cells were recovered from frozen master stocks. Cells were cultured in RPMI-1640 supplemented with 10% FCS and 1% pen-strep. Cells were counted using a Vi-cell cell counter (Beckman Coulter) on days 3 and 7.

Staining of MLEC

A total of 2 × 10⁵ MLEC cells were plated on a coverslip in a 12-well plate. The coverslips were fixed with 4% formaldehyde for 30 minutes and washed with PBS (3 times for 5 minutes each). Following fixation, the cells were permeabilized with 0.1% Triton X-100 for 20 minutes and washed again in PBS. The coverslips were incubated with (1:200) rabbit IgG (R&D), (1: 300) Endomucin antibody (Santa Cruz Sc-65495). IL6Rα antibody (1:200; Santa Cruz C-20, Sc-661) overnight at 4°C. The following day, the coverslips were incubated with secondary (1:2000) goat anti-rabbit Alexa 488 (Life Technologies, A-11034) for 2 hours at room temperature and then mounted on slides with Prolong Gold DAPI containing medium (Invitrogen, P36931). The images were taken using confocal microscopy (Zeiss LSM 510 META).

Western blotting

A total of 2 × 10⁵ MLEC or HUVECs were plated in a 6-well plate with 2 ml MLEC medium (10%FBS). The following day, the supernatant was removed and the cells were treated with VEGF (30 ng/mL), human IL6 (30 ng/mL), or mouse IL6 (30 ng/mL) in 2 ml of serum-free MLEC medium for 6 hours or 24 hours. Cells were then washed with PBS and harvested using RIPA Buffer (R0278, Sigma) with 1 × proteinase inhibitors. Protein quantification was performed using the Bradford reagent (Sigma-Aldrich), according to the manufacturer’s instructions. Cell extracts (25 μg) were run on a NuPAGE Novex 4% to 12% Bis-Tris Gels, 1.5 mm and transferred to a nylon membrane. The membrane was blocked overnight (4°C in PBS with 0.1% Tween and 5% milk powder) and probed using the following antibodies: Jagged1 (1:1000, Abcam ab7771). DLL4 (1:1000, Abcam ab7280). Ang2 (1:500, Abcam ab8452). Hey1 (1:500, Abcam ab22614), Phospho-Stat3 (Tyr705) (1:1000, Cell Signaling Technology 9145), Stat3 (1:1000, Cell Signaling Technology 4904), p-ERK (1:1000, Santa Cruz Biotechnology sc-7383), ERK (1:1000, Cell Signaling Technology 9102), β-Actin (1:5000, Sigma A5316). A rabbit or mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare) incubation allowed visualization using enhanced chemiluminescence (GE Healthcare). Protein concentration equivalence was confirmed by anti-β-actin antibody.

Scratch wound migration assay

Confluent monolayers of MLEC cells were scratched with a p20 pipette tip, cells were then washed twice with PBS before the addition of serum-free growth medium with VEGF 100 ng/mL, hIL6 100 ng/mL, and mIL6 100 ng/mL. Wounds were monitored by time lapse microscopy using an Olympus IX81 Microscope Hamamatsu Orca ER digital camera. Images were acquired every 30 minutes and subsequently analyzed using ImageJ software.

Cell proliferation assay

Of note, 70,000 MLEC cells were seeded in a 24-well plate. The following day cells were treated with either 50 or 100 ng/mL of VEGF, human IL6, or mouse IL6. The plate was then incubated for 72 hours at 37°C and 5% CO2. After incubation, the cells were trypsinized and counted using the Vi-cell counter (Beckman Coulter). Each condition was repeated in triplicate.

In vivo IGROV-1 xenografts

A total of 1 × 10⁷ luciferase-expressing IGROV-1 cells (IGROV-1luc) were injected intraperitoneally (i.p.) into 20 g 6- to 8-week-old female BALB/c nu/nu mice (purchased from Charles River, UK Ltd.). After 24 hours, anti-IL6 antibody (MEDI5117) was prepared at a concentration of 20 mg/kg in sterile, endotoxin-free PBS and mice were injected i.p with 200 μL of this solution twice weekly for 4 weeks. Control mice were injected with 200 μL of 20 mg/kg IgG control antibody. MEDI5117 is a human IgG1 monoclonal antibody that binds to IL6 with sub-pM affinity and neutralizes it by preventing the binding to IL6Rα. MEDI5117 was generated using phage display technology. It bears a triple mutation (referred to as YTE) in the Fc domain of the heavy chain that extends its half-life in circulation. MEDI5117 is active in several preclinical cancer models, including NSCLC, prostate cancer, breast cancer, and ovarian cancer (H. Zhong and colleagues; Mol Cancer Ther, in revision). An IgG1 isotype control antibody was
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Figure 2.
IL6 has direct effects on mouse lung endothelial cells. A, immunocytochemistry staining for endomucin (red) and IL6Rx (green) on MLEC. B, 70,000 MLECs were plated and treated with either PBS (control) or indicated concentrations of VEGF or IL6 for 72 hours. The cells were then trypsinized and counted using a cell counter and the mean of the triplicates was calculated. Proliferation assay shows a significant increase in proliferation with VEGF (50 or 100 ng/mL), human IL6 (100 ng/mL), and mouse IL6 (50 or 100 ng/mL). C, scratch assay using a time-lapse microscope was used to measure the migration of MLEC after treatment with VEGF and IL6. Significant increases in cell migration were observed with VEGF or IL6-treated MLEC after 16 hours. Statistical analysis carried out using Student t test is shown as **, *P < 0.05; **, *P < 0.01; ***, *P < 0.001. D, Western blot analysis of protein extracted from MLEC treated with IL6 (30 ng/mL) or VEGF (30 ng/mL) for 24 hours. IL6-induced downstream pSTAT3 and VEGF-induced downstream pERK levels, indicating both pathways are independent of each other for signaling in MLEC.

Figure 1.
IL6 stimulates angiogenesis in the aortic ring assay. A, phase contrast images of aortic rings embedded in collagen I, with the indicated concentrations of VEGF or human IL6. Human and mouse IL6 experiments were carried out with aortas isolated from wild-type C57BL/6 mice (8–12 weeks) and rat IL6 experiments were carried out with aortas isolated from Wistar rats weighing 180 to 200 g. Angiogenic sprouts were counted after a week in culture for mouse aortic rings or after 4 days in culture for the rat aortic rings following treatment with VEGF, hIL6 or mIL6, or rIL6. Significant increases in microvessel sprouting were observed in VEGF and IL6-treated rings (n = 9 per group) compared with PBS-treated controls; however, no difference in number of sprouts was observed between VEGF and IL6 treated rings. The P value for medium versus VEGF (P < 0.058) and medium versus VEGF (0.054) just failed to reach significance. Statistical analysis carried out using Student t test is shown as **, *P < 0.05; ***, *P < 0.001. C, length of sprouts was measured using ImageJ analysis. Mean length of sprouts (n = 9 per group) shows no significant difference between the VEGF and IL6-treated sprouts. D, 10 nmol/L of VEGFRi inhibited vessel sprouting in VEGF (10 ng/mL)-treated rings but not in rIL6 (10 ng/mL)-treated rings. Statistical analysis carried out using Student t test is shown as **, *P < 0.05; ***, *P < 0.001. E, aortic ring vessel stained for endothelial cells using BS1 lectin (green) and for IL6R (red). F, Western blot analysis of protein extracted from mouse aortas treated with VEGF (30 ng/mL) or mouse IL6 (30 ng/mL). Mouse IL6 activated downstream pSTAT3 and VEGF induced downstream pERK levels.
Centrifuged at 1,500 rpm for 2 minutes. Samples are always kept on ice between procedures. Next, using a probe sonicator set at 40% amplitude, tissues were sonicated for 5 to 15 seconds bursts. Sonicated samples were then rotated for 30 minutes at 4°C followed by a centrifugation for 15 minutes at 13,200 rpm at 4°C. The pellet was discarded and protein concentration measured. Lysates were frozen at −80°C until loaded on gels.

IHC
Paraffin-embedded sections of tumor sections collected from IGROV-1 mouse xenograft were stained with antibodies for Jagged1 (1:100, R&D Systems, AF1277), DLL4 (1:100, R&D Systems, AF1389), and Ang2 (1:50, Abcam, AB8452). Slides were counterstained with hematoxylin. Negative controls were isotype matched. The conditions used for staining with individual antibodies were in accord with manufacturers’ recommendations.

Gene expression analysis
Table generated from a heatmap established in a previous study (Coward and colleagues; ref. 16).

Statistical analysis
Statistical analyses were carried out using Prism GraphPad software. Statistical significance was calculated using Student t test and χ² test. Findings are presented as SEM.

Results
IL6 stimulates angiogenesis in the aortic ring assay
As we had found that anti-human IL6 reduced tumor blood vessel density in human tumor xenografts (7), we tested the activity of human IL6 in the aortic ring assay. This is an ex vivo model of angiogenesis that studies the effects of mediators on normal vessel sprouting. Optimal vessel sprouting was observed in the mouse aortic ring assay 7 to 10 days after treatment with 30 ng/mL VEGF or 50 ng/mL hIL6 (Fig. 1A). Mouse IL6 (30 ng/mL) in mouse aortas and rat IL6 (10 ng/mL) in rat aortas also stimulated vessel sprouting and there was no significant difference in the number of sprouts between the VEGF and IL6 treatments (Fig. 1B). VEGF and IL6 treatments also gave similar results in terms of length of vessel sprouts (Fig. 1C).

Other reports have described that IL6 has indirect angiogenic activity via stimulation of VEGF production, and thus inhibition of VEGF action would also block IL6 activity (17). We therefore tested the action of the VEGF receptor inhibitor cediranib in the rat aortic ring assay. Cediranib significantly inhibited the sprouting activity of VEGF but had no significant activity on the actions of IL6 in this model (Fig. 1D). This result suggested that in the aortic ring model IL6 may stimulate vessel sprout formation without inducing VEGF.

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We also found that the endothelial cells of the aortic ring vessels expressed IL6 receptor, staining positive for the gp80 IL6R<sup>a</sup>; Fig. 1E). Furthermore, Western blot analyses of lysates from the aortic ring assay showed strong induction of STAT3 phosphorylation by mouse IL6 but only weak induction by VEGF (Fig. 1F and Supplementary Fig. S1). In contrast, there was strong phosphorylation of ERK in VEGF-stimulated aortic ring lysates but IL6 had no effect (Fig. 1F and Supplementary Fig. S1).

As the aortic ring assay has a mixture of cells that could complicate any analysis of signaling pathways, we used a simpler experimental system in our next set of experiments, the MLEC mouse endothelial cell line (16).

**Pericyte coverage is defective in IL6-stimulated vessels**

Over the 7 to 10 days mouse aortic ring assay incubation period, and the 4 days of the rat aortic ring assay, pericytes also develop around the endothelial cells of the vessel sprouts when they are stimulated by VEGF. Using α-SMA as a pericyte marker, we treated mouse aortic rings with VEGF, mouse or human IL6, and rat aortic rings with rat IL6, and we noticed that pericyte coverage was diminished in the IL6-stimulated cultures. Overall, there were less pericytes attached to the tips of the IL6-stimulated vessels compared with the VEGF cultures and many detached pericytes were observed in the presence of IL6 (Fig. 3A–C). This difference between number of pericytes associated with the sprout (data not shown). We also found that the endothelial cells of the aortic ring vessels expressed IL6 receptor, staining positive for the gp80 IL6R<sup>a</sup>; Fig. 1E). Furthermore, Western blot analyses of lysates from the aortic ring assay showed strong induction of STAT3 phosphorylation by mouse IL6 but only weak induction by VEGF (Fig. 1F and Supplementary Fig. S1). In contrast, there was strong phosphorylation of ERK in VEGF-stimulated aortic ring lysates but IL6 had no effect (Fig. 1F and Supplementary Fig. S1).

As the aortic ring assay has a mixture of cells that could complicate any analysis of signaling pathways, we used a simpler experimental system in our next set of experiments, the MLEC mouse endothelial cell line (16).

**IL6 has direct effects on endothelial cells**

MLECs stained with the endothelial cell marker endomucin express IL6R<sup>x</sup> (Fig. 2A), and both human and mouse IL6 at 100 ng/mL stimulated MLEC proliferation (Fig. 2B). The IL6 effect was not as potent as that of VEGF but was still significant. VEGF and human and mouse IL6 were equally potent in the "scratch" assay, which measured MLEC migration (Fig. 2C). We then used Western blotting to study the VEGF and IL6 signaling pathways in these cells. As expected, IL6 increased STAT3 phosphorylation and VEGF increased ERK phosphorylation, but IL6 stimulation did not affect phospho-ERK levels and VEGF had no effect on phospho-STAT3 (Fig. 2D and Supplementary Fig. S2) after 24 hours of treatment. Similar results were observed in HUVEC after treatment with human IL6 (hIL6) or VEGF (Supplementary Fig. S3). Earlier time points (2 and 6 hours) were also studied with similar results (data not shown). Thus, we concluded that in both the aortic ring assay and in cultures of MLEC cells, IL6 has direct effects on endothelial cells.

**Pericyte coverage is defective in IL6-stimulated vessels**

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Figure 5.
Effects of anti-IL6 antibody on in vivo IGROV-1 vasculature. A total of $1 \times 10^7$ IGROV-1-luc cells were injected i.p. into 8 weeks old Balb/c nude female mice. After 24 hours, the mice were treated with either control IgG antibody (20 mg/kg) or anti-IL6 antibody (20 mg/kg) twice a week for 4 weeks. Following this, three of the animals from each group were injected with TRITC-conjugated Bandeiraea via the tail vein, 3 minutes before being perfused with 4% paraformaldehyde. The lectin-stained frozen sections were stained for α-SMA (green; A) or NG2 (green; B). (Continued on the following page.)
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In addition, IHC of the anti-IL6–treated tumors showed decreased Jagged 1 and Ang2 staining and increased DLL4 protein (Fig. 5D). The IHC results were scored blind and the results are shown in Supplementary Fig. S7B. This was confirmed in Western blot analyses of tumor lysates (Fig. 5E and Supplementary Fig. S7C).

Correlations from human ovarian cancer biopsies

Finally, we revisited a previously published analysis of mRNAs that are associated with high IL6 expression (7) using publicly available datasets of ovarian cancer. Ang2 and Jagged1 had significant a coefficient of correlation with high IL6 expression ($P = 0.015$ and $P = 0.001$, respectively; Fig. 6A). Further in-depth analysis of these associations was carried out on RNAseq data from 27 samples of omental metastases from patients with stage III/IV high-grade serous ovarian cancer (HGSC). The samples were divided into three groups based on histologic analysis: uninvolved omental tissue, established tumor, and stroma with low tumor burden postchemotherapy. Figure 6B shows typical histology of each group. Only within the established tumor groups was a strong and significant correlation (Spearman $r$) seen between IL6, Jagged 1, and Ang2 mRNA (Fig. 6C). There was no significant correlation between IL6 and DLL4 mRNA in the same samples (data not shown).

Discussion

A role for IL6 in pathogenic angiogenesis has been suggested in diseases such as stroke, rheumatoid arthritis, and various cancers (1, 30, 31). In a previous publication, we showed that treatment of ovarian cancer xenografts with an anti-IL6 antibody reduced the tumor vasculature with concomitant inhibition of the NOTCH ligand Jagged1, which has been implicated in vessel sprouting (7).

Collectively, the published literature suggested that IL6 could drive abnormal angiogenesis and that the anti-IL6 antibody had a potential as an antiangiogenic agent. Thus, we investigated the direct effects of IL6 on normal angiogenesis using endothelial in vitro and ex vivo studies, and used the findings from those studies to explore its importance in tumor angiogenesis using peritoneal models of ovarian cancer and ovarian cancer biopsies.

We found that IL6 is as potent as VEGF in inducing vessel sprouting in the aortic ring assay and was also able to stimulate endothelial cell migration and proliferation in MLEC cells. VEGFR inhibition studies in the aortic ring model and protein analysis of downstream VEGF and IL6 signaling indicate that the angiogenic effects observed with IL6 may not depend on VEGF in endothelial cells. The effects on IL6 on malignant cells may be different because of the complex autocrine signaling networks generated in cancer cells. Preliminary experiments on malignant cell lines showed that, in contrast with endothelial cells, IL6 can stimulate VEGF signaling and vice versa. In addition, in the RNAseq experiments of Fig. 6, we found that there was a positive association between IL6 and VEGF mRNA levels, but this would be expected in the contralateral tumor side.
when studying isolates from a complex multicellular tumor microenvironment. Hence, we suspect that IL6 may have different effects on malignant cells and endothelial cells.

We found that the angiogenesis stimulated by IL6 leads to formation of vasculature with defective pericyte coverage, and that anti-IL6 treatment of the peritoneal ovarian cancer xenografts leads to restoration of pericytes on the blood vessels. Investigating the mechanism of action of IL6 and VEGF in inducing this different phenotype of vessel maturation has shown roles for Notch ligands and Ang2.

This is, to our knowledge, the first study showing that IL6 can induce a type of vessel sprouting with abnormal pericyte coverage compared with VEGF. These observations have clinical implications for malignant and other diseases, especially as studies in various cancers suggest that pericyte depletion leads to increased metastasis (23, 32, 33). The ability of anti-IL6 treatment to improve pericyte coverage of vessels in xenograft models suggests that in the tumor microenvironment, defective pericyte coverage may be due to the action of IL6 in those tumors with high levels of this cytokine.

The regulation of Ang2 by IL6 is another interesting finding as Ang2 expression has been shown to correlate with lymph node metastasis in various cancers (34–36). Moreover, the angiopoietin inhibitor trebananib increased progression-free survival in patients with recurrent ovarian cancer (37).

As STAT3 signaling is implicated in the treatment failure of various anti-angiogenic agents (13, 38–40), combinations of IL6 and angiogenesis antagonists may be worthy of further study.

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

Authors' Contributions
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Grant Support

This work was supported by Arthritis Research UK Grant 19207, Cancer Research UK Programme Grant CS87/A16354, and European Research Council Advanced Grant 322566.

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Received May 11, 2015; accepted May 13, 2015; published OnlineFirst June 16, 2015.

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doi:10.1158/0008-5472.CAN-15-1227

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