Adaptive Upregulation of EGFR Limits Attenuation of Tumor Growth by Neutralizing IL6 Antibodies, with Implications for Combined Therapy in Ovarian Cancer


Abstract

Excess production of the proinflammatory IL6 has both local and systemic tumor-promoting activity in many cancers, including ovarian cancer. However, treatment of advanced ovarian cancer patients with a neutralizing IL6 antibody yielded little efficacy in a previous phase II clinical trial. Here, we report results that may explain this outcome, based on the finding that neutralizing antibodies to IL6 and STAT3 inhibition are sufficient to upregulate the EGFR pathway in high-grade serous and other ovarian cancer cells. Cell treatment with the EGFR inhibitor gefitinib abolished upregulation of the EGFR pathway. Combining neutralizing IL6 antibodies and gefitinib inhibited malignant cell growth in 2D and 3D culture. We found that ErbB-1 was localized predominantly in the nucleus of ovarian cancer cells examined, contrasting with plasma membrane localization in lung cancer cells. Treatment with anti-IL6, gefitinib, or their combination all led to partial restoration of ErbB-1 on the plasma membrane. In vivo experiments confirmed the effects of IL6 inhibition on the EGFR pathway and the enhanced activity of a combination of anti-IL6 antibodies and gefitinib on malignant cell growth. Taken together, our results offer a preclinical rationale to combine anti-IL6 and gefitinib to treat patients with advanced stage ovarian cancer.

Introduction

Abnormal regulation of IL6 and its major downstream transcription factor STAT3 is a feature of many human cancers (1). Constitutive production of IL6 and STAT3 occurs downstream of some oncogenic mutations in malignant cells and there is strong evidence that IL6 is tumor promoting in many different experimental cancer models (2).

IL6 is implicated in the pathophysiology of high-grade serous ovarian cancer (HGSC) and clear cell ovarian cancer (3–5). We previously demonstrated that constitutive IL6 production by malignant cells is a major regulator of cancer-related inflammation and cytokine networks in HGSC, having important local and systemic tumor-promoting actions (3, 4, 6).

In mouse models, anti-IL6 antibodies have antitumor activity, inhibiting communication between malignant cells and stroma, reducing the leukocyte infiltrate and angiogenesis, with evidence of vessel normalization (3). We reported some activity in a phase II clinical trial of an anti-IL6 antibody in patients with advanced HGSC, but sustained responses were not achieved (3). One of the 17 HGSC patients treated had a partial response to anti-IL6 therapy, 7 others had periods of disease stabilization, and systemic levels of some cytokines, inflammatory, and tumor biomarkers were reduced during the therapy but rose as the patients regressed.

There could be many reasons for the low efficacy of IL6 blockade in patients with HGSC. Our previous research would suggest that a major factor might be the complexity of malignant cell cytokine production. As we have shown that inflammatory cytokines such as IL6 interact in networks with other inflammatory mediators and growth factors in ovarian cancer cells (4, 7, 8), we hypothesized that inhibiting constitutive IL6 production by malignant cells may induce reciprocal feedback regulation in other signaling pathways that compensates for their action and reduces efficacy of neutralizing anti-IL6 antibodies.

To investigate this hypothesis, we treated ovarian cancer cells with neutralizing anti-IL6 antibodies and studied changes in intracellular signaling pathways. We found that inhibiting IL6 signaling in these cells and ovarian cancer xenografts upregulated EGF signaling and ERK activation. A combination of EGFR inhibition by gefitinib and neutralizing anti-IL6 antibodies had enhanced anticancer activity.
Materials and Methods

Ovarian cancer cell lines

The IGROV-1 line was recently characterized as a hypermutated line but does have TP53 and BRCA2 mutations typical of HGSC (9). The AOC51 cell line was established from a patient diagnosed with HGSC, Silverberg grade 3, with <1cm residual disease after primary surgery. The patient had 18 months progression-free survival after six cycles carboplatin and paclitaxel adjuvant chemotherapy but showed no response to Line 2 liposomal doxorubicin. The cell line AOC51 was established from material taken at second relapse. AOC51 stains with antibodies to EPCAM and Pax8. The G33 cell line was established in our laboratory from a patient with HGSC after chemotherapy. It has a p53 mutation W146* and is positive for EPCAM and Pax8. Quality control of all cell lines was carried out by frequent STR analysis (Eurofins MWG), mycoplasma testing (InvivoGen), and cell lines were used for 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% penicillin/streptomycin. Cells were counted using a Vi-cell cell counter (Beckman Coulter) on days 3 and 7. All technical and experimental replicates were repeated in triplicate.

Primary cancer cells

All the tissue samples were obtained under the guidelines of the Human Tissue Authority Act 2004, and all patients had given prior consent under the Research Ethical Committee Project reference 10/H0304/14. A total of 10^4 cells were plated on coverslips (Nalge Nunc) in PBS with 0.1% Tween and 5% milk powder and incubated for 24 hours in culture media before drug treatment. Cells were stained with antibodies to EPCAM for 30 minutes at 4°C using 500 μL of antibody (Medi5117; Medimmune). Medi5117 is a human IgG1κ monoclonal antibody that binds to IL6 with sub-pM affinity and neutralizes it by preventing the binding to IL6R. Medi5117 was used for phage display technology. It bears a triple mutation (referred to as YTE) in the Fc domain of the heavy chain that extends its stability in circulation. Medi5117 is active in several preclinical cancer models, including non–small cell lung cancer, prostate cancer, breast cancer, and ovarian cancer xenografts in mice (Manuscript in preparation, Medimmune). An IgG1 isotype control antibody was generated by Medimmune and was used at the same concentration as anti-IL6 antibody (10 μg/mL).

Protein extraction from mouse tumors

Of note, 75 mg of tumor tissue was lysed with 1 mL of ice-cold lysis buffer (150 mmol/L NaCl 20mmol/L Tris, pH 7.5, 1 mmol/L EDTA 1 mmol/L EGTA 1% Triton X-100) with protease and phosphatase inhibitors. Samples were then dissociated using gentleMACS Dissociator. After dissociation, samples were centrifuged at 1,500 rpm for 2 minutes. Samples were 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.

Western blotting

Cells were 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 Gel, 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: Phospho-Stat3 (Tyr705; 1:1,000 #9145, Cell Signaling Technology), Stat3 (1:1,000 #4904, Cell Signaling Technology), p-ERK (1:1,000 sc-7383, Santa Cruz Biotechnology), ERK (1:1,000 #9102, Cell Signaling Technology), phospho-ErbB-1 (1:1,000 #2220, Cell Signaling Technology), ErbB-1 (1:1,000 2232, Cell Signaling Technology), phospho-ErbB-4 (1:500 #3790, Cell Signaling Technology), ERB-B4 (1:1,000 #4795, Cell Signaling Technology), TNFα (1:1,000 ab6671, Abcam), Jagged1 (1:1,000 SC-8303, Santa Cruz Biotechnology), α-tubulin (1:2,000 sc-8035, Santa Cruz Biotechnology), lamin A/C (1:2,000 R0278, Sigma), ERK (1:1,000 #4904, Cell Signaling Technology), phospho-ERK (1:1,000 #9145, Cell Signaling Technology), phospho-ErbB-1 (1:1,000 #2220, Cell Signaling Technology), phospho-ErbB-4 (1:500 #3790, Cell Signaling Technology), phospho-ErbB-1 (1:1,000 #2220, Cell Signaling Technology), phospho-ErbB-4 (1:500 #3790, Cell Signaling Technology), ErbB-1 (1:1,000 2232, Cell Signaling Technology), phospho-ErbB-4 (1:500 #3790, Cell Signaling Technology), and β-actin (1:5,000 A5316, Sigma). A rabbit or mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare) incubation allowed visualization using enhanced chemiluminescence (ECL; GE Healthcare). Protein concentration equivalence was confirmed by anti-β-actin antibody.

Quantifying Western blot analyses

Western blot analyses were quantified using ImageJ analysis. The images were set to grayscale 8-bit and a rectangular box was drawn to enclose a single lane. The box was then selected as the first lane and the same process was repeated for the remaining of the lanes. The relative density of the contents of each lane was then plotted as histograms and a straight line was drawn underneath where the peak ends to subtract any background. The wand tool was then used to get the measurements of each profile. The ratios were obtained by normalizing the density of each band to its total protein or loading control (10).

Immunoprecipitation

A total of 2 × 10^7 cells were homogenized in 1 mL of RIPA buffer (R0278, Sigma) containing phosphate inhibitor cocktail (P-5726, Sigma) and incubated on ice for 10 minutes. Samples were centrifuged at 10,000 g for 10 minutes at 4°C, and supernatant was then transferred to a new tube. Protein concentration of the supernatant was determined. Lysates were cleaned using Rabbit IgG control and 20 μL of Protein A/G PLUS-Agarose beads (sc-2033; Santa Cruz Biotechnology) for 30 minutes at 4°C. Beads were pelleted by centrifugation at 2,500 rpm for 5 minutes and lysates transferred to a clean tube. Samples were immunoprecipitated for 2 hours at 4°C using 500 μg of total lysate and 2 μg of appropriate antibody (Erbb-1 or STAT3). Lysates were then rotated overnight at 4°C with 20 μL of A/G PLUS Agarose beads. The next day, the Agarose beads were washed four times in RIPA buffer followed by a 5 minute centrifugation at 1,000 × g at 4°C. After final wash, supernatant was discarded and pellet was resuspended in 40 μL of 1× electrophoresis buffer, boiled for 5 minutes at 70°C before separation using SDS-PAGE.

Immunofluorescence staining and nuclear quantification

A total of 1 × 10^6 cells were plated on coverslips (Nalge Nunc International, ThermoFisher Scientific) and kept in culture with...
RPMI-1640 medium supplemented with 10%FCS and 1% penicillin/streptomycin. Next day, medium was replaced with serum-free medium. Cells were treated with 10 μg/mL of anti-IL6 antibody and/or 1 μmol/L gefitinib and incubated at 37°C, 5% CO2 for 24 hours. Supernatants were discarded and cells were fixed with 4% paraformaldehyde for 30 minutes, permeabilized in 0.5% Triton X-100 for 30 minutes, and washed three times in 0.1% Triton X-100. Staining was performed overnight with ErbB-1 (1:50 Alexa Fluor 488, Santa Cruz Biotechnology) or isotype control (1:200, R&D systems). Primary antibody was washed with PBS. Slides were counterstained with DAPI Prolong Gold Antifade (Media Cybernetics).

Nuclear and cytoplasmic quantification of ErbB-1 immunofluorescent data was done using MetaMorph Microscopy Automation & Image Analysis Software following the instructions provided by the manufacturer.

siRNA transfection

A total of 2 × 10⁴ cells were plated in a 6-well plate and transfected with 100 pmol of siRNA pool of four different targeting sequences or nonspecific scramble control (stock concentration at 50 pmol/μL) in 250 μL serum-free medium. Of note, 4 μL of lipofectamine 2000 (Life Technologies) was also added to the mix. Samples were vortexed and incubated at room temperature for 15 minutes. After 6 to 8 hours, medium was removed and replaced with fresh RPMI-1640 supplemented with 10% FCS. Twenty-four hours later, protein quantification was accessed and 20 μg of cell lysate was analyzed by Western blot analysis.

IHC

Paraffin-embedded sections of tumors collected from mouse xenograft were stained with antibodies for Phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204; Cell Signaling Technology, 9106S), Tyr705 phospho-STAT3 (Cell Signaling Technology, 9145), and Y1068 phospho-EGFR (Cell Signaling Technology, 8543S). Slides were counterstained with hematoxylin. Negative controls were isotype matched. The conditions used for staining with individual antibodies were in accord with the manufacturers’ recommendations.

Growth of tumors in mice and bioluminescence imaging

A total of 10 × 10⁶ 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). These cell lines were generated as previously published (11). Mice were observed daily for tumor growth and killed once they reached end points as defined in our Home Office Project Licence PPL 70/7411 (20% increase in abdominal distension). All observable peritoneal tumors were dissected and weighed at the end of the experiment. There were 8 mice in each group and two separate experiments were conducted.

Statistical analysis

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

Results

IL6 inhibition activates EGFR signaling in ovarian cancer cells

We initially tested the effect of neutralizing antibodies to IL6 on newly established high-grade serous ovarian cancer cell lines (AOCS1, G33), on freshly isolated cells from HGSC ascites and on IGROV-1, a previously characterized IL6 producing ovarian cancer line of uncertain histotype but with TP53 and BRCA2 mutations (9). We first asked whether treating cells with a neutralizing antibody to IL6 had an impact on other intracellular signaling pathways that were likely to be active in these cells, with a focus on ErbB signaling. Cells were cultured for 3 and 7 days in the presence of an anti-IL6 antibody (MEDI5117) or an IgG isotype control (Fig. 1A, for AOCS1 and primary cells; Supplementary Fig. S1A for IGROV-1 cells).

Western blot analysis and quantification showed, as expected, that inhibition of IL6 reduced STAT3 phosphorylation (Supplementary Fig. S1A–S1C). However, in the presence of IL6 inhibition, the cells activated alternative signaling pathways through

![Figure 1](https://example.com/image1.png)

**Figure 1.** IL6 inhibition activates the EGFR signaling pathway. A, 2 × 10⁵ HGSC cells (AOCS1) and primary cells were treated with anti-IL6 antibody (10 μg/mL) for 3 and 7 days and lysates were analyzed by Western blotting. Data shown are typical of three independent experiments. B, 2 × 10⁵ AOCS1 and G33 HGSC cells were treated with anti-IL6 antibody (10 μg/mL), gefitinib (1 μmol/L) alone, or with a combination of both inhibitors.
ErbB-1, ErbB-4, and ERK phosphorylation (Fig. 1A and Supplementary Fig. S1). The results in Fig. 1 and Supplementary Fig. S1 were consistent in repeated experiments. To demonstrate this, we show mean results of the key finding from three experiments with the AOCS1 cell line (See Supplementary Fig. S2). We also observed that IL6 inhibition leads to a modest increase in release of EGFR ligands (HB-EGF, TGF\(_\alpha\), and EGF) by the cells (data not shown).

EGFR family members are overexpressed in approximately 70% of ovarian cancers and this is associated with a poor prognosis (12, 13). However, single-agent targeting of ErbB-1 has shown minimal activity in patients with ovarian cancer (13, 14). Our results suggested that combination therapies targeting both the EGFR and IL6 pathways might have therapeutic potential. To investigate this, cells were treated with gefitinib, a selective inhibitor of the EGFR tyrosine kinase by binding to the ATP-binding site of the enzyme (15). Gefitinib treatment alone decreased phosphorylation of ErbB-1 and ERK but induced pSTAT3 activation (Fig. 1B), as previously described (16). In AOCS1, a combination of gefitinib and the anti-IL6 antibody also decreased ErbB-1 phosphorylation and pERK levels, but in this instance, pSTAT3 activation decreased as well (Fig. 1B). Similar results were obtained with the G33 cell line (Fig. 1B, right) and IGROV-1 cells (data not shown). Quantification of these blots can be found in Supplementary Fig. S3.

**Combined therapy that targets IL6 and EGFR pathways reduces proliferation in 2D and 3D cultures**

To investigate the biologic relevance of these observations, we studied growth of the cell lines in the presence of anti-IL6 and gefitinib. AOCS1 and IGROV-1 cells were treated with the anti-IL6 antibody, gefitinib, or their combination. In 2D culture (Fig. 2A and B), IL6 inhibition had no effect on cell proliferation as we have previously reported (3). Gefitinib significantly reduced cell counts and this growth inhibition was greater in the combination treatment group (Fig. 2A and B).

We next used a 3D cell culture system adapted from (17) that mimics the microenvironment of the human omentum, a major site of peritoneal cancer metastasis. The model consisted of fibroblasts embedded in a collagen matrix covered by a layer of mesothelial cells. AOCS1 cells were seeded on top of a layer of mesothelial cells and fibroblasts embedded in collagen.

![Figure 2](image_url)

**Figure 2.** Anti-IL6 combined with gefitinib reduces proliferation in vitro and tumor growth area on 3D culture models. A and B, AOCS1 and IGROV-1 cells were treated with an anti-IL6 antibody (10 \(\mu\)g/mL) and/or gefitinib (1 \(\mu\)mol/L) for 7 days. C and D, H&E staining sections of 3D cultures of AOCS1 cells. AOCS1 cells were seeded on top of a layer of mesothelial cells and fibroblasts embedded in collagen. Data in A, B, and D are shown as mean ± SEM of three independent experiments. Statistical analysis was performed and is shown as *: \(P \leq 0.05\); **: \(P \leq 0.01\); ***: \(P \leq 0.001\).
primary human mesothelial cells. AOCS1 cells were grown for 2 weeks in this system and then the area of malignant cell growth was measured using ImageJ (Fig. 2C and D). Both anti-IL6 and gefitinib reduced malignant cell growth in the organotypic cultures and the greatest inhibition was seen in the combination treatment group (Fig. 2D).

**ErbB-1 has a nuclear localization in ovarian cancer cells and this is altered by IL6 inhibition**

We also investigated the effects of anti-IL6 on EGFR in the cells using immunofluorescence and confocal microscopy. Confocal images and z-stack analysis of the images revealed that ErbB-1 was primarily located in the nucleus (nErbB-1) in all three lines (Fig. 3A–C and Supplementary Fig. S4A, z-stack top). This observation has been previously reported in some ovarian cancer cell lines and human ovarian cancer biopsies (18). Upon treatment with anti-IL6 antibody for 24 hours, nErbB-1 staining was reduced and in some of the cells analyzed, relocated to the plasma membrane (Fig. 3A–C and Supplementary Fig. S4A, z-stack bottom). As an experimental control, we also studied A549 cells, a lung adenocarcinoma cell line with well-characterized cell surface expression of ErbB1. ErbB-1 remained at the plasma membrane whether or not the cells were treated with anti-IL6 (Fig. 3D). In a control for the specificity of antibody staining, IGROV-1 cells were treated with siRNA to ErbB-1 or a scramble control, resulting in an almost complete removed staining in both nucleus and cytoplasm (Fig. 3E and F). We then carried out nuclear and cytoplasmic fractionation of cells treated either with IgG control antibody or the anti-IL6 antibody. Anti-IL6 treatment resulted in a reduction of ErbB-1 in the nuclear fraction of IGROV-1 cells at 24 hours and 3 days treatment with anti-IL6 (Fig. 3G and H and Supplementary Fig. S4B). Similar results were obtained with AOCS1 (Supplementary Fig. S5A). Tubulin and nuclear lamin antibodies were used to test separation of cytoplasmic and nuclear fraction, respectively, confirming the purity of the fractions (Fig. 3G and H and Supplementary Fig. S5A).

**Anti-IL6 antibodies and β-importin silencing both disrupt ErbB-1 nuclear localization**

Next, we tested the effects of gefitinib plus/minus anti-IL6 on nErbB-1 (Fig. 4A). Gefitinib, anti-IL6 treatment, and their combination relocalized ErbB-1 expression to the membrane.

The presence of growth factor receptors in the nucleus is well documented in several human cancers and nuclear translocation of EGFR family members has been extensively described (19–21), but the mechanism of translocation and the role of EGF receptor family members in the nucleus of malignant cells is still unclear (22–24). Others have reported that ErbB-1 colocalizes and interacts with β-importin in thyroid cells to be translocated into the nucleus (19, 22, 24). We therefore treated AOCS1 cells with β-importin siRNA. Knockdown of β-importin reduced nErbB-1; this was not observed when AOCS1 cells were treated with scrambled control siRNA (Fig. 4A).

Data from the above experiments were quantified using meta-morph analysis software and correspond to an average of five microscope fields per condition (Fig. 4B). The intensity of nErbB-1 was reduced after treating the cells with either anti-IL6 antibody, gefitinib, or the combination of both agents (Fig. 4B). Studies have suggested that ErbB-1 interacts with other transcription factors, such as STAT3, in the nucleus (25). Thus, we next performed coimmunoprecipitation to see whether Erbb1 colocalized with STAT3. Figure 4C shows that immunoprecipitation of ErbB-1 pulls down STAT3 in AOCS1 cells, thus suggesting that IL6 inhibition and consequent STAT3 reduction in the nucleus might offer a possible explanation for the reduction of ErbB-1 in the nucleus.

Our work so far has shown that ovarian cancer cells are able to compensate for reduced autocrine IL6 signaling by upregulating the EGF signaling pathway. In addition, it appears that in HGSC cell lines, ErbB1 is located to the nucleus and that treatment with either anti-IL6 antibody or gefitinib, or their combination, leads to relocalization of Erbb1 on the plasma membrane of AOCS1, G33, and IGROV-1 cells.

To test whether treatment of ovarian cancer cells with both gefitinib and anti-IL6 antibodies was able to enhance antitumor activity, we conducted experiments in peritoneal xenografts.

**Gefitinib in combination with anti-IL6 reduces tumor weight in mouse xenografts**

We grew IGROV-1 cells as intraperitoneal xenografts in nude mice and treated the mice with the anti-human IL6 antibody, gefitinib, or both in combination. Results of two independent experiments were combined and data are shown in Fig. 5.

Twenty-one days after treatment, tumors were extracted and weighed. IL6 inhibition reduced tumor weight as previously shown (Fig. 5A; ref. 3). Gefitinib treatment alone was able to significantly reduce tumor weight compared with the IgG control group. However, the combination of anti-IL6 antibodies and gefitinib was the most effective in reducing tumor weight (Fig. 5A).

Histologic analysis of tumors collected from mice revealed a reduction of pSTAT3 with IL6 inhibition, which was accompanied by an increase in pERK and pErbB-1 (Fig. 5B). We next analyzed the cellular localization of ErbB-1 in the xenograft sections. Results show that tumors from control mice have nuclear ErbB-1. Tumors from mice treated with anti-IL6, gefitinib, or the combination have mainly plasma membrane ErbB-1 (Fig. 5C) as previously suggested in Fig. 3. To assess further the effect of anti-IL6 on the EGFR and IL6 pathways in vivo, we extracted protein from the mouse tumors and assessed the expression of pSTAT3, pErbB-1, and pERK. IL6 inhibition in vivo effectively reduced STAT3 activation and led to an increase in pErbB-1 and pERK (Fig. 5D). In addition, we used these lysates to demonstrate that in vivo, IL6 inhibition reduces TNFα and Jagged1 levels (Fig. 5E), two components of the IL6 tumor cytokine network that we previously described (3).

A summary diagram of our results is shown in Fig. 6.

**Discussion**

Although many preclinical studies and clinical observations suggest that IL6 inhibitors would be of therapeutic benefit in patients with advanced cancers (1, 3, 6, 8), clinical trials have not shown conclusive evidence of activity, apart from in patients with Castleman disease, which is primarily driven by IL6. In our clinical trial of a therapeutic anti-IL6 antibody in patients with HGSC, we found some evidence of disease stabilization but this was not sustained (3). All our evidence to
date would suggest that the effects of IL6 in this disease are driven by constitutive malignant cell production of IL6 (3, 6–8). Thus we investigated the possibility that IL6 inhibition might induce some reciprocal feedback regulation of other pathways.

In our study, treatment with the anti-IL6 antibody did not completely abrogate STAT3 phosphorylation. IL6 is not the only cytokine/growth factor that activates STAT3, hence neutralizing IL6 would not always be expected to completely abrogate STAT3 phosphorylation. However, experimental data...
in the literature would suggest that partial STAT3 abrogation is therapeutically significant. For example, tumor cells that become dependent on persistent STAT3 signaling are more sensitive to STAT3 inhibition than normal cells (26). In experiments with a lymphoma model, partial downregulation of STAT3 (40%) was responsible for strong antitumor effects in vivo (27) and in colon cancer xenografts, partial inhibition of JAK/STAT3 signaling pathway is enough to suppress the growth of colon cancer xenografts (28).

In addition, our previous research on cytokine networks in malignant cells (7) would suggest that rational combination of therapies targeting the malignant cells and their interaction with the microenvironment is worthy of investigation. Links between the IL6 and EGFR pathway have already been reported (29–32). For instance, EGFR stimulation of ovarian cancer cell lines increased IL6 production (29), but none of the cell lines used in the latter paper are now thought to be of HGSC origin (9). In addition, in human lung adenocarcinomas, mutations in the EGFR kinase domain stimulated STAT3 activation via IL6 production (30).

Hence we decided to study the influence of IL6 inhibition on EGFR signaling. The cell lines that we used in our experiments did not have EGFR mutations (data not shown). In this paper, we have used cell lines and primary tumor cells from peritoneal cancers that are collectively termed “ovarian” cancers. The primary cancer cells used in our experiments were from pathologically confirmed HGSC patient ascites. The cancer of origin of the ovarian cancer cell line IGROV-1 is uncertain (33). We have included it in our experiments as an example of an IL6 producing cell line that forms peritoneal xenografts in nude mice (3).

Our results led us to test combinations of anti-IL6 and gefitinib and our experiments suggested that this may have therapeutic potential. However, our recent increased understanding of the natural history and genetic drivers of HGSC (34–36) means that some of the cell lines and syngeneic models (e.g., ID8 cells) are not relevant models of the disease. A new genetic model of HGSC has recently been published (37) and in the next few years as this, or transplantable cell lines derived from such mice, become more widely used, we should have more appropriate models of HGSC for preclinical experiments. IL6 produced by malignant cells has paracrine actions not only on angiogenesis, but also on the extent and phenotype of the leukocyte infiltrate (3, 7). Hence, a combination of IL6 and EGFR inhibition may be more powerful in a syngeneic model or in patients with HGSC compared with the results described here using immunocompromised mouse xenograft models.

Figure 4. Gefitinib and anti-IL6 reduces nuclear ErbB-1 in HGSC cells. A, confocal analysis of AOCS1 cells treated with IgG control, anti-IL6 antibody, gefitinib, gefitinib+anti-IL6 antibody, scramble siRNA or β-importin siRNA. After 24 hours, cells were fixed and stained for ErbB-1. Results observed were validated in five independent experiments. B, nErbB-1 intensity was quantified using methamorph analysis software. Data correspond to the average of 5HPF analyzed per group. Statistical analysis was performed and is shown as ***P < 0.001. C, STAT3 Western blot analysis after ErbbB1 immunoprecipitation in AOCS1 cells.

Nuclear EGFR signaling is thought to confer resistance to various anticancer therapies (23) and may explain the poor response of patients with ovarian cancer to gefitinib (38). All the cells used in this paper were resistant to the anti-EGFR antibody cetuximab (data not shown) and there was no interaction between cetuximab and anti-IL6. The nuclear expression of
ErbB-1 may explain this finding (20). The partial restoration of membrane-localized ErbB-1 by anti-IL6 antibody treatment may not be sufficient to permit inhibition by cetuximab.

As both gefitinib and therapeutic anti-IL6 antibodies have been given safely to patients with ovarian cancer, and both targets are implicated in this disease, specifically HGSC, we

Figure 5.
Effects of inhibition of IL6 and/or ErbB-1 in mouse ovarian cancer xenografts. A, weight of dissected peritoneal tumors collected from mice injected intraperitoneally with IGROV-1 and treated with either an IgG antibody, an anti-IL6 antibody, gefitinib, or their combination. Data in A are shown as mean ± SEM of two independent experiments, with a total of 8 mice per experimental group in each experiment. Statistical analysis was performed and is shown as *, \( P \leq 0.05; **, P \leq 0.01; *** P \leq 0.001. B, pSTAT3, pERK, and pErbB-1 staining on mouse tumor sections from the above experiment. C, ErbB-1 staining on mouse tumor sections. D, Western blot analysis and quantification of pSTAT3, pErbB-1, and pERK in protein extracts from mouse xenografts treated with an anti-IL6 antibody. E, Western blot analysis and quantification of TNF\(\alpha\) and Jagged1 in protein extracts from mouse xenografts treated with an anti-IL6 antibody. Quantification was done using ImageJ software and is always relative to the total protein. Results represented on graphs correspond to fold increase.
suggest that our data provide a rationale for testing their combination.

Disclosure of Potential Conflicts of Interest

R.E. Hollingsworth is the senior director, oncology research in MedImmune. No potential conflicts of interest were disclosed by the other authors.

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References


Adaptive Upregulation of EGFR Limits Attenuation of Tumor Growth by Neutralizing IL6 Antibodies, with Implications for Combined Therapy in Ovarian Cancer

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