Resistance to Anti-VEGF Therapy Mediated by Autocrine IL6/STAT3 Signaling and Overcome by IL6 Blockade

Alexandra Eichten, Jia Su, Alexander P. Adler, Li Zhang, Ella Ioffe, Asma A. Parveen, George D. Yancopoulos, John Rudge, Israel Lowy, Hsin Chieh Lin, Douglas MacDonald, Christopher Daly, Xunbao Duan, and Gavin Thurston

Abstract

Anti-VEGF therapies benefit several cancer types, but drug resistance that limits therapeutic response can emerge. We generated cell lines from anti-VEGF–resistant tumor xenografts to investigate the mechanisms by which resistance develops. Of all tumor cells tested, only A431 (A431-V) epidermoid carcinoma cells developed partial resistance to the VEGF inhibitor afibercept. Compared with the parental tumors, A431-V tumors secreted greater amounts of IL6 and exhibited higher levels of phospho-STAT3. Notably, combined blockade of IL6 receptor (IL6R) and VEGF resulted in enhanced activity against A431-V tumors. Similarly, inhibition of IL6R enhanced the antitumor effects of afibercept in DU145 prostate tumor cells that displays high endogenous IL6R activity. In addition, post hoc stratification of data obtained from a clinical trial investigating afibercept efficacy in ovarian cancer showed poorer survival in patients with high levels of circulating IL6. These results suggest that the activation of the IL6/STAT3 pathway in tumor cells may provide a survival advantage during anti-VEGF treatment, suggesting its utility as a source of response biomarkers and as a therapeutic target to heighten efficacious results.

Introduction

VEGF plays a key role in physiologic and pathologic angiogenesis, including tumor angiogenesis. Blockade of the VEGF pathway is effective at inhibiting angiogenesis in many tumors (1–3). Several VEGF pathway inhibitors, including the monoclonal anti-VEGF antibody bevacizumab, the soluble receptor afibercept (VEGF Trap, known as ziv-afibercept in the United States), and the mAb to VEGF receptor 2 (ramucirumab) delay tumor growth in preclinical tumor models (4–6) and extend the survival of cancer patients (7–11).

Despite showing broad activity in both preclinical and clinical settings, not all tumors respond to anti-VEGF therapies, and those that do may eventually become resistant. Clinical and preclinical studies suggest that resistance of tumors to anti-VEGF therapies can occur via several mechanisms: (i) changes in the tumor microenvironment resulting in upregulation of various proangiogenic factors, which lead to vessels that are less sensitive to VEGF blockade and/or (ii) changes in the characteristics of cancer cells, such as mutations and epigenetic changes, which provide the cells with a survival advantage and/or increased invasive potential in the environment of reduced tumor vessels (12–14). These resistance mechanisms cause insensitivity of tumor vasculature to anti-VEGF treatments and/or decreased dependence of tumors on angiogenesis. Combination treatments targeting VEGF and other angiogenic pathways such as angiopoietin-2 (Ang2) or Delta-like 4 (DLL4) are being evaluated to increase the efficacy of angiogenic blockade. However, the underlying mechanisms of tumor cells becoming less dependent on angiogenesis in response to anti-VEGF therapies also need to be investigated.

To begin addressing this, we aimed to identify molecules involved in mediating anti-VEGF resistance using xenograft tumor models with acquired resistance to VEGF blockade. Afibercept is a recombinant fusion protein that potently binds all isoforms of human and murine VEGF-A, VEGF-B and placental growth factor (PIGF; ref. 4). A431 human epidermoid cell tumor xenografts are sensitive to afibercept, but can acquire resistance when treated for longer time periods (several weeks or more). We established a stable variant cell line (A431-V), which is partially resistant to afibercept and allowed the direct comparison of resistant tumors to their sensitive parental A431 (A431-P) counterparts. Studies using these two cell lines revealed that A431-V tumors have increased levels of proinflammatory mediators including IL6, as well as activation of the STAT3 signaling pathway. The activation of the IL6/STAT3 pathway led us to investigate the effects of an anti-IL6R antibody (sarilumab), which blocks IL6 receptor signaling on human cells. Importantly, sarilumab does not bind or block murine IL6R. Treatment of A431-V tumor cells with sarilumab reduced the phosphorylation of STAT3, and enhanced the antitumor activity of afibercept against A431-V tumors in vivo. Similarly, IL6R inhibition enhanced the antitumor activity of afibercept in another tumor model with high endogenous IL6R activity, namely DU145 prostate tumors. In addition, IL6...
overexpression rendered sensitive A431 cells resistant to aflibercept. Finally, IL6R blockade decreased the number of A431 tumors that escaped long-term aflibercept treatment. To assess potential clinical relevance, we determined whether circulating levels of IL6 were associated with poorer outcome in a phase II clinical trial of ovarian cancer patients treated with aflibercept. A correlation between high levels of IL6 and poorer tumor response to anti-VEGF therapy was observed. Taken together, these data suggest that resistance to VEGF blockade can be mediated at least in part by increased IL6/STAT3 signaling in tumor cells, and that blockade of IL6 signaling on tumor cells can overcome this resistance.

Materials and Methods

Tumor cells and reagents

Tumor cells including A431 human epidermoid cell skin carcinoma and Du145 human prostate carcinoma were obtained from the ATCC and grown according to ATCC guidelines. All cell lines were authenticated between 2012 and 2015 using the STR Profiling Test by ATCC. A431 parental (P) and variant (V) cells were obtained by in vivo passaging, as described in Fig. 1. Aflibercept (also known as VEGF Trap or ziv-aflibercept in the United States) is a recombinant fusion protein that potently binds all isoforms of human and murineVEGF-A, VEGF-B, and PIGF. Sarilumab (REGN88) is a fully human IgG1 mAb that binds human IL6R and prevents binding of IL6. Cetuximab is a chimeric mouse-human (30:70) IgG1 mAb that competitively inhibits the binding of EGF to its receptor EGFR.

Cell culture and in vitro cell growth

A431 and Du145 cells were cultured in 10% FBS, DMEM, and MEM, respectively. Preparation of conditioned media (CM) was done by the following method: A431-P and A431-V cells were grown in serum-free media for 16 hours followed by media done by the following method: A431-P and A431-V cells were grown on a 6-well plate at a density of 1 \times 10^5 cells/well or on 8-well chamber slides at a density of 30,000 cells/well were serum starved for 16 hours followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour. Cells were either lysed to detect p-STAT3 status by Western blot analysis or followed by exposure to CM for 1 hour.
secondary antibody (Vector Laboratories, catalog # PI1000), and 3,3′-diaminobenzidine (DAB, Sigma) was done. For Ki-67 IHC, antigen retrieval was done and blocking was performed with 0.1% Triton X-100, 5% goat serum, 2.5% BSA in PBS, Ki-67 antibody (BD, catalog # 556003), biotinylated antibody (Vector Laboratories, catalog # BA-2001), ABC-ELITE (Vector Laboratories; ABC...

Figure 1. Generation and characterization of aflibercept-resistant A431 variants. A, schematic of A431-P and -V tumor cell line generation. B, SCID mice bearing A431 tumors (50–100 mm³) were treated with aflibercept or human Fc control protein for up to 7 weeks. C, growth kinetics and response to aflibercept treatment of tumors grown from in vivo passaged A431-V and A431-P cell lines. The average tumor volume ± SD is plotted over the course of treatment. *** P < 0.0001 two-way ANOVA, Bonferroni post hoc test. D, representative images of cell proliferation assessed by Ki-67 IHC in A431-P and A431-V tumors treated with human Fc control protein or aflibercept for 14 days. Scale bar, 50 μm. E, representative images of cell apoptosis assessed by TUNEL staining in A431-P and A431-V tumors treated with human Fc control protein or aflibercept (aflib) for 24 hours. Scale bar, 10 μm. Quantitative analysis of apoptotic index based on TUNEL-positive (green) and -negative nuclei in A431-P and A431-V tumor tissue. *** P < 0.001 one-way ANOVA with Bonferroni post hoc test.
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VectorStain Elite), and 3,3’-diaminobenzidine (DAB, Sigma). Tissue sections were counterstained with hematoxylin.

**Immunocytochemistry.** Cells were fixed in 4% PFA for 20 minutes, blocked for 45 minutes in 10% donkey serum/0.3% Triton X-100, incubated with rabbit anti-human phopho-STAT3 (Y705) antibody (R&D Systems, catalog # AF4607), anti-rabbit NL557-conjugated antibody (R&D Systems, catalog # NL004), and DAPI. Slides were mounted in ProLong Gold.

**Immunoblotting analysis and ELISA assay.**

Tumor cells or tissues were harvested in lysis buffer [50 mmol/L Heps pH 8.0, 10% Glycerol, 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 1.5 mmol/L MgCl2, 100 mmol/L NaF, 10 mmol/L Na2P2O7, freshly supplemented with protease inhibitor tablet (Roche catalog # 1836153001) and 1 mmol/L NaVO4]. Tissues were homogenized on ice and tissue or cell lysates were run on a SDS-PAGE gel and Western blot analysis was conducted with the following antibodies overnight at 4°C: Cell Signaling Technology: p-STAT3 (catalog # 9145), STAT3 (catalog # 9139), p44/p42 MAPK (ERK1/2; catalog # 4370), phospho-p44/p42 MAPK (ERK1/2; catalog # 4370), tubulin (catalog # 2125), Santa Cruz Biotechnology: p-STAT3 (catalog # sc-80056), and sc52948), SAA (catalog # sc-52214), IL36G (catalog # sc-80056), and β-actin (BD Transduction, catalog # 610077). HRP-conjugated anti-rabbit (Cell Signaling Technology, catalog # 7074), anti-mouse (Pierce, catalog # 31437), and anti-rat (Santa Cruz Biotechnology, catalog # sc-20068) antibodies were applied for 1 hour at room temperature. Blots developed with ECL. Densitometry analysis was performed using Carestream Software (Molecular Imaging).

**Patient samples.**

Collection and testing of plasma for exploratory biomarker analysis was specified in the amended clinical trial protocol for ARDS122/AVE0005, a phase II, multicenter, randomized, double-blind, parallel-arm, two-stage study of albirecept in patients with platinum-resistant and topotecan- and/or liposomal doxorubicin-resistant advanced ovarian cancer. Informed consent for sample testing was obtained following protocol approval by local Institutional Review Boards.

**Statistical analysis.**

Tumor sizes at the different time points from control and treatment groups were compared using two-way ANOVA and Bonferroni’s multiple comparison tests (Prism software version 5).

**Results.**

**Generation and characterization of an albirecept-resistant A431 tumor cell line.**

Human xenograft tumors grown in mice show different degrees of response to VEGF blockade. To study resistance mechanisms, we aimed to derive tumor cell lines with acquired resistance to albirecept from albirecept-sensitive tumors. For this aim, we selected four albirecept-sensitive tumor lines, namely A498, Colo205, 786-0, and A431, and treated tumor-bearing mice with high-dose albirecept (25 mg/kg, twice weekly), for up to 7 weeks. For three of the four tumor lines, we observed complete growth stasis (Colo205, A498) or gradual incremental tumor growth (786-0), but no outgrowth of resistant tumors based on our criteria (Supplementary Fig. S1). However, A431 human epidermoid cell carcinoma tumors exposed to long-term high-dose albirecept showed complete and prolonged tumor growth inhibition for approximately 6 weeks followed by conspicuous outgrowth of some of the tumors (Fig. 1B and Supplementary Fig. S1 for individual tumor growth).

One of the outgrowing A431 tumors was harvested, and fragments of viable tumor tissue were reimplanted subcutaneously into other SCID mice (the procedure is outlined in Fig. 1A). In some cases, reimplanted A431 tumors that were treated with albirecept continued to grow. This reimplantation and selection procedure was repeated once more, and we again observed tumor growth in the presence of albirecept. In parallel, we performed a similar reimplantation procedure with A431 tumors treated with human control protein (Fc fragment only, Fig. 1A). After in vivo passaging and selection, the albirecept and control-treated tumors were harvested, minced, and transferred to tissue culture dishes for propagation in vitro. The cell lines derived from control and albirecept-treated tumors were called A431-P (parental) and A431-V (variant), respectively (Fig. 1A).

To confirm that the isolated A431-V cells retained resistance to albirecept, tumors from A431-V and A431-P cell lines were studied for growth kinetics and response to albirecept. We observed that control treated A431-V tumors grew slightly faster than control-treated A431-P tumors (Fig. 1C). In addition, A431-V tumors continued to grow during treatment with albirecept, albeit at a slower rate (Fig. 1C), suggesting that A431-V tumors are inherently resistant to VEGF blockade. These differences in tumor growth were mirrored when assessing cell proliferation in tissue sections by detecting the proliferation marker Ki-67 (Fig. 1D), with changes being apparent as early as 24 hours after albirecept treatment (Supplementary Fig. S2A). In contrast, albirecept treatment had a much smaller effect on cell proliferation in A431-V tumors (Fig. 1D). Similarly, TUNEL staining revealed that albirecept treatment increased apoptotic cells in A431-P tumors, but not in A431-V tumors (Fig. 1E).

Interestingly, the growth rates of A431-P and A431-V cells in vitro did not differ significantly (Fig. 2A). In addition, A431-V tumors had increased vessel density compared with A431-P tumors, although vessel density after VEGF blockade was similar in A431-P and A431-V tumors (Supplementary Fig. S2B and S2C). A431-P and A431-V tumor lysates did not show differences in murine (20.5 ± 2 and 20.8 ± 5 pg/mg, respectively) or human VEGF (13 ± 1.9 and 12.1 ± 4 ng/mg, respectively), suggesting that VEGF levels were not responsible for the difference in vessel density.

We next wanted to determine whether the resistance of A431-V tumors was specific to anti-VEGF agents, or whether it was a more generalized resistance to targeted therapy. A431 cells express high
levels of EGFR and are normally sensitive to EGFR antibodies. Variants of A431 that are resistant to EGFR blockers have been described previously (15). EGFR activation was not altered in A431-V cells, as no difference was observed in a phospho-protein array (Fig. 3A). To determine EGFR activity, we assessed phospho-ERK1/2 levels by Western blot analysis and observed that P-ERK1/2 in A431-V was somewhat lower compared with phospho-ERK1/2 levels by Western blot analysis. To determine EGFR activity, we assessed phosphorylation levels ERK1/2 were assessed in A431-P and A431-V cells by Western blot analysis. C, tumor growth kinetics of A431-P and A431-V tumors (100–150 mm\(^3\)) treated with human Fc control protein, afiblercept, or cetuximab for 14 days. The average tumor volume ± SD is plotted over the course of treatment. D, tumor growth changes of A431-P and A431-V tumors treated with control protein, afiblercept (afib), or cetuximab (cetux) from start of treatment. **, P < 0.01; ****, P < 0.0001 one-way ANOVA, Bonferroni post hoc test.

Identification of pathways/factors mediating afiblercept resistance in A431-V tumors

To explore the mechanisms of afiblercept resistance in A431-V tumors, we first utilized a phospho-protein array to compare the activities of 28 receptor tyrosine kinases and 11 downstream signaling molecules in A431-V and A431-P cell lines (Fig. 3A). The most distinct difference between the resistant and sensitive tumor cells was a marked increase in the phosphorylation of STAT3 at Tyr705 (Fig. 3A). The increase in phospho-STAT3 was confirmed by Luminex as well as Western blot analysis (Fig. 3B and C, respectively). STAT3 can be phosphorylated in response to various secreted cytokines and chemokines, including IL6, IL10, and LIF (16). To determine whether the phosphorylation of STAT3 in A431-V cells is mediated by secreted factors, we exposed A431-P cells to CM from A431-V cells. We observed that phospho-STAT3 was increased, and was localized in the nucleus, in A431-P cells treated with CM from A431-V cells (Fig. 3D, third lane and E). In contrast, phospho-STAT3 in A431-V cells was notably reduced after incubation with A431-P CM (Fig. 3D, fourth lane and E), suggesting that secreted factor(s) mediate the phosphorylation of STAT3 in A431-V cells.

To identify the factors secreted by A431-V cells that could mediate STAT3 activation, we compared the relative expression levels of secreted proteins in A431-P and A431-V cells using a quantitative proteomics technique called SILAC (stable isotope labeling with amino acids in cell culture; Supplementary Fig. S3A and S3B). We observed increased expression of 80 proteins and decreased expression of 98 proteins in the secretome of A431-V cells compared with A431-P cells (Supplementary Table S1). As many secreted signaling proteins, such as cytokines, are below the current detection limit of mass spectrometry, a multiplex Luminex assay was performed in parallel to measure the concentrations of 42 cytokines in A431-P and
A total of 13 cytokines could be detected (Supplementary Table S2), of which IL8, CXCL1, GM-CSF, IL1α, and IL6 were significantly increased in CM from A431-V cells compared with that from A431-P cells (Fig. 3F).

Of these cytokines, CXCL-1 and IL6 are known to activate STAT3 in various cell types (16–20). To assess the role of CXCL-1 in aflibercept resistance, we generated A431 cells over-expressing CXCL-1 and assessed the response to aflibercept in vivo. CXCL-1 overexpression provided a slight growth advantage to A431 cells, but did not confer resistance to aflibercept (Supplementary Fig. S4A and S4B), suggesting that CXCL-1 is not a key player in mediating aflibercept resistance in this model.

We next focused on IL6 and Ingenuity Pathway Analysis of the 186 dysregulated proteins (SILAC plus Luminex) revealed the IL6

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Protein fold change</th>
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<tbody>
<tr>
<td>IL8</td>
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<tr>
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<tr>
<td>GM-CSF</td>
<td>25.7</td>
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<tr>
<td>IL1α</td>
<td>24.5</td>
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<td>IL6</td>
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Figure 3. Constitutive STAT3 activation in A431-V cells. A, phospho-RTK signaling antibody array was used to examine phosphorylation status of 28 receptor tyrosine kinases and 11 downstream signaling molecules in A431-P and A431-V cell lysates. Each RTK or signaling node is spotted in duplicate. B, Tyr705 phosphorylation level of STAT3 in A431-P and A431-V cells assessed by Luminex. C, Tyr705 phosphorylation level of STAT3 in A431-P and A431-V cells assessed by Western blot analysis. D, A431-P and A431-V cells were exposed to CM from A431-P and A431-V cells as indicated by the red arrows and cell lysates were analyzed for phospho-STAT3 detection by Western blot analysis. E, A431-P and A431-V cells were exposed to CM from A431-P and A431-V cells as indicated and analyzed and analyzed for phospho-STAT3 by IHC. Scale bar, 10 µm. F, top five cytokines with higher concentrations in A431-V conditioned media compared with A431-P CM, as measured by Luminex. G, increased expression of the IL6/STAT3-dependent protein SAA, S100A7, fibronectin, and IL36G in A431-V cell-conditioned media was confirmed by Western blot analysis.
IL6/STAT3 Signaling Mediates Anti-VEGF Resistance

Blockade of IL6 signaling by anti-IL6R antibody partially attenuates STAT3 activity in vitro and completely overcomes aflibercept resistance in vivo

To assess whether elevated STAT3 activation in A431-V cells was due to increased IL6 signaling, we determined whether A431-V cells and tumors have higher IL6 levels than their A431-P counterparts. Indeed, IL6 protein levels in both A431-V cell CM and A431-V tumor lysates were more than 3-fold higher than those in A431-P counterparts (Fig. 4A). In contrast to IL6, levels of IL6R were similar on A431-P and -V tumor cells (Fig. 4B). To determine whether increased phosphorylation of STAT3 in A431-V tumors was due to the increased IL6, we used an IL6 receptor antibody (sarilumab) that blocks IL6 binding to IL6R (25). As shown in Fig. 4C, STAT3 phosphorylation in cultured A431-V cells was noticeably reduced by blocking IL6R with sarilumab, although not to the same level as seen in A431-P cells.

To test whether elevated IL6/STAT3 signaling in A431-V tumors resists aflibercept treatment, we treated tumor-bearing mice with sarilumab and/or aflibercept to assess the effects on A431-V and A431-P tumor growth. Sarilumab binds and blocks human IL6R but not murine IL6R, so its actions are specific to the A431 human tumor cells. Sarilumab had no effect on parental A431-P tumor growth and did not enhance the effects of aflibercept, indicating that the growth of A431-P tumors is not dependent on IL6 signaling, irrespective of whether VEGF activity is inhibited (Fig. 4D, left). While single-agent sarilumab had no effect on the growth of A431-V tumors, the combination of sarilumab plus aflibercept had a significantly greater effect than aflibercept alone, completely inhibiting A431-V tumor growth (Fig. 4D, right). This finding indicates that IL6 signaling limits the effectiveness of aflibercept in A431-V tumors. The changes in growth kinetics of A431-V tumors upon aflibercept, sarilumab, or aflibercept plus sarilumab were reflected in differences in proliferation, as assessed by Ki-67 IHC (Fig. 4E). Consistent with a more prominent role for IL6/STAT3 signaling in A431-V tumors, Western blot analysis revealed that A431-V tumors have elevated levels of phosphorylated STAT3 compared to A431-P tumors, and that this STAT3 phosphorylation can be blocked by sarilumab treatment (Fig. 4F). These findings confirm that aflibercept resistance in A431-V cells is attributable to the increased STAT3 phosphorylation and IL6 signaling.

To extend our findings on the role of IL6/STAT3 signaling in limiting aflibercept efficacy, we tested the effects of sarilumab as a single agent and in combination with aflibercept on the growth of Du145 prostate tumor xenografts. Cultured Du145 cells exhibit constitutive STAT3 phosphorylation that can be inhibited by blocking IL6R with sarilumab, indicating the presence of an autocrine IL6/STAT3 signaling pathway (Fig. 5A) similar to that in A431-V cells (Fig. 4C). As shown in Fig. 5B, sarilumab as a single agent caused a significant growth delay of Du145 tumors, suggesting a role for autocrine IL6 signaling in Du145 tumor growth. Aflibercept as a single agent had a more dramatic effect than single-agent sarilumab, causing some tumor regression (Fig. 5B). However, the combination of aflibercept plus sarilumab caused a more rapid and pronounced tumor regression than aflibercept alone (Fig. 5B), further supporting the possibility that IL6 signaling limits the effectiveness of VEGF blockade in some tumors. Consistent with active IL6/STAT3 signaling in Du145 tumors, sarilumab decreased STAT3 phosphorylation as assessed by IHC of tumor sections (Fig. 5C).

Overexpression of IL6 promotes aflibercept resistance, whereas long-term blockade of both IL6 and VEGF decreases the emergence of aflibercept resistance

To determine whether high IL6 expression is sufficient to induce resistance to aflibercept, A431 cells were transduced with a lentiviral vector expressing human IL6 or an empty control vector. Ectopic IL6 expression resulted in accelerated tumor growth compared with control tumors (Supplementary Fig. S6A). The high levels of ectopic IL6 expression also resulted in increased circulating IL6 protein level (~100–200 ng/mL; data not shown), which was associated with body weight loss and early morbidity of tumor-bearing mice (Supplementary Fig. S6B). Aflibercept treatment starting when tumors were approximately 100 mm³ had no effect on tumor growth in IL6-overexpressing A431 tumors, while being very effective in A431 empty vector control tumors (Fig. 6A and B). These data indicate that elevated IL6 expression in tumor cells is sufficient to induce resistance to aflibercept in A431 xenograft tumors.

To investigate whether blockade of the IL6 pathway could prevent the emergence of aflibercept-resistant A431 tumors, we treated A431 tumor-bearing mice with control protein, aflibercept, sarilumab, or a combination of aflibercept plus sarilumab when the tumors reached a volume of approximately 100 mm³. Single-agent sarilumab treatment did not delay A431 tumor growth, whereas both aflibercept single agent and aflibercept plus sarilumab combination treatments resulted in prolonged growth stasis (Fig. 6C and D) for 6 weeks. As observed before, a subset of A431 tumors (5/7 tumors; Fig. 6D top, Supplementary Fig. S1) began to grow significantly after 6 weeks of continuous aflibercept treatment. In contrast, no tumors showed steady growth during the latter phases of continuous treatment with aflibercept plus sarilumab (0/7 tumors; Fig. 6D, bottom). These results suggest that changes in the IL6 pathway are not only relevant after aflibercept resistance occurred, but also play a role in the development of anti-VEGF resistance in A431 xenograft tumors.

IL6 serum levels may be predictive for outcome in ovarian cancer patients treated with aflibercept

Taken together, our preclinical results show that activation of the IL6/STAT3 pathway can provide evasive resistance to treatment with VEGF inhibitors such as aflibercept. To assess whether this finding is clinically relevant, we examined the levels of IL6 in ovarian cancer patients treated with single-agent aflibercept (26). Serum levels of IL6 were measured in a group of patients in an international, double blind, phase II study of advanced ovarian cancer, in which two different doses of aflibercept were used as monotherapy. Ninety-six patients were stratified into those with high and low circulating IL6 (cutoff 3.86 pg/mL). Although the study showed only a modest overall response rate to aflibercept,
Figure 4.
Effect of blocking cancer cell IL6 signaling pathway with sarilumab on the response of A431-P and A431-V tumors to aflibercept treatment. A, concentration of human IL6 was measured in CM from cultured A431-P or A431-V cells (left) and lysates from A431-P or A431-V tumor xenografts (right) by ELISA. **, P < 0.01, unpaired t test. B, IL6R was detected on A431-P and A431-V cells by flow cytometry. C, A431-P and A431-V cells were treated with human Fc control or sarilumab in vitro and cell lysates were used for Western blot analysis detecting STAT3 and phospho-STAT3. Densitometry analysis was performed to calculate the ratio of phospho-STAT3 to total STAT3 (p/t STAT3). D, A431-P or A431-V tumor-bearing mice were treated with human Fc, sarilumab, aflibercept, or the combination of sarilumab plus aflibercept for 14 days. The average tumor volume ± SD is plotted over the course of treatment. *, P < 0.05; **, P < 0.01; ***, P < 0.0001, two-way ANOVA with Bonferroni post hoc test. E, representative images of cell proliferation assessed by Ki-67 IHC in A431-V tumors treated with control Fc, sarilumab, aflibercept, or the combination of sarilumab plus aflibercept for 14 days. Scale bar, 10 μm; S, stroma; T, tumor; N, necrosis. F, A431-P or A431-V tumor-bearing mice were treated with a single dose of human Fc or sarilumab and tumor lysates were prepared 18 hours after treatment. Phospho-STAT3 (p-STAT3) and total STAT3 (t-STAT3) levels were determined by Western blot analysis. Densitometry analysis was performed to calculate the ratio of phospho-STAT3 to total STAT3 (p/t STAT3). *, P < 0.05, one-way ANOVA with Bonferroni post hoc test.
we observed that patients with high IL6 serum levels (> median) show significantly poorer overall survival compared with those with low IL6 levels (< median; Fig. 7). These results suggest a correlation between high levels of IL6 and poorer tumor response to anti-VEGF therapy.

Discussion

To uncover mechanisms underlying resistance to anti-VEGF therapies, we used serial passaging and selection in vivo to generate A431-V that is partially resistant to afibercept, and compared this line to the afibercept-sensitive parental counterpart A431-P. Our studies found that increased levels of proinflammatory mediators, such as IL6, were associated with STAT3 pathway activation in A431-V tumors, and can contribute to afibercept resistance. Blockade of the IL6/STAT3 pathway with an anti-IL6R antibody (sarilumab), which is specific to human IL6R, rendered A431-V tumors more sensitive to afibercept. Similarly, IL6R inhibition enhanced the activity of afibercept in another tumor model with strong endogenous IL6/STAT3 activity, namely Du145 prostate tumors. In addition, IL6 overexpression rendered A431 tumors resistant to afibercept, suggesting that elevated IL6 expression is sufficient to provide a survival advantage to tumor cells during afibercept treatment. In these experiments, activation of IL6R/STAT3 signaling in cancer cells is mediated by human IL6 because mouse IL6 does not activate the human IL6R (27), and murine IL6R is not inhibited by sarilumab, a human-specific IL6R antibody. Limitations of working with human tumor cells in immunocompromised mouse include the lack of species cross-activity of some factors such as IL6, thus potentially limiting tumor–stromal interactions that could induce STAT3 signaling in other tumor compartments (43).

Nevertheless, based on these findings, we conclude that in A431-V tumors, afibercept resistance is mediated to a large extent by increased IL6/STAT3 signaling in tumor cells, and that blockade of IL6R can overcome resistance to afibercept.

Various clinical studies have correlated high pretreatment serum IL6 level with poor outcome in patients with different types of cancer including head and neck squamous cell carcinoma (28), stage II and III gastric carcinoma (29), prostate cancer (30), metastatic renal cell carcinoma (31), metastatic breast cancer (32), and ovarian cancer (33). Elevated levels of IL6 in cancer patients could be due to increased expression in normal immune cells as a consequence of chronic (systemic) inflammation, and/or from stromal or cancer cells within tumors. The resulting activation of the STAT3 transcription factor in cancer cells is essential for IL6-dependent oncogenic activities such as promoting cancer cell proliferation and survival (34). STAT3 activation has been shown to support cancer cell survival in the conditions of growth factor deprivation (35) and to promote chemoresistance in cancer cells exposed to hypoxia (36), suggesting that STAT3 may be a potentially important cancer cell survival factor in tumors treated with antiangiogenic therapies. Our findings that patients with high IL6 serum levels showed poorer survival than those with low IL6 levels in a phase II study of advanced ovarian cancer where afibercept was used as monotherapy (26), suggests that our preclinical findings could have clinical significance and warrant further investigation.
Resistance to anti-VEGF therapies can occur via several mechanisms: (i) upregulation of various proangiogenic factors resulting in vessels that are less sensitive to VEGF blockade and/or (ii) changes in the characteristics of cancer cells, which provide the cells with a survival advantage and/or increased invasive potential in the environment of reduced tumor vessels (12–14). Our approach to induce variants of A431 tumor cells that are resistant to aflibercept allowed direct comparisons between parental and variant tumors. For example, screening of phospho-protein arrays found that phospho-STAT3 was the major difference between parental and variant A431 tumor cells. We also used unbiased SILAC screens to test for overall differences in the secreted protein profile (secretome) of parental and variant cells. Detailed expression analysis revealed upregulation of various potentially proangiogenic factors, including IL8 and CXCL1 in A431-V tumors, which could contribute to the increased vessel density. A key role for CXCL1 in mediating aflibercept resistance was ruled out in our model system. Given the STAT3 activation we observed in A431-V tumors, we decided to focus on IL6, rather than IL8 in subsequent studies. A large body of literature suggests that IL6 activates STAT3 (37, 38) and, in addition, our studies implicate a key role for the IL6/STAT3 pathway in resistance to anti-VEGF therapies.

Figure 6.
Overexpression of IL6 or long-term blockade of the IL6 pathway affects the response of A431 tumor xenografts to aflibercept treatment. A, A431 tumor cells were engineered to overexpress IL6 and tumor growth kinetics and aflibercept response was compared with A431 empty vector tumors for 7 days. The average tumor volume ± SD is plotted over the course of treatment. B, tumor growth changes of IL6 or empty vector expressing tumors in response to human Fc control protein or aflibercept from start of treatment. ****, P < 0.0001 one-way ANOVA, Bonferroni post hoc test. C, SCID mice bearing A431 tumors (100–150 mm³) were treated with human Fc control protein, sarilumab, aflibercept, or a combination of aflibercept + sarilumab for up to 7 weeks. D, individual tumor growth kinetics for the aflibercept and aflibercept + sarilumab-treated groups (Combo). The tumors that exhibit a “late escape,” defined as a tumor showing 40% or more increase in tumor volume between day 42 and day 49, are indicated by a red star next to the tumor growth curve. One combination-treated tumor, as indicated by a black # symbol, showed intermittent growth throughout the experiment, but did not show escape as defined above. One combination-treated tumor grew at the end of the experiment, but only showed an increase in tumor volume of 33% between day 42 and 49, indicated by a blue star next to the tumor growth curve.

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Notably, our studies on the role of IL6 in resistance to anti-VEGF therapies focus on autocrine IL6/STAT3 signaling in the cancer cells. Autocrine IL6 signaling has been reported in various cancer types, including multiple myeloma (39), prostate cancer (40), lung cancer (41), and breast cancer (42). In our studies on A431-V and Du145 xenograft tumors, activation of IL6R/STAT3...
signalizing in cancer cells is solely mediated by IL6 derived from the human cancer cells, but not the murine stromal cells, because mouse IL6 does not activate the human IL6R (27). Although human IL6 activates mouse IL-6R on stromal cells, this interaction is not inhibited by sarilumab, which is a human-specific IL6R antibody. Therefore, the effects of sarilumab reported here are due to the interruption of autocrine IL6/STAT3 signaling in cancer cells. Although other studies have reported that increased activation of tumor or stromal STAT3 can lead to enhanced STAT3 signaling in tumor endothelial cells (43), such a mechanism is apparently not at play in the A431-V model. Instead, our data highlight a mechanism of resistance to anti-VEGF therapy, in which the cancer cell characteristics change and provide a survival advantage in the environment of reduced tumor vessels.

Although IL6 has previously been implicated as a potential biomarker for targeted anti-VEGF therapy, the results have been inconsistent. When treated with bevacizumab plus a chemotherapeutic regimen, a better outcome was reported for metastatic colorectal cancer (mCRC) patients with a signature that included lower than average IL6 serum levels (44), whereas in another study of mCRC, a worse outcome was associated with a signature that included lower than average IL6 serum levels (45). High baseline IL6 levels were also reported to predict shorter progression-free survival (PFS) and overall survival (OS) in patients with advanced hepatocellular carcinomas (46). In line with these later studies, we observed in an ovarian cancer trial that aflibercept-treated patients with high IL6 had poorer survival than patients with low IL6 serum levels. However, caution should be taken when using serum IL6 levels as a biomarker, as our studies suggest that IL6R signaling on tumor cells is the relevant feature that confers resistance to anti-VEGF therapy.

IL6 could be a potential biomarker for anti-VEGF therapies, but our data now suggest that IL6 can also mediate anti-VEGF resistance and thus might be a valid therapeutic target when combined with anti-VEGF therapies in cancer patients. Although the data presented in this article do not provide details on how IL6/STAT3 activation in the tumor cells can confer resistance to aflibercept treatment, we hypothesize that a double hit of targeting the vasculature, thus diminishing oxygen and nutrient supply for the tumor cells, and blocking IL6, thereby shutting down the IL6/STAT3--regulated survival and proliferation pathway in the tumor cells and limiting IL6-mediated inflammation in the tumor microenvironment, can overcome anti-VEGF resistance in tumors with measurable IL6/STAT3 signaling. Further studies and clinical trials are necessary to validate or refute the role of IL6 in anti-VEGF resistance in different types of cancer.

Disclosure of Potential Conflicts of Interest
E. Ioffe is an employee and stock holder at Regeneron Pharmaceuticals. I. Lowy is a vice president and has ownership interest (including patents) in Regeneron. H.C. Lin is a senior director, molecular profiling, and reports receiving a commercial research grant and other commercial research support from Regeneron Pharmaceuticals Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: A. Eichten, J. Su, A. Adler, L. Zhang, E. Ioffe, G.D. Yancopoulos, D. MacDonald, G. Thurston
Development of methodology: A. Adler, E. Ioffe, A.A. Parveen, X. Duan, G. Thurston
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Su, A. Adler, X. Duan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Eichten, J. Su, A. Adler, L. Zhang, I. Lowy, C. Lin, C. Daly, G. Thurston
Writing, review, and/or revision of the manuscript: A. Eichten, J. Su, A. Adler, L. Zhang, I. Lowy, C. Lin, D. MacDonald, X. Duan, G. Thurston
Eichten et al.

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Resistance to Anti-VEGF Therapy Mediated by Autocrine IL6/STAT3 Signaling and Overcome by IL6 Blockade


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