Effects of Siltuximab on the IL-6 Induced Signaling Pathway in Ovarian Cancer

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Abbreviations: IL-6, interleukin-6; Stat3, signal transducers and activators of transcription 3; MDR, multidrug resistance

Running title: Siltuximab targeting IL-6 in ovarian cancer

Key words: Siltuximab, IL-6, ovarian cancer, drug resistance

Translational Relevance

IL-6 production is increased in drug resistant ovarian cancer cells, and in the serum and ascites of ovarian cancer patients. Siltuximab is a chimeric murine anti-human IL-6 monoclonal antibody and has been studied in different types of human cancers. We found that metastatic and drug resistant recurrent tumors have significantly higher IL-6 expression when compared to the matched primary tumors. Siltuximab specifically suppressed IL-6 induced Stat3 phosphorylation and Stat3 nuclear translocation. Treatment with siltuximab significantly decreased Stat3 downstream protein levels, including MCL-1, Bcl-X\textsubscript{L} and survivin. Siltuximab also increased the cytotoxic effects of paclitaxel in a paclitaxel resistant ovarian cancer cell line \textit{in vitro}. Therefore, we believe inhibition of IL-6 signaling may be beneficial for ovarian cancer treatment although siltuximab itself did not show significant effects on the growth of paclitaxel resistant tumor \textit{in vivo}.

Abstract

\textbf{Purpose:} To explore potential therapeutic strategies for interrupting the IL-6 signaling pathway, we measured IL-6 expression in ovarian cancer tissues, and evaluated the effects of a monoclonal anti-IL-6 antibody, siltuximab (CNTO 328), on levels of IL-6 induced Stat3 phosphorylation, Stat3
nuclear translocation, and Stat3 downstream antiapoptotic genes. We then looked for enhancing paclitaxel sensitivity in multidrug resistant ovarian cancer cell lines.

**Experimental Design:** Expressions of IL-6 in ovarian cancer patient specimens were assessed by immunohistochemistry. Effects of siltuximab on IL-6 induced activation of Stat3 in an ovarian cancer cell line were determined by Western blot and real-time analysis of Stat3 nucleocytoplasmic translocation. Influence of combination of siltuximab and paclitaxel on tumor growth was evaluated in a xenograft mouse mode *in vivo.*

**Results:** Metastatic and drug-resistant recurrent tumors have significantly higher IL-6 expression when compared to the matched primary tumors. Siltuximab specifically suppressed IL-6 induced Stat3 phosphorylation and Stat3 nuclear translocation. Treatment with siltuximab significantly decreased the levels of Stat3 downstream proteins such as MCL-1, Bcl-XL and survivin. Treatment with siltuximab reduced expression of multiple IL-6-induced genes in these cell lines. Furthermore, siltuximab increased the cytotoxic effects of paclitaxel in a paclitaxel resistant ovarian cancer cell line *in vitro,* but combination therapy with siltuximab did not have a significant effect on paclitaxel resistant tumor growth *in vivo.*

**Conclusions:** These results demonstrated that siltuximab effectively block the IL-6 signaling pathways and IL-6-induced gene expression. Blockage of IL-6 signaling may provide benefits for the treatment of ovarian cancer.
Ovarian cancer is the fourth most lethal cancer among women and the leading cause of gynecological cancer deaths in the United States (1). Approximately 26,000 new cases of epithelial ovarian cancers are diagnosed and over 16,000 women die of this disease in the U.S. annually (1, 2). Standard chemotherapy for ovarian cancer is combination of paclitaxel and carboplatin. Unfortunately, the efficacy of both of these agents is limited by the eventual development of multidrug resistance (3). This multidrug resistance may be due to overexpression of a plasma membrane glycoprotein Pgp, alterations in specific proteins targeted by these drugs (such as tubulin mutations at the site of paclitaxel binding) or due to changes in the apoptotic threshold (4-6). Therapeutic strategies that manipulate the apoptotic threshold offer a mechanistic approach to reversing multidrug resistance.

Interleukin-6 (IL-6) was initially identified as an antigen non-specific B-cell differentiation factor in the culture supernatants of mitogen or antigen stimulated peripheral blood mononuclear cells, which were capable of inducing B cells to produce immunoglobulins (7). IL-6 has subsequently been identified as a pleiotropic cytokine with a wide range of biologic activities in various cells including tumor cells (8, 9). IL-6 activates several target genes involved in differentiation, survival, apoptosis and proliferation (7). Considerable evidence implicates IL-6 in the progression of cancer, in particular in multiple myeloma, where it inhibits apoptosis induced by serum starvation, dexamethasone, and Fas ligand (7, 10). Several studies have shown that the anti-apoptotic effects of IL-6 were associated with the expression of the Bcl-2 family proteins(10, 11). For example, in myeloma cell lines and myeloma cells obtained from patients, disease progression was correlated with upregulation of Bcl-X\textsubscript{L} protein and increasing serum IL-6 levels (11). IL-6 has
also been implicated in the tumor biology of breast, prostate and ovarian cancer (12-15). Breast cancer cells that are sensitive to drug treatment do not express IL-6, whereas multidrug resistant breast cancer cells produce high levels of IL-6, suggesting that autocrine production of IL-6 is capable of modifying drug resistance (16). Additional evidence suggests that IL-6 is an autocrine and paracrine growth factor for prostate cancer cell lines and serves as a resistance factor for cisplatin-mediated cytotoxicity (14). Similarly, treatment of cisplatin resistant renal carcinoma cell lines with cisplatin in combination with an anti-IL-6 or anti-IL-6 receptor (IL-6R) antibody reversed the resistance to cisplatin in vitro (17). In ovarian cancer, IL-6 is present at high levels in serum and overexpression of IL-6 has also been observed in a genetically defined human ovarian cancer model (13, 18, 19). Although IL-6 is less sensitive than CA125 as a tumor marker for ovarian cancer, its production may be related to tumor aggressiveness (13, 18). Specifically, patients with high IL-6 levels have a shorter survival than patients presenting with normal or low IL-6 values (18).

Moreover, recent evidence suggests that therapeutic strategies directed at modulating the IL-6 pathway may have a direct impact on cellular resistance to cisplatin and doxorubicin (20). For instance, exogenous IL-6 treatment rendered tumor cells resistant to apoptosis induced by several cytotoxic agents including doxorubicin, VP-16 and cisplatin. In contrast, specific blockade of IL-6 by antisense oligonucleotide sensitized tumor cells to these drugs (20). Finally, we and others have demonstrated that IL-6 production is increased in drug resistant ovarian cancer cell lines and also in the serum and ascites of ovarian cancer patients (5, 9, 13, 18). Together these data strongly support the theory that IL-6 is a potent and clinically important regulator of anti-apoptotic gene expression and drug resistance.
Recent studies have demonstrated that siltuximab (CNTO 328), a chimeric murine anti-human IL-6 monoclonal antibody (mAb), can neutralize IL-6 function, an effect that has been investigated in different types of human cancers (21-23). It has been used in clinical trials in which it was well tolerated in patients with cancer (24, 25). In this study, we investigated the effect of siltuximab on the IL-6 induced signaling pathway and IL-6-dependent gene expression in paclitaxel-resistant ovarian cancer cells. Our study provided further evidence for the development of the paclitaxel and siltuximab combination therapy for patients with ovarian cancer.

Material and Methods

Cell lines, antibodies and drugs. Human ovarian cancer cell line SKOV-3 and, CAOV-3 cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD). The paclitaxel-resistant SKOV-3_{TR} and CAOV-3_{TR} were established in this laboratory as described previously (5, 26). Briefly, the paclitaxel-resistant cell lines were selected over a period of 8 months by continuous culture in media containing step-wise increases in paclitaxel. The fresh paclitaxel was obtained from the pharmacy at the Massachusetts General Hospital and stored at –20°C. The rabbit polyclonal antibodies to Bcl-X_{L}, MCL-1 and the mouse monoclonal antibodies to phosphorylated-Stat3, survivin were purchased from Cell Signaling Technologies (Cambridge, MA). The mouse mAb to actin and MTT were purchased from Sigma-Aldrich (St. Louis, MO). The IL-6 mAb siltuximab and control non-specific antibody F105 were produced by Ortho Biotech Oncology Research & Development, Division of Centocor.
**Cell culture.** All the cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin and streptomycin at 100 units/ml and 100 mg/ml, respectively (Invitrogen). Cells were incubated at 37°C in 5% CO₂-95% air atmosphere and passaged twice every 6 days. Paclitaxel resistant cell lines were periodically cultured in paclitaxel (0.2µM) to confirm their drug resistance. Cells were free on mycoplasma contamination as tested by MycoAlert(R) MycoplasmaDetection Kit from Cambrex (Rockland, ME).

**Immunohistochemistry.** Expressions of IL-6 in ovarian cancer patient specimens were assessed by immunohistochemistry. Human ovarian cancer tissue microarray was generated by the Tissue Microarray Core at the Dana-Farber/Harvard Cancer Center using matched sample sets obtained from 26 individual ovarian cancer patients as previously described (6) (Approved IRB Protocol 03-348). In brief, each set was composed of the primary ovarian tumor, a metastatic tumor obtained at the time of the primary surgery, and a recurrent tumor. Slides of 5-µm sections of the relevant arrays were baked at 60°C for 2 hour, deparaffinized in xylene for 15 minutes, transferred through 100% ethanol for 5 minutes, and then rehydrated with graded ethanol. Endogenous peroxidase activity was quenched by a 10-minute incubation in 3% hydrogen peroxide in methanol. Antigen retrieval was processed with Target Retrieval Solution (Vector Laboratories, Burlingame, CA) following the instruction of the manufacturer. After antigen retrieval, the slides were washed with PBS (pH7.5) thrice at room temperature. Protein blocking was done by incubating the slides in 5% normal goat serum and 1% bovine serum albumin in PBS for 1 hour. Primary antibody of IL-6 (R&D Systems, immunohistochemistry validated antibody) was applied at 4°C overnight (1:100 dilution) in 1% bovine serum albumin with 5% normal goat serum. After 2-minute rinses in PBS...
thrice, bound antibody was detected with the Vectastain ABC kit (Vector Laboratories) and visualized with 3, 3′-diaminobenzidine high-sensitivity substrate from Vector Laboratories. Finally, the slides were counterstained with hematoxylin QS (Vector Laboratories) and mounted with VectaMount AQ (Vector Laboratories) for long-term preservation. IL-6 staining intensity was graded into 4 groups: no staining (0), weak staining (1+), moderate staining (2+) and intense staining (3+). In each sample, three different areas and 100 cells per area were evaluated microscopically with a X 40 objective magnification. The percentage of cells at each intensity within these areas was determined at different times by two investigators blinded to the source of the samples, and the average score was used.

**Evaluation of ovarian cancer cell line’s response to IL-6.** To confirm IL-6 regulation of pStat3 protein expression, ovarian cancer cell line SKOV-3 and kidney cell line BHKpEGFP-Stat3 were cultured for 24 hours without IL-6. Recombinant human IL-6 (R&D Systems) was used to treat these cells. The cells were cultured in the presence of exogenous IL-6 (30 ng/mL) for 1 hour. After the treatment period, the cells were harvested, washed, lysed in 1× RIPA lysis buffer (Upstate), and pStat3 protein levels were determined by Western blotting.

**Effect of siltuximab on IL-6 induced activation of Stat3 in ovarian cancer cell line.** SKOV-3 cells were seeded onto 6-well plates and cultured overnight. The cells were then treated with either IL-6 (30ng/mL) alone or in the presence of different doses of siltuximab for 1 hour. After the treatment period, the cells were harvested, washed, lysed in 1× RIPA assay lysis buffer, and pStat3 protein levels were determined by Western blotting.

**Effect of siltuximab on Stat3 signaling in paclitaxel resistant cell lines.** SKOV-3_TR and Caov-3_TR cells were treated with siltuximab at the dose of 0, 0.001, 0.01, 0.1, 1.0, 10.0 µg/ml overnight. Cellular proteins were harvested with 1× RIPA lysis buffer. Expression levels of Stat3,
pStat3, BcL-XL, MCL-1, survivin, or β-actin was determined by Western blotting with specific antibodies.

**Western Blotting.** Total cell lysates were prepared, and Western blot analysis was performed as previously described (27). In brief, the cells were lysed in 1× RIPA lysis buffer (Upstate Biotechnology, Charlottesville, VA) and protein concentration was determined by the DC Protein Assay (Bio-Rad). Twenty-five micrograms of total protein were resolved on NuPage™ 4–12% Bis-Tris Gels (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS, pH 7.4, with 0.1% Tween-20 and with gentle agitation overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) were incubated in TBS, pH 7.4, with 5% nonfat milk (Bio-Rad) and 0.1% Tween-20, at a 1:2,000 dilution for 1 h at room temperature with gentle agitation. Positive immunoreactions were detected by using SuperSingal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

**Real-time analysis of the effect of siltuximab on Stat3 nucleocytoplasmic translocation.**

To study the effects of siltuximab on Stat3 nuclear translocation in live cells, a real-time cell-based assay was used as described below. Stable transfection of expressing the EGFP-Stat3 was generated in hamster kidney cell line BHK-21 (BHKpEGFP-Stat3) and in ovarian cancer cell line SKOV-3 (SKOV-3pEGFP-Stat3) using standard lipofectmine (Invitrogen) transfection techniques with G418 selection. The pEGFP-Stat3 stable expression cell line is a cell-based assay system examining the IL-6/Stat3 signaling pathway, which allows to directly visualizing nuclear transport of Stat3 as a result of IL-6R binding to IL-6 ligand. Cells which stably express the EGFP-Stat3 fusion protein were seeded at a density of 4,000 cells per well in 96-well flat bottom plates and incubated overnight at 37°C. The cells were then incubated for 4 hours with siltuximab at 0, 0.001, 0.01, 0.1, 1.0, 10.0 µg/ml followed immediately thereafter with the addition of IL-6 to a final concentration of 30 ng/ml.
Subcellular localization of the fusion protein was analyzed using a Nikon Eclipse Ti-U fluorescence microscope (Nikon Corp.) equipped with a SPOT RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Transcriptional profiling and analysis.** The SKOV-3 and Caov-3 ovarian cancer cell lines were seeded onto the 100mm cell culture plate overnight. The cells were then incubated with IL-6 (30ng/ml) alone for one hour or pre-treated with siltuximab for four hours and then treated with IL-6 for one hour. Total RNA was collected from these cells using TRIzol® Reagent (GIBCO Grand Island, NY) according to the manufacturer’s instructions. To account for and eliminate biologic noise, RNA was isolated from three distinct flasks of each cell line and pooled. RNA quality was determined via ethidium bromide staining following agarose/formaldehyde gel electrophoresis. Total RNA was processed and hybridized to Affymetrix Genechip U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) by the Gene Array Technology Center at Harvard Medical School (http://genome.med.harvard.edu). Affymetrix Gene Chip U133 Plus 2.0 is the first and most comprehensive whole human genome expression array. This array completes coverage of the whole human Genome with over 47,000 transcripts. The expression level of each mRNA is quantified by measuring its hybridization to these 23-mers in comparison to its hybridization to a one-base mismatch oligonucleotide. GeneSifter was used to analyze the microarray data (http://www.genesifter.net/web/).

**TaqMan reverse transcription-PCR for validation of differentially expressed gene.** Real-time RT-PCR was performed to validate differentially expressed genes. For gene expression detection, cDNA reverse transcription was performed from total RNA samples using specific oligo dT primers from the Taq Man RNA Assays and reagents from the TaqMan RNA Reverse
Transcription kit (Applied Biosystems, Foster City, CA). The resulting cDNA was amplified by PCR using Taq Man UGT2B4 (NM_021139), ATP2B2 (X63575) and β-actin gene assay primers with the Taq Man Universal PCR Master Mix and analyzed with a StepOnePlus Real time PCR System according to the manufacturer’s instructions (Applied Biosystems). β-actin gene expression was used as a control. The relative levels of gene expression were calculated from the relevant signals by normalization with the signal for actin expression. PCR reaction mixtures contained Taq Man human UGT2B4, or ATP2B2 and β-actin probes in Universal PCR Master Mix in a total volume of 20µl. Cycling variables were as follows: 95ºC for 10 min followed by 40 cycles at 95ºC (15 s) and annealing/extension at 60ºC (1 min). All reactions were performed in triplicate.

**Cytotoxicity assay.** The cytotoxic effects of siltuximab and paclitaxel on SKOV-3TR, Caov-3TR, were assessed using the MTT assay as previously described (28). Briefly, 2,000 cells/well were seeded in 96-well plates in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin) containing different concentrations of siltuximab. After 96 hours of culture, 10µl MTT (5 mg/ml in PBS) were added to each well and the plates were incubated for 4 h. The resulting formazan product was dissolved with acid-isopropanol and the absorbance at a wavelength of 490 nm (A490) was read on a SPECTRAmax Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). The absorbance values were normalized by assigning the value of the control line in the medium with F105. Experiments were performed in triplicate. Response curves were fitted with use of GraphPad PRISM 4 software (GraphPad Software). The IC50 was defined as the paclitaxel concentration required decreasing the A490 value to 50%.
**Effect of combination siltuximab and paclitaxel on tumor growth in vivo.** The protocol for animal use in this project has been approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (SRAC) under the protocol number 2004-N000296/1. The Crl:SHO-Prkd<sup>SCID</sup>Hr<sup>hr</sup> nude female mice at approximately 3-4 weeks of age were purchased from The Charles River Laboratories (Ann Arbor, MI). To determine the effect of siltuximab on paclitaxel sensitivity in xenograft model, 5 x 10<sup>6</sup> SKOV-3<sub>TR</sub> cells were injected subcutaneously with matrigel (BD Biosciences, San Jose, CA) on day one. Two weeks after injection, the mice were divided into 6 groups. Group one received intraperitoneal injection with normal saline, group two with non-specific antibody F105 (20 mg/kg), group three with siltuximab (20 mg/kg), group four with paclitaxel (20 mg/kg), group five with paclitaxel (20 mg/kg body weight) and siltuximab (20 mg/kg). The final group of mice received paclitaxel (20 mg/kg) and non-specific antibody F105 (20 mg/kg). The dose of paclitaxel (20 mg/kg) and siltuximab (20 mg/kg) was based on previous studies (29-32). All six groups were treated twice a week for five weeks. The health of the mice and evidence of tumor growth were examined daily. Tumors were measured twice a week with a digital caliper. Tumor volume (mm<sup>3</sup>) was calculated as (W<sup>2</sup> x L) / 2, where W is width and L is length.

**Statistical data Analysis.** A two-sided Student's t test (GraphPad PRISM 4 software, San Diego, CA) was used to compare the IL-6 staining intensity scores among primary tumors, recurrent tumors, and tumors with metastasis and P values < 0.05 were accepted as a significant difference between means. Effect of treatment siltuximab on paclitaxel sensitivities in multidrug resistant cells were also evaluated using the two-sided Student's t test). Error bars are SD of averaged results and P
values < 0.05 were accepted as a significant difference between means. Values are representative of triplicate determinations in two or more experiments.

Results

**Increased expression of IL-6 in metastatic and recurrent ovarian cancer.** IL-6 has been found at high levels in serum and ascites of ovarian cancer patients. Patients with high IL-6 levels have a shorter survival rate when compared to patients with low IL-6 levels. In this study, we further examined IL-6 expression in primary, metastatic, and recurrent ovarian cancer tissues. This analysis was done in a tumor tissue microarray that included the primary tumor, a synchronous metastasis, and a metachronous metastasis from the same patient with the metachronous metastasis being collected at the time of tumor recurrence after treatment with platinum- and taxane-based chemotherapy. Immunohistochemical analyses determined that all tumors present on the microarray had weak, moderate and strong staining for IL-6. The relative levels of IL-6 staining in the tumor sample sets from 28 individual patients were scored as weak staining (1+), moderate staining (2+) or intense staining (3+). There were trends toward greater IL-6 expression in the recurrent tumors as compared with the matched primary tumors (Fig. 1). There was also an increase in the intensity of IL-6 expression in the recurrent metastatic lesion as compared with the primary metastasis.

**IL-6 enhances Stat3 phosphorylation levels.** To test whether Stat3 phosphorylation levels could be stimulated by IL-6, a BHKpEGFP-Stat3 stable expression cell line and an ovarian cancer cell line SKOV-3 were treated with IL-6 and the levels of Stat3 phosphorylation were measured by Western blotting. As illustrated in Figure 2A, a clear increase in Stat3 phosphorylation levels was
observed in the IL-6-treated cell lines. Notably, both phosphor-Stat3 (pStat3, endogenous protein) and pEGFP-Stat3 (fusion protein) were upregulated in BHKpEGFP-Stat3 treated cell lines (Fig.2A).

**Siltuximab inhibits IL-6 induced Stat3 phosphorylation.** Because IL-6 activity is mediated by Stat3 phosphorylation, we then investigated whether siltuximab inhibits IL-6 induced Stat3 phosphorylation in SKOV-3. When the cells were incubated with either IL-6 alone or with siltuximab at a range of concentrations of 0, 0.01, 0.1, 1.0 and 10 ug/ml for one hour, Western blot analysis demonstrated that siltuximab significantly reduced phospho-Stat3 (pStat3) expression in the treated cell lines (Fig.2B).

**Siltuximab inhibits Stat3-mediated anti-apoptotic protein expression.** IL-6 exerts its actions through activation of Stat3 and then through Stat3 downstream anti-apoptotic regulatory genes BcL-X\textsubscript{L}, MCL-1 and survivin. Inhibition of Stat3 phosphorylation would be predicted to effect Stat3 downstream genes expression. Therefore, we examined whether exposure of paclitaxel-resistant ovarian cancer cells line SKOV-3\textsubscript{TR} and Caov-3\textsubscript{TR} to siltuximab would result in decreased expression of the anti-apoptotic proteins Bcl-X\textsubscript{L}, MCL-1 and survivin. Incubation with siltuximab overnight significantly reduced levels of BcL-X\textsubscript{L}, MCL-1 and survivin expression in SKOV-3\textsubscript{TR} and Caov-3\textsubscript{TR} (Fig.3). In the control experiments, siltuximab did not significantly alter the level of total Stat3. These results thus suggest siltuximab could effectively inhibit IL-6 induced antiapoptotic gene expression.

**Siltuximab inhibits Stat3 nucleocytoplasmic translocation.** In resting cells, most Stat3 remains localized to the cytoplasm until it interacts with IL-6 activated Jak kinase, which induces a series of events, including phosphorylation and translocation of Stat3 molecules to the nucleus. In an
attempt to access whether siltuximab could interrupt IL-6-dependent Stat3 nuclear translocation, a real-time cell-based method was applied to image an EGFP-Stat3 chimera in the nucleus and cytoplasm in SKOV-3pEGFP-Stat3 cells. In resting cells, most of EGFP-Stat3 remains cytoplasm until the addition of human IL-6, which promptly induced the translocation of fluorescent Stat3 molecules to the nucleus. Exposure of cells to siltuximab for 4 h, followed by an hour-long incubation in IL-6 significantly blocked IL-6- or Stat3 nucleocytoplasmic translocation (Fig.4A). Similar results have been found in BHKpEGFP-Stat3 cells (Fig.4B).

**Differentially expressed genes in IL-6 treated ovarian cancer cells.** IL-6-induced Stat3 activation is a well known pathway which is regulated by a series of anti-apoptotic (anti-death) signals. However, the details of IL-6 signaling in ovarian cancer are still unknown. A better understanding of the mechanisms that underlie IL-6 signaling in ovarian cancer may allow for more effective treatments. In this study, Human Affymetrix Gene Chip U133 Plus 2.0 arrays were used to examine relative gene expression levels in IL-6 treated ovarian cancer cell lines SKOV-3 and Caov-3. The array data have been submitted to Gene Expression Omnibus* and have been assigned a GEO accession number as GSE20272. The expression profiles were evaluated by Genesifter. We found that a large number of genes had significantly different levels of expression in IL-6 treated cell lines as compared to untreated cell lines. To focus on genes with only significant changes in expression levels, we identified genes with a ten-fold or greater change in expression levels. Using this criteria, 506 (SKOV-3/IL-6) and 674 (Caov-3/IL-6) genes exhibited more than ten-fold overexpression in the IL-6 treated cell lines relative to their expression in the untreated lines (Supplementary Fig. 1A). The top 20 most highly overexpressed genes in SKOV-3/IL-6 and Caov-3/IL-6 as compared with SKOV-
3 and Caov-3 are summarized in Supplementary Table 1. These genes include UDP glucuronosyltransferase 2 family, polypeptide B4 (UGT2B4), ATPase calcium transporting plasma membrane 2 (ATP2B2) and several hypothetical genes with unknown function. On the other hand, 1002 (SKOV-3/IL-6) and 1148 (Caov-3/IL-6) transcripts were more than ten-fold downregulated in the IL-6 treated cell lines when compared to the parental cell line. Fifty four genes exhibited more than ten-fold down regulation in both SKOV-3/IL-6 and Caov-3/IL-6 cell lines. The top 20 most highly down regulated genes in SKOV-3/IL-6 and Caov-3/IL-6 as compared with SKOV-3 and Caov-3 are summarized in Supplementary Table 2. These genes include glycine receptor, cadherin, and protein tyrosine phosphatase (lymphoid).

Although both SKOV-3 and Caov-3 are ovarian cancer cell lines, the transcripts identified with altered expression in each IL-6 treated cell line were largely non-overlapping and encode proteins with a wide variety of functions (Supplementary Fig. 1A, Supplementary Table 1). Venn diagram (Supplementary Fig. 1A) shows that 27 genes exhibited more than ten-fold overexpression in both SKOV-3/IL-6 and Caov-3/IL-6 cell lines.

**Effect of siltuximab on IL-6 induced gene expression.** To determine the effect of siltuximab on IL-6 induced gene expression in ovarian cancer, the gene expression profiles were compared between cell lines treated with IL-6 alone and those treated with both siltuximab and IL-6. We found a large number of genes had significantly decreased expression when treated with siltuximab. 634 (SKOV-3/IL-6/ siltuximab) and 679 (Caov-3/IL-6/ siltuximab) genes were downregulated more than ten-fold as compared with SKOV-3/IL-6 and Caov-3/IL-6 (Supplementary Fig. 1B). 31 genes
exhibited more than ten-fold downexpression in both SKOV-3/IL-6/ siltuximab and Caov-3/IL-6/ siltuximab cell lines (Supplementary Fig.1B).

**Confirmation of differentially expressed genes by real-time PCR.** UGT2B4 and ATP2B2 genes were selected for further study as these two genes are overexpressed in both SKOV-3/IL-6 and Caov-3/IL-6 cells lines and on the top 20 genes list (Supplementary Table 3). The real time RT-PCR results demonstrated that the expressions of UGT2B4 and ATP2B2 genes were blocked by treatment with siltuximab (Fig. 5).

**Siltuximab increases paclitaxel sensitivity in paclitaxel resistant ovarian cancer cells in vitro.** Inhibition of Stat3 signaling in SKOV-3<sub>TR</sub> or Caov-3<sub>TR</sub> would be predicted to inhibit ovarian cancer cell proliferation and increase drug sensitivity to paclitaxel. MTT cytotoxicity assay demonstrated that siltuximab increased paclitaxel-induced cell death and partially overcame paclitaxel resistance (Fig. 6). The paclitaxel IC<sub>50</sub> value of siltuximab treated SKOV-3<sub>TR</sub> cells were two to four-fold lower than those of untreated cells (Fig.6A). Statistical analysis of MTT cell viabilities data also showed inhibit IL-6 with siltuximab enhances the cytotoxicity of paclitaxel (Fig.6B and Fig.6D).

**Combination therapy with siltuximab has no significant effect on tumor growth in vivo.** To further evaluate the effects of siltuximab and paclitaxel combination therapy on the tumor growth of ovarian cancer resistant cells, the growth of xenograft tumors were examined. Data showed that paclitaxel and siltuximab have no significant effect on tumor growth on paclitaxel resistant tumors when compared with treatment with individual agents (Supplementary Fig.2).
Discussion

The development of multidrug resistance (MDR) in ovarian cancer creates a major obstacle for treatment of ovarian cancer patients. Understanding the biology of MDR in ovarian cancer may help identify new therapeutic strategies. Previously, we and others have found that IL-6 as well as IL-6 activated Stat3 is associated with development of MDR in ovarian cancer (5, 6). This pathway is often over expressed and activated in many paclitaxel-resistant ovarian cancer cells (5, 6, 33). Higher serum and ascites levels of IL-6 have been found in patients with ovarian cancer than in patients with other malignancies, and levels have been shown to correlate with extent of disease and poor clinical outcome (8, 13-15, 34). As yet it is not clear whether increased IL-6 levels in patients with ovarian cancer are produced by the tumor itself or mainly by host tissues, in this study, we provide further evidence suggesting that IL-6 is associated with ovarian cancer progression as demonstrated by IL-6 is expressed at higher levels in metastatic and recurrent as compared with primary tumors. These data suggest that ovarian cancer cells themselves produce IL-6 in paracrine and autocrine fashion, and IL-6 secretion may be associated with the higher histological grade, greater invasion of tissues, and worse overall survival. IL-6 exerts its actions by activation of Stat3 and then through Stat3 downstream anti-apoptotic proteins. There are multiple lines of evidence demonstrating that IL-6 production and IL-6 induced Stat3 activation may prevent cell death and lead to immunosuppression and drug resistance through upregulation of survival proteins in ovarian cancer. Therefore, IL-6 and Stat3 proteins emerged as important targets for ovarian cancer therapy.
Antibody-mediated therapy offers a unique opportunity to inhibit the activity of specific proteins that are critical for tumor growth and metastasis. Several clinical investigations of mAbs to IL-6 in the treatment of cancer have been reported (35-37). In the early clinical trials with IL-6 neutralizing anti-human IL-6 mAb (BE-8, a murine anti-human IL-6 mAb) in multiple myeloma, the results showed IL-6 therapy could help patients with early-stage disease (35, 36). In this preclinical study, we demonstrated that siltuximab specifically inhibited IL-6 induced Stat3 activation, Stat3 nuclear translocation, and Stat3 downstream genes expression in ovarian cancer cells. Western blot analysis demonstrated that siltuximab treatment resulted in the down regulation of anti-apoptotic proteins in paclitaxel-resistant cells. The ability of siltuximab to affect IL-6-Stat3 signaling points to the potential for its application in the treatment of ovarian cancer.

Understanding the details of IL-6-dependent gene expression cascade may lead to development of novel therapeutic strategies to overcome drug resistance. To further elucidate the IL-6 induced signaling pathways and gene expressions in ovarian cancer, we first evaluated the IL-6 dependent gene expression profiles in ovarian cancer. As we demonstrate here, treatment of ovarian cancer cell lines with IL-6 displayed a large number of alterations in gene expression. In accordance with the importance of Stat3 signaling in ovarian cancer cells, our Affymetrix studies revealed an induction of gene participation in the Jak/Stat3 signaling pathway by IL-6. These results suggest the existence of IL-6-dependent and Stat3-mediated mechanism triggering survival of ovarian cancer cells. In addition to Jak/Stat3 genes, there are two genes, UGT2B4 and ATP2B2, which are overexpressed in both SKOV-3 and Caov-3 treated cell lines. Siltuximab can effectively block IL-6 induced expression of UGT2B4 and ATP2B2 in these cell lines as confirmed by real-time PCR.
UGT2B4 is a member of UDP-glucuronosyltransferase family and plays a role in detoxification (38). ATP2B2 is a magnesium-dependent enzyme catalyzes the hydrolysis of ATP coupled with the transport of calcium out of the cell (39). Notably, none of these genes have yet to be found associated with IL-6 signaling previously.

Siltuximab has shown promising results in early clinical trials for the treatment of several types of human cancers including ovarian cancer (22, 37, 40, 41). Because siltuximab showed it specifically blocks IL-6 signaling, we then assessed the effects of siltuximab on the IL-6 induced gene expression in ovarian cancer. We observed that pretreatment of cells with siltuximab lead to downregulation of IL-6 induced gene expressions including MCL-1, BcL-XL and survivin. Taken together, our results provide strong evidence that siltuximab specifically inhibits IL-6 induced gene expression. Similar results have been found in studying siltuximab effects on prostate cancer cell lines (41, 42). Based on the observation that siltuximab specifically inhibit IL-6 signaling pathway, we examined whether disruption of the IL-6 pathway would also increase paclitaxel sensitivity in ovarian cancer. We found that siltuximab could modest increase the sensitivity of ovarian cancer drug resistant cells to paclitaxel in vitro. These studies provided a proof of principle that siltuximab is active against drug resistant ovarian cancer cell lines.

To further determine the effect of siltuximab and paclitaxel in vitro, we also evaluated combination therapy in the xenograft mouse model using SKOV-3TR. Although combination therapy with siltuximab showed modest increases of paclitaxel sensitivity in resistant cell lines in vitro, our in vivo results suggest that a combination of siltuximab with paclitaxel have no significant effect on xenograft tumor growth. There are several possible reasons for the failure of combination therapy.
with siltuximab *in vivo*. One possibility may be that the paclitaxel resistant cell line, SKOV-3\textsubscript{TR} used in this study is 100-fold more resistant as compared to the sensitive SKOV-3 cell line, and the treatment dose (20mg/Kg) chosen was based on the sensitive cell line (29, 30). This dose (20mg/kg) may have an insufficient drug effect on resistant tumor growth. Higher doses of paclitaxel could be administrated for better verification, but unfortunately, the mice’s tolerance to paclitaxel is limited. The other possibility is that the siltuximab antibody dose (20mg/kg) was also selected based on reported data and this may not be the optimal dosage amount for resistant cells *in vivo*. Finally, the siltuximab antibody may have not penetrated into the tumor site effectively.

The ability to modulate paclitaxel resistance has been complicated by the fact that drug resistant tumors simultaneously exhibit multiple resistance mechanisms (3, 43). Although the effect of siltuximab on reversing the paclitaxel is modest *in vitro* and there is an absence of an effect *in vivo*, we believe that the usefulness of siltuximab in clinical drug resistance cannot be discounted as clinically irrelevant because most patients with cancer who progress after chemotherapy therapy have levels of acquired drug resistance of no more than 2–3-fold (44, 45). Because most cytotoxic drugs have a low therapeutic index, low fold changes in drug resistance may be sufficient to cause clinical treatment failure (44, 45). Since most cytotoxic drugs have a low therapeutic index, low fold changes in drug resistance may be sufficient to cause clinical treatment failure (44-46). Evaluations of these possibilities may be considered to the clinical development of siltuximab/paclitaxel treatment of drug resistant ovarian cancer.
Acknowledgements

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Figure Legends

Fig. 1. Comparison of IL-6 expression in primary, metastatic, and recurrent ovarian cancer. (A) Distribution of Stat3 immunohistochemical staining scores in primary, metastatic, and recurrent ovarian cancer. Statistical analysis performed by a Student's t test (between two groups) to compare the IL-6 staining intensity scores among primary tumors, recurrent tumors, and tumors with metastasis and p values < 0.05 were accepted as a significant difference between means. (B) Representative expression of IL-6 in matched primary, metastatic, and recurrent ovarian cancers.

Fig. 2. IL-6 dependent up-regulation of pStat3 expression and effect of siltuximab. Hamster kidney BHKpEGFP-Stat3 and ovarian cancer cell line SKOV-3 cells were treated with 30 ng/mL IL-6 for 1 hour as indicated. Cell lysates were subjected to Western blot analysis with anti-pStat3, or anti-β-actin antibodies as described in Materials and Methods.
Fig. 3. Effect of siltuximab on Stat3 signaling in paclitaxel resistant cell lines.

Siltuximab inhibits phosphorylation of Stat3 levels and down-regulated Stat3-targeted antiapoptotic proteins MCL-1, Bcl-XL and survivin in paclitaxel resistant ovarian cancer cell lines SKOV-3_{TR} and Caov-3_{TR}. SKOV-3_{TR} and Caov-3_{TR} cells were treated with different doses of siltuximab overnight and proteins were harvested. For Western blot analysis, 25 μg of total cellular proteins was subjected to immunoblotting with specific antibodies to Stat3, pStat3, Bcl-XL, MCL-1, survivin, or β-actin. The results were detected by a chemiluminescence detection system as described in Materials and Methods.

Fig. 4. Siltuximab inhibits IL-6 induced EGFP-Stat3 nuclear translocation. SKOV-3 pEGFP-Stat3 and BHKpEGFP-Stat3 cells which stably express the EGFP-Stat3 fusion protein were incubated for 4 hours with siltuximab (0, 0.001, 0.1, 1.0, 10.0 μg/ml) followed immediately thereafter with the addition of IL-6 to a final concentration of 30 ng/mL. Subcellular localization of the fusion protein was assessed by fluorescence microscopy. (A) Effects of siltuximab on IL-6 induced stat3 nuclear translocation in SKOV-3pEGFP-Stat3 cells. (B) Effects of siltuximab on IL-6 induced stat3 nuclear translocation in BHKpEGFP-Stat3 cells. Arrow labels (←) show the IL-6 induced Sta3 nucleocytoplasmic shuttling and arrow labels (→→→) show the effect of siltuximab (at different doses) on the IL-6 induced Stat3 nuclear translocation.

Fig. 5. Confirmation of UGT2B4 and ATP2B2 mRNA expression in IL-6/ siltuximab treated cell lines by real-time RT-PCR. Real-time RT-PCR was performed as described in Materials and Methods.
Method. The relative levels of gene expression were calculated from the relevant signals by normalization with the signal for actin expression.

**Fig. 6.** Effect of siltuximab on paclitaxel sensitivity in ovarian cancer drug resistant cells. SKOV-3\textsubscript{TR} and Caov-3\textsubscript{TR} cells at $2 \times 10^3$ cells per well were plated in 96-well plates in culture medium containing different concentrations of siltuximab and paclitaxel. After 96 hours of culture, effect of siltuximab on paclitaxel-induced cell death were determined by MTT assay as described in Materials and Methods. (A) Effect of siltuximab on paclitaxel sensitivity in SKOV-3\textsubscript{TR}. (C) Effect of siltuximab on paclitaxel sensitivity in Caov-3\textsubscript{TR}. Statistical analysis was performed by a two-sided Student's $t$ test in SKOV-3\textsubscript{TR} (B) and Caov-3\textsubscript{TR} (D). Cell viability was assessed using the MTT and recorded as percent viability relative to the untreated control cells. Error bars are SD of averaged results and P values < 0.05 were accepted as a significant difference between means. Values are representative of triplicate determinations in two or more experiments.

**Supplementary Fig. 1.** Differentially expressed genes in IL-6 treated ovarian cancer cells and effect of siltuximab on IL-6 induced gene expression. Venn diagram of upregulated genes expression in IL-6 treated SKOV-3/IL-6 or Caov-3/IL-6 cells as compared with SKOV-3 and Caov-3(A). Down regulated genes expression in both siltuximab and IL-6 treated cells as compared with IL-6 alone treated cells (B). Genes differentially expressed in more than one cell line are indicated in the overlapping regions of the circles.
Supplementary Fig. 2. Effect of combination treatment with Siltuximab and paclitaxel on tumor growth in vivo. Tumors were measured twice a week with a digital caliper. Tumor volume (mm$^3$) was calculated as $(W^2 \times L) / 2$, where $W$ is width and $L$ is length.
Fig. 1

A

B

Score 1, weak staining

Score 2, moderate staining

Score 3, strong staining
Fig. 2

A

- pEGFP-Stat3
- pStat3
- Stat3
- Actin

B

<table>
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<th>Siltuximab (µg/ml)</th>
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<th>-</th>
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<th>0.1</th>
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- pStat3
- Stat3
- Actin
Fig. 3

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<tr>
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</table>

Research. on November 11, 2017. © 2010 American Association for Cancer Research.
Fig. 4

A

(a) No Treatment
(b) IL-6 (30 ng/ml)
(c) IL-6 (30 ng/ml), 0.001 \( \mu \)g/ml siltuximab
(d) IL-6 (30 ng/ml), 0.1 \( \mu \)g/ml siltuximab
(e) IL-6 (30 ng/ml), 1.0 \( \mu \)g/ml siltuximab

B

(a) No treatment
(b) IL-6 (30 ng/ml)
(c) IL-6 (30 ng/ml), 0.001 \( \mu \)g/ml siltuximab
(d) IL-6 (30 ng/ml), 0.1 \( \mu \)g/ml siltuximab
(e) IL-6 (30 ng/ml), 1.0 \( \mu \)g/ml siltuximab

(c) IL-6 (30 ng/ml), 10.0 \( \mu \)g/ml siltuximab
Fig. 5

A

Relative expression of UGT2B4

1: SKOV-3
2: SKOV-3+IL-6
3: SKOV-3+IL-6+siltuximab
4: Caov-3
5: Caov-3+IL-6
6: Caov-3+IL-6+siltuximab

B

Relative expression of ATP2B2

1: SKOV-3
2: SKOV-3+IL-6
3: SKOV-3+IL-6+siltuximab
4: Caov-3
5: Caov-3+IL-6
6: Caov-3+IL-6+siltuximab
Fig. 6

A

Absorbance (O.D)

SKOV-3TR
SKOV-3TR + 1 µg/ml siltuximab
SKOV-3TR + 10 µg/ml siltuximab

Paclitaxel (µM)

B

Cell Viability (%)

SKOV-3TR
SKOV-3TR + 1 µg/ml siltuximab
SKOV-3TR + 10 µg/ml siltuximab

Paclitaxel (0.2 µM)

C

Absorbance (O.D)

Caov-3TR
Caov-3TR + 1 µg/ml siltuximab
Caov-3TR + 10 µg/ml siltuximab

Paclitaxel (µM)

D

Cell Viability (%)

Caov-3TR
Caov-3TR + 1 µg/ml siltuximab
Caov-3TR + 10 µg/ml siltuximab

Paclitaxel (0.0006 µM)
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Yuqi Guo, Jeffrey A Nemeth, Colin O'Brien, et al.

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