A Novel IL6 Antibody Sensitizes Multiple Tumor Types to Chemotherapy Including Trastuzumab-Resistant Tumors

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Abstract

Elevated levels of the proinflammatory cytokine IL6 are associated with poor survival outcomes in many cancers. Antibodies targeting IL6 and its receptor have been developed for chronic inflammatory disease, but they have not yet been shown to clearly benefit cancer patients, possibly due to antibody potency or the settings in which they have been tested. In this study, we describe the development of a novel high-affinity anti-IL6 antibody, MEDI5117, which features an extended half-life and potent inhibitory effects on IL6 biologic activity. MEDI5117 inhibited IL6-mediated activation of STAT3, suppressing the growth of several tumor types driven by IL6 autocrine signaling. In the same models, MEDI5117 displayed superior preclinical activity relative to a previously tested. In this study, we describe the development of a novel anti-IL6 antibody. Consistent with roles for IL6 in promoting tumor angiogenesis, we found that MEDI5117 inhibited the growth of endothelial cells, which can produce IL6 and support tumorigenesis. Notably, in tumor xenograft assays in mice, we documented the ability of MEDI5117 to enhance the antitumor activities of chemotherapy or gefitinib in combination treatment regimens. MEDI5117 also displayed robust activity on its own against trastuzumab-resistant HER2+ tumor cells by targeting the CD44+CD24- cancer stem cell population. Collectively, our findings extend the evidence of important pleiotropic roles of IL6 in tumorigenesis and drug resistance, and offer a preclinical proof of concept for the use of IL6 antibodies in combination regimens to heighten therapeutic responses and overcome drug resistance. Cancer Res; 76(2): 480–90. ©2016 AACR.

Introduction

IL6 is produced by lymphoid and nonlymphoid cells including T and B cells, monocytes, fibroblasts, keratinocytes, as well as endothelial, mesangial, and tumor cells (1, 2). Its pleiotropic roles include the activation of T cells, induction of the acute phase inflammatory response, differentiation, and survival of plasma B cells, synovial fibroblasts, and osteoclasts, and stimulation of growth and differentiation of hematopoietic precursor cells (3). IL6 acts on target cells by binding to the IL6 receptor (IL6R), which consists of two membrane-bound proteins, an 80 kDa ligand-binding protein (IL6Rα chain, CD126) and a 130 kDa signal transduction glycoprotein (gp130). IL6 binds to the transmembrane IL6Rα protein and the IL6/IL6Rα complex binds to two molecules of gp130, which then activates an intracellular signaling cascade. This mechanism is known as classical signaling. IL6Rα also exists as a soluble receptor (sIL6Rα), which can combine with IL6 to trigger membrane-bound gp130 dimerization and thus mediate the so-called trans-signaling (3, 4). Because of the ubiquitous expression of gp130 on different cell types, the trans-signaling mechanism is attributed to the broad array of IL6 functions in the body. Activated gp130 stimulates JAK tyrosine kinases, which phosphorylate and recruit the transcription factor STAT3 to regulate gene expression (5).

Many cancers exploit IL6 as a growth factor as well as a modifier of the tumor microenvironment. Solid tumors such as lung, ovarian, breast, and colon carcinomas produce IL6. IL6Rα and gp130, allowing them to constitutively stimulate their own growth in an autocrine manner (6–8). Other cancers, such as multiple myeloma and neuroblastoma, do not produce IL6, but do express IL6Rα and gp130; these tumors respond to IL6 produced in the tumor microenvironment in a paracrine manner (9). IL6 plays multiple roles in tumor progression and drug resistance (10–12). It can act directly on tumor cells, or function by interacting with normal cells in the tumor microenvironment, including endothelial, immune, and inflammatory cells. Targeting IL6 and its pathway therefore is expected to inhibit tumors through multiple mechanisms. Several anti-IL6 antibodies are in clinical development for cancer therapy. Among these, siltuximab (CT0328) is...
the most advanced and is being tested in the clinic for multiple types of cancer. Published data suggest that anti-IL6 antibody therapies have acceptable safety and tolerability (13-15). In this study, we further elucidate the critical role of IL6 in cancer biology in multiple solid tumor types and provide evidence that IL6 is an anticancer target in mono- and especially combination therapy settings. MEDI5117 is a human monoclonal antibody (mAb) that potently binds and neutralizes human IL6. It was engineered to have increased persistence in circulation compared with unmodified antibodies through incorporation of YTE mutations in the Fc region (16, 17). MEDI5117 inhibited IL6 signaling and suppressed the growth of lung, breast, and ovarian tumor cells in vitro and in vivo with higher potency than siltuximab. Inhibition of tumor growth was significantly increased when MEDI5117 was used in combination with gefitinib, taxanes, or other chemotherapeutics. Furthermore, MEDI5117 suppressed the growth of IL6-dependent trastuzumab-resistant breast tumors, and this was associated with a reduction of cancer stem cells (CSC). These results extend our understanding of the role of IL6 in tumor biology and demonstrate the potential of MEDI5117 for the treatment of multiple cancers.

**Materials and Methods**

**Cells and reagents**

Cancer cell lines were obtained from the ATCC. Cell line authentication was conducted by STR-based DNA fingerprinting and multiplex PCR. IMPACT tests were also performed on all cell lines.

CellTiterGlo reagents were obtained from Promega. An enzyme linked immunosorbent assay (ELISA) was developed in our laboratories to detect free IL6 in tumor lysates. Human IL6 and sIL6R ELISA kits were purchased from R&D Systems. Recombinant human IL6 and sIL6R proteins were obtained from R&D Systems. Antibodies to detect STAT3 and phospho-STAT3-Tyr705 were purchased from Cell Signaling Technology and gp130 antibody was obtained from Santa Cruz Biotechnology. MCF-7/IL6 overexpressing cell line was kindly provided by Dr. Mercedes Rincone (University of Vermont, Burlington, VT; ref. 18). Siltuximab (CINTO328) was generated inhouse based on published sequence information.

**siRNA Knockdown**

A reverse transcription protocol was used to analyze the effects of IL6 and IL6R knockdown. Briefly, siRNA pools targeting IL6 and IL6R (Dharmacon) were prepared at a final concentration of 50 nmol/L in optiMEM (Invitrogen). DU145 cells were then added to the siRNAs at a density of 5,000 cells per well in 96-well plates in medium containing 10% heat-inactivated FBS (Life Technologies). After 48 hours, transfected cells were washed with ice-cold PBS and then lysed by adding Laemmli reducing buffer (Boston BioProducts). Cell lysates were then subjected to Western blot analysis for phosphorylated STAT3 (pSTAT3). Conditioned medium was analyzed in duplicate with human IL6 and soluble IL6R ELISA kits (R&D Systems). For proliferation assays, CellTiter Glo reagent was used five days after transfection and luminescence was measured using an EnVision 2104 Multilabel Reader (PerkinElmer).

**Cell proliferation analysis**

For DU145 cells, cell proliferation was assessed by three-dimensional tumor spheroid assay. Cells were plated at a density of 8,000 cells per well. Plates were spun down and incubated overnight to allow cells to assemble into compact spheroids. Two-day-old spheroids were treated with human recombinant IL6 or sIL6R proteins (R&D Systems) with MEDI5117 at varying concentrations. After 7-day incubation, proliferation was quantified using the CellTiter-Glo assay using an EnVision 2104 Multilabel Reader (PerkinElmer).

For human umbilical vein endothelial cells (HUVEC), cells were cultured as monolayers. Recombinant proteins and antibody were added on next day. After 72 hour incubation, proliferation was quantified using the CellTiter-Glo assay.

**mRNA profiling**

Total RNA was extracted from snap-frozen xenograft tumor samples using the ZR RNA MicroPrep Kit (Zymo Research). Xenograft tumors were treated with 30 mg/kg of MEDI5117 or IgG isotype control IgG1 for a total of two doses. Generating biotin-labeled amplified cRNA was accomplished using the MessageAmpTM Premier RNA Amplification Kit (Ambion) and used for gene expression with Affymetrix Human Genome U133 Plus 2.0 GeneChip microarrays. Canonical pathway enrichment analyses were conducted using Ingenuity Pathway Analysis. All microarray data are deposited in GEO repository under accession number GSE62941: (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ujajyuyowbqdyf&acc=GSE62941).

**Mouse xenograft studies**

All animal procedures were conducted in accordance with all appropriate regulatory standards under protocols approved by the Medimmune Institutional Animal Care and Use Committee. For the MCF-7 xenograft study, 0.36 mg of an estrogen pellet was implanted subcutaneously into the left flank of each female athymic nude mouse. The effect of the estrogen pellet lasted for 60 days in vivo. Two million MCF7 or MCF7/IL6 tumor cells were suspended in 100 µL of BME (6 mg/mL) before injection. All mice received an orthotopic inoculation of tumor cells into the mammary fat pad. For in vivo efficacy studies, 5 million NCI-H1650, KPL-4, DU145, or MDAH2774 cells in 50% Matrigel were inoculated subcutaneously into each female athymic nude mouse. When tumors reached approximately 150 to 200 mm3, mice were randomly assigned into groups (10 mice per group). MEDI5117 or siltuximab was administered intraperitoneally (i.p.) twice per week at indicated doses. Tumor volumes were measured twice weekly with calipers. Tumor growth inhibition was calculated on the last day of study relative to the initial and final mean tumor volume of the control group.

For in vivo mechanism of action and pharmacodynamic studies, a single dose of MEDI5117 was administered when tumors reached approximately 400 mm3. Tumor and serum samples were collected 4 hours after dosing.

**CSC analyses**

For tumorsphere assays, single cells were plated on ultra-low attachment plates at a density of 1 × 10^3/mL and grown for 7 days in a mammmocult medium ( Stem Cell Technologies). After the treatment of primary spheres with drugs, they were dissociated into single-cell suspension and plated at a density of 5 × 10^2–1 × 10^3/mL. Secondary spheres were counted after 5 to 7 days in culture and evaluated the efficacy of the drug treatment.
For CD44+CD24− CSC flow cytometry analyses, single-cell suspensions of residual tumors cells isolated from drug-treated mice were incubated with fluorophore-conjugated CD44 or CD24 antibodies alone or in combination on ice for 30 minutes, washed with Hank’s Balanced Salt Solution (HBSS), and resuspended in DAPI containing HBSS buffer for flow cytometry analyses. Gate compensation was performed with single-color–stained cells and the CD44+CD24− phenotype analyses were performed using the double stained cells.

Tumor reimplantation studies were conducted using cells recovered from the residual tumors of treated animals. Tumors were excised, chopped, and processed with Hank’s Balanced Salt Solution (HBSS), and resuspended in Hank’s Balanced Salt Solution (HBSS) buffer for flow cytometry analyses. Gate compensation was performed with single-color–stained cells and the CD44+CD24− phenotype analyses were performed using the double stained cells.

An anti-IL6 antibody, MEDI5117, inhibits cancer cell and endothelial cell proliferation

MEDI5117 is a human, high affinity, IgG1 monoclonal antibody (mAb) that neutralizes human IL6. The parental antibody (CAT-6001) was obtained by screening phage display human antibody libraries for binding to recombinant human IL6 and for inhibition of the IL6/IL6R interaction (14). We then created MEDI5117 by incorporating three amino acid substitutions, M252Y, S254T, and T256E (YTE), into the Fc region of CAT-6001 to increase the antibody’s half life in vivo. Pharmacokinetic studies in cynomolgus monkeys showed that the half-life of MEDI5117 was extended by 3-fold (28.4 vs. 8.4 days) and clearance was reduced by 4-fold (3.02 vs. 12.1 mL/kg/day) when compared with CAT-6001 (16, 17). The half-life of MEDI5117 in humans was 84 days, significantly longer than other anti-IL6 antibodies, such as siltuximab whose half-life was 17.8 days. MEDI5117 binds IL6 with high affinity. In the KinExa assay, MEDI5117 had an $K_d < 1$ pmol/L, while siltuximab had a $K_d = 6.25$ pmol/L. By ELISA, MEDI5117 had an EC50 = 6.5 pmol/L for human IL6 binding as measured, more potent than that of siltuximab ($EC_{50} = 14.1$ pmol/L). MEDI5117 cross reacts with cynomolgus monkey IL6, but not with IL6 from other species, including dogs, rats, and mice.

The effect of MEDI5117 on the proliferation of cancer cells was investigated in DU145 prostate cancer cells using a spheroid formation assay. Stimulating DU145 cells with recombinant human IL6 resulted in increased cell proliferation as measured by the number of spheroids grown in serum-free medium for seven days. The addition of sIL6R alone had little effect on DU145 cell proliferation. Addition of IL6 in combination with sIL6R stimulated cell growth to the same extent as IL6 treatment alone. This finding indicates that the proliferative effect of IL6 on
DU145 spheroids occurs through classical signaling involving the membrane-bound receptor. Treatment with MEDI5117 completely blocked IL6-induced spheroid growth (Fig. 2A, left). Proliferation was not blocked by treatment with a control isotype-matched IgG1 mAb.

HUVEC cells do not express membrane bound IL6Rα (20), and therefore IL6 stimulation of HUVECs relies on trans-signaling (21). Adding recombinant human IL6 and sIL6R alone had only modest effects on HUVEC proliferation. However, adding IL6 with sIL6R together resulted in significantly increased HUVEC proliferation (Fig. 2A, right). Treatment with MEDI5117 blocked the HUVEC proliferation induced by IL6 and sIL6R. Thus, MEDI5117 can inhibit proliferation of cancer cells by blocking classical IL6 signaling and proliferation of endothelial cells by blocking IL6 trans-signaling in vitro.

The inhibitory activities of MEDI5117 and siltuximab were compared in both DU145 cells and TF-1 (erythroleukemic) cells. Treatment with MEDI5117 or siltuximab blocked IL6-induced cell proliferation in a dose-dependent manner (Fig. 2B); however, MEDI5117 was more potent than siltuximab based on IC50 values.
IL6 signaling involves activation of the transcription factor, STAT3. To analyze effects on STAT3, several different cancer cell lines and HUVECs were treated with MEDI5117, siltuximab, or a control IgG1 mAb (Fig. 2C, left). Basal levels of pSTAT3 (its activated form) were suppressed by MEDI5117 in each of the cell lines; treatment with siltuximab resulted in less efficient suppression. Addition of recombinant human IL6 activated STAT3 in both the MDAH2774 and DU145 cancer cell lines, and pretreatment with MEDI5117 blocked this activation (Fig. 2C, right). In DU145 cells, the level of pSTAT3 in MEDI5117-treated cells was even lower than the basal level of pSTAT3 in untreated cells. MEDI5117 treatment reduced basal pSTAT3 levels, and reversed IL6 or IL6/sIL6R induced STAT3 activation in HUVECs (Fig. 2D). These results corroborate that MEDI5117 can inhibit both classical and trans-signaling stimulated by IL6.

Neutralization of IL6 by MEDI5117 inhibits tumor growth in xenograft models

MEDI5117 was evaluated in NSCLC (NCI-H1650), ovarian (MDAH-2774), and prostate (DU145) xenograft models in vivo (Fig. 3A and Supplementary Fig. S3). These tumor models produce IL6 and bear cell surface IL6R. MEDI5117 at 30 mg/kg alone caused tumor growth inhibition (ΔTGI) of 51% (P = 0.004) in NCI-H1650 and 59% ΔTGI (P = 0.007) in MDAH2774. Doses between 3 and 30 mg/kg showed comparable ΔTGI with the 30 mg/kg dose. A low dose of 1 mg/kg was insufficient to inhibit growth.
tumor growth. MEDI5117 treatment was well tolerated in mice as no body weight loss was observed compared with control groups. The antitumor activity of MEDI5117 was also found to be superior to that of siltuximab in DU145 and MDAMB231 xenograft models (Supplementary Fig. S3).

Free human IL6 levels were measured in NCI-H1650 xenograft tumors in both control IgG1 and MEDI5117-treated mice. MEDI5117 treatment suppressed human IL6 levels in both serum and tumor lysates in vivo. In addition, MEDI5117 treatment resulted in more than 50% reduction of pSTAT3 levels in tumor cell lysates as detected by ELISA (Fig. 3B). Immunohistochemistry confirmed this reduction, and also showed pSTAT3 reduction in tumor cells and stromal cells within and surrounding the tumor (Fig. 3C). Cell proliferation was also reduced, as assessed by immunohistochemistry with anti-Ki67 antibodies (Fig. 3D).

It is important to note that MEDI5117 does not bind to systemic mouse IL6, which is known to regulate tumor microenvironment and contribute to xenograft tumor growth. This may explain the partial tumor growth inhibition we observed in vivo, and also suggests that our results underestimate the antitumor effects that MEDI5117 would exert in cancer patients. To confirm that MEDI5117 could inhibit systemic IL6 in vivo, a model was generated in which human IL6 was administered by intraperitoneal injection into male C57/B6J mice. Systemic administration of IL6 is known to trigger an elevation of acute phase proteins such as haptoglobin. MEDI5117 dose dependently inhibited the haptoglobin levels induced by human IL6, with significant inhibition being noted at doses of 78 g/kg and higher. Siltuximab had a similar effect at a dose of 266 g/kg, much higher than that of MEDI5117 (Supplementary Fig. S4). Therefore, MEDI5117 was more potent than siltuximab in inhibiting human IL6 in vivo.

MEDI5117 was also tested in the MDA-MB-468 model, which does not secrete IL6, and the U87MG model, which produces IL6 but does not express surface IL6R. No TGI was observed in these models (data not shown), suggesting an active IL6 autocrine loop is the key determinant of sensitivity to anti-IL6 cancer activity in xenograft models.

MEDI5117 inhibits expression of IL6 target genes and angiogenic genes

To further understand the mechanism of action of MEDI5117, whole genome mRNA profiling was performed on untreated or MEDI5117-treated tumor xenograft samples. Table 1 lists the genes whose expression was most downregulated with MEDI5117 treatment in the three responsive models (DU145, NCI-H1650, and KPL-4). All of these genes are known to be transcriptional regulation targets of the IL6/JAK/STAT3, AKT, and MAPK pathways. Among these, SOCS3 (suppression of cytokine signaling 3) mRNA was found to be consistently downregulated by MEDI5117 in all three models. The expression of SOCS3 is induced by cytokines such as IL6, IL10, and IFNγ. In contrast, no modulation of IL6–related pathway gene expression was observed in the nonresponsive models (NCI-H1975, U87MG; data not shown).

We explored effects on gene expression further using a human angiogenesis gene array. MEDI5117 treatment inhibited the expression of several genes involved in angiogenesis such as KDR, PECAM-1, and VEGF as shown in Table 2. The downregulation of KDR and PECAM-1 expression was confirmed by qRT-PCR analysis (Fig. 3E). We also found reductions in CD31 expression, blood microvessel density, and the size of vessel lumens in NCI-H1650 tumors after MEDI5117 treatment (Fig. 3F). This demonstrates that IL6 is involved in angiogenesis in some tumor types, and extends our previous findings studying ovarian cancer cells (22). Thus, the antitumor effects of MEDI5117 involve inhibition of tumor cell growth and suppression of angiogenesis.

Neutralization of IL6 with MEDI5117 enhances antitumor activities of chemotherapeutic agents and gefitinib

We tested the antitumor efficacy of MEDI5117 in combination with several standard-of-care therapies in various mouse cancer models. All of the combination treatments we tested were well tolerated. The effect of combining MEDI5117 with taxol, a standard-of-care for breast, ovarian, NSCLC, and prostate cancers (23), was evaluated in the NCI-H1650 xenograft models. Treatment with either MEDI5117 or taxol alone inhibited tumor growth being noted at doses of 50% and 92% at the end of treatment, respectively. Combination treatment with MEDI5117 plus taxol resulted in

| Table 1. MEDI5117 treatment conferred specific inhibition of IL6-related signaling pathways |
|-----------------|-----------------|
| Gene | Log(P value) | Ratio |
| SOCS3 | HP | C3 | C4B | CP | SERPINA1 | FGB | FGA |
| IL6ST | MAP2K6 | SOCS3 | TCFA | THBS1 | MAP2K7 | SERPING1 | FNI |
| TNFRSF1A | RRS | SERPINA3 | PIK3R3 | JKX8K | HMOS | SHC1 |
| JUN | CRABP2 | AKT3 | SERPINAI | OSMR | SERPIN | RBP5 |
| SOCS3 | SOD2 | C4 | SERPINA5 | OSMR | FGB | LBP | FGA |

| Table 2. MEDI5117 treatment inhibited angiogenic gene expression |
|-----------------|-----------------|
| Gene | % Inhibition with MEDI5117 treatment |
| CD44 | 41% |
| CD85 | 31% |
| COL5A1 | 48% |
| FGFR2 | 35% |
| IFNβ | 45% |
| KDR | 44% |
| NR2F2 | 25% |
| TNF | 32% |
| VEGF | 22% |
| COL4A2 | 35% |
| FBLN5 | 39% |
| FLTS | 29% |
| HEY1 | 34% |
| IL12A | 45% |
| ITGAV/β3 | 38% |
| PDGFRb | 42% |
| PECAM1 | 56% |
| PROX1 | 47% |
| SERPINC1 | 56% |
| TEK | 52% |
| TGFα | 24% |
| TIMP2 | 32% |

NOTE: DU145 cells were treated with IgG1 control mAb or MEDI5117. mRNA was isolated and was then subjected to a human angiogenesis array.
tumor regression (ΔTGI = 102%), with 2 of 10 mice experiencing complete tumor regression. Tumor regrowth was monitored following the discontinuation of the treatments. Animals in the taxol only treatment group showed rapid regrowth of their tumors. However, the regrowth of the tumors in mice treated with the combination of MEDI5117 and taxol was significantly delayed. The two mice that had complete tumor regression remained tumor free at the end of the regrowth phase (Fig. 4A, left). Free human IL6 and pSTAT3 levels were measured in NCI-H1650 xenograft mice treated with MEDI5117, taxol, and a combination of both (Fig. 4A, middle and right). Taxol treatment resulted in upregulation of human IL6 and pSTAT3 compared with baseline levels, indicating activation of the IL6R inflammatory pathway. In contrast, treatment with MEDI5117 suppressed basal IL6 and pSTAT3 and counteracted taxol-stimulated pathway activation.

The combination of MEDI5117 with taxotere was evaluated in the KPL-4 breast cancer orthotopic model (Fig. 4B) and DU145 prostate xenograft model (Supplementary Fig. S5A). MEDI5117 alone had a modest effect on KPL-4 tumor growth (ΔTGI = 29%). Taxotere was highly efficacious causing regression during the dosing phase; however, tumors regrew once the treatment was discontinued. Combining MEDI5117 with taxotere resulted in complete tumor regression, which lasted for more than two months (Fig. 4B).

MEDI5117 was also tested in combination with other non-taxane chemotherapeutics including gemcitabine + cisplatin, alimta + carboplatin, doxorubicin, and topotecan (Supplementary Fig. S5). Combination treatments led to enhanced inhibition or delay in tumor growth compared with either agent alone. In some cases (Supplementary Figs. S4B and S4D), the combination efficacy was less pronounced compared with that seen in other experiments, and in these cases we observed lower induction of IL6 secretion and signaling by the chemotherapy agent. This reinforces the hypothesis that patients could be selected for IL6 combination therapy based on assessment of therapy-induced IL6 upregulation.

The effects of combining MEDI5117 and gefitinib were examined in the NCI-H1650 xenograft model (Fig. 4C). Both MEDI5117 alone and gefitinib alone inhibited tumor growth, although gefitinib was more effective during the treatment phase (53% ΔTGI for gefitinib vs. 39% ΔTGI for MEDI5117). The combination of MEDI5117 and gefitinib enhanced antitumor activity (70% ΔTGI). In addition, the combination treatment led to the strongest posttreatment antitumor activity.

MEDI5117 inhibits a trastuzumab-resistant HER2+ breast tumor model

We previously reported that activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2-overexpressing breast cancer cells (24, 25). BT474 cells, which contain amplified HER2, are sensitive to trastuzumab, but PTEN knockdown and long-term trastuzumab treatment–generated cells (called BT474-PTEN-LIT cells) that resisted killing by trastuzumab. Compared
with the parental BT474, BT474-PTEN-LTT cells displayed an aggressive metastatic phenotype. This was due to the induction of an epithelial-to-mesenchymal transition (EMT) and CSC expansion mediated by autocrine IL6 secretion. As in our previous studies, trastuzumab significantly inhibited the growth of parental BT474 cells in vitro but failed to do so for BT474-PTEN-LTT cells (Fig. 5A). In contrast, MEDIS117 suppressed the growth of BT474-PTEN-LTT cells but did not affect the growth of BT474 cells, suggesting that this inhibitory activity is specific to the IL6-producing BT474-PTEN-LTT cells.

We reasoned that MEDIS117 might exert these effects on the BT474-PTEN-LTT cells by sequestering free IL6 and indirectly reducing the levels of related inflammatory cytokines. Elevated levels of IL6, IL8, TGFβ, sIL6R, and surface IL6R in BT474-PTEN-LTT cells compared with the parental BT474 were demonstrated by ELISA (Supplementary Figs. S6A and S6B). These proteins were significantly reduced following a 48-hour MEDI5117 treatment in vitro (Fig. 5B), confirming suppression of the IL6-related inflammatory response.

Similar to results from our in vitro studies, parental BT474 tumors were very sensitive to trastuzumab treatment in mouse xenografts, while they failed to respond to the MEDIS117 treatment (data not shown). In contrast, MEDIS117 exerted a strong inhibition of BT474-PTEN-LTT tumor growth (Fig. 5C), whereas this tumor was resistant to trastuzumab treatment in vivo (Fig. 5D). In addition, we utilized an NF-kB reporter-expressing BT474-PTEN-LTT cell line to monitor the IL6-mediated NF-kB activity in vivo (24). MEDIS117 treatment significantly reduced NF-kB activity in growing tumors compared with the negative control (R347) or trastuzumab-treated animals (Fig. 5E). The fact that the NF-kB activity correlated with tumor growth suggests a critical role for IL6-mediated NF-kB signaling in tumor growth.

**IL6 neutralization by MEDIS117 inhibits trastuzumab-resistant breast cancer stem cells**

Compared with BT474 cells, trastuzumab-resistant BT474-PTEN-LTT cells displayed an expanded CSC population characterized by a CD44+/CD24− phenotype (24). To determine whether MEDIS117 can target breast CSCs, we performed in vitro tumorsphere assays. BT474-PTEN-LTT cells displayed considerably higher tumorsphere forming capacity compared with the parental BT474 tumorspheres but significantly reduced tumorsphere formation by BT474-PTEN-LTT cells, indicating the latter depends on IL6. We also characterized the proportion of CD44+/CD24− CSCs in BT474-PTEN-LTT xenografts with or without MEDIS117 treatment by flow cytometry. Although parental BT474 lacks CD44+/CD24− cells, these CSCs represented compared with the parental BT474 formed spontaneous metastases primarily in the lungs, which were inhibited by MEDI5117 treatment (Fig. 5D). In addition, we utilized an NF-kB reporter-expressing BT474-PTEN-LTT cell line to monitor the IL6-mediated NF-kB activity in vivo (24). MEDIS117 treatment significantly reduced NF-kB activity in growing tumors compared with the negative control (R347) or trastuzumab-treated animals (Fig. 5E). The fact that the NF-kB activity correlated with tumor growth suggests a critical role for IL6-mediated NF-kB signaling in tumor growth.

**IL6 Blockade in Combination and Drug-Resistant Settings**

Figure 5.

MEDIS117 shows specific therapeutic activity against a trastuzumab-resistant breast tumor model. A, sensitivity of BT474 cells and BT474-PTEN-LTT cells to trastuzumab and MEDIS117 analyzed in vitro. B, IL6, IL8, and TGFβ levels measured by ELISA in BT474-PTEN-LTT cells treated with MEDIS117 in vitro. C, in vivo growth inhibition of BT474-PTEN-LTT tumors treated with trastuzumab and MEDIS117. D, H&E staining of primary tumors and lungs from animals bearing BT474 cells, BT474-PTEN-LTT cells, or BT474-PTEN-LTT cells treated with MEDIS117. E, an NF-kB reporter-expressing BT474-PTEN-LTT cell line was used to measure the activation of the NF-kB pathway by MEDIS117 or trastuzumab in vivo. F, body weights of BT474-PTEN-LTT cells treated with MEDIS117, trastuzumab, or an isotype control antibody, R347.

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about 75% of the BT474-PTEN-LTT cell population (Fig. 6B). In line with our in vitro tumourosphere data, the number of CD44+ CD24− CSCs was substantially lower in BT474-PTEN-LTT xenografts when animals were treated with MEDI5117 compared with those treated with the negative control antibody, R347. It is unclear whether, in addition to reducing the CD44+CD24− cell fraction, MEDI5117 also drives differentiation to the CD44+ CD24+ phenotype.

To further determine the effect of the MEDI5117 on CSCs, we next performed reimplantation assays using cells taken from BT474-PTEN-LTT tumors treated for eight weeks with the negative control antibody, trastuzumab, or MEDI5117. Recipient mice were implanted with either 500 or 5,000 cells and monitored for 12 weeks. Under these conditions, both trastuzumab and control-treated tumor cells were able to initiate secondary tumors, although tumors did not grow in all recipient mice (Fig. 6C). In contrast, MEDI5117-treated primary tumors failed to grow upon secondary reimplantation in any of the recipient mice, suggesting elimination of the CSC population. Data from this experiment was used to estimate the frequency of CSCs after each treatment as well (Fig. 6D). These results demonstrate that IL6 depletion by MEDI5117 diminishes the CSC population, which contributes to overall tumor growth suppression of our trastuzumab-resistant model.

Discussion

IL6 is a pleiotropic cytokine that was initially characterized as a key component of inflammatory responses. Therapeutics targeting the IL6 pathway are being developed for inflammatory and autoimmune diseases. Tocilizumab, an anti-IL6 receptor antibody, was approved in 2009 for the treatment of rheumatoid arthritis (26, 27). Siltuximab, a chimeric mouse-human anti-IL6 antibody, was approved to treat patients with Castleman disease (FDA.gov press release for siltuximab approval, April 24, 2014).

Numerous studies have revealed a role for IL6 in various types of cancer as well. Elevated levels of IL6 are associated with poor prognosis in cancers of the lung, colon, kidney, bladder, and others (28–30). Thus, therapeutics targeting IL6 and IL6 signaling components are also being tested in clinical trials for cancer. Siltuximab has been shown to have modest benefit in treating cancer-related fatigue and cachexia in NSCLC patients (33, 34). Elsilimab (BE-8), a murine anti-IL6 mAb, could not efficiently block IL6 levels due to its short half-life and neutralization by a human anti-mouse immune response, and has generated mixed results in clinical trials (35). MEDI5117 is a fully human mAb with no potential immunogenicity issues in human and has a higher binding affinity for human IL6 (Kd < 1 pmol/L) relative to other anti-IL6 mAbs. Compared with siltuximab, MEDI5117 showed enhanced in vitro cell killing (Fig. 2B) and pathway suppression (Fig. 2C) and inhibited systemic IL6 in vivo more efficiently (Supplementary Fig. S3). In mouse xenograft models, MEDI5117 treatment resulted in an improvement in tumor growth inhibition compared with siltuximab (Supplementary Fig. S3). In mouse xenograft models, MEDI5117 targets the breast cancer stem cell population in vitro and in mouse xenografts. A, breast tumourosphere formation of BT474 cells BT474-PTEN-LTT cells treated with MEDI5117 or an isotype control antibody. B, residual tumors from isotype or MEDI5117 treated animals were evaluated by flow cytometry for the presence of CD44+CD24− breast CSCs. C, secondary tumor relinitation was assessed by removing BT474-PTEN-LTT cells removed from tumor-bearing mice that were treated for eight weeks with an isotype control antibody, trastuzumab, or MEDI5117 and reimplanted into new recipient mice. The table lists number of mice in which secondary tumors were formed out of the five mice in each treatment group. D, the frequency of breast CSC was calculated on the basis of the secondary tumor initiation capacity of residual tumors.

Figure 6. MEDI5117 targets the breast cancer stem cell population in vitro and in mouse xenografts. A, breast tumourosphere formation of BT474 cells BT474-PTEN-LTT cells treated with MEDI5117 or an isotype control antibody. B, residual tumors from isotype or MEDI5117 treated animals were evaluated by flow cytometry for the presence of CD44+CD24− breast CSCs. C, secondary tumor relinitation was assessed by removing BT474-PTEN-LTT cells removed from tumor-bearing mice that were treated for eight weeks with an isotype control antibody, trastuzumab, or MEDI5117 and reimplanted into new recipient mice. The table lists number of mice in which secondary tumors were formed out of the five mice in each treatment group. D, the frequency of breast CSC was calculated on the basis of the secondary tumor initiation capacity of residual tumors.
we found that MEDI5117 treatment of several tumor types in vivo could reduce tumor angiogenesis by downregulation of various genes including VEGF, KDR, and PECAM-1. Thus, the activity of MEDI5117 in inhibiting tumor growth reflects the pleiotropic roles of IL6 in cancer.

IL6 overproduction occurs in response to various cancer drug treatments and in drug-resistant tumors, and thus the full benefits of suppressing IL6 may be achieved in these situations. We found that taxanes upregulated IL6 production in NSCLC and prostate cancer cells, and that the combination of taxanes with MEDI5117 was significantly more efficacious than either agent alone. This efficacy was associated with suppression of activated STAT3, the downstream effector of the IL6/JAK signaling axis. Similarly, MEDI5117 combined with taxotere resulted in durable tumor regression in a breast cancer model, whereas treatment with either agent alone did not. Activating mutations in the EGFR can promote IL6 production and activate JAK/STAT3 signaling in human lung adenocarcinomas (38). Increased IL6 secretion and consequent autocrine survival signaling were also found to be a cause of drug resistance to the EGFR inhibitor, erlotinib, in NSCLC (12). Conversely, IL6 antibody treatment can upregulate EGFR expression and limit ovarian tumor growth inhibition (39). This suggests that a combined approach blocking both the EGFR and IL6 pathways may improve response and counteract resistance. Our results showing that MEDI5117 in combination with gefitinib enhanced antitumor efficacy in the H1650 NSCLC model supports this hypothesis. We also generated gefitinib-resistant H1650 cells and found that MEDI5117 treatment restored sensitivity to this drug (data not shown).

Our previous work showed that activation of an IL6 feedback loop may play a role in trastuzumab resistance in PTEN-deleted HER2+ tumors (24). We therefore tested the activity of MEDI5117 in our trastuzumab-resistant breast cancer model, which produces high levels of IL6. Neutralization of IL6 by MEDI5117 potently inhibited the growth of this trastuzumab resistant tumor. In contrast, the antibody had no activity against matched EGFR-sensitive (PTEN wild-type) tumor cells that do not overproduce IL6. Growth inhibition of the trastuzumab-resistant tumor model by MEDI5117 was associated with suppression of downstream NF-kB signaling and lung metastasis. Biopoulos and colleagues demonstrated that IL6 activation of NF-kB in turn drives further IL6 production, creating an inflammatory positive feedback loop (40). Thus, interrupting this IL6/NF-kB activation loop may be particularly important in some drug-resistant cancers that depend on IL6.

IL6 is known to regulate the growth of some cancer stem cells, and this may contribute to treatment resistance and relapse (41). For example, IL6 stimulates the tumorigenic capacity of CSCs isolated from head and neck squamous tumors (42). Similarly, glioma stem cells express IL6R, and targeting IL6 decreases glioma stem cell survival and tumor growth (43). This stimulation of CSC growth has also been found to contribute to resistance to trastuzumab. Yang, and colleagues reported that resistance of gastric cancer cells with CSC characteristics to trastuzumab is associated with activation of an IL6/STAT3/Jagged-1/Notch positive feedback loop (44). The results described in this report extend our previous findings (24) by showing that neutralization of IL6 by MEDI5117 can inhibit CSCs and that this may contribute to overcoming trastuzumab resistance in breast cancer. Together with the enhanced efficacy we observed when paired with other therapeutics, these results encourage further study of MEDI5117 in combination treatment scenarios and in drug-resistant cancers.

Disclosures of Potential Conflicts of Interest

J. Huang has ownership interest (including patents) and is an employee at Medimmune/AstraZeneca. R.E. Hollingsworth is a Senior Director at MedImmune. No potential conflicts of interest were disclosed by the other authors.

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