Activation of an IL6 Inflammatory Loop Mediates Trastuzumab Resistance in HER2+ Breast Cancer by Expanding the Cancer Stem Cell Population

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SUMMARY

Although inactivation of the PTEN gene has been implicated in the development of resistance to the HER2 targeting antibody trastuzumab, the mechanisms mediating this resistance remain elusive. We generated trastuzumab resistant cells by knocking down PTEN expression in HER2 overexpressing breast cancer cell lines and demonstrate that development of trastuzumab resistance in these cells is mediated by activation of an IL6 inflammatory feedback loop leading to expansion of the cancer stem cell (CSC) population. Long term trastuzumab treatment generates highly enriched CSCs which display an EMT phenotype secreting over 100-fold more IL6 than parental cells. An IL6 receptor antibody interrupted this inflammatory feedback loop reducing the cancer stem cell population resulting in decreased tumor growth and metastasis in mouse xenographs. These studies demonstrate that trastuzumab resistance may be mediated by an IL6 inflammatory loop and suggest that blocking this loop may provide alternative strategy to overcome trastuzumab resistance.

INTRODUCTION

The HER2 gene is amplified in approximately 20%–25% of human breast cancers, which are characterized by an aggressive clinical course (Slamon et al., 1987). The development of HER2-targeted therapeutic agents, such as trastuzumab, has dramatically altered the course of this disease. However, despite the clinical benefits of these HER2-targeted therapies, almost 50% of patients with HER2-amplified cancers fail to respond to trastuzumab, and the vast majority of tumors that respond to trastuzumab develop resistance within 1 to 2 years of treatment (Lan et al., 2005). Although a number of mechanisms that mediate de novo, or “acquired,” trastuzumab resistance have been proposed, the most common molecular alteration associated with this resistance is inactivation of the tumor suppressor PTEN, found in over 40% of HER2-positive breast cancers (Nagata et al., 2004). Reduced PTEN activity mediates trastuzumab resistance via activation of the downstream signaling molecule Akt, bypassing the requirement for HER2 activation (Berns et al., 2007). In addition, we and others have shown that both HER2 and PTEN are important regulators of subpopulations of breast cancer cells that display stem cell properties (Cicalese et al., 2009; Korkaya et al., 2009). There is increasing evidence that these cancer stem cells (CSCs) mediate tumor growth and metastasis and, by virtue of their relative resistance to chemotherapy and radiation therapy, may also contribute to tumor recurrence (Eyler and Rich, 2008). However, it is unknown if CSCs play a role in de novo or acquired trastuzumab resistance.

Interleukins 6 (IL6) and 8 (IL8) have also been demonstrated to regulate the breast CSC self-renewal (Ginestier et al., 2010; Iliopoulos et al., 2011). Although these cytokines are regulated by multiple factors, HER2 overexpression in breast CSCs has been shown to increase IL6 production (Hartman et al., 2011). IL6 links inflammation to malignant transformation by activating the NF-κB pathway which, in turn, drives constitutive IL6 production, generating a positive feedback loop. In addition, IL6 is able to induce an epithelial-mesenchymal transition (EMT), which has been implicated in generation of a stem cell phenotype (Iliopoulos et al., 2011; Mani et al., 2008; Sullivan et al., 2009). The clinical relevance of these studies is demonstrated by the strong association between serum IL6 levels and poor clinical outcome in breast cancer patients, including those with HER2-amplified breast tumors (Bachelot et al., 2003; Salgado et al., 2003). Together, these studies suggest the possibility that the generation of inflammatory feedback loops regulating CSCs may play a role in mediating trastuzumab resistance in HER2-overexpressing breast cancers. To determine whether this is the case, we examined the activation of these pathways and their effects on CSC populations in genetically engineered breast cancer cell lines and mouse xenograft models. We demonstrate that PTEN deletion in HER2-overexpressing breast cancer cells activates an IL6 mediated inflammatory feedback loop. This feedback loop expands the CSC population displaying an EMT phenotype through both autocrine and paracrine mechanisms, which in turn...
confers trastuzumab resistance. In addition, we demonstrate that interfering with this feedback loop utilizing an IL6 receptor (IL6R) antibody reduces the CSC population inhibiting tumor growth and metastasis. These studies define an alternative mechanism of trastuzumab resistance and suggest an effective therapeutic strategy to overcome this resistance.

RESULTS

PTEN Downregulation in HER2-Overexpressing Breast Cancer Cells Increases the Proportion of Invasive CSCs

Since PTEN inactivation frequently occurs in the context of HER2 amplification, a phenotype associated with trastuzumab resistance, we examined the effect of PTEN knockdown on CSC-like populations in HER2 overexpressing breast cancer cell lines. The efficiency of HER2 overexpression and PTEN knockdown utilizing lentiviral shRNAs or control vector is demonstrated (Figures 1A and 1F). We first assessed the effect of these molecular alterations on CSCs by tumorsphere assay, which was shown to enrich for CSCs (Singh et al., 2003). PTEN deletion and HER2 overexpression resulted in a significant increase in tumorsphere formation (Figures 1B and 1G). PTEN knockdown in HER2-overexpressing cells resulted in a 2- to 3-fold increase in sphere formation and a 6-fold increase over the parental cells. To confirm and extend these observations, we examined the effect of PTEN knockdown and HER2 overexpression on the CSC markers, such as the expression of aldehyde dehydrogenase (ALDH) or the CD44+/CD24− phenotype (Al-Hajj et al., 2003; Ginestier et al., 2007). There was a stepwise increase in the Aldefluor-positive and CD44+/CD24− populations in parental MCF7-DsRed, MCF7-PTEN−, MCF7-HER2+, and MCF7-HER2+PTEN− cells, respectively, when they were analyzed by the Aldefluor assay or CD44+/CD24− expression (Figures 1C and 1E). SUM159 cells are composed of over 90% CD44+/CD24− cells, precluding the use of these markers to identify CSC in this cell line. However, there was a 2-fold increase in the Aldefluor-positive population in SUM159-HER2+PTEN− cells as compared to SUM159-HER2+ cells (Figure 1H). Together, these results demonstrate that the increase in the CSC population induced by HER2 overexpression is further enhanced by PTEN deletion.

Next we assessed the effect of HER2 overexpression and PTEN deletion on invasion of tumor cells through matrigel. As shown in Figures 1D and 1I, PTEN deletion in HER2-overexpressing MCF7 or SUM159 cells enhanced in vitro invasive capacity, as compared to cells with either HER2 overexpression or PTEN deletion alone.

PTEN Downregulation Generates a Trastuzumab-Resistant CSC Population

We determined the effect of the HER2 blockade using the HER2-targeting antibody trastuzumab on CSC populations as assessed by tumorsphere formation or by ALDH expression. Primary tumorspheres for each indicated cell line were treated with trastuzumab during the course of 5–7 days, and the effects of this treatment on the ability to form secondary tumorspheres was assessed. There was no significant effect of trastuzumab on tumorsphere formation in parental cells. However, HER2 overexpression rendered the tumorsphere-forming population sensitive to trastuzumab as reflected by a 50% reduction in tumorsphere formation in MCF7-HER2+ and SUM159-HER2+ cells upon trastuzumab treatment. In contrast, trastuzumab had no significant effect on secondary tumorsphere formation in MCF7-HER2+PTEN− and SUM159-HER2+PTEN− cells (Figures 2A and 2D). In addition, trastuzumab reduced the Aldefluor-positive population by 75% and 50% in MCF7-HER2+ and SUM159-HER2+ cells, respectively (Figures 2B and 2E). In contrast, there was a modest nonstatistically significant increase in the Aldefluor-positive population in both MCF7-HER2+PTEN− and SUM159-HER2+PTEN− cells (Figures 2B and 2E) upon trastuzumab treatment.

PTEN Downregulation in HER2-Overexpressing Cells Generates Trastuzumab-Resistant Metastatic Tumors in NOD/SCID Mice

We next examined the biological consequences of increased CSC populations generated by HER2 overexpression and PTEN knockdown by implanting these cells into the mammary fat pads of NOD/SCID mice. Although parental MCF7 and SUM159 xenografts were able to grow in the mammary fat pads of these mice, they failed to generate metastasis in distant organs (data not shown). In contrast, MCF7-HER2+PTEN− and SUM159-HER2+PTEN− cells generated larger, primary tumors that extensively metastasized to lymph nodes, liver, and lung when compared to MCF7-HER2+ and SUM159-HER2+ cells, which displayed only occasional metastasis to lung and liver (Figures 2C and 2F). Furthermore, while MCF7-HER2+ xenografts in mice were responsive to trastuzumab treatment leading to reduced tumor size (Figure 2H), MCF7-HER2+PTEN− xenografts demonstrated de novo resistance to trastuzumab (Figure 2H). The effects of trastuzumab on tumor weight were paralleled by effects on the CSC populations as assessed by the Aldefluor assay. The percentage of Aldefluor-positive tumor cells was reduced by over 50% by trastuzumab treatment in MCF7-HER2+ cells (Figure 2I). In contrast, trastuzumab actually caused a slight increase in the Aldefluor-positive populations in MCF7-HER2+PTEN− cells, demonstrating that PTEN deletion in HER2-overexpressing breast cancer cells generates a trastuzumab-resistant CSC population (Figure 2I).

PTEN Downregulation and HER2 Overexpression Synergize to Increase Expression of the Cytokines IL6, IL8, and CCL5/RANTES

Recent studies have demonstrated that a number of cytokines, including IL6, IL8, and CCL5/RANTES, play a role in CSC regulation as well as in invasion and metastasis (Korkaya et al., 2011). Utilizing an antibody cytokine array, we determined the effects of HER2 overexpression, PTEN deletion, or the combination on levels of cytokine expression in MCF7 cells. We detected a stepwise increase in IL6, IL8, and CCL5, as well as platelet-derived growth factor B (PDGF-B) secreted from MCF7-DsRed, MCF7-HER2+, MCF7-PTEN−, and MCF7-HER2+PTEN− cells (Figure 3A). As assessed by densitometry of the cytokine blots and utilizing an ELISA, we further confirmed that secretion of these cytokines was increased by 2- to 3-fold in MCF7-HER2+ or MCF7-PTEN− cells, compared to parental cells, and by 10-
Figure 1. PTEN Downregulation and HER2 Overexpression Synergize to Increase the CSC Population In Vitro

(A–G) Downregulation of PTEN and/or overexpression of HER2 in MCF7 or Sum159 cells is demonstrated by western blotting in (A) and (F). PTEN downregulation in HER2-overexpressing cells increased the tumorsphere formation (B and G), Aldefluor-positive cell populations (C and H), as well as invasion compared to either HER2 overexpression or PTEN downregulation in vitro (D and I). MCF7-HER2+/PTEN- cells showed a significant increase in the proportion of CD44+/CD24- cells as compared to the MCF7-HER2+ or MCF7-PTEN- cells (E). Error bars represent the mean and SD of two independent experiments performed in duplicate samples. *p ≤ 0.05, #p ≤ 0.01.
20-fold in MCF7-HER2+/PTEN− cells, compared to parental MCF7-DsRed cells (Figures 3B and 3C). This dramatic elevation of cytokines in the MCF7-HER2+/PTEN− cells suggests a synergistic effect resulting from PTEN deletion and HER2 overexpression. Although parental MCF7-DsRed cells secreted detectable levels of IL8, CCL5, and PDGF-B, which were increased in
IL6 Feedback Loop Regulates Trastuzumab Resistance

Figure 3. PTEN Downregulation in HER2-Overexpressing Cells Activates an IL6/NF-κB-Mediated Inflammatory Feedback Loop

(A–E) MCF7-HER2+PTEN- cells secreted 3- to 5-fold higher levels of IL6, IL8, and CCL5 compared to MCF7-HER2+ or MCF7-PTEN- cells as determined by RayBio human cytokine antibody Array 5 (A). The intensity of each blot compared to control was determined by Kodak image analyzer (B) and confirmed by ELISA (C). Downregulation of PTEN in HER2-amplified breast cancer cells, BT474, SKBR3, HCC1954, and Sum159-HER2+ (D) results in increased levels of these cytokines in vitro (E).

(F–H) Secretion of all three cytokines in Sum159-HER2+PTEN- cells were completely inhibited by the NF-κB inhibitor (5 μM Bay11-7082), or combined inhibition of Akt and Stat3, while Akt (5 μM perfosine) or Stat3 (1 μM Stat3 Inhibitor VII) reduced the levels of these cytokines by 50%. Addition of recombinant IL6 to Sum159-HER2+PTEN- cells stimulated the levels of all three cytokines, while blocking IL6 using the IL6R antibody (at 5 μg/ml) reduced their levels by more than 50%.

(I) IL6 activated Akt, Stat3, and NF-κB pathways while suppressing PTEN expression.

(J) An IL6 feedback loop is schematically illustrated. Scale bar, 100 μm. Error bars represent the mean and SD of two independent experiments performed in duplicate samples. *p ≤ 0.05. **p ≤ 0.01.
Figure 4. An IL6 Mediated Inflammatory Loop Expands the CSC Population that Displays Characteristics of EMT

(A) Parental BT474-DsRed cells demonstrated a modest decrease in IL6 levels after 3 days of trastuzumab treatment with a 2-fold increase after LTT (more than 3 weeks); however, treatment of BT474-PTEN− cells resulted in an increase of more than 10-fold in IL6 levels after 3 days and more than 100-fold after 3 weeks, reaching to an increase of several 100-fold in LTT cells.

(B) Trastuzumab treatment gradually increased the percentage of cells expressing the CD44+/CD24− markers in BT474-PTEN− cells compared to parental BT474-DsRed cells that are predominantly CD44−CD24+. Blocking IL6R in early stage inhibited this process.
MCF7-HER2\(^+\), MCF7-PTEN\(^-\), and MCF7-HER2\(^+\)PTEN\(^-\) cells, there was no detectable expression of IL6 in parental MCF7 cells. To confirm that PTEN deletion increased cytokine production in HER2-overexpressing cells, we performed PTEN knockdown in BT474, SKBR3, and HCC1954 cells, all of which display endogenous HER2 gene amplification as well as in SUM159-HER2\(^+\) cells. The efficiency of PTEN knockdown in these cells is demonstrated by western blotting (Figure 3D). The PTEN knockdown in these HER2-amplified cell lines and in SUM159-HER2\(^+\)PTEN\(^-\) cells increased IL6 production (Figure 3E).

The NF-κB transcription factor is known to transcribe a number of cytokine genes including IL6, IL8, and CCL5 (Yu et al., 2010). Furthermore, Iliopoulos et al. recently reported that IL6-activated NF-κB signaling is mediated by Stat3 and Akt signaling pathways (Iliopoulos et al., 2009). Consistent with these studies, recombinant IL6 activated Akt, Stat3, and NF-κB pathways while suppressing PTEN expression as shown by western blotting (Figure 3F). We utilized inhibitors of NF-κB, Akt, and Stat3 to determine their effects on cytokine production in HER2\(^+\)PTEN\(^-\) cells. A Stat3 inhibitor or the Akt inhibitor perifosine only partially inhibited secretion of all three cytokines (Figures 3G–3I). In contrast, inhibition of NF-κB using Bay11 or combined inhibition of Akt and Stat3 pathways completely suppressed secretion of these cytokines (Figures 3G–3I). We next determined the effect of recombinant cytokines or cytokine-blocking antibodies on cytokine production. IL6, but not IL8, increased the production of all three cytokines, an effect that was completely inhibited by anti-IL6R antibody. In contrast, addition of recombinant IL8, or an IL8 blocking antibody, had no significant effect on production of the other cytokines (Figures 3G–3I). Together, these results suggest that Stat3 and Akt signaling through NF-κB increases the production of cytokines including IL6, a cytokine whose production is necessary to maintain a positive feedback loop as illustrated in Figure 3J.

**Trastuzumab Treatment of PTEN-Deleted Cells Activates an IL6 Inflammatory Loop Expanding the CSC Population**

We next examined the effects of trastuzumab treatment on secretion of cytokines in trastuzumab-sensitive and trastuzumab-resistant cells. Trastuzumab-sensitive BT474-DsRed cells showed a modest decrease in IL6 levels after 3 days of trastuzumab treatment. However, there was approximately a 2-fold increase in IL6 secretion when cells were cultured for 3 weeks or longer (Figure 4A). In contrast, trastuzumab treatment of resistant BT474-PTEN\(^-\) cells resulted in greater than 10-fold increase in IL6 after 3 days and several 100-fold after 3 weeks of trastuzumab treatment (Figure 4A). The effects of trastuzumab-mediated IL6 production on CSCs was also tested. Parental BT474-DsRed cells contain no detectable CD44\(^+\)/CD24\(^+\) cells, a situation that was not significantly altered after trastuzumab treatment. In contrast, downregulation of PTEN in these cells generated a population that contained approximately 10% CD44\(^+\)/CD24\(^+\) cells (Figure 4B). Furthermore, culture of BT474-PTEN\(^-\) cells in the presence of trastuzumab further increased the proportion of CD44\(^+\)/CD24\(^-\) cells to 32% after 3 days, 51% after 3 weeks, and 70% when these cells were cultured for a month in the presence of trastuzumab (long-term treatment [LTT]) (Figure 4B). It is interesting that BT474-PTEN LTT cells maintained this phenotype even in the absence of trastuzumab in subsequent passages. To determine whether induction of the CSC phenotype was dependent on IL6 production, we assessed the effect of addition of IL6 receptor antibody on induction of the CSC phenotype. Addition of this antibody not only reduced the CD44\(^+\)/CD24\(^-\) CSC population in BT474-PTEN\(^-\) cells but, more important, completely blocked the increase in this population induced by trastuzumab (Figure 4C). We examined the ability of the anti-IL6R antibody to affect the CD44\(^+\)/CD24\(^-\) population in Sum159 cells, which lack the expression of the luminal CD24 marker. Anti-IL6R antibody treatment of these cells for 5 days resulted in generation of CD24\(^+\) cells (11%) and substantial growth arrest (Figures S1A and S1B available online). We further confirmed the effect of IL6 in different cell lines (MCF7, Sum159, BT474, and SKBR3) representing different breast cancer subtypes by analyzing both Aldefluor and CD44\(^+\)/CD24\(^-\) phenotypes. Although IL6 induced the CD44\(^+\)/CD24\(^-\) phenotype in all cell lines, it only increased the Aldefluor-positive population in MCF7 and Sum159 cells while slightly reducing in HER2-amplified BT474 and SKBR3 cell lines (Figures S1C and S1D). This discrepancy may be explained by the existence of different stem cell populations in different breast cancer subtypes.

To determine whether NF-κB activation was involved in these processes, we assessed p65 NF-κB phosphorylation. We found increased NF-κB phosphorylation following the trastuzumab treatment in BT474-PTEN\(^-\) and SKBR3-PTEN\(^-\) cells, compared to parental cells, which was further enhanced in trastuzumab LTT cells (Figure 4D). Stepwise activation of NF-κB over time of trastuzumab treatment was also confirmed utilizing an NF-κB reporter assay (Figure 4E).

Since EMT has been linked to the CSC phenotype (Mani et al., 2008), a population known to be regulated by IL6, we examined whether the dramatic increase in IL6 production resulted in induction of EMT markers. In addition to a several 1,000-fold increase in the IL6 and IL8 transcripts, mRNA expression of EMT markers, TGFR, Vimentin, and Twist were upregulated by 5- to 25-fold in BT474-PTEN\(^-\) cells but not in parental BT474 cells upon trastuzumab treatment (Figure 4F). Furthermore, trastuzumab treatment of BT474-PTEN\(^-\) cells resulted in downregulation of epithelial-associated genes including E-cadherin, Epcam, and Claudin (Figure 4G).

**Paracrine Induction of a Trastuzumab-Resistant CSC Phenotype in Parental BT474 Cells**

To determine whether paracrine factors could also act upon PTEN wild-type (BT474 cells), we cocultured parental BT474 cells with parental BT474-DsRed cells upon trastuzumab treatment. There was no detectable expression of IL6 in parental MCF7 cells. To confirm that PTEN deletion increased cytokine production in HER2-overexpressing cells, we performed PTEN knockdown in BT474, SKBR3, and HCC1954 cells, all of which display endogenous HER2 gene amplification as well as in SUM159-HER2\(^+\) cells. The efficiency of PTEN knockdown in these cells is demonstrated by western blotting (Figure 3D). The PTEN knockdown in these HER2-amplified cell lines and in SUM159-HER2\(^+\)PTEN\(^-\) cells increased IL6 production (Figure 3E). Furthermore, culture of BT474-PTEN\(^-\) cells in the presence of trastuzumab further increased the proportion of CD44\(^+\)/CD24\(^-\) cells to 32% after 3 days, 51% after 3 weeks, and 70% when these cells were cultured for a month in the presence of trastuzumab (long-term treatment [LTT]) (Figure 4B). It is interesting that BT474-PTEN LTT cells maintained this phenotype even in the absence of trastuzumab in subsequent passages. To determine whether induction of the CSC phenotype was dependent on IL6 production, we assessed the effect of addition of IL6 receptor antibody on induction of the CSC phenotype. Addition of this antibody not only reduced the CD44\(^+\)/CD24\(^-\) CSC population in BT474-PTEN\(^-\) cells but, more important, completely blocked the increase in this population induced by trastuzumab (Figure 4C). We examined the ability of the anti-IL6R antibody to affect the CD44\(^+\)/CD24\(^-\) population in Sum159 cells, which lack the expression of the luminal CD24 marker. Anti-IL6R antibody treatment of these cells for 5 days resulted in generation of CD24\(^+\) cells (11%) and substantial growth arrest (Figures S1A and S1B available online). We further confirmed the effect of IL6 in different cell lines (MCF7, Sum159, BT474, and SKBR3) representing different breast cancer subtypes by analyzing both Aldefluor and CD44\(^+\)/CD24\(^-\) phenotypes. Although IL6 induced the CD44\(^+\)/CD24\(^-\) phenotype in all cell lines, it only increased the Aldefluor-positive population in MCF7 and Sum159 cells while slightly reducing in HER2-amplified BT474 and SKBR3 cell lines (Figures S1C and S1D). This discrepancy may be explained by the existence of different stem cell populations in different breast cancer subtypes.

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cells with BT474-PTEN LTT cells that had been cultured for 4 weeks in the presence of trastuzumab. The green fluorescent protein (GFP) label in the parental (PTEN wild-type) cells allowed separation by flow cytometry of cell populations following coculture. It is interesting that coculture of parental BT474-GFP cells with BT474-PTEN LTT cells increased the percentage of CD44+/CD24− phenotype, they were primarily CD44+/CD24−, which is distinctly different from the control BT474 cells (Figure S2A). Furthermore, addition of anti-IL6R antibody had no effect in parental cells (Figure 5A). To examine the direct role of IL6 in these processes, we stimulated parental BT474 or SKBR3 cells with recombinant IL6 or conditioned medium (CM) from BT474-PTEN LTT cells in the presence or absence of anti-IL6R antibody. Either IL6 or CM treatment of cells for 5 days induced a mesenchymal phenotype associated with a 10-fold increase in the expression of the CD44 marker, while the anti-IL6R antibody was able to reverse these phenotypic changes (Figures 5B and 5C). Longer exposure of these cells to CM or IL6 (10 days) further increased the CD44+/CD24− and CD44+/CD24− populations (Figures S2B and S2C), suggesting that, over time, these populations are enriched.

We next examined whether the IL6 renders the CSC population resistant to trastuzumab in parental BT474 cells in vitro. While IL6 or CM significantly increased the number tumorspheres in suspension cultures of BT474 cells, trastuzumab treatment reduced the sphere forming cells by more than 50% (Figure 5D). In contrast, trastuzumab failed to inhibit sphere formation in the presence of IL6 or CM, while addition of anti-IL6R antibody reduced the number of tumorspheres induced by CM in BT474 cells (Figure 5D).

BT474 cells were stimulated by CM or IL6 in the presence or absence of trastuzumab and/or anti-IL6R antibody. While parental BT474 cell growth was reduced by 50% following 48 hr of trastuzumab treatment, BT474-PTEN− cells were unaffected (Figure 5E). Addition of CM from BT474-PTEN− LTT cells or recombinant IL6 rendered parental BT474 cells resistant to trastuzumab, an effect that was blocked by anti-IL6R antibody (Figure 5E).

Blocking the IL6 Receptor Inhibits the CSC Population Reducing Tumor Growth and Metastasis in Trastuzumab-Resistant Mouse Xenografts
To demonstrate the in vivo relevance of these findings, we assessed the effects of inhibition of IL6 signaling utilizing anti-IL6R antibody, and Akt signaling using perifosine in SUM159-HER2+ PTEN− luciferase labeled trastuzumab-resistant xenografts (Figures 2E and 2F). The effects of these treatments on tumor growth were assessed by luminescent imaging or by weighing tumors after mice were sacrificed. Treatments were started on the day of tumor inoculation (early) (Figure 6B) or delayed until palpable tumors were established, at approximately 0.4 cm in size (late). The effects of the chemotherapeutic agent docetaxel, the Akt inhibitor perifosine, anti-IL6R antibody, or perifosine plus anti-IL6R antibody were assessed after 8 weeks of treatment (Figures 6B, 6D, and 6E). In contrast to the chemotherapeutic agent docetaxel, perifosine, or anti-IL6R antibody significantly inhibited tumor growth while the combination of perifosine and anti-IL6R antibody showed greatest inhibition of tumor growth with complete inhibition of tumors in 50% of animals in which treatments were begun early (Figures 6B–6E). Furthermore, while control and docetaxel-treated mice lost body weight, those treated with anti-IL6R antibody alone or in combination with perifosine maintained normal body weight (Figure 6F). The effects of these treatments on the CSC populations were assessed by the Aldefluor assay. Although docetaxel had no significant effect on the percent of Aldefluor-positive cells, both perifosine and anti-IL6R antibody significantly reduced this population. Furthermore, the combination of perifosine and anti-IL6R antibody resulted in the greatest reduction in the Aldefluor-positive population with more than 80% reduction compared to control or docetaxel-treated tumors (Figure 6G). To determine whether these treatments affected tumor cytokine production, we determined the level of human cytokines in the serum of treated mice utilizing a human specific ELISA. Although docetaxel had no significant effect on serum IL6 or IL8 levels, perifosine or anti-IL6R antibody treatment significantly reduced the levels of these cytokines. Furthermore, the combination of perifosine and anti-IL6R antibody produced a reduction greater than 80% in levels of human serum IL6 compared to untreated or docetaxel-treated mice (Figure 6H).

Anti-IL6 Receptor Antibody Overcomes Acquired and De Novo Trastuzumab Resistance
We next examined the effect of anti-IL6R antibody on trastuzumab-sensitive BT474-DSRed and trastuzumab-resistant BT474-PTEN− xenografts. The growth of parental BT474-DSRed xenografts was significantly inhibited by trastuzumab treatment compared to saline treatment over a 7-week period (Figure 7A). However, tumors in trastuzumab-treated mice began to grow by Week 5, demonstrating acquired trastuzumab resistance. Although the anti-IL6R antibody had little effect on tumor growth on its own, when added to trastuzumab, it completely blocked tumor growth up to Week 7 of follow-up (Figure 7A). In contrast, trastuzumab had no effect on the growth of BT474-PTEN− xenografts while the anti-IL6R antibody completely blocked tumor growth when given alone or in combination with trastuzumab (Figure 7B). Addition of anti-IL6R antibody to established tumors (late treatment) completely blocked tumor growth as assessed by tumor weight at sacrifice (Figure 7C). To determine the effects of these treatments on the development of metastasis, we excised primary BT474-PTEN− tumors after 8 weeks of treatment and assessed subsequent development of local and distant metastasis by luciferase imaging (Figure 7D). While control or trastuzumab-treated mice quickly developed secondary tumors and distant metastasis requiring euthanization, there were no distant metastasis detected in anti-IL6R antibody-treated
Figure 5. An IL6 Inflammatory Loop Mediates Trastuzumab Resistance through Autocrine and Paracrine Mechanisms

(A–C) When cocultured with BT474-PTEN/LTT cells for 2 weeks, parental BT474-DsRed cells acquired a CD44+/CD24- phenotype, a transition that was inhibited by the addition of anti-IL6R antibody at the beginning of coculture (A and B). However, once cells acquired the CD44+/CD24- phenotype, they became resistant to anti-IL6R antibody (late). CM from BT474-PTEN/LTT cells or recombinant IL6 was able to induce mesenchymal phenotype and CD44 expression in both parental BT474-DsRed and SKBR3-DsRed cells (B and C).

(D) CM from BT474-PTEN/LTT cells or recombinant human IL6 increased the sphere formation of BT474-DsRed cells providing resistance to trastuzumab and that effect was reversed by anti-IL6R antibody.

(E) Trastuzumab reduced the number of viable BT474-DsRed cells by 50%, while it had no effect on the viability of BT474-PTEN- cells or BT474-DsRed cells when they were grown in the presence of CM or IL6, an effect reversed by anti-IL6R antibody. Error bars represent the mean and SD of two independent experiments performed in duplicate samples. *p ≤ 0.05. #p ≤ 0.01. See Figure S2.
mice (Figure 7D). The effects of these treatments were also reflected in human cytokine levels secreted in the bloodstream of these animals as determined by ELISA (Figure 7E).

One of the hallmarks of CSC model is their ability to initiate tumors in secondary reimplantation assays (Clarke et al., 2006). We therefore utilized a reimplantation assay to determine...
Figure 7. The IL6 Receptor Antibody Overcomes De Novo and Acquired Trastuzumab Resistance in Mouse Xenografts

(A–C) Combining anti-IL6R antibody with trastuzumab completely suppresses tumor growth in mice bearing trastuzumab-sensitive BT474-DsRed tumors (A) and overcomes de novo trastuzumab resistance in BT474-PTEN/C0 xenografts (B and C).

(D) Anti-IL6R antibody completely inhibited the development of secondary metastasis in distant organs after the primary tumors were excised in NOD-SCID mice.

(E) Serum IL6 levels were significantly higher in trastuzumab-treated mice while anti-IL6R antibody-treated mice showed the lowest levels of serum IL6.

(F) Frequencies of CSC were calculated in serial reimplantation of residual tumors from treated mice, showing significantly lower CSCs in anti-IL6R antibody alone or combination treatments. Error bars represent the mean and SD of five independent mice data. *p ≤ 0.05, #p ≤ 0.01.
the frequency of breast CSCs (tumor-initiating cells) in residual tumors from control mice and mice treated with trastuzumab or anti-IL6R, alone or in combination. Reimplantation of residual tumor cells into secondary mice showed that the frequency of breast CSCs was actually increased by trastuzumab treatment alone (Figure 7F). However, the frequency of CSCs in anti-IL6R antibody or anti-IL6R antibody + trastuzumab-treated tumors were reduced by more than 50% and 90%, respectively (Figure 7F), suggesting that anti-IL6R antibody targets the tumorigenic CSC population.

**DISCUSSION**

There is increasing evidence that many human cancers, including breast cancer, are driven and maintained by a population of cells that display stem cell properties. In addition to mediating tumor invasion and metastasis, the relative resistance of CSCs to cytotoxic chemotherapy and radiation therapy may also contribute to treatment resistance (Morrison et al., 2011). The HER2 gene, which is amplified in approximately 20% of human breast cancers, is an important regulator of breast CSCs. Despite the demonstrated clinical efficacy of HER2 blockade, the existence of de novo and acquired resistance to HER2-targeted therapeutics remains a major therapeutic challenge. Although multiple mechanisms may contribute to this resistant phenotype, inactivation of the PTEN tumor suppressor gene occurs in over 40% of HER2-amplified breast cancers, an alteration associated with trastuzumab resistance (Nagata et al., 2004). We have previously reported that PTEN downregulation increases the breast CSC population via Akt activation of the Wnt signaling pathway (Korkaya et al., 2009). The Akt inhibitor perifosine was able to partially block this pathway, reducing CSC populations.

Recently, Iliopoulos et al. demonstrated that IL6 links inflammation to malignant transformation by constitutively activating the NF-κB pathway, which, in turn, drives further IL6 production creating a positive feedback loop (Iliopoulos et al., 2009). IL6 also has previously been reported to be capable of expanding the CSC populations, as well as inducing EMT, both of which are implicated in tumor metastasis and therapeutic resistance (Iliopoulos et al., 2011; Sansone et al., 2007; Sullivan et al., 2009). Resistance to EGFR and Notch-targeted therapies in lung cancer may be regulated by IL6 (He et al., 2011; Yao et al., 2010). Elevated levels of IL6 have been shown to be associated with chronic inflammation, obesity, and increased risk for developing malignancies (Bromberg and Wang, 2009; Scheller et al., 2006). The clinical relevance of these findings is suggested by the correlation of serum IL6 levels and poor outcome in breast cancer patients (Yao et al., 2010). Furthermore, an IL6 knockout mouse failed to generate glioma tumors (Weissenberger et al., 2004). In the present studies, we demonstrate that an IL6 driven inflammatory loop mediates both de novo and acquired trastuzumab resistance. We generated trastuzumab-resistant breast cancer cell lines by overexpressing HER2 and/or downregulating PTEN. In addition to inducing trastuzumab resistance, PTEN downregulation in HER2-overexpressing cells expanded the CSC population, resulting in cells that were highly metastatic compared to HER2-overexpressing cells with wild-type PTEN.

We demonstrated that PTEN deletion in multiple HER2-amplified breast cancer cell lines resulted in substantial increases in production of several cytokines, including IL6. IL6, in turn, was necessary to maintain a positive feedback loop that also expanded the CSC population as assessed by the Aldefluor assay or by expression of the cancer stem cell markers CD44+/CD24−. It is interesting that, when HER2-amplified PTEN-deleted cells were cultured in the presence of the HER2-blocking antibody, trastuzumab, they demonstrated progressive increase in cytokine production as well as in the proportion of CSCs. LTTL with trastuzumab of BT474-PTEN−/− cells resulted in a several 100-fold increase in IL6 production associated with an increase in the CD44+/CD24− cells from less than 1% to over 70%. It is unclear whether this increase results from expansion of a preexisting CSC population or induction of this phenotype in non-CSC cells. Further studies will be necessary to distinguish between these possibilities. However, appearance of a CD44+ cell population in HER2-amplified BT474 and SKBR3 breast cancer lines suggests that IL6 may induce the CSC phenotype in non-CSC cells. In addition to increasing the CSC population, these cells assumed a mesenchymal appearance with increased expression of EMT markers such as vimentin, TGF-β, and Twist and decreased expression of epithelial markers including E-cadherin, EpCAM, and Claudin. IL6 has previously been reported to be an inducer of EMT, a state also associated with CSCs (Mani et al., 2008). We demonstrated that an IL6 receptor blocking antibody was able to prevent the increase in CSCs, EMT, and cytokine production, demonstrating a critical role for IL6 in maintaining this feedback loop. Long-term trastuzumab-treated cells became resistant to anti-IL6R antibody, suggesting that these cells may have undergone additional epigenetic changes.

We also demonstrated that the IL6 inflammatory loop is dependent on NF-κB signaling. This transcription factor is known to regulate the production of a number of cytokines including IL6, IL8, and CCL5 (Yu et al., 2010). Iliopoulos et al., recently reported that IL6-activated NF-κB signaling is mediated by both STAT3 and Akt pathways (Iliopoulos et al., 2009, 2010). Consistent with this, we demonstrate that, although combined inhibition of Akt and STAT3 pathways was required to completely inhibit production of these cytokines, blocking IL6 or inhibiting the NF-κB pathway with Bay11 almost completely blocked cytokine production. We also utilized an NF-κB reporter to demonstrate that there was an increase of greater than 3-fold in the proportion of NF-κB-activated cells upon PTEN downregulation. The aforementioned experiments suggest that activation of an IL6 inflammatory loop plays an important role in both de novo and acquired trastuzumab resistance. Activation of this inflammatory loop was dependent upon PTEN downregulation. However, unexpectedly, we found that, when parental BT474 cells were cocultured with BT474/PTEN−/−LTT (cultured long term in the presence of trastuzumab), the IL6 inflammatory loop was activated in parental BT474 cells with wild-type PTEN expression via an IL6-dependent paracrine mechanism. These experiments suggest that once the IL6 inflammatory loop is activated in PTEN-deleted cells, paracrine factors, including IL6, are able to activate similar loops in neighboring cells, even in the absence of genetic alterations. Suppression of PTEN expression by IL6...
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IL6 Feedback Loop Regulates Trastuzumab Resistance

has previously been shown to be mediated by the microRNA miR21 (Ililopoulos et al., 2010). Consistent with this, we show that IL6 activates the Akt, Stat3, and NF-κB pathways while suppressing PTEN expression. Furthermore, IL6 has been shown to induce epigenetic alterations such as methylation in a number of genes including CD44, which is induced by IL6-mediated hypomethylation, resulting in basal/stem cell phenotype (D’Anello et al., 2010). This may explain stable phenotypic changes in parental BT474 or SKBR3 cells upon IL6 treatment.

The role of an IL6-mediated inflammatory loop in trastuzumab resistance was confirmed utilizing NOD/SCID xenograft models. Both SUM159/HER2+/PTEN- and HER2 amplified BT474/PTEN- cells generated rapidly growing highly metastatic tumors in NOD/SCID mice that exhibited de novo resistance to trastuzumab treatment. Furthermore, the addition of anti-IL6R antibody to trastuzumab prevented development of acquired trastuzumab resistance in mice bearing parental BT474 xenografts. As was the case in vitro, trastuzumab treatment of mice actually accelerated BT474-PTEN tumor growth and CSC frequency, as well as markedly increasing the level of secreted cytokines IL6 and IL8 as demonstrated by a human specific ELISA. This suggests that the IL6 inflammatory loop not only mediates de novo trastuzumab resistance, but also that further amplification of this loop is involved in acquired trastuzumab resistance.

The functional importance of this inflammatory loop was demonstrated by our studies, indicating that the IL6R antibody, alone or in combination with trastuzumab or the Akt inhibitor perifosine, not only decreased the population of CSCs in primary tumor but also completely inhibited development of distant metastasis. Furthermore, primary tumors treated with anti-IL6R alone or in combination with trastuzumab reduced the frequency of CSCs, while trastuzumab treatment alone resulted in enrichment of CSCs as demonstrated by serial dilution reimplantation assays. These results are consistent with our findings that IL6 regulates the CSC population, as well as the process of EMT, both of which have been linked to tumor metastasis.

Anti-IL6R (Tocilizumab) is currently approved by the Food and Drug Administration for the treatment of rheumatoid arthritis, a condition in which IL6 plays a role in joint inflammation. Our results suggest that addition of agents targeting the IL6 pathway, such as anti-IL6R antibody, may prove a valuable addition to HER2-targeted agents for treatment of HER2+ breast cancer.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**

MCF7, BT474, SKBR3, and HCC1954 cell lines were maintained by ATCC guidelines. The SUM159 cell line was maintained in Ham’s F12 medium supplemented (with 5% fetal bovine serum, 5 μg/ml insulin, 1 μg/ml hydrocortisone and antibiotic/antimycotic 10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B).

Perifosine, Akt inhibitor was obtained from Keryx Biopharmaceuticals Inc., and docetaxel (Taxotere) was from Sanofi Aventis (Bridgewater, NJ). Anti-IL6R antibody (Tocilizumab) was obtained from Chugai Pharmaceuticals Co. Ltd. (Shizuoka, Japan). Trastuzumab was purchased from the University of Michigan Cancer Center Pharmacy. NF-κB inhibitor, Bay11-7082, and Stat3 Inhibitor VII were purchased from EMD Chemicals (Gibbstown, NJ).

The PTEN antibody was purchased from Cell Signaling Technology Inc., the α-Tubulin antibody was from Santa Cruz Biotechnology Inc., and the phospho-NF-κB (p65) antibody was from Cell Applications. Fluorescent-conjugated antibodies to CD44, CD24, CD49f, and EpCAM are from BD Biosciences (San Jose, CA).

**Cytokine Antibody Array and ELISA**

The equal numbers of cells were plated and cultured for 3 days. Subsequently, conditioned media from these cell cultures were collected and analyzed by the RayBio Human Cytokine Antibody Array 5 (RayBiotech, Inc. Norcross, GA). ELISA assay was performed using the conditioned medium collected from two day cultures of cells seeded at 200,000 cells/plate. Blood samples were drawn through orbital vein just before sacrificing the mice. Plasma separated from whole blood by centrifugation at 14,000 rpm at 4°C. Plasma from tumor bearing mice and the conditioned medium from in vitro cultures were then analyzed for the indicated cytokines by UM Cytokine Core facility.

**Tumorsphere Assay**

Single cells were plated on ultra-low attachment plates at a density of 1 x 10^3/ml and grown for 7 days in a mammocont medium (StemCell Technologies). After the treatment of primary spheres, they were dissociated into single-cell suspension and plated at a density of 5 x 10^3–1 x 10^4/ml for the subsequent passages. Secondary spheres were counted after 5–7 days in culture.

**Lentiviral Constructs and Infection of NMECs and Breast Cancer Cell Lines**

The construction of lentiviral shRNA, pLL3.7-shPTEN targeting the human PTEN gene and pLenti-RSV-HER2 overexpressing HER2 gene were previously described (Korkaya et al., 2008; Korkaya et al., 2009). Using both pLL3.7-shPTEN and pLenti-RSV-HER2, we coinfect SUM159 and MCF7 cell lines to generate MCF7-HER2+/PTEN- and Sum159-HER2+/PTEN- cells. Stable clones of MCF7-HER2+ Sum159-HER2+ MCF7-PTEN+ and Sum159-PTEN+ cells were previously generated (Korkaya et al., 2008; Korkaya et al., 2009).

**Aldefluor Assay and Flow Cytometry**

To measure ALDH activity, the Aldefluor assay was carried out according to manufacturer’s guidelines (StemCell Technologies, Inc., Durham, NC). Indicated cells were incubated with fluorophore-conjugated CD44 or CD24 antibodies alone or in combination on ice for 30 min, washed with Hank’s balanced salt solution (HBSS), and resuspended in DAPI containing HBSS buffer for flow cytometry analyses.

**Implantation of Cells in NOD/SCID Mice and Drug Treatments**

In mouse xenografts, we utilized the luciferase-expressing breast cancer cell lines for in vivo bioluminescence imaging using the Caliper IVIS imaging system. Breast cancer cells expressing the luciferase gene were implanted into the fat pads of 5-week-old NOD/SCID mice. These mice were imaged the following day to ensure the implantation of tumor cells.

Early drug treatments were started right after the implantation of cells in mice; trastuzumab was given at 20 mg/kg dose i.p. once per week, docetaxel was given at 10 mg/kg dose i.p. once per week, anti-IL6R antibody was given at 10 mg/kg once a week, and perifosine was given at 20 mg/kg twice per week. Treatments for all early settings were 8 weeks long.

Late treatments were started after the establishment of primary tumors (roughly when they reached 0.4 cm in size). Drug doses were as described in early setting.

All mice were housed in the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International)-accredited specific pathogen-free rodent facilities at the University of Michigan. Mice were housed on sterilized, ventilated racks and supplied with commercial chow and sterile water, both previously autoclaved. All experiments involving live mice were conducted in accordance with standard operating procedures approved by the University Committee on the Use and Care of Animals at the University of Michigan.

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Immunoblotting and immunostaining assays were performed as previously described (Korkaya et al., 2009).

Statistical Analyses

Statistical differences for the number of spheres, GFP-positive cells, Aldefluor assays, and tumor growths were determined using Student’s t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2012.06.014.

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