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Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the pro-inflammatory cytokines IL-6 and IL-8

Zachary C. Hartman¹, Graham M. Poage¹, Petra den Hollander¹, Anna Tsimelzon², Jamal Hill¹, Nattapon Panupinthu³, Yun Zhang¹, Abhijit Mazumdar¹, Susan G. Hilsenbeck², Gordon B. Mills³, and Powel H. Brown¹

¹Department of Clinical Cancer Prevention, The University of Texas - MD Anderson Cancer Center, Houston, TX

²Lester and Sue Smith Breast Center, Department of Medicine, Baylor College of Medicine, Houston, TX

³Department of Systems Biology, MD Anderson Cancer Center, Houston, TX

Abstract

Triple-negative breast cancers (TNBCs) are aggressive with no effective targeted therapies. A combined database analysis identified 32 inflammation-related genes differentially expressed in TNBCs, 10 proved critical for anchorage-independent growth. In TNBC cells a LPA-LPAR2-EZH2 NF-kappaB signaling cascade was essential for expression of IL-6, IL-8 and CXCL1. Concurrent inhibition of IL-6 and IL-8 expression dramatically inhibited colony formation and cell survival *in vitro* and stanching tumor engraftment and growth *in vivo*. A Cox multivariable analysis of patient specimens revealed that IL-6 and IL-8 expression predicted patient survival times. Together these findings offer a rationale for dual inhibition of IL-6/IL-8 signaling as a therapeutic strategy to improve outcomes for TNBC patients.

Keywords

Triple-Negative Breast Cancer; IL-6; IL-8; EZH2; LPA

Corresponding Author: Correspondence: Powel H. Brown, MD, PhD, Professor, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030. Phone: 713-792-4509, phbrown@mdanderson.org.

Author contributions:

Conception and design: ZC Hartman & PH Brown

Development of methodology: ZC Hartman, GM Poage, SG Hilsenbeck, GB Mills & PH Brown (general methodology); Brown lab members (method-based discussions)

Acquisition of data: ZC Hartman, P den Hollander, GM Poage

Analysis and interpretation of data: ZC Hartman, GM Poage, P den Hollander, A Tsimelzon, SG Hilsenbeck, GB Mills & PH Brown

Writing, review and/or revision of the manuscript: ZC Hartman, GM Poage, P den Hollander, SG Hilsenbeck, GB Mills, MI Savage & PH Brown

Administrative, technical, or material support: J Hill, Y Zhang, PH Brown & N Panupinthu

Study supervision: PH Brown

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PH Brown is on the Scientific Advisory Board of Susan G. Komen for the Cure. All remaining authors declare no actual, potential, or perceived conflict of interest that would prejudice the impartiality of this article.

INTRODUCTION

Breast cancer is a heterogeneous disease that can be subdivided into distinct tumor types based upon expression of molecular markers predicting patient outcomes and response to therapy. 60-70% of breast cancers overexpress estrogen receptor (ER) alpha, while 15-20% overexpress human epidermal growth factor receptor-2 (HER2). ER-positive tumors respond to hormonal therapies (1), while HER2-positive tumors respond to HER2-specific antibodies and inhibitors (such as trastuzumab or lapatinib) (2, 3). However, targeted therapy is not available for triple-negative breast cancers (TNBCs), which lack ER, progesterone receptor (PR) and HER2 expression. Recent studies have demonstrated that TNBCs are genomically heterogeneous and rarely possess activating mutations in oncogenes (4, 5). There is an urgent need to identify critical oncogenic events that promote growth and transformation within this subgroup.

While oncogenic pathways driving tumorigenesis in different lineages vary considerably, a wide variety of oncogenes trigger inflammatory signaling critical to their transforming capacity. Inflammatory signaling from Ras, epidermal growth factor receptor (EGFR), Src, Myc, myeloid differentiation primary response gene 88 (MyD88), and HER2 inflammatory signaling cascades contribute to the transformation of solid and blood cell malignancies, including breast carcinomas (6-10). Oncogenic induction of inflammation appears context-dependent, as aberrant activation can trigger senescence and clearance of primary cells, or induce transformation of cells carrying tumor suppressor mutations (11, 12). The duality of protective and transformative inflammatory signaling may represent an opportunity for therapeutic manipulation. We hypothesized that a set of inflammatory genes is critical for growth and tumorigenicity of TNBCs, and that pathways activated by these cytokines would offer a common platform for treatment of this heterogeneous group of cancers.

MATERIALS AND METHODS

Cell lines

Cell lines were obtained from ATCC (Manassas, VA), and maintained according to their recommendations. Cell lines were PCR-tested for Mycoplasma and validated by STR DNA fingerprinting using the AmpF/STR Identifiler kit (Life Technologies, Foster City, CA) protocol. Profiles were compared to known ATCC fingerprints, (CLIMA), and the MD Anderson fingerprint database in 2012.

Inhibitors and agonists

CXCR1/2 inhibitor (SB225002), NF- κ B inhibitors (BAY 11-7082 and BAY 11-7085), and paclitaxel were obtained from Sigma (St. Louis, MO). JAK1/2 inhibitor (ruxolitinib) was obtained from LC laboratories (Woburn, MA). Lysophosphatidic Acid (LPA 18:1) and LPA inhibitors (VPC32183 and VPC12249) were obtained from Avanti Polar Lipids (Alabaster, AL).

siRNA transfection

EZH2 siRNAs and LPAR2 were purchased from Sigma (St. Louis, MO). siRNA transfection was performed using a final concentration of 10nM using DharmaFECT1 (Dharmacon, Inc., Chicago, IL), according to manufacturer's instructions.

Viral vectors and cloning

Gene specific shRNA vectors were obtained from Open Biosystems (Huntsville, AL) and cloned into tetracycline-inducible vectors. pTRIPZ, TRIBZ, and pTRINZ vectors were created by modification of pTRIPZ. Unique shRNAs were constructed using PAGE-purified

oligos. Lentiviral vectors were prepared by co-transfection (GAG, POL, TAT, and VSV-G helper plasmids, and shuttle vectors) into 293T cells, followed by viral concentration. Viruses were titered by serial dilution and cells were infected at a multiplicity of infection of 1. Selection was staged 48hr post-infection.

Luciferase assays

Firefly luciferase reporters were purchased from Qiagen (Valencia, CA) and assays performed using the Dual-Luciferase Assay Kit (Promega, Madison, WI). Measurements were performed using a FLUOstar Omega (BMG Labtech, Ortenberg, Germany) according to manufacturer's recommendations.

Microarray and quantitative RT-PCR (qRT-PCR) assessments

Breast cancer patient and cell line datasets were obtained from the Gene Express Omnibus Repository or ArrayExpress Archive databases. The Richardson (GSE5460, n=129) (13), Bild (GSE3143, n=158) (14), Wang (E-TABM-158, n=287) (15), Børresen-Dale (GSE19783, n=115) (16), Chin (GSE2034, n=119) (17), and TCGA (18) patient datasets were compared to the GSE12777 (n=51) (19), GSE16795 (n=39) (20), and E-TABM-157 (n=51) (21) breast tumor line datasets. Microarray analysis of KEGG cytokine gene sets was performed using the Biometric Research Branch Array Tools. Due to limited availability of HER2 IHC in multiple patient datasets, we performed univariate Student's *t*-tests (unpaired with unequal variance) to determine which genes were more highly expressed in ER-negative versus ER-positive breast cancers. Tests were considered significant at a stringency level of $p < 0.001$. Gene expression levels were validated by extracting RNA using the RNeasy kit (QIAGEN Inc., Valencia, CA), followed by reverse-transcription (Invitrogen, Carlsbad, CA). Q-PCR assays (quadruplicate) were performed using an ABI PRISM 7900 (Life Technologies).

Cell proliferation and anchorage-independent growth assays

Measurement of proliferation was performed as previously described (22). Anchorage-independent growth assays were performed by plating 5,000 cells per well on 12-well plates in 0.35% agar (FMC Corporation, Rockland, ME). Cells were grown for a period of 1-5 weeks in their recommended media. Colonies were fed with media weekly and measured using the GelCount system (Oxford Optronix, Oxford, UK), with colonies 50-450µm in diameter scored. Data points were performed in quadruplicate, with results reported as average number $\pm$ SD.

Apoptosis assays

Apoptosis assays were performed using Annexin V staining (eBioscience, San Diego, CA) following manufacturer's instructions, replacing propidium iodide (PI) with DAPI staining. 100,000 cells per well were plated on 6-well plates in the presence or absence of doxycycline (2µg/ml). Both 3 and 4 days post-treatment, cells were treated with the described agents or vehicle. Cells were harvested, stained, and analyzed using a Gallios flow cytometer (Beckman Coulter, Brea, CA) and performed in triplicate. Results were reported as average percentage of each population $\pm$ SD. Cell viability was determined by MTS assay when using chemical inhibitors. 2,000 cells per well were plated on 96-well plates. Absorbance was measured at 490nm according to manufacturer's instructions. All data points were performed in heptuplicate with results reported as average absorption $\pm$ SD.

ELISA

Supernatants of 5×10^5 cells were harvested at 24hr. ELISAs were performed using IL-6, IL-8 (BioLegend, San Diego, CA) and CXCL1 kits (R&D Systems, Minneapolis, MN),

according to the manufacturer's recommendations. For TRIPZ-inducible knockdown experiments, cells were treated for 3 days $\pm$ doxycycline (2 μ g/ml). On day 4, cells were plated in $\pm$ doxycycline. Supernatants were harvested (24hr after media replacement and 4 days after doxycycline treatment) and assessed for cytokines by ELISA. Experimental data points were performed in quintuplicate or triplicate with results reported as average number $\pm$ SD.

Mouse experiments

Nude mice (Charles River, Wilmington, MA) experiments were performed in accordance with MD Anderson IACUC-approved protocols. SUM159 cells were injected into mammary fat pads of nude mice (5×10^6 per animal in 100 μ l 1:1 Matrigel (BD Biosciences, San Jose, CA)) and tumor size was measured at the indicated time points. In pre-treatment experiments, cells were pre-treated for 4 days with doxycycline (2 μ g/ml), and mice treated at the indicated time points with doxycycline-containing water (20 μ g/ml in 30% Sucrose). Control animals were treated with 30% sucrose water. In tumor treatment experiments, mice were injected and monitored for growth. When tumors reached $>30\text{mm}^3$ in size, mice were randomized to doxycycline-treatment or control groups and monitored for tumor growth. Tumors were measured with digital calipers and volumes calculated ($v = \text{width} \times \text{width} \times (\text{length}/2)$). Statistical differences were calculated by comparison of growth slopes from log transformed tumor volumes plotted versus time.

Survival Analysis

The prognostic importance of IL-6, and IL-8 was evaluated using gene expression profiles and survival data generated by Curtis *et al.* (4), and Kao *et al.* (23). R statistical software was used to analyze data obtained from the Oncomine database to generate Kaplan-Meier survival curves and determine statistical significance using the log rank (Mantel-Cox) method and perform Cox proportional hazards models analyses. For each gene, patients were dichotomized at the mean expression level.

RESULTS

To identify inflammatory pathways critical for the growth of TNBC cells, we focused on the 226 inflammatory genes within the cytokine and cytokine receptor signaling pathways, as defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG). We performed analyses of gene expression across patient and cell line datasets. (**Figure S1**). In 5 mRNA expression datasets, we performed a locus-by-locus analysis with univariate Student's *t*-tests ($p < 0.001$) between ER-negative versus ER-positive tumors and identified 65 genes (51 upregulated, 14 downregulated) that were differentially expressed in at least two datasets (Representative dataset **Figure 1A**). We next investigated cytokine gene expression differences between basal-like and luminal-like breast cancer cell lines using 3 different datasets. Forty-four genes were significantly dysregulated (37 upregulated, 7 downregulated) (**Figure 1B**). The intersection of differentially expressed cytokines in breast tumors and cell lines revealed 24 genes that were highly expressed in both ER-negative patient samples and in ER-negative/basal-like breast cancer lines (**Figure 1C, Table S1**).

We next investigated whether these 24 inflammatory genes were critical for anchorage-independent colony formation in TNBC cell lines. Using lentiviral shRNA vectors (verified for efficacy **Table S2**), we investigated the effect of gene knockdown on growth of 4 different TNBC lines (SUM159, MDA-MB-231, MDA-MB-468, MDA-MB-436). A group of 10 genes whose inhibition decreased colony formation across tumor lines by greater than 50% were identified (**Figure 1D**). Only inhibition of two genes, EGFR and TNFRSF19, resulted in greater than 50% inhibition of colony formation of ER-positive/luminal-like

MCF-7 and T47D cells, suggesting the remaining 8 genes are required specifically for the growth of TNBCs. Furthermore, analysis of the TCGA dataset (24) revealed that 9 of 10 inflammatory genes identified were significantly differentially expressed in patient TNBCs (**Figure 1E**).

As the majority of these critical inflammatory genes are cytokine/chemokine factors, we focused on Interleukin-6 (IL-6), Interleukin-8 (IL-8), and chemokine (C-X-C motif) ligand 1 (CXCL1), three inflammatory mediators whose inhibition caused the most suppression of anchorage-independent growth. We found that 12 TNBC cell lines had both higher levels of mRNA transcripts (**Figure S2**) and of chemokines/cytokines release into the media compared to 6 ER-positive/luminal-like breast cancer lines (**Figure 2A**).

To ascertain whether this coordinated inflammatory protein secretion was caused by external or internal stimuli, we serum-starved TNBCs and assayed IL-6, IL-8, and CXCL1 levels. In 7/8 cell lines, cytokine levels were dependent upon serum stimulation (**Figure 2B**), suggesting that these factors are produced for autocrine signaling.

Since stimulation with heat-inactivated serum was strongly associated with the secretion of IL-6, IL-8, and CXCL1 in TNBCs, we focused on the components of serum responsible for induction of inflammatory signaling. Lysophosphatidic acid (LPA) (25), and the LPA receptor (LPAR) family have been shown to mediate cytokine secretion in cancer cells (26, 27). In addition, strong associations between LPA receptors and TNBC have been reported (24, 28, 29). Further, expression the three major LPA receptors, or the enzyme producing LPA in mammary epithelium, leads to increased mammary tumors associated with production of inflammatory cytokines (24). To determine whether LPA stimulation elicits IL-6, IL-8, and CXCL1 production in TNBCs, we treated serum-starved cells with LPA, and measured IL-6, IL-8, and CXCL1 secretion. LPA was sufficient to completely or partially restore cytokine secretion in TNBC cell lines (**Figure 2C**). To determine the receptors responsible for LPA signaling, we measured cytokine secretion associated with knockdown of different LPA receptors by small molecule inhibition (**Figures 2D**) and by RNAi (data not shown). These studies revealed that combined inhibition of LPAR1, LPAR2, and LPAR3 suppressed IL-6, IL-8, and CXCL1 expression. However, inhibition of LPAR2 alone was sufficient to suppress IL-6 and IL-8 expression, suggesting that LPAR2 signaling is responsible for inflammatory gene expression in TNBCs. LPAR2 is more highly expressed in basal, compared to luminal cancers and cell lines, and knockdown of LPAR2 resulted in loss of IL-6, IL-8 and CXCL1 expression (**Figure S3**), demonstrating that LPAR2 is required for cytokine/chemokine expression in serum containing media. This is consistent with previous studies showing that LPAR2 is the main receptor responsible for cytokine secretion (30, 31).

To identify the association between LPA stimulation and upregulation of these inflammatory genes, we confirmed that LPA stimulation resulted in a significant increase in IL-6, IL-8, and CXCL1 mRNA levels in SUM159 cells after serum starvation (**Figure 3A-C**). These genes share several critical transcription factor binding sites in their proximal promoters, including AP-1, NF- κ B and C/EBP binding sites (32-36). Therefore, to determine which transcription factors were induced by LPA stimulation, we measured luciferase activity of transcription factor-specific reporters in serum-starved TNBCs (**Figure 3D**). After NF- κ B inhibition, serum-elicited and LPA-elicited IL-6, IL-8, and CXCL1 production was also blocked (**Figure 3E and F**), thus establishing the importance of a LPA-LPAR2-NF- κ B signaling cascade in the production of autocrine inflammatory factors in TNBCs.

Recently, EZH2 was identified as a critical regulator of NF- κ B-induced inflammatory gene expression after TNF- α stimulation in TNBCs (37). We hypothesized that EZH2 plays a

central role in LPA-mediated cytokine secretion and found that EZH2 was highly expressed in TNBCs compared to ER-positive breast cancer cells, consistent with a role in production of inflammatory cytokines in TNBC (**Figure 3G**). We then determined the effect of EZH2 siRNA on secretion of IL-6, IL-8, and CXCL1 and observed that EZH2 was required for optimal cytokine production (**Figures 3H**). Consistent with the secretion of cytokines by TNBCs, ectopic overexpression of ER α in TNBC cells inhibited EZH2 expression, and this was associated with a strong inhibition of baseline or LPA-induced expression of inflammatory genes (**Figure S4**). Finally, we inhibited EZH2 expression and discovered that reduction of EZH2 suppressed colony formation of TNBCs (**Figure 3I**). These results demonstrate that EZH2 is critical for anchorage-independent growth of TNBCs, and that this LPA-LPAR2-EZH2-NF- κ B-dependent signaling axis is critical for the expression of tumorigenic inflammatory cytokines and chemokines in TNBCs.

Since IL-6, CXCL1, and IL-8 are highly expressed in and secreted by TNBCs, we investigated their role in regulating anchorage-independent growth. Using doxycycline-inducible IL-6, IL-8, and CXCL1 shRNA, we found that inhibition of IL-6, IL-8, and CXCL1 strongly suppressed TNBC anchorage-independent colony formation (**Figures 4A-C**) consistent with the effects of EZH2 and NF- κ B inhibition.

To determine whether tandem inhibition of inflammatory gene combinations enhanced the suppression of TNBC colony formation, we generated SUM159 cell lines with suppressed IL-6, CXCL1, and IL-8 expression (**Figure S5**). Tandem inhibition of IL-6 and IL-8 or CXCL1 caused a more pronounced inhibition than with inhibition of either cytokine alone or of CXCL1 and IL-8 together (**Figure 4D**). This strong effect of tandem autocrine IL-6 and IL-8 inhibition on colony growth was confirmed in two additional TNBC cell lines, MDA-MB-231 and MDA-MB-468 (**Figures 4E, S5**), highlighting the importance of autocrine expression of these genes in TNBCs.

To confirm that inhibition of colony growth is dependent upon autocrine signaling through known IL-6 and IL-8 pathways, we used SUM159 and MDA-MB-231 cells expressing shRNA to IL-6 and IL-8 receptors (shIL-6ST, shCXCR1, and shCXCR2) (**Figure S6**), and assessed the effects of IL-6 and IL-8 receptor signaling suppression on colony formation. Inhibition of these receptors had a significant effect on ligand-induced anchorage-independent growth (**Figure 4F**). JAK1/2 and CXCR1/2 inhibitors demonstrated significant inhibition of anchorage-independent growth of triple-negative, but not ER-positive breast cancer cells (**Figure S6**) consistent with the cytokine signaling cascade being critical for anchorage independent growth. Finally, we found that dual suppression of IL-6 and IL-8 receptors significantly inhibited colony formation more than either receptor component alone (**Figure 4G**). Collectively, these results demonstrate the critical non-redundant nature of autocrine cytokine/chemokine signaling through the IL-6 and IL-8 pathways in TNBCs.

Since our results showed that IL-6 and IL-8 signaling are critical for anchorage-independent growth, we next investigated the effects of IL-6 and IL-8 on apoptosis in TNBCs. Inhibition of IL-6 or IL-8 had no significant effect, however, coordinate IL-6-IL-8 inhibition caused a significant induction in apoptosis (**Figure 4H**). In addition, only dual IL-6-IL-8 inhibition sensitized cells to staurosporine-induced apoptosis (**Figure 4I**). To determine whether cytokine knockdown sensitizes cells to a chemotherapeutic agent used for TNBCs, we studied the sensitivity to paclitaxel. In contrast to single-gene inhibition, combined IL-6 and IL-8 inhibition enhanced paclitaxel-induced apoptosis (**Figure 4J**). These results suggest that concurrent IL-6 and IL-8 signaling plays a critical role in TNBC resistance to apoptosis.

Based on these *in vitro* results, we posited that IL-6 and IL-8 are critical for *in vivo* TNBC growth. To test this hypothesis, we employed two complementary approaches (**Figure 5A**):

one to determine if IL-6 and IL-8 depletion altered TNBC cell engraftment and tumor outgrowth, and another to determine if these proteins are critical for growth of established tumors. In our first approach, we depleted cells of IL-6 and IL-8 expression prior to injection. Mice injected with control or shIL-6 cells all formed tumors, while 3/5 mice injected with shIL-8 cells formed tumors, and mice injected with dual shIL-6/shIL-8 tumor cells failed to form palpable tumors. Further analysis showed that mice injected with shIL-6 cells formed tumors with delayed kinetics and at a decreased overall growth rate in comparison to their non-doxycycline treated counterparts (**Figure 5B**). In our second approach, we injected mice with cells and began doxycycline after tumors had established (greater than 30mm³). Inhibition of IL-6 or IL-8 did not affect tumor growth of established tumors, however, coordinate inhibition of IL-6 and IL-8 significantly suppressed tumor growth (**Figure 5C**). Together, these data demonstrate that inhibition of both IL-6 and IL-8 is necessary to inhibit TNBC tumor growth *in vivo*.

We next hypothesized that these cytokines contribute to more rapid tumor growth in humans and are associated with poor overall survival. Kaplan-Meier analysis of patients dichotomized on the median expression value of IL-6 demonstrated a poorer prognosis (log-rank $p=5.8e-5$) for patients with high tumor expression of IL-6 compared to those expressing lower levels (**Figure 5D**). A similar significant (log-rank $p=2.2e-5$) result was observed with high IL-8 levels (**Figure 5E**). When women were stratified according to combined expression of both genes, patients in the group expressing high levels of both IL-6 and IL-8 had the worst prognoses (log-rank $p=7.5e-5$, **Figure 5F**). To control for PAM50 intrinsic molecular subtype, tumor grade, and nodal involvement, we performed a Cox proportional hazards model and found that coordinated high expression of IL-6 and IL-8 was a significant and independent predictor of poor prognosis (HR:1.47, $p=7.5e-5$, **Table 1**). This hazard ratio estimate was comparable to Cox models from the Kao (23) dataset, however these did not reach statistical significance (data not shown). A subset analysis of only TNBCs revealed a similar hazard ratio of 1.42 that did not reach statistical significance (data not shown).

We propose a model for autocrine IL-6 and IL-8 action in the progression of TNBC (**Figure 6**). Trophic factors present in serum (for example LPA, through LPAR2) induce activation of NF- κ B. Subsequently, NF- κ B activation combined with high EZH2 expression stimulates transcription of inflammatory genes, such as IL-6 and IL-8. These genes are produced and secreted by tumor cells, but act in an autocrine feedback loop through IL6ST and CXCR1 to stimulate growth and survival through multiple downstream pathways. The findings suggest that targeting the autocrine synergies between these and other inflammatory factors could potentially represent a novel approach for the treatment of triple-negative breast cancer.

DISCUSSION

In our examination of TNBC inflammatory-related genes, we discovered that many inflammatory genes are produced by ER-negative cancers, and that a subset of these genes is critical for anchorage-independent growth of TNBC cells. We found that the production of these cytokines most important for anchorage-independent growth was regulated by a LPAR2-EZH2-NF- κ B-dependent axis. In addition, our investigation identified two of these highly expressed cytokines; IL-6 and IL-8 are both critical for growth of TNBCs, but not ER-positive breast cancer cells. IL-6 and IL-8 are also critical for anchorage-independent colony formation, as well as resistance to apoptosis. Moreover, our investigations revealed that the tandem expression of IL-6 and IL-8 is critical for xenograft tumor growth and that combined overexpression of these genes correlates with poor prognoses in breast cancer patients. Thus, our study demonstrates that coordinated cytokine expression and signaling is critical for the growth of TNBCs.

There is extensive evidence that autocrine production of IL-6 and IL-8 occurs in immune and other stromal cells which, in turn, act in a paracrine fashion to enhance neovascularization and inflammation-dependent carcinogenesis (38). Based on this rationale, clinical trials are now underway to target IL-6 in metastatic prostate cancers using chimeric antibodies (ClinicalTrials.gov Identifier: NCT00433446). Our results demonstrate that autocrine cytokine production and usage within breast epithelium is the key requirement for TNBC cell growth in the absence of supporting cells. These two concepts are not mutually exclusive; the function of secreted factors in the tumor microenvironment would reinforce growth signaling within malignant cells. In addition to pro-proliferative properties, IL-6 and IL-8 are mediators of Notch signaling that promote cancer stem cell self-renewal. Marotta *et al.* described a gene signature which includes IL6 that is preferentially activated in stem-like breast cancer cells (39). In the setting of HER2-positive breast cancer, amplified IL-6 signaling is known to facilitate Trastuzumab resistance by stabilizing the cancer stem cell population (40). Likewise, prognostic signatures that include IL-8 metagene have also been identified in TNBC (41). Our observation that coordinate overexpression is critical for triple-negative tumor growth, is consistent with literature demonstrating an enrichment of cancer stem cells among TNBCs (42). These include a majority of the claudin-low molecular subtype tumors, known to possess cancer stem cell characteristics (43). It is possible that the generation and utilization of these cytokines is compartmentalized within the same tissue. Previous studies have shown differential expression of CXCR1 in the stem cell compartment in TNBC lines (44), and similar results for the IL6 receptor have been published. Our data supports the paradigm, wherein targeting of individual cytokines does not substantially affect tumor growth, but simultaneous inhibition of ligands induces stem cell apoptosis and alters their propensity for tumor initiation. Further studies are required to determine the mechanism of dual requirement for these cytokines with a single population of cells, but these data do suggest that the inhibition of pro-inflammatory cytokines would have a profound effect on the ability of TNBCs to progress or develop resistance to therapeutic regimens.

The observation that coordinated expression of inflammatory cytokines is induced by LPA-LPAR2 is consistent with previous observations (26, 30, 45, 46). The tumorigenic significance of the LPA inflammatory axis likely applies to TNBC as well as ovarian cancer, melanoma, hepatocellular and colon carcinoma (47-49). Further, we have demonstrated that expression of ATX, the enzyme that produces LPA or the LPA2 receptor, is sufficient to induce mammary cancers that are associated with a high degree of inflammatory cytokines (24). Our identification of the critical role of EZH2 in activating LPA and LPA2 is supported by a recent study documenting EZH2 involvement in stimulated TNBCs (37). These findings link EZH2 to LPA-induced signaling and support its function as a critical complex for the expression of NF- κ B mediated inflammatory genes. The observation that the LPAR2-EZH2 inflammatory signaling axis is critical for growth of TNBC suggests that this pathway may be targeted for the treatment of these aggressive cancers. As many tumor types overexpress EZH2, there is strong interest in developing strategies that target EZH2. However current drug development studies target the SET domain. Our identification of EZH2 expression as a requirement for inflammatory response induction in TNBCs, combined with that of others demonstrating the dispensability of the EZH2 SET domain for its capacity to induce an inflammatory response, suggest that EZH2 inhibitors may be ineffective (37, 50). However, this problem could be overcome by using tandem inhibitors targeting different critical downstream inflammatory genes, such as IL-6 and IL-8. The results presented here support such an approach for the treatment of triple-negative breast cancer.

Our results strongly suggest that for heterogeneous triple-negative breast cancers, which currently have few targeted therapeutic options, combined inhibition of IL-6 and IL-8 would be a novel and effective treatment strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. An Inflammatory gene signature is critical for anchorage-independent growth of triple-negative breast cancer

A-B) Heat maps of significantly different ($p < 0.001$, hierarchically clustered) KEGG cytokine-chemokine genes in: ER-negative versus ER-positive tumors (GSE5460 dataset, $n=129$) (**A**); and basal versus non-basal cancer cell lines (GSE12777 dataset, $n=51$) (**B**). **C**) Consensus Venn diagram of overexpressed inflammatory genes in patient and cell line datasets. **D**) Anchorage-independent growth results for inflammatory genes in triple-negative and ER-positive breast cancer cells. Results are shown as growth compared to control-infected cells. **E**) Heat map of gene expression for critical inflammatory genes in the TCGA dataset.

Figure 2. IL-6, IL-8, and CXCL1 are highly expressed in an LPA-dependent manner in triple-negative breast cancer cells

ELISA assays performed on triple-negative/basal-like and ER-positive/luminal-like breast cancer cell lines assessing (A) secreted protein expression of IL-6, IL-8, and CXCL1 (B) effects of serum starvation (40hrs) (C) rescue of serum starvation by LPA induction (D). ELISA determination of cytokine levels in SUM159 cells after 10 μ M treatment with pan-LPAR1-3 inhibitor Ki16425. Statistically significant changes: * p <0.05 and ** p <0.01.

Figure 3. LPA induction of NF-κB activation is critical for IL-6, IL-8, and CXCL1 expression and is dependent upon high EZH2 expression in TNBC cells
 qRT-PCR determination of: IL-6 (A), IL-8 (B), and CXCL1 (C) RNA expression in SUM159 cells after 24 hrs of LPA-stimulation (5μM) (n=3). D) Induction of transcription factors determined by dual luciferase assays in LPA-stimulated (5μM) SUM159 cells. E-F) ELISA determination of IL-6, IL-8, and CXCL1 protein expression in SUM159 cells after 24 hrs of NF-κB inhibition (10μM) (n=6) (E); or treated with LPA (5μM) and inhibitors (F). G) qRT-PCR expression of EZH2 in triple-negative/basal-like and ER-positive/luminal-like breast cancer cell lines (n=4). H) ELISA determination of IL-6, IL-8, and CXCL1 in SUM159 cells after treatment with siEZH2 (n=3). I) Colony formation with EZH2 inhibition in SUM159 cells (n=4). Statistically significant inhibition compared to control is denoted with * $p < 0.05$ and ** $p < 0.01$.

Figure 4. Tandem autocrine expression and signaling mediated by IL-6, IL-8, and CXCL1 are critical for anchorage-independent growth and resistance to apoptosis in TNBCs
Colony formation in SUM159 and MDA-MB-231 cells with inducible IL-6 (A), IL-8 (B), and CXCL1 (C) inhibition. D) Soft agar colony formation of SUM159 cells following tandem inhibition. E) Tandem inhibition of IL-6 and IL-8 in MDA-MB-231 and MDA-MB-468 cells. F-G) Colony formation following: inducible IL-6ST, CXCR1, and CXCR2 inhibition in SUM159 cells (F); and tandem IL-6ST-CXCR1 knockdown in SUM159 and MDA-MB-231 cells (G). H) Apoptosis assessed by AnnexinV-FITC and DAPI staining in SUM159 cells with inducible shIL-6, shIL-8, shIL-6-shIL-8, or control after 4-days treatment (n=3). I-J) Cell viability of SUM159 cells after doxycycline induction and treatment with staurosporine (n=6) (I) or pre-treated with paclitaxel (n=6) (J). Statistically significant inhibition compared to control is denoted with * <0.05 and ** <0.01 .

Figure 5. IL-6 and IL-8 inhibition suppresses TNBC tumor formation and growth *in vivo* and is independently prognostic for human breast cancers

A) Experimental set-up using SUM159-inducible shIL-6, shIL-8, or shIL-6-IL-8 cell lines. **(B)** shRNAs were induced for 4 days and 5×10^6 cells were injected into mammary fat pads of nude mice. **(C)** Cells were injected and tumors were allowed to reach 30mm^3 , at which time mice were randomized to receive doxycycline or vehicle. Growth was assessed every 3-4 days. **(D-F)** Kaplan-Meier overall survival analyses of patient data from the Curtis *et al.* dataset (n = 1699). Patients were classified to high or low IL-6 **(D)**, IL-8 **(E)**, and combined IL-6/IL-8 **(F)** groups

Figure 6. Role of autocrine IL-6 and IL-8 in eliciting the tumorigenicity of triple-negative breast cancer cells

In TNBCs, factors in serum, such as LPA, elicit NF-κB activation (which is enhanced by high EZH2 expression), thereby promoting autocrine production of IL-6 and IL-8. Subsequently, these factors stimulate activation of TNBCs through multiple pathways eliciting both tumor growth and resistance to apoptosis.

Table 1

Multivariable Cox Proportional Hazards Analysis

Predictor	HR	<i>p</i> -value
Meta-Expression Group		
IL6 Low/ IL8 Low	Reference	
IL6 Low/ IL8 High	1.16	0.19
IL6 High/ IL8 Low	1.19	0.09
IL6 High/ IL8 High	1.47	2.50E-04
PAM50 Subtype		
Luminal A	Reference	
Luminal B	1.41	5.80E-04
HER2+	1.45	1.90E-03
Basal-like	1.26	0.05
Normal-like	1.2	0.24
Tumor Grade		
Grade 1	Reference	
Grade 2	1.21	0.3
Grade 3	1.3	0.17
Nodal Status		
N0	Reference	
N1 +	1.96	1.30E-20

HR = Hazard Ratio