Tumor and Stem Cell Biology

Growth of Triple-Negative Breast Cancer Cells Relies upon Coordinate Autocrine Expression of the Proinflammatory Cytokines IL-6 and IL-8

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Abstract

Triple-negative breast cancers (TNBC) are aggressive with no effective targeted therapies. A combined database analysis identified 32 inflammation-related genes differentially expressed in TNBCs and 10 proved critical for anchorage-independent growth. In TNBC cells, an LPA-LPAR2-EZH2 NF-κB signaling cascade was essential for expression of interleukin (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 stanchened 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 patients with TNBCs. Cancer Res; 73(11): 1−11. ©2013 AACR.

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. About 60% to 70% of breast cancers overexpress estrogen receptor (ER)-α, whereas 15% to 20% overexpress human EGF receptor-2 (HER2). ER-positive tumors respond to hormonal therapies (1), whereas HER2-positive tumors respond to HER2-specific antibodies and inhibitors (such as trastuzumab or lapatinib; refs. 2, 3). However, targeted therapy is not available for triple-negative breast cancers (TNBC), which lack ER, progesterone receptor (PR), and HER2 expression. Recent studies have shown 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, EGF receptor (EGFR), Src, Myc, myeloid differentiation primary response gene 88 (MyD88), and HER2 inactivation is critical for expression of interleukin (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 stanchened 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 patients with TNBCs.

Materials and Methods

Cell lines

Cell lines were obtained from American Type Culture Collection (ATCC), and maintained according to their recommendations. Cell lines were PCR-tested for mycoplasma and validated by short tandem repeat (STR) DNA fingerprinting using the AmpF/STR Identifiler kit (Life Technologies) 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-κB inhibitors (BAY 11-7082 and BAY 11-7085), and paclitaxel were obtained from Sigma. JAK1/2 inhibitor (ruxolitinib) was obtained from LC Laboratories. Lyosphosphatidic Acid (LPA 18:1) and LPA inhibitors (VPC32183 and VPC12249) were obtained from Avanti Polar Lipids.

siRNA transfection

EZH2 siRNAs and LPAR2 were purchased from Sigma. siRNA transfection was conducted using a final concentration of...
10 nmol/L using DharmaFECT1 (Dharmacon, Inc.), according to manufacturer’s instructions.

**Viral vectors and cloning**
Gene-specific short hairpin RNA (shRNA) vectors were obtained from Open Biosystems 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 cotransfection (GAG, POL, TAT, VSV-G helper plasmids, and shuttle vectors) into 293T cells, followed by viral concentration. Viruses were tittered by serial dilution, and cells were infected at a multiplicity of infection of 1. Selection was staged 48 hours postinfection.

**Luciferase assays**
Firefly luciferase reporters were purchased from Qiagen and assays conducted using the Dual-Luciferase Assay Kit (Promega). Measurements were conducted using a FLUOS- tar Omega (BMG Labtech) according to manufacturer’s recommendations.

**Microarray and quantitative real-time polymerase chain reaction (qRT-PCR) assays**
Breast cancer patient and cell line datasets were obtained from the Gene Express Omnibus Repository or ArrayExpress Archive databases. The Richardson (GSE5460, n = 129; ref. 13), Bild (GSE3143, n = 158; ref. 14), Wang (E-TABM-158, n = 287; ref. 15), Borresen-Dale (GSE19783, n = 115; ref. 16), Chin (GSE2034, n = 119; ref. 17), and TCGA (18) patient datasets were compared with the GSE12777 (n = 51; ref. 19), GSE16795 (n = 39; ref. 20), and E-TABM-157 (n = 51; ref. 21) breast tumor line datasets. Microarray analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) cytokine gene sets was conducted using the Biometric Research Branch Array Tools. Because of limited availability of HER2 immunohistochemistry in multiple patient datasets, we conducted univariate Student 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.), followed by reverse transcription (Invitrogen). Quantitative PCR assays (quadruplicate) were conducted using an ABI PRISM 7900 (Life Technologies).

**Cell proliferation and anchorage-independent growth assays**
Measurement of proliferation was conducted as previously described (22). Anchorage-independent growth assays were conducted by plating 5,000 cells per well on 12-well plates in 0.35% agar (FMC Corporation). Cells were grown for a period of 1 to 5 weeks in their recommended media. Colonies were fed with media weekly and measured using the GelCount system (Oxford Optronix), with colonies 30 to 450 μm in diameter scored. Data points were conducted in quadruplicate, with results reported as average number ± SD.

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

**ELISA**
Supernatants of 5 × 10⁵ cells were harvested at 24 hours. ELISAs were conducted using interleukin (IL)-6, IL-8 (BioLegend) and CXCL1 kits (R&D Systems), according to the manufacturer’s recommendations. For TRIPZ-inducible knockdown experiments, cells were treated for 3 days with/without doxycycline (2 μg/mL). On day 4, cells were plated with/without doxycycline. Supernatants were harvested (24 hours after media replacement and 4 days after doxycycline treatment) and assessed for cytokines by ELISA. Experimental data points were conducted in quintuplicate or triplicate with results reported as average number ± SD.

**Mouse experiments**
Nude mice (Charles River) experiments were carried out in accordance with MD Anderson IACUC-approved protocols. SUM159 cells were injected into mammary fat pads of nude mice (5 × 10⁶ per animal in 100 μL 1:1 Matrigel; BD Biosciences), and tumor size was measured at the indicated time points. In pretreatment experiments, cells were pretreated for 4 days with doxycycline (2 μg/mL), and mice treated at the indicated time points with doxycycline-containing water (20 μg/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 >30mm³ 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 = width × width × (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 and colleagues (4) and Kao and colleagues (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 conduct 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 KEGG. We conducted analyses of gene expression across patient and cell line datasets (Supplementary Fig. S1). In 5 mRNA expression datasets, we conducted a locus-by-locus analysis with univariate Student 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 2 datasets (representative dataset in Fig. 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; Fig. 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 (Fig. 1C, Supplementary Table S1).
Figure 2. IL-6, IL-8, and CXCL1 are highly expressed in an LPA-dependent manner in TNBC cells. ELISA assays conducted on triple-negative/basal-like and ER-positive/luminal-like breast cancer cell lines assessing secreted protein expression of IL-6, IL-8, and CXCL1 (A); effects of serum starvation (40 hours; B); and rescue of serum starvation by LPA induction (C). D, ELISA determination of cytokine levels in SUM159 cells after 10 μmol/L treatment with pan-LPAR1-3 inhibitor Ki16425. Statistically significant changes, *P < 0.05; **P < 0.01.
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, Supplementary 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 (Fig. 1D). Only inhibition of 2 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 with TNBCs (Fig. 1E).

As the majority of these critical inflammatory genes are cytokine/chemokine factors, we focused on IL-6, IL-8, and CXCL1 (C-X-C motif) ligand 1 (CXCL1), 3 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 (Supplementary Fig. S2) and of chemokines/cytokines release into the media compared to 6 ER-positive/luminal-like breast cancer cell lines (Fig. 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 of 8 cell lines, cytokine levels were dependent upon serum stimulation (Fig. 2B), suggesting that these factors are produced for autocrine signaling.

As 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) 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 hours of LPA-stimulation (5 μmol/L; n = 3).

D, induction of transcription factors determined by dual luciferase assays in LPA-stimulated (5 μmol/L) SUM159 cells. E and F, ELISA determination of IL-6, IL-8, and CXCL1 protein expression in SUM159 cells after 24 hours of NF-κB inhibition (10 μmol/L; n = 6) or treated with LPA (5 μmol/L) 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 with control is denoted with *, P < 0.05; **, 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 hours of LPA-stimulation (5 μmol/L; n = 3).

D, induction of transcription factors determined by dual luciferase assays in LPA-stimulated (5 μmol/L) SUM159 cells. E and F, ELISA determination of IL-6, IL-8, and CXCL1 protein expression in SUM159 cells after 24 hours of NF-κB inhibition (10 μmol/L; n = 6) or treated with LPA (5 μmol/L) 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 with control is denoted with *, P < 0.05; **, P < 0.01.
acid (LPA; ref. 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). Furthermore, expression of the 3 major LPA receptors, or the enzyme-producing enzyme 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 (Fig. 2C). To determine the receptors responsible for LPA signaling, we measured cytokine secretion associated with knockdown of different LPA receptors by small-molecule inhibition (Fig. 2D) and by RNA interference (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 with luminal cancers and cell lines, and knockdown of LPAR2 resulted in loss of IL-6, IL-8, and CXCL1 expression (Supplementary Fig. S3), showing 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 (Fig. 3A–C). These genes share several critical transcription factor–binding sites in their proximal promoters including AP-1, NF-kB-, 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 (Fig. 3D). After NF-kB inhibition, serum-elicited and LPA-elicited IL-6, IL-8, and CXCL1 production was also blocked (Fig. 3E and F), thus establishing the importance of a LPA-LPAR2-NF-kB signaling cascade in the production of autocrine inflammatory factors in TNBCs.

Recently, EZH2 was identified as a critical regulator of NF-kB–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 with ER-negative breast cancer cells, consistent with a role in production of inflammatory cytokines in TNBCs (Fig. 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 (Fig. 3H). Consistent with the secretion of cytokines by TNBCs, ectopic overexpression of Erb in TNBC cells inhibited EZH2 expression, and this was associated with a strong inhibition of baseline or LPA-induced expression of inflammatory genes (Supplementary Fig. S4). Finally, we inhibited EZH2 expression and discovered that reduction of EZH2 suppressed colony formation of TNBCs (Fig. 3I). These results show that EZH2 is critical for anchorage-independent growth of TNBCs and that this LPA-LPAR2-EZH2-NF-kB–dependent signaling axis is critical for the expression of tumorigenic inflammatory cytokines and chemokines in TNBCs.

As 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 (Fig. 4A–C) consistent with the effects of EZH2 and NF-kB 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 (Supplementary Fig. 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 (Fig. 4D). This strong effect of tandem autocrine IL-6 and IL-8 inhibition on colony growth was confirmed in 2 additional TNBC cell lines, MDA-MB-231 and MDA-MB-468 (Fig. 4E, Supplementary Fig. 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; Supplementary Fig. 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 (Fig. 4F). JAK1/2 and CXCR1/2 inhibitors showed Table 1. Multivariable Cox proportional hazards analysis

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NOTE: Bold text, significantly poor prognosis (HR > 1.35).
significant inhibition of anchorage-independent growth of triple-negative but not ER-positive breast cancer cells (Supplementary Fig. 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 (Fig. 4G). Collectively, these results show the critical nonredundant nature of autocrine cytokine/chemokine signaling through the IL-6 and IL-8 pathways in TNBCs.

As 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. 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 and 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 Annexin V-FITC and DAPI staining in SUM159 cells with inducible shIL-6, shIL-8, shIL-6-shIL-8, or control after 4 days of treatment (n = 3). I and J, cell viability of SUM159 cells after doxycycline induction and treatment with staurosporine (n = 6; I) or pretreated with paclitaxel (n = 6; J). Statistically significant inhibition compared with control is denoted with * or **, P < 0.05; ***, 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 and 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 Annexin V-FITC and DAPI staining in SUM159 cells with inducible shIL-6, shIL-8, shIL-6-shIL-8, or control after 4 days of treatment (n = 3). I and J, cell viability of SUM159 cells after doxycycline induction and treatment with staurosporine (n = 6; I) or pretreated with paclitaxel (n = 6; J). Statistically significant inhibition compared with control is denoted with * or **, P < 0.05; ***, P < 0.01.
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 (Fig. 5B). In our second approach, we injected mice with cells and began doxycycline after tumors had established (>30 mm3). 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 (Fig. 5C). Together, these data show 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 showed a poorer prognosis (log-rank: P = 5.8e-5) for patients with high tumor expression of IL-6 compared with those expressing lower levels (Fig. 5D). A similar significant (log-rank: P = 2.2e-5) result was observed with high IL-8 levels (Fig. 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; Fig. 5F). To control for PAM50 intrinsic molecular subtype, tumor grade, and nodal involvement, we conducted 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 HR 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 HR 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 (Fig. 6). Trophic factors present in serum (e.g., LPA, through LPAR2) induce activation of NF-kB. Subsequently, NF-kB 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 TNBC.

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-κB-dependent axis. In addition, our investigation identified 2 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 patients with breast cancer. Thus, our study shows that coordinated cytokine expression and signaling is critical for the growth of TNBCs.

There is extensive evidence that autocrine IL-6 and IL-8 in eliciting the tumorigenicity of TNBC 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.

Figure 6. Role of autocrine IL-6 and IL-8 in eliciting the tumorigenicity of TNBC 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.
same tissue. Previous studies have shown differential expression of CXCR1 in the stem cell compartment in TNBC lines (44), and similar results for the IL-6 receptor have been published. Our data support 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 proinflammatory 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-LPARK2 is consistent with previous observations (26, 30, 45, 46). The tumorigenic significance of the LPA inflammatory axis likely applies to TNBCs as well as ovarian cancer, melanoma, hepatocellular, and colon carcinoma (47–49). Furthermore, we have shown that expression of ATX, the enzyme that produces LPA or the LPAR2 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 LPARC 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-κB–mediated inflammatory genes. The observation that the LPARC-EZH2 inflammatory signaling axis is critical for growth of TNBCs 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 showing 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 TNBC.

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

Disclosure of Potential Conflicts of Interest

G.B. Mills has a commercial research grant from AstraZeneca, Celgene, CeMinex, Exelixis/Ono, GSK, Roche, and Wyeth/Pfizer/Puma; has ownership interest (including patents) in Catena Pharma, PTV Ventures, Spindle Top Ventures; and is a consultant/advisory board member of AstraZeneca, Catena Pharm, Tau Therapeutics, Critical Outcome Tech, Daiichi Pharm., Targeted Molecular Diagnostics LLC, Foundation Medicine, Han Alblo Korea, Komen Foundation, Novartis, Symphogen. P. Brown is a consultant/advisory board member of Susan G. Komen for the Cure. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: Z.C. Hartman, A. Mazumdar, P.H. Brown.


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