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TITLE: Regulation of Survival by IKKe in Inflammatory Breast Cancer Involves EpCAM

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Regulation of Survival by IKKe in Inflammatory Breast Cancer Involves EpCAM

Although triple negative breast cancers (TNBC) consistently lack hormone receptor expression and ERBB2 amplification, several lines of evidence suggest that these cancers are heterogeneous. Here we find that aberrant expression of the IkB kinase (IKK) related-kinase IKKe drives a specific subset of TNBC and are maintained by an autocrine cytokine circuit involving JAK/STAT pathway activation. We identify CYT387 as a novel potent inhibitor of IKKe and JAK signaling that disrupts this circuit and preferentially impairs the proliferation of IKKe-driven breast cancer cells in vitro. CYT387 treatment inhibits both NF-kB and STAT activation and disrupts expression of the pro-tumorigenic cytokines CCL5 and IL-6 in these breast cancer cells. When CYT387 is combined with MEK inhibition, mouse models of triple negative breast cancer (TNBC) are effectively treated. As CYT387 and MEK inhibitors are already in advanced human clinical trials, this combination therapy has the potential to make a positive impact on patients suffering from TNBC.
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1. INTRODUCTION:

Breast cancer is the leading cause of cancer incidence and second leading cause of cancer deaths in women in the United States (1). Despite tremendous advances in screening, surgical management, and targeted therapies such as endocrine and HER2-directed treatments, the prognosis for women with advanced disease remains poor.

The IKK-related kinases IkB kinase epsilon (IKKe) and TANK binding kinase 1 (TBK1) represent an emerging link between inflammation and cancer (2). IKKe is overexpressed and/or amplified in approximately 30% of breast carcinomas (3-5), where it induces survival signaling associated with NF-kB pathway activation. Aberrant IKKe expression facilitates cell transformation, whereas suppression of IKKe in breast cancer cell lines that harbor IKKe amplification results in cell death (4). IKKe phosphorylates CYLD and TRAF2 in breast cancer cells, which contributes to NF-kB activation and promotes tumorigenesis (6, 7). IKKe also directly phosphorylates and activates specific STAT transcription factors (8, 9). Furthermore, cytokines produced by TBK1/IKKe can engage downstream JAK/STAT signaling in an autocrine or paracrine fashion (10).

Advances in targeted therapy for patients with breast cancers that express ER/PR and/or ERBB2 have improved survival. Limited treatment options exist, however, for the 15-20% of patients with triple negative breast cancers (TNBC). Although TNBC may respond to chemotherapy, tumors frequently relapse, resulting in decreased survival compared with other breast cancer subtypes (11). Activation of NF-kB and JAK/STAT signaling has been implicated in an immune subtype of TNBC (12-16). In preliminary studies we found that, in addition to its genomic amplification in luminal breast tumors, IKKe is aberrantly overexpressed in immunomodulatory TNBC. IKKe coordinately activates NF-kB and STAT signaling in these cells and sustains protumorigenic cytokine production. CYT387, a dual IKKe/TBK1 and JAK inhibitor, potently disrupts this inflammatory signaling circuit and impairs tumor progression in preclinical mouse models of TNBC, identifying a novel therapeutic strategy for this refractory breast cancer subtype.

2. KEYWORDS:

Epithelial cell adhesion molecule (EpCAM), IKKe, Mitogen-activated protein kinase kinase enzymes MEK1 inhibitor, targeted therapies, cytokine signaling

3. ACCOMPLISHMENTS:

Summary of key research accomplishments:

- Identified IKKe as a novel driver of an inflammatory subtype of TNBC that maintains features of epithelial differentiation.
- Characterized specific NF-κB, STAT3, and cytokine signaling pathways that contribute to IKKe mediated tumorigenesis.
- Discovered CYT387 as a potent IKKe and JAK inhibitor that inhibits this breast cancer subtype in vitro.
- Identified therapeutic activity of CYT387 in IKKe driven TNBC patient derived xenografts, particularly when combined with MEK inhibition.
3a. What were the major goals of the project?

Major Goal 1: Define the mechanism(s) by which EpCAM is regulated by IKKε.

Major Goal 2: Define the ability of small molecule inhibitors of IKKε to inhibit breast cancer growth and invasion.


3b. What was accomplished under these goals?

Major Goal 1: Define the mechanism(s) by which EpCAM is regulated by IKKε.

Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein that is expressed on normal epithelial cells and over-expressed in a subset of carcinomas, including breast and ovarian cancer (17). It has attracted recent attention as a tool for capture-based detection of circulating cells (18), as well as a marker for stem cell-like tumor initiating cells (19). Emerging evidence from the Gillanders’ laboratory also supports the concept that EpCAM is not simply a passive cell surface marker, but rather actively regulates breast cancer proliferation and invasion (5, 6).

To gain even further insight into EpCAM regulation and function, we first explored EPCAM gene expression across a panel of 1062 primary breast cancers (20-22) and cell lines (http://www.broadinstitute.org/ccle/home) to identify the specific molecular subtypes of breast cancer in which it is over-expressed. This analysis showed that EPCAM expression was enriched in an inflammatory subtype of triple negative breast cancer. Interestingly, this subtype of breast cancer is characterized by over-expression of several immune associated genes, including the non-canonical IκB kinase IKBKE (encoding IKKε). IKKε and its homologue TBK1 represent an emerging link between inflammation and cancer (2). IKKε is overexpressed and/or amplified in approximately 30% of breast carcinomas (3-5), where it induces survival signaling associated with NF-κB pathway activation.

Aberrant IKKε expression facilitates cell transformation, whereas suppression of IKKε in breast cancer cell lines that harbor IKKε amplification results in cell death (4). IKKε phosphorylates CYLD and TRAF2 in breast cancer cells, which contributes to NF-κB activation and promotes tumorigenesis (6, 7). IKKε also directly phosphorylates and activates specific STAT transcription factors (8, 9). Furthermore, cytokines produced by TBK1/IKKε can engage downstream JAK/STAT signaling in an autocrine or paracrine fashion (10).

Activation of NF-κB and JAK/STAT signaling has also been strongly implicated in this subtype of TNBC (12-16). IKKε coordinately activates NF-κB and STAT signaling in these cells and sustains protumorigenic cytokine production. CYT387, a dual TBK1/IKKε and JAK inhibitor, potently disrupts this inflammatory signaling circuit and impairs tumor progression in preclinical mouse models of TNBC, identifying a novel therapeutic strategy for this refractory breast cancer subtype.

In addition to being overexpressed in a subset of luminal/ER+ breast cancers as previously reported (4), we also found that IKKε mRNA was highly expressed in this EpCAM positive subset of ER- breast cancers and particularly in TNBC (Figure 1A). Induction of IKKε mRNA in this subset of TNBC tumors was more closely associated with IL-1 pathway activation, as evidenced by co-expression of an IL-1 signature (23) (Fig. 1A). Hierarchical clustering with previously reported gene expression subtypes (24) further revealed that IKKε expression and IL-1 activation were associated most closely with the immunomodulatory subtype of TNBC.
We next identified TNBC cell lines with elevated IKKε levels using gene-expression data from the Broad/Novartis Cell Line Encyclopedia (25) and validated that these cell lines expressed high levels of IKKε protein (Fig 1B, C). Using two independent IKKε-specific shRNAs, we found that the TNBC cell lines MDA-MB-468 cells and MDA-MB-231 were sensitive to suppression of IKKε, whereas specific ablation of IKKε failed to affect the proliferation of non-transformed MCF10A cells (Fig. 1B). These findings revealed that IKKε is not only overexpressed, but also contributes to the proliferation and survival of this subset of TNBC.

When we examined the relationship between IKKε and STAT3 activation (as measured by Y705 pSTAT3 levels), we observed a strong correlation between elevated IKKε levels and activated STAT3 in TNBC cell lines (Fig. 1C). Further, IKKε overexpression in HEK-293T cells not only induced NF-κB pathway activation as measured by S933 pNF-κB p105 levels, but also STAT3 activation as reflected by increased Y705 pSTAT3 levels (Fig. 1D), as well as CCL5 and IL-6 expression (data not shown). Taken together, these findings confirm that IKKε signaling promotes NF-κB, STAT3 and cytokine activation.

Despite our preliminary observations that EpCAM was linked with IKKε expression in this subtype of TNBC, subsequent experiments following IKKε suppression or over-expression failed to give consistent results. While we still suspect that EpCAM expression in these tumors reflects epithelial differentiation within this inflammatory subset of tumors, it is not clear that the relationship with IKKε is direct. Although we remain interested in the specific features that delineate this tumor cell state as a means to identify predictive biomarkers, our focus has shifted to Aims #2 and #3, in which we have made significant progress towards a novel therapeutic strategy for this breast cancer subtype.

Major Goal 2: Define the ability of small molecule inhibitors of IKKε to inhibit breast cancer growth and invasion.

Since IKKε expressing TNBC cells exhibited STAT3 activation, we considered the possibility that inhibition of JAK/STAT signaling by treatment with the clinically advanced JAK inhibitors Ruxolitinib (26) or CYT387 (27) might impact their proliferation and survival.

Treatment of MDA-MB-468 cells with several different doses of Ruxolitinib or CYT387 inhibited STAT3 phosphorylation (Fig. 1E). However, when we treated multiple different cell lines with 5 µM Ruxolitinib, which completely inhibited pSTAT3, we failed to observe any effect on cell viability in contrast to CYT387 (Fig. 1F, G). These findings suggested an additional activity of CYT387.

Since CYT387 inhibits the IKKε homologue TBK1 (28), we next assessed whether IKKε signaling was inhibited by CYT387. Both CYT387 and Ruxolitinib inhibited IKKε-induced Y705 pSTAT3 (Fig. 2A). However, CYT387 alone inhibited IKKε-induced NF-κB (Fig. 2B) and also directly impaired IKKε expression itself (Fig. 2C). We also collected media from 293T cells following transfection with EGFP or IKKε and analyzed levels of 36 different cytokines and chemokines using a antibody array. Expression of IKKε potently induced CCL5 levels in the media, which was completely abrogated by CYT387 but not Ruxolitinib treatment (Fig. 2D). We confirmed by ELISA that IKKε-induced CCL5 and IL-6 were preferentially inhibited by CYT387 (data not shown). Thus, the unique activity of CYT387 in IKKε-driven TNBC relates to its activity as a TBK1/IKKε inhibitor.

Inhibition of TNBC patient-derived xenograft (PDX) growth by CYT387 therapy in vivo. CYT387 has been evaluated in mouse models of myelofibrosis and is undergoing phase II trials for this indication in humans. In addition, Ruxolitinib was recently FDA approved for myelofibrosis based upon phase III clinical trial data demonstrating significant improvement in splenomegaly. Thus, the favorable pharmacokinetic properties, therapeutic window, and elucidation of maximum tolerated dosing (MTD) for each compound enables preclinical testing with these compounds in mice. We therefore next explored the therapeutic impact of CYT387 therapy in clinically relevant models in vivo. After tumors were established in nude mice, CYT387 was administered via daily oral gavage at a dose of 100 mg/kg. CYT387 treatment impaired the growth of established MDA-MB-468 xenografts, as well as in the Washington University Human-in Mouse (WHIM) line WHIM21, derived from a patient with IKKε expressing TNBC (Fig. 2E, Fig. 3A,B). CYT387 treatment suppressed IKKε expression in WHIM21 patient derived xenografts in vivo, potently inhibited CCL5 and IL-6 expression, and suppressed activated STAT3 (Fig 2F, data not shown). Thus, CYT387 effectively inhibits IKKε and JAK signaling in vivo, suppresses protumorigenic cytokine expression, and exhibits therapeutic potential for IKKε-driven TNBC.

Since CYT387 inhibits IKKε and JAK and is effective as a single agent in TNBC, we considered it might synergize even more potently with inhibitors of PI3K/mTOR or MEK/ERK signaling (29). Indeed, we found that combination treatment with CYT387 (50 mg/kg) and the MEK inhibitor trametinib (2.5 mg/kg), results in a dramatic reduction in tumor size in WHIM21 (Fig. 2G). In addition to WHIM21, another in vivo model of TNBC with IKKε expression (WHIM4) showed a similar impressive response to combination therapy (Fig. 4A). We also observed moderate responsiveness in another TNBC line (WHIM12E) that expressed lower levels of IKKε, suggesting that the synergy with MEK inhibition might extend more broadly across TNBC subtypes (Fig. 4B), but not to luminal tumors, which remained unresponsive (Fig. 4C). We note that the WHIM21 and WHIM4 TNBC models were obtained from patients who succumbed to their disease after multiple chemotherapies. Thus, CYT387/trametinib may represent a highly effective combination therapy for chemotherapy resistant TNBCs. Furthermore, in preclinical studies, these WHIM models have been used to evaluate the drug efficacy of multiple other targeted approaches that have been unsuccessful to date.

Combination therapy with CYT387/trametinib results in tumor necrosis of TNBC mouse PDX models. In addition to a significant decrease in size of the TNBC xenografts treated with CYT387/trametinib, the tumors also appeared particularly pale when compared with vehicle or single agent treated tumors (Fig. 2G, 3B, 4A, 4B). We therefore performed a detailed histologic examination of the WHIM21 treated tumors, including measures of angiogenesis. Whereas single agent treatment with trametinib showed preferential impairment of proliferation as measured by Ki-67 staining, and CYT387 modestly reduced microvascular density, the combination therapy resulted in a striking inhibition of angiogenesis and profound tumor necrosis (Fig. 5). Therefore, antitumor activity of this drug combination was not only direct but was also related to the synergistic effects of cytokine and MEK inhibition on angiogenesis. Taken together, combined CYT387/trametinib treatment impairs tumor progression and angiogenesis, representing a promising novel therapy for TNBC.

Combination therapy with CYT387/trametinib results in inhibition of a cytokine circuit as demonstrated in a 3D microfluidic cell culture system. We previously utilized a novel cell culture system to demonstrate the role of CCL5/IL6 in promoting tumor cell spheroid dispersal and endothelial cell migration, and the activity of CYT387 in vitro (Fig. 6A, B). By incorporating the 3D culture of TNBC cell line spheroids in a collagen matrix, along with endothelial cell co-culture to recapitulate the tumor microenvironment more faithfully than traditional 2D culture systems, this system enables more physiologic analysis of the effects of these cytokines on tumor and endothelial cell biology as they occur in vivo. Recently, we further adapted this system to the culture of actual primary tumor explants. By correlating drug therapy responses in this model with identical studies of engrafted tumors in mice, the goal is to validate this cost-effective system as a way to supplant PDX systems for performing predictive patient-based therapeutic studies. To assess whether this system is able to recapitulate the PDX response to CYT387 and trametinib treatment ex vivo, we compared control with CYT387
(1 mM), trametinib (10 nM), or combination treatment of a TNBC PDX in the device and monitored the effect on spheroid dispersal/viability using phase contrast microscopy. Notably, this PDX was obtained after only one passage in mice, and the derived spheroids still retained human immune cells (T cells, dendritic cells, and monocytes) by flow cytometric analysis (data not shown). Interestingly, similar to what we observed in vivo (Figs. 4, 5), we found that treatment with CYT387 or trametinib alone for 5 d inhibited spheroid dispersal with remaining viable cells. However, combination treatment resulted in potent synergy leaving only residual cellular debris (Fig. 7).

In short, these data demonstrate promising response of TNBC preclinical models to CYT387, particularly when combined with MEK inhibition. Given that this IKKe inhibitor is currently in human clinical trials for the treatment of myelofibrosis and has a favorable side-effect profile, we are working to translate these findings into a clinical trial, with the hopes of achieving a positive patient-related outcome for its use in breast cancer.

3c. What opportunities for training and professional development has the project provided?
This award has allowed me to obtain mentorship in basic science research as a breast cancer surgeon. It also allowed me to attend multiple basic science research conferences including the annual conferences at AACR and ASCO.

3d. How were the results disseminated to communities of interest?
Nothing to report.

3e. What do you plan to do during the next reporting period to accomplish these goals?
Nothing to report.

4. IMPACT
4a. What was the impact on the development of the principal discipline(s) of the project?
These data have not only identified a novel approach to target a subset of TNBC patients, but also one with realistic clinical potential. We are working with Gilead Sciences to extend these preclinical findings, with the hope of moving this drug combination forward into an actual clinical trial in advanced refractory metastatic TNBC patients. Thus, our data have significant potential to have a positive impact on patients suffering from this terrible disease.

4b. What was the impact on other disciplines?
Nothing to report.

4c. What was the impact on technology transfer?
Nothing to report.

4d. What was the impact on society beyond science and technology?
Nothing to report.

5. CHANGES/PROBLEMS
5a. Changes in approach and reasons for change: Nothing to report.
5b. Actual or anticipated problems or delays and actions or plans to resolve them: Nothing to report.
5c. Changes that had a significant impact on expenditures: Nothing to report.
5d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: Nothing to report.
5e. Significant changes in use or care of human subjects: Nothing to report.
5f. Significant changes in use or care of vertebrate animals: Nothing to report.
5g. Significant changes in use of biohazards and/or select agents: Nothing to report.

6. PRODUCTS:
6a. Publications, conference papers, and presentations:

   Journal publications

   Other publications, conference papers, and presentations

6b. Website or other internet site:
Nothing to report.

6c. Technologies or techniques:
Nothing to report.

6d. Inventions, patent applications, and/or licenses:
Nothing to report.

6e. Other Products:
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7a. What individuals have worked on the project?
Name: Thanh Barbie, M.D.
Project Role: Principal Investigator
Researcher Identifier: Does not have an ORCID
Nearest person month worked: 3
Contribution to project: Design, execution, and reporting of the research overall.
Funding support: N/A (this award).

7b. Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period?
Yes. The subject grant W81XWH-13-0029 has ended, and the following award was received since the last report:
Robert T. Osteen Fellowship (PI: Barbie) 07/01/15 – 06/30/16 (No effort commitment)
Harvard Medical School/BWH Department of Surgery $30,000

Therapeutic targeting of triple negative breast cancer by dual cytokine/MEK inhibition

Goals: Although triple negative breast cancers (TNBC) are defined by the absence of hormone receptor expression and ERBB2 amplification, they represent a heterogeneous set of cancers. We recently found that inducible expression of the IkB kinase (IKK) related-kinase IKKe and JAK/STAT pathway activation underlies a cytokine signaling network in the immune activated subset of TNBCs. CYT387, a novel potent inhibitor of TBK1/IKKe and JAK signaling, disrupts this circuit. Combined therapy with the MEK inhibitor trametinib is particularly effective, abrogating tumor growth and angiogenesis. The goal of this research is to perform additional mechanistic studies to determine the subset of patients who will respond to this combination therapy.

Specific Aims: 1) Correlates of response in momelotinib/trametinib TNBC clinical trial; 2) Prospective analysis of momelotinib/trametinib therapy in TNBC PDX models; 3) Development of an in vitro culture system for studies of patient-derived explants.

Funding Agency Contact: Brigham and Women’s Hospital, Department of Surgery, 75 Francis St., Boston, MA

7c. What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS
N/A
9. APPENDICES

References:


Figures:

Figure 1. A. Heatmap of IKKε mRNA levels vs amplification or IL-1 signature expression in TCGA breast tumor data. B. TNBC cell line dependence on IKKε expression. C. STAT3 activation correlates with IKKε over-expression in TNBC cell lines. D. IKKε directly induces NF-κB and STAT3 activation. E. Ruxolitinib or CYT387 treatment inhibits STAT3 activation in TNBC cells. F. Phase contrast images of TNBC cells treated with 5 µM Ruxolitinib or CYT387. G. Cell viability data following JAK inhibitor treatment of TNBC cells.
**Figure 2.**

A. Immuno-blot of IKKe, pSTAT3, STAT3, and Actin levels following EGFP or IKKe expression and inhibitor treatment of 293T cells. B. Immunoblot of p-p105, p105 and Actin levels following EGFP or IKKe expression and inhibitor treatment of 293T cells. C. IKKe and Actin levels following treatment of TNBC cells with inhibitors. D. Cytokine levels of 293T cell media following IKKe expression and inhibitor treatment. E. Effects of CYT387 treatment on cell-line or patient-derived TNBC xenografts. F. IKKe, CCL5, and IL-6 levels in treated tumors. G. Effects of combination CYT387 and trametinib therapy on TNBC PDX growth.
Figure 3.  A. Identification of a Washington University human-in-mouse line (WHIM21) derived from a human TNBC that overexpressed IKKe.
B. Established WHIM21 tumors were then treated with vehicle only, CYT387 at 50mg/kg/d, Trametinib at 2.5 mg/kg/d, or combination therapy with CYT387 and trametinib.
Figure 4. **A.** Established WHIM4 (TNBC with high IKKε expression) were treated with control or vehicle only versus combination therapy with CYT387/trametinib. **B.** Established WHIM12E (TNBC with low IKKε expression) were treated with vehicle only versus combination therapy with CYT387/trametinib. **C.** Established WHIM20 (Luminal breast cancer with low IKKε expression) were treated with vehicle only versus combination therapy with CYT387/trametinib.
Figure 5. Histologic examination of WHIM21 treated tumors with control, CYT387 alone, Trametinib alone, or combination therapy. Markers of proliferation (Ki-67) and angiogenesis (CD31 and ERG) were examined. Whereas, trametinib treatment had a more profound effect on proliferation and CYT387 on microvascular density, the combination resulted in striking inhibition of angiogenesis and profound tumor necrosis.
Figure 6. A. MDA-MB-468 spheroids in microfluidic 3D culture 0, 24 and 48 h after addition of CCL5/IL6, EGF or both. In contrast to DMSO, CYT387 treatment inhibited EGF-induced spheroid dispersal but was rescued by CCL5 and IL6. B. Compared with control media, CCL5/IL6 attracted HUVECs into collagen over 24 h. Cotreatment trametinib (MEKi) strongly inhibited this effect. Mean and SD of cell migration from 3 independent devices shown.
Figure 7. TNBC PDX treatment response \textit{ex vivo} in microfluidic 3D culture. Phase contrast images 5 d following treatment of TNBC PDX derived tumor spheroids with DMSO control, CYT387, trametinib, or combination. Whereas cells from control treated spheroids dispersed, proliferated, and migrated throughout the collagen over time, those from single agent CYT387 or trametinib treated devices were inhibited and failed to disperse. Combination of CYT387 and trametinib synergized and resulted in strong cell death, leaving only residual cellular debris. Images are representative of spheroids analyzed from 2 independent devices for each condition.
Targeting an IKBKE cytokine network impairs triple-negative breast cancer growth

Thanh U. Barbie, Gabriela Alexe, Amir R. Aref, Shunqiang Li, Zehua Zhu, Xiuli Zhang, Yu Imamura, Tran C. Thai, Ying Huang, Michaela Bowden, John Herndon, Travis J. Cohoon, Timothy Fleming, Pablo Tamayo, Jill P. Mesirov, Shuji Ogino, Kwok-Kin Wong, Matthew J. Ellis, William C. Hahn, David A. Barbie, William E. Gillanders

Introduction

Advances in targeted therapy for patients with breast cancers that express estrogen/progesterone receptors and/or HER2 have improved patient outcomes and survival. Limited treatment options exist, however, for the 15% to 20% of patients with triple-negative breast cancers (TNBCs). Although TNBCs may respond to anthracycline-based chemotherapy or cisplatin, tumors frequently relapse, resulting in decreased disease-free and overall survival compared with other breast cancer subtypes (1).

The diversity of somatic mutations, gene amplifications, and deletions observed in TNBC has hampered efforts to elucidate a common drug target in this breast cancer subtype (2). Recent evidence suggests that a significant fraction of TNBCs exhibit immune cell infiltration, with features of stem cells and epithelial-mesenchymal transition (3–5). Indeed, a refined classification of TNBC based on gene expression profiling recently identified an immunomodulatory (IM) subtype that corresponds with this category of tumors (6). However, the specific genetic drivers of this and other TNBC subtypes remain poorly defined.

Conflict of interest: David A. Barbie is a consultant for N-of-One. William C. Hahn is a consultant for Novartis. Submitted: February 19, 2014; Accepted: September 30, 2014.


Triple-negative breast cancers (TNBCs) are a heterogeneous set of cancers that are defined by the absence of hormone receptor expression and HER2 amplification. Here, we found that inducible IxkB kinase–related (IKK-related) kinase IKBKE expression and JAK/STAT pathway activation compose a cytokine signaling network in the immune-activated subset of TNBC. We found that treatment of cultured IKBKE-driven breast cancer cells with CYT387, a potent inhibitor of TBK1/IKBKE and JAK signaling, impairs proliferation, while inhibition of JAK alone does not. CYT387 treatment inhibited activation of both NF-κB and STAT and disrupted expression of the protumorigenic cytokines CCL5 and IL-6 in these IKBKE-driven breast cancer cells. Moreover, in 3D culture models, the addition of CCL5 and IL-6 to the media not only promoted tumor spheroid dispersal but also stimulated proliferation and migration of endothelial cells. Interruption of cytokine signaling by CYT387 in vivo impaired the growth of an IKBKE-driven TNBC cell line and patient-derived xenografts (PDXs). A combination of CYT387 therapy with a MEK inhibitor was particularly effective, abrogating tumor growth and angiogenesis in an aggressive PDX model of TNBC. Together, these findings reveal that IKBKE–associated cytokine signaling promotes tumorigenicity of immune-driven TNBC and identify a potential therapeutic strategy using clinically available compounds.

The IxB kinase–related (IKK-related) kinases TANK-binding kinase 1 (TBK1) and IxB kinase ε (IKBKE, also known as IKKe) represent an emerging link between inflammation and cancer (7). In response to pathogen exposure, induction of IKBKE reinforces TBK1 signaling and promotes sustained activation of the type 1 interferon pathway (8–11). Furthermore, IKBKE directly phosphorylates and activates specific STAT transcription factors (12, 13), and cytokines produced by TBK1/IKBKE can engage downstream JAK/STAT signaling in an autocrine or paracrine fashion (14).

IKBKE is also aberrantly expressed and/or amplified in approximately 30% of breast carcinomas (15–17), in which it induces survival signaling associated with NF-κB pathway activation. IKBKE activation facilitates cell transformation, whereas suppression of IKBKE in breast cancer cell lines that harbor IKBKE amplification or overexpression results in cell death (16). IKBKE phosphorylates CYLD and TRAF2 in breast cancer cells, which induces NF-κB activation and contributes to cell transformation (18, 19). However, a comprehensive understanding of how IKBKE promotes tumorigenicity is lacking, and the therapeutic efficacy of targeting IKBKE signaling in vivo has yet to be defined.

Activation of NF-κB and JAK/STAT signaling has been strongly implicated in the pathogenesis of certain TNBCs and closely related basal-like breast cancers (20–24). Markers of JAK/STAT pathway activation are particularly enriched in the IM TNBC gene expres-
Identification of an IKBKE-driven TNBC subtype. IKBKE is amplified in approximately 30% of human breast tumors, and luminal breast cancer cell lines that harbor IKBKE copy gain are dependent upon its expression (16). IKBKE overexpression has also been observed in breast cell lines and cancers without IKBKE amplification, such as the TNBC cell lines, MDA-MB-231 and MDA-MB-468 (17). To gain further insight into IKBKE regulation and function in breast cancer, we analyzed gene expression data from primary breast cancers profiled in the The Cancer Genome Atlas (TCGA) data set (2). Whereas IKBKE expression was linked with IKBKE amplification in luminal tumors, a substantial additional fraction of breast cancers overexpressed IKBKE in the absence of gene amplification (Figure 1A). Since IKBKE is also induced by multiple different cytokines (25), we examined correlation between the levels of several different cytokine gene expression signatures and IKBKE mRNA expression across these samples (26, 27). Among these signatures, IL-1 induction correlated most strongly with high IKBKE levels in a subtype of TNBC, followed by TGFα (P < 0.001 for both, normalized mutual information (NMI) statistic) (Figure 1A and Supplemental Figure 1, A and B). Hierarchical clustering with previously reported gene expression subtypes (6) and B lymphocyte markers (28) further revealed that IKBKE expression and IL-1 activation most closely associated with the IM subtype of TNBC (Figure 1D). These findings recapitulated what we had observed in primary tumors and confirmed that IKBKE is not only amplified in luminal breast cancers but also aberrantly overexpressed in a subset of TNBC.

IKBKE-amplified ZR751 cells depend on IKBKE expression for their proliferation and survival (16). Using 2 independent IKBKE-specific shRNAs, we found that TNBC MDA-MB-468 cells were at least as sensitive to suppression of IKBKE as ZR751 cells (Figure 1C). Indeed, whereas specific depletion of IKBKE failed to affect the proliferation of nontransformed MCF10A cells, we confirmed that suppression of IKBKE expression inhibited the proliferation of multiple IKBKE-amplified (ZR751 and MCF7) and IKBKE-overexpressing TNBC cell lines (MDA-MB-231 and MDA-MB-468) (Figure 1D). These findings revealed that IKBKE is not only overexpressed but also contributes to the proliferation and survival of this subset of TNBC.

IKBKE expression in TNBC is associated with STAT3 activation and cytokine production. IKBKE promotes NF-κB (7) and STAT signaling (12, 13) both directly and indirectly via autocrine cytokine production (14). Indeed, we confirmed that IKBKE overexpression in HEK-293T (293T) cells not only induced NF-κB pathway activation, as measured by S93 phosphorylated NF-κB p105 levels, but also STAT3 activation, as reflected by increased Y705 phosphorylated STAT3 (pSTAT3) levels (Figure 2A). Activation of these signaling pathways by IKBKE was associated with induction of CCL5 expression in a kinase-dependent manner (Figure 2B). When we measured IKBKE levels and activated STAT3 (as measured by Y705 pSTAT3 levels) across breast cancer cell lines, we observed correlation preferentially in TNBC cell lines (Figure 2C). These findings suggested that engagement of IKBKE signaling in TNBC occurred within the context of a broader cytokine signaling network. Since elevated IKBKE expression in TNBC tumors correlated with IL-1 and other markers of inflammation (Figure 1A and Supplemental Figures 1, A–C), we assessed the role of IL-1β in engaging IKBKE signaling in this context. Indeed, treatment of multiple TNBC cell lines with IL-1β led to a substantial further increase in IKBKE protein levels (Figure 2D) and enhanced the secretion of CCL5 (Figure 2E). Depletion of IKBKE alone in MDA-MB-468 cells failed to prevent IL-1β-induced CCL5 production but modestly reduced IL-6 levels (Supplemental Figure 3, A and B). These observations support the view that functional redundancy exists between multiple components of this network, including TBK1, which together with IKBKE promotes CCL5 and IL-6 production (30).

Sensitivity of IKBKE-driven TNBC cells to CYT387 treatment. We next compared the effects of selective inhibition of JAK/STAT signaling on TNBC cell proliferation and survival by treatment with the JAK inhibitor ruxolitinib (31) or the multitargeted JAK/TKR2/1/IBK inhibitor CYT387 (30, 32, 33). Treatment of MDA-MB-468 cells with ruxolitinib or CYT387 over a range of doses inhibited STAT3 phosphorylation (Figure 3A). Despite comparable inhibition of JAK signaling, treatment of these cells with CYT387 but not ruxolitinib impaired the viability of multiple different TNBC cell lines (Figure 3, B and C).

Table 1. IKBKE copy number in ZR-751- and IKBKE-expressing TNBC cell lines from the Broad/Novartis Cell Line Encyclopedia

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IKBKE CN (log_2 [CN/2])</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR751</td>
<td>1.23</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0.12</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>0.36</td>
</tr>
<tr>
<td>HCC70</td>
<td>0.47</td>
</tr>
<tr>
<td>HCC1143</td>
<td>0.49</td>
</tr>
<tr>
<td>HCC1167</td>
<td>0.53</td>
</tr>
</tbody>
</table>

CN, copy number.
IL-1β and found that CYT387 treatment inhibited proliferation and caused cells to aggregate (Supplemental Figure 4B). These findings demonstrate that CYT387 treatment uniquely impairs not only cell viability in 2D culture but also growth factor- and cytokine-driven TNBC cell proliferation and dispersal in 3D culture.

Next, we treated a panel of 15 breast cancer cell lines with CYT387 over a range of concentrations and found that TNBCs that exhibited high levels of IKBKE and pSTAT3 exhibited the greatest sensitivity.

We further examined the effects of CYT387 treatment on MDA-MB-468 cells in a 3D culture tumor spheroid dispersal assay that captures features of the tumor microenvironment and also models aspects of the epithelial-mesenchymal transition (34). EGF-induced proliferation of MDA-MB-468 breast cancer cells in this assay was completely suppressed by CYT387 treatment at concentrations as low as 800 nM (Supplemental Figure 4A). We also cultured several other TNBC cell lines in 3D suspension together with IL-1β and found that CYT387 treatment inhibited proliferation and caused cells to aggregate (Supplemental Figure 4B). These findings demonstrate that CYT387 treatment uniquely impairs not only cell viability in 2D culture but also growth factor- and cytokine-driven TNBC cell proliferation and dispersal in 3D culture.
MDA-MB-468 cells with CYT387 or ruxolitinib, we were unable to observe significant changes in this marker, which suggests a lack of a direct relationship between p38α and IKBKE or JAK activity (Supplemental Figure 5, C and D).

We next examined the effects of CYT387 or ruxolitinib treatment on IKBKE-induced NF-κB signaling. IKBKE-driven TNBC cell lines were more sensitive to CYT387 treatment than isogenic cells expressing a control vector, whereas ruxolitinib exposure had no effect on these cells (Figure 2B). Taken together, these observations reveal that CYT387, unlike ruxolitinib, selectively impairs TNBC cell viability in a manner that correlates with IKBKE expression.

Figure 2. IKBKE promotes inflammatory signaling and is induced by IL-1β in TNBC cells. (A) Immunoblot of IKBKE, S933 p-p105, total p105, Y705 pSTAT3, total STAT3, and β-actin in 293T cells transiently transfected with IKBKE or a control EGFP-expressing vector. (B) CCL5 mRNA expression in 293T cells following transient transfection with EGFP, IKBKE-WT, and IKBKE-K38A. Values were normalized to EGFP and represent the mean and SEM of triplicate samples. (C) Immunoblot of IKBKE, Y705 pSTAT3, total STAT3, and β-actin in a panel of 15 breast cancer cell lines. (D) Immunoblot of IKBKE and β-actin in a panel of TNBC cell lines with or without exogenous IL-1β (25 ng/ml) for 24 hours. (E) CCL5 levels in the media measured by ELISA following IL-1β (25 ng/ml) treatment of IKBKE-expressing TNBC cell lines for 24 hours. Values represent mean and SD of duplicate samples.

suggestive of a relationship between IKBKE activation and CYT387 treatment (Figure 2A and Figure 3D). Corroborating these findings, immortalized human mammary epithelial cells that expressed IKBKE (16) were more sensitive to CYT387 treatment than isogenic cells expressing a control vector, whereas ruxolitinib exposure had no effect on these cells (Figure 3E). Taken together, these observations reveal that CYT387, unlike ruxolitinib, selectively impairs TNBC cell viability in a manner that correlates with IKBKE expression.

Activity of CYT387 in TNBC directly involves inhibition of IKBKE signaling. To assess the direct consequences of CYT387 treatment on IKBKE activity, beyond CYT387’s TBK1-specific effects (30), we transiently transfected 293T cells with IKBKE and measured downstream signaling pathways in the absence or presence of this inhibitor. Compared with expression of an EGFP control vector, exogenous overexpression of IKBKE primarily activated multiple STAT family members as well as p38α, and these effects were inhibited by CYT387 treatment (Supplemental Figure 5A). Both CYT387 and ruxolitinib inhibited IKBKE-induced Y705 pSTAT3 levels, consistent with suppression of autocrine cytokine signaling through JAK kinases (Figure 4A). IKBKE-induced pSTAT3 was also inhibited by CYT387 and ruxolitinib treatment (Supplemental Figure 5B). In contrast, when we measured phosphorylated p38α levels following treatment of IKBKE-expressing 293T cells or MDA-MB-468 cells with CYT387 or ruxolitinib, we were unable to observe significant changes in this marker, which suggests a lack of a direct relationship between p38α and IKBKE or JAK activity (Supplemental Figure 5, C and D).

We next examined the effects of CYT387 or ruxolitinib treatment on IKBKE-induced NF-κB signaling. IKBKE-induced p105 phosphorylation in 293T cells was inhibited by CYT387 treatment but not ruxolitinib treatment (Figure 4B). We confirmed that p105 was phosphorylated at a baseline low level in both MDA-MB-468 cells and MDA-MB231 cells and that CYT387 treatment also selectively inhibited phosphorylated p105 (p-p105) levels in these TNBC cell lines compared with ruxolitinib treatment (Figure 4C). IL-1β stimulation further induced S933 p105 phosphorylation in MDA-MB-468 cells, which was also selectively inhibited by CYT387 treatment, in contrast to ruxolitinib treatment, and resulted in p105 stabilization (Figure 4D). CYT387 treatment also suppressed IKBKE expression in MDA-MB-468 cells, in contrast to that of IKKβ or IKKα (Figure 4E). We further confirmed that CYT387 treatment inhibited p-p105, pSTAT3, and IKBKE levels in multiple other IKBKE-driven TNBC cell lines (Supplemental Figure 5, E and F). Thus, in contrast to ruxolitinib treatment, CYT387 treatment inhibits multiple components of the inflammatory signaling network that sustain proliferation and survival of this TNBC subtype.
CYT387 treatment disrupts IKBKE-induced protumorigenic cytokine expression. Given the unique ability of CYT387 to target this signaling network, we next tested its impact on autocrine cytokine expression. We collected media from 293T cells 24 hours following transient transfection with EGFP or IKBKE and analyzed levels of 36 different cytokines and chemokines using a cytokine antibody array. Enforced expression of IKBKE potently induced CCL5 levels in the media, consistent with what was observed at the mRNA level (Figure 2B), and was the dominant secreted factor at this time point (Figure 5A). IKBKE-dependent CCL5 production was completely abrogated by CYT387 treatment but was negligibly affected by ruxolitinib treatment (Figure 5A).

To confirm these observations, we used ELISA to measure CCL5 levels in addition to those of IL-6 and found that IKBKE-induced CCL5 and IL-6 were strongly inhibited by CYT387 treatment, whereas they were only partially suppressed by ruxolitinib treat-

To examine more directly the role of IKBKE inhibition by CYT387 in TNBC proliferation and survival, we used a CYT387-resistant allele, IKBKE-Y88C, identified by homology to JAK2 (30, 35). We stably expressed the IKBKE-Y88C allele in MDA-MB-468 cells and selected the cells in the presence of 2.5 μM CYT387 for 3 weeks. We confirmed that the cells that emerged markedly overexpressed IKBKE-Y88C compared with control EGFP-expressing MDA-MB-468 cells (Figure 4F). CYT387 treatment of MDA-MB-468-IKBKE-Y88C cells failed to suppress IKBKE expression or baseline levels of p-p105, consistent with downstream resistance to this activity (Figure 4F). Treatment of MDA-MB-468-IKBKE-Y88C cells with CYT387 resulted in enhanced proliferation and survival in vitro compared with control cells that expressed EGFP (Figure 4G). These observations confirm that inhibition of IKBKE by CYT387 directly contributes to its antiproliferative activity in IKBKE-driven TNBC cells.
Figure 4. Inhibition of JAK and IKBKE signaling by CYT387. (A) Immunoblot of IKBKE, Y705 pSTAT3, total STAT3, and β-actin following transient transfection of 293T cells with IKBKE and treatment with 5 μM ruxolitinib or CYT387. Lysates were obtained 24 hours after transfection and 12-hour inhibitor treatment. (B) Immunoblot of S933 p-p105, p105, and β-actin following IKBKE overexpression in 293T cells and 5 μM ruxolitinib or CYT387 treatment. (C) Immunoblot of S933 p-p105, p105, and β-actin in MDA-MB-468 or MDA-MB-231 cells with endogenous IKBKE overexpression following 1-hour treatment with 5 μM ruxolitinib or CYT387. (D) Immunoblot of S933 p-p105, p105, p50, and β-actin in MDA-MB-468 cells pretreated with DMSO, 5 μM ruxolitinib, or 5 μM CYT387 for 1 hour and stimulated with IL-1β for the indicated times. (E) Immunoblot of IKBKE, IKKa, and IKKβ in MDA-MB-468 cells 24 hours following treatment with DMSO, ruxolitinib, or CYT387 at the indicated concentrations, each compared with β-actin as a loading control. (F) MDA-MB-468 cells were stably infected with IKBKE-Y88C and selected in 2.5 μM CYT387 for 3 weeks. Immunoblot shows IKBKE, S933 p-p105, p105, and β-actin in these cells compared with control EGFP-expressing MDA-MB-468 cells following treatment with DMSO or CYT387. (G) Cell viability measured by CTG on day 3 or day 10 following treatment of MDA-MB-468-EGFP or IKBKE-Y88C cells with DMSO or CYT387. Values were normalized to DMSO as a control and represent mean ± SEM of triplicate samples. Crystal violet-stained wells are shown below.
ment (Figure 5B). These findings reveal that CYT387 treatment not only inhibits both STAT3- and IKBKE-induced p105 phosphorylation but also uniquely ablates the production of CCL5 and IL-6 following IKBKE overexpression.

We next assessed whether CYT387 inhibition of this network also impaired the production of CCL5 and IL-6 in TNBC cell lines. Treatment of MDA-MB468, MDA-MB231, HCC1187, or HCC70 cells with CYT387 in general prevented IL-1β-induced CCL5 and IL-6 (Figure 5C and Supplemental Figure 6A). To examine the consequences downstream of CCL5 and IL-6 production on TNBC proliferation, we first tested whether the addition of exogenous CCL5 and/or IL-6 rescued the viability of CYT387-treated MDA-MB-468 cells in 2D culture. We observed a modest but significant rescue following treatment with either cytokine or the combination of both (P < 0.001) (Supplemental Figure 6B). In contrast, in 3D culture, CCL5 and IL-6 not only promoted MDA-MB-468 cell migration and proliferation as effectively as EGF but they also completely rescued the inhibition of spheroid dispersal by CYT387 (Figure 5D). Taken together, these observations demonstrate that IKBKE-driven CCL5 and IL-6 directly contribute to TNBC migration and proliferation of tumor spheroids, which is disrupted by CYT387 treatment.

TBK1/IKBKE-regulated cytokines also influence the tumor microenvironment and angiogenesis in particular (36). We therefore used another 3D device optimized to study the effects of IKBKE-induced CCL5/IL-6 on HUVEC behavior in collagen (Figure 5E and ref. 37). First, we overexpressed IKBKE-WT in 293T cells, seeded them in the opposing channel, and found that expression of IKBKE-WT induced HUVEC migration, in contrast to EGFP and IKBKE-KD controls (Supplemental Figure 6C). Next, we directly supplemented media with CCL5 and IL-6 and observed that these cytokines induced both endothelial cell migration and proliferation (Figure 5F and Supplemental Figure 6D). Because of the proliferation, we tested whether cotreatment of CCL5/IL-6 with the MEK inhibitor GSK1120212 prevented this phenotype, and indeed HUVEC migration was abrogated (Figure 5F). Taken together, IKBKE-regulated CCL5 and IL-6 induce the proliferation and migration of TNBC and endothelial cells, consistent with both autocrine and paracrine tumor-promoting activities.

Inhibition of IKBKE by CYT387 contributes to its therapeutic potential in vivo. To determine efficacy of CYT387-based treatment in vivo, we first tested its therapeutic impact on MDA-MB-468 tumor xenograft growth and the relationship with IKBKE inhibition. After tumors were established in immuno-deficient mice at an average volume of 50 mm³, CYT387 was administered via daily oral gavage at a dose of 100 mg/kg (33). Compared with a vehicle control, CYT387 treatment at this dose effectively inhibited pSTAT3 expression in tumors (Figure 6A) and strongly suppressed tumor progression (Figure 6B). In consonance with our observations in vitro, CYT387 treatment did not affect the growth of MDA-MB-468 IKBKE-Y88C xenografts (Figure 6B).

We next explored single-agent CYT387 activity in a system that more closely recapitulates human tumor physiology using patient-derived breast cancer xenografts (PDXs). First, we examined therapy in two different Washington University human-in-mouse (WHIM) lines (WHIM4 and WHIM21) that were derived from patients with TNBC that overexpressed IKBKE (ref. 38 and Supplemental Figure 7A). Similar to what we observed following treatment of MDA-MB-468 xenografts, CYT387 treatment impaired the growth of established PDX WHIM4 tumors and WHIM21 tumors, the latter a particularly aggressive model that recurred rapidly following neoadjuvant doxorubicin/cyclophosphamide and paclitaxel chemotherapy (ref. 38 and Figure 6C). Inhibition of WHIM21 PDX growth was associated with disruption of human IKBKE, CCL5, and IL6 expression, confirming effective interruption of autocrine cytokine signaling in these tumors (Figure 6D). Taken together, these findings reveal that inhibition of TBK1/IKBKE and JAK signaling by CYT387 suppresses protumorigenic cytokine expression and exhibits therapeutic potential for IKBKE-driven TNBC.

Synergistic response to combined CYT387 and GSK1120212 therapy. MEK inhibition in TNBC not only results in feedback activation of receptor tyrosine kinases but also induces cytokine expression, suggesting the possibility of synergy with CYT387 treatment (39). In addition the requirement of MEK signaling for CCL5/IL-6–induced proliferation/migration of endothelial cells (Figure 5F) indicated the potential for dual impairment of angiogenesis. We therefore treated established WHIM21 tumors with CYT387 (50 mg/kg/d), GSK1120212 (2.5 mg/kg/d), or combination CYT387/GSK1120212 therapy by oral gavage. The drug combination was well tolerated, and, in contrast to either of the single agents, markedly impaired tumor progression (Figure 7A). Indeed, several of the largest established tumors also showed evidence of tumor regression (Supplemental Figure 7B). We confirmed that dual CYT387 and GSK1120212 treatment effectively inhibited both phosphorylated ERK (pERK) and pSTAT3 levels in treated WHIM21 tumors, confirming suppression of multiple pathways by this drug combination in vivo (Figure 7B).

To assess the dose-dependent effect of this impressive activity, we further reduced CYT387 to 10 mg/kg daily and compared results with vehicle or high-dose ruxolitinib treatment (Supplemental Figure 6A). Treatment of WHIM21 tumors with just a 2-week course of low-dose CYT387/GSK1120212 led to marked and persistent inhibition of tumor progression at 4 weeks, in contrast to continuous vehicle or ruxolitinib treatment at 100 mg/kg daily over the entire time period (Supplemental Figure 8A). Response to this low-dose CYT387 regimen was also examined in WHIM12 PDX tumors, derived from a patient with TNBC with low IKBKE levels (Supplemental Figure 6A). WHIM12 tumors responded to CYT387/GSK1120212 treatment though not as dramatically as WHIM21 tumors, with some tumors progressing despite therapy (Supplemental Figure 8B).

In addition to their small size, we also noted that WHIM21 tumors treated with the combination of CYT387 and GSK-1120212 appeared particularly pale compared with vehicle- or single-agent–treated tumors (Figure 7C and Supplemental Figure 7C). We therefore performed a detailed histologic examination of treated tumors, including measures of angiogenesis. Whereas single-agent treatment with GSK1120212 showed preferential impairment of proliferation, as measured by Ki67 staining, and CYT387 modestly reduced microvascular density, the combination resulted in a striking inhibition of angiogene-
Figure 5. Inhibition of this network by CYT387 suppresses protumorigenic cytokines. (A) Cytokine antibody array incubated with media from 293T cells transfected with EGFP or IKBKE for 24 hours and pretreated with DMSO, 5 μM CYT387, or 5 μM ruxolitinib for 12 hours. Circles represent the location of CCL5, the predominant cytokine induced by IKBKE and inhibited by CYT387, compared with ruxolitinib. (B) ELISA measurement of CCL5 or IL-6 levels in 293T cells expressing IKBKE and treated with DMSO, 5 μM ruxolitinib, or 5 μM CYT387. Mean and SD of duplicate samples shown. (C) ELISA measuring CCL5 or IL-6 levels in MDA-MB-468 cells or MDA-MB-231 cells stimulated with IL-1β for 24 hours following pretreatment with DMSO or 5 μM CYT387 for 1 hour. Mean and SD of duplicate samples shown. (D) Phase-contrast images (original magnification, ×20) of MDA-MB-468 spheroids in microfluidic 3D culture at baseline and 24 and 48 hours following addition of CCL5/IL-6, EGF, or the combination of both, together with DMSO as a control (left). Treatment with 1 μM CYT387 inhibited EGF-induced MDA-MB-468 spheroid dispersal, but this was rescued by the addition of CCL5 and IL-6 (right). (E) Schematic of angiogenesis microfluidic device. HUVECs were seeded in central channel and subjected to cytokine/chemokine diffusion as indicated. (F) Compared with control media, diffusion of CCL5/IL-6–attracted HUVECs into collagen (original magnification, ×20) over the course of 24 hours. Cotreatment with the MEK inhibitor (MEKi), GSK1120212, at 10 nM strongly inhibited this effect. Mean and SD of cell migration per number from 3 independent devices shown.
naling and lymphocytic infiltration. Despite engagement of the JAK/STAT pathway (24), treatment with the potent and selective JAK1/2 inhibitor ruxolitinib was insufficient to impair viability of these TNBCs. Instead, another clinical stage JAK inhibitor, CYT387, impaired the proliferation of TNBC cells in vitro and prevented tumor spheroid dispersal in 3D culture. The efficacy of CYT387 was directly related to its additional ability to inhibit IKBKE activity and the production of protumorigenic cytokines, since exogenous CCL5 and/or IL-6, or expression of a CYT387 inhibitor–resistant allele of IKBKE, rescued these effects. These observations suggest a promising therapeutic option for a subset of patients with IKBKE-driven TNBC.

Integrative genomic studies identified a key role for aberrant IKBKE activation in breast cancer by virtue of its amplification in a subset of luminal tumors (16). IKBKE is unique among IKK family members in that cytokines such as IL-1 that promote NF-κB signaling (25) and STAT3 activation (41), which induces its expression. The finding that high level IKBKE expression in the IM subtype of TNBC was linked more closely to engagement of inflammatory signaling and profound tumor necrosis (Figure 7D and Supplemental Figure 9). Thus, antitumor activity of this drug combination was not only direct but was also related to the synergistic effects of cytokine and MEK inhibition on angiogenesis. Taken together, combined CYT387 and GSK1120212 treatment impairs tumor progression and angiogenesis and represents a promising novel therapy for this IKBKE-driven subtype of TNBC.

Discussion

TNBC has been defined by the lack of ER and HER2 expression, but several lines of evidence suggest that TNBCs are a heterogeneous set of breast cancers (40). Here, we identify a specific TNBC subset characterized by aberrant expression of the IKK-related kinase IKBKE and production of protumorigenic cytokines CCL5 and IL-6. These tumors show substantial overlap with the IM subtype of TNBC, recently identified by gene expression profiling studies (6). In contrast to luminal tumors, which exhibit IKBKE amplification (16), these triple-negative tumors exhibit inducible IKBKE expression associated with markers of IL-1 sig-
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Tropic effects on NF-κB target gene expression (43) and AKT activation (44, 45) and, like IL-6, directly engages JAK/STAT signaling (46). Thus, NF-κB and STAT3 not only induce the production of CCL5 and IL-6, but they also engage these same pathways and activate IKBKE expression itself (41) to amplify and sustain their expression as components of an inflammatory circuit (30). Induction of CCL5, which promotes cell survival and metastasis, has also been observed in breast cancer following coculture with mesenchymal stem cells (44). These findings suggest that paracrine effects due to interactions within tumor microenvironment likely facilitate engagement of this signaling pathway. Since we observed an important role of IL-1 signaling in driving this phenotype, it will be interesting to examine whether signaling than to genomic amplification reveals an alternative route to oncogenic IKBKE activation in TNBC, similar to what was recently described in a subset of lung cancers (41). While IKBKE drives the expression of these cytokines, engagement of other kinases, including TBK1, likely also contributes to inflammatory signaling in this subtype, since multitargeted IKBKE, TBK1, and JAK signaling was required to disrupt this circuit. Since other non-TNBC breast cancers also overexpress IKBKE and also activate TBK1 signaling (42), such tumors could also respond to TBK1/IKBKE and JAK inhibition by CYT387.

Our studies also identified key downstream roles for CCL5 and IL-6 as IKBKE-driven mediators of cell proliferation, survival, and migration of breast cancer cells. CCL5 induces pleiotropic effects on NF-κB target gene expression (43) and AKT activation (44, 45) and, like IL-6, directly engages JAK/STAT signaling (46). Thus, NF-κB and STAT3 not only induce the production of CCL5 and IL-6, but they also engage these same pathways and activate IKBKE expression itself (41) to amplify and sustain their expression as components of an inflammatory circuit (30). Induction of CCL5, which promotes cell survival and metastasis, has also been observed in breast cancer following coculture with mesenchymal stem cells (44). These findings suggest that paracrine effects due to interactions within tumor microenvironment likely facilitate engagement of this signaling pathway. Since we observed an important role of IL-1 signaling in driving this phenotype, it will be interesting to examine whether
the source of this cytokine in primary breast tumors is derived from mesenchymal stem cells, tumor-associated macrophages, and/or other cell types in the tumor microenvironment.

IKBKE-induced CCL5 and IL-6 expression also stimulated HUVEC proliferation, consistent with a previous report showing that conditioned media from TBK1-transfected cells promotes vascular cell proliferation (36). Our findings confirm and extend these data, revealing a particular role for MEK signaling downstream of these cytokines in mediating endothelial cell proliferation and identifying synergistic inhibition of angiogenesis by CYT387 and MEK inhibition in vivo. Cytokines such as CCL5 may also promote TNBC growth by influencing the local immune microenvironment, since it also influences recruitment of myeloid-derived suppressor cells to tumors and promotes local immunosuppression (47). Analysis of such cells is challenging in PDX models, given the altered immune background of nude mice, but will be important to evaluate in future studies. Thus, CYT387 therapy may be particularly effective in vivo due to the additional disruption of these tumor-stromal interactions.

Clinical trials of selective JAK1/2 inhibitors such as ruxolitinib have been initiated in patients with breast cancer (48). While JAK/STAT signaling is clearly active in this subset of TNBC, our data suggest that JAK inhibition alone may not be sufficient to disrupt this cytokine circuit. Furthermore, although certain markers, such as CD44+CD24– positivity or the IM gene, provide a strong rationale for pursuing clinical trials of this drug combination in patients with TNBC.

Methods

Gene expression profiling. Analyses were performed using TCGA data (2) and applied single-sample gene set enrichment analysis of an IL-1 signature as described previously (26, 52). For details, see the Supplemental Methods.

Cell culture. Breast cancer cell lines and 293T cells were cultured using standard conditions. MDA-MB-468 cells were maintained in the absence of CO2. MDA-MB-468 tumor spheroids were generated and assayed in 3D culture as described previously (34). Detailed methods are described in the Supplemental Methods.

Immunoblotting and ELISA. Immunoblotting was performed according to standard protocols. Proteome Profiler and Cytokine Antibody Arrays were from R&D Systems. The Proteome Profiler Human Cytokine Array Kit, Panel A (catalog no. ARY005), the Human CCL5/Rantes Quantikine ELISA Kit (catalog no. DRN00B), and the Human IL-6 ELISA Kit (catalog no. D6050) were also purchased from R&D Systems. Details are provided in the Supplemental Methods.

ORF and shRNA expression. 293T cells were transiently transfected with the indicated ORF expression constructs using FuGENE 6 (Promega). Using stable lentiviral transduction as previously described (52), shRNA (shIKBKE-1, shIKBKE-2, shGFP) was successfully expressed and its effects on the various breast cancer cell lines were analyzed using stable lentiviral transduction as described previously (52). For detailed methods and shRNA sequences see the Supplemental Methods.

Quantitative real-time PCR. mRNA was purified and qRT-PCR was performed according to a standard protocol using the LightCycler 480 SYBR Green I Master (Roche). Data were normalized to 36B4. For detailed methods and primer sequences see the Supplemental Methods.

Animal studies. Patient-derived human breast xenografts were cultured as described previously (38, 53). pSTAT3 immunohistochemistry and pSTAT3/pERK immunoblotting were performed following short-term treatment with CYT387. Tumor measurement was conducted in a blinded fashion over time. Details are provided in the Supplemental Methods.

Statistics. Statistical analysis was carried out using an IBM software package, SPSS V.22.0. Cell viability data are presented as mean ± SEM. Histology data are presented as mean ± SD of independent results. Overall differences among the 4 groups (vehicle, GSK1120212, CYT387, and CYT387 plus GSK1120120) for all variables were determined by ANOVA. Differences between groups were examined using the nonparametric independent-samples t test to determine the statistical significance. Two-sided P values of less than 0.05 were considered statistically significant.

Study approval. Human breast cancer tissues for the present studies were obtained via core needle, skin punch biopsy, or surgical resection following informed consent and processed in compliance with NIH regulations and with approval from the Institutional Review Board at Washington University in St. Louis. All mouse experiments were conducted in accord with a Washington University Institutional Animal Care and Use Committee-approved protocol.

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