Notch Shapes the Innate Immunophenotype in Breast Cancer

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ABSTRACT

Notch activation, which is associated with basal-like breast cancer (BLBC), normally directs tissue patterning, suggesting that it may shape the tumor microenvironment. Here, we show that Notch in tumor cells regulates the expression of two powerful proinflammatory cytokines, IL1β and CCL2, and the recruitment of tumor-associated macrophages (TAM). Notch also regulates TGFβ-mediated activation of tumor cells by TAMs, closing a Notch-dependent paracrine signaling loop between these two cell types. We use a mouse model in which Notch can be regulated in spontaneous mammary carcinoma to confirm that IL1β and CCL2 production, and macrophage recruitment are Notch-dependent. In human disease, expression array analyses demonstrate a striking association between Notch activation, IL1β and CCL2 production, macrophage infiltration, and BLBC. These findings place Notch at the nexus of a vicious cycle of macrophage infiltration and amplified cytokine secretion and provide immunotherapeutic opportunities in BLBC.

SIGNIFICANCE: BLBC is aggressive and has an unmet need for effective targeted treatment. Our data highlight immunotherapeutic opportunities in Notch-activated BLBC. Effective IL1β and CCL2 antagonists are currently in clinical review to treat benign inflammatory disease, and their transition to the cancer clinic could have a rapid impact. Cancer Discov; 7(11): 1320–35. ©2017 AACR.

INTRODUCTION

Breast cancer is the most common cancer in women worldwide and remains a leading cause of cancer death (1). It is a heterogeneous disease with multiple subtypes that display different patterns of gene expression, prognosis, and response to treatment (2). Clinically, breast cancer can be subtyped into those expressing estrogen receptor (ER) and/or progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2)–amplified breast cancer, and a third group, triple-negative breast cancer (TNBC; lacking ER, PR, or HER2), which overlaps significantly with the basal-like breast cancer (BLBC) molecular subtype. Although molecular targeted therapies have substantially improved survival in ER/PR-positive and HER2-amplified breast cancer, BLBC is without effective targeted treatment. This presents a significant clinical problem: BLBC is aggressive, with poor prognosis, and comprises 15% of all breast cancers, primarily affecting young women, people of African or Hispanic ancestry, and those with BRCA1 mutations.

The Notch signaling pathway plays a crucial role in intercellular signaling and tissue patterning during development (3). Mammals have four Notch receptors (NOTCH1–4) that interact with five Notch ligands (DLL1, DLL3, DLL4, JAG1, and JAG2). Notch ligand–receptor interaction on neighboring cells leads to a series of proteolytic events, including a presenilin–protease (γ-secretase)-mediated cleavage that liberates the active cytoplasmic domain fragment, intracellular NOTCH (Nγ), from the plasma membrane. Nγ translocates to the nucleus where it engages the DNA-binding protein CBF1/RBPJκ resulting in replacement of a multiprotein corepressor complex with a coactivator complex, initiating transcription of target genes (4). Notch activation is a hallmark of the TNBC/BLBC subtype (5–7) and contributes to the pathogenesis of human breast cancer by affecting multiple cellular processes, including cancer stem cell maintenance, cell fate specification, differentiation, proliferation, motility, and survival (8). Whether Notch is involved in tumor morphogenesis and patterning of the tumor microenvironment (TME) is relatively unexplored.

In addition to cancer cells, the TME is composed of diverse nonmalignant lineages, including cancer-associated fibroblasts, endothelial cells, and cells of both the innate and adaptive immune systems. Tumor-associated macrophages (TAM), which are part of the adaptive immune response, constitute a major portion of the leukocyte infiltrate found in malignant tumors. Clinically, elevated TAM counts are linked to poor outcome in cancer and promote numerous tumor-promoting activities such as angiogenesis, enhanced tumor cell migration and invasiveness, and suppression of adaptive antitumor immunity (9, 10). In breast cancer, TAM infiltration is associated with the BLBC subtype (11), and numerous clinical studies (12, 13) and animal models (14) indicate a strong correlation between elevated macrophage infiltration and poor prognosis.

Infiltration of the tumor with nonmalignant cells is accompanied by expression of numerous inflammatory cytokines, including TGFβ, IL1β, and CCL2. TGFβ has a dual role in tumor progression, displaying antiproliferative properties...
early in tumorigenesis with later subversion to tumor-promoting activity as disease progresses (15). Stromal expression of TGFβ can promote the development of malignant lesions from ostensibly normal mammary epithelium, underscoring a key role for this cytokine in mediating the stromal–epithelial interface (16). IL1β is a pleiotropic cytokine implicated in tumor progression through effects on proliferation, invasion, metastases, myeloid cell recruitment, and angiogenesis (17, 18). In breast cancer, IL1β expression is associated with receptor-negative disease, macrophage infiltration, and poor outcome (19–21). Animal models suggest that CCL2 is critical to macrophage recruitment to the TME (22) and enhanced metastases (23). Indeed, elevated CCL2 in breast cancer is associated with enhanced macrophage infiltration and decreased survival (24).

We inferred from the role that Notch normally plays in cell–cell communication during tissue patterning and development that it may regulate interactions between cells in the TME. In this study, we demonstrate that, in BLBC, TAMs induce Notch-dependent TGFβ, IL1β, and CCL2 paracrine signaling, resulting in a progressive cycle of TAM recruitment, amplified cytokine expression, and tumor progression.

RESULTS

Notch Regulates Cytokine Expression in BLBC Cell Lines

To identify Notch target genes that may be involved in shaping tumor–stromal interactions, expression arrays were used to identify genes whose expression was dependent upon the Notch ligand JAG1 or the receptors NOTCH1 and NOTCH3 (25). The focus on these Notch molecules was based on the finding that their expression correlated with poor outcome in breast cancer (6). Among the Notch target genes identified were those encoding cytokines and chemokines (Supplementary Table S1), including IL1β and CCL2 (Fig. 1A). An analysis of expression data from breast cancer cell lines (26) suggested an association between the BLBC subtype, Notch pathway activation, and IL1β and CCL2 expression (Fig. 1B). An independent study of four breast cancer cell lines confirmed a correlation between elevated expression of JAG1, IL1β, and CCL2 and the BLBC subtype (Fig. 1C). Additionally, siRNA-mediated knockdown of JAG1 or NOTCH1 and NOTCH3 diminished IL1β and CCL2 expression (Fig. 1D and E) and cytokine secretion (Fig. 1F). These findings suggest that Notch regulates IL1β and CCL2 expression in the BLBC subtype.

IL1β Expression Depends upon Canonical Notch Signaling

Typically, Notch-dependent processes require the release of N1C from the plasma membrane and translocation to the nucleus. There, N1C participates in transcriptional regulation at CBF1/RBPjk DNA-binding sites (CBS). To explore the role of so-called “canonical” Notch, IL1β expression was studied further. Canonical Notch signaling was demonstrated in a rescue of IL1β expression by N1C, after JAG1 knockdown. MDA MB231 cells containing a stable doxycycline-inducible shRNAi targeting JAG1 were transduced with adenovirus expressing activated, cleaved Notch receptor (N1C or N3C). N1C and N3C induced mRNA upregulation of both IL1β and the classic, canonical Notch target HES1 (Fig. 2A). JAG1 inhibition through doxycycline treatment reduced IL1β mRNA to undetectable levels, and this was rescued by ectopic expression of N1C or N3C. Appropriately, N1C and N3C induced upregulation of pro-IL1β and of JAG1 protein, itself a target of activated Notch (Fig. 2B; compare lanes 1 and 2 with lanes 3 and 4). JAG1 inhibition reduced Notch activation (N1C and N3C) and pro-IL1β to undetectable levels (Fig. 2B; lanes 5 and 6), and this was rescued by ectopic N1C or N3C expression (Fig. 2D; lanes 7 and 8).

Further implicating canonical Notch, multiple putative high-affinity CBS (27) were identified within the promoter/enhancer regions of both the IL1β and CCL2 genes (Fig. 2C; Supplementary Fig. S1). Complementary approaches were taken to test whether the IL1β gene is a direct Notch transcriptional target. Chromatin immunoprecipitation (ChIP) assays were undertaken with lysates from MDA MB231 cells using antibodies against N1C (Fig. 2D). The immunoprecipitated chromatin fragments were analyzed by PCR using primer sets targeting the high-affinity sites A, D, and F. Enrichment for N1C–chromatin complexes was observed for all three sites. An electrophoretic mobility shift assay (EMSA) was used to test two of these sites (D and F) for direct CBF1 binding and both were found to be capable of forming DNA–protein complexes within MDA MB231 lysates (Fig. 2E). These complexes could be competitively inhibited with excess molar amounts of unlabeled wild-type probe. The specificity of site D was found to be competitively inhibited with excess molar amounts of unlabeled wild-type, but not mutant, probe D (Fig. 2F). The identity of CBF1 in the probe D-protein complex was confirmed by demonstrating the inability of the complex to form in nuclear extracts from cells that had undergone siRNA-mediated CBF1 knockdown. These data show that JAG1, N1C, and N3C are required for IL1β production and identify at least one CBS (at ~2,085 from the translation start site) in the IL1β gene. Whether the canonical pathway drives the expression of CCL2 or other Notch-dependent cytokines remains to be explored.

IL1β Production in Breast Cancer Cells Requires the Inflammasome

Classically, IL1β is produced by activated macrophages in a two-step process. The first step involves the induction of mRNA and protein production of an inactive IL1β proprotein (pro-IL1β). In the second step, pro-IL1β is activated by caspase-1, which is contained together with the Nod-like (NALP) and apoptosis-associated speck-like (ASC) proteins in an activated multiprotein complex called the inflammasome (28). Compared with cells of the mononuclear phagocyte lineage where lipopolysaccharide (LPS) serves as a traditional priming signal for IL1β mRNA expression, LPS stimulation had a marginal effect on IL1β mRNA expression in MDA MB231 cells (130-fold vs. 3-fold increase, respectively; Supplementary Fig. S2A). Notably, baseline IL1β expression was approximately 20-fold higher in MDA MB231 cells compared with THP-1 cells, likely owing to activated Notch signaling within the breast cancer line (29). Like cells of the myeloid lineage, breast cancer cells contain inflammasome components, whose expression is Notch-independent. Inflammasome activation,
Figure 1. Notch drives IL1β and CCL2 expression in the BLBC subtype. A, Box plots of normalized IL1β and CCL2 mRNA after treatment of HCC1143 cells with siRNAs targeting JAG1 (siJ1), NOTCH1 and NOTCH3 (siN1/3), or scrambled control (siScr; n = 3/group). **, P < 0.01. B, Supervised clustering of Notch-activated signature genes (yellow box) associated with the BLBC (basal) subtype in 51 breast cancer cell lines (26) and magnified data from JAG1, IL1β, and CCL2. Color bar, fold change relative to the median value. Spearman correlations (P) of JAG1, UPA, IL1β, and CCL2 to the Notch signature (nearest mean centroid) are shown. C, Relative mRNA expression of JAG1, IL1β, and CCL2 in the indicated luminal (black) and basal-like (red) breast cancer cell lines (n = 3/group). *, P < 0.05; **, P < 0.01. D–F, IL1β (MDA MB231) and CCL2 (HCC1143) mRNA (D), immunoblots (E), and ELISA assays from conditioned media (F) after treatment of cells with siScr, siJ1, siCCL2, siN1/3, or siJ1 (n = 5/group). *, P < 0.05; **, P < 0.01 are significant compared with siScr. MW markers are shown in kilodaltons. β-actin is included as a loading control.
Figure 2. Canonical Notch regulates IL1β expression. A, RNA qRT-PCR of the canonical Notch target HES1, or IL1β in uninfected (-) MDA MB231 cells stably carrying a doxycycline-inducible shRNA-targeting JAG1 (MB231/shJ1), or after infection (MOI = 50) with adenovirus (Ad) expressing LacZ, Notch1 intracellular domain (N1IC), or N3IC, in the absence (-Dox) or presence (+Dox) of doxycycline (n = 3/group). mRNA levels are expressed relative to uninfected (no Ad) MB231/shJ1 cells (−Dox) and are normalized according to the β-actin expression level. Significance is indicated by *, P < 0.05; **, P < 0.01.

B, Immunoblot of N1IC, N3IC, JAG1, and pro-IL1β in uninfected (Ad. -) or adenovirus-infected (expressing LacZ, N1IC, or N3IC) MB231/shJ1 cells in −Dox or +Dox conditions. MW markers are shown in kilodaltons. β-actin is included as a loading control.

C, The human IL1β promoter/enhancer indicating putative high-affinity (YGTGRGAA, black triangles) and low-affinity (RTGRGAR, gray triangles) CBS. The 5' locations of CBS (minus strand; bold, light blue) are indicated relative to the start of translation of IL1β. ChIP primers (arrows) and wild-type (WT) and mutant (mut) EMSA probes are shown. D, ChIP using NOTCH1 (anti-N1) or control (IgG) antisera and primers that target sites F, D, A of the IL1β promoter, the CCND1 [14B] and PLAU promoters [25; positive controls], and GAPDH [negative control]. E, EMSA of probes D and F in the absence (-) or presence (+) of nuclear extract (lysate) or unlabeled (cold) WT probe. F, EMSA of Probe D in the presence of WT or mut cold probe, or using a nuclear extract from CBF1 siRNA-treated cells.
Notch Signaling and IL1β/CCL2 Expression in Breast Cancer

**Figure 3.** Protumorigenic macrophages induce tumor cell Notch-dependent cytokines that promote monocyte recruitment. **A,** In vitro adhesion assays using conditioned media (CM) from cells previously transfected with either siScr, siIL1β, siNOTCH1 and siNOTCH3 (siN1/3), or siJAG1 (siJ1; 500 pg/mL IL1β supplementation). Ratios relative to the control (unconditioned media or MDA MB231 treated with siScr) are shown (n = 3/group; *, P < 0.05, compared with the control; **, P < 0.01). **B,** In vitro extravasation assays using CM from cells previously transfected with either siScr, siIL1β, siN1/3, or siJ1 (±1 ng/mL CCL2 supplementation; n = 3/group); **C,** Immunoblots of pro-IL1β or CCL2 from human [MDA MB231/THP-1–derived M2 macrophages (M2)] or murine [4T1-luc/bone marrow-derived M2 macrophages (BM2)] monocultures or cocultures. Mw markers are shown in kilodaltons. β-actin is included as a loading control. **D and E,** Immunoblots and ELISAs of IL1β (MDA MB231) or CCL2 (HCC1143) in cells expressing a doxycycline-inducible JAG1 shRNA (shJ1), with (+) or without (-) doxycycline (Dox), in monoculture or coculture with M2 macrophages. *, P < 0.05; **, P < 0.01 indicate significance.

as indicated by CASP1 p20, is unchanged by NOTCH1/3 or JAG1 knockdown (Supplementary Fig. S2B). However, inflammasome components are required for the secretion of mature IL1β (Supplementary Fig. S2C and S2D). Our study provides the first evidence that in BLBC Notch provides the priming signal for the production of pro-IL1β and that processing to mature IL1β requires the classic inflammasome.

**Notch Promotes Macrophage Recruitment and TAM-Dependent Cytokine Secretion**

To investigate Notch-dependent paracrine interactions between TAMs and cancer cells, the TME was modeled in vitro. TAMs are derived from inflammatory monocytes that are recruited to the TME (22, 30). IL1β participates in recruitment by mediating monocyte adhesion to blood vessel endothelium (31–33) and CCL2 promotes extravasation of these cells into the tumor where they differentiate into tissue macrophages (22, 34). In vitro assays for monocyte adhesion (Fig. 3A) and extravasation (Fig. 3B) confirmed that breast cancer cells produce Notch-dependent cytokines that are capable of recruiting monocytes. Notch knockdown was phenocopied by IL1β or CCL2 knockdown in these assays and could be rescued by the addition of exogenous cytokine. In both human and rodent coculture systems, M2 macrophages...
(Supplementary Fig. S3), the predominant protumorigenic TAM subtype (35), stimulated IL1β and CCL2 expression (Fig. 3C), and this was dependent upon tumor cell JAG1 (Fig. 3D and E). siRNA-induced IL1β knockdown in either cancer or M2 cells revealed both cell types as significant sources of cytokine production induced by coculture (Supplementary Fig. S4; compare lane 4 with lanes 5 and 7, respectively). IL1β expression in coculture after IL1β knockdown in either tumor or M2 cells was higher than IL1β expression in either cell type in monoculture, indicating reciprocal paracrine signaling between these two cell types. TAM differentiation is dependent upon Notch/RBPJκ (30), raising the possibility that tumor cell JAG1 drives cytokine expression in TAMs through Notch. Together, these in vitro data suggest a paracrine positive feedback circuit where TAMs induce tumor cell Notch-dependent secretion of cytokines that are capable of macrophage recruitment.

**Notch Regulates TGFβRI and Sensitizes Cells to TAM-Derived TGFβ**

To investigate the mechanism by which TAMs induce Notch-dependent IL1β and CCL2 production in tumor cells, we confirmed, as previously shown (36), that TGFβ promotes JAG1 expression and Notch receptor activation in breast cancer cells (Fig. 4A). Based on gene expression array analyses (25), we hypothesized that tumor cell Notch may also control TGFβ effects by regulating TGFβRI expression. Indeed, siRNA-mediated knockdown of JAG1 or NOTCH1 and NOTCH3 in human and murine BLBC cells resulted in decreased TGFβRI mRNA (Fig. 4B) and protein (Fig. 4C).
In accordance with this, Notch knockdown reduced TGFβ-mediated SMAD2/3 phosphorylation (Supplementary Fig. S5A). Notch knockdown also abrogated TGFβ-mediated expression of the Notch target gene UPA, confirming the central role of Notch.

Although M2 cells in monoculture produce latent TGFβ, tumor cell–M2 coculture resulted in the production of mature, active TGFβ in conditioned media (Fig. 4D), and this could be inhibited by knockdown of JAG1, NOTCH1, and NOTCH3 or UPA in tumor cells (Fig. 4E). These data fit with the role of Notch as a key regulator of UPA secretion and NOTCH3 or UPA in tumor cells (Fig. 4E). Further support for the importance of TGFβ signaling in TAM–cancer cell cross-talk, M2-induced Notch activation and expression of the Notch targets UPA, pro-IL1β and CCL2 required TGFβ signaling in tumor cells (Fig. 4F) and could be reduced with neutralizing anti-TGFβ antibody (Supplementary Fig. S5C).

Together, these data suggest a second Notch-centric paracrine feedback loop, where M2-derived TGFβ activates Notch in tumor cells, and Notch in turn potentiates TGFβ signaling by promoting UPA-dependent production of mature TGFβ and by priming a response in tumor cells through upregulation of TGFβR1. In this context, whether the primary role of Notch is to directly drive UPA production, to provide the conversion of latent TGFβ to its active form (37) shown schematically (Supplementary Fig. S5B). Further supporting the importance of TGFβ signaling in TAM–cancer cell cross-talk, M2-induced Notch activation and expression of the Notch targets UPA, pro-IL1β and CCL2 were inhibited by knockdown of JAG1, NOTCH1, and NOTCH3 or UPA in tumor cells (Fig. 4E). These data fit with the role of Notch as a key regulator of UPA secretion and NOTCH3 or UPA in tumor cells (Fig. 4E). These data fit with the role of Notch as a key regulator of UPA secretion and NOTCH3 or UPA in tumor cells (Fig. 4E).

Notch Induces Macrophage Recruitment in Mouse Mammary Carcinoma Transplant and Xenograft Models

To validate our findings in vivo, murine 4T1 TNBC cells expressing doxycycline-inducible shRNAs that target murine NOTCH3 and JAG1 (4T1-luc shN/J) were grafted into mammary fat pads of BALB/c mice (Fig. 5A). Compared with untreated animals, the addition of doxycycline to the drinking water resulted in smaller tumors (Fig. 5B and C) with reduced expression of the shRNA targets (Supplementary Fig. S6A) and concomitant IL1β and CCL2 downregulation (Fig. 5D and E). Accordingly, recruitment of endogenous F4/80- and CD11b-positive macrophages was decreased by 60% in doxycycline-treated, Notch-inhibited animals (Fig. 5F). This effect could be rescued with exogenous, recombinant IL1β and CCL2 (Supplementary Fig. S6B). To rule out effects of doxycycline alone, mice with 4T1-luc tumors were treated with or without doxycycline, resulting in no significant difference in tumor weight or macrophage recruitment (Supplementary Fig. S6C). Because JAG1 or NOTCH1/3 potentially regulate other cytokines that may influence the inflammatory infiltrate in the TME (Supplementary Table S1), we surveyed additional immune cells, including neutrophils and regulatory (Tregs), CD8 and CD4 T cells, without significant Notch-dependent findings (Supplementary Fig. S7).

To rule out the possibility that reduced macrophage recruitment was a property of smaller tumor size, and to explore our findings in a second mouse model, a complementarity xenograft approach was undertaken (Fig. 5G). Female nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice were injected orthotopically with MDA MB231 cells that were stably transfected with a doxycycline-inducible shRNA vector targeting human JAG1. After allowing xenograft tumor growth for 8 weeks, doxycycline was administered for a relatively short period (9 days) without affecting tumor size at sacrifice (Fig. 5H). Four days prior to sacrifice, labeled THP-1 monocytes were injected via the tail vein. JAG1 knockdown, Notch inhibition (reduced N1IC), reduced IL1β, CCL2 expression (Fig. 5I and J), and reduced THP-1 recruitment to tumor (Fig. 5K and L) were confirmed in the treated animals.

The RBPJκIND-MMTVMMTV-PyMT Mouse Model Demonstrates Notch-Dependent Cytokine Expression and Macrophage Recruitment

Given the obvious limitation of tissue culture and tumor transplant models to predict complex Notch-driven cell–cell interactions in a tumor, we created a transgenic mouse (RBPJκIND-MMTV) where Notch activity could be regulated in mammary tissues. RBPJκIND-MMTV mice contain endogenous CBF1/RBPJκ flanked by loxP (FL) sites (RBPJκFL/FL), mouse mammary tumor virus (MMTV)-cre, ROSA26-stop/loxPrtTa-IRES-GFP, and pTetO5RBPJκ-HA transgenes (Fig. 6A). Here, mammary gland–specific cre expression through the MMTV promoter silences endogenous RBPJκ and allows Dox-dependent rtTA-mediated Notch responsiveness through expression of the RBPJκ transgene. In this unique model, Notch signaling is allowed by regulating the availability of RBPJκ. Cre activity and rtTA expression are followed by GFP in mammary epithelium, and Dox-induced RBPJκ expression is tracked with anti-HA or anti-RBPJκ immunohistochemistry. We noted that RBPJκ and HA were only expressed in mammary ducts in the presence of doxycycline (Fig. 6B). RBPJκIND-MMTV mice were crossed with MMTV-Polyoma Virus middle T (PyMT) mice (RBPJκIND-MMTVMMTV-PyMT), which develop macrophage-infiltrated, Notch-activated mammary tumors within 16 weeks of age (39). Unlike xenograft and allograft transplantation models, here mammary carcinomas occurred spontaneously in an immune-competent host, allowing us to study Notch-dependent immune interactions during the evolution of the TME. Confirming the importance of Notch, doxycycline withdrawal resulted in tumors with reduced Notch responsiveness (decreased HA and RBPJκ; Fig. 6C), reduced expression of RBPJκ, IL1β, and CCL2 (Fig. 6D), reduced recruitment of activated, CD163-positive (40) M2 macrophages (Fig. 6E and F), and a trend toward reduced tumor size (Fig. 6G).

Together with the tumor transplantation models, these data demonstrate that in breast tumors, Notch regulates the expression of inflammatory cytokines and the recruitment of activated macrophages to the TME.

The Inflammasome and Macrophage Infiltration Characterize BLBC/TNBC

To confirm our findings in humans, breast cancer cell lines (Fig. 7A) and primary tumors (Fig. 7B) were tested for the presence of inflammasome components. Strikingly, the expression of CASP1 and NALP1 was associated with Notch-activated BLBC cell lines and tumors. Macrophage infiltration was determined using a colony-stimulating factor 1 (CSF1) signature validated in breast cancer (41). When collapsing arrays into centroids and performing Spearman correlations,
Figure 5. Notch induces cytokine secretion and macrophage infiltration in mouse transplantation models of mammary cancer. A, BALB/c mice underwent orthotopic mammary fat pad injection of 4T1-luc cells stably expressing doxycycline-inducible shRNAs targeting NOTCH3 and JAG1 (4T1-luc shN/J1). Prior to tumor engraftment, doxycycline was administered to half of the animals (+Dox; three experimental replicates; representative data from n = 9/group shown). B and C, Two weeks after engraftment, relative tumor luciferase activities (B) and weights (C) were compared between −Dox and +Dox mice. Statistical significance is indicated by *, P < 0.05; **, P < 0.01. D, Representative immunoblot of JAG1, Pro-IL1β, IL1β, and CCL2 in tumor tissue from −Dox and +Dox mice. M.W markers are shown in kilodaltons. β-actin is included as a loading control. E, ELISA of tumor tissue IL1β and CCL2. F, Representative flow-cytometric analysis of single-cell suspensions from −Dox or +Dox tumors (infiltrated THP-1 cells = P4; MB231/shJ1 cells = P5; murine tumor stromal cells = P6). G, Six-week-old female NOD/SCID mice underwent orthotopic mammary fat pad injection of MB231/shJ1 cells, which stably express eGFP and shJ1. Eight weeks after tumor engraftment and 9 days prior to sacrifice, doxycycline (Dox) was administered to half of the animals to induce shJ1 expression (four experimental replicates; data from n = 15/group shown). 4 days prior to sacrifice CellTracker Red CMTPX-labeled THP-1 monocytes underwent tail vein injection (TVi). H, Tumor weight of −Dox and +Dox-treated animals. I, Representative immunoblot analysis of JAG1, IL1β, Pro-IL1β, IL1β, and CCL2 in −Dox and +Dox tumors. J, ELISA assays of tumor tissue IL1β and CCL2 in −Dox and +Dox-treated animals. K, Representative flow-cytometric analyses of single-cell suspensions from −Dox or +Dox tumors (infiltrated THP-1 cells = P4; MB231/shJ1 cells = P5; murine tumor stromal cells = P6). L, Relative THP-1 infiltration (corrected for per gram of tumor tissue) in −Dox and +Dox tumors.
Figure 6. Notch induces cytokine secretion and macrophage infiltration in the RBPJκ<sup>IND-MMTV</sup>MMTV-PyMT mouse model of spontaneous breast cancer.  
A, RBPJ<sup>FL/FL</sup>MMTV-cre<sup>ROSA26-stoplox-rtTA-IRES-GFP</sup> Transgenic mouse. B, Anti-GFP, anti-HA, and anti-RBPJ<sup>κ</sup> immunohistochemistry (IHC, arrowheads) of mammary ducts in doxycycline-treated (+Dox) and untreated (−Dox) animals. C, Anti-GFP, anti-HA, and anti-RBPJ<sup>κ</sup>IHC of mammary tumors from +Dox and −Dox RBPJ<sup>κ</sup>IND-MMTVMMTV-PyMT mice. D, RBPJ<sup>κ</sup>(), IL1β, and CCL2 mRNA expression in tumor tissue from +Dox and −Dox mice (n = 5/group). E, Anti-RBPJ<sup>κ</sup> and anti-CD163 IHC of mammary tumors from +Dox and −Dox mice. F and G, Relative macrophage (MØ) infiltration quantified from IHC (F) and weights (G) of 5 pairs of −Dox and +Dox mice. Statistical significance is indicated by *, P < 0.05.
we observed a tight correlation between Notch activation, IL1β and CCL2 expression, macrophage infiltration, and the BLBC subtype. Using gene set enrichment analyses (GSEA), Notch activation showed a statistically significant, concordant difference as a function of macrophage infiltration (NES = 2.2 and \( p < 0.001 \); Fig. 7C).

Collectively, our data demonstrate that in the BLBC/TNBC subtype Notch is the nexus of paracrine interactions between tumor cells and TAMs. This suggests a model whereby Notch potentiates TGFβ-mediated activation of tumor cells by macrophages, which in turn induce Notch-dependent cytokine secretion and further monocyte recruitment (Fig. 7D).

**DISCUSSION**

The current understanding is that Notch promotes tumorigenesis through cell-cycle deregulation, inhibition of apoptosis, and by reprogramming differentiation; tumor progression is facilitated by Notch through epithelial-mesenchymal transition and by regulating self-renewal of cancer stem cells (42). It is becoming increasingly recognized that tumor development depends not only upon drivers within premalignant or malignant cells, but also upon the activities of nonmalignant cells that populate the TME (10, 43). Therefore, understanding cancer and identifying technologies for its effective treatment will require a deeper knowledge of the cell types and complex cytokine networks that reside within the tumor. Here, we show that Notch designs the TME by regulating cytokine activities that control bidirectional signaling between tumor cells and TAMs. Our data suggest that Notch not only regulates cancer cell expression of the mononuclear cell chemokines IL1β and CCL2, but also facilitates TGFβ-mediated activation of tumor cells by TAMs, closing a signaling loop between these two cell types.

Our data may shed light upon previously unlinked observations that characterize the BLBC/TNBC subtype; these tumors are Notch activated and they are highly infiltrated by macrophages. We and others have shown that elevated expression of the Notch ligand JAG1 (6, 44), Notch pathway activation (45–47), and expression of Notch target genes (5, 25, 48–50) are defining features of poor-prognosis BLBC. There is strong clinical evidence that high TAM density in breast cancer correlates with theBLBC/TNBC subtypes, poor prognosis, and high risk of metastasis (11, 51). Putting these independent findings together, our *in vitro* models demonstrate that Notch drives the expression of IL1β and CCL2 in cancer cells, two cytokines with pleiotropic effects including direct or indirect promotion of myeloid cell recruitment. In *in vitro* models confirm a correlation between Notch-dependent cytokine expression and TAM recruitment. Supporting these findings, in human disease we find a tight correlation between Notch activation, cytokine expression, macrophage infiltration, and breast cancer subtype, with Notch-activated BLBC being the most infiltrated by macrophages. TAMs are critical regulators of the TME, promoting neoplastic transformation, tumor cell migration and metastases, angiogenesis, immune evasion, and the recruitment of other tumor-promoting leukocytes, resulting in therapeutic resistance and poor outcome (52). Addressing the mechanisms that control macrophage recruitment, as our paper has, will identify important targets for anticancer therapy.

IL1β and the inflammasome are key mediators of the innate immune response in infection and autoimmune disease, but their role in malignancy is controversial (53). The properties of IL1β/inflammasome in cancer may be context dependent. In models of colitis-associated colon cancer, the inflammasome is protective, likely owing to IL18 production, a cytokine critical to intestinal tissue healing and remodeling (54). In an epithelial skin carcinogenesis model, the inflammasome adapter ASC is protective when expressed in keratinocytes, but functions as a tumor promotor in myeloid cells (55). Asbestos induction of the inflammasome is likely critical to mesothelioma development (56). IL1β/inflammasome pathway activation in nonmalignant tissues, including the myeloid compartment, induces myeloid recruitment and tumor progression in murine mammary carcinoma (18). Our study specifically addresses IL1β/inflammasome activation in tumor cells, showing that unlike myeloid cells where Toll-like receptor ligands or endogenous danger signals induce the expression of pro-IL1β, in cancer cells Notch is required. It is also notable that among breast cancer subtypes, BLBC/TNBC cells are uniquely poised to convert pro-IL1β to mature IL1β through their production of the inflammasome components, CASP1 and NALP1. Although the association between IL1β expression and poor-outcome breast cancer is well recognized, this is the first report describing the mechanism of IL1β production by tumor cells.

Cells that have undergone senescence, a stress response that induces proliferative arrest in premalignant tissues, have a profound effect on their microenvironment through the senescence-associated secretory phenotype (SASP; ref. 57). Our work may link findings from two recent reports, the first demonstrating that Notch regulates transition of...
the SASP between TGFβ and proinflammatory cytokine states (58), and the second identifying CCL2 as the key proinflammatory cytokine required for the recruitment of CCR2+ myeloid cells to the senescent niche (59). Although a role for CCL2 in senescence is emerging, the effects of CCL2 in invasive disease are better understood. CCL2 exerts its tumorigenic effects through mononuclear cell recruitment to the tumor and through its proangiogenic activities, which may be either direct (60) or secondary to macrophage accrual (61). CCL2 may also promote seeding to metastatic sites through enhanced retention of metastasis-associated macrophages at these sites (22, 62). Thus, the tumorigenic effect of Notch on CCL2 expression and recruitment of TAMs may run the gamut from premalignant to metastatic disease.

The work described here provides a rationale for the continued development of Notch inhibitors, some of which are currently in the pharmaceutical pipeline. However, the development of anti-Notch compounds has encountered the usual barriers to drug development. As Notch is ubiquitously expressed, and current anti-Notch drugs are nonspecific, achieving antitumor levels of drug results in substantial on- and off-target side effects (63–66). The tight link between Notch activation, IL1β and CCL2 expression, and the BLBC subtype highlight immunotherapeutic opportunities. Effective and safe antagonists that target IL1β and/or CCL2 are in either clinical trial or clinical use to treat inflammatory disease and could be rapidly repurposed for breast cancer treatment. For these agents, information is available on their pharmacology, formulation, and toxicity. Anakinra (Kineret; Amgen) is a human recombinant form of the IL1β receptor antagonist (IL1Ra) and effectively treats rheumatoid arthritis and other inflammatory diseases (67). Emapritcap pegol (NOX-E6; Nioxon Pharma) effectively binds and neutralizes CCL2, and in a phase IIa exploratory study in humans has shown significant and sustained amelioration of CCL2/macrophase-mediated diabetic nephropathy (68). Although promising, a recent report cautions that cessation of CCL2 inhibition accelerates breast cancer metastases in a mouse model, reinforcing that principled introduction of these compounds within an appropriate clinical trial setting will be imperative (69).

Immunotherapies that stimulate antitumor immunity have provided promising results (70), but so far the most significant advances have been made in the realm of the adaptive immune system (71). Here, we provide evidence supporting the development of therapies that target the innate immune response through cytokine inhibition in Notch-activated BLBC. It is almost certain that immunotherapies attacking tumor-promoting cytokines such as IL1β or CCL2, either alone or in combination with other therapies, will become a tumor-promoting cytokines such as IL1β and/or CCL2, either

per group. The Notch activation signature was generated by combining genes that showed 1.5-fold or greater downregulation in HCC1143 cells treated with siRNAs targeting both JAG1 and combined NOTCH1/3. The macrophage CSF1 signature is defined by Beck and colleagues (41).

Cell Lines
Human breast cancer cell lines MDA MB231, HCC1143, T47D, and MCF-7 and human acute monoblastic leukemia THP-1 cells were from ATCC and were authenticated using short-tandem repeat profiling. Primary human lung microvascular endothelial cells (HMVEC-L) were obtained from Lonza. The mouse mammary tumor cell line 4T1-luc that stably expresses firefly luciferase was a kind gift from Dr. Rakesh K. Singh (University of Nebraska Medical Center). All cell lines were obtained between 2011 and 2016 and maintained in growth media according to the manufacturer’s or provider’s instruction, unless otherwise specified. Cell lines were never passaged for greater than 6 months.

Mouse 4T1 Mammary Tumor Model
Six-week-old female BALB/c mice were purchased from The Charles River Laboratory. Doxycycline was administered through the drinking water (2 mg/mL) in half of the animals that were randomly allocated. 4T1-luc shNh/J1 cells, which stably express firefly luciferase and doxycycline-inducible shRNAs targeting murine NOTCH1 and JAG1, were injected (106 cells in 30 μL PBS) into the mammary fat pad as previously described (72, 73). Rescue experiments were conducted using 4T1-luc shNh/J1 cells in 30 μL Matrigel/PBS (1:1 mix) with or without recombinant mouse IL1β (0.25 ng/mL) and CCL2 (1 ng/mL). Doxycycline-containing water was changed every 2 to 3 days. After 2 weeks, tumor-engrafted mice were imaged using an IVIS system (Xenogen) and then euthanized, followed by excision of tumors.

Xenograft Tumor Model
Six-week-old female NOD/SCID (NOD.CB17-Prkdcscid/J; University Health System Animal Resources Centre) mice underwent mammary fat pad injection with MDA MB231 cells containing a doxycycline-inducible shRNA targeting human JAG1 (MB231/ShJ1, 3 x 106 cells in 30 μL PBS). Mice were then randomly assigned to two groups before tumors were palpable. After 8 weeks, doxycycline was administered via the drinking water (2 mg/mL) to half of the tumor-engrafted mice, and 5 days later CellTracker Red CMTPX-labeled THP-1 cells were administered via the tail vein for all mice. Four days after monocyte administration, the animals were euthanized and tumors were analyzed.

Transgenic Mouse Model
To create RBPJKO2–MMTV mice (RBPJKO2+/−; ROSA26-stop:lacz-rTa-IRES-GFP +/−; pTetOS-RBPJKO2HA +/−; MMTV-cre +/−), RBPJKO2 mice, which contain an endogenous RBPJx flanked by IoxP (FL) sites (RBPJKO2+/−; refs. 74, 75), ROSA26-stop:lacz-rTa-IRES-GFP (76) and pTetOS-RBPJKO2HA transgenes were further crossed with MMTV-cre (C57BL/6) mice. Full description of generation of pTetOS-RBPJKO2HA and characterization of the RBPJKO2 mouse will be provided elsewhere (P.K. Thompson and colleagues, manuscript in preparation). Expression of RBPJx from the transgene, and thus Notch responsiveness, is controlled with doxycycline administration to the animals. To generate spontaneous mammary tumors, RBPJKO2+/− mice were crossed with MMTV-Polyoma Viruses middle T (MMTV-PyMT) mice. The genotype of all animals was confirmed by PCR analyses of the tail tips of 4-week-old female mice, using genotyping primer sets (Table S2) as previously described (74–76). After RBPJKO2+/MMTV-PyMT mice developed mammary tumors (approximately 16 weeks), doxycycline administration was withdrawn to deplete Notch signaling.

METHODS
Gene Signatures
Agilent 44k Whole Human Genome chips were used to analyze gene expression levels after treatment of basal-like breast cancer HCC1143 cells with siRNAs targeting JAG1 or both NOTCH1 and NOTCH3 (25). Three replicate measurements were performed
in mammary tumors. Mice were euthanized at 18 weeks and tumors were excised for further analysis. All animal procedures, including the above-described 4T1 tumor study and xenograft tumor study, were approved by the University Health Network Institutional Animal Care and Use Committee.

**Supplementary Methods**

Additional detailed methods are available in the Supplementary Materials.

**Disclosure of Potential Conflicts of Interest**

J.C. Züitiga-Pflücker is a consultant/advisory board member for Intellia and Realist. No potential conflicts of interest were disclosed by the other authors.

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**Grant Support**

This study was supported by funds to M. Reedijk and P.S. Ohashi from Susan G. Komen for the Cure (Award ID IIR13264436). This research was funded in part by the Ontario Ministry of Health and Long Term Care.

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Received January 11, 2017; revised May 1, 2017; accepted August 2, 2017; published OnlineFirst August 8, 2017.

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Notch Shapes the Innate Immunophenotype in Breast Cancer


*Cancer Discov* 2017;7:1320-1335. Published OnlineFirst August 8, 2017.

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