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 (TME). Here we show that Notch in tumor cells regulates the expression of two powerful pro-inflammatory cytokines, IL1β and CCL2, and the recruitment of tumor-associated macrophages (TAMs). 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 novel 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 novel immunotherapeutic opportunities in BLBC.
SIGNIFICANCE

BLBC is aggressive and has an unmet need for effective targeted treatment. Our data highlight novel 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.
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 sub-typed 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 BLBC molecular subtype. While 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 cancer, primarily affecting young women, people of African or Hispanic ancestry, and those with BRCA1 mutations.

The Notch signaling pathway plays a crucial role in inter-cellular signaling and tissue patterning during development (3). Mammals have four Notch receptors (NOTCH1-4) that interact with five Notch ligands (DLL1, 3, 4 and JAG1, 2). 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 (NIC) from the plasma membrane. NIC translocates to the nucleus where it engages the DNA binding protein CBF-1/RBPJκ resulting in replacement of a multiprotein co-repressor complex with a co-activator 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 comprised of diverse non-malignant lineages including cancer associated fibroblasts, endothelial cells, and cells of both the innate and adaptive immune systems. Tumor-associated macrophages (TAMs), 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 non-malignant cells is accompanied with expression of numerous inflammatory cytokines including TGFβ, IL1β and CCL2. TGFβ has a dual role in tumor progression displaying anti-proliferative 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 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 the present 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 sub-type, 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 N\textsuperscript{IC} from the plasma membrane and translocation to the nucleus. There, N\textsuperscript{IC} participates in transcriptional regulation at CBF-1/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 N\textsuperscript{IC}, after JAG1 knockdown. MDA
MB231 cells containing a stable doxycycline-inducible small hairpin (sh)RNAi targeting JAG1 were transduced with adenovirus expressing activated, cleaved Notch receptor (N1IC or N3IC). N1IC and N3IC induced mRNA up-regulation of both IL1β and the classical, 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 N1IC or N3IC. Appropriately, N1IC and N3IC induced up-regulation of pro-IL1β and of JAG1 protein, itself a target of activated Notch (Fig. 2B, compare lanes 1 and 2 to lanes 3 and 4). JAG1 inhibition reduced Notch activation (N1IC and N3IC) and pro-IL1β to undetectable levels (Fig. 2B; lanes 5 and 6) and this was rescued by ectopic N1IC or N3IC 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 N1IC (Fig. 2D). The immunoprecipitated chromatin fragments were analyzed by PCR using primer sets targeting the high-affinity sites A, D and F. Enrichment for N1IC-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 CBF-1 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 CBF-1 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 CBF-1 knockdown. These data show that JAG1, N1IC and N3IC are required for IL1β production and identify at least one CBS (at -2085 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 pro-protein (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 to cells of the mononuclear phagocyte lineage where lipopolysaccharide (LPS) serves as a traditional priming signal for pro-IL1β mRNA expression, LPS stimulation had a marginal effect on pro-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, as indicated by caspase 1 p20, is unchanged by NOTCH1/3 or JAG1 knock-down (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 classical 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 knock-down was phenocopied by IL1β or CCL2 knock-down in these assays and could be rescued by the addition of exogenous cytokine. In both human and rodent co-culture systems M2 macrophages (Supplementary Fig. S3), the predominant pro-tumorigenic 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 knock-down in either cancer or M2 cells revealed both cell types as significant sources of cytokine production induced by co-culture (Supplementary Fig. S4; compare lane 4 to lanes 5 and 7, respectively). IL1β expression in co-culture after IL1β knock-down in either tumor or M2 cells was
higher than IL1β expression in either cell type in mono-culture, 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 TGFβR1 expression. Indeed, siRNA-mediated knock-down of JAG1 or NOTCH1 and NOTCH3 in human and murine BLBC cells resulted in decreased TGFβR1 mRNA (Fig. 4B) and protein (Fig. 4C). In accordance with this, Notch knock-down reduced TGFβ-mediated Smad2/3 phosphorylation (Supplementary Fig. S5A). Notch knock-down also abrogated TGFβ-mediated expression of the Notch target gene uPA, confirming the central role of Notch.

While M2 cells in monoculture produce latent TGFβ, tumor cell-M2 co-culture resulted in the production of mature, active TGFβ in conditioned media (Fig. 4D) and this could be inhibited by knock-down 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 from tumor cells (25) and with reports that uPA catalyzes 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, required TGFβR1 expression 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 up-regulation of TGFβR1. In this context, whether the primary role of Notch is to directly drive uPA production, to provide TGFβR1 for TGFβ-mediated uPA production (38) or both, is to be determined.

**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/J1) were grafted into mammary fat pads of BALB/c mice (Fig. 5A). Compared with untreated animals, 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 down-regulation (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). Since 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, 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
complementary xenograft approach was undertaken (Fig. 5G). Female non-obese
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. 6H). Four days prior to sacrifice, labeled THP-1
monocytes were injected via the tail vein. JAG1 knockdown, Notch inhibition (reduced
N1\text{IC}), reduced IL1\beta, CCL2 expression (Fig. 5I and J) and reduced THP-1 recruitment to
tumor (Fig. 5K and L) were confirmed in the treated animals.

The \textit{RBPJK}\textsuperscript{IND-MMTV}\textit{MMTV-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 (RBPJκ\textsuperscript{IND-MMTV}) where Notch activity could be regulated in mammary tissues. RBPJκ\textsuperscript{IND-MMTV} mice contain endogenous CBF-1/RBPJκ flanked by loxP (FL) sites (RBPJκ\textsuperscript{FL/FL}), mouse mammary tumor virus (MMTV)-cre, ROSA26-stop/lox-rtTa-IRES-GFP and pTetOS-RBPJκ-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 green fluorescent protein (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). RBPJκ\textsuperscript{IND-MMTV} mice were crossed with MMTV-Polyoma Virus middle T (PyMT) mice (RBPJκ\textsuperscript{IND-MMTV}MMTV-PyMT), which develop macrophage-infiltrated, Notch-activated mammary tumors by sixteen 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 towards 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 were associated with Notch-activated BLBC cell lines and tumors. Macrophage infiltration was determined using a colony stimulating factor 1 signature validated in breast cancer (41). When collapsing arrays into centroids and performing Spearman correlations, we observed a tight correlation between Notch activation, IL1β and CCL2 expression, macrophage infiltration and the BLBC sub-type. 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 not only depends upon drivers within premalignant or malignant cells, but also upon the activities of non-malignant 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 for the first time, that Notch designs the TME by regulating cytokine activities that control bi-directional 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 the BLBC/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 vivo 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 sub-type, 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 anti-cancer 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 promoter in myeloid cells (55). Asbestos induction of the inflammasome is likely critical to mesothelioma development (56). IL1β/inflammasome pathway activation in non-malignant 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) (57). Our work may link findings from two recent reports: the first, demonstrating that Notch regulates transition of the SASP between TGFβ and pro-inflammatory cytokine states (58) and the second, identifying CCL2 as the key pro-inflammatory cytokine required for the recruitment of CCR2+ myeloid cells to the senescent niche (59). While 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 pre-malignant to metastatic disease.

The work described here provides 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 novel
drug development. Since Notch is ubiquitously expressed, and current anti-Notch drugs are non-specific, achieving anti-tumor 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 novel 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). Emapticap pegol (NOX-E36, Noxxon pharma) effectively binds and neutralizes CCL2 and in a Phase 2a exploratory study in humans has shown significant and sustained amelioration of CCL2/macrophage-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 anti-tumor 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 key component of personalized cancer care.
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 siRNA(s) targeting JAG1 or both NOTCH1 and NOTCH3 (25). Three replicate measurements were performed per group. The Notch activation signature was generated by combining genes that showed 1.5 or greater fold down-regulation in HCC1143 cells treated with siRNAs targeting both JAG1 and combined NOTCH1/3. The macrophage CSF1 signature is defined by Beck et al. (41).

**Cell lines**

Human breast cancer cell lines MDA MB231, HCC1143, T47D, MCF-7, and human acute monocytic leukemia THP-1 cells were from American Type Culture Collection (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 manufacturers’ 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 Charles River Laboratory. Doxycycline was administered through the drinking water (2 mg/mL) in half of the animals that were randomized allocated. 4T1-luc shN/J1 cells, which stably express firefly luciferase and doxycycline-inducible shRNAs targeting murine Notch3 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 shN/J1 cells in 30μL Matrigel/PBS (1:1 mix) with or without recombinant mouse IL1β (0.25ng/mL) and CCL2 (1ng/mL). Doxycycline-containing water was changed every 2-3 days. Two weeks after tumor engraftment 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 Network Animal Resources Centre) 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 2 groups before tumors were palpable. Eight weeks after engraftment doxycycline was administered via the drinking water (2 mg/mL) for half of the mice and 5 days later CellTracker Red CMTPX-labeled THP-1 cells were administrated 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 RBPJκIND-MMTV mice (RBPJκ FL/FL; ROSA26-stop/lox-rtTa-IRES-GFP +/-; pTetOS- RBPJκ -HA +/-; MMTV-cre +/-), RBPJκIND mice, which contain an endogenous RBPJκ flanked by loxP (FL) sites (RBPJκ FL/FL) (74,75) ROSA26-stop/lox-rtTa-IRES-GFP (76) and pTetOS- RBPJκ -HA transgenes, were further crossed with MMTV-cre (C57BL/6) mice. Full description of generation of pTetOS- RBPJκ -HA and characterization of the RBPJκIND mouse will be provided elsewhere (Thompson, P.K. et al., manuscript in preparation). Expression of RBPJκ from the transgene, and thus Notch responsiveness, is controlled with doxycycline administration to the animals. To generate spontaneous mammary tumors, RBPJκIND-MMTV mice were crossed with MMTV-Polyoma Virus 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 RBPJκIND-MMTV MMTV-PyMT mice developed mammary tumors (approximately 16 weeks), doxycycline administration was withdrawn to deplete Notch signaling in mammary tumors. Mice were euthanized at 18 weeks and tumors were excised for further analysis. All animal procedures, including 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.
REFERENCES


FIGURE LEGENDS

Figure 1. Notch Drives IL1β and CCL2 Expression in the BLBC Subtype. A, Boxplots of normalized IL1β and CCL2 mRNA after treatment of HCC1143 cells with siRNA 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, the Notch target uPA (25), 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, siILβ1, 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 small hairpin RNA targeting JAG1 (MB231/shJ1), or after infection (MOI = 50) with adenovirus (Ad) expressing LacZ, Notch 1 intracellular domain (N1 IC) 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 N1 IC, N3 IC, JAG1 and pro-IL1β in uninfected (Ad. -) or adenovirus-infected (expressing LacZ, N1 IC or N3 IC) 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; grey triangles) CBS. The 5’ locations of CBS (minus strand; bold, light blue) are indicated relative to the start of translation of IL1β. Chromatin immunoprecipitation (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 Cyclin D1 (48) and uPA 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 CBF-1 siRNA-treated cells.
Figure 3. Pro-Tumorigenic 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, siN1/3 or siJ1 (+/- 50 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<0.05, compared with un-rescued counterparts). 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) or murine (4T1-luc/bone marrow-derived M2 macrophages) mono- or co-cultures. 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 mono- or co-culture with M2 macrophages. * P<0.05, ** P<0.01 indicate significance.
**Figure 4.** Notch Potentiates TGFβ Signaling Between Tumor and M2 cells. **A,** Immunoblot of JAG1 or N1IC after stimulation of MDA MB231 cells with 1 ng/ml recombinant TGFβ for the indicated times. Mw markers are shown in kilodaltons. β-actin is included as a loading control. **B,** QRT PCR expression analysis of TGFβR1 mRNA in MDA MB231 or 4T1-luc cells treated with either siscr, siN1/3 or siJ1 (n=3-5/group). Statistical significance is indicated by * P<0.05, ** P<0.01. **C,** Immunoblot of TGFβR1 protein expression in MDA MB231 or 4T1-luc cells treated with either siscr, siN1/3 or siJ1. **D,** Immunoblot of latent or mature (under reducing or non-reducing conditions) forms of TGFβ in serum-free conditioned media from MDA MB231 or M2 cells cultured either alone or in co-culture. **E,** Immunoblot of latent or mature forms of TGFβ in serum-free conditioned media from MDA MB231 or M2 cells cultured either alone or in co-culture. MDA MB231 cells were treated with either siscr, siuPA, siN1/3 or siJ1. **F,** Immunoblot of TGFβR1, JAG1, N1IC uPA, Pro-IL1β and CCL2 in MDA MB231 cells (treated with either siscr or siTGFβR1) cultured either alone (-) or in co-culture (+) with M2-like cells.
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. Mw markers are shown in kilodaltons. β-actin is included as a loading control. E, ELISA of tumor tissue IL1β and CCL2. F, Summary of flow cytometric analysis for macrophage (MØ; F4/80, CD11b) infiltration (corrected for per gram of tumor tissue) in -Dox and +Dox tumors. G, Six-week old female NOD/SCID mice underwent orthotopic mammary fat pad injection of MB231/shJ1 cells, which stably express eGFP and shRNAJ1. Eight weeks after tumor engraftment and 9 days prior to sacrifice, doxycycline (Dox) was administered to half of the animals to induce shJAG1 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, NlIC, 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, Representive flowcytometric 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-<i>MMTV</i></sup>-<i>MMTV-PyMT</i> Mouse Model of Spontaneous Breast Cancer. A, RBPJκ<sup>IND-</sup><i>MMTV</i> mouse model - see text. B, Anti-GFP, anti-HA and anti-RBPJκ immunohistochemistry (IHC; arrowheads) of mammary ducts in doxycycline-treated (+Dox) and untreated (-Dox) animals. C, Anti-GFP, anti-HA and anti-RBPJκ IHC of mammary tumors from +Dox and -Dox RBPJκ<sup>IND-<i>MMTV</i></sup>-<i>MMTV-PyMT</i> mice. D, RBPJκ, IL1β and CCL2 mRNA expression in tumor tissue from +Dox and -Dox mice (n=5/group). E, Anti-RBPJκ 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.
Figure 7. Notch Activation, Cytokine Expression and Macrophage Infiltration Characterize Human BLBC. A, Supervised clustering of Notch-activated signature genes in the Genentech dataset of 51 breast cancer cell lines (26). Shown is a heatmap of gene expression patterns from the entire set of Notch signature genes and magnified data from the inflammasome genes CASP1, NALP1, ASC, NALP3 and NALP4. The yellow box identifies the BLBC cell lines. Fold-change relative to the median value is indicated by the color bar. Shown are \( p \) values for gene set enrichment analyses (GSEA) comparing inflammasome component genes with the Notch-activated signature. B, Supervised clustering of the Notch activation and macrophage gene (Mac) signatures (41) in a dataset of 263 primary breast tumors (77) (yellow box). Fold-change relative to the median value is indicated by the color bar. The Spearman correlations (\( p \)) of multiple genes (including the IL1\( \beta \) cluster CASP1, NALP1, ASC, NALP3, NALP4 and IL1\( \beta \)) and the centroid of the macrophage signature to the Notch-activated signature genes (represented by the nearest mean centroid) is shown. C, GSEA of the Notch-activation signature as a function of the macrophage signature centroid. \( P \), nominal \( P \) value; NES, normalized enrichment score. D, A positive paracrine feedback loop exists where TAM-derived TGF\( \beta \) activates Notch signaling between BLBC cells. Notch-dependent cytokines promote further recruitment of mononuclear cells to the TME and Notch potentiates TGF\( \beta \) signaling through uPA-dependent production of active TGF\( \beta \), and by priming a response in tumor cells through TGF\( \beta \)R1 up-regulation.
Figure 2

A

Fold compared to No Ad minus Dox (actin corrected)

B

Hes1
IL1β

Dox

Ad -

N1IC

LacZ

N3IC

+Dox

C

Start codon

D

Input

Anti-N1

F

D

A

Cyclin D1

uPA

GAPDH

E

Lysate

cold wt probe

F

Lyse

cold wt probe D

cold mut probe D

siRNA

CBF-1
Figure 3

A

B

C

D

E
**A.** 4T1 transplantation model

**B.** Relative luciferase activity

**C.** Tumor weight (g)

**D.** Western blot analysis for JAG1, Pro-IL1β, IL1β, CCL2, and β-actin proteins.

**E.** Tumor tissue IL1β (pg/mL) levels.

**F.** Relative MO infiltration.

**G.** Xenograft tumor model

**H.** Tumor weight (g)

**I.** Western blot analysis for JAG1, N1IC, Pro-IL1β, IL1β, CCL2, and β-actin proteins.

**J.** Tumor tissue IL1β (pg/mL) levels.

**K.** Relative THP-1 infiltration.

**L.** Tumor tissue CCL2 (pg/mL) levels.