

## Tumoral Lymphocytic Infiltration and Expression of the Chemokine CXCL10 in Breast Cancers from the Ontario Familial Breast Cancer Registry

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### Abstract

**Purpose:** Breast carcinomas, including basal and hereditary cases, often present with a prominent tumoral lymphocytic infiltrate. Chemokines could play a role in attracting these cells and contribute to tumor progression. We explored tumoral expression of CXCL10 and determined the relationship between CXCL10 and lymphocytic infiltrate in a cohort of breast cancers.

**Experimental Design:** Using tissue microarrays of 364 breast tumors, we evaluated expression of CXCL10 and its receptor, CXCR3, in relation to histopathologic features, biomarkers, and lymphocyte markers. In addition, we overexpressed CXCL10 and CXCR3 in MCF7 breast cancer cells and monitored T-lymphocyte migration and invasion.

**Results:** Forty-five percent of tumors expressed CXCL10, and a significant association was found with CXCR3 and lymphocytic infiltrate. Further characterization of the lymphocytic infiltrate revealed an association with CXCL10 expression for peritumoral CD4+ and CD8+ lymphocytes. CD8+ intratumoral lymphocytes, FOXP3+ regulatory T cells (Tregs), and T-BET+ T<sub>H</sub>1 cells were associated with *BRCA1* and basal tumors. Conditioned media from MCF7 cells overexpressing both CXCL10 and CXCR3 increased T-lymphocyte migration and invasion.

**Conclusions:** Our findings suggest that CXCL10 may act in a paracrine manner, affecting the tumor microenvironment, and in an autocrine manner, acting on the tumor cells themselves and may play a role in tumor invasiveness and progression. The CXCL10-CXCR3 axis can serve as a potential target in *BRCA1* and basal breast cancers, which present with a prominent lymphocytic infiltrate and a poor prognosis. *Clin Cancer Res*; 19(2); 336–46. ©2012 AACR.

### Introduction

Important roles for chemokines have been shown in inflammation, infection, injury, allergy, and cancer (1).

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Apart from their role in recruiting leukocytes to the site of inflammation, chemokines are also known to function as regulators of cell proliferation, differentiation, migration, and invasion. In gene expression profiling, we have previously identified the chemokine, CXCL10 (IFN- $\gamma$ -inducible protein 10), as being significantly overexpressed in basal tumors as compared with estrogen receptor (ER)-positive tumors (Andrulis and colleagues, manuscript in preparation). Secreted by activated T lymphocytes, endothelial cells, fibroblasts, monocytes, and keratinocytes, it attracts other immune cells, such as activated T lymphocytes, natural killer cells, and monocytes, which express its receptor, CXCR3.

Studies have shown a role for CXCL10 as well as its receptor in the progression of certain cancers (2). Both ligand and receptor expression have also been detected in a number of human breast cancer cell lines suggesting that the ligand acts on cells within the breast tissue microenvironment, and also acts on the tumor cells themselves in an autocrine manner to promote tumor growth (3). Conversely, CXCL10 has also been shown to inhibit tumor progression by its recruitment of mononuclear cells in

### Translational Relevance

CXCL10 expression was detected in primary breast cancers and found to be associated with tumoral lymphocytic infiltrate and expression of its receptor, CXCR3. Analysis of the infiltrate revealed association of CXCL10 with peritumoral CD4+ and CD8+ lymphocytes, intratumoral CD8+ lymphocytes, as well as FOXP3+ regulatory T cells (Tregs) and T-BET+ T<sub>H</sub>1 cells. The latter were associated with the *BRCA1* subgroup and basal subtype. Overexpression of this chemokine axis in MCF7 cells increased *in vitro* T-lymphocyte migration and invasion. These results suggest that CXCL10 may act both in a paracrine manner, affecting the tumor microenvironment, as well as an autocrine manner, acting on the tumor cells themselves. The host immune response is believed to be important in the outcome of many cancers. The CXCL10-CXCR3 axis may play a role and serve as a potential drug target for *BRCA1* and basal breast cancers, which present with a prominent lymphocytic infiltrate and a poor prognosis.

hepatocellular carcinoma (4), and through antagonizing angiogenic factors and endothelial cells in renal cell carcinoma (5).

Carriers of germline mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* have an increased lifetime risk of developing breast cancer (6). *BRCA1*-associated cancers are poorly differentiated, are typically negative for ER, progesterone receptor (PR) and HER2 (7). In addition, *BRCA1*-associated cancers show a specific morphologic phenotype: high mitotic count, a prominent lymphocytic infiltrate, and pushing margins (8–9); features that are shared with basal-like breast cancers. *BRCA2*-associated breast cancers have also been shown to have a prominent lymphocytic infiltrate (10). Despite the presence of a prominent host immune response, patients with hereditary breast cancers do not fare better. Furthermore, basal tumors, in patients unselected for family history, behave aggressively (11–14). We hypothesize that chemokines play a role in attracting these immune cells, and once present, these cells contribute to tumor progression.

Given the prominent lymphocytic infiltrate found in a large proportion of basal and familial breast cancers, we investigated the expression of CXCL10 in a cohort of pathologically well-characterized breast cancers from the Ontario site of the Breast Cancer Family Registry and sought to determine the relationship between CXCL10 expression and tumoral lymphocytic infiltrate as well as molecular biomarkers and other morphologic and clinical parameters. The make-up of the lymphocytic infiltrate was further characterized by immunohistochemical staining for the T-lymphocyte markers CD4, CD8, FOXP3, T-BET, and for the B-lymphocyte marker CD20, and determining the association of these markers with CXCL10 expression, as well as morphologic and clinical parameters. In addition, we eval-

uated the tumoral expression of the CXCL10 receptor, CXCR3, using immunohistochemistry, to investigate a possible autocrine role for the ligand. This autocrine role was further investigated *in vitro* by coexpressing CXCL10 and CXCR3 in the MCF7 human breast cancer cell line and using conditioned media from these cells to study the migration and Matrigel invasion of T lymphocytes towards this chemotactic source.

### Materials and Methods

#### Study population

Three hundred and sixty four subjects were selected from the Ontario site of the Breast Cancer Family Registry (15) including 58 patients with known *BRCA1* germline mutations and 64 patients with known *BRCA2* germline mutations detected as previously described (16–18).

#### Tumor morphology

As part of the Breast Cancer Family Registry, tumors from consenting patients undergo a centralized pathology review by an expert in breast pathology, using a standardized pathology reporting form (19). Included in this review are assessments of established pathologic prognostic factors including size, nodal status, tumor type, tumor grade, and its individual components as well as specific tumor morphologic features. From this database, information on morphologic features was abstracted.

#### Mutational analysis of *BRCA1* and *BRCA2*

Mutational analysis for *BRCA1* and *BRCA2* was conducted using an RNA/DNA-based protein truncation test with complementary 5' sequencing or complete gene sequencing by Myriad Genetics. Mutations were confirmed by DNA sequencing, and were classified as deleterious if they were protein truncating, missense mutations (rare), or splice-site mutations as defined by the Breast Informatics Consortium (<http://research.nhgri.nih.gov/bic/>).

#### Tissue microarray construction and immunohistochemical staining

Tissue microarrays (TMA; Beecher Instruments) were constructed using duplicate 0.6 mm cores of tumor. Four-micron TMA sections were cut and used for immunohistochemical staining using methods as listed in Supplementary Table S1. Staining for hormone receptors (ER, PR), HER2, basal (CK5, CK14) cytokeratins, EGFR, p53, Ki-67, CD4, CD8, CD20, FOXP3, T-BET, CXCL10, and CXCR3 was conducted. Microwave antigen retrieval was carried out in a Micromed T/T Mega Microwave Processing Lab Station (ESBE Scientific). Sections were developed with diaminobenzidine tetrahydrochloride and counterstained in Mayer's hematoxylin.

#### Interpretation and scoring of immunohistochemistry

CD4, CD8, and CD20-positive lymphocytes were scored as present or absent and categorized as peritumoral or intratumoral. The latter were defined as those lymphocytes located within the epithelial component of the carcinoma.

Absolute counts of T-BET+ lymphocytes and FOXP3+ lymphocytes were conducted and these were categorized as intratumoral [when within the epithelial nests or within close proximity (the distance between positive lymphocyte and tumor nest is equal to or less than the size of one tumor cell)] or peritumoral (at a distance from the epithelial nests). For FOXP3 and T-BET, an absolute count of 10 positive lymphocytes (within or within close proximity of the epithelial cell nests) was used as the cutoff for positivity (Supplementary Fig. S3).

The remaining immunohistochemical-stained sections were scored using the Allred scoring method (20), which adds the intensity of staining (absent: 0, weak: 1, moderate: 2, and strong: 3) to the percentage of cells stained (none: 0, <1%: 1, 1%–10%: 2, 11%–33%: 3, 34%–66%: 4, and 67%–100%: 5) to yield a "raw" score of 0 or 2 to 8. Nuclear staining was scored for ER, PR, p53, and Ki-67 using previously validated cutoffs for ER and PR (>2 = positive; refs. 21–22) and p53 (>3 = positive; ref. 23). A cutoff of 4 was used for Ki-67. Moderate to strong complete membranous staining was assessed for HER2 and the validated cutoff of 5 or more was used to indicate positivity with this antibody (24). Membranous and/or cytoplasmic staining was scored for the remaining antibodies and a score of 4 or more was arbitrarily considered positive for CXCL10, whereas a score of 3 or more was considered positive for CXCR3. Only expression in tumor cells was evaluated. The raw score data were reformatted using a TMA deconvoluter software program (25) into a format suitable for statistical analysis. Interpretable scores were obtained in 79% to 92% of tumors. Tumors from each group were assigned to molecular subgroups based on previous publications (26–28). Tumors that were positive for HER2 protein overexpression were assigned to the HER2 group. Tumors that were negative for HER2 but positive for ER or PR were assigned to the luminal group. Tumors that were negative for HER2, ER, and PR, and positive for one or more of the following: CK5, CK14, or EGFR, were assigned to the basal subtype.

### Cell culture

The MCF7 cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS (both from Gibco), 10 µg/mL bovine insulin (Sigma), and 100 µg/mL penicillin–streptomycin (Gibco). This cell line passed authentication and is 100% match to American Type Culture Collection's HTB-22 (MCF7) short tandem repeat profile. Media for transfected cells were supplemented with 550 µg/mL G418 (MultiCell Technologies). T lymphocytes were grown in Iscove's modified Dulbecco's medium supplemented with 10% human serum (Gemini), 100 µg/mL penicillin–streptomycin, 10 µg/mL gentamicin (Gibco),  $5.5 \times 10^{-5}$  mol/L β-mercaptoethanol (Sigma), and 200 U/mL recombinant human IL-2 (Chiron Corp.).

### Gene transfection

pC-CXCL10 and pC-CXCR3 constructs were created by inserting the full-length human CXCL10 and CXCR3

cDNA, respectively, into the multiple cloning site of the pcDNA 3.1 TOPO vector (Invitrogen). CXCL10 cDNA was amplified from the RPMI-8226 cell line, whereas the CXCR3 sequence was commercially purchased (Open Biosystems Inc.). MCF7 cells were cotransfected with pC-CXCL10 and pC-CXCR3, or the empty vector using FuGene transfection reagent (Roche). Transfected cells were selected for and maintained with 550 µg/mL G418.

### T-lymphocyte isolation and activation

Fresh blood was collected from consenting healthy donors, and peripheral blood mononuclear cells (PBMC) were isolated using CPT Vacutainer tubes according to the manufacturer's instructions (BD). CD4+ T lymphocytes were further isolated from the PBMCs through positive selection using magnetic bead sorting with a cell type-specific antibody according to the manufacturer's protocol (Miltenyi Biotec). The lymphocytes were activated over a 15-day period. The cells were cultured in complete media supplemented with 200 U/mL IL-2, and 1 µg/mL phytohemagglutinin was added on the first day of plating only. Flow cytometry was used to verify CXCR3 upregulation in the activated T lymphocytes.

### ELISA

A CXCL10-specific ELISA development kit was used to confirm CXCL10 overexpression in conditioned media from the CXCL10-CXCR3 cells according to the manufacturer's instructions (Peprotech). Conditioned media were generated by plating  $1 \times 10^7$  cells in complete media in 10-cm plates for 24 hours followed by washing in serum-free medium (SFM) and a further incubation in SFM for 24 hours. Conditioned media were then collected, centrifuged at  $1,000 \times g$  for 10 minutes, and frozen for later use in ELISA or Transwell assays.

### Flow cytometry

To evaluate the expression of CXCR3 on CXCL10-CXCR3 cells and activated CD4+ T lymphocytes, the cells were stained with anti-CXCR3-PE or an IgG phycoerythrin (PE) isotype-matched control (BD Biosciences). In addition, the CD4+ T lymphocytes were costained with anti-CD4-FITC and anti-CD45RO-APC to confirm the positive selection and activation status (BD Biosciences). Cells were washed and fixed with 2% paraformaldehyde. Data were acquired on the FACScalibur analytic flow cytometer (Becton Dickinson) and analysis was done using FlowJo software (Tree Star Inc.). Gating was done using forward scatter and side scatter, and both the percentage of CXCR3-positive cells and the mean fluorescence intensity for CXCR3 were determined.

### Transwell migration and invasion assays

For T lymphocyte Transwell assays,  $1 \times 10^5$  cells per well in 0.5% bovine serum albumin SFM were loaded in triplicate into the top chambers of 24-well inserts (5.0 µm pore size, Costar). The bottom well contained conditioned

media from either CXCL10-CXCR3 or empty vector control cells. Plates were incubated at 37°C for 2 hours, at which point, the cells from the underside of the filter and from the lower chamber were collected. The CyQuant NF cell proliferation assay kit was used to quantify cell number (Invitrogen). Cell invasion was determined by coating the filters with Matrigel before cell placement (BD Biosciences). For CXCL10 neutralization experiments, 120 ng/mL of anti-CXCL10 antibody (R&D Systems) was added to the conditioned media for 1 hour at 37°C before T lymphocyte addition.

### Statistical analysis

Tumor expression associations were tested using  $\chi^2$  or Fisher exact test. For *in vitro* experiments, significance was tested using the student *t* test. As a further exploration, the overall survival (OS-died from breast cancer) differences in 4 combination groups of CXCL10/CXCR3 were studied as well as those in FOXP3-positive versus negative and T-BET-positive versus negative tumors. The follow-up data were collected until November 24, 2011. Excluding the patients lost to follow-up and those with deaths, the minimum follow-up time was 12 months after surgery and the median follow-up time was 148 months. Patient status on November 24, 2011 determined the OS time and censoring status. Kaplan–Meier curves in combination with log-rank tests were used to compare survival of the groups. All tests were 2 sided. A test with a *P* value <0.05 was considered statistically significant. All statistical analyses were conducted using SAS 9.2 software (SAS Inc.) with *P* values unadjusted for multiple testing and Kaplan–Meier plots were carried out by R statistical software version 2.3.0 (<http://www.r-project.org/>).

### Study approval

Approval of the study protocol was obtained from the research ethics boards of Mount Sinai Hospital (Toronto, Canada) and the University Health Network (Toronto, Canada), and written informed consent was received from all study participants.

## Results

### Expression of CXCL10 and clinicopathologic parameters

Forty-five percent of tumors were positive for CXCL10 expression (Fig. 1 and Supplementary Fig. S1). The histopathologic features of the tumors in this cohort are provided in Supplementary Table S2. The relationship between the expression of CXCL10 and histopathologic characteristics of the tumors was examined. CXCL10 expression was found to be significantly associated with the presence of lymphocytic infiltrate (80.1% vs. 65.4%, *P* = 0.0053) and with higher tumor grade (36.4% vs. 26.4%, *P* = 0.0215; Table 1). In addition, expression of CXCL10 was negatively associated with margin circumscription (81.7% vs. 69.8%, *P* = 0.0189). No significant associations were identified between tumor expression of CXCL10 and the genetic subgroups (data not shown) or with tumor size, lymphatic invasion, mitotic score, or lymph node metastasis (Table 1).

### Association of lymphocyte markers with CXCL10 and clinicopathologic parameters

The cellular make-up of the lymphocytic infiltrate was assessed by determining cell surface expression of T-lymphocyte (CD4+ and CD8+) and B-lymphocyte (CD20+) markers in the intratumoral and peritumoral tissue areas (Supplementary Fig. S2 for CD4 and CD8 images). The presence of peritumoral CD4+ as well as CD8+ lymphocytes was positively associated with CXCL10 expression (80.0% vs. 43.4%, *P* = 0.0002 for peritumoral CD4+ lymphocytes; 49.6% vs. 0%, *P* = 0.01 for peritumoral CD8+ lymphocytes; Supplementary Table S3). Intratumoral CD8+ lymphocytes were positively associated with both the *BRCA1* subgroup (20.0% vs. 7.3%, *P* = 0.02) and the basal subtype (31.9% vs. 9.4%, *P* < 0.0001), and negatively associated with ER expression (48.1% vs. 28.0%, *P* = 0.002; data not shown). Intratumoral CD20+ lymphocytes were positively associated with the basal subtype (55.9% vs. 17.3%, *P* < 0.0001), and negatively associated with ER expression (73.5% vs. 34.5%, *P* < 0.0001); however, an

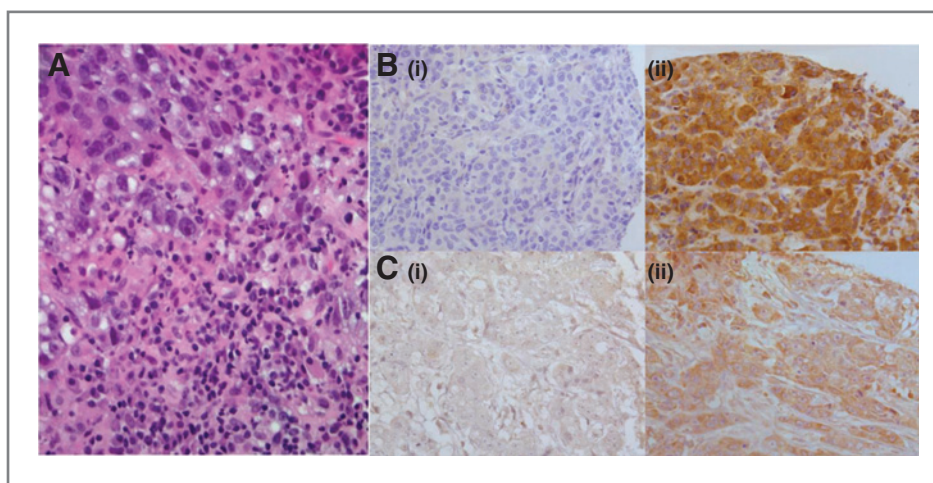


Figure 1. A, invasive breast cancer showing a prominent tumoral lymphocytic infiltrate. B, negative (i) and positive (ii) CXCL10 tumoral expression. C, negative (i) and positive (ii) CXCR3 tumoral expression.

**Table 1.** Association between CXCL10 and clinicopathologic parameters

Tumor characteristics	CXCL10		P <sup>a</sup>
	Positive n (%)	Negative n (%)	
Grade			
High	73 (55.3)	90 (55.2)	0.0215
Moderate	48 (36.4)	43 (26.4)	
Low	11 (8.3)	30 (18.4)	
Size (mm)			
0–20	78 (59.1)	92 (56.8)	0.6911
>20	54 (40.9)	70 (43.2)	
Lymphatic invasion			
Positive	60 (45.4)	61 (37.7)	0.1764
Negative	72 (54.6)	101 (62.3)	
Mitotic score			
1	54 (40.9)	55 (33.8)	0.2048
2 or 3	78 (59.1)	108 (66.2)	
Number of lymph nodes positive			
0	67 (55.4)	82 (54.0)	0.8143
>0	54 (44.6)	70 (46.0)	
Margin circumscription			
Positive	24 (18.3)	49 (30.2)	0.0189
Negative	107 (81.7)	113 (69.8)	
Lymphocytic infiltrate			
Absent	26 (19.9)	56 (34.6)	0.0053
Present	105 (80.1)	106 (65.4)	

<sup>a</sup>P values are from  $\chi^2$  or Fisher exact test and are not adjusted for multiple testing.

association with CXCL10 was not seen (data not shown). Altogether, these results suggest that T lymphocytes, but not B lymphocytes, are attracted by CXCL10 to the tumor site.

#### Association of CXCL10 expression with molecular biomarkers

The expression of CXCL10 was found to be associated with 2 features that are also shared with the basal molecular subtype—p53 expression (40.0% vs. 26.7%,  $P = 0.0191$ ) and a high proliferation index, as determined by Ki-67 (58.5% vs. 35.5%,  $P = 0.0001$ ; Table 2). CXCL10 expression was neither related to the expression of ER, PR, HER2, CK5, CK14, or EGFR (Table 2), nor to molecular (HER2, luminal, and basal) subgroups, as previously defined (data not shown).

#### CXCR3 expression and association with clinicopathologic parameters and molecular biomarkers

Twenty-one percent of tumors were positive for CXCR3 expression (Fig. 1 and Supplementary Fig. S1). CXCR3 expression was associated with low tumor grade (60.8% vs. 40.0%,  $P = 0.0065$ ) and a low mitotic score (51.7% vs. 30.8%,  $P = 0.0102$ ; data not shown). Further analysis

stratified by CXCL10 status revealed that CXCR3 expression was associated with low tumor grade (43.8% vs. 13.6%,  $P = 0.0118$ ) only in the CXCL10 positive group. Furthermore, CXCR3 expression was negatively associated with lymphatic invasion (75.0% vs. 54.9%,  $P = 0.0048$ ; data not shown). No significant association was found between CXCR3 expression and tumor size, lymph node metastasis, margin circumscription, lymphocytic infiltrate, molecular biomarkers, or the molecular or genetic subgroups (data not shown).

#### Coexpression of CXCL10 and CXCR3 in breast cancers

We hypothesized that CXCL10 tumor expression might be related to expression of CXCR3 and found this to be the case (29.8% vs. 11.3%,  $P = 0.0002$ ) as shown in Table 2. On further analysis of tumors that coexpressed both CXCL10 and CXCR3, CXCL10+/CXCR3+ was significantly associated with CK14 expression (29.4% vs. 9.4%,  $P = 0.0027$ );

**Table 2.** Association between CXCL10 and molecular biomarkers

Biomarker	CXCL10		P <sup>a</sup>
	Positive n (%)	Negative n (%)	
ER			
Positive	78 (59.1)	88 (56.4)	0.6464
Negative	54 (40.9)	68 (43.6)	
PR			
Positive	58 (44.3)	81 (51.9)	0.1966
Negative	73 (55.7)	75 (48.1)	
HER2			
Positive	16 (12.0)	12 (7.8)	0.2347
Negative	117 (88.0)	141 (92.2)	
p53			
Positive	52 (40.0)	39 (26.7)	0.0191
Negative	78 (60.0)	107 (73.3)	
CK5			
Positive	40 (31.2)	36 (22.9)	0.1141
Negative	88 (68.8)	121 (77.1)	
CK14			
Positive	19 (15.2)	16 (10.2)	0.2050
Negative	106 (84.8)	141 (89.8)	
EGFR			
Positive	18 (13.9)	19 (13.1)	0.8570
Negative	112 (86.1)	126 (86.9)	
Ki-67			
Positive	76 (58.5)	55 (35.5)	0.0001
Negative	54 (41.5)	100 (64.5)	
CXCR3			
Positive	36 (29.8)	16 (11.3)	0.0002
Negative	85 (70.2)	126 (88.7)	

<sup>a</sup>P values are from  $\chi^2$  or Fisher exact test and are not adjusted for multiple testing.

other clinicopathologic parameters did not show significant associations. We also examined expression of CXCL9 and CXCL11, the other ligands for the CXCR3 receptor, by real time PCR but did not detect significant differences in expression in basal versus ER+ tumors.

#### FOXP3+ expression and association with clinicopathologic parameters and molecular biomarkers

In an effort to determine whether the immune response observed is pro- or antitumor, we examined the expression of FOXP3 as an immunohistochemical method to identify regulatory T cells (Tregs). We found that 32% (73/226) of the tumors were positive for FOXP3 (Supplementary Fig. S3). An association with CXCL10 expression was not seen (Supplementary Table S4). This suggested that at least in terms of Treg cells, CXCL10 is not specific in recruiting this component of the immune system. However, FOXP3 expression was found to be significantly associated with the basal molecular subtype and *BRCA1* genetic group ( $P = 0.0002$  and  $P = 0.0015$ , respectively, Supplementary Table S5), as well as morphologic features characteristic of such tumors including high grade (87.5% vs. 46.7%,  $P < 0.0001$ ), p53 expression (48.6% vs. 29.2%,  $P = 0.0054$ ), ER negativity (56.3% vs. 35.6%,  $P = 0.0036$ ), PR negativity (67.1% vs. 44.7%,  $P = 0.0019$ ), CK5 positivity (38.4% vs. 20.7%,  $P = 0.005$ ), EGF receptor (EGFR) positivity (26.4% vs. 6.1%,  $P < 0.0001$ ), and a moderate lymphocytic infiltrate (44.4% vs. 13.2%,  $P < 0.0001$ ; Supplementary Tables S4 and S6). These results suggest that FOXP3+ cells could also be an important component of the lymphocytic infiltrates of basal and *BRCA1*-associated tumors separate from the immune cells that are specifically chemoattracted by CXCL10.

#### T-BET+ T<sub>H</sub>1 lymphocyte expression and association with clinicopathologic parameters and molecular biomarkers

To further characterize the CD4+ lymphocytic infiltrate in the tumors, we conducted immunohistochemical staining and determined the expression of the T<sub>H</sub>1-associated transcription factor T-BET as CXCL10 is mainly a T<sub>H</sub>1-type chemokine. We found that 19% (40/209) of the tumors were positive for tumoral infiltration by T-BET-positive lymphocytes (Supplementary Fig. S3). T-BET expression was also found to be associated with the basal molecular subtype and *BRCA1* genetic group ( $P = 0.0002$  and  $P = 0.0329$ , respectively; Supplementary Table S5), as well as morphologic features characteristic of such tumors including high grade (84.6% vs. 54.2%,  $P = 0.0021$ ), p53 expression (56.4% vs. 30.4%,  $P = 0.0024$ ), ER negativity (64.1% vs. 39.3%,  $P = 0.0050$ ), PR negativity (74.4% vs. 48.2%,  $P = 0.0032$ ), CK5 positivity (47.5% vs. 23.8%,  $P = 0.0029$ ), EGFR positivity (30.0% vs. 8.6%,  $P = 0.0003$ ), and a moderate lymphocytic infiltrate (48.7% vs. 19.9%,  $P < 0.0001$ ; Supplementary Tables S4 and S6). In addition, when considering only those tumors that had T<sub>H</sub>1 cells present (T-BET positive), 62% (23/37 tumors) were CXCL10 positive ( $P = 0.1390$ ). Even though not statistically

significant, this result supports the T<sub>H</sub>1-type chemokine status of CXCL10, and suggests that CXCL10 could be specifically attracting this particular CD4+ cell type to the tumor.

#### Expression of CXCL10, CXCR3, FOXP3, and T-BET and patient outcome

Finally, in an exploratory analysis, OS differences were examined between patients that fall into the following groups: tumors expressing (i) ligand and receptor (CXCL10+/CXCR3+), (ii) either ligand or receptor (CXCL10+/CXCR3-; CXCL10-/CXCR3+), or (iii) neither ligand nor receptor (CXCL10-/CXCR3-). As shown in Fig. 2A, in the CXCL10+/CXCR3+ group ( $n = 35$ ) we observed 13 deaths (37%) as compared with 54 (24%) in the other 3 groups ( $n = 222$ ). Patients with CXCL10+/CXCR3+ tumors exhibited poor OS compared with the other 3 groups, although the difference did not reach significance ( $P = 0.1421$ ). In addition, we examined OS differences in FOXP3-positive versus negative tumors and T-BET-positive versus negative tumors. While FOXP3 was not associated with a survival difference, T-BET, despite its positive correlation with an aggressive phenotype, was associated with an improved prognosis (Fig. 2B). This finding requires further study as it may identify a population of patients with tumors showing a basal-like phenotype (both sporadic and *BRCA1*-related) that could have a favorable prognosis.

#### Activated CD4+ T lymphocytes show increased migration and invasion towards conditioned media from CXCL10-CXCR3-expressing cells

Because CXCL10 is both secreted by and mainly chemoattracts activated CD4+ T lymphocytes, it was of interest to determine whether these cells would show increased migration and invasion to conditioned media from CXCL10-CXCR3 cells. Both *CXCL10* and *CXCR3* expression vectors were created and simultaneously transfected into the MCF7 human breast cancer cell line, which we previously determined, does not express either of the genes endogenously. ELISA was used to verify CXCL10 overexpression, whereas flow cytometry was used to verify CXCR3 overexpression (Supplementary Fig. S4). After 2 hours of incubation, conditioned media from CXCL10-CXCR3 cells were able to significantly increase the migration (Fig. 3A) as well as Matrigel invasion (Fig. 3B) of the CD4+ T lymphocytes as compared with their migration and invasion towards empty vector conditioned media.

To show that the presence of CXCL10 in the CXCL10-CXCR3 conditioned media was contributing to these results, an anti-CXCL10 antibody was used to neutralize the activity of CXCL10 in the media before starting the Transwell migration. There was no effect on the number of cells that migrated towards empty vector media. However, there was a 33% decrease in the number of cells that migrated to CXCL10-CXCR3 media (Fig. 3C). Even after neutralization, there were still significantly more cells migrating to the CXCL10-CXCR3 media compared with the empty vector media suggesting that neutralization was

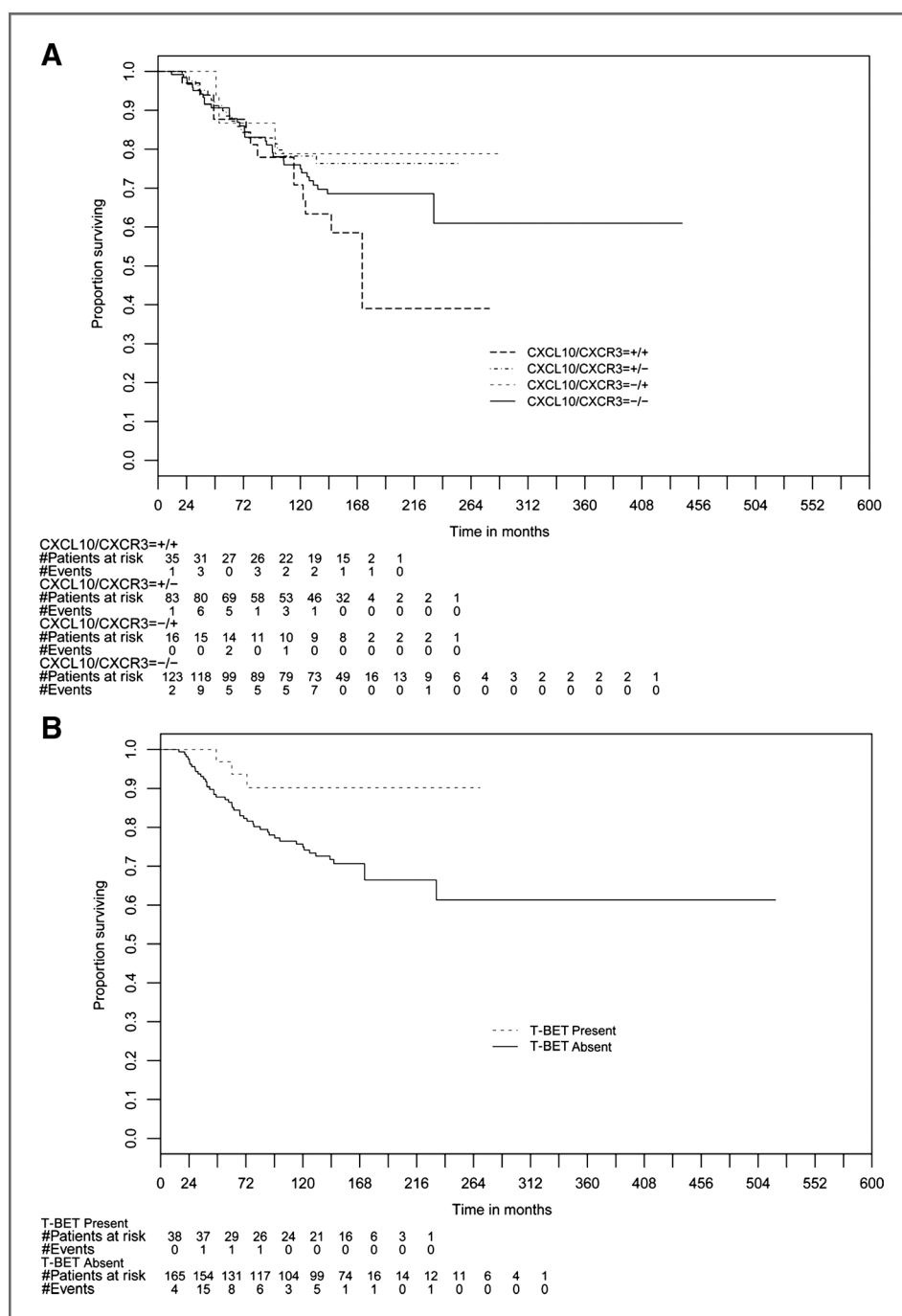


Figure 2. A, Kaplan-Meier survival curves according to expression of CXCL10 and CXCR3. B, Kaplan-Meier survival curves according to the expression of T-BET.

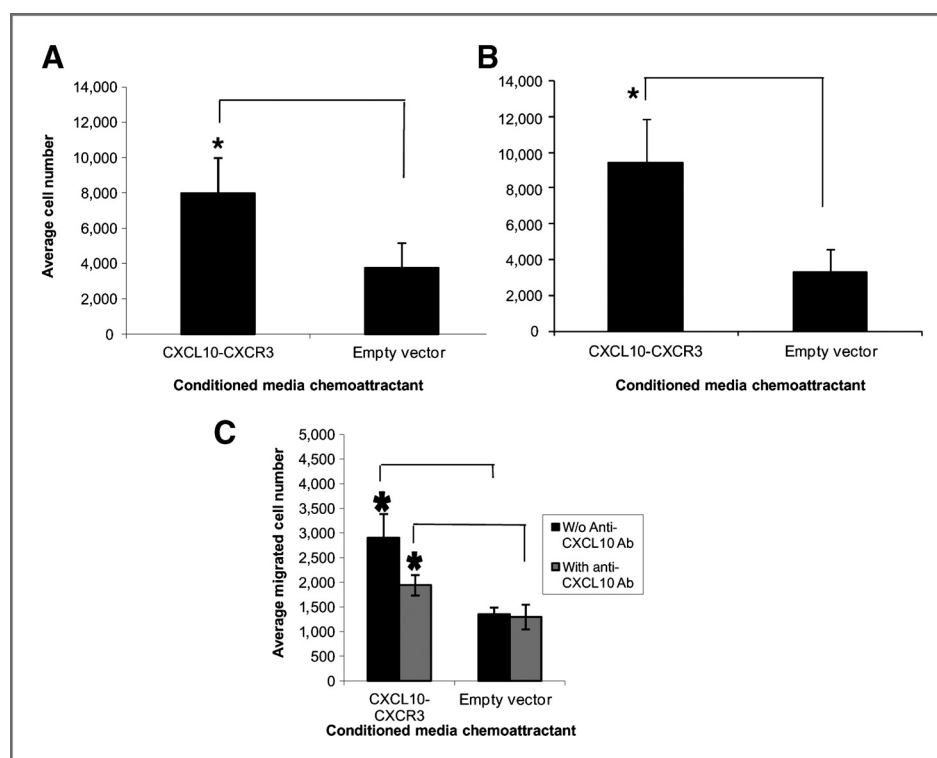
not complete or other factors present in the media were still mediating this effect, though to a lower extent, as neutralizing CXCL10 activity did decrease the number of cells migrating.

**Discussion**

Few studies have examined the expression of CXCL10 and/or its receptor, CXCR3, in breast tumors (29–30). This is the largest study to date to examine this chemokine axis and its association with various clinical and pathologic

parameters in invasive breast carcinoma and, to our knowledge, the only one to examine it in familial breast cancer. We found that CXCL10 expression was significantly associated with lymphocytic infiltrate, suggesting that tumoral expression serves to attract the various CXCR3-expressing immune cells. When we further characterized the cellular make-up of the lymphocytic infiltrate by determining CD4 and CD8 (T lymphocyte) and CD20 (B lymphocyte) cell surface expression, we found that it was only the presence of peritumoral CD4+ and CD8+ T lymphocytes that was significantly

**Figure 3.** Activated CD4+ T lymphocytes show increased migration and invasion towards CXCL10-CXCR3 conditioned media. A, Transwell migration (no Matrigel) of T lymphocytes toward conditioned media from CXCL10-CXCR3 and empty vector cells. Mean  $\pm$  SE ( $n = 9$  replicates); \*,  $P = 0.02$ . B, Transwell invasion (with Matrigel) of T lymphocytes toward conditioned media from CXCL10-CXCR3 and empty vector cells. Mean  $\pm$  SE ( $n = 9$  replicates); \*,  $P = 0.004$ . C, Transwell migration of T lymphocytes toward conditioned media from CXCL10-CXCR3 and empty vector cells incubated without or with anti-CXCL10 antibody for 1 hour before the start of 2-hour cell migration. Mean  $\pm$  SE ( $n = 6$  replicates); \*,  $P < 0.05$ .



associated with CXCL10 positivity, and no association was found with the presence of B lymphocytes. Our characterization of the lymphocytic infiltrate also supported the increased immune cell infiltration known to exist in the basal subtype and with *BRCA1*-associated carcinomas, as the presence of intratumoral CD8+ lymphocytes, as well as FOXP3+ Tregs and T-BET+ T<sub>H</sub>1 cells, was associated with both these groups. Such an association has also been found in other cancers. For example, Clarke and colleagues have shown that the presence of intraepithelial CD8+ T cells significantly correlated with mutation or loss of expression of *BRCA1* in ovarian carcinoma (31). In addition, Mahmoud and colleagues found the total number of FOXP3+ tumor cells in invasive breast cancer to correlate with higher grade, ER negativity, and the basal subtype (32). While we did find Tregs in the tumors, they were not associated with CXCL10 expression. However, their association with both *BRCA1*-associated and basal tumors suggests that they could also be an important component of the lymphocytic infiltrate of these tumors separate from the immune cells that are specifically chemoattracted by CXCL10. Studies suggest that within the tumor microenvironment, Tregs may serve to suppress anticancer cell immunity and to have a tumor-promoting role in the progression of established tumors (33–34). Thus, the presence of these cells in these tumors could serve a protumor role.

Our *in vitro* results showing that conditioned media from MCF7 cells overexpressing CXCL10 and CXCR3 significantly increased both the migration and invasion of activated CD4+ T lymphocytes as compared with empty vector media further supports the possibility that CXCL10 is responsible

for the presence of CXCR3-positive immune cells. Our results also show that the presence of CXCL10 itself in the conditioned media may contribute to this increased migration. These findings are consistent with those of other studies. For example, CXCL10 expression was only found in hepatocellular carcinomas in which there was marked lymphocyte infiltration (4), and CXCL10 expression in melanoma metastases was found to be associated with CD8+ T lymphocyte recruitment (35). In addition, tumor-infiltrating CD8+ T lymphocytes significantly increased with stage progression in a study of patients with stage I–III breast cancer, and Matkowski and colleagues found that patients with high expression of CD4 or CD8 had distinctly worse cancer-specific OS (36–37). In ductal cancers, however, increased CD4 lymphocyte infiltration was linked to more aggressive histologic features, such as higher grade and ER-negative status, and univariate, but not multivariate, analysis was associated with significantly shorter survival (38). Therefore, it is possible that in our cohort, it is the intratumoral CD8+ T lymphocytes in particular that are contributing more to breast cancer progression, and this is the cell type that we have found to associate with the basal molecular subtype and *BRCA1* genetic group.

Lymphocytic infiltration within the cancer milieu has been shown to be of mixed significance. Some reports have shown lymphocytic infiltrate to play an important role in anticancer immunity (39–41) with others showing a role for immune cells in tumor progression (42–43). Studies have shown a role for CXCL10 in the proliferation of certain cell types that express endogenous CXCR3, including



vascular pericytes and smooth muscle cells (44–45). We found CXCL10 tumor cell expression to be associated with higher grade and a high proliferation index. In contrast, expression of its receptor, CXCR3, was found to be associated with favorable prognostic factors. The prognostic significance of tumoral CXCR3 expression has been previously examined with varying results. In breast cancer, Ma and colleagues (30) have found CXCR3 expression to be associated with a poorer OS in early-stage disease. Our apparently contradictory result may be due to the CXCR3-positive tumors not having the same extent of lymphocytic infiltrate, as we did not find an association with lymphocytic infiltrate in those tumors, and thus the inflammatory microenvironment that may contribute to prognosis is not established. Furthermore, when we examined the OS in an exploratory analysis, a possible survival difference was suggested between the CXCL10+/CXCR3+ group and the other 3 groups of ligand/receptor expression. We have found that rather than downregulating expression of its receptor, tumoral CXCL10 expression was positively associated with tumoral CXCR3 expression and, interestingly, the group of patients with both CXCL10- and CXCR3-positive breast cancer showed a worse prognosis. Therefore, it is possible that not only is CXCL10 acting to shape the tumor microenvironment via a paracrine circuit, but also that it is acting in an autocrine manner on the tumor cells themselves, and the presence of both ligand and receptor could contribute to tumor progression. However, as our study was exploratory, and the OS result did not reach statistical significance, further studies to include a larger number of tumor samples and multivariate analysis to control for other factors are necessary to support this hypothesis.

FOXP3 expression has been reported to be associated with poor, good, and neutral outcomes (46). We did not find FOXP3 to be associated with a survival difference and the effect of FOXP3 on outcome remains unclear. Of interest, we found that the presence of T-BET+ lymphocytes was associated with a good prognosis. Recently, Ladoire and colleagues reported that the presence of T-BET+ lymphocytes in peritumoral lymphoid structures post neoadjuvant therapy was associated with improved survival in trastuzumab–taxane–treated patients with HER2-positive breast cancer (47). Our novel finding requires further study as it may identify a population of patients with tumors showing a basal-like phenotype (both sporadic and *BRCA1* related) that could have a favorable prognosis.

Ligand binding to the CXCR3 receptor leads to the activation of the phosphoinositide-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (44, 48), and previous work has shown that the activation of these pathways by CXCL10 binding results in changes in cell shape via actin polymerization, increased cell motility, chemotaxis, migration, and invasion (44, 48–50). It is therefore possible that the CXCL10-CXCR3 axis is acting in an autocrine fashion to increase the migratory capacity of these coexpressing cancer cells and plays a role in their invasiveness. Interestingly, we found CXCL10 protein expression to be negatively associated with margin circum-

scription, suggesting that the tumoral expression of CXCL10 and/or the presence of the associated host inflammatory response, may contribute to increased invasiveness of the leading tumor edge, which, in turn, may contribute to cancer progression.

We have found that tumor expression of CXCL10 is associated with lymphocytic infiltration; this lymphocytic infiltrate was shown to be composed of both CD4+ and CD8+ T lymphocytes, including Tregs and T<sub>H</sub>1 cells. Furthermore, we found that CXCL10 was associated with increased expression of its receptor on tumor cells, and that conditioned media from CXCL10 and CXCR3 coexpressing cells increased the migration and Matrigel invasion of T lymphocytes towards this chemotactic source. Our data suggest that CXCL10 may act in both a paracrine manner, affecting the tumor microenvironment, as well as in an autocrine manner, acting on the tumor cells themselves. Our novel observation that T-BET expression is associated with a better outcome requires further study as it may identify a population of patients with tumors showing a basal-like phenotype (both sporadic and *BRCA1* related) that could have a favorable prognosis. The CXCL10-CXCR3 axis can serve as a potential drug target for *BRCA1*-associated and basal breast cancers, which present with a prominent lymphocytic infiltrate and a poor prognosis and are currently in need of targeted therapy.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Disclaimer

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