Published in final edited form as:

Clin Cancer Res. 2013 January 15; 19(2): 336–346. doi:10.1158/1078-0432.CCR-11-3314.

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

Anna Marie Mulligan^{1,2,*}, Irene Raitman^{3,8,*†}, Linda Feeley⁴, Dushanthi Pinnaduwa³, Linh T. Nguyen⁵, Frances P. O'Malley^{1,2}, Pamela S. Ohashi^{5,6}, and Irene L. Andrulis^{2,3,7,8}

¹Keenan Research Centre, Li Ka Shing Knowledge Institute of St. Michael's Hospital, Toronto, Ontario, Canada ²Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada ³Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada ⁴Department of Pathology, Cork University Hospital, Cork, Ireland ⁵The Campbell Family Institute for Breast Cancer Research, Princess Margaret Hospital, Toronto, Ontario, Canada ⁶Departments of Immunology and Medical Biophysics, University of Toronto, Toronto, Ontario, Canada ⁷Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada ⁸Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada

Abstract

Purpose—Breast carcinomas, including basal and hereditary cases, often present with a prominent tumoral lymphocytic infiltrate (LI). 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 LI 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. Additionally, 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 LI. Further characterization of the LI revealed an association with CXCL10 expression for peritumoral CD4+ and CD8+ lymphocytes. CD8+ intratumoral lymphocytes, FOXP3+ Tregs and T-BET+ Th1 cells were associated with BRCA1 and basal

Corresponding Author: Dr. Irene L. Andrulis, Mount Sinai Hospital, 600 University Avenue, Room 984, Toronto, Ontario, Canada, M5G 1X5.

*Co-first authors

†Department of Molecular Biology, Princeton University, Princeton, New Jersey, United States (Current Affiliation)

Competing Interests: The author(s) declare that they have no competing interests.

Author's Contributions

AMM contributed to study design, interpretation and scoring of biomarkers, interpretation of data, manuscript writing and final approval of manuscript. IR contributed to study design, performing and analysis of *in-vitro* experiments, interpretation of data, manuscript writing and final approval of manuscript. LF contributed to study design, interpretation and scoring of biomarkers, interpretation of data, manuscript writing and final approval of manuscript. DP contributed to collection and assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. LTN contributed to study design of T-lymphocyte experiments, lymphocyte isolation design, flow cytometry, and final approval of manuscript. FOM contributed to conception and design, provision of study material, and revision and final approval of manuscript. PSO contributed to study design of T-lymphocyte experiments, lymphocyte isolation design, and revision and final approval of manuscript. ILA contributed to conception and design, financial support, provision of study material, interpretation of data, manuscript writing and final approval of manuscript.

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 LI and a poor prognosis.

Keywords

breast cancer; tumor-stromal cell interactions; CXCL10; CXCR3; lymphocytic infiltrate; CD4; CD8; FOXP3; T-BET

Introduction

Important roles for chemokines have been demonstrated in inflammation, infection, injury, allergy, as well as in cancer(1). Aside 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 (Interferon-gamma inducible protein 10), as being significantly overexpressed in basal tumors as compared to estrogen receptor (ER) positive tumors (Andrulis et al., in preparation). Secreted by activated T-lymphocytes, endothelial cells, fibroblasts, monocytes, and keratinocytes, it attracts other immune cells, such as activated T-lymphocytes, NK 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 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 (LI) 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 LI(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 LI 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 LI as well as molecular biomarkers and other morphologic and clinical parameters. The make-up of the LI 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. Additionally, we evaluated 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 co-expressing 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 sixty four subjects were selected from the Ontario site of the Breast Cancer Family Registry(15) including 58 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 assessment 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 performed 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, Sun Prairie, WI) were constructed using duplicate 0.6mm 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 performed. Microwave antigen retrieval was carried out in a Micromed T/T Mega Microwave Processing Lab Station (ESBE Scientific, Markham, Ontario, Canada). 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 performed 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 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 Figure S3).

The remaining immunohistochemical stained sections were scored using Allred's 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 2–8. Nuclear staining was scored for ER, PR, p53, and Ki-67 using previously validated cut-offs for ER and PR (>2 = positive) (21–22) and p53 (>3 = positive)(23). A cut-off of 4 was used for Ki-67. Moderate to strong complete membranous staining was assessed for HER2 and the validated cut-off of 5 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 was arbitrarily considered positive for CXCL10 while a score of 3 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 on 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), 10µg/ml bovine insulin (Sigma), and 100µg/ml penicillin-streptomycin (Gibco). This cell line passed authentication and is 100% match to ATCC's HTB-22 (MCF7) STR profile. Media for transfected cells was supplemented with 550µg/ml G418 (MultiCell Technologies). T-lymphocytes were grown in Iscove's Modified Dulbecco's Medium supplemented with 10% human serum (Gemini), 100µg/ml penicillin-streptomycin, 10µg/ml gentamicin (Gibco), 5.5×10^{-5} M 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, while the *CXCR3* sequence was commercially purchased (Open Biosystems Inc.). MCF7 cells were co-transfected with pC-*CXCL10* and pC-*CXCR3*, or the empty vector using FuGene transfection reagent (Roche). Transfected cells were selected for and maintained with 550µg/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 1µg/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 was generated by plating 1×10^7 cells in complete media in 10-cm plates for 24h followed by washing in serum free medium (SFM) and a further incubation in SFM for 24h. Conditioned media was then collected, centrifuged at $1000 \times g$ for 10min, 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 PE isotype-matched control (BD Biosciences). Additionally, the CD4+ T-lymphocytes were co-stained 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 (MFI) for CXCR3 were determined.

Transwell Migration and Invasion Assays

For T-lymphocyte transwell assays 1×10^5 cells/well in 0.5% BSA serum free media were loaded in triplicate into the top chambers of 24-well inserts (5.0 μ m 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 2h, 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 prior to cell placement (BD Biosciences). For CXCL10 neutralization experiments 120ng/ml of anti-CXCL10 antibody (R&D Systems) was added to the conditioned media for 1h at 37°C prior to T-lymphocyte addition.

Statistical Analysis

Tumor expression associations were tested using Chi-Square or Fisher's exact test. For *in-vitro* experiments significance was tested using the student's t-test. As a further exploration, the overall survival (OS-died from breast cancer) differences in the four 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 was to the end of 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 OS time and censoring status. Kaplan Meier (K-M) curves in combination with log rank tests were used to compare survival of the groups. All tests were two-sided. A test with a P-value <0.05 was considered statistically significant. All statistical analyses were performed using SAS 9.2 software (SAS Inc., Cary, NC, USA) with P-values unadjusted for multiple testing and K-M plots were done 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 and the University Health Network, Toronto 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 (Figure 1 and Supplementary Figure 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 using TMAs. CXCL10 expression was found to be significantly associated with the presence of LI (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 LI 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 Figure 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 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 two 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 not related to 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 (Figure 1 and Supplementary Figure 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- 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, LI, molecular biomarkers, or the molecular or genetic subgroups (data not shown).

Co-expression 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 co-expressed both CXCL10 and CXCR3, CXCL10+/CXCR3+ was significantly associated with CK14 expression (29.4% vs. 9.4%, $P=0.0027$); other clinicopathologic parameters did not show significant associations. We also examined expression of CXCL9 and CXCL11, the other ligands for the CXCR3 receptor, by RT-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 if the immune response observed is pro or anti-tumor we examined the expression of FOXP3 as an immunohistochemical method to identify regulatory T cells (Tregs). We found that 32% (73 out of 226) of the tumors were positive for FOXP3 (Supplementary Figure 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%, $P=0.005$), EGFR positivity (26.4% vs. 6.1%, $P<0.0001$), and a moderate LI (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 tumors separate from the immune cells that are specifically chemoattracted by CXCL10.

T-BET+ Th1 Lymphocyte Expression and Association with Clinicopathologic Parameters and Molecular Biomarkers

To further characterize the CD4 positive LI in the tumors we performed immunohistochemical staining and determined the expression of the Th1-associated transcription factor T-BET as CXCL10 is mainly a Th1-type chemokine. We found that 19% (40 out of 209) of the tumors were positive for tumoral infiltration by T-BET-positive lymphocytes (Supplementary Figure 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 morphological 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 LI (48.7% vs. 19.9%, $P<0.0001$) (Supplementary Tables S4 and S6). Additionally, when considering only those tumors that had Th1 cells present (T-BET positive), 62% (23 out of 37 tumors) were CXCL10 positive ($p=0.1390$). Even though not statistically significant, this result supports the Th1-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, overall survival (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 Figure 2A, in the CXCL10+/CXCR3+ group (n=35) we observed 13 deaths (37%) as compared to 54 (24%) in the other three groups (n=222). Patients with CXCL10+/CXCR3+ tumors exhibited poorer OS compared to the other three groups, although the difference did not reach significance (P=0.1421). Additionally, 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 (Figure 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) who could have a favorable prognosis.

Activated CD4+ T-lymphocytes Show Increased Migration and Invasion towards Conditioned Media from CXCL10-CXCR3 Expressing Cells

Since CXCL10 is both secreted by and mainly chemoattracts activated CD4+ T-lymphocytes, it was of interest to determine if 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, while flow cytometry was used to verify CXCR3 overexpression (Supplementary Figure S4). After 2h of incubation conditioned media from CXCL10-CXCR3 cells was able to significantly increase the migration (Figure 3A) as well as Matrigel invasion (Figure 3B) of the CD4+ T-lymphocytes as compared to 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 prior to 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 (Figure 3C). Even after neutralization there were still significantly more cells migrating to the CXCL10-CXCR3 media compared to the empty vector media suggesting that neutralization was not complete or other factors present in the media were still mediating this effect, though to a lower extent, since 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 LI, suggesting that tumoral expression serves to attract the various CXCR3-expressing immune cells. When we further characterized the cellular make-up of the LI 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 associated with CXCL10 positivity, and no association was found with the presence of B lymphocytes. Our characterization of the LI also supported the increased immune cell infiltration known to exist in the basal subtype and with BRCA1 carcinomas since the presence of intratumoral CD8+ lymphocytes, as well as FOXP3+ Tregs and T-BET+ Th1 cells, was associated with both these groups. Such an association has also been found in other cancers. For example,

Clarke et al. have shown that the presence of intraepithelial CD8+ T cells significantly correlated with mutation or loss of expression of BRCA1 in ovarian carcinoma(31). Additionally, Mahmoud et al. 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 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 anti-cancer 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 pro-tumor 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 to 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). Additionally, tumor infiltrating CD8+ T lymphocytes significantly increased with stage progression in a study of stage I–III breast cancer patients, and Matkowski et al. found that patients with high expression of CD4 or CD8 had distinctly worse cancer specific overall survival(36–37). In ductal cancers however increased CD4 lymphocyte infiltration was linked to more aggressive histological features, such as higher grade and ER-negative status, and in 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 these are the cell type 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 LI to play an important role in anti-cancer immunity(39–41) with others demonstrating 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 et al.(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 LI since we did not find an association with LI 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 three groups of ligand/receptor expression. We have found that rather than down-regulating 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, since 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 both 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 et al. reported that the presence of T-BET+ lymphocytes in peritumoral lymphoid structures post neo-adjuvant 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) who could have a favorable prognosis.

Ligand binding to the CXCR3 receptor leads to activation of the PI3K and MAPK pathways(44, 48) and previous work has shown that 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 co-expressing cancer cells and plays a role in their invasiveness. Interestingly, we found CXCL10 protein expression to be negatively associated with margin circumscription, 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 LI was shown to be composed of both CD4+ and CD8+ T lymphocytes, including Tregs and Th1 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 co-expressing 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) who could have a favorable prognosis. The CXCL10-CXCR3 axis can serve as a potential drug target for BRCA1 and basal breast cancers which present with a prominent lymphocytic infiltrate and a poor prognosis and are currently in need of targeted therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Nayana Weerasooriya for her assistance with data preparation.

Funding

This work was supported by the National Cancer Institute, National Institutes of Health under RFA # CA- 06-503 and through cooperative agreements with members of the Breast Cancer Family Registry and Principal Investigators, including Cancer Care Ontario (U01 CA69467). The content of this manuscript does not necessarily reflect the views or policies of the National Cancer Institute or any of the collaborating centers in the BCFR, nor

does mention of trade names, commercial products, or organizations imply endorsement by the US Government or the BCFR.

List of Abbreviations

ER	Estrogen receptor
PR	Progesterone receptor
LI	Lymphocytic infiltrate
TMA	Tissue microarray
EGFR	Epidermal growth factor receptor
HER2	Human epidermal growth factor receptor 2
CK5	Cytokeratin 5
CK14	Cytokeratin 14
OS	Overall survival

References

1. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002; 420:860–867. [PubMed: 12490959]
2. Zipin-Roitman A, Meshel T, Sagi-Assif O, Shalmon B, Avivi C, Pfeffer RM, et al. CXCL10 promotes invasion-related properties in human colorectal carcinoma cells. *Cancer research*. 2007; 67:3396–3405. [PubMed: 17409450]
3. Goldberg-Bittman L, Neumark E, Sagi-Assif O, Azenshtein E, Meshel T, Witz IP, et al. The expression of the chemokine receptor CXCR3 and its ligand, CXCL10, in human breast adenocarcinoma cell lines. *Immunol Lett*. 2004; 92:171–178. [PubMed: 15081542]
4. Hirano S, Iwashita Y, Sasaki A, Kai S, Ohta M, Kitano S. Increased mRNA expression of chemokines in hepatocellular carcinoma with tumor-infiltrating lymphocytes. *J Gastroenterol Hepatol*. 2007; 22:690–696. [PubMed: 17444857]
5. Kondo T, Ito F, Nakazawa H, Horita S, Osaka Y, Toma H. High expression of chemokine gene as a favorable prognostic factor in renal cell carcinoma. *J Urol*. 2004; 171:2171–2175. [PubMed: 15126779]
6. Wooster R, Weber BL. Breast and ovarian cancer. *N Engl J Med*. 2003; 348:2339–2347. [PubMed: 12788999]
7. Lakhani SR, Van De Vijver MJ, Jacquemier J, Anderson TJ, Osin PP, McGuffog L, et al. The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. *J Clin Oncol*. 2002; 20:2310–2318. [PubMed: 11981002]
8. Pathology of familial breast cancer: differences between breast cancers in carriers of BRCA1 or BRCA2 mutations and sporadic cases. Breast Cancer Linkage Consortium. *Lancet*. 1997; 349:1505–1510. [PubMed: 9167459]
9. Lakhani SR, Jacquemier J, Sloane JP, Gusterson BA, Anderson TJ, van de Vijver MJ, et al. Multifactorial analysis of differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations. *J Natl Cancer Inst*. 1998; 90:1138–1145. [PubMed: 9701363]
10. Bane AL, Beck JC, Bleiweiss I, Buys SS, Catalano E, Daly MB, et al. BRCA2 mutation-associated breast cancers exhibit a distinguishing phenotype based on morphology and molecular profiles from tissue microarrays. *Am J Surg Pathol*. 2007; 31:121–128. [PubMed: 17197928]
11. Abd El-Rehim DM, Ball G, Pinder SE, Rakha E, Paish C, Robertson JF, et al. High-throughput protein expression analysis using tissue microarray technology of a large well-characterized series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. *Int J Cancer*. 2005; 116:340–350. [PubMed: 15818618]

12. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*. 2001; 98:10869–10874. [PubMed: 11553815]
13. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, et al. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci U S A*. 2003; 100:10393–10398. [PubMed: 12917485]
14. van de Rijn M, Perou CM, Tibshirani R, Haas P, Kallioniemi O, Kononen J, et al. Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. *Am J Pathol*. 2002; 161:1991–1996. [PubMed: 12466114]
15. John EM, Hopper JL, Beck JC, Knight JA, Neuhausen SL, Senie RT, et al. The Breast Cancer Family Registry: an infrastructure for cooperative multinational, interdisciplinary and translational studies of the genetic epidemiology of breast cancer. *Breast Cancer Res*. 2004; 6:R375–R389. [PubMed: 15217505]
16. Ozcelik H, Antebi YJ, Cole DE, Andrulis IL. Heteroduplex and protein truncation analysis of the BRCA1 185delAG mutation. *Hum Genet*. 1996; 98:310–312. [PubMed: 8707299]
17. Yazici H, Bitisik O, Akisik E, Cabioglu N, Saip P, Muslumanoglu M, et al. BRCA1 and BRCA2 mutations in Turkish breast/ovarian families and young breast cancer patients. *Br J Cancer*. 2000; 83:737–742. [PubMed: 10952777]
18. Ozcelik H, Schmocker B, Di Nicola N, Shi XH, Langer B, Moore M, et al. Germline BRCA2 6174delT mutations in Ashkenazi Jewish pancreatic cancer patients. *Nat Genet*. 1997; 16:17–18. [PubMed: 9140390]
19. Longacre TA, Ennis M, Quenneville LA, Bane AL, Bleiweiss IJ, Carter BA, et al. Interobserver agreement and reproducibility in classification of invasive breast carcinoma: an NCI breast cancer family registry study. *Mod Pathol*. 2006; 19:195–207. [PubMed: 16341153]
20. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol*. 1998; 11:155–168. [PubMed: 9504686]
21. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol*. 1999; 17:1474–1481. [PubMed: 10334533]
22. Mohsin SK, Weiss H, Havighurst T, Clark GM, Berardo M, Roanh le D, et al. Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study. *Mod Pathol*. 2004; 17:1545–1554. [PubMed: 15272277]
23. Done SJ, Arneson CR, Ozcelik H, Redston M, Andrulis IL. P53 protein accumulation in non-invasive lesions surrounding p53 mutation positive invasive breast cancers. *Breast Cancer Res Treat*. 2001; 65:111–118. [PubMed: 11261826]
24. O'Malley FP, Parkes R, Latta E, Tjan S, Zadro T, Mueller R, et al. Comparison of HER2/neu status assessed by quantitative polymerase chain reaction and immunohistochemistry. *Am J Clin Pathol*. 2001; 115:504–511. [PubMed: 11293897]
25. Liu CL, Prapong W, Natkunam Y, Alizadeh A, Montgomery K, Gilks CB, et al. Software tools for high-throughput analysis and archiving of immunohistochemistry staining data obtained with tissue microarrays. *Am J Pathol*. 2002; 161:1557–1565. [PubMed: 12414504]
26. Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, et al. Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. *Mod Pathol*. 2006; 19:264–271. [PubMed: 16341146]
27. Kornegoor R, Verschuur-Maes AH, Buerger H, Hogenes MC, de Bruin PC, Oudejans JJ, et al. Molecular subtyping of male breast cancer by immunohistochemistry. *Mod Pathol*. 2012; 25:398–404. [PubMed: 22056953]
28. Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, Kennecke H. Breast cancer subtypes and the risk of local and regional relapse. *J Clin Oncol*. 2010; 28:1684–1691. [PubMed: 20194857]
29. Datta D, Flaxenburg JA, Laxmanan S, Geehan C, Grimm M, Waaga-Gasser AM, et al. Ras-induced modulation of CXCL10 and its receptor splice variant CXCR3-B in MDA-MB-435 and MCF-7 cells: relevance for the development of human breast cancer. *Cancer Research*. 2006; 66:9509–9518. [PubMed: 17018607]

30. Ma X, Norsworthy K, Kundu N, Rodgers WH, Gimotty PA, Goloubeva O, et al. CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model. *Mol Cancer Ther.* 2009; 8:490–498. [PubMed: 19276169]
31. Clarke B, Tinker AV, Lee CH, Subramanian S, van de Rijn M, Turbin D, et al. Intraepithelial T cells and prognosis in ovarian carcinoma: novel associations with stage, tumor type, and BRCA1 loss. *Mod Pathol.* 2009; 22:393–402. [PubMed: 19060844]
32. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Lee AH, Ellis IO, et al. An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. *Breast Cancer Res Treat.* 2011; 127:99–108. [PubMed: 20556505]
33. Somasundaram R, Jacob L, Swoboda R, Caputo L, Song H, Basak S, et al. Inhibition of cytolytic T lymphocyte proliferation by autologous CD4+/CD25+ regulatory T cells in a colorectal carcinoma patient is mediated by transforming growth factor-beta. *Cancer Res.* 2002; 62:5267–5272. [PubMed: 12234995]
34. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res.* 2001; 61:4766–4772. [PubMed: 11406550]
35. Harlin H, Meng Y, Peterson AC, Zha Y, Tretiakova M, Slingluff C, et al. Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. *Cancer Res.* 2009; 69:3077–3085. [PubMed: 19293190]
36. Sheu BC, Kuo WH, Chen RJ, Huang SC, Chang KJ, Chow SN. Clinical significance of tumor-infiltrating lymphocytes in neoplastic progression and lymph node metastasis of human breast cancer. *Breast.* 2008; 17:604–610. [PubMed: 18656354]
37. Matkowski R, Gisterek I, Halon A, Lacko A, Szewczyk K, Staszek U, et al. The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer. *Anticancer Res.* 2009; 29:2445–2451. [PubMed: 19596912]
38. Drieser R, Zlobec I, Kilic E, Güth U, Heberer M, Spagnoli G, et al. Differential pattern and prognostic significance of CD4+, FOXP3+ and IL-17+ tumor infiltrating lymphocytes in ductal and lobular breast cancers. *BMC Cancer.* 2012; 12:134. [PubMed: 22471961]
39. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *New England Journal of Medicine.* 2003; 348:203–213. [PubMed: 12529460]
40. Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, et al. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med.* 2008; 14:518–527. [PubMed: 18438415]
41. Teschendorff AE, Miremadi A, Pinder SE, Ellis IO, Caldas C. An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. *Genome Biol.* 2007; 8:R157. [PubMed: 17683518]
42. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell.* 2008; 141:39–51. [PubMed: 20371344]
43. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer.* 2006; 6:24–37. [PubMed: 16397525]
44. Bonacchi A, Romagnani P, Romanelli RG, Efsen E, Annunziato F, Lasagni L, et al. Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes. *J Biol Chem.* 2001; 276:9945–9954. [PubMed: 11136732]
45. Wang X, Yue TL, Ohlstein EH, Sung CP, Feuerstein GZ. Interferon-inducible protein-10 involves vascular smooth muscle cell migration, proliferation, and inflammatory response. *J Biol Chem.* 1996; 271:24286–24293. [PubMed: 8798675]
46. deLeeuw RJ, Kost SE, Kakal JA, Nelson BH. The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature. *Clin Cancer Res.* 2012; 18:3022–3029. [PubMed: 22510350]
47. Ladoire S, Arnould L, Mignot G, Apetoh L, Rébé C, Martin F, et al. T-bet expression in intratumoral lymphoid structures after neoadjuvant trastuzumab plus docetaxel for HER2-overexpressing breast carcinoma predicts survival. *Br J Cancer.* 2011; 105:366–371. [PubMed: 21750556]

48. Kouroumalis A, Nibbs RJ, Aptel H, Wright KL, Kolios G, Ward SG. The chemokines CXCL9, CXCL10, and CXCL11 differentially stimulate G alpha i-independent signaling and actin responses in human intestinal myofibroblasts. *J Immunol.* 2005; 175:5403–5411. [PubMed: 16210647]
49. Kelsen SG, Aksoy MO, Yang Y, Shahabuddin S, Litvin J, Safadi F, et al. The chemokine receptor CXCR3 and its splice variant are expressed in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol.* 2004; 287:L584–L591. [PubMed: 15155273]
50. Shahabuddin S, Ji R, Wang P, Brailoiu E, Dun N, Yang Y, et al. CXCR3 chemokine receptor-induced chemotaxis in human airway epithelial cells: role of p38 MAPK and PI3K signaling pathways. *Am J Physiol Cell Physiol.* 2006; 291:C34–C39. [PubMed: 16467404]

Statement of 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+ Tregs and T-BET+ Th1 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 in both 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 outcome in 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.

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.

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

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

Table 1

Association between CXCL10 and Clinicopathologic parameters

Tumor Characteristic	CXCL10				P* -value
	Positive		Negative		
	n	%	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 LN_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	

* P-values are from Chi-square or Fisher's exact test and are not adjusted for multiple testing.

Table 2

Association between CXCL10 and Molecular Biomarkers

Biomarker	CXCL10				P [*] -value
	Positive		Negative		
	n	%	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

\$watermark-text

\$watermark-text

\$watermark-text

Biomarker	CXCL10				P*-value
	Positive		Negative		
	n	%	n	%	
Negative	85	70.2	126	88.7	

* P-values are from Chi-square or Fisher's exact test and are not adjusted for multiple testing.