

Prognostic Value of Measurable Th₁ Immune Response in the Tumors and Lymph Nodes of Patients with Lymph Node Positive and Lymph Node Negative Breast Cancer

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Abstract

Background

The functional significance of immune cell subsets may provide important information for maximizing the prognostic value of immunoscore.

Objective

We hypothesize that immune cell population and T cell activation status (Th₁ type) in the resected breast tumors and sentinel Lymph Nodes (LN) may serve as immunological biomarkers of tumor aggressiveness and offer useful prognostic information to facilitate specific therapy related clinical decision.

Methods

Biopsies from two groups of patients were examined for T cell activation status, 1) lymph node negative Breast Cancer (BC) and; 2) lymph node positive BC. Tissue slides from sentinel node biopsies and tumor resections from twenty four women who underwent sentinel node biopsy with invasive breast cancer resection were dual stained for CD3/IL-10, CD3/IFN- γ or triple stained for CD3/CD20/CD68. Slides were imaged using a Digital Scanner, the density and intensity of each cell type and cytokine was recorded as the number of positive cells per unit tissue surface area.

Results

Our data show that patients who had no evidence of disease after 5 years showed significantly higher IFN- γ expression in BC ($p < 0.02$) and LN ($p < 0.03$) as well as increased infiltration of T cells in both BC ($p < 0.03$) and LN ($p < 0.02$) biopsies while patients who progressed rapidly had no expression of IFN- γ in the BC or in the LN.

Conclusions

Understanding of the immune cell environment within the tumors and lymph nodes may have implications for improving clinical outcome of cancer patients.

Keywords

Tumor Infiltrating Lymphocytes; Breast Cancer; Immunohistochemistry; Immunoscore

Introduction

Although T cell infiltration is recognized to be of positive prognostic significance in a broad range of solid tumors [1-11]. Early analyses in colorectal carcinoma show that the majority of patients with stage I and stage II cancer who lack a T cell infiltrate develop disease recurrence within 5 years, whereas the presence of a T cell infiltrate in patients with stage III cancer can predict an unusually long disease-free interval [4]. A subset of patients with breast cancer, renal cell carcinoma, melanoma, ovarian cancer and gastrointestinal stromal tumors with spontaneous T cell infiltrate have similar positive prognostic value

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[12-16]. A high ratio of CD8⁺ T cells to Foxp3⁺ regulatory T cells (T_{reg} cells) in the ovarian tumor microenvironment has been associated with a favorable clinical outcome [17]. Immune scoring of lymph nodes (LN) continues to evolve and is not clearly defined for sentinel lymph nodes in breast cancer (BC). Identifying and quantitating the number and Th₁ (interferon-gamma, IFN- γ) and Th₂ (interleukin-10, IL-10) phenotypes in the sentinel LN is likely to more clearly delineate the functional significance of T cells and various immune cell subsets, and may provide important information for maximizing the prognostic value of immunoscore. There was a 20-30% relapse rate in early stage breast cancer patients with no detectable lymph node invasion, while one-third of LN positive BC patients remain free of distant metastasis [18]. In other words, the prognostic gap remains and the vast majority of the patients in both groups had no prognostic marker to assist in driving clinical management based on risk factors. Understanding of the immune cell environment within the tumors and lymph nodes may have implications for improving clinical outcome of cancer patients.

In this report we asked a question if there is a quantifiable immune response in the tumors and sentinel lymph nodes of breast cancer patients and whether immunoscore and immune profiling is suggestive of clinical outcomes of cancer patients. Tissue sections from breast cancer biopsies along with auxiliary lymph nodes were stained for cancer-infiltrating immune cells (T and B lymphocytes and macrophages) as well as IFN- γ and IL-10, which can stimulate or suppress anti-tumor immune mechanisms, respectively.

Since the nodal immune environment is influenced in part by tumor-derived factors [19], we tested a small cohort for cytoplasmic expression of IFN- γ and IL-10 to explore the association between Th₁ and Th₂ status of the CD3⁺ T cells along with the CD20⁺ B cells and CD68⁺ macrophages in tumor and lymph node biopsies with clinical outcomes of breast cancer patients. Our exploratory studies showed that patients who had no evidence of disease after 5 years showed IFN- γ expression while patients who progressed rapidly had no expression of IFN- γ by T cells in the tumors or in the lymph nodes.

Material and Methods

Patients and tumor tissues

Breast cancer patients included in this study (n = 24) were seen at the Karmanos Cancer Institute, patients who underwent surgery signed the Institutional Review Board-approved informed consent releasing the use of specimens (tissues) for research purposes. The tissues were processed and banked at the Wayne State University Tissue Procurement Facility, tumor and lymph node slides were obtained from 24 patients undergoing surgery for treatment of primary disease. None of the patients received neoadjuvant chemotherapy.

Immunohistochemical staining

All tissue blocks were sectioned at 4 μ m and mounted on positively charged glass slides. Tissue sections were stained with monoclonal antibodies recognizing CD3 (T cells), CD20 (B-cells), and CD68 (Macrophages) for immune cell population scoring. For cytokine detection, antibodies recognizing IFN- γ and IL-10 were used. Standardization of the dilution, incubation, and development times appropriate for each antibody allowed an accurate comparison of expression levels in all cases. In the entire antibody staining studies conducted, positive and negative control slides were analyzed in parallel. Before use, all mAbs were titrated using breast biopsy and lymph node sections. Staining was carried out on paraffin embedded resected breast tissue sections by dual staining for Th₁ using anti-CD3/anti-IFN- γ and Th₂ using anti-CD3/anti-IL-10 expression. For double staining, HRP Polymer anti-Mouse secondary antibody and AP Polymer anti-Rabbit secondary antibody was used (GBI Labs Polink DS-MR-Hu A1 Kit). Color was developed using DAB Chromogen and GBI Permanent Red Chromogen followed by counter stain with hematoxylin. Isotype-matched mouse monoclonal

antibodies were used as negative controls. Second set of slides were triple stained with anti-CD3/anti-CD19/anti-CD68 antibody cocktail, using automated protocol on a Ventana Discovery Ultra instrument (Roche Diagnostics, Ventana Medical Systems, Tucson, AZ). Sections of human tumor known to contain abundant CD8⁺ lymphocytes were used as positive controls. Slides were imaged using a Panoramic Digital Scanner and Viewer (Caliper Life Sciences) for semi-quantitative evaluation of the density and intensity of each cell type and cytokine. A breast pathologist at the KCI reviewed H&E and all stained slides from tumor and LN biopsies for scoring.

Manual scoring criteria for Intensities and Cell Densities of Immunostained Samples

Immunostaining for cytokines and different cell populations were scored semi-quantitatively for both the intensity and the density of immune cell populations by 2 trained personnel for all sample sets in a blinded fashion. Scoring for staining intensity and cell densities are shown Table 1. Semi-quantitative analysis of IHC stained slides for tumor infiltrating immune cells (TIIC) was performed on paired tumor and lymph node sections. The TIIC were evaluated for overall impression through microscopic magnification (40x) followed by a higher magnification (100x-200x) fields for tumor margin, tumor stained stroma and tumor center. Cell densities for triple stained slides were scored as 0 (-) for the absence of specific immune cells, 1 (+) for sparse 2% - 10% positive staining, 2 (++) for moderately dense = 11% - 30% positive staining, 3 (+++) for dense = 31% - 60% positive staining and 4 (+++++) for very dense = 61% - 100% positive staining of CD3⁺ T cells [Purple or brown in dual stained samples], CD20⁺ B cells [Brown] and CD68⁺ macrophages [Yellow]. Intensities were scored as 0 (-) for absence of IL-10 and IFN- γ , 1 (+) for weak staining, 2 (++) for moderate staining, 3 (+++) for strong and 4 (+++++) for very strong expression of extracellular and intracellular staining of IFN- γ and IL-10.

Algorithm to analyze staining densities and Intensities of stained sections

The Python programming language (version 3.5) was chosen for this project due to its open source, high readability, quick to develop and well-supported by computing packages. The third-party packages we used include Num Py for efficient multidimensional array and linear algebra operations, Matplotlib for visualizations, and Scikit-Image for image operations and several computer vision algorithm implementations. The source code and documentation are accessible through a repository on GitHub (search "Micro Deconvolution"). The digital images were imported into Micro Deconvolution software and analyzed using the program's ability to differentiate immunostained cells and hematoxylin-stained nuclei as well as extracellular staining. Software based scoring was called Digital Scoring (DS).

Validation

To validate DS results, we first performed an acceptability check of the DS data; high-level visual assessment was performed at high resolution for each slide to identify major errors in the DS, such as

Intensity Score	Interpretation
0	Not present, no staining noted or completely non-specific (based on isotype control)
1	Weak staining (cell surface/cytoplasmic and/or extracellular staining)
2	Moderate staining (cell surface/cytoplasmic and/or extracellular staining)
3	Strong staining (cell surface/cytoplasmic and/or extracellular staining)
4	Very strong staining (cell surface/cytoplasmic and/or extracellular staining)

Table 1: Shows the manual scoring criteria for intensity

incorrect exclusion of histological artifacts etc. In parallel stained slides were validated by a pathologist and two scientists blindly manually for Scoring (MS).

Results

Manual scoring of cell densities and expression intensities of immunostained sections

In Representative IHC results for several paired samples are presented in Figures. 1A-1D showing negative (-) or + to +++ scores for BC and LN specimens. In general patients with progressive disease (PD) show low infiltration of T cells (Figure 1A) and complete lack of IFN- γ compared to patients with no evidence of disease (NED) (Figure 1B). One progressive disease case (P-636) showed a large infiltration of CD3+ T cells (+++) and macrophages (++) but complete lack of IFN- γ (Figure 1A) suggesting that Th₁ response may be of better prognostic significance (Figure 1A and 1B). Figure 1C shows various intensities of CD3+ T cells, IFN- γ and IL-10 scored as + to +++ for BC and LN specimens. Figure 1D shows densities of CD+ T cells, B cells and macrophages (Top panel) and intensities of CD3+ T cells,

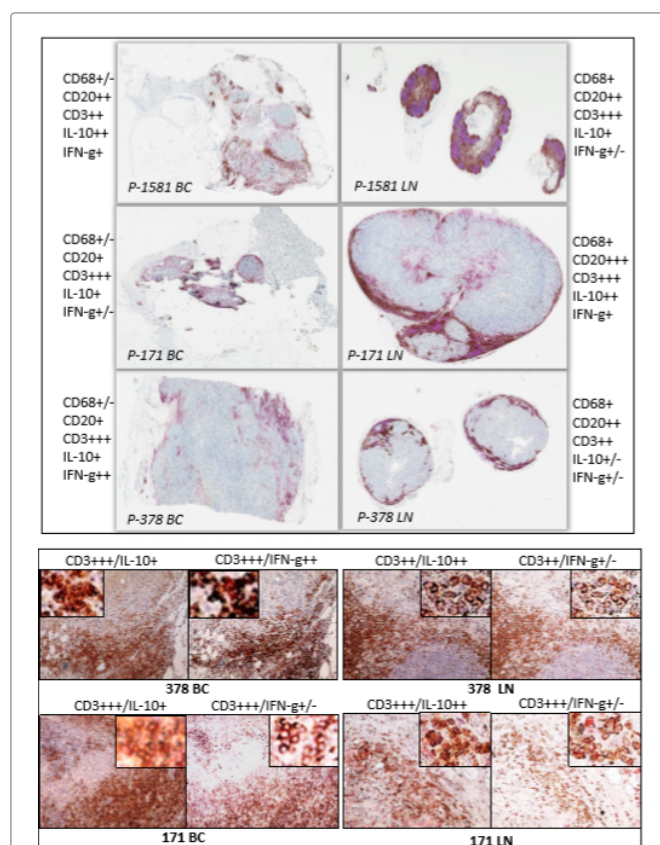
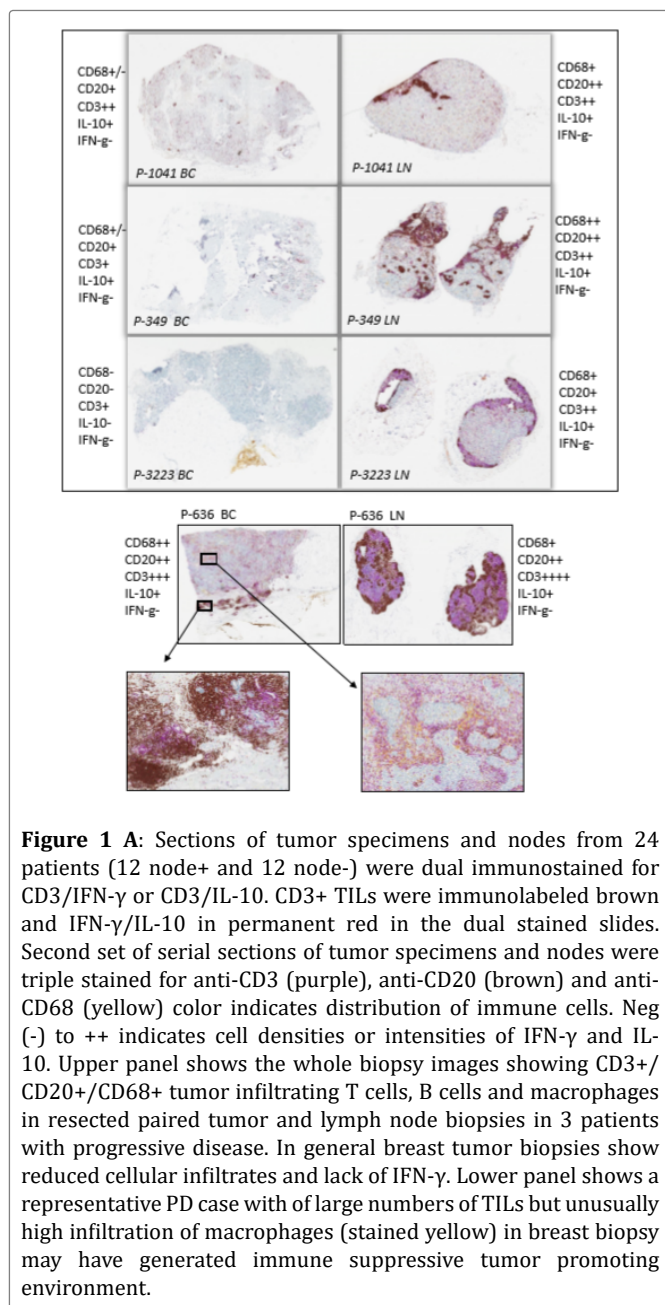
IFN- γ and IL-10 scored as + to +++ in LN specimens (Lower panel).

Digital scoring (DS) and validation of cd3+ t cells in tumor and lymph node sections

We used automated computer vision algorithms that provided a unique ability to analyze IHC dual or multicolor stained specimens fast and accurately. Using color deconvolution and random walker segmentation, when combined in series, showed consistent segmentation ability. The numbers of CD3+ lymphocytes determined by algorithm based DS were compared with those scored semi-quantitatively by the 2 trained personnel for all sample sets in a blinded fashion. We found that the majority of CD3+ lymphocytes that were evident microscopically were detected by the software (Figure 2). The overall numbers of CD3+ cells counted by our software and microscopic evaluation based scoring (manual scoring) showed good agreement (Table 3).

Validation of digital scoring of dual stained CD3/IFN- γ and CD3/IL-10 tumor and lymph node sections

We compared the DAB global threshold with intensity conservation at T = 0.5 (50%) and, adaptive thresholds to correct the regional variations in lighting by utilizing localized intensity information to determine the threshold value for each pixel based on the defined regional block size [20,21]. We concluded that our DS could accurately measure CD3+ TILs and IFN- γ or IL-10 as part of



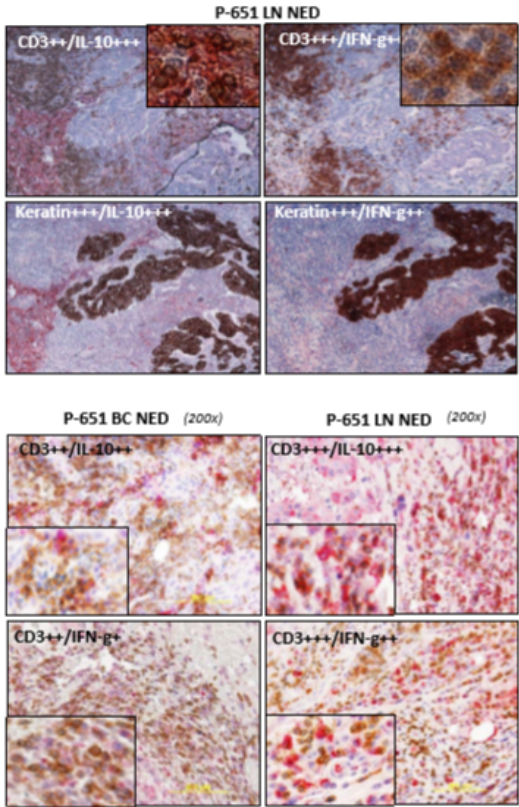


Figure 1 C: Upper panel show serial sections of tumor specimens and nodes dual immunostained for CD3/IL-10 or CD3/IFN-γ and Keratin/IL-10 or Keratin/IFN-γ densities and intensities, respectively, from a representative NED patient. CD3/Keratin was immunolabeled brown and IFN-γ/IL-10 in permanent red in the dual stained slides. Lower panel shows the TILs dual immunostained for CD3/IL-10 (Top two images) and bottom two images show CD3/IFN-γ in BC biopsy (left) and LN biopsy (right).

the dual stain and was comparable to manual scoring. These findings support the use of DS to analyze cell densities, intracellular and extracellular intensities in the TME. The DS algorithm we developed should, however, provide a useful analytical tool for multi-color staining analysis (Figures 3 and 4).

Comparison of tumor and LN biopsies in patients with no evidence of disease versus progressive disease

We scored IHC stained slides for cellular infiltrates and cytokines in paired tumor (n=24) and LN (n=24) biopsies for CD3, CD20, CD68, IL-10 and IFN-γ. *Tumor biopsies.* Tumor (BC) biopsies of patients with no evidence of disease had significantly increased infiltration of CD3+ T cells (p<0.03) compared to patients with progressive disease (median score for T cells, 2.5 vs 1.0) and significantly lower macrophage infiltration (p<0.03) compared to patients with progressive disease (median score for macrophages, 1.0 vs 2.0). Median score of B cells was 1.5 fold higher in patients with no evidence of disease. Striking difference was seen for Th₁ cytokine IFN-γ (p<0.02), all eight patients with progressive diseases showed no expression of IFN- γ in tumor biopsies while scores ranged between 0.5-1.0 in 8/16 patients with no evidence of disease (median score of 0.25). No changes were observed for IL-10 expression between two groups. *LN biopsies.* In LN biopsies, significantly higher relative score for T cells (p<0.02) and increased B-cell infiltration in patients with no evidence of disease compared to patients with progressive disease. No changes were observed for macrophages between two groups. In order to determine the functional basis of immune cell infiltration profiles we analyzed the IFN- γ levels, a marker of Th₁ response, and IL-10, a Th₂ mediator, by IHC in node

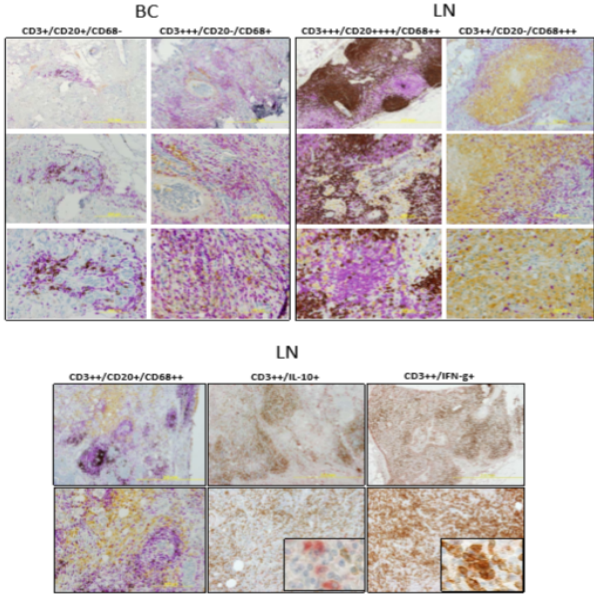


Figure 1 D: Upper panel show serial sections of tumor specimens and nodes triple immunostained for T cells (CD3, purple), B cells (CD20, brown) and macrophages (CD68, yellow) showing low to high score of CD3+ T cells, CD20+ B cell and CD68+ macrophages at 40x, 100x and 200x magnification (Top to bottom). Lower panel shows the nodes triple immunostaining for TILs and macrophages (Left at 100x and 200x magnification). Middle and right images show dual immunostained for CD3/IL-10 (middle) and CD3/IFN-γ in LN biopsy (right) with overall score of 1(+) for both IL-10 and IFN-γ.

Density Score	Interpretation
0	No Staining
1	Sparse = 2% - 10% Positive staining
2	Moderately Dense =11% - 30% Positive staining
3	Dense =31% - 60% Positive staining
4	Very Dense =61% - 100% Positive staining

Table 2. Manual density scoring criteria.

positive and node negative breast and LN biopsies. IFN- γ / IL-10 expression was scored negative or + to +++. Similar to tumor (BC) biopsies, IFN-γ expression was significantly higher (p<0.03) in patients with no evidence of disease compared to patients with progressive disease (median score of 1 vs 0.2). IL-10 was also higher in no evidence of disease patients compared to patients with progressive disease (1.5 vs 1). Figure 5 show the IHC scores for paired tumor (BC)/LN samples. These data suggest that patients with no evidence of disease showed significantly higher IFN- γ expression, consistent with a dominant Th₁ response profile, compared to patients with progressive disease (Table 3).

Discussion

We conducted a pilot study to determine if there is a measurable immune response in the tumors and lymph nodes of patients with lymph node positive and lymph node negative breast cancer. To best of our knowledge, there has not been a study looking at both metastasis positive and metastasis negative lymph nodes in patients with clinical outcomes and comparing the T cells, *T cell activation status*, B cells and macrophage infiltration between them by IHC. Lymph node invasion is one of the most powerful clinical factors

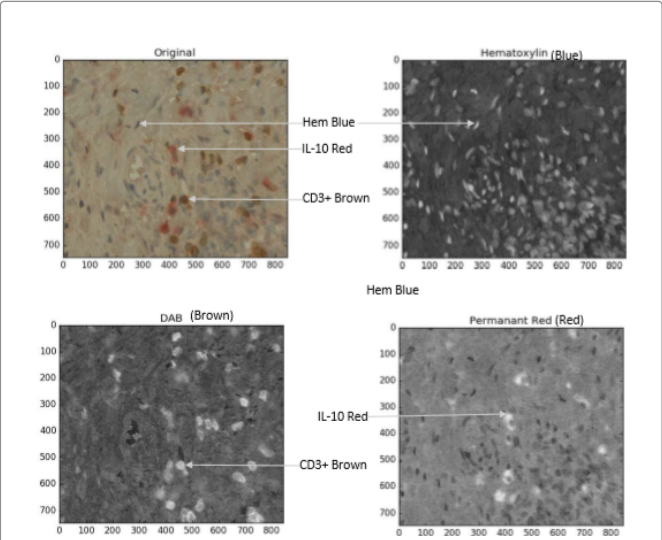


Figure 2: Shows color deconvolution for three stain channels DAB (brown for CD3+ T cells), Permanent red (IL-10) and Hematoxylin for nuclear staining (blue). White signifies high intensity. IHC specimen stained for CD3 with DAB, Hematoxylin, and IL-10 with permanent red. After the stain intensity map is generated for each stain, global threshold segmentation algorithm was used to quantify the resulting stain. In this process a threshold number is pre-defined and all pixels below that value of intensity are marked as negative. The pixels above that value can either be marked as positive to produce a binarized result or the intensity information can be conserved for analysis.

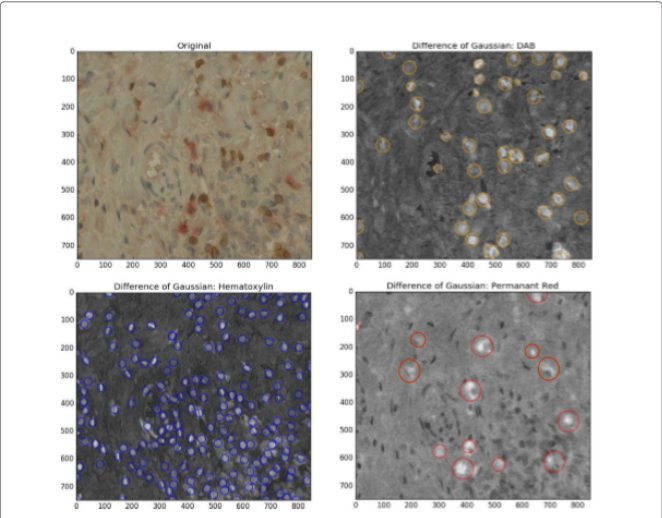


Figure 3: Shows T-cell, cell nuclei, and IL-10 blob detection. Comparison of DAB global threshold with intensity conservation at T = 0.5 (50%). Adaptive thresholds were used to correct for regional variations in lighting by utilizing localized intensity information to determine the threshold value for each pixel based on the defined regional block size. IHC specimen stained for CD3 with DAB (upper right), Hematoxylin (lower left), and IL-10 (lower right) with permanent red. Specimen stained for CD3 with DAB shows 42 stained cells, IL-10 stained with permanent red show 14 cells expressing IL-10, and a hematoxylin counterstain shows 188 nuclei staining.

in cancer prognosis. Most of the studies examined the immune environment surrounding the primary breast tumor, but the immune environment in lymph nodes, which are removed as part of the surgical management of invasive breast cancer, have not been studied

CD3 in BC		CD3 in LN	
DS	MS	DS	MS
1	1	3	3
2	2	4	4
4	4	4	4
3	3	4	4
3	3	2	1
2	2	3	3
1	2	3	3
2	2	1	1
3	3	1	1
2	2	3	4
2	1	3	2
1	1	3	3
3	3	4	4
4	4	3	2
3	3	4	4
3	3	4	4
1	1	2	1
2	1	4	3
1	1	1	2
1	1	1	2
1	1	1	1
1	2	2	2
2	2	2	3
3	3	2	3
P value: NS		P value: NS	

Table 3: Compares the digital scoring (DS) with manual scoring (MS) for CD3+ T cells in tumors (BC) and lymph nodes (LN)

extensively. Understanding of the immune cell environment within the tumors and lymph nodes may have implications for improving clinical outcome of cancer patients. There are indications that the immune environment within axillary lymph nodes can be used for prognostication [22,23].

We obtained the FFPE blocks from twenty four women with invasive breast cancer who underwent tumor resection with sentinel node biopsy from Pathology Core Services, Wayne State University, Detroit during 2008. This study reports a comprehensive investigation the T cell activation status by measuring Th₁ (IFN- γ or Th₂ (IL-10) cytokines by immunohistochemical staining for CD3/IFN- γ or CD3/IL-10 in dual staining fashion as well as spatial configuration and ratios of CD3/CD20/CD68 not only in the TME but also in the sentinel lymph nodes by triple immunostaining, which may play a role in promoting or limiting metastasis. We scored the dual stained CD3+ T cells in the context of IL-10 and IFN- γ and scores were then compared to the DS of the same 24 tumor and lymph node sections. The DS results showed that the overall numbers of CD3+ TILs detected in the dual staining were highly comparable to those detected manually (Table 3), suggesting that our DS could accurately measure surface stained CD3+ T cells as well as intra and extra-cellular expression of IL-10 or IFN- γ .

In spite of a small sample size, our pilot study show that BC patients with no evidence of disease (NED) had significantly increased expression of T cells and IFN- γ and decreased infiltration of macrophages in BC biopsies. Similarly in LN biopsies, CD3+ T cells

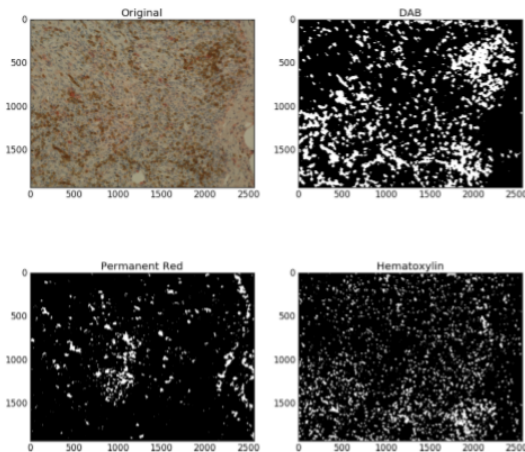


Figure 4: Program output parameter calculated value show DAB (CD3) percent coverage of 20.3%; Permanent red (IL-10) percent converge of 5.1%; Hematoxylin percent coverage of 10.8% and DAB & hematoxylin area combined 27.9%.

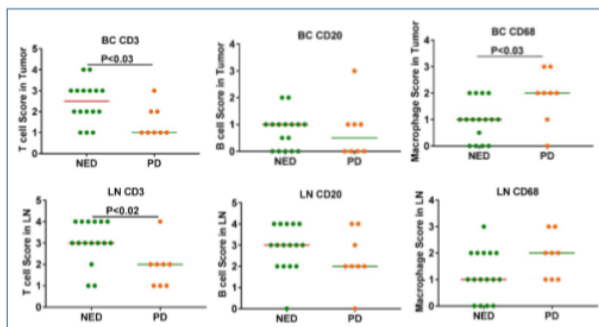
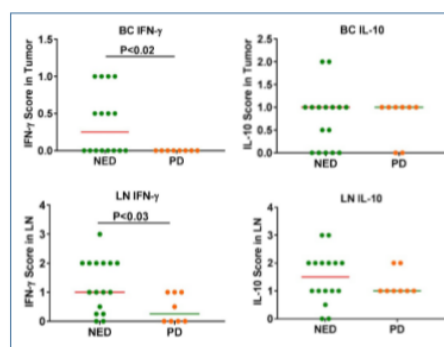


Figure 5: Shows the disease status based immune landscape in paired tumor and LN biopsies. Top panel shows that in patients with NED had enhanced Th1 response indicated by significantly high IFN- γ scores for both BC ($p<0.02$) and LN ($p<0.03$) biopsies compared to patient population with PD. Among cellular infiltrates, patients with NED had significantly higher infiltration of T cells in BC ($p<0.03$) and LN ($p<0.02$) and significantly reduced infiltration of macrophages ($p<0.03$).

infiltration increased significantly along with significantly increased expression of IFN- γ in patients with no evidence of disease compared with patients with progressive disease. These data suggest that Th₁ response may be important in controlling disease progression. Our data is in concurrence with earlier studies showing that type-I helper T-cells (characterized by systemic CD4⁺ T-helper type-1 responses by peripheral blood EliSpot assay) represent a favorable antitumor

immunity in breast cancer [24,25]. Several studies have confirmed the presence of tumor-infiltrating lymphocytes (TILs) in highly proliferative triple-negative breast cancer and HER2 positive breast cancers and their associated pathologic response to neoadjuvant therapy as well as disease-free (DFS) and Overall Survival (OS) after adjuvant chemotherapy [10,11,26]. Other studies have also established that tumors associated with a lymphocytic infiltrate have a better prognosis [4-6].

In this study, we did not see any significant changes in B cells infiltration in either BC or LN biopsies, but there was an increased infiltration of macrophages in BC biopsies in patients with progressive disease compared to patients with no evidence of disease. The role of B-cell infiltrates in BC tissues is not clear, studies report both positive and negative prognostic significance associated with B cell infiltration [27-32]. The role of macrophages has been suggested to depend on their phenotype that can either inhibit tumor growth or promote tumor growth and metastasis [33-38]. Mantovani et al. [33] have suggested that Tumor-Associated Macrophages is biased towards the M2 type that supports tumor growth, invasion and metastasis [33-35]. A number of biomarker studies in breast cancer showed that the presence of macrophages has been correlated with poor prognosis. Increased infiltration of CD68⁺ macrophages were significantly associated with worse breast cancer-specific survival and shorter DFS [37,38].

In summary, our findings show that increased expression of IFN- γ by T cells in BC and LN biopsies in patients with no evidence of disease compared to patients with progressive disease suggest that Th₁ response may be important in controlling disease progression.

Future Directions

The Th₁/Th₂ scoring approach may help clinicians predict outcomes as part of a clinical trial that will guide the treatment decisions. Given the predictive value of TILs, the next logical question is how to enhance a host immune response in patients with breast cancer and how to modulate existing TILs that are either ineffective or being suppressed. Can immune phenotype and immune profile be changed to benefit patient outcome? Dieci et al. [39] reported that 85% of patients had 60% or more TILs in tumors after neoadjuvant chemotherapy and had increased TIL densities from those found at baseline, suggesting that cytotoxic chemotherapy can favorably modify the tumor immune microenvironment, perhaps by altering the T effector/T regulatory cell ratio, removing myeloid-derived suppressor cells [40-43]. Another study showed that the quantity of TILs present in the residual disease at surgery was significantly associated with a better prognosis as well as a smaller amount of invasive disease and less nodal involvement at surgery. The combination of radiotherapy with immunotherapies, such as anti-PD-1 agents, may further augment antitumor immunity [44].

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Authors' Contributions

AT, LGL and LC conceived and designed the study, performed statistical analysis, and wrote the manuscript. DLS, GC, TL, JU PT and SB helped in drafting the manuscript. DLS, GC, TL, JU, PP performed the experiments and participated in the data analysis. All authors read and approved the final manuscript.

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Originality Disclosure

The data presented in this manuscript are original and have not been published elsewhere except in the form of abstracts and poster presentations at symposium and meetings.

Conflict of Interest

LGL is co-founder of Transtarget Inc; AT is co-founder of Nova Immune Platform Inc.; DLS, TL, GC, JU, LC, PT and SB have no conflicts of interest.

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