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Biology of Human Tumors

Clinical Cancer Research

Alterations in the Immune Cell Composition in Premalignant Breast Tissue that Precede Breast Cancer Development

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Abstract

Purpose: Little is known about the role of the immune system in the earliest stages of breast carcinogenesis. We studied quantitative differences in immune cell types between breast tissues from normal donors and those from women with benign breast disease (BBD).

Experimental Design: A breast tissue matched case–control study was created from donors to the Susan G. Komen for the Cure Tissue Bank (KTB) and from women diagnosed with BBD at Mayo Clinic (Rochester, MN) who either subsequently developed cancer (BBD cases) or remained cancer-free (BBD controls). Serial tissue sections underwent immunostaining and digital quantification of cell number per mm^2 for $CD4^+$ T cells, $CD8^+$ T cells, $CD20^+$ B cells, and $CD68^+$ macrophages and quantification of positive pixel measure for CD11c (dendritic cells).

Results: In 94 age-matched triplets, BBD lobules showed greater densities of $CD8^+$ T cells, $CD11c^+$ dendritic cells, $CD20^+$ B cells, and $CD68^+$ macrophages compared with KTB normals. Relative to BBD controls, BBD cases had lower $CD20^+$ cell density ($P = 0.04$). Nearly 42% of BBD cases had no $CD20^+$ B cells in evaluated lobules compared with 28% of BBD controls ($P = 0.02$). The absence of $CD20^+$ cells versus the presence in all lobules showed an adjusted OR of 5.7 (95% confidence interval, 1.4–23.1) for subsequent breast cancer risk.

Conclusions: Elevated infiltration of both innate and adaptive immune effectors in BBD tissues suggests an immunogenic microenvironment. The reduced B-cell infiltration in women with later breast cancer suggests a role for B cells in preventing disease progression and as a possible biomarker for breast cancer risk. *Clin Cancer Res; 23(14); 3945–52. ©2017 AACR.*

Introduction

Benign breast disease (BBD) refers to a variety of benign pathologic findings in the breast. These can include abnormalities of both the epithelium and the supporting stroma. As a group, women with BBD are at an increased risk of breast cancer compared with the general population, with the degree of risk stratified on the basis of the degree of epithelial proliferation (1, 2). In addition to the glandular epithelial cells and underlying stroma that enable lactation, the normal mammary gland contains a mucosal immune system (3), making it similar to other mucosal organs, such as the gastrointestinal tract and the lung. Integration of the immune system into the mammary gland is essential for multiple reasons, including provision of secretory immunoglobulin A (IgA) for neonatal protection, protection of the gland from microbial infection, and maintenance of normal glandular structure and function (4–9). The observations that immunodeficient mice develop breast adenomas and cancers more frequently than their immunocompetent counterparts suggest that the immune system plays a critical role in protection from malignancy (10). In contrast, deregulation of the mucosal immune system may promote subclinical tumor-promoting chronic inflammation similar to other mucosal environments (11). While the role of the immune system in breast cancer progression has been actively studied, its role in breast carcinogenesis is much less studied despite its potential for impacting cancer prevention.

Our objective for this project was to examine patterns of immune cell infiltration in normal and BBD breast tissues and to investigate associations with breast cancer risk. With about 1 million women undergoing a breast biopsy with benign findings in the United States every year, an immune profile of risk could help improve risk stratification, identify pathologic immune effectors, and inform novel immune-related breast cancer prevention strategies.

Materials and Methods

Study design and breast tissue samples

Institutional Review Board approval was obtained before conducting this research. We planned a case–control study to perform

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Little is known about the role of the immune system in the earliest stages of breast carcinogenesis. We studied quantitative differences in immune cell types in normal and benign biopsy breast tissues and evaluated associations with breast cancer risk. We found that lobules in benign breast disease tissues have quantitatively higher densities of multiple immune cell types than normal breast tissues, especially dendritic cells and macrophages. Among women with benign breast disease, a lack of B cells was associated with increased breast cancer risk. These results provide a necessary initial characterization of basic immune cell components within breast tissues in normal and abnormal benign states and identify promising lines for further investigation, particularly the role of macrophages and B cells in inhibiting or promoting breast cancer from the premalignant state.

histologic quantification of immune cell infiltrates in breast tissues from 2 tissue sources. Normal breast tissues were obtained from the Susan G. Komen for the Cure Tissue Bank at IU Simon Cancer Center (i.e., Komen Tissue Bank, KTB) and breast tissues with benign disease were obtained from the Mayo BBD Cohort. The normal breast tissue samples from the KTB are a unique resource of tissue from donor women with no known clinical breast abnormalities. Prior histologic review of a large sample of these tissues confirms that the majority indeed have no histologic abnormalities (12).

The Mayo BBD Cohort is a unique cohort of approximately 15,000 women who had a benign breast biopsy at Mayo Clinic from 1967–2001. Cohort resources include data on risk factors, later breast cancer events, and archived benign biopsy tissues. Women who developed breast cancer subsequent to their benign biopsy are defined as cases, with controls defined as women with similar length of follow-up who did not develop breast cancer. Within the Mayo BBD Cohort, a nested set of 100 cases and 100 controls was randomly selected from the latter portion of the Mayo BBD cohort (1992–2001) to be nearer the years during which KTB samples were collected and were matched on age, year at biopsy, and length of follow-up. Once these case–control pairs were established, an age-matched normal breast tissue donor was randomly selected from the KTB samples available as of June 2012 (~2,500 total available) for each BBD case–control pair to create an age-matched triplet: KTB normal tissue donor, BBD case, and BBD control. Groups were also frequency-matched to ensure a similar distribution of first-degree family history of breast cancer across the 3 groups. Six of the selected BBD case–control pairs involved a subject later excluded from the BBD cohort based on additional data review; 2 due to the history of bilateral prophylactic mastectomy prior to the benign breast biopsy and 4 due to breast cancer identified at the time of the BBD biopsy. Thus, the final analysis sample excluded the 6 affected triplets resulting in a set of 94 triplets totaling 282 subjects.

Histology and immune cell quantitation

For each study sample, serial formalin-fixed, paraffin-embedded tissue sections were stained immunohistochemically using previously described methods (13). Immunostains of paired samples (KTB, BBD case, BBD control) were done within the same batch to minimize batch effects when comparing quantitative results between pairs. The following immunostains were performed with the following antibodies: CD4 (Leica Novocastra NCL-CD4-368-L-CE at 1:50), CD8 (DAKO M7103 at 1:20), CD11c (Leica Novocastra NCL-L-CD11c-563 at 1:25), CD20 (DAKO M0755 at 1:60), CD45(DAKO M0710 at 1:1,500), and CD68 (DAKO, M0876 at 1:100). Slides were digitally scanned with the Aperio ScanScope XT Slide Scanner (Leica Biosystems) using the 20× objective lens.

Each hematoxylin and eosin (H&E) digital image was assessed by the study breast pathologist (D.W. Visscher) for an overall histologic impression of the greatest severity of abnormality according to established categories of benign breast lesions: no histologic abnormality, nonproliferative changes, proliferative changes without atypia, or atypical hyperplasia. From each digital H&E image, 10 representative lobules (or all lobules if <10 present) were selected. These same lobules were then identified, circled digitally, and annotated on successive immunostain sections for immune cell quantitation.

Digital images were analyzed using Aperio ImageScope Software, version 12.1.0.5029 (Leica Biosystems), and quantitative image analysis was performed using methods on the basis of the FDA-approved algorithms optimized as previously described (3). The area of each circled lobule was calculated, and digital quantitation was used to enumerate the number of positively staining cells per mm^2 within all selected lobules. For dendritic cell quantitation, CD11c was measured as ratio of positive to total pixels due to a more diffuse pattern of particle staining (see Fig. 1).

Statistical analysis

Cell densities were calculated as the number of positively stained cells per mm^2 of lobule area for all immunostains except CD11c, where the positive:total pixel ratio was multiplied by 100 to express it as a percentage. Multiple lobules measured within each sample were condensed to a single measure per subject by taking the median across lobules within a sample. Immune cell measures were compared between groups using Wilcoxon signed-rank tests for univariate analysis on the matched sets. Multivariable analysis was performed using conditional logistic regression with a stratification variable to account for matched sets. Continuous immune cell measures were transformed using the Van der Waerden transformation before fitting statistical models (14). Because of a large proportion of lobules with zero counts for some immune cell types, a secondary analysis categorized each sample according to whether all lobules in the sample had a zero count, some lobules had zero count, or no lobules had a zero count; analysis using this variable was then performed using conditional logistic regression as described above. Analysis was performed using SAS (SAS Institute Inc., Version 9.3); graphs were drawn using R software (R Foundation, Version 3.0.2). $P < 0.05$ was considered statistically significant. Because the goal of this exploratory study was to provide the first detailed characterization of multiple immune cells types in premalignant breast tissues with differing levels of risk and because pairwise comparisons for the 3 risk groups were planned *a priori*, no corrections for multiple comparisons were performed to limit the possibility of type II error. However, a modified Bonferroni-corrected α level for 3 pairwise comparisons would be 0.0167 if applied.

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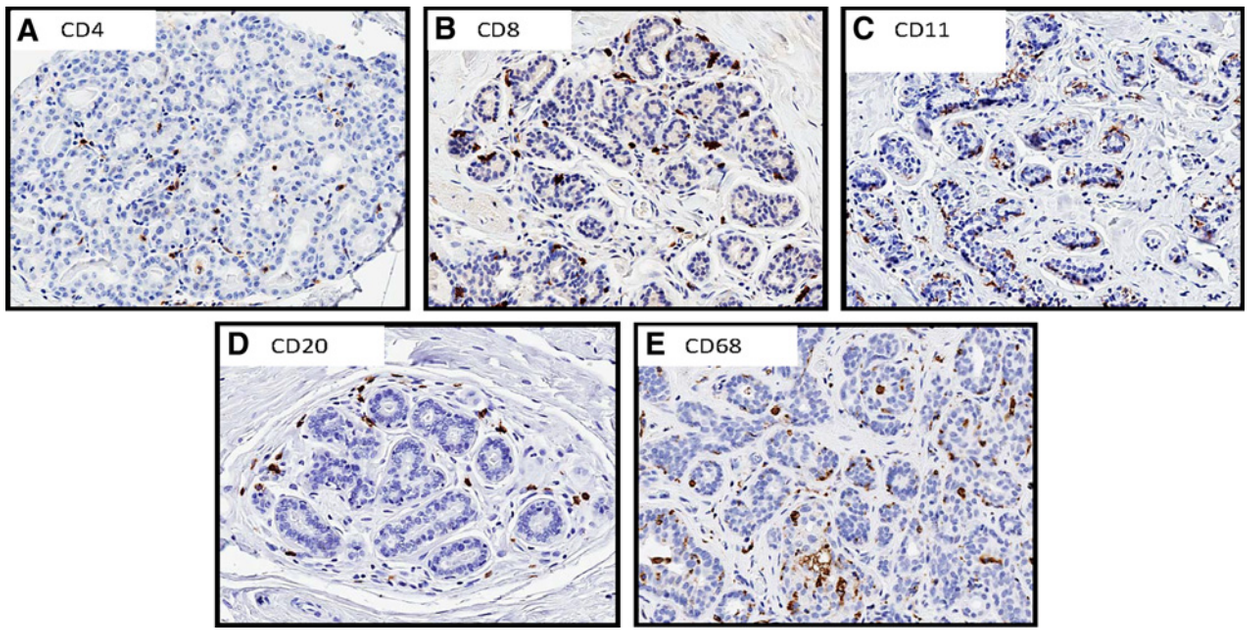

Figure 1.
Photomicrograph of each cell type: CD4, CD8, CD11c, CD20, CD68.

Results

Characteristics of subjects and tissue samples

In the final age-matched study set of 94 triplet samples (n = 282), the mean age was 54 years (Table 1). Among these 282 samples, 2,687 lobules were assessed (898 BBD cases, 922 BBD controls, 867 KTB) to characterize the immune cell presence. Among KTB normal tissue donors, histologic characteristics were similar to those of the larger previously assessed sample (12), with no histologic abnormality in 63.0%, nonproliferative fibrocystic changes in 27.2%, and proliferative findings (±atypia) in 9.8%. Comparing BBD cases and controls, cases had a greater frequency of atypical hyperplasia and less nonproliferative changes, consistent with the higher breast cancer risk associated with these benign lesions (2). Among BBD cases, the subsequent breast cancers were: 26% ductal carcinoma *in situ* (DCIS), 46% invasive ductal cancer, 10% invasive lobular cancer, 6% invasive mixed ductal/lobular, and 12% other invasive histologies; 75% of invasive cancers were estrogen receptor–positive.

Microanatomic patterns of immune cell distribution

On the basis of qualitative review of histologic images, we observed characteristic patterns of immune cell distribution (Fig. 1, A–E). The $CD4^+$ cells were located both in the intralobular stroma between acini and also interspersed among the epithelial cells (Fig. 1A). The $CD8^+$ cells were scattered uniformly across lobules, with the majority of $CD8^+$ cells in close association with the basal aspect of the epithelium in most acini of the lobule (Fig. 1B), although occasional $CD8^+$ cells were also observed in the intralobular stroma. The CD11c staining formed a reticular staining pattern, highlighting dendritic cell processes, outlining lobular acini, also primarily and closely associated with the basal aspect of the epithelium (Fig. 1C). When present, $CD20^+$ cells were more likely to be located in the intralobular stroma rather than in direct association with the epithelium (Fig. 1D). The $CD68^+$ macrophages had a less compartmentalized pattern and were located in acinar epithelium, within acinar lumens, and also within intralobular stroma (Fig. 1E).

Table 1. Characteristics of n = 94 triplets included in final analysis set

Variable	BBD case (N = 94)	BBD ctrl (N = 94)	KTB (N = 94)	P
Age at benign biopsy				0.84
Mean (SD)	54.4 (10.4)	54.4 (10.4)	53.6 (9.7)	
Median (range)	53 (35–79)	53 (36–78)	53 (35–74)	
Age category				0.85
<45 y	20 (21.3%)	20 (21.3%)	20 (21.3%)	
45–55 y	31 (33.0%)	30 (31.9%)	35 (37.2%)	
>55 y	43 (45.7%)	44 (46.8%)	39 (41.5%)	
Histologic impression				<0.0001
Missing	0	0	2	
No histologic abnormality	0 (0.0%)	0 (0.0%)	58 (63.0%)	
Nonproliferative BBD	28 (29.8%)	41 (43.6%)	25 (27.2%)	
Proliferative BBD w/o atypia	45 (47.9%)	39 (41.5%)	7 (7.6%)	
Atypia	21 (22.3%)	14 (14.9%)	2 (2.2%)	

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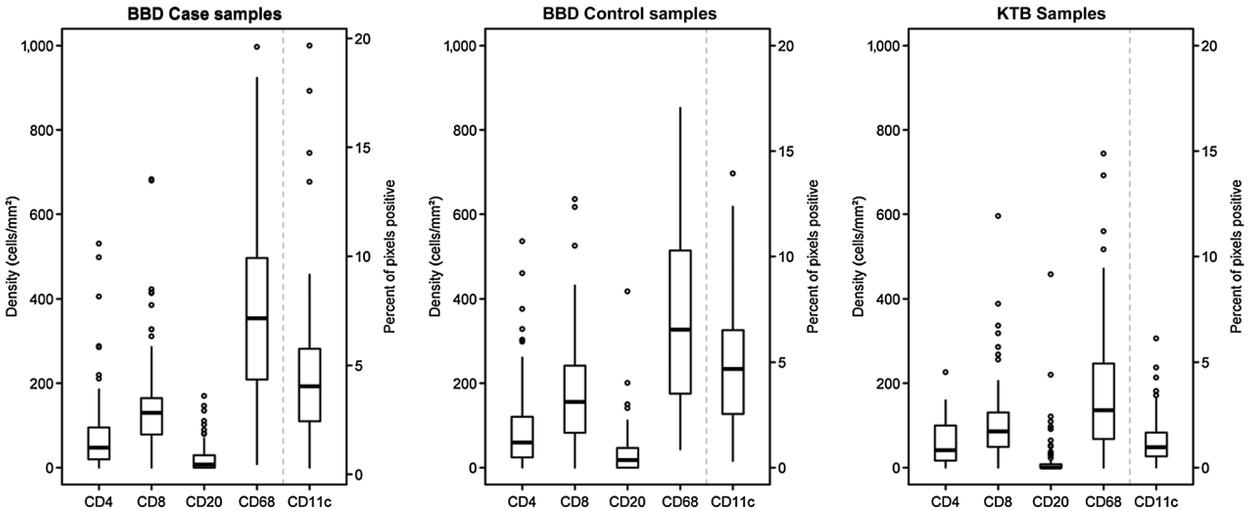

Figure 2.
Boxplots showing the distribution and relative frequency of different immune cell types within each risk group.

Relative frequencies of immune cell types are consistent across tissue groups

Boxplots comparing densities of the various immune cell types across the 3 tissue groups demonstrate a similar relative frequency of the five immune cell types within each group (Fig. 2; Supplementary Table S1). In all 3 groups, $CD68^+$ macrophages were most frequent, followed by $CD8^+$, $CD4^+$, and $CD20^+$ lymphocytes, respectively (CD11c is not directly comparable to these other cell types due to the percent-positive pixel measure). Compared with KTB normal samples, BBD samples (both cases and controls) showed generally higher densities of all immune cell types, but there was substantial variability within each group.

BBD samples have increased macrophages and dendritic cells relative to KTB

Differences in immune cell densities between sample groups were evaluated by calculating pairwise differences of the median values between age-matched samples of different groups (KTB, BBD cases, BBD controls). In unadjusted analysis, BBD cases and controls had elevated levels of all immune cell types compared with KTB normal tissues with the exception of the $CD4^+$ cell density comparison between BBD cases and KTB samples, which was not statistically significant. The largest effects were seen with $CD68^+$ and $CD11c^+$ cells (Table 2 and Fig. 3). $CD68^+$ cell density was significantly elevated after adjustment for histologic impression ($P = 0.02$ for BBD cases and $P = 0.005$ for BBD controls compared with KTB). Similarly, $CD11c^+$ pixel percentage was higher in BBD cases and controls than in KTB samples and remained significant after adjustment ($P = 0.01$ and $P = 0.006$, respectively).

Because we observed that many lobules lacked several immune cell subtypes (most notably $CD4^+$ T cells and $CD20^+$ B cells), we also analyzed findings on the basis of the percentage of samples having all, some, or none of the lobules with a value of zero immune cells (Table 3). Similar to analysis of median cell

Table 2. Paired comparisons of cell density or percent

	N	Median percent difference[a]	Median paired difference (95% CI)	*P*	
				Unadjusted	Adjusted[b]
Comparing BBD cases vs. KTB					
CD4	88	21.9%	7.8 (−4.4 to 21.8)	0.21	0.62
CD8	87	21.2%	27.0 (5.8–60.1)	0.001	0.33
CD11c	91	74.0%	2.5 (2.2–3.4)	<0.0001	0.01
CD20	89	10.9%	0.7 (0.0–10.3)	0.009	0.67
CD68	93	59.0%	171.0 (129.3–228.8)	<0.0001	0.02
Comparing BBD controls vs. KTB					
CD4	88	28.2%	13.9 (0.0–29.4)	0.01	0.14
CD8	87	45.4%	74.5 (41.3–100.3)	<0.0001	0.01
CD11c	90	80.6%	3.6 (2.5–4.5)	<0.0001	0.006
CD20	89	81.9%	13.9 (0.4–19.9)	<0.0001	0.08
CD68	93	57.8%	170.0 (138.0–219.0)	<0.0001	0.005
Comparing BBD cases vs. BBD controls					
CD4	94	−19.3%	−9.1 (−26.0 to 4.0)	0.11	0.14
CD8	92	−37.0%	−39.5 (−65.8 to 0.0)	0.009	0.09
CD11c	89	−16.0%	−0.8 (−1.6 to 0.0)	0.04	0.11
CD20	94	−10.9%	−5.1 (−11.3 to 0.0)	0.04	0.02
CD68	94	−4.9%	−9.3 (−50.0 to 53.0)	0.91	0.93

[a]A small constant (10^{-6}) was added to the denominator to avoid division by zero.
[b]Adjusted for histologic impression.

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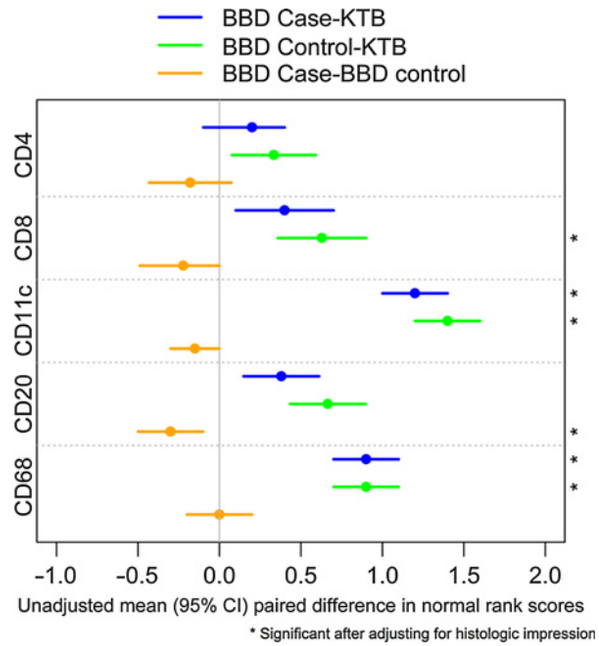

Figure 3.

Paired comparisons of cell density or percent at the per sample level (using the median calculated across lobules within each sample).

densities, unadjusted analysis of zero densities showed that $CD68^+$, $CD4^+$, $CD8^+$, and $CD20^+$ cells were significantly more prevalent in BBD than in KTB samples. As virtually, all lobules demonstrated presence of $CD11c^+$ pixels regardless of sample group, the analysis of zero counts was not informative for this cell type. After adjustment for histologic impression, only $CD68^+$ macrophages remained significantly different in separate comparisons of KTB with BBD cases and controls.

Absence of B cells is associated with increased risk of subsequent breast cancer development

Comparing median cell densities between BBD cases and controls, BBD cases generally had lower immune cell densities than controls, but the differences were smaller in magnitude compared with the differences between BBD and KTB samples (Table 2 and Fig. 3). Notably, median CD68 cell density did not differ between BBD cases and controls. In unadjusted analyses of median cell density, BBD cases showed significantly lower cell densities than BBD controls for $CD8^+$ cells ($P = 0.009$), $CD11c^+$ cells ($P = 0.04$), and $CD20^+$ cells ($P = 0.04$). After adjustment for histologic impression, differences remained significant only for $CD20^+$ B-cell density ($P = 0.02$).

Comparing BBD cases with controls using the zero cells approach (Table 3), $CD20^+$ B cells emerged as the only immune cell type that differed significantly, with all lobules having zero counts in 41.5% of case samples compared with 27.7% of controls, $P = 0.02$ ($P = 0.006$ after adjustment). A $CD20^+$ B-cell count of zero across all lobules, versus no lobules zero within a sample, showed an unadjusted OR of 4.1 [95% confidence interval (CI), 1.1–15.6] for association with BBD cases versus controls, and some lobules zero versus no lobules zero showed OR of 2.0 (95% CI, 0.6–6.7). After adjusting for histologic impression, these ORs increased to 5.7 (95% CI, 1.4–23.1) and 2.4 (95% CI, 0.7–8.2), respectively. Thus, the absence of $CD20^+$ B cells in BBD tissues was associated with increased risk of progression to breast cancer.

Discussion

In this study, we enumerated major immune cell subsets in mammary gland lobules in normal and BBD tissues, and we evaluated associations with breast cancer risk. Two main findings emerged from this work. (i) In general, BBD tissue has higher densities of $CD4^+$ and $CD8^+$ T cells, dendritic cells, $CD20^+$ B cells, and $CD68^+$ macrophages compared with normal breast tissues, with the strongest associations for dendritic cells and macrophages. (ii) Tissues from BBD cases who later developed breast cancer had lower levels of $CD20^+$ B cells than matched BBD controls who did not develop breast cancer. Each of these findings advances our understanding about the possible role of the immune system in tumor immunosurveillance and early breast carcinogenesis.

The increased immune cell infiltration observed in BBD tissues relative to normal mammary gland tissue suggests there is a local immune response which may be antigen-specific, given increased numbers of T and B cells. However, it is unclear whether the increased immune infiltration in BBD tissues is induced by existing fibrocystic stromal and epithelial abnormalities (supporting the tumor surveillance hypothesis) or whether immune cell infiltration promotes chronic inflammation and cancer development. The century-old immunosurveillance hypothesis has garnered support recently with the demonstration that immunodeficient mice have increased incidence of colon and breast adenocarcinomas (10). In humans, evidence for immunosurveillance includes reports of spontaneous tumor rejection, increased malignancy in immunodeficient patients, and elevated tumor antigen-specific T cells and antibodies in newly diagnosed patients with cancer (15–17).

The idea of a protective immune response is further supported by our observation that B-cell infiltration was associated with a decreased risk of breast cancer. B cells produce antigen-specific antibodies in an adaptive immune response coordinated with antigen-presenting cells and T cells. In breast cancer, naturally occurring B-cell responses include serum antibodies, tumor-infiltrating B cells, and tumor-reactive lymph node B cells (18). Regardless of mechanism, a lack of B cells in benign breast tissue may be a useful biomarker of breast cancer risk among women with benign breast biopsies.

Alternatively, the increased immune response may contribute to breast abnormalities through tumor-promoting chronic inflammation, with oxidative processes initiating malignant progression via inactivating mutations in tumor suppressor genes or posttranslational modifications in proteins involved in apoptosis and DNA repair (19). We found that macrophages are more common in lobules of BBD than in normal tissues, suggesting greater inflammation in tissues with higher cancer risk. Macrophages are a dominant component of chronic inflammation, producing cytokines that promote epithelial abnormalities (20). In breast cancer, tumor-associated macrophages affect virtually all aspects of disease progression including metabolism, angiogenesis, invasion, and metastasis. (21–24). Although we found similar macrophage densities in BBD cases and controls, suggesting no association with cancer risk, there may be biologically relevant differences in macrophage phenotypes [specifically

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Table 3. Summary of lobules with zero cell counts

Variable	BBD case ($N = 94$)	BBD control ($N = 94$)	KTB ($N = 94$)		P Unadjusted	P Adjusted[a]
CD4				CD4		
Zero count for all lobules in sample	7 (7.4%)	8 (8.5%)	14 (15.9%)	BBD case vs. KTB	0.0003	0.52
Zero count for some lobules in sample	49 (52.1%)	55 (58.5%)	63 (71.6%)	BBC control vs. KTB	0.003	0.19
Non-zero count for each lobule in sample	38 (40.4%)	31 (33.0%)	11 (12.5%)	BBD case vs. control	0.26	0.26
Missing	0	0	6			
CD8				CD8		
Zero count for all lobules in sample	3 (3.3%)	2 (2.2%)	3 (3.4%)	BBD case vs. KTB	0.0003	0.12
Zero count for some lobules in sample	23 (25.0%)	20 (21.7%)	49 (56.3%)	BBC control vs. KTB	<0.0001	0.02
Non-zero count for each lobule in sample	66 (71.7%)	70 (76.1%)	35 (40.2%)	BBD case vs. control	0.45	0.33
Missing	2	2	7			
CD11c				CD11c		
Zero count for all lobules in sample	0	0	0	BBD case vs. KTB	0.99	N/A
Zero count for some lobules in sample	0 (0.0%)	1 (1.1%)	3 (3.2%)	BBC control vs. KTB	0.34	N/A
Non-zero count for each lobule in sample	92 (100.0%)	90 (98.9%)	90 (96.8%)	BBD case vs. control	0.99	N/A
Missing	2	3	1			
CD20				CD20		
Zero count for all lobules in sample	39 (41.5%)	26 (27.7%)	59 (66.3%)	BBD case vs. KTB	0.001	0.64
Zero count for some lobules in sample	49 (52.1%)	57 (60.6%)	28 (31.5%)	BBC control vs. KTB	<0.0001	0.02
Non-zero count for each lobule in sample	6 (6.4%)	11 (11.7%)	2 (2.2%)	BBD case vs. control	0.02	0.006
Missing	0	0	5			
CD68				CD68		
Zero count for all lobules in sample	0 (0.0%)	0 (0.0%)	10 (10.8%)	BBD case vs. KTB	<0.0001	0.02
Zero count for some lobules in sample	13 (13.8%)	13 (13.8%)	38 (40.9%)	BBC control vs. KTB	<0.0001	0.02
Non-zero count for each lobule in sample	81 (86.2%)	81 (86.2%)	45 (48.4%)	BBD case vs. control	1.0	0.99
Missing	0	0	1			

[a]Adjusted for histologic impression.

proinflammatory (M1) or immunosuppressive (M2); refs. 21–25] and resulting inflammation that is either pro- or antitumorigenic (26). Further study will be required to identify the polarization state of macrophages in BBD tissue.

Other evidence supports a role for chronic inflammation in breast cancer development. Population-based studies show that long-term use of aspirin and ibuprofen is associated with reduced incidence of breast cancer (27–31). Individuals with higher C-reactive protein (CRP) levels have increased risk of breast cancer (32), and chronically immunosuppressed solid organ transplant recipients also have reduced numbers of breast cancers (33). Our observed predominance of macrophage and dendritic cells in BBD tissues suggest chronic inflammatory responses of unknown etiology, possibly in response to persistent irritants, bacteria, or viruses (24–37). Inflammation has also been recognized as tumor-promoting in multiple other epithelial cancer types, especially of the gastrointestinal tract where aspirin is associated with reduced risk of colorectal cancer (38). Similar to our finding of inflammation in BBD tissues with premalignant potential, research on colorectal adenomas has shown that precancerous polyps have higher infiltration of T cells and macrophages compared with non-polypoid lesions (39). Mechanisms of inflammation-induced carcinogenesis that are supported by current research across multiple tumor types include NF-κB–driven production of proinflammatory cytokines in immune cells (which promote neoplastic transformation of epithelial cells) and generation of free radicals with resulting epithelial DNA damage (40).

Limitations of our findings include the lack of functional data on immune cell types, although our results form the foundation for future studies and are a notable improvement over previously published data in this field. Prior studies on immune cell subsets in nonmalignant breast tissue (41–43) involved much smaller sample sizes and lacked information on subsequent cancer risk. Other limitations of our study include: (i) only 10 lobules were studied per sample and (ii) quantitation was limited to breast lobules and intralobular stroma; we did not evaluate interlobular stroma. Both factors were related to the time-intensive nature of the cell quantitation and may be overcome in the future by technological advances with multiplexing immunostains on a single-tissue section, allowing better assessment of immune cell function via multiple markers. Finally, the older age of BBD tissues and different tissue processing protocols between BBD and KTB tissues could impact immune cell findings between these 2 groups. This seems unlikely, as immunostains used for major immune cell types are robust and generally immune cells were more abundant in BBD tissues, suggesting that there was not a lack of antigen retrieval in these older samples. Strengths of our study include a systematic and detailed quantitation of major immune cell subsets in both normal and BBD tissues, which has not been previously reported.

In conclusion, we found that lobules in BBD tissues have quantitatively higher densities of multiple immune cell types than normal breast tissues, especially dendritic cells and macrophages. Among women with BBD, a lack of B cells appears to be associated with increased breast cancer risk. Although these data are limited to quantitative cell counts without functional status, the results presented here provide a necessary initial characterization of basic immune cell components within breast tissues in normal and abnormal benign states and identify promising lines for further investigation, particularly the role of macrophages and B cells in inhibiting or promoting breast cancer from the premalignant state.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.C. Degnim, T.L. Hoskin, K.L. Knutson, D.W. Visscher
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References

1. Dupont WD, Page DL. Risk factors for breast cancer in women with proliferative breast disease. N Engl J Med 1985;312:146–51.
2. Hartmann LC, Sellers TA, Frost MH, Lingle WL, Degnim AC, Ghosh K, et al. Benign breast disease and the risk of breast cancer. N Engl J Med 2005;353:229–37.
3. Degnim AC, Brahmbhatt RD, Radisky DC, Hoskin TL, Stallings-Mann M, Laudenschlager M, et al. Immune cell quantitation in normal breast tissue lobules with and without lobulitis. Breast Cancer Res Treat 2014;144:539–49.
4. Brandtzaeg P. The mucosal immune system and its integration with the mammary glands. J Pediatr 2010;156:S8–15.
5. Cheroutre H, Madakamutil L. Acquired and natural memory T cells join forces at the mucosal front line. Nat Rev Immunol 2004;4:290–300.
6. Ghajar CM. On leukocytes in mammary development and cancer. Cold Spring Harb Perspect Biol 2012;4. pii: a013276.
7. Goldman AS. The immune system of human milk: antimicrobial, antiinflammatory and immunomodulating properties. Pediatr Infect Dis J 1993;12:664–71.
8. Reed JR, Schwertfeger KL. Immune cell location and function during postnatal mammary gland development. J Mammary Gland Biol Neoplasia 2010;15:329–39.
9. Spencer JP. Management of mastitis in breastfeeding women. Am Fam Physician 2008;78:727–31.
10. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001;410:1107–11.
11. Trinchieri G. Cancer and inflammation: an old intuition with rapidly evolving new concepts. Annu Rev Immunol 2012;30:677–706.
12. Degnim AC, Visscher DW, Hoskin TL, Frost MH, Vierkant RA, Vachon CM, et al. Histologic findings in normal breast tissues: comparison to reduction mammaplasty and benign breast disease tissues. Breast Cancer Res Treat 2012;133:169–77.
13. Radisky DC, Santisteban M, Berman HK, Gauthier ML, Frost MH, Reynolds CA, et al. p16(INK4a) expression and breast cancer risk in women with atypical hyperplasia. Cancer Prev Res (Phila) 2011;4:1953–60.
14. van der Waerden BL. Order tests for the two-sample problem and their power. Proc Koninklijke Nederlandse Akademie van Wetenschappen 1952;55:453–8.
15. Teng M, Galon J, Fridman W, Smyth M. From mice to humans: developments in cancer immunoediting. J Clin Invest 2015;125:3338–46.
16. Vesely M, Kershaw M, Schreiber R, Smyth M. Natural innate and adaptive immunity to cancer. Annu Rev Immunol 2011;29:235–71.
17. Zahl PH, Maehlen J, Welch HG. The natural history of invasive breast cancers detected by screening mammography. Arch Intern Med 2008;168:2311–6.
18. Coronella-Wood JA, Hersh EM. Naturally occurring B-cell responses to breast cancer. Cancer Immunol Immunother 2003;52:715–38.
19. Hussain M, Cunnick GH. Management of lobular carcinoma in-situ and atypical lobular hyperplasia of the breast–a review. Eur J Surg Oncol 2011;37:279–89.
20. Philip M, Rowley DA, Schreiber H. Inflammation as a tumor promoter in cancer induction. Semin Cancer Biol 2004;14:433–9.
21. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005;5:953–64.
22. Laoui D, Movahedi K, Van Overmeire E, Van den Bossche J, Schouppe E, Mommer C, et al. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. Int J Dev Biol 2011;55:861–7.
23. Mantovani A, Germano G, Marchesi F, Locatelli M, Biswas SK. Cancer-promoting tumor-associated macrophages: new vistas and open questions. Eur J Immunol 2011;41:2522–5.
24. Mukhtar RA, Nseyo O, Campbell MJ, Esserman LJ. Tumor-associated macrophages in breast cancer as potential biomarkers for new treatments and diagnostics. Expert Rev Mol Diagn 2011;11:91–100.
25. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002;23:549–55.
26. Palucka K, Coussens LM, O'Shaughnessy J. Dendritic cells, inflammation, and breast cancer. Cancer J 2013;19:511–6.
27. Bardia A, Olson JE, Vachon CM, Lazovich D, Vierkant RA, Wang AH, et al. Effect of aspirin and other NSAIDs on postmenopausal breast cancer incidence by hormone receptor status: results from a prospective cohort study. Breast Cancer Res Treat 2011;126:149–55.
28. Brasky TM, Bonner MR, Moysich KB, Ambrosone CB, Nie J, Tao MH, et al. Non-steroidal anti-inflammatory drug (NSAID) use and breast cancer risk in the Western New York exposures and breast cancer (WEB) Study. Cancer Causes Control 2010;21:1503–12.
29. Sangthawan P, Fox J, Atkins RC, Kerr PG. Increased incidence of benign breast disease in female renal transplant patients receiving cyclosporin. ANZ J Surg 2002;72:222–5.
30. Takkouche B, Regueira-Mendez C, Etminan M. Breast cancer and use of nonsteroidal anti-inflammatory drugs: a meta-analysis. J Natl Cancer Inst 2008;100:1439–47.
31. Zhao YS, Zhu S, Li XW, Wang F, Hu FL, Li DD, et al. Association between NSAIDs use and breast cancer risk: a systematic review and meta-analysis. Breast Cancer Res Treat 2009;117:141–50.
32. Siemes C, Visser LE, Coebergh J-WW, Splinter TAW, Witteman JCM, Uitterlinden AG, et al. C-reactive protein levels, variation in the C-reactive protein gene, and cancer risk: the Rotterdam Study. J Clin Oncol 2006;24:5216–22.
33. Stewart T, Tsai SC, Grayson H, Henderson R, Opelz G. Incidence of de-novo breast cancer in women chronically immunosuppressed after organ transplantation. Lancet 1995;346:796–8.

34. Fernandez SV, Russo J. Estrogen and xenoestrogens in breast cancer. Toxicol Pathol 2010;38:110–22.
35. Joshi D, Buehring GC. Are viruses associated with human breast cancer? Scrutinizing the molecular evidence. Breast Cancer Res Treat 2012; 135:1–15.
36. Wang Y, Holland JF, Bleiweiss IJ, Melana S, Liu X, Pelisson I, et al. Detection of mammary tumor virus env gene-like sequences in human breast cancer. Cancer Res 1995;55:5173–9.
37. Xuan C, Shamonki JM, Chung A, Dinome ML, Chung M, Sieling PA, et al. Microbial dysbiosis is associated with human breast cancer. PLoS One 2014;9:e83744.
38. Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. Lancet Oncol 2012; 13:518–27.
39. Maglietta A, Maglietta R, Staiano T, Bertoni R, Ancona N, Marra G, et al. The immune landscapes of polypoid and nonpolypoid precancerous colorectal lesions. PLoS ONE 2016;11:e0159373.
40. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell 2005;7:211–7.
41. Ben-Hur H, Cohen O, Schneider D, Gurevich P, Halperin R, Bala U, et al. The role of lymphocytes and macrophages in human breast tumorigenesis: an immunohistochemical and morphometric study. Anticancer Res 2002; 22:1231–8.
42. Giorno R. Mononuclear cells in malignant and benign human breast tissue. Arch Pathol Lab Med 1983;107:415–7.
43. Lwin KY, Zuccarini O, Sloane JP, Beverley PC. An immunohistological study of leukocyte localization in benign and malignant breast tissue. Int J Cancer 1985;36:433–8.

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