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

Short Running Title: Immune cells and premalignant breast tissue

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Statement of 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 compared to 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.

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 women with benign breast disease (BBD).

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 who either subsequently developed cancer (BBD cases) or remained cancer-free (BBD controls). Serial tissue sections underwent immunostaining and digital quantification of cell #/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 to 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 to 28% of BBD controls, $p = 0.02$. The absence of CD20+ cells versus presence in all lobules showed an adjusted odds ratio of 5.7 (95% CI: 1.4-23.1) for subsequent breast cancer risk.

Conclusion

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.

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 to the general population, with the degree of risk stratified based on 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 suggests 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 ~1 million women undergoing a breast biopsy with benign findings in the United States every year, an immune profile of risk could help to improve risk stratification, identify pathologic immune effectors, and inform novel immune-related breast cancer prevention strategies.

Methods

Study design and breast tissue samples

Institutional Review Board approval was obtained prior to conducting this research. We planned a case-control study to perform histologic quantification of immune cell infiltrates in breast tissues from two 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 were 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 (~2500 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 three groups. Six of the selected BBD case-control pairs involved a subject later excluded from the BBD cohort based on additional data review; two due to history of bilateral prophylactic mastectomy prior to the benign breast biopsy and four due to breast cancer identified at the time of the BBD biopsy. Thus, the final analysis sample excluded the six 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 1:1500) and CD68 (DAKO, M0876 at 1:100). Slides were digitally scanned with the Aperio ScanScope® XT slide scanner (Leica Biosystems®, Buffalo Grove, IL) using the 20X objective lens.

Each H&E digital image was assessed by the study breast pathologist (DWV) 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®, Buffalo Grove, IL), and quantitative image analysis was performed using methods based on 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 DC quantitation, CD11c was measured as ratio of positive to total pixels due to a more diffuse pattern of particle staining (see **Figure 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 prior to fitting statistical models(14). Due to 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, Vienna, Austria, Version 3.0.2). P-values < 0.05 were considered statistically significant. Because the goal of this exploratory study was to provide the first detailed characterization of multiple immune cells types in pre-malignant breast tissues with differing levels of risk and because pairwise comparisons for the three risk groups were planned *a priori*, no corrections for multiple comparisons were performed in order to limit the possibility of type II error. However, a modified Bonferroni-corrected alpha-level for three pairwise comparisons would be 0.0167 if applied.

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 **(Table 1).** Among these 282 samples, 2687 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% 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

Based upon qualitative review of histologic images, we observed characteristic patterns of immune cell distribution (**Figure 1, A-E**). The CD4+ positive cells were located both in the intralobular stroma between acini and also interspersed among the epithelial cells (**Figure 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 (**Figure 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 (**Figure 1C**). When present, CD20+ cells were more likely to be located in the intralobular stroma rather than in direct association with the epithelium (**Figure 1D**). The CD68+ macrophages had a less compartmentalized pattern and were located in acinar epithelium, within acinar lumens, and also within intralobular stroma (**Figure 1E**).

Relative frequencies of immune cell types are consistent across tissue groups

Boxplots comparing densities of the various immune cell types across the three tissue groups demonstrate a similar relative frequency of the five immune cell types within each group (**Figure 2, Supplemental Table 1**)**.** In all three 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 to 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 to 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 Figure 3**). CD68+ cell density significantly elevated after adjustment for histologic impression ($p = 0.02$ for BBD cases and $p = 0.005$ for BBD controls compared to KTB). Similarly, CD11c+ pixel percent was higher in BBD cases and controls compared to 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 based on 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 densities, unadjusted analysis of zero densities showed that CD68+, CD4+, CD8+, and CD20+ cells were significantly more prevalent in BBD compared to 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 compared to controls but the differences were smaller in magnitude compared to the differences between BBD and KTB samples (**Table 2 and Figure 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 compared to 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 to 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% 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: 1) In general, BBD tissue has higher densities of CD4+ and CD8+ T cells, DCs, CD20+ B cells, and CD68+ macrophages compared to normal breast tissues, with the strongest associations for DCs and macrophages; and 2) Tissues from BBD cases who later developed breast cancer had lower levels of CD20+ B cells compared to 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 if the increased immune infiltration in BBD tissues is induced by existing fibrocystic stromal and epithelial abnormalities (supporting the tumor surveillance hypothesis), or if 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 cancer patients(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 post-translational modifications in proteins involved in apoptosis and DNA repair(19). We found that macrophages are more common in lobules of BBD compared to 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 pro-inflammatory [M1] or immunosuppressive [M2](21-25)) and resulting inflammation that is either pro- or anti-tumorigenic(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 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 GI 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 to non-polypoid lesions(39). Mechanisms of inflammation-induced carcinogenesis that are supported by current

research across multiple tumor types include NF-KB 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 non-malignant breast tissue(41-43) involved much smaller sample sizes and lacked information on subsequent cancer risk. Other limitations of our study include 1) only 10 lobules were studied per sample, and 2) 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. Lastly, the older age of BBD tissues and different tissue processing protocols between BBD and KTB tissues could impact immune cell findings between these two 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 benign breast disease tissues, which has not been previously reported.

In conclusion, we found that lobules in benign breast disease tissues have quantitatively higher densities of multiple immune cell types compared to normal breast tissues, especially dendritic cells and macrophages. Among women with benign breast disease, 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.

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Disclosure/Duality of Interest

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Figure Legend:

Figure 1. Photomicrograph of each cell type- CD4, 8, 11c, 20, 68 (see separate attached file of revised figure)

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

Figure 3. Paired comparisons of cell density or percent at the per sample level (using the median calculated across lobules within each sample).

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-value
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 years	20 (21.3%)	20 (21.3%)	20 (21.3%)	
45-55 years	31 (33.0%)	30 (31.9%)	35 (37.2%)	
>55 years	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%)	
Non-proliferative 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%)	

Table 2. Paired comparisons of cell density or percent.

	N	Median percent difference[a]	Median paired difference (95% CI)		p-value	
					Unadjusted	Adjusted[b]
Comparing BBD cases vs KTB						
CD4	88	21.9%	7.8 (-4.4, 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, 4.0)		0.11	0.14
CD8	92	-37.0%	-39.5 (-65.8, 0.0)		0.009	0.09
CD11c	89	-16.0%	-0.8 (-1.6), 0.0)		0.04	0.11
CD20	94	-10.9%	-5.1 (-11.3, 0.0)		0.04	0.02
CD68	94	-4.9%	-9.3 (-50.0, 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

Table 3. Summary of lobules with zero cell counts.

Variable	BBD case (N=94)	BBD control (N=94)	KTB (N=94)		P-values	
					Unadjusted	Adjusted*
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

Variable	BBD case (N=94)	BBD control (N=94)	KTB (N=94)			P-values	
						Unadjusted	Adjusted*
Zero count for some lobules in samples	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				

* Adjusted for histologic impression

Figure 1

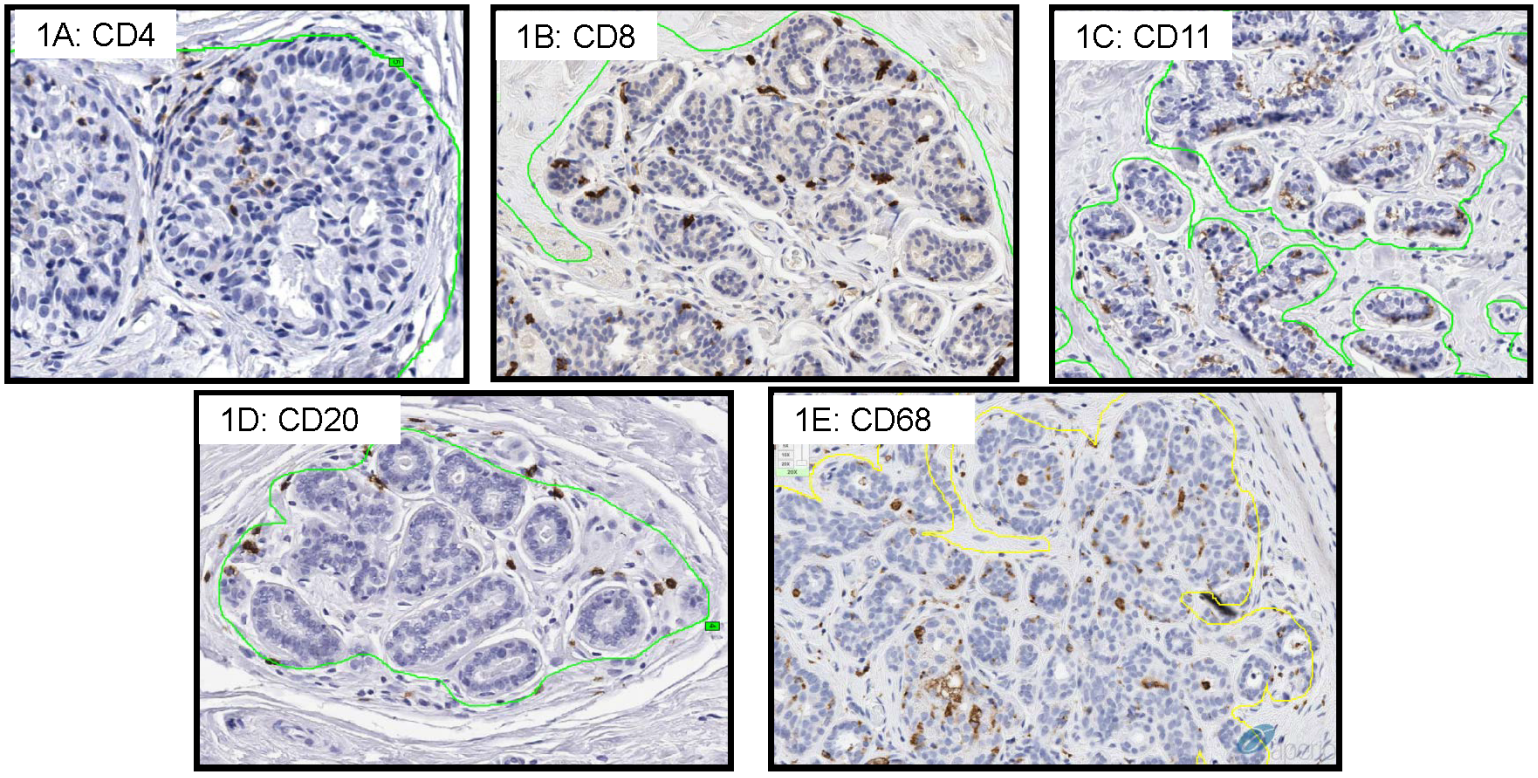
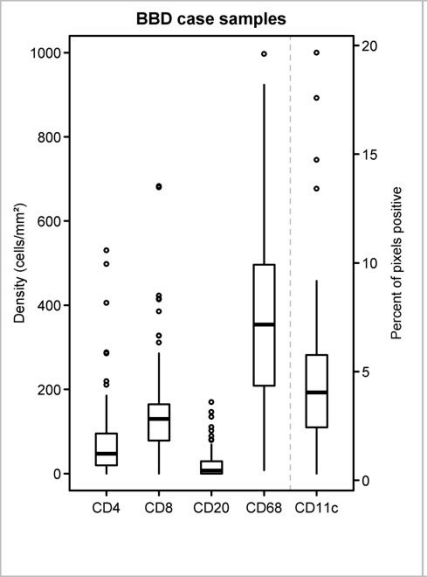
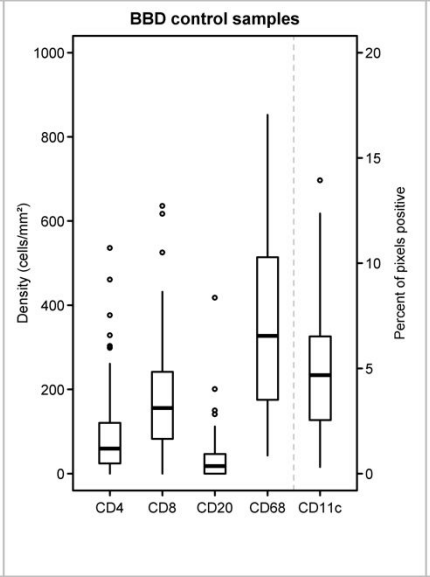
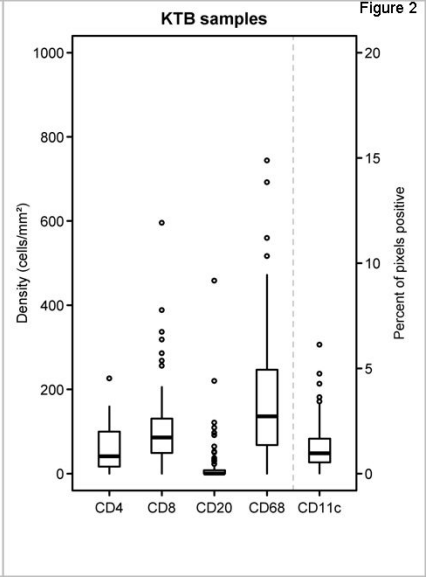
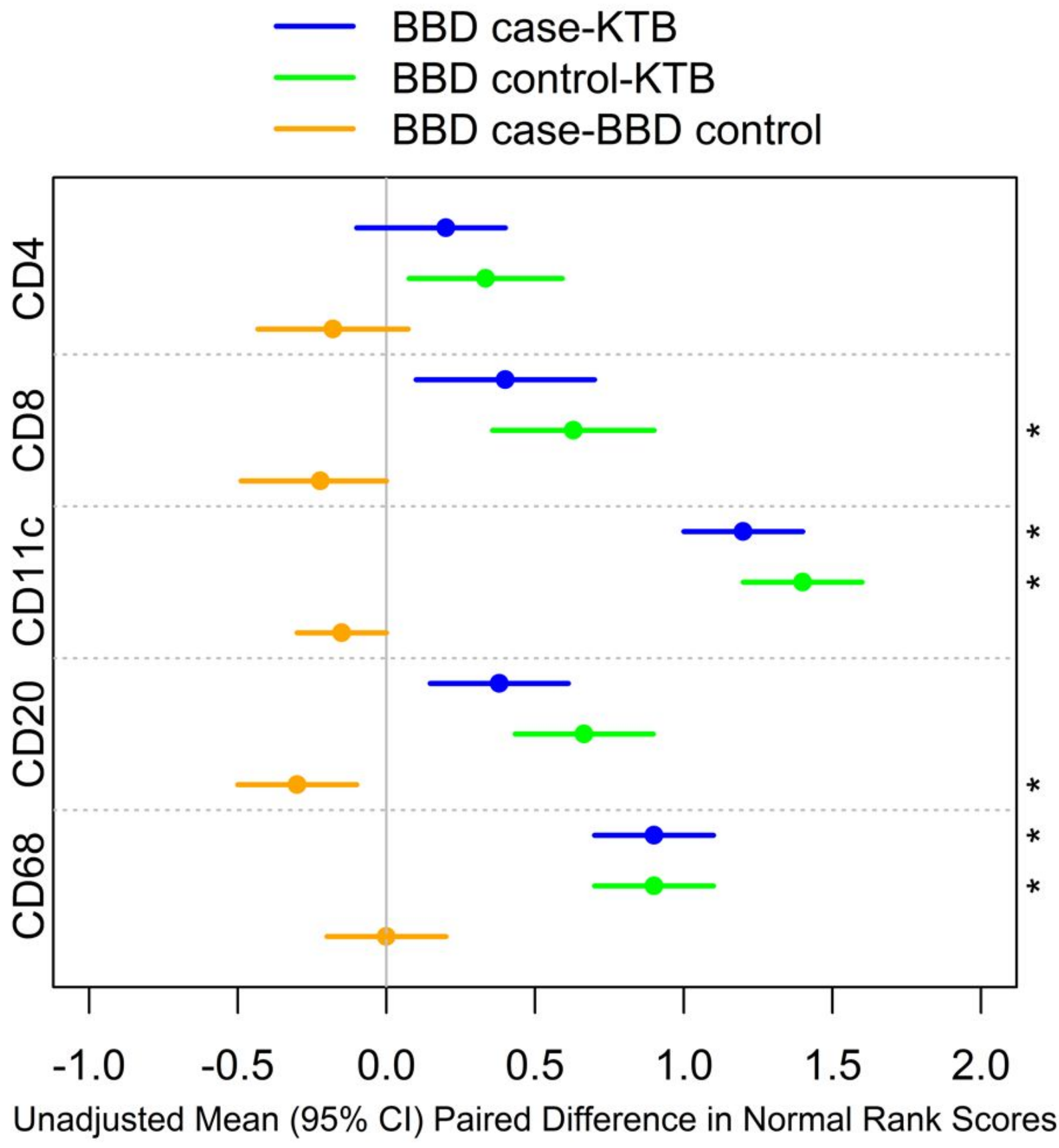


Figure 2


BBD case samples
Density (cells/mm²)
Percent of pixels positive
0
200
400
600
800
1000
0
5
10
15
20
CD4
CD8
CD20
CD68
CD11c


BBD control samples
Density (cells/mm²)
Percent of pixels positive
0
200
400
600
800
1000
0
5
10
15
20
CD4
CD8
CD20
CD68
CD11c


KTB samples
Density (cells/mm²)
Percent of pixels positive
0
200
400
600
800
1000
0
5
10
15
20
CD4
CD8
CD20
CD68
CD11c



* Significant after adjusting for histologic impression