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Graphical abstract

TUMOR INFILTRATING B-CELLS SIGNAL FUNCTIONAL HUMORAL IMMUNE RESPONSES IN BREAST CANCER

Authors and affiliations

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

Abstract

Tumor-infiltrating B-cells (TIL-B) in breast cancer (BC) have previously been associated with improved clinical outcomes; however, their role(s) in tumor immunity is not currently well known. This study confirms and extends the correlation between higher TIL-B densities and positive outcomes through an analysis of HER2-positive and triple-negative BC patients from the BIG 02-98 clinical trial (10yr median follow-up). Fresh tissue analyses identify an increase in TIL-B density in untreated primary BC compared to normal breast tissues, which is associated with global, $CD4^+$ and $CD8^+$ TIL, higher tumor grades, higher proliferation and hormone receptor negativity. All B-cell differentiation stages are detectable but significant increases in memory TIL-B are consistently present. BC with higher infiltrates are specifically characterized by germinal center TIL-B, which in turn are correlated with T_{FH} TIL and antibody-secreting TIL-B principally located in tertiary lymphoid structures. Some TIL-B also interact directly with tumor cells. Functional analyses reveal TIL-B are responsive to BCR stimulation *ex vivo*, express activation markers and produce cytokines and immunoglobulins (Igs) despite reduced expression of the antigen-presenting molecules HLA-DR and CD40. Overall, these data support the concept that ongoing humoral immune responses are generated by TIL-B and help to promote effective anti-tumor immunity at the tumor site.

Introduction

The key roles of the immune response in cancer progression have only recently come to the forefront despite reports of immune cells infiltrating tumors that date to 1863 (1). Studies of human breast cancer (BC) show that tumor infiltrating lymphocytes (TIL) are more frequently observed in the triple-negative (TN) and HER2-positive (HER2+) subtypes. A TIL presence at diagnosis has been associated with improved disease free (DFS) and overall survival (OS) after adjuvant chemotherapy and pathological complete responses to neoadjuvant therapy in both TN and HER2+ BC (2-6).

Tumor infiltrates contain a heterogeneous population of immune cells frequently dominated by T-cells but also containing B-cells, NK-cells and myeloid-lineage cells including macrophages, mast-cells and neutrophils (7-9). Our laboratory used flow cytometric analysis (FACS) to identify three TIL density groups by setting thresholds based on parallel analyses of normal breast tissues, finding differences among the lymphocyte subpopulations in these TIL density groups (10). TIL^{hi} tumors are distinguished by significantly higher $CD4^{+}$ TIL and B-cell TIL (TIL-B) densities with a corresponding reduced frequency of $CD8^{+}$ TIL (10). T-cell TIL have an activated phenotype characterized by CD69 and HLA-DR upregulation, constitutive expression of the costimulatory receptor CD28 and loss of naïve T-cell markers, including CD45RA and CCR7 (7). $PD\text{-}1^{+}CD4^{+}$ and $PD\text{-}1^{+}CD8^{+}$ T-cell frequencies increase in BC compared to normal breast tissues with up to 40% of the $PD\text{-}1^{+}CD4^{+}$ T-cells characterized as $CD4^{+}$ T follicular helper (T_{FH}) cells located in tertiary lymphoid structures (TLS)(11,12). Current data indicate that in human BC an important $CD4^{+}$ Th1 plus T_{FH} and $CD8^{+}$ TIL presence sustains anti-tumor immune responses while increasing balance in favor of $FOXP3^{+}$ regulatory T-cell (Treg) TIL is generally associated with worse clinical outcomes (11,13-17). Although many experimental animal models and human studies have documented a key role for T-cell TIL in anti-tumor immunity, relatively little is known about the role of TIL-B.

TIL-B have been identified in many tumor types, including high-grade serous ovarian cancer (18), pancreatic adenocarcinoma (19), tongue squamous cell carcinoma (20), colorectal cancer (21) and cutaneous primary melanomas (22). They are principally enriched at the invasive margin where they form dense aggregates opposite the tumor or are resident in TLS. A small fraction of TIL-B also sporadically infiltrates the peri-tumoral areas. TIL-B in TLS have been detected in 30% of melanomas and 8% of colorectal carcinoma where they are correlated with earlier disease stages (21,22). One BC study of TIL-B identified TLS in ~25% of tumors where they incorporated up to 40% of total TIL (23). Our previous work found BC-associated TLS contain TIL-B and are detectable in 60% of all BC and 70% of TIL^{pos} BC (10). We further identified T-cell TIL as principally memory T-cells, while only 55% of TIL-B are memory B-cells, although this is a significant increase over normal breast tissues (10). TLS have been reported to be prognostic for many types of cancer, including NSCLC (24,25), colorectal cancer (26), renal cell carcinoma (27), melanoma (28), pancreatic cancer (29) and BC (11).

Early data from B-cell-deficient mice suggested that TIL-B inhibit T-cell-mediated regression of established tumors (30); however, in humans it is now generally accepted that TIL-B signal a good prognosis for the majority of solid tumor types (31). High TIL-B have been associated with better survival in patients with cutaneous primary melanoma (22), colorectal carcinoma (21), tongue squamous cell carcinoma (20), high-grade serous ovarian cancer (HGSOC)(18), non-small cell lung cancer (NSCLC)(32) and lower relapse rates in cervical cancer (33). Moreover, the prognostic significance of TIL-B and plasma cells (PC) was generally similar to that of $CD3^+$ and/or $CD8^+$ T-cells and increased the prognostic effect of T-cells (31). Interestingly, TIL-B resident in TLS were associated with better survival in pancreatic adenocarcinoma patients, but when randomly scattered in the tumor bed they were not (19). Additionally, TIL-B resident in TLS were also correlated with higher $CD8^+$ TIL infiltration in these tumors.

In BC, a B-cell metagene was shown to have prognostic value for high proliferative node-negative BC (34). Subsequently, a B-cell/PC metagene exhibited a strong prognostic value in high proliferative estrogen receptor positive (ER+) BC, with lower prognostic value for ER– (negative) BC and no prognostic value for low proliferative ER+ BC (35). This was followed by an IHC study associating TIL-B with better survival in grade 3, ER–, basal-like and HER2+ tumors (36). Denkert *et al.* demonstrated that 70% of LPBC are infiltrated with TIL-B and their presence is linked to pathological complete responses (2). Higher expression of B-cell markers has also been correlated with a pathologic complete response in TNBC patients treated with neoadjuvant chemotherapy (37). In contrast to invasive BC, higher numbers of TIL-B detected in pre-invasive ductal carcinoma *in situ* were associated with shorter recurrence free survival (38).

B-cell immune functions extend well beyond being factories for antibody production to include cytokine production, antigen presentation, co-stimulation, and participation in the development of lymphoid tissue architecture. Conversely, B-cells can perform immunosuppressive roles when differentiated to regulatory B-cells (Breg) via their control of cellular immune responses. Prognostic and predictive values for TIL-B have also been reported in human cancer; however, their role is still controversial. TIL-B have been shown to mount tumor-specific autoantibody responses directed against tumor associated antigens (TAA). Our recent work revealed that >84% of the BC patients examined produce autoantibodies to one or more of the antigens present on a 91-TAA microarray (39). We further identified eight antigens that elicit BC-associated autoantibody responses (39). In esophago-gastric adenocarcinoma, 48% of plasma samples were positive for one or more TAA (40). The ability of TIL-B to present antigen to $CD4^+$ TIL have also been demonstrated to function in NSCLC and alter the $CD4^+$ TIL phenotype (41). Direct tumor-cell-killing by TIL-B via antibody-independent mechanisms has also been reported (42). In hepatocellular carcinoma, margin-infiltrating B-cells were found to mainly produce IFN-γ and IL-12p40 rather than IL-2, IL-4, IL-6 or IL-10 (43). The functionality of TIL-B in BC is currently unknown.

This study focuses on BC TIL-B because, in contrast to T-cell TIL, their nature, phenotypic characteristics and functional properties are not well characterized. Our data confirm and extend the correlation between TIL-B and improved clinical outcome in HER2+ and TNBC. We show that TIL-B densities increase in all tumors compared to normal breast tissue. TIL-B are associated with global TIL ($CD45^+$), $CD4^+$ and $CD8^+$ TIL, higher tumor grade and proliferation and hormone receptor negativity. Our data demonstrate that highly infiltrated BC is distinguished by increased germinal center (GC) TIL-B, T_{FH} TIL and antibody-secreting TIL-B, principally resident in TLS. Finally, our functional analyses show that TIL-B not only express activation markers but they respond to BCR stimulation and produce B-cell cytokines and Igs *in situ*.

Results

The prognostic value of TIL-B in HER2+ and TNBC

The long term prognostic value of TIL-B was examined in node-positive HER2+ (N=136) and TNBC (N=113) BC patients from the BIG 02-98 phase III clinical trial (44). Enrollment accrued between 1998-2001 (prior to HER2+ BC patients receiving adjuvant trastuzumab), with a median follow up of 10 years. Dual CD3/CD20 IHC staining performed on full-face tissue sections was independently scored for the percentage of global T-cell TIL and TIL-B by two pathologists (45). Globally, TIL-B were associated with hormone receptor negativity and high histological grade and proliferation (Table S1). No significant associations were identified between TIL-B and age, surgery, histology, positive lymph nodes, tumor size, laterality, treatment or radiotherapy. The median (50th percentile) IHC scores for TIL-B were 2.0% (interquartile range, 0.9% to 4.5%) and 2.5% (interquartile range, 1.0% to 6.25%) in the HER2+ and TNBC cohorts, respectively.

The optimal cut-off for TIL-B positivity in HER2+ tumors was 5.5%, which grouped 84% (N=113) as TIL-B^{neg} and 16% (N=22) as TIL-B^{pos}. Events in the HER2+ cohort at 10 years were 58 (43%) for invasive disease-free survival (iDFS) and 42 (31%) for OS. TNBC had an optimal cutoff at 2.75%, which categorized 51% (N=58) as TIL-B^{neg} and 49% (N=55) as TIL-B^{pos}. The number of events in the TNBC cohort were 51 (35%) for I-DFS and 40 (31%) for OS at 10 years. The Kaplan-Meier curves (Figure 1) show that a TIL-B presence is significantly associated with a better prognosis for both HER2+ and TNBC. In the HER2+ cohort the 10 year iDFS for TIL-B^{pos} was 80% versus 52% for TIL-B^{neg} [Hazard ratio (HR), 0.34; 95%CI 0.12-0.95, p=0.03] and OS for TIL-B^{pos} was 90% versus 66% for TIL-B^{neg} (HR, 0.25; 95%CI 0.06-1.02, p=0.04). In the TNBC cohort the 10 year iDFS for TIL-B^{pos} was 70% versus 40% for TIL-B^{neg} (HR, 0.40; 95%CI 0.22-

0.72, p=0.002) and OS for TIL-B^{pos} was 78% versus 54% for TIL-B^{neg} (HR, 0.44; 95%CI 0.23-0.85, p=0.01). We further found that iDFS and OS for both the HER2+ and TNBC cohorts plateaued before 5 years and was extended to 10 years, suggesting that the greatest impact of a TIL-B presence occurs in the first few years after diagnosis. Determination of the likelihood ratio (Table S2) found that addition of TIL-B (CD20) to T-cell TIL (CD3) for HER2+ and inversely T-cell TIL to TIL-B for TNBC added further prognostic information in multivariate analysis. Overall, these data confirm previous findings (46) and add new weight to the positive role of TIL-B, particularly when linked with T cell TIL, on long-term clinical outcomes.

B-cells infiltrating normal and malignant breast tissues

B-cells infiltrating fresh tissue specimens from normal (n=62), non-adjacent non-tumor (NANT; n=312), benign tumor (n=21), untreated invasive ductal carcinoma (IDC; n=241) and untreated invasive lobular carcinoma (ILC; n=62) were analyzed by FACS (Figure 2). The clinicopathological characteristics of the patients are detailed in Table 1 and the FACS gating strategies shown are in Figure S1 (47). TIL-B were detected at significantly higher densities (absolute number/mg tissue) and percentages (% of total CD45^{+} cells) in invasive carcinomas (IDC and ILC) compared to normal breast tissues (Figure 2A-B). B-cell infiltration in NANT was similar to normal tissues. Alternatively, benign tumors contain significant increases in TIL-B density compared to normal and NANT tissues, although as a percentage of total TIL they are not markedly different. We also analyzed TIL-B as a function of disease stage. The accrual of TIL-B in IDC BC ends abruptly with stage IV tumors and local relapses, which are both poorly infiltrated and show no significant differences compared to normal tissues (Figure 2C-D).

IDC and ILC patients were next grouped on the basis of TIL density ($CD45^+$ TIL/mg) as previously defined: 1) TIL^{neg} with TIL densities equivalent to normal breast tissues; and TIL^{pos} split into 2) TIL^{int}, which falls between the normal tissue and NANT thresholds; and 3) TIL^{hi}, with densities above the NANT threshold (10). TIL-B increase significantly from TIL^{neg} to TIL^{int} to TIL^{hi} in IDC and TIL^{int} to TIL^{hi} in ILC compared to NANT, both as absolute numbers and as a percentage of $CD45^+$ cells (Figure 2E-H). Interestingly, only TIL-B densities but not their percentage within the $CD45^+$ cell compartment increase significantly between the IDC TIL groups (Figure 2E). Thus, apart from a higher set point, TIL-B generally parallel global $CD45^+$ TIL (Table S3A) with TIL^{hi} tumors characterized by their extensive presence.

FACS quantification of $CD4^+$ and $CD8^+$ TIL subpopulations in the same fresh BC specimens (n=311) reveals that their presence is highly correlated with TIL-B (Table S3A). Because the majority of published studies identified TIL-B on IHC-stained tissues, we selected a subset of full-face blocks (n=25) from our IDC BC cohort to comparatively score TIL on CD3/CD20 and CD4/CD8 dual IHC-stained tissues for comparison. The correlations between TIL-B quantified on paired data from $CD19^+$ TIL (FACS) and CD20 (IHC) were statistically significant (Table S3B). Once again these data reveal that TIL-B are positively correlated with global, intra-stromal and intra-tumoral TIL, $CD3^+$ TIL and the number of aggregates or TLS in the BC microenvironment. CD4 and CD8 scores from IHC-stained tissues were less well correlated, most likely due to some technical limitations with this stain. Clinicopathological parameters were correlated with TIL-B densities (determined by FACS) showing that high TIL-B densities are significantly associated with higher histological grade, higher tumor cell proliferation (Ki67) and hormone receptor negativity (Table 1). This is consistent with our previous observations for global $CD45^+$ TIL densities, with the exception of age (10). Overall, these data reveal higher infiltration of TIL-B in early BC in association with global TIL.

The immunophenotype of BC TIL-B

$CD19^+CD45^+$ B-cells from peripheral blood mononuclear cells (PBMC), lymph nodes (LN) and breast tissues were FACS analyzed for the expression of various B-cell differentiation and activation markers. CD38 and IgD, confirmed markers for defining naïve to memory B-cells, were used to identify and quantify B-cell differentiation stages (Figure 3, Figure S2). The analysis of BC patient compared to healthy donor (HD) PBMC detected no significant differences between any of the B-cell subpopulations (Figure S2A) and therefore functions as an internal baseline control for each patient. Involved LNs show a significant increase in early memory and memory TIL-B compared to reactive tonsils (Figure S2B). In breast tissues, the frequency of naïve B-cells (within total $CD19^+$ B-cells) was significantly reduced in parallel with an increase in memory B-cells in benign, IDC and ILC compared to normal breast (Figure 3A-B)(*note: B-cell immunophenotypes in NANT tissues were not analyzed because of their low absolute numbers combined with limited tissue availability*). Furthermore, the low TIL densities in stage IV IDC tissues were distinguished by a higher prevalence of memory TIL-B compare to IDC.

GC TIL-B significantly increase in TIL^{hi} compared with TIL^{neg} IDC, suggesting TLS are likely present in these highly infiltrated tumors (11,12). Furthermore, GC TIL-B and T_{FH} TIL are consistently, well correlated with one another [Figure 3C-D and Table S4; T_{FH} TIL are identified as $CD3^+CD4^+CD45^+CD200^{hi}PD\text{-}1^{hi}$ T-cells because the canonical T_{FH} marker CXCR5 is downregulated on a significant number of these specialized TIL in BC (11,12)]. GC TIL-B frequencies (centroblasts and centrocytes) are also positively correlated with $PD1^+CD4^+$ and $PD\text{-}1^+CD8^+$ T-cell TIL and antibody-secreting cells (ASC) but inversely correlated with memory

TIL-B. Overall, these data characterize TIL^{hi} BC tissue as a receptive site for cooperative interactions between various active adaptive immune functions.

Analysis of the late stages of TIL-B differentiation reveal that >20% of $CD19^+$ TIL-B express surface IgG in line with an affinity-matured response; however, significant differences between normal and tumor tissues were not detected (Figure S2C). Tumor tissues also contain $CD5^+$ TIL-B (approximately 15% of TIL-B) but at lower frequencies than in normal tissues. ASC TIL-B, including $CD27^{hi}CD38^{hi}$ plasmablasts and PC as well as $CD38^{hi}CD138^+$ PC were identified but represent only minor TIL-B subpopulations. While no significant differences in ASC TIL-B were seen between normal and tumor tissues, a trend for more ASC in association with increased TIL infiltration was observed in IDC BC. These data show that humoral immune responses are generated at the tumor site with GC TIL-B characteristic of more extensively infiltrated tumors and correlated with T_{FH} TIL, which are indicative of a TLS presence.

TIL-B composition, activation and organization in BC

Our previous work determined that BC with extensive infiltrates are distinguished by both intra-tumoral and stromal TLS (11,12,48). This study focused on the spatial distribution and organization of TIL-B in TLS by analyzing BC (Figure 4) and tonsil (control; Figure S3) FFPE tissues. Immunofluorescence (IF) confocal microscopy shows that TLS have a higher order organization that resembles secondary lymphoid organs (SLO), including a B-cell follicle surrounded or adjacent to a T-cell zone (Figure 4A; Figure S3A). The T-cell zone is principally composed of $CD4^+$ TIL with some $CD8^+$ TIL and IgD^+ follicular mantle TIL-B. T_{FH} cells, characterized as PD-1^{hi} cells, are detected in the light zone of tonsillar GC and in TLS (Figure 4B; Figure S3A). In contrast, TIL aggregates are characterized by randomly distributed TIL-B, $CD4^+$ and $CD8^+$ T-cell TIL (Figure 4A).

GC in SLO are key sites for generating humoral immune responses, which led us to use markers of active GC to determine whether TLS house similar activities. BC TLS are built on an interdigitating network of follicular dendritic cells ($CD35^+$ FDC) similar to a tonsillar GC (Figure 4C; (Figure S3B). IgM^+CD20^+ TIL-B surround the GC with proliferating TIL-B ($Ki67^+$) at its center, again similar to tonsil tissue. $CD138^+$ PC are scarce and principally detected at the periphery of a BC TLS or tonsil GC, suggesting that once matured they quickly move into the periphery. B-cell activation factor (BAFF; *TNFSF13B*) is a cytokine that potently activates B-cells and together with its receptor (BAFF-R; *TNFRSF13C*) plays a role in B-cell differentiation and survival (49-51). BAFF is ubiquitously expressed throughout BC TLS and tonsillar GC while BAFF-R expression is restricted to the mantle zone in tonsillar GC with similar but less structured expression in BC TLS (Figure 4D; Figure S3B).

An analysis of TIL-B interactions with other cells in the tumor microenvironment was accomplished using markers for subpopulations of T-cell TIL (CD4, FOXP3 and CD8), TIL-B (CD20), macrophages (CD68) and tumor cells (panCK) in a multiplex IHC assay (mIHC; Figure 4E; Figure S3B; Figure S4). Interestingly, some TIL-B directly interact with tumor cells as well as $CD8^+$ TIL and macrophages, both in aggregates, primary lymphoid follicles and fully formed TLS. A region of BC aggregates was selected for analysis using the Phenoptr package of InForm® software and identified 41% and 29% of TIL-B touching tumor cells and CD8-TIL, respectively (Figure S4). These data show that specific TIL-B immunophenotypes are in contact with other immune cell subpopulations in BC-associated TLS and indicative of active humoral immune responses developing at the tumor site.

The functionality of TIL-B in BC

We evaluated the functional attributes of TIL-B beginning with the balance of Ig isotypes present in fresh breast tissue supernatants (47) and plasma. Plasma from HD and BC patients, the latter stratified on TIL levels, were remarkably similar except for an increase in IgG3 detected in the TIL^{neg} group (Figure 5A). In the tissue supernatants, total Igs as well as the individual isotypes (including IgG subclasses) generally increase in parallel to the extent of immune infiltration from normal breast tissue to TIL^{hi} BC (Figure 5B). Statistically significant increases in IgG1, IgG2, IgG3 and IgM were detected between normal and malignant breast tissues as well as in association with increasing TIL in BC. Interestingly, IgA was significantly increased in TIL^{int} compared to TIL^{neg} and TIL^{hi} BC, suggesting that the balance of isotypes may be influenced by immune activities associated with the level of infiltration in the tumor microenvironment.

B-cells can also influence immune responses via soluble factors with their subpopulations characterized by the cytokines they produce, similar to T-cells. For example, effector B-cells (Be1 and Be2) can prime naïve $CD4^+$ Th1 or Th2 differentiation via their secretion of the canonical polarizing cytokines, IFNγ and IL-4, respectively (52). Cytokines, including IFNγ, TNFα, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-21 and IL-22, were analyzed in fresh breast tissue supernatants (mammary reductions, NANT and tumors) together with plasma from HD and BC patients. In plasma, most of these cytokines were detectable (except IL-1β, IL-2 and IL-5; data not shown) with elevated IFNγ, TNFα, IL-4, IL-13, IL-17A and significant increases in IL-22 and IL-10 distinguishing BC patients from HD (Figure S5A). Interestingly, a substantial increase in IL-6 was observed in plasma from TIL^{hi} BC compared to other BC patients and HD. Analysis of tissue supernatants again detected all of the cytokines tested (except IL-17A, data not shown) in normal and abnormal breast tissues (Figure S5B). The tissue milieus from normal breast, NANT and BC are reflected in the composition of their supernatant, which generally followed a pattern of increased expression from normal to NANT to BC. Statistically significant increases were found for TNFβ, IL-1β, IL-2, IL-13 and IL-22 in tumor relative to normal tissues. No significant differences were detected between normal breast and BC patient NANT tissues except for IL-10, which was expressed at very low levels but interestingly increased in both NANT and tumor relative to normal breast tissues.

The previous experiments clearly demonstrate that the BC tissue microenvironment is bathed in higher levels of many cytokines; however, our primary goal here was to identify those specifically produced by TIL-B. A comparative analysis of B-cells sorted from tonsillar, lymph node and BC tissues shows that TIL-B express a multitude of cytokines except for IL-17A, IL-21 and IL-22, which are very low compared to B-cells from tonsils and lymph nodes (Figure 6). Interestingly, TIL-B express higher levels of type 1 cytokines (*IFNG* and *TNFA*) compared to B-cells from secondary lymphoid tissues. Type 2 cytokines were more diverse with BC tissues characterized by higher *IL4*, equivalent *IL5* and lower *IL6* and *IL13* expression levels. The immunosuppressive cytokines *IL10* and *TGFB* were also expressed by TIL-B with *TGFB2* significantly increased compared to tonsillar B-cells. These data suggest that TIL-B favor a type 1 cellular immune response and could thereby help to drive Th1 responses when present in the BC microenvironment.

While B-cells are well known for their role as antibody producers, they also perform a variety of other immunological functions, including the induction of antigen-specific T-cell activation via antigen presentation. The expression of co-stimulatory molecules such as CD40, CD80 and CD86 together with HLA-DR, an antigen presenting cell/activation marker, were analyzed on PBMC, tonsillar B-cells and BC TIL-B (Figure 7A). HLA-DR and CD40 expression are significantly downregulated on TIL-B from IDC BC compared to PBMC B-cells from HD and patients. A similar trend was also observed for TIL-B from ILC. Tonsillar B-cells generally had low HLA-DR expression while CD40 expression was comparable to PBMC. No significant differences were detected in the percentage of $CD80^+$ and $CD86^+$ TIL-B compared with PBMC or tonsillar B-cells.

Decreased HLA-DR and CD40 expression could result from specific biological processes such as: 1) B-cell differentiation to plasmablasts/PC, 2) the presence of exhausted tissue-like memory (TLM) B-cells [recently described to have reduced CD40 expression (53) and/or 3) a tissue microenvironment favoring immunosuppression as described for BC dendritic cells (54-56). Similar to previous findings (57), our study did not detect increases in plasmablasts or PC in tumor tissues compared to normal breast tissues and peripheral blood (Figure 7B). Furthermore, while TLM B-cells ($CD19^+CD27^{lo}CD21^{lo}$) were detected in malignant breast tissues (Figure 7C), their frequency was only slightly increased in IDC and ILC BC compared to peripheral blood and tonsils, which was not statistically significant.

The functional consequences of the immunophenotypic changes we detected in HLA-DR and CD40 were evaluated by examining TIL-B intracellular calcium mobilization in response to stimulation with anti-BCR antibodies (ionomycin was used as a positive control; Figure 8). PBMCs and BC tissue homogenates (47) were rested overnight prior to loading Fluo-8, followed by FACS measurement of intracellular calcium levels in the $CD19^+$ B-cell gate following the addition of BCR stimuli. TIL-B and HD B-cells responded similarly with an immediate increase in intracellular calcium upon stimulation. This rapid increase of intracellular calcium suggests that the downregulation of HLA-DR and CD40 on TIL-B does not reflect an irreversible anergic state. Overall, these data show the functionality and multiple facets of TIL-B in anti-tumor immune responses, not only in the context of antibody production but also Th1 responses and antigen presentation.

Discussion

The data presented in this study show that TIL-B increase in invasive BC compared to normal breast tissues, which is particularly marked in extensively infiltrated tumors (TIL^{hi}). TIL-B are significantly associated with T-cell TIL, histological grade, proliferation ($Ki67^+$) and ER/PR status. While all B-cell subsets are present, TIL^{hi} BC is specifically characterized by increased GC B-cells in tight correlation with T_{FH} cells and ASC resident in TLS. These TIL-B are functionally responsive to antigen-receptor stimulation and produce cytokines and Igs despite their lower expression of APC markers. Overall, our data support the concept that ongoing humoral immune responses are associated with elevated and well-organized TIL, signaling that TIL-B play important role(s) in anti-tumor immunity.

Early studies variably associated the presence of TIL-B with either positive or negative clinical outcomes. More recently, Schmidt *et al*. reported that a B-cell metagene had a positive prognostic value for metastasis free-survival in node-negative BC patients (N=200; diagnosed between 1988-1998) (34). They further demonstrated that their B-cell metagene provided independent prognostic information for high proliferative BC. Subsequently, Mahmoud *et al.* examined IHC-stained tissue microarrays from BC patients treated in the adjuvant setting (N=1902; diagnosed between 1987-1998), finding that global TIL-B were associated with better long term BC specific survival, particularly in hormone receptor negative, high grade tumors (36). Interestingly, these data are also consistent with a human pancreatic adenocarcinoma study finding a correlation between a TIL-B/$CD8^+$ TIL presence and a more favorable prognosis in a tumor type that is generally lethal (19). In this study, we examined the impact of a TIL-B presence on the long-term survival of HER2+ and TNBC patients diagnosed between 1998-2001 and treated in the adjuvant BIG 02-98 phase III clinical trial with a median follow up of 10 years (45). TIL-B, scored on IHC-stained full-face sections, confirm the earlier gene expression and TMA data that global TIL-B density is associated with hormone receptor negativity, higher proliferation, higher histological grades and higher stages. We further demonstrate that iDFS and OS in the uniformly treated TIL-B^{pos} HER2+ (without trastuzumab) and TNBC patients was remarkably high at 5 years with OS remaining stable up to 10 years. Interestingly, we demonstrated added prognostic value for TIL-B plus T-cell TIL in HER2+ and T-cell TIL plus TIL-B in TN BC, which is consistent with pre-existing adaptive immune responses in some patients (58). Overall, these studies identify TIL-B as an important element of long-term BC survival; however, clinical outcome alone is not sufficient to establish a critical functional role for TIL-B in anti-tumor immunity.

Our earlier study of fresh breast tissues reported that immune infiltrates (CD45$^+$ TIL) in BC form a continuum from very low to exceedingly high immune activities at the tumor site (10). Further, we used normal breast tissues to set thresholds for stratifying TIL densities into TIL^{neg}, TIL^{int} and TIL^{hi} BC. TIL^{hi} tumors contain significantly lower frequencies of CD8$^+$ TIL offset by higher CD4$^+$ TIL and TIL-B. The present study, using our extended patient cohort, found that similar to total CD45$^+$ TIL, TIL-B densities and frequencies increase continuously above the thresholds for normal breast and TIL^{neg} (BC). We further observed that benign tumors (principally fibroadenomas) have an important TIL-B infiltrate. Miligy *et al.* reported that pure DCIS tumors have higher numbers of TIL-B compared to DCIS-associated with invasive BC (38). In their study, the TIL-B in pure DCIS were associated with larger tumor size, hormone receptor negativity, HER2+ positivity and a shorter recurrence free interval. Together, these data support the concept that TIL-B may either actively contribute to BC development and/or be attempting to help contain the developing malignancy.

TIL-B differentiation and maturation states have been studied in other human solid tumor types. NSCLC were found to contain all stages of B-cell differentiation although they were dominated by IgD^{-}CD38$^{+/-}$ memory B-cells (25). The frequency of GC B-cells was similar in blood, distant lung and tumor tissues from individual patients but generally PC specifically increased in lung tumors compared to blood or lymph nodes. We previously demonstrated that CD19^{+}CD27^{-} naïve B-cells are the major subpopulation (65% of B-cells) in normal breast tissues with enrichment to a majority of CD19^{+}CD27^{+} memory B-cells (55% of TIL-B) in BC (10). In contrast to our BC data, no differences in B-cell differentiation stages were detected between distant lung and NSCLC (25). We show here that ASC (CD27^{hi}CD38^{hi}) and PC (CD38^{hi}CD138^{+}) are minor subpopulations of BC TIL-B with no significant differences observed between normal and tumor tissues. In contrast to NSCLC, our data parallel those from high-grade serous ovarian cancer where a small population of IgD^{-}CD38^{+} B-cells, consistent with GC B-cells, together with an absence of CD38^{+}CD138^{+} PC was observed (57). This study also found that 80% of the ovarian tumors analyzed had small numbers of CD38^{+}CD138^{-} plasmablasts. The data from breast and ovarian cancer suggest that once matured, PC and ASC do not remain in normal or malignant breast tissues and therefore have likely moved to the periphery.

In addition to the increases in early and late memory B-cells, we detected higher frequencies of GC TIL-B in association with T_{FH} cells and ASC in benign and invasive TIL^{hi} BC. Detailed characterization of TIL-B in nine ovarian tumors found an overwhelming majority of IgD^{-}CD38$^{-/lo}$ TIL-B, indicative of a memory B-cell phenotype; however, these TIL-B were all CD27 negative (57). These atypical memory B-cells have also been reported in ovarian metastases in association with memory B-cells with a more classical phenotype (59). In chronic infections characterized by an inability to eradicate the pathogen, B-cells with an atypical memory phenotype have been shown to accumulate in patients (60). These data suggest that atypical memory TIL-B could be a subset of CD27^{-} memory or TLM B-cells arising in chronic inflammatory microenvironments. Tissue resident memory T-cells were initially described more than a decade ago in viral infection studies and are now viewed as the primary T-cell subpopulation responsible for peripheral tissue surveillance and defense (61). Furthermore, these resident memory T-cells not only serve in pathogen defenses but have recently been shown to be functionally important in sustaining tumor dormancy by maintaining the equilibrium between malignant progression and immune surveillance (62). Similarly, TLM B-cells were identified in virus studies where they were described as having an exhausted phenotype in viremic HIV patient blood (63-65). TLM B-cells express low levels of some distinguishing markers, exemplified by CD21 and CD27, but also high levels of inhibitory receptors, including Fc-receptor-like-4 (FCRL4), together with homing receptors specific for inflammatory sites. B-cells with a TLM phenotype have been detected in tonsils, the blood of elderly individuals as well as patients with microbial infections and autoimmune diseases (60). Bruno *et al.* recently identified $CD69^{+}HLA\text{-}DR^{+}CD27^{-}$ TIL-B in NSCLC, finding they were associated with Treg TIL (41). In this study, we detected $CD21^{lo}CD27^{lo}$ TLM TIL-B in BC and positively correlated their presence with $PD1^{+}CD4^{+}$ and $PD1^{+}CD8^{+}$ TIL, all of which could potentially be functionally exhausted TIL.

T-cell or B-cell exhaustion, distinct from anergy or senescence, is a reversible state of lymphocyte dysfunction characterized by deficient effector functions. Curtailing immune responses is an important regulatory mechanism for day-to-day immune activities; however, in situations of chronic antigen exposure, seen in autoimmunity, infection and cancer, this often results in an inability to control disease. These dysfunctional T-cells and B-cells are greatly diminished in their response to antigen receptor stimulation, leading to decreased proliferation, cytokine secretion and/or antibody production (63,66). In immune responsive tumors, the accumulation of T-cell TIL and TIL-B with a dysfunctional phenotype could reflect multiple rounds of antigenic-stimulation and efforts, albeit unsuccessful, to eradicate the tumor. Our previous study found that TIL-B are principally located in TLS (10) and while these structures may provide an order of protection from tumor-mediated suppression, they are also sites of chronic antigenic stimulation. Some of these tumors resemble an excluded phenotype where TIL accumulate at the tumor bed border in aggregates or TLS. TLS-positive tumors have been shown to contain both active and inactive structures suggesting that in the chronic inflammatory tumor microenvironment they may be driven from functional to non-functional over time (67,68).

TLS formation and maintenance in tumors is just beginning to be understood and because their composition and organization is similar to secondary lymphoid organs it suggests they potentially evolved via common mechanisms of lymphoid organogenesis. A variety of cell types, including lymphoid tissue inducer cells, stromal cells, dendritic cells, B-cells and T-cell subpopulations, are critical partners in TLS formation. Cytokines and chemokines, including lymphotoxin A, CXCL13, CCL19, CCL21, IL-17, IL-22, and IL-23, have also been implicated in the initiation and organization of TLS (reviewed in (69)). Our laboratory recently demonstrated that the transcription factor FOXP1 is an important negative regulator of immune cell migration in BC via its impact on tumor cell cytokine and chemokine expression (70). Further, we found that CXCL13-producing Tfh (named TfhX13) TIL promote local memory TIL-B differentiation and are potentially an early trigger of TLS formation in BC (12). The present study extends our observations on TLS formation by showing that mantle zone TIL-B express high levels of BAFF and its receptor, which reflects the pattern seen in secondary lymphoid organs. This data is also consistent with a study demonstrating BAFF's critical role in the formation and compartmentalization of renal TLS (71). Our data suggest that CXCL13-producing Tfh cells guide TIL-B to inflammatory sites for TLS formation, where local concentrations of BAFF promote their survival, proliferation and ultimate differentiation to memory cells, PC or ASC.

The migration, differentiation and maturation of TIL-B in tumor-associated TLS potentially drives the production of Igs that specifically recognize tumor antigens, thereby eliciting functional anti-tumor humoral immunity. This study examined Ig isotypes and IgG subclasses in plasma and primary tissue supernatants from normal and malignant breast tissues finding that Ig concentrations were associated with TIL infiltration levels and characterized by a significant increase in IgG1 in TIL^{hi} BC. The link between polarization toward specific Igs and downstream effects on anti- or pro-tumor immunity is currently unknown. In melanoma, polarization to IgG4 under Th2 inflammatory conditions was associated with poor clinical outcomes (72). This study found that melanoma antigen-specific IgG4 antibodies impaired the ability of the corresponding IgG1 to induce antibody-dependent tumor cell phagocytosis. Alternatively, anti-HER2 autoantibodies have been associated with a protective effect for primary or recurrent BC development (73). Thus, polarization of Ig isotypes and/or subclasses is likely to have a significant impact on tumor immunity.

TIL-B are functional, reflected by their production of autoantibodies specific for tumor-associated antigens, with a previous demonstration that BC TIL-B expand oligoclonally (74). Our recent analysis of paired primary tissue supernatant and plasma samples detected autoantibodies in >84% of our BC cohort to one or more antigens on a 91-TAA microarray (39). The three most frequently recognized proteins were ANKRD30BL (Ankyrin repeat domain 30B like), COPS4 (COP9 Signalosome Subunit 4) and CTAG1B (also known as NY-ESO-1). This work also found higher IgG but not IgA responses to BC-associated antigens were associated with shorter recurrence-free survival and lower CD8$^+$ TIL suggesting Ig isotypes may provide different biological functions in the tumor microenvironment (39). In the present study, global

Ig production and specifically IgG1 levels increase in the tumor tissue supernatant as TIL levels graduate from low to intermediate to high, but this was also associated with a bump in IgA in the TIL^{int} group. Although there is no current precedent for interpreting these findings, a recent case report found an accumulation of PC and IgG in a metastatic lymph node from a lung cancer patient who achieved a partial response to immune checkpoint blockade (75). Alternatively, in primary melanoma an increasing abundance of PC's predominantly producing IgG and IgA was associated with a worse prognosis (76). IgA production was further associated with melanoma progression, an effect that might be reflected in higher IgA in the TIL^{int} BC group. The importance of changing balances in Ig isotypes and IgG subclasses remains unknown; however, our data have identified a clear correlation between increased GC TIL-B and Tfh TIL, both expected to drive increased memory B-cell and PC differentiation leading to Ig production.

The versatility of B-cells extends to their regulation of immune responses via cytokine production. Effector B-cell subpopulations (Be1 and Be2), influenced by their surrounding milieu, produce distinct sets of cytokines [IFNG (Be1) or IL-4 (Be2)], which primes them to regulate naïve CD4^{+} T-cell differentiation and polarize them toward Th1 or Th2, respectively (52,77). First described in hepatocellular carcinoma, margin-infiltrating effector TIL-B were found to principally produce IFNγ and IL-12p40 but not IL-2, IL-4, IL-6 or IL-10 (43). We also primarily detected type 1 cytokines (IFNγ and TNα) in BC TIL-B with significantly lower amounts of type 2 cytokines (IL-5, IL-6 and IL-13). These effector TIL-B in association with CD8^{+} T-cells were linked with improved survival both in hepatocellular carcinoma and ovarian cancer (57), suggesting that type 1 TIL-B may help to drive cytotoxic T-cell responses. There is also accumulating evidence that TIL-B contain Breg, whose function is to regulate effector immune responses. Through their production of immunosuppressive cytokines, including IL-

10 and TGFβ, the balance between effector TIL-B and Breg TIL-B could play an important role in suppressing anti-tumor immune responses. Consistent with previous studies in other human tumor types, we found that BC TIL-B can express IL-10 and TGFβ. Generally, these data support the notion that interactions between TIL-B and T-cell TIL, particularly in the intimate microenvironment of a TLS, may be a driving force in activating and regulating humoral and cell-mediated anti-tumor immunity in human cancer.

B-cells play important roles in immune responses that extend well beyond their canonical functions as antibody producers and include cytokine production, antigen presentation, co-stimulation and contributions to lymphoid tissue development. Initial studies in B-cell-deficient mice suggested that TIL-B generally inhibit T-cell-mediated regression of established tumors (30); however, other murine tumor model studies found that TIL-B are necessary for optimal T-cell activation and cellular immunity (78). These positive and negative effects on cellular immune responses directed by TIL-B could be due to differences in activation status, maturation stages and B-cell functions in different murine model systems. In this human study, we show that TIL-B express surface APC and costimulatory markers and are capable of responding to antigen engagement ex vivo. In ovarian cancer, TIL-B also express APC markers and were shown to co-localize with $CD8^+$ T-cells, which suggests they could be functioning as APC (57)*.* This is supported by as recent NSCLC study demonstrating efficient antigen presentation to $CD4^+$ TIL by TIL-B (41). While most TIL-B express MHC class II (HLA-DR), three types of $CD4^+$ TIL responses to TIL-B have been identified: 1) activated where they spontaneously present endogenous tumor antigens to $CD4^+$ TIL; 2) antigen-associated, following re-stimulation with antigen; or 3) non-responsive, where no response is detectable. Based on previously published work together with the data presented here, we suggest that TIL-B can present antigen to $CD4^+$ TIL and thereby promote anti-tumor immune responses.

Overall, this study shows that BC TIL-B are associated with an improved clinical prognosis and their presence primarily reflects an active, functional humoral immune response that likely transpires in BC-associated TLS.

Methods

Patient population and clinical samples

Fresh breast tissues, including tumor, normal tissue from mammary reduction and nonadjacent non tumor (NANT) tissues, were obtained at the day of surgery from consenting BC patients diagnosed and treated at the Institut Jules Bordet between August 2012 and January 216. The clinicopathological characteristics of BC patients are detailed in Table 1. Each fresh sample was measured, weighed and a hematoxylin and eosin stain were performed. Among a total number of 636 recruited tumors, only 56% were infiltrated with a sufficient number of B-cells to perform ex vivo studies. Peripheral blood samples were obtained from 102 patients undergoing tumor resection for BC the day before the surgery. Peripheral blood samples from 23 healthy female adults were used as controls. Plasma were taken after centrifugation, clarified and stored at -80°C. All specimens were acquired after signed informed consent using procedures approved by the Institut Jules Bordet's Medical Ethics Committee.

For the retrospective study, TIL-B were assessed on samples from 136 patients with HER2 and 113 patients with TNBC included in the BIG 02-98 adjuvant phase III trial. TNBC subtype was defined as estrogen receptor (ER)-negative, progesterone (PR)-negative and HER2-negative based on central IHC review that determined ER, PR and HER2 status. HER2 subtype was defined as HER2 overexpression based on +3 by immunohistochemistry or +2 by immunohistochemistry and confirmed positive by fluorescent *in situ* hybridization. All samples were collected at baseline from the surgical specimen. Patients enrolled onto this study consented for use of their tumor tissue for future research purposes. Patient characteristics are detailed in (45).

Tumor infiltrated leukocytes and PBMC isolation

Dissected tumor fragments from fresh surgical specimens were directly transferred into 3ml of X-VIVO 20 (Lonza). Tissue was manually minced before two rapid rounds of mechanical dissociation with the GentleMACS™ Dissociator (Myltenyi Biotec). The resulting cell suspension was filtered following each dissociator run using a 40µm cell strainer (BD Falcon), washed with X-VIVO 20, centrifuged 15min at 600g, and resuspended in X-VIVO 20 before FACS analysis. The tumor supernatant was the initial 3ml of X-VIVO 20 recovered after the first round of dissociation, which was subsequently clarified by centrifugation for 15 min at 13000g (47).

PBMC were purified by density gradient centrifugation over Lymphoprep™ and washed three times before FACS staining.

Flow cytometry and cell sorting

Cell suspensions were incubated with manufacturer's suggested dilutions of fluorescently labeled primary monoclonal antibodies (Table S5) for 1 hour at 4°C in 100ul of X-VIVO 20 followed by washing with of PBS. After washing once, a lysing solution is used for the lysis of red blood cells in cell suspension from breast tissues (VersaLyse, Beckman Coulter). Cells were then immediately acquired on a GALLIOS 10/3 cytometer, and analyzed on Kaluza Flow Cytometry Analysis v1.2 software (Beckman Coulter).

Cell sorting was performed on breast tissue cell suspension using a Moflo ASTRIOS EQ 12/4 sorter (Beckman Coulter). B-cells were sorted on CD3$^-$CD19$^+$ lymphocyte gate. Cells were then lysed in TRIzol (Invitrogen) for RNA extraction and qRT-PCR.

Immunohistochemistry staining and pathologic assessment

FFPE tissue sections (4 µm) were immunohistochemically stained for CD3/CD20 and CD4/CD8 dual staining on a Ventana Benchmark XT automated staining instrument (Ventana Medical Systems). A detailed protocol for the dual IHC stains is described in Buisseret et al. For CD3/CD20 dual staining, the slides were incubated with the ready-to-use polyclonal rabbit anti-CD3 primary antibody (IR50361-2, Agilent) and the ready-to-use mouse monoclonal anti-CD20 primary antibody (IR60461-2, Agilent). For CD4/CD8 dual staining, the slides were incubated with the ready-to-use monoclonal rabbit anti-CD4 primary antibody (BSB5150, BioSB) and the ready-to-use mouse monoclonal anti-CD8 primary antibody (IR62361-2, Agilent). Scoring TIL infiltration, lymphocyte subpopulation markers and TLS on IHC-stained tissues was independently performed by two trained pathologists (RdW, GVdE) who were blinded to the clinical and experimental data.

Immunofluorescence microscopy

4µm sections of FFPE tissues were dewaxed twice in 100% xylene for 10 minutes, once in ethanol 100%, once with 90% ethanol, once with 70% ethanol and twice in H2O for 5 minutes each baths. Then slides were transferred in pre-warmed sodium citrate buffer for antigen retrieval and incubated 30 minutes in the bath at 95°C, 20 minutes on the bench and 5 minutes in running water. Tissues were blocked with immunofluorescence (IF) buffer (PBS plus 1% bovine serum albumin and 2% fetal bovine serum) for 30 minutes at room temperature prior incubation of primary antibodies diluted in IF buffer overnight at 4°C. After three washes of 5 minutes in PBS, secondary antibodies diluted in IF buffer were incubated 2 hours at room temperature. After washing, slides were mounting mounted with ProLong Gold anti-fade mounting medium (Invitrogen) overnight, and were visualized on a Zeiss LSM 710 confocal microscope equipped with a ×20/0.8 Plan-Apochromat dry objective (Carl Zeiss).

Multiplex immunohistochemistry

FFPE tissue sections (4 µm) were processed manually for mIHC. Briefly, slides were heated at 37°C overnight, then deparaffinized and fixed in Neutral-buffered 10% formalin. Slides were labeled for CD4 (helper T-cells), CD8 (cytotoxic T-cells), CD20 (B-cells), FOXP3 (Treg), CD68 (macrophages), pan-cytokeratin (cancer cells), and DAPI (all nuclei) using a serial same-species fluorescence-labeling approach that employs tyramide signal amplification and micro- wave-based antigen retrieval and antibody stripping according to the manufacturer's instructions (Opal 7 Solid Tumor Immunology kit, PerkinElmer). Slides were mounted with Vectashield Hardset Antifade Mounting Medium (VectorLaboratories). Samples were visualized on a Zeiss LSM 710 confocal microscope equipped with PMT spectral 34 canaux QUASAR (Carl Zeiss).

Quantitative real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was extracted using TRIzol Reagent (Invitrogen) and reverse transcribed into cDNA using High Capacity RNA-to-cDNA (Applied Biosystems) following standard procedures. qRT-PCR reactions were performed using iTaq™ SYBR® Green Supermix with ROX (Bio-Rad) on an ABI 7900HT Prism sequence detector (Applied Biosystems). The relative mRNA expression levels calculated using the $2^{-\Delta\Delta ct}$ method.

Cytokines/Ig Measurement by Multiplex Bead Array

Soluble Igs and cytokines were determined by FACS using the multiple analyte detection system FlowCytomix™ (Bender MedSystems GmbH) performed according to the manufacturers' instructions. Plasma and tumor supernatant samples were clarified prior to use in the assay. The detection limits for IgG1, IgG2, IgG3, IgG4, IgA, IgM, IgE were 0.28, 1.15, 0.29, 1.20, 0.18, 10.17 and 0.02 ng/ml, respectively and for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-22, IFNG, TNFA were 4.2, 16.4, 20.8, 1.6, 1.2, 1.5, 1.9, 1.5, 4.5, 2.5, 43.3, 1.6, 3.2 pg/ml. Quantification was done by FlowCytomix Pro Software (Bender MedSystems GmbH) and normalized to the tissue size (mm^3).

Flow cytometric analysis of calcium flux

PBMC and TIL were incubated overnight in RPMI 1640 media supplemented with 10% FBS, 2mM glutamine and antibiotics (50µg/ml streptomycin and 50U/ml penicillin) at 37°C and 5% CO_2. Cells were loaded with 5µM Fluo-8 calcium indicator (Abcam) and 0.02% Pluronic F-127 (Life Technologies) and incubated at 37°C for 30 min, then at room temperature for another 30 min. Loaded cells were washed and subsequently stained with anti-CD4-APC and anti-CD19-APCVio770 (Miltenyi) for 15 min at room temperature. Cells were washed and resuspended in HHBS buffer at 37°C for FACS analysis. After establishment of the baseline for

30 seconds, cells were stimulated with 50µg/ml goat anti-human IgA+IgG+IgM (Jackson ImmunoResearch), and the resulting calcium release was recorded for 150 seconds. A total of 2µg/ml ionomycin (Sigma-Aldrich) served to elicit the maximum response over the last minute in all assays. The relative concentration of intracellular free calcium was measured as the median fluorescence ratio of the maximum peak poststimulation to baseline (79,80).

Statistics

Unpaired data sets were compared by using the nonparametric One-Way ANOVA test (GraphPad Prism4 software, San Diego, CA, USA). All values are expressed as mean± SEM. In the BIG2-98 series, we used the the Mann-Whitney-Wilcoxon test and the chi-square or Fisher exact test to assess differences in continuous and categorical variables, respectively. The association between two continuous variables was assessed with Spearman correlation, and the confidence intervals were calculated using Fisher's z transformation. Survival curves were visualized using the Kaplan-Meier Method and the logrank test was used to compare two survival curves. The hazard ratio with 95% CI was calculated using the Cox's proportional hazards model. The optimal cutoff of TIL-B in association to iDFS and OS was determined by the method of Contal and O'Quigley using the SASmacro %findcut (81), separately in the HER2+ BC and TNBC groups. The outer 20% of the continuous variable distribution were excluded in this analysis to avoid having small numbers in one of the groups following dichotomization, in order to prevent substantial losses in statistical power. The considered cutoffs were from 0.50 to 9.00 with increases of 0.25 (i.e. 0.50, 0.75, 1.00, 1.25, 1.50, …. 8.75, 9.00). The added prognostic value of a variable was evaluated using the likelihood ratio test. All statistical analyses were performed two sided by using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). A p-values less than 0.05 were considered statistically significant (*p<0.05; **p<0.01; ***p<0.001).

Study approval

All human specimens were acquired using a protocol approved by the Medical Ethics Committee of the Institut Jules Bordet with written informed consent obtained from each patient and donor prior to inclusion in the study (CE1981).

Author contributions

SG conceived the research and designed experiments with support from **KWG**; **SG** performed the majority of experiments with specialized help from **LB**, **CS**, **CGT**, **CN**, **J-NL**, **AB** and **HD**; **LB** and **CS** collected clinical case data; **AdW** and **GVdE** performed pathological evaluations; **SG**, **LB** and **KWG** analyzed the data; **SG**, **LB**, **CGT** and **KWG** discussed the data; **SG** and **KWG** interpreted the data; **LC**, **GvDE**, **IV** and **DL** recruited and sampled patients; **SG**, **AL** and **MP** performed statistical analyses; **MPG** proposed important concepts; **KWG** supervised the research; **SG** and **KWG** wrote and revised the manuscript with all authors subsequently providing advice and approving the final manuscript.

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Figures and figure legends

Figure 1
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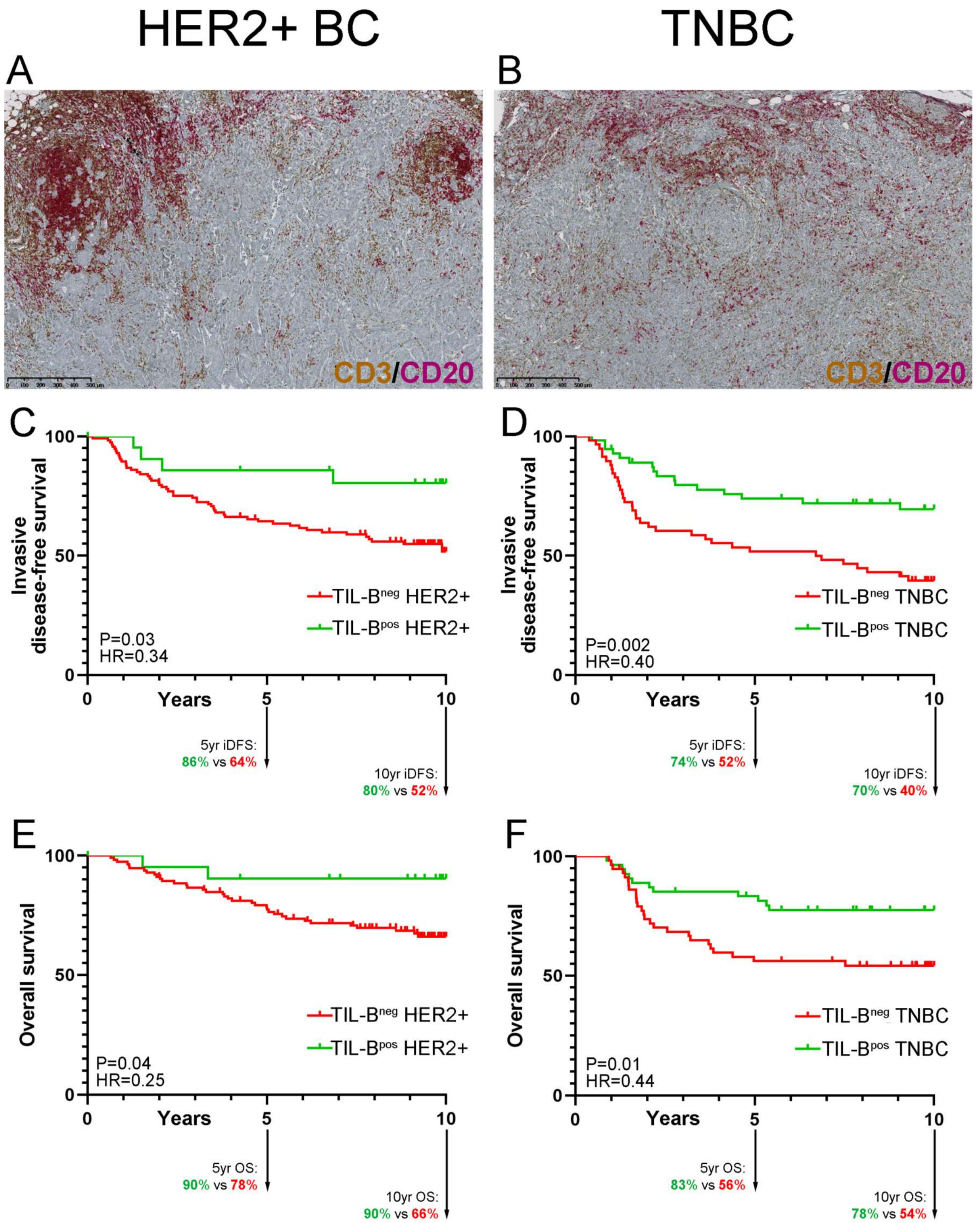

Figure 1. Prognostic value of tumor-infiltrating B-cells in breast cancer. (A-B) Representative sections of HER2 (A) and TN (B) breast cancer with extensive TIL stained with CD3/20. (C-F) Kaplan-Meyer survival curves of 10-year (C) invasive disease free survival (iDFS) for 136 patients with HER2-positive disease, (D) iDFS for 113 TN disease, (E) overall survival (OS) for 136 patients with HER2-positive disease, and (F) OS for 113 TN disease. Statistical analysis: log-rank (Mantel-Cox) test. See also Supplemental Table 1 and 2.

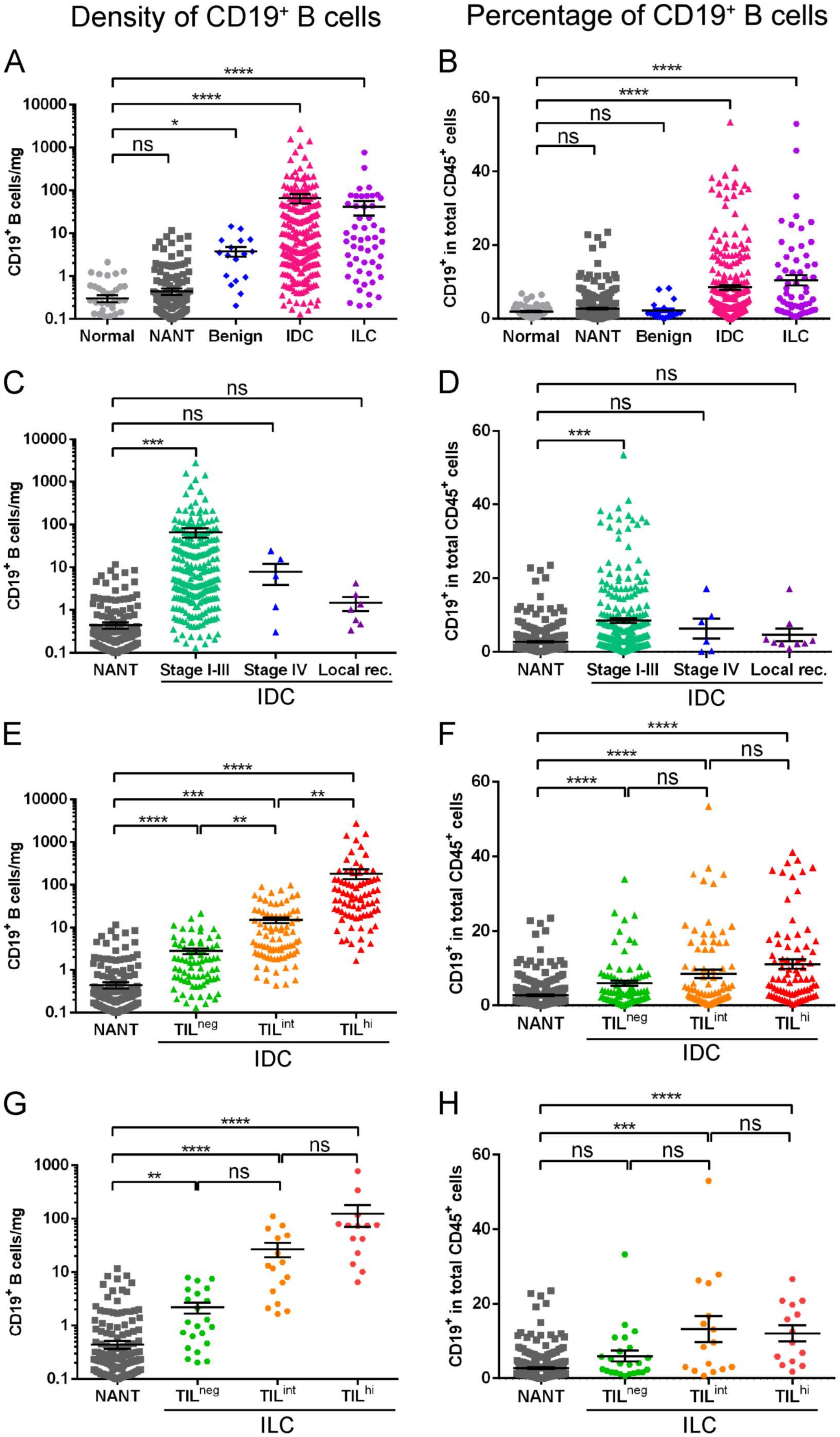
Density of CD19⁺ B cells
Percentage of CD19⁺ B cells
A
B
C
D
E
F
G
H
CD19⁺ B cells/mg
CD19⁺ in total CD45⁺ cells
Normal
NANT
Benign
IDC
ILC
Stage I-III
Stage IV
Local rec.
TIL neg
TIL int
TIL hi
ns
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Figure 2. B-cells infiltration in breast cancer tissues. (A-H) B-cell infiltration was determined as an absolute number normalized to the weight (mg) of the tissue sample (A, C, E and G) and as a percentage (B,D, F and H) of CD45+ TIL in the tissue by FACS. B-cell infiltration was analyzed according to breast tissue type and cancer histology (A and B), BC stage (C and D), global TIL infiltration in IDC (E and F), and in ILC (G and H). Data represent a combination of experiments involving individual patients and are displayed as the mean ± SEM by One-Way ANOVA with Dunn's multiple comparisons test (Normal, *n* = 62; NANT, *n* = 312; Benign, *n* = 21; IDC, *n* = 241; ILC, *n* = 62; Stage I-III, *n* = 241; Stage IV, *n* = 6, Local rec., *n* = 9; IDC TIL^{neg}, *n* = 78; IDC TIL^{int}, *n* = 84; IDC TIL^{hi}, *n* = 79; ILC TIL^{neg}, *n* = 21; ILC TIL^{int}, *n* = 16; ILC TIL^{hi}, *n* = 14) . *P < 0.05, **P < 0.01, ***P < 0.001, and ****P< 0.0001. Abbreviations: NANT, non-adjacent non-tumor; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; Rec, recurrence; TIL, tumor-infiltrating lymphocytes. See also Supplemental Table 3, and Figure 1.

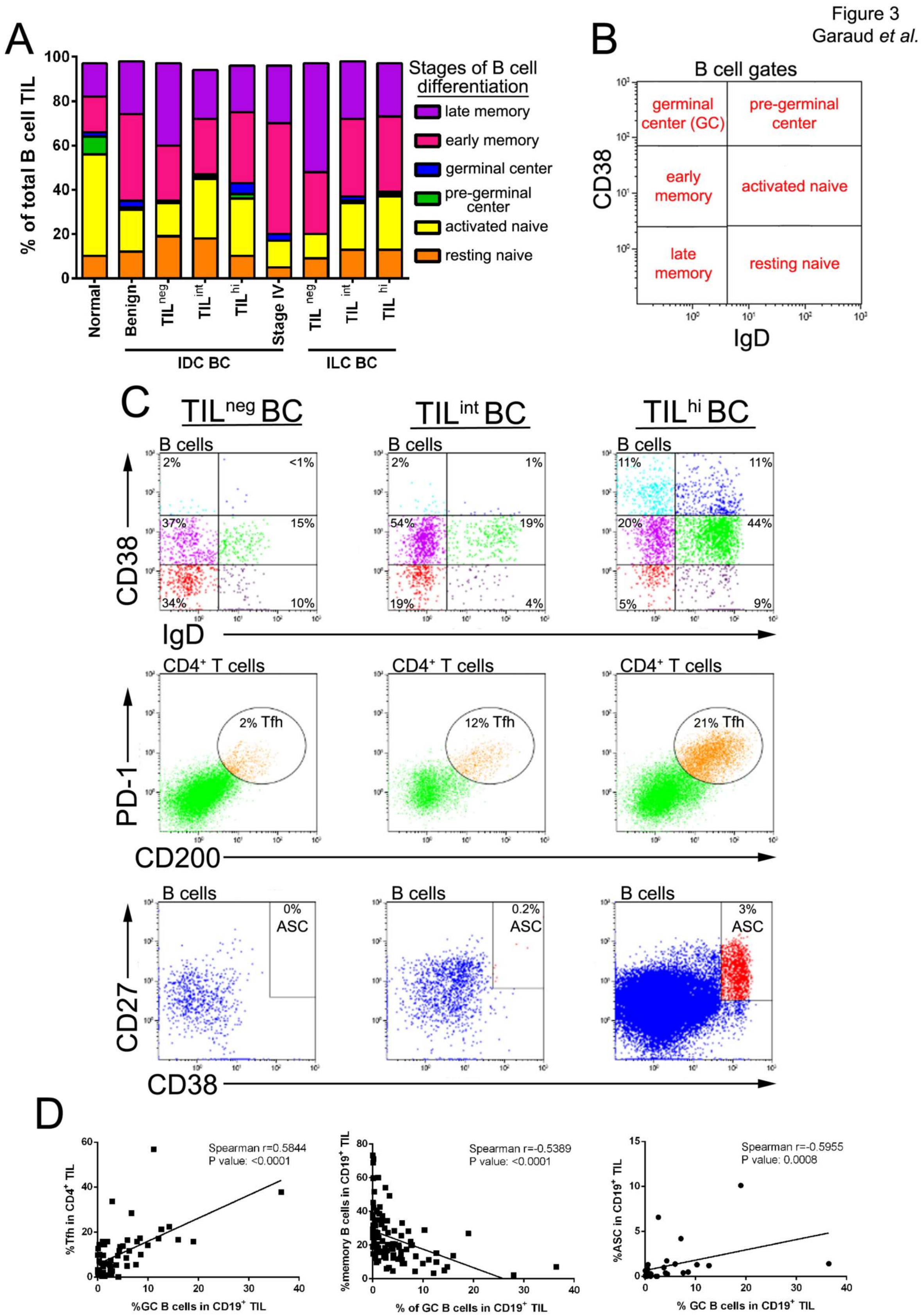

Figure 3. Phenotypic characterization of TIL-B in breast cancer tissues. (A and B) Stacked bars show the percentage of TIL-B subsets in total TIL-B according to the Bm classification (CD38

and IgD) (Normal, n = 22; Benign, n = 6; IDC TIL^{neg}, n = 9; IDC TIL^{int}, n = 27; IDC TIL^{hi}, n = 56; Stage IV, n = 3; ILC TIL^{neg}, n = 4; ILC TIL^{int}, n = 10; ILC TIL^{hi}, n = 12). Data represent a combination of experiments involving individual patients and are displayed as the mean by Chi-square test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P< 0.0001. (C) Representative dot plots show percentages of Bm subsets, T_{FH}, and ASC in TIL^{neg}, TIL^{int}, and TIL^{hi} patients. (C) Graphs show the correlation between Bm3-4 and T_{FH} (n = 57), Bm5 (n = 101) and ASC (n = 28). Data represent a combination of experiments involving individual patients and are displayed as single value by Sperman test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P< 0.0001. Abbreviations: Bm, mature B-cells ; T_{FH}, $CD4^+$ T follicular helper ; ASC, antibody-secreting B-cells; recurrence; TIL, tumor-infiltrating lymphocytes; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma. See also Supplemental Figure 2 and Supplemental Table 4.

Figure 4
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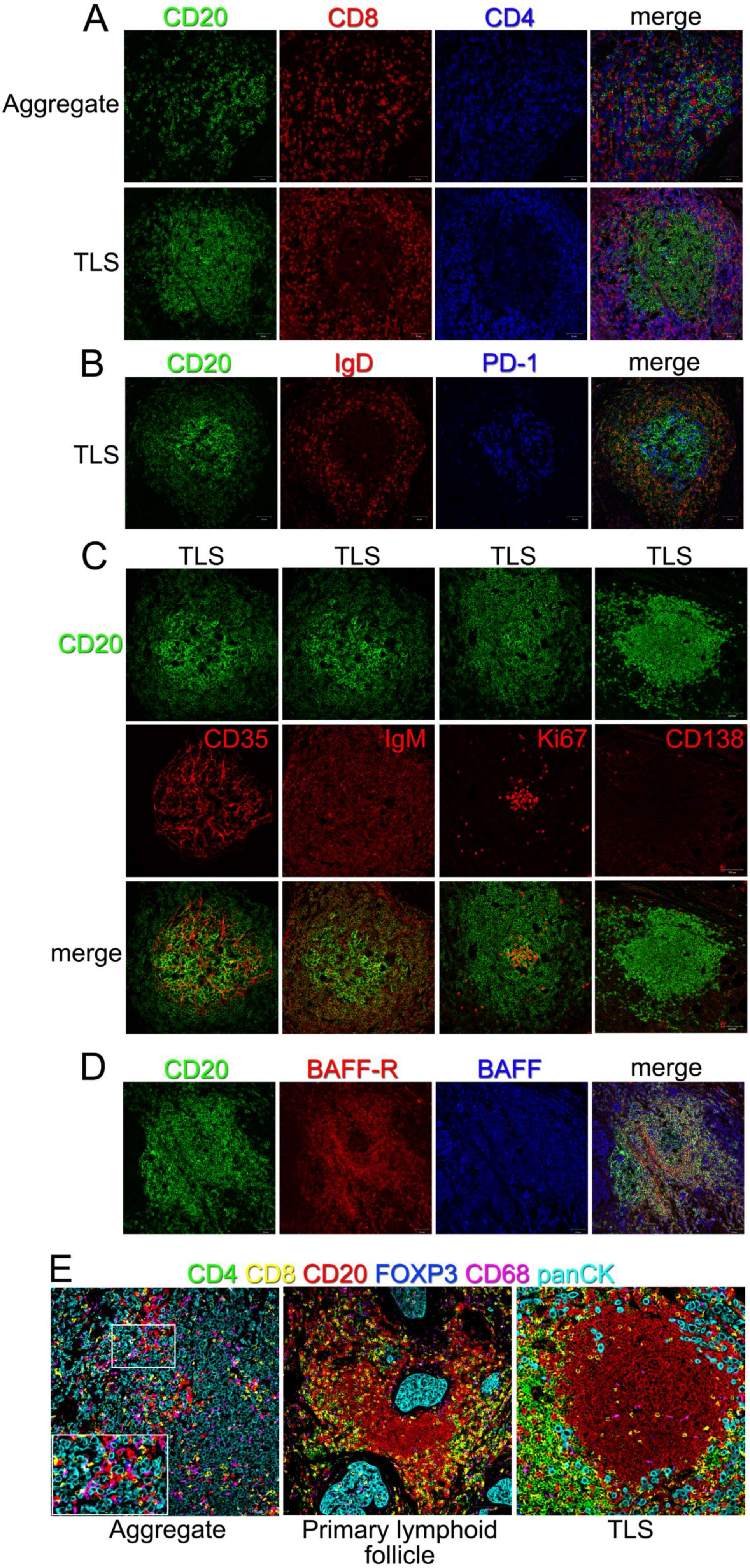

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Figure 4. B-cell organization in breast cancer tissues. (A-D) Immunofluorescent staining of tumor-associated aggregates and TLS on FFPE sections (20x magnification). Tumor-infiltrating lymphocytes were revealed by CD20 (green), CD8 (red) and CD4 (blue) (A). Germinal center were identified by the absence of naïve IgD+ B-cells (red) and the presence of PD-1+ cells (blue) (B), CD35+ (red) follicular dendritic cells, IgM+ (red) memory B-cells, Ki67+ (red) proliferating cells, and CD138+ (red) plasma cells (C). BAFF (blue) and its receptor (red) were detected in germinal center of breast cancer (D). (E) Representative multiplexed IHC images of breast cancer. Tumor-infiltrating lymphocytes were revealed by CD20 (red), CD8 (yellow) and CD4 (green), FOXP3 (blue), CD68 (magenta), and tumor cells by panCK (cyan) (20x magnification). See also Supplemental Figure 3.

Figure 5
Garaud *et al.*

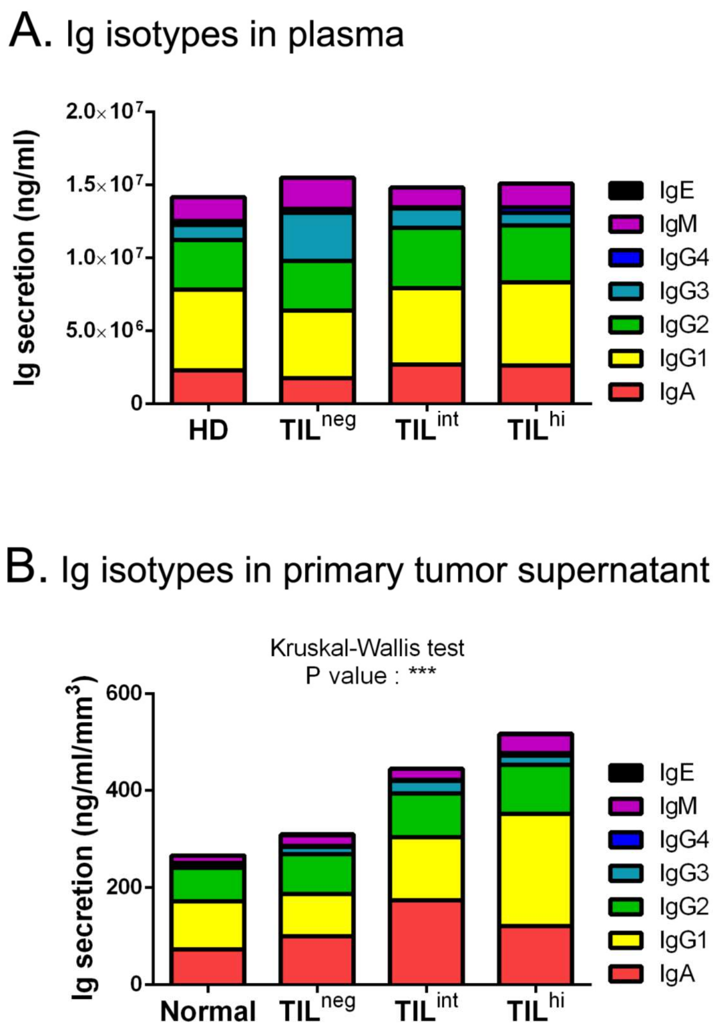

Figure 5. Ig repertoire in breast cancer. (A-B) Stacked bars quantify Ig subclasses in plasma from breast cancer patients and HD (A) and in breast tissue supernatant (B) (HD, *n* = 8; Normal, *n*= 10; TIL^{neg}, *n* = 8; TIL^{int}, *n* = 4; TIL^{hi}, *n* = 9). Data represent a combination of experiments involving individual patients and are displayed as the mean ± SEM by One-Way ANOVA with Kruskal-Wallis test. ***P < 0.001. Abbreviations: HD, healthy donors; TIL, tumor-infiltrating lymphocytes.

Figure 6
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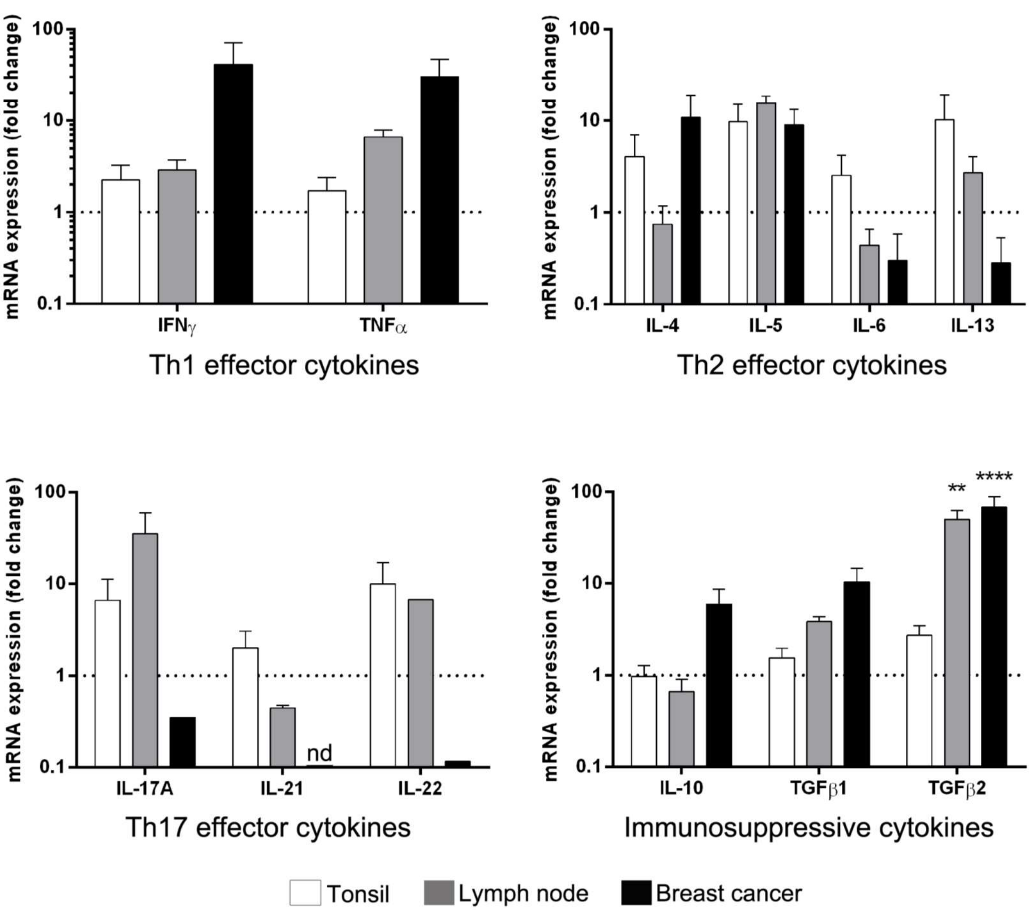

Figure 6. Cytokine profile of tumor-infiltrating B-cells in breast cancer. Interleaved bars show the transcript levels of cytokines in sorted B-cells from lymph node (*n* = 6) and BC (*n* = 6), normalized to MLN51, and relative to B-cells from tonsil (*n* = 5). Data represent a combination of experiments involving individual patients and are displayed as mean ± SEM by One-Way ANOVA with Tukey multiple comparisons test. **P < 0.01, and ****P< 0.0001. Abbreviations: IL, interleukin; LN, lymph node; BC, breast cancer. See also Supplemental Figure 4.

Figure 7
Garaud *et al*.

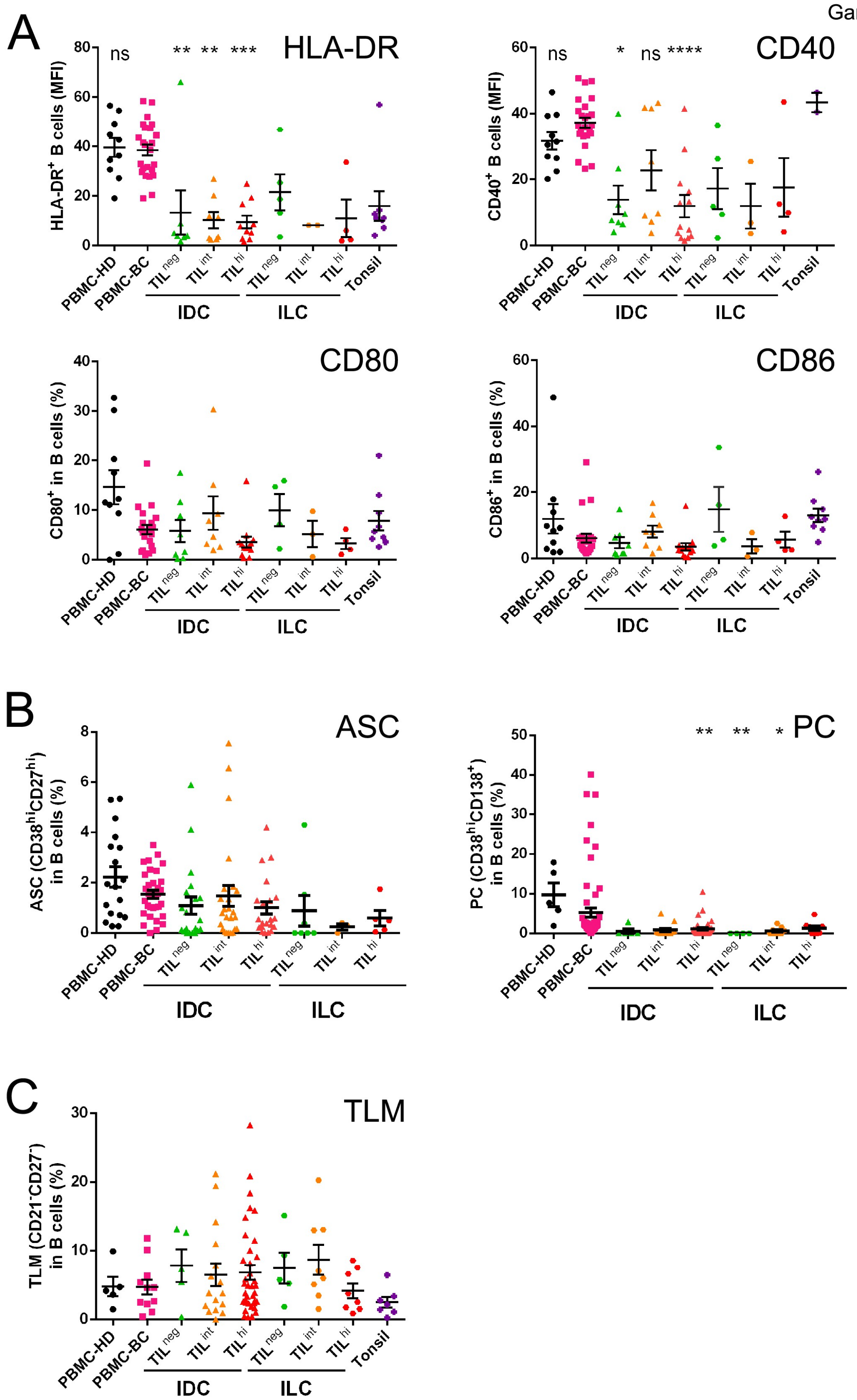

Figure 7. Antigen-presenting cells functions of B-cells in breast cancer. (A) Scatter plots display the mean of fluorescent intensity of HLA-DR, CD40, and the percentage of CD80 and CD86 on TIL-B (PBMC-HD, *n* = 10; PBMC-BC, *n* = 24; IDC TIL^{neg}, *n* = 8; IDC TIL^{int}, *n* = 8; IDC TIL^{hi}, *n* = 13; ILC TIL^{neg}, *n* = 5; ILC TIL^{int}, *n* = 3; ILC TIL^{hi}, *n* = 4: Tonsil, *n* = 9). (B) Scatter plots display the percentage of ASC (PBMC-HD, *n* = 18; PBMC-BC, *n* = 33; IDC TIL^{neg}, *n* = 20; IDC TIL^{int}, *n* = 25; IDC TIL^{hi}, *n* = 21; ILC TIL^{neg}, *n* = 7; ILC TIL^{int}, *n* = 3; ILC TIL^{hi}, *n* = 5) and PC in B-cells (PBMC-HD, *n* = 5; PBMC-BC, *n* = 63; IDC TIL^{neg}, *n* = 5; IDC TIL^{int}, *n* = 14; IDC TIL^{hi}, *n* = 36; ILC TIL^{neg}, *n* = 4; ILC TIL^{int}, *n* = 9; ILC TIL^{hi}, *n* = 8). (C) Scatter plot display the percentage of tissue-like memory B-cells using $CD21^{-}CD27^{-}CD19^{+}$ in B-cells (PBMC-HD, *n* = 5; PBMC-BC, *n* = 11; IDC TIL^{neg}, *n* = 5; IDC TIL^{int}, *n* = 16; IDC TIL^{hi}, *n* = 37; ILC TIL^{neg}, *n* = 5; ILC TIL^{int}, *n* = 8; ILC TIL^{hi}, *n* = 8: Tonsil, *n* = 7). Data represent a combination of experiments involving individual patients and are displayed as mean ± SEM by One-Way ANOVA with Dunn's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P< 0.0001. Abbreviations: PBMC, peripheral blood mononuclear cells; HD, healthy donors; BC, breast cancer; TIL, tumor-infiltrating lymphocytes; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ASC, antibody-secreting cells; PC, plasma cells; TLM; tissue-like memory B-cells.

Figure 8
Garaud, *et al.*

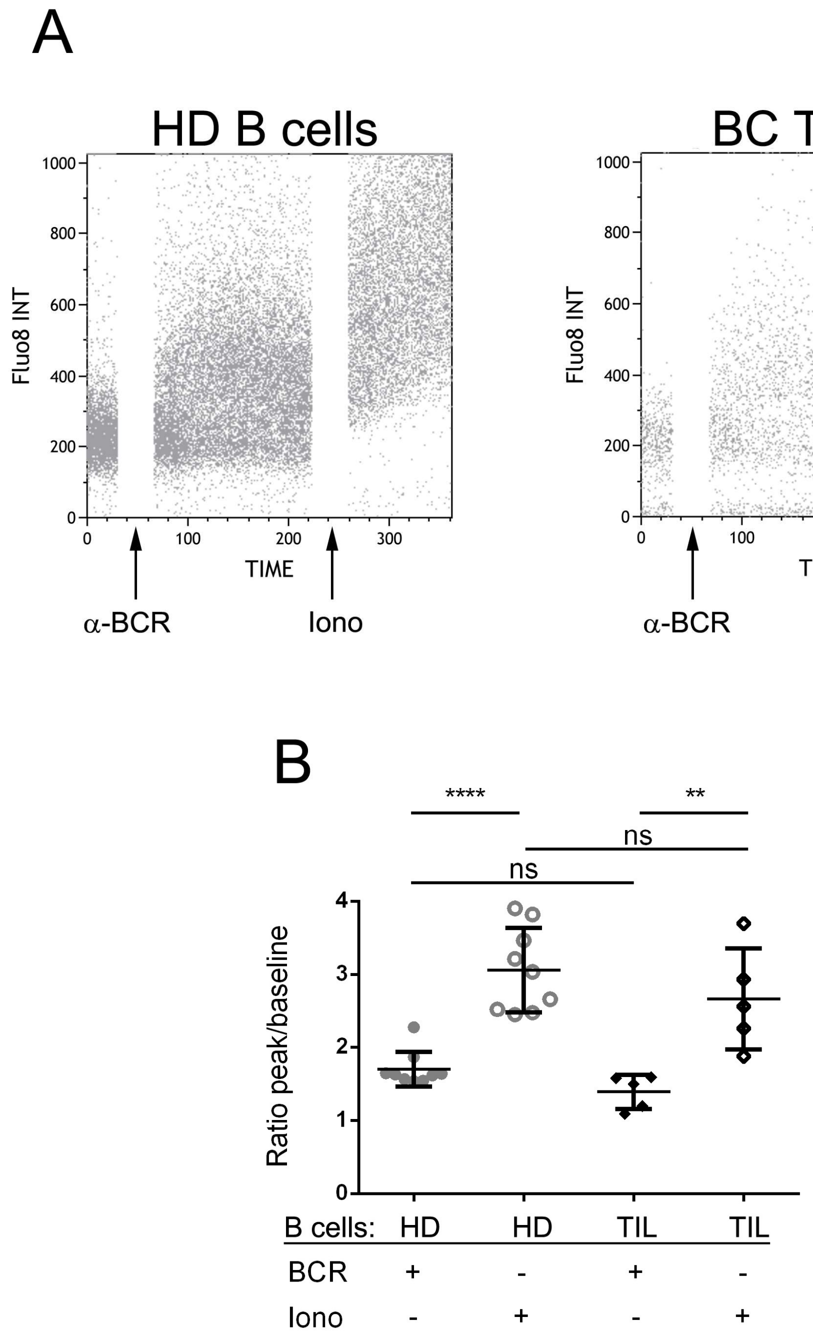

Figure 8. BCR-mediated activation in tumor-infiltrating B-cells. (A and B) TIL-B were loaded with Fluo-8 and BCR stimulated. Ionomycin was used as a positive control. (A) Representative dot plots show the calcium influx in B-cells from HD and BC patients. (B) Scatter plot shows the ratio of calcium peak to baseline (HD, *n* = 9; TIL, *n* = 5). Data represent a combination of

experiments involving individual patients and are displayed as mean ± SEM by One-Way ANOVA with Tukey multiple comparisons test. NS, Not significant. Abbreviations: TIL, tumor-infiltrating lymphocytes; PBMC, peripheral blood mononuclear cells; BCR, B-cell receptor; iono, ionomycin.

Tables

Table1. TIL-B density association with clinicopathological parameters of the patients in our breast cancer cohort.

	Number (%)	Median (Q1-Q3)	*p* Value
Age (years)			
<50	74 (23.3)	11.14 (2.854-44.25)	0.4314
≥50	243 (76.7)	4.77 (1.488-34.07)	
Histology			
Ductal	241 (79.5)	6.06 (1.865-37.16)	0.7259
Lobular	54 (17.6)	6.44 (1.116-42.89)	
Mixed (ductal+lobular)	4 (1.3)	3.92 (0.9773-9.15)	
Other‡	8 (2.6)	5.53 (0.7638-86.71)	
Node status			
Negative	154 (49.7)	6.12 (1.814-36.11)	0.2546
Positive	156 (50.3)	5.41 (1.492-36.82)	
Stage (AJCC staging)			
I	126 (44.4)	7.65 (1.94-39.49)	0.2583
II	116 (40.8)	5.02 (1.108-32.23)	
III	35 (12.3)	8.95 (2.299-48.85)	
IV	7 (2.5)	6.30 (0.3055-15.50)	
Histological grade			
1	62 (20.0)	4.91 (1.053-15.63)	**0.0023**
2	129 (41.6)	3.95 (1.326-19.71)	
3	119 (38.4)	16.63 (2.376-50.98)	
Ki67-Proliferation index (IHC)			
<20%	173 (55.8)	3.91 (1.02-15.06)	**0.006**
≥20%	137 (44.2)	17.49 (2.36-54.58)	
Lymphovascular Embolism			
Absent	198 (65.1)	5.08 (1.478-39.33)	0.1559
Present	106 (34.9)	5.32 (2.023-26.47)	
Hormonal receptors (IHC)			
ER negative	49 (15.6)	34.07 (8.141-159.9)	**0.005**
ER positive	266 (84.4)	4.25 (1.329-22.75)	
PR negative	76 (24.2)	19.95 (3.086-94.27)	**0.0047**
PR positive	238 (75.8)	4.28 (1.473-22.5)	
HER-2 receptor (FISH)			
HER-2 negative	263 (84.3)	5.16 (1.627-32.04)	0.3678
HER-2 positive	49 (15.7)	8.95 (2.057-49.92)	
Size			
<20mm	163 (51.7)	8.01 (2.057-43.04)	0.1301
≥20mm	152 (48.3)	4.55 (1.04-25.44)	
Menopause			
Absent	92 (30.8)	10.59 (2.925-43.67)	0.7161
Present	207 (69.2)	4.29 (1.331-34.07)	

The p value was determined using the Wilcoxon rank or Kruskal-Wallis test when appropriate. Bold type indicates significant ($p<0.05$). Q1 and Q3. The first and the third quartile, respectively.

†. Data from flow cytometric analysis.

‡. Other includes medullar, metaplastic, micropapillary, mucinous and tubullar BC.