Antigen Specificity and Clinical Significance of IgG and IgA Autoantibodies Produced in situ by Tumor-Infiltrating B Cells in Breast Cancer

Soizic Garaud¹, Pawel Zayakin², Laurence Buisseret³, Undine Rulle³, Karina Silina⁴, Alexandre de Wind⁵, Gert Van den Eyden⁶, Denis Larsimont⁵, Karen Willard-Gallo¹ and Aija Linē²,⁷

¹Molecular Immunology Unit, Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium, ²Cancer Biomarker and Immunotherapy Group, Latvian Biomedical Research and Study Centre, Riga, Latvia, ³Institute of Pathology and Molecular Pathology, University Hospital Zurich, Zurich, Switzerland, ⁴Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland, ⁵Department of Pathology, Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium, ⁶Translational Cancer Research Unit Antwerp, Oncology Centre, General Hospital Sint Augustinus, Wilrijk, Belgium, ⁷Faculty of Biology, University of Latvia, Riga, Latvia

An important role for tumor infiltrating B lymphocytes (TIL-B) in the immune response to cancer is emerging; however, very little is known about the antigen specificity of antibodies produced in situ. The presence of IgA antibodies in the tumor microenvironment has been noted although their biological functions and clinical significance are unknown. This study used a 91-antigen microarray to examine the IgG and IgA autoantibody repertoires in breast cancer (BC). Tumor and adjacent breast tissue supernatants and plasma from BC patients together with normal breast tissue supernatants and plasma from healthy controls (patients undergoing mammary reduction and healthy blood donors) were analyzed to investigate relationships between autoantibodies and the clinical, histological and immunological features of tumors. Our data show that >84% of the BC samples tested contain autoantibodies to one or more antigens on the array, with ANKRD30BL, COPS4, and CTAG1B being most frequently reactive. Ex vivo TIL-B responses were uncoupled from systemic humoral responses in the majority of cases. A comparison of autoantibody frequencies in supernatants and plasma from patients and controls identified eight antigens that elicit BC-associated autoantibody responses. The overall prevalence of IgG and IgA autoantibodies was similar and while IgG and IgA responses were not linked they did correlate with distinct clinical, pathological and immunological features. Higher levels of ex vivo IgG responses to BC-associated antigens were associated with shorter recurrence-free survival (RFS), HER2 overexpression and lower tumor-infiltrating CD8⁺ T cell counts. Higher IgA levels were associated with estrogen and progesterone receptor-negative cancers but were not significantly associated with RFS. Furthermore, ex vivo IgA but not IgG autoantibodies reactive to BC-associated antigens were linked with germinal center and early memory
INTRODUCTION

Breast cancer (BC) is a profoundly heterogeneous disease not only in respect of its molecular architecture and clinical course but also to the hosts’ immune responses against the tumor (1). The intensity and mode of anti-tumor immune response depends on the cellular composition, density, functional phenotype and spatial distribution of tumor-infiltrating immune cells (2). While higher density of tumor-infiltrating CD8+ T cells is consistently associated with favorable clinical outcomes in various solid tumors, including BC (3, 4), the functional role and clinical significance of tumor-infiltrating B lymphocytes (TIL-B) is far less clear (5). Several studies have shown that higher number of CD20+ TIL-B is associated with better BC specific survival and longer recurrence-free survival (RFS) (6–8), or predicts pathologic complete response in BC patients treated with neoadjuvant chemotherapy (9, 10). On the contrary, higher counts of CD138+ B lymphocytes and plasma cells (PC) were associated with poor cancer-specific survival in invasive ductal BC (6, 11), whereas higher counts of CD38+ PC have been found to be associated with better disease-free survival in triple negative BC (12). Another recent study showed that higher CD19+ and CD20+ TIL-B but not CD138+ PC counts are associated with larger tumor size and shorter RFS in patients with ductal carcinoma in situ (13). At the same time, a meta-analysis of gene expression signatures in ~18,000 various human tumors revealed PC-gene expression signature as one of the most significant predictors of favorable survival across various solid tumors (14). The reasons for these controversies are poorly understood so far.

Important aspect for gaining a better understanding into the TIL-B effector functions is the antigen specificity of the antibodies they produce. Most of the studies addressing the repertoire of autoantibodies have been focused on plasma or serum IgG class antibodies, while the repertoire of antibodies produced in situ by TIL-B is poorly characterized. Moreover, although the presence of high amounts of IgA class antibodies in BC tissues has been reported (15), the antigen specificity and functions of IgAs has not been studied so far. Earlier studies of TIL-B antigen specificity were based on the analysis of immunoglobulin genes and suggested that TIL-B undergo antigen-driven oligoclonal proliferation, somatic hypermutation and affinity maturation in situ (16–18). However, only a few antigens recognized by TIL-B have been identified in BC so far and include the cytoskeletal protein β-actin (19), ganglioside D3 (20), and CEA, MUC1, and FN1 (ED-B domain) (21).

In the current study, we characterized the repertoire of IgG and IgA autoantibodies present in the BC and normal breast tissue supernatants and plasma of BC patients and controls using our in-house 91-antigen microarrays, and analyzed the relationship between the presence of autoantibodies and clinical, histological and immunological features of tumors.

MATERIALS AND METHODS

Samples and Study Population

Paired tumor and adjacent breast tissue specimens and plasma samples were collected from 66 primary BC patients diagnosed and treated at the Institut Jules Bordet, Brussels between July 2012 and October 2013 and followed up until January 2018. Inclusion criteria: untreated invasive primary carcinomas, stage I to III at diagnosis. The characteristics of the study population are provided in Table 1. Normal breast tissue specimens were collected from 23 women undergoing mammary reduction surgery. Fresh breast tissues were obtained at the day of surgery and dissociated (without enzymatic digestion) using the GentleMacs Dissociator (Miltenyi Biotec) (22). The resulting cell suspension was filtered following each dissociation run using a 40 µm cell strainer (BD Falcon), washed with X-VIVO 20, centrifuged 15 min at 600 g, and resuspended in X-VIVO 20 before flow cytometric analysis. The tissue supernatant was the initial 3 ml of X-VIVO 20 recovered after the first round of dissociation, which was subsequently clarified by centrifugation for 15 min at 13,000 g.

Plasma samples from 200 cancer-free women were obtained from the Latvian Genome Database (Riga cohort) and additional 18 plasma samples were collected from cancer-free women at the Institute Jules Bordet (Brussels cohort). Plasma samples from the BC patients were collected before the surgery. The blood samples were collected in EDTA-treated tubes (Riga cohort) or heparin-treated tubes (Brussels cohort) and processed within 24 h. The blood tubes were centrifuged at 200 g for 10 min, the plasma was aliquoted and stored at −80°C until use.

The study was conducted according to the Declaration of Helsinki. The specimens were collected after the patients’ informed written consent was obtained and the research on anonymized patients’ samples was approved by the Institut Jules Bordet’s Medical Ethics Committee (Accepted project CE 1981) and the ethical committee of the University of Latvia, Institute of Experimental and Clinical Medicine.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded tissue sections (4 µm) were immunohistochemically stained for CD3 (pan T cells; Dako) and CD20 (pan B cells; Dako) on a Ventana Benchmark XT automated staining instrument (Ventana Medical Systems). A detailed protocol for the dual CD3/CD20 immunohistochemical stain is described in Buisseret et al. (23).
Flow Cytometry
Cell suspensions were incubated with manufacturer’s suggested dilutions of fluorescently labeled primary monoclonal antibodies (Supplementary Table S1) for 1 h at 4°C in 100 µl of X-VIVO 20 followed by washing with PBS. After washing once, a lysing solution was used for the lysis of red blood cells in cell suspension from breast tissues (VersaLyse, BC). Fluorescently labeled cells were acquired on a GALLIOS 10/3 cytometer and the data were analyzed using Kaluza Flow Cytometry Analysis v1.2 software.

Production and Processing of Antigen Microarray
For the production of antigen array, cDNA fragments encoding 91 antigens were cloned into the pFN19A (HaloTag®) T7 SP6 Flexi bacterial expression vector (Promega, USA) as described before (24). A His-tag coding sequence was introduced upstream the Halo-tag and the cDNAs encoding the antigens were inserted at the 3’ end of the Halo tag. The recombinant proteins were expressed in E. coli XL Blue cells, purified using Ni-TED columns (Macherey-Nagel, Germany) and quality-checked by Western blot analysis. The proteins were diluted to 0.03–0.1 mg/ml in PBS, 2% glycerol and printed on nickel chelate coated glass slides (Xenopore) in duplicates using QArray Mini microarray printer (Genetix, UK). His-Halo tags were used as negative controls.

The slides were processed as described by Zayakin et al. with some modifications (25). Briefly, the slides were blocked in 7% (w/v) milk powder in TBS, 0.05% Tween-20 and incubated with 1:2 diluted tissue culture supernatants or 1:50 diluted plasma samples that had been pre-absorbed with His-Halo-tag expressing E. coli lysates. The slides were washed overnight and incubated with 1:500 diluted rabbit anti-Halo-tag antibody (G9281, Promega, USA). IgG and IgA autoantibodies were detected with Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:750) (109-605-011, Jackson ImmunoResearch, USA) and Cy5-conjugated mouse anti-human IgG secondary antibody (1:2,000) (111-165-046, Jackson ImmunoResearch, USA). His-Halo tags were used as negative controls.

The arrays were scanned at 10 µm resolution in PowerScanner (Tecan, Switzerland). The results were recorded as TIFF files and the raw data were processed with GenePix™ Pro 4.0 software.

---

**TABLE 1**| Association of the IgG-TAA and IgA-TAA scores with clinicopathological parameters.

| Parameter | Mean IgG-TAA score | Mean IgA-TAA score | Parameter | Mean IgG-TAA score | Mean IgA-TAA score |
|-----------|--------------------|--------------------|-----------|--------------------|--------------------|
| **Age**   |                    |                    | **PR status** |                   |                    |
| <50 years, n = 17 | 0.68 | 0.44               | PR+, n = 45 | 0.5     | 0.36               |
| ≥50 years, n = 49 | 0.90 | 0.52               | PR-, n = 21 | 1.57    | 0.81               |
| M-WU, P-value | 0.66 | 0.96               | M-WU, P-value | 0.22   | 0.026              |
| **Histology** |                   |                    | **HER2 status** |               |                    |
| Ductal, n = 51 | 0.87 | 0.53               | HER2+, n = 12 | 1.34   | 0.66               |
| Lobular, n = 13 | 0.83 | 0.43               | HER2-, n = 53 | 0.74    | 0.47               |
| M-WU, P-value | 0.69 | 0.27               | M-WU, P-value | 0.032   | 0.23               |
| **Subtype** |                   |                    | **Ki67 status** |               |                    |
| Lobular, n = 11 | 0.71 | 0.35               | <20, n = 37 | 0.55     | 0.40               |
| Luminal A, n = 23 | 0.49 | 0.43               | ≥20, n = 29 | 0.12     | 0.13               |
| Luminal B, n = 15 | 0.28 | 0.31               | ≥50, n = 3 | 0.78     | 0.12               |
| HER2+, n = 12 | 1.34 | 0.66               |              | 0.30     | 0.58               |
| **Stage**   |                   |                    | **Tumor size, mm** |               |                    |
| I, n = 29    | 0.29 | 0.40               | <20, n = 37 | 0.51     | 0.48               |
| II, n = 29   | 1.29 | 0.60               | 20–49, n = 26 | 1.32   | 0.58               |
| III, n = 7   | 1.1  | 0.31               | ≥50, n = 3 | 0.78     | 0.12               |
| **Grade**   |                   |                    |              | 0.30     | 0.58               |
| 1, n = 10    | 0.47 | 0.42               | 0, n = 39 | 0.86     | 0.65               |
| 2, n = 24    | 0.50 | 0.31               | 1–4, n = 20 | 0.71     | 0.47               |
| 3, n = 31    | 1.25 | 0.69               | ≥5, n = 6 | 1.28     | 0.36               |
| **ER status** |               |                    | **in situ** |                   |                    |
| ER+, n = 57  | 0.60 | 0.4                | No, n = 15 | 0.96     | 0.48               |
| ER-, n = 9   | 2.4  | 1.12               | M-WU, P-value | 0.99   | 0.69               |
| M-WU, P-value | 0.25 | 0.027             |                    |                    |                    |

M-WU, Mann-Whitney U test, KW, Kruskal-Wallis test. Statistically significant findings are highlighted in bold.
(Molecular Devices, USA) and further analyzed using an ad hoc program composed in R language.

**Microarray Data Processing and Statistical Analyses**

For each antigen, the mean Cy5 and Cy3 signals were background subtracted and the Cy5/Cy3 ratios were averaged between replicates. Next, the Cy5/Cy3 values were normalized by applying quantile normalization to 25th and 75th percentiles between all samples. In order to define reactive antigens in each array, the values were log transformed and the cut-off value was set at median plus 10 SDs of trimmed (25th to 75th percentiles) signals in each sample. This cut-off could clearly and reproducibly distinguish background reactivity from the reactive antigens (outliers deviating from normal distribution). The frequencies of reactive antigens were calculated in each group of samples. BC-associated antigens were defined by comparing combined IgG and IgA reactivity in SN-BC and PB-BC samples vs. SN-MR and PB-HD samples. The Fisher’s exact test and was applied to determine the level of significance and Benjamini-Hochberg (BH) procedure was used for adjusting the false discovery rate for multiple comparisons. In order to assess the combined reactivity against BC-associated antigens, IgG-TAA and IgA-TAA scores were calculated by summing of signal intensities (above the cut-off) of all BC-associated antigens in each sample.

Statistical analyses were performed using an ad hoc program composed in R language. The prognostic significance of IgG and IgA production, IgG-TAA and IgA-TAA scores were considered to be significantly associated with BC (Table 2). Among them were several previously known cancer antigens as well as novel peptides. CTAG1B, BRCA2, and SPAG8 have been previously shown to induce humoral immune response in BC (27–29) and other cancer patients (25, 27, 30), ANKRD30BL and COPS4 were previously found to elicit autoantibody response in gastric and thyroid cancer and melanoma (24, 26). Artificial peptides API292, API1775 and API1361 were reacting with sera from gastric cancer patients in our previous studies (24) and may represent neoantigens or mimotopes of other cancer-associated antigens.

In total, 86.4% of SN-BC samples had IgG and ~84.8% IgA autoantibodies against one or more antigens included in the antigen array. The most frequently reacting antigens (ranked by the frequency of combined IgG and IgA responses in SN-BC samples) are listed in Table 2 and include both BC-associated antigens and BC non-related antigens. The frequencies of autoantibodies in SN-AT samples were only slightly lower than in SN-BC samples; however it is not clear if these antibodies are produced ex-vivo by B cells present in the adjacent normal tissues or have diffused from the tumor tissues. Most of these antibodies, except for anti-AP1292 and SOX2, were also found in SN-MR samples, though at lower frequencies.

**Prevalence of IgG and IgA Class Autoantibodies in Tissues and Plasma of BC Patients**

To assess combined levels of BC-associated autoantibody production, IgG-TAA and IgA-TAA scores were calculated for each of the SN-BC samples based on the signal intensities against the BC-associated antigens. The mean scores in tissue supernatant and plasma samples are shown in Figures 1A,B, respectively.

As shown in Table 2, the majority of antigens were recognized by both IgG and IgA autoantibodies at similar frequencies, except for 3 antigens. CTAG1B and API292 more frequently elicited IgG responses, while ANKRD30BL–IgA responses (Fisher’s exact test P-value < 0.01). Furthermore, a fraction of patients mounted both IgG and IgA responses against the same antigen, while others produced exclusively IgG or IgA class antibodies (selected
antigens shown in Figure 1C; all BC-associated antigens shown in Figure S2).

To assess whether the occurrence of autoantibodies in tissue supernatants is correlated with that in plasma, we compared the prevalence of IgG and IgA responses to each of the BC-associated antigens in SN-BC, SN-AT, and PB-BC samples. We found that only a minor fraction of patients had autoantibodies to a given antigen both in plasma and tissue supernatants, while in the majority of patients the presence of autoantibodies in the tissue supernatants was uncoupled from that in plasma (selected antigens shown in Figure 1D; all BC-associated antigens shown in Figure S3A and total frequencies of BC-associated IgG and IgA responses are shown in Figure S3B).

**Association of TIL-B Antibodies With Histological and Clinical Features**

In order to gain an insight into the clinical significance of TIL-B-produced antibodies, the IgG-TAA and IgA-TAA scores were correlated with the clinical and histological features including histological type, subtype, stage and grade of BC, and hormone receptor status (Table 1).

IgG-TAA score was significantly higher in HER2-positive tumors as compared to HER2-negative cancers (mean fold change (FC) = 1.81; \( P = 0.032 \)) and showed a tendency to be increased in estrogen receptor (ER) and progesterone receptor (PR) negative cancers (Figure 2A). Furthermore, the IgG-TAA score showed a tendency to increase with the grade and stage of the disease. IgA-TAA score was significantly higher in HER2-positive lobular BC. IgG-TAA score showed a tendency to increase with the grade and stage of the disease. IgA-TAA score was significantly higher in HER2-positive lobular BC. The scores were not significantly associated with age, tumor size, number of positive lymph nodes, Ki67 levels nor differed between ductal and lobular BC.

**IgA Production Is Linked to B Cell Maturation Stages**

To gain a better understanding into the phenotype of TIL-B producing autoantibodies in situ, we correlated the levels of IgG
and IgA against individual antigens and the IgG and IgA-TAA scores with the density (cells/mm³ of tissue) of CD19⁺ B cells, plasmablasts/plasma cells (PB/PC), CD4⁺ and CD8⁺ T cells, and the maturation stage of B cells determined by flow cytometry. PB/PC were defined as CD45⁺CD19⁺CD27⁺CD38⁺⁺ cells, naïve B cells were defined as CD19⁺CD27⁻ cells and memory
B cells—as CD19+CD27+ cells. The gating strategies used to define these cell types and B cell maturation stages are shown in Figure S4. Additionally, the density of tertiary lymphoid structures (TLS) was determined by IHC and defined as the number of dense B-cell aggregates with an adjacent T-cell zone relative to the tumor area (per mm²) (23).

Unexpectedly, the levels of IgG or IgA antibodies against individual antigens or the TAA scores were not significantly different in groups of patients stratified by CD19+ B cell, PB/PC, CD4+ and CD8+ T cell density, and percentage of naïve and memory B cells (Figure S5). Similarly, no significant correlation was found by Spearman rank test. Mean PB/PC density tended to be higher in patients with anti-CTAG1B, API292 IgG and higher IgG-TAA score, and anti-CTAG1B, COPS4 IgA and higher IgA-TAA score in SN-BC; however, the differences did not reach statistical significance (Figure S6). With the exception of anti-COPS4 IgA, the autoantibody levels were not significantly associated with the presence of TLS (Figure S5). The mean anti-COPS4 IgA level, however, was 7.5-fold higher (P = 0.019) in TLS-positive tumors (Figures 3A,B). Anti-COPS4 IgA was present in 11 out of 41 TLS+ tumors and only in 1 out of 25 TLS- tumors (OR 8.8, Fisher test P = 0.023). This suggests that the presence of TLS may be required for the production of some IgA specificities in situ. Furthermore, we found that the production of IgA but not IgG against 4 TAAs (CTAG1B, ANKRD30BL, COPS4, and SPAG8) as well as the IgA-TAA score was linked to the B cell maturation stages: the percentage of pre-germinal center (Bm2 defined as CD19+IgD+CD38hi), germinal center (Bm3-4 defined as CD19+IgD−CD38hi) and early memory (eBm5 defined as CD19+IgD−CD38+)) B cells was increased but the percentage of late memory B cells (Bm5 defined as CD19+IgD−CD38−) was similar or decreased in the IgA-positive as compared to IgA-negative BC samples (Figures 3C,D).

The IgG-TAA Score Is Associated With Poor Recurrence-Free Survival and Lower Tumor Infiltrating CD8+ T Cell Counts

To assess the prognostic significance of the ex vivo-produced autoantibodies, the patients were separated into groups with high and low IgG and IgA-TAA scores using median as a threshold. Kaplan-Meier analysis was performed to assess the importance of the scores with RFS. The analysis revealed that high IgG-TAA score is associated with shorter RFS (Log rank test, P = 0.032; Figure 4A), whereas the IgA-TAA score is not (Figure S7). At the same time, CD19+ B cell count within the tumor was not associated with RFS in our cohort (Figure 4B), while higher number of CD19+ B cells in the adjacent tissues was correlated with significantly increased RFS (Figure 4C).

To search for the possible reasons of the adverse prognostic significance of the IgG-TAA score, we compared the count of CD4+ and CD8+ T cells in groups of IgG-positive and negative samples. We found that the presence of anti-CTAG1B, ANKRD30BL, COPS4, SPAG8 antibodies, and high IgG-TAA score had a tendency to be associated with lower mean counts of CD8+ T cells, which reached statistical significance for anti-COPS4 and SPAG8 antibodies (Figure 4D). No such association was observed for IgA class antibodies.

DISCUSSION

To the best of our knowledge, this is the first study comprehensively examining the antigen specificity of antibodies produced in situ and correlating their presence with clinical and histological features and immunologic makeup of tumors. Furthermore, we simultaneously examined IgG and IgA responses thus providing the first insight into the prevalence and repertoire of IgA autoantibodies in BC patients and a head-to-head comparison of clinical significance of IgG and IgA responses.

Although some earlier studies suggested that non-lymphoid cells and cancers can produce immunoglobulin molecules (31, 32), a confirmation that these immunoglobulins are functional, affinity-matured antibodies capable of binding to tumor antigens is still lacking. Therefore, we assume that the autoantibodies analyzed in this study are produced by TIL-B.

We report here that over 84% of BC tissue specimens have autoantibodies against at least one antigen represented on our in-house generated 91-feature antigen array. The top three antigens most frequently recognized by the TIL-B autoantibodies are ANKRD30BL, COPS4, and CTAG1B. ANKRD30BL (Ankyrin repeat domain 30B like) is a poorly characterized protein that shows a partial similarity (65%, E value 1e-101) to ANKRD30A (also known as NY-BR-1)—a breast differentiation antigen that is frequently overexpressed in BC tissues (33) and has been shown to elicit spontaneous humoral and cellular immune response in BC patients (34). According to the NCBI's Gene database, ANKRD30BL expression in normal tissues is restricted to testis (35). Here, we for the first time show that it elicits BC-associated humoral immune response. Hence it may represent a novel CT antigen, however assessment of its expression pattern in tumor tissues is required in order to consider it as a potential immunotherapeutic target. COPS4 is a ubiquitously expressed intracellular protein, a subunit of COP9 signalosome that functions as an important regulator in multiple signaling pathways. We have previously found anti-COPS4 IgG in gastric and thyroid cancer and melanoma (19, 21), however the reasons for its immunogenicity are not clear. CTAG1B (also known as NY-ESO-1) is a well-known CT antigen eliciting humoral and cellular immune response in patients with various cancers including BC (25, 28, 36). Interestingly, ANKRD30BL predominantly elicited IgA response, while CTAG1B more frequently elicited IgG response. Autoantibodies against these antigens, however, were not strictly specific to BC and were also found in the normal breast tissues obtained from patients undergoing mammary reduction, though at lower frequencies. The only antigen showing strictly BC-specific response was AP1292—a 14 amino acid peptide encoded by a human intergenic DNA fragment sharing 77% identity with aminepeptidase of Clostridium peptidivorans. Of note, BC tissues have been recently shown to contain a variety.
of bacterial genera including *Clostridium* (37). Hence, AP1292 likely represents a mimotope of a bacterial antigen, which elicits B response specifically in BC patients. Moreover, the frequency of anti-AP1292 antibodies was significantly higher in tissues than in plasma. This suggests that *in situ* TIL-B responses may be directed not only against autoantigens but also against BC microbiome, however our antigen array technology does not allow a comprehensive characterization of such responses.

Comparison of the prevalence of autoantibodies in paired BC tissue and plasma samples showed that less than a half of patients had autoantibodies against the same antigen both in plasma and tissues. These results suggest that the *in situ*-produced autoantibodies and the respective TIL-B often remain sequestered in the tissue microenvironment and do not significantly contribute to the plasma autoantibody levels. This is in line with the observation that CD20+ TILs do not contribute significantly to the systemic level of anti-NY-ESO-1...
and TP53 antibodies in patients with ovarian cancer (38). The opposite was also true, the presence of given autoantibodies in the circulation was not indicative of the autoantibody production in situ. Whether such B cells were activated in draining lymph nodes as it has been recently demonstrated for anti-CTAG1B B cells in BC patients (39) and never infiltrated the tumors or whether they were activated in BC-associated TLS but left the tissue is not known.

We and others have reported that BC tissues contain substantial amounts of IgA immunoglobulins (15, 23), however the antigen specificity and biological functions of these antibodies remained unknown. Here we show that IgA present in the BC tissues recognize BC-associated antigens as well as cancer-non-related antigens and the overall prevalence of IgA is similar to that of IgG class autoantibodies. However, the IgA and IgG responses were not concordant and their presence was associated with distinct clinical and histological features.

We found that high IgG-TAA score (but not IgA-TAA score) is associated with shorter RFS in our cohort of BC patients. This finding is in line with a number of other studies showing that systemic IgG responses to tumor-associated antigens are associated with poor prognosis and shorter overall or progression free survival in patients with melanoma (40), gastric cancer (41), hepatocellular carcinoma (42) and breast cancer (43). Our data suggest that the presence of in situ IgG responses is associated with more aggressive pathologic features, such as HER2 overexpression and later stage of BC, as well as lower mean counts of tumor infiltrating CD8+ T cells, however the causal relationship between the IgG responses and these features remains unclear. One possible explanation is that the cytokine/chemokine profile in the tumor microenvironment that promotes IgG production inhibits the cytotoxic function of T cells, hence the presence of IgG may be a marker of Th2-polarized microenvironment. Another possibility is that IgG have tumor-promoting properties by themselves. For example, IgG have been shown to promote cancer development by activating Fc-γ receptors on myeloid cells that in turn regulate recruitment, composition, and effector functions of TILs in a mouse model of squamous carcinogenesis (44). Furthermore, IgG4 subclass autoantibodies have been found to impair IgG1-mediated antitumor immunity and to promote Th2-biased inflammation in melanoma (45). Alternatively, the prognostic significance may be related to the expression of the respective antigens not the presence of autoantibodies itself.
It also has to be noted that the presence and intensity of the IgG analyzed in this study was not directly proportional to the density of TIL-B. Moreover, the count of intratumoral TIL-B was not associated with RFS in our cohort, whereas higher number of peritumoral TIL-B was associated with better RFS.

It’s also possible that only a minor fraction of CD19+ TIL-B secretes antibodies and the antibody production may not be their main effector function. Alternatively, TIL-B may serve as antigen-presenting cells, secrete polarizing cytokines and foster the formation of TLS as it has been shown in serous ovarian cancer and lung squamous cell carcinoma (46–48) and more recently in BC (submitted for publication by Garaud et al. submitted).

We hypothesized that the production of autoantibodies in situ may be associated with the formation and maturation of TLS in the tumor microenvironment, therefore we correlated the presence of IgG and IgA with the presence of TLS and phenotypic features of TIL-B. Since we have previously shown that the development of tumor-associated TLS follows sequential stages analogous to the formation of germinal centers (GC) in lymph nodes (48), we classified the TIL-B as resting naïve, activated naïve, pre-GC, GC, early memory, and late memory B cells according to the B cell maturation stages during the formation of GCs in lymph nodes. Our data show that the proportion of pre-GC, GC, and early memory B cells is increased in tumors with IgAs against BC-associated antigens and high IgA-TAA scores as compared with IgA-negative tumors. Furthermore, the level of anti-COPS4 IgA was significantly higher in tumors with TLSs as compared with TLS-negative tumors. Whereas, no association of IgG-class antibodies with the presence of TLS or B cell maturation stages was found. These results suggest that the activation of naïve B cells and Ig class switching to IgA may take place in the TLS. These findings are in agreement with a study by Cipponi et al. showing that TLS-associated B cells found in the metastatic lesions of melanoma undergo clonal amplification and somatic hypermutations, and produce IgM, IgG, and IgA class antibodies (49). However, why only anti-COPS4 IgA but no other antibodies correlated with the presence of TLS in our cohort is unclear. One explanation of this observation could be that the formation of TLS is highly dynamic and the structures are short-lived, thus obscuring the link between the presence of TLS and autoantibodies. Alternatively, it could be possible that only a fraction of TIL-B is de novo activated in the TLS, while other antigen-experienced B cells have migrated into the tumor tissue from secondary lymphoid organs, and thus produce antibodies independently of TLS. Finally, the absence of correlation with the presence of TLS could be explained by the concept of T cell-independent antigens. The production of antibodies in the absence of T cell help is largely mediated by marginal zone B cells, which differentiate into short-lived plasma cells, with limited isotype switching to some IgG subtypes and also to IgA (50, 51).

In summary, this study for the first time examined the antigen specificity of antibodies produced by TIL-B and showed that they are directed against BC-associated antigens, BC non-specific autoantigens and possibly also BC-associated microbial antigens. Their frequency in BC tissues is higher than that in normal breast tissues and their production in situ is uncoupled from systemic humoral responses. Although the prevalence of IgG and IgA class autoantibodies was similar, IgG and IgA responses were not discordant and they correlated with distinct clinical, pathological and immunological features possibly reflecting distinct signals required for their production and different biological functions in the tumor microenvironment.

**AUTHOR CONTRIBUTIONS**

KW-G, KS, SG, LB, and AL designed research, SG, KS, PZ, and UR performed research and participated in analysis and interpretation of the results, PZ performed the microarray data analysis and statistical analyses, SG and LB collected clinical data, GV and DL provided human samples, AdW and GV performed the pathological evaluations, AL wrote the manuscript, KW-G, KS, and SG revised the manuscript. All authors have read and approved the manuscript.

**FUNDING**

This work was funded by the Latvian National Research Programme BIOMEDICINE 2014–2017, the Belgian Fund for Scientific Research (FNRS), FNRS-OpérationTélèvie, Les Amis de l’Institut Bordet, Medic Foundation, Plan Cancer of Belgium, Fonds J. C. Heuson and Fonds Lambeau-Marteaux. SG is a post-doc of the FNRS-Operation Télèvie.

**ACKNOWLEDGMENTS**

We thank Genome Database of Latvian population for providing plasma samples of healthy women.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02660/full#supplementary-material

**REFERENCES**

1. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol.* (2015) 26:259–71. doi: 10.1093/annonc/mdu450

2. Fridman WH, Zitvogel L, Sautes-Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. *Nat Rev Clin Oncol.* (2017) 14:717–34. doi: 10.1038/nrclinonc.2017.101

3. Mao Y, Qu Q, Chen X, Huang O, Wu J, Shen K. The prognostic value of tumor-infiltrating lymphocytes in breast cancer: a systematic review and meta-analysis. *PLoS ONE* (2018) 11:e0152500. doi: 10.1371/journal.pone.0152500
11. Mohammed ZM, Going JJ, Edwards J, Elsberger B, McMillan DC. Yeong J, Lim JCT, Lee B, Li H, Chia N, Ong CCH, et al. High densities of Garaud et al. Repertoire of TIL-B Autoantibodies in BC
10. Song IH, Heo SH, Bang WS, Park HS, Park IA, Kim YA, et al. Predictive value of Welinder C, Baldetorp B, Blixt O, Grabau D, Jansson B. Primary breast cancer. Breast Cancer Res Treat. (2012) 152:545–53. doi: 10.1007/s10549-011-1620-1
9. Brown JR, Wimberly H, Lannin DR, Nixon C, Rimml DL, Bossuyt V. Multiplexed quantitative analysis of CD3, CD8, and CD20 predicts response to neoadjuvant chemotherapy in breast cancer. Clin Cancer Res. (2014) 20:5995–6005. doi: 10.1158/1078-0432.CCR-14-1622
8. Arias-Pulido H, Cimino-Mathews A, Chaher N, Qualls C, Joste N, Colpaert F, et al. The combined presence of CD20 + B cells and PD-L1 + tumor-infiltrating lymphocytes in inflammatory breast cancer is prognostic of improved patient outcome. Breast Cancer Res Treat. (2018) 17:273–82. doi: 10.1007/s10549-018-4834-7
7. Mahmoud SM, Lee AH, Paish EC, Macmillan RD, Ellis IO, Green AR. The prognostic significance of B lymphocytes in invasive carcinoma of the breast. Breast Cancer Res Treat. (2012) 132:545–53. doi: 10.1007/s10549-011-1620-1
6. Mohammed ZM, Going JJ, Edwards J, Elsberger B, Doughty JC, McMillan DC. The relationship between components of tumour inflammatory cell infiltrate and clinicopathological factors and survival in patients with primary operable invasive ductal breast cancer. Br J Cancer (2012) 107:864–73. doi: 10.1038/bjc.2012.347
prognosis in ovarian cancer. *Clin Cancer Res.* (2012) 18:3281–92. doi: 10.1158/1078-0432.CCR-12-0234

39. McDaniel JR, Pero SC, Voss WN, Shukla GS, Sun Y, Schaetzle S, et al. Identification of tumor-reactive B cells and systemic IgG in breast cancer based on clonal frequency in the sentinel lymph node. *Cancer Immunol Immunother.* (2018) 67:729–38. doi: 10.1007/s00262-018-2123-2

40. Zornig I, Halama N, Lorenzo Bermejo I, Ziegelmeier C, Dickes E, Migdoll A, et al. Prognostic significance of spontaneous antibody responses against tumor-associated antigens in malignant melanoma patients. *Int J Cancer* (2015) 136:138–51. doi: 10.1002/ijc.28980

41. Silina K, Zayakin P, Ancans G, Kalnina Z, Meistere I, Pismennaya A, et al. (Eds.). Autoantibody biomarkers for early detection and prognosis of gastric cancer. In: *15th International Congress of Immunology (ICI)*, 22 Aug 2013, Milan, Italy, Front. Immunol. Conference Abstract (2013).

42. Ying X, Zhao Y, Wang JL, Zhou X, Zhao J, He CC, et al. Serum anti-osteopontin autoantibody as a novel diagnostic and prognostic biomarker in patients with hepatocellular carcinoma. *Oncol Rep.* (2014) 32:1550–6. doi: 10.3892/or.2014.3367

43. Kulic A, Sirotkovic-Skerlev M, Jelisavac-Cosic S, Herceg D, Kovac Z, Vrbanez D. Anti-p53 antibodies in serum: relationship to tumor biology and prognosis of breast cancer patients. *Med Oncol.* (2010) 27:887–93. doi: 10.1007/s12032-009-9301-1

44. Andreu P, Johansson M, Affara NL, Pucci F, Tan T, Junankar S, et al. FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* (2010) 17:121–34. doi: 10.1016/j.ccr.2009.12.019

45. Karagiannis P, Gilbert AE, Josephs DH, Ali N, Dodev T, Saul L, et al. IgG4 subclass antibodies impair antitumor immunity in melanoma. *J Clin Invest.* (2013) 123:1457–74. doi: 10.1172/JCI65579

46. Nielsen JS, Nelson BH. Tumor-infiltrating B cells and T cells: working together to promote patient survival. *Oncoimmunology* (2012) 1:1623–5. doi: 10.4161/onci.21650

47. Kroeger DR, Milne K, Nelson BH. Tumor-infiltrating plasma cells are associated with tertiary lymphoid structures, cytolytic T-cell responses, and superior prognosis in ovarian cancer. *Clin Cancer Res.* (2016) 22:3005–15. doi: 10.1158/1078-0432.CCR-15-2762

48. Silina K, Soltermann A, Attar FM, Casanova R, Uckeley ZM, Thut H, et al. Germinal centers determine the prognostic relevance of tertiary lymphoid structures and are impaired by corticosteroids in lung squamous cell carcinoma. *Cancer Res.* (2018) 78:1308–20. doi: 10.1158/0008-5472.CAN-17-1987

49. Cipponi A, Mercier M, Seremet T, Baurain JF, Theate I, van den Oord J, et al. Neogenesis of lymphoid structures and antibody responses occur in human melanoma metastases. *Cancer Res.* (2012) 72:3997–4007. doi: 10.1158/0008-5472.CAN-12-1377

50. Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol.* (2013) 13:118–32. doi: 10.1038/nri3383

51. Carson PJ, Schut RL, Simpson ML, O’Brien J, Janoff EN. Antibody class and subclass responses to pneumococcal polysaccharides following immunization of human immunodeficiency virus-infected patients. *J Infect Dis.* (1995) 172:340–5.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Garaud, Zayakin, Buisseret, Rulle, Silina, de Wind, Van den Eyden, Larsimont, Willard-Gallo and Liné. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.