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Immune landscape of inflammatory breast cancer suggests vulnerability to immune checkpoint inhibitors

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ABSTRACT

Background. Anti-PD1/PDL1 immune checkpoint inhibitors (ICIs) showed promising results in breast cancer, and exploration of additional actionable immune checkpoints is ongoing. Inflammatory breast cancer (IBC) is an aggressive form of disease, the immune tumor microenvironment (TME) of which is poorly known. We aimed at providing the first comprehensive immune portrait of IBCs.

Methods. From the gene expression profiles of 137 IBC and 252 non-IBC clinical samples, we measured the fractions of 22 immune cell types, expression of signatures associated with tertiary lymphoid structures (TLS) and with the response to ICIs (T cell-inflamed signature: TIS) and of 18 genes coding for major actionable immune checkpoints. The IBC/non-IBC comparison was adjusted upon the clinicopathological variables.

Results. The immune profiles of IBCs were heterogeneous. CIBERSORT analysis showed profiles rich in macrophages, CD8+ and CD4 + T-cells, with remarkable similarity with melanoma TME. The comparison with non-IBCs showed significant enrichment in M1 macrophages, γδ T-cells, and memory B-cells. IBCs showed higher expression of TLS and TIS signatures. The TIS signature displayed values in IBCs close to those observed in other cancers sensitive to ICIs. Two-thirds of actionable immune genes (*HAVCR2/TIM3, CD27, CD70, CTLA4, ICOS, IDO1, LAG3, PDCD1, TNFRSF9, PVRIG, CD274/PDL1*, and *TIGIT*) were overexpressed in IBCs as compared to normal breast and two-thirds were overexpressed in IBCs *versus* non-IBCs, with very frequent co-overexpression. For most of them, the overexpression was associated with better pathological response to chemotherapy.

Conclusion. Our results suggest the potential higher vulnerability of IBC to ICIs. Clinical trials.

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Introduction

Inflammatory breast cancer (IBC) is a highly metastatic form of breast cancer,[1] with a poorer prognosis than non-IBC. Although its incidence represents only 2–4% of breast cancers, it accounts for 8–10% of breast cancer-related mortality. However currently, the treatment remains similar to that of non-IBC and is based on anthracycline/taxane-based neoadjuvant chemotherapy (and anti-HER2 agents for HER2+ cases), followed by surgery and radiotherapy, and then adjuvant therapy including capecitabine in the case of residual tumor for triple-negative tumors, hormone therapy for estrogen receptor (ER)-positive tumors and anti-HER2 agents for HER2 + tumors.[2] Because of this lack of specific treatment, the 5-year survival remains inferior to 50%. Identification of new therapeutic targets is crucial, justifying the biological studies published for many decades.[3,4] The largest "omics" series reported to date is the World IBC Consortium's series,[5–7] in which we showed the overrepresentation of high-risk molecular subtypes (basal, HER2-enriched, luminal B) in IBC, underscoring the need to adjust the IBC/non-IBC comparison upon the molecular subtypes.[7]

During the last decade, immunotherapy, based on the targeting of co-inhibitory immune checkpoints such as PD1/PDL1 and CTLA4, has become important in treating many cancers, including melanoma, and lung or head/neck carcinomas. Immune checkpoint inhibitors (ICIs) are increasingly being tested in many other cancers, but despite major advances, only a minority of patients benefit from these, and most of them develop secondary resistance. The immune response is a highly coordinated and complex process involving numerous specialized cell types and immune regulator proteins, including immune checkpoints. Resistance to ICIs may be due to the expression of alternative inhibitory immune checkpoints that dampen the T-cell responses, leading to severe T-cell exhaustion not rescuable by anti-PD1/PDL1 alone.[8,9] Another mechanism is the lack of efficient immune cell infiltration, which may be improved by antibodies targeting co-stimulating immune checkpoints. These mechanisms

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led to the testing of the combination of anti-PD1/PDL1 with drugs targeting other co-inhibitory and/or co-stimulating immune checkpoints. Today, numerous clinical trials of immunotherapy are ongoing,[10] based on the targeting of co-inhibitory and/or co-stimulating immune checkpoints.

Regarding breast cancer, the importance of immunity emerged recently. Several immune variables have been associated with survival and/or response to chemotherapy in non-IBC, including the quantification of tumor-infiltrating lymphocytes (TILs), gene expression signatures reflecting the abundance and/or the functionality of immune cells, expression of immune checkpoints such as IDO1[11] or CD274/PDL1.[12–24] In the last few years, anti-PD1/PDL1 ICIs have shown interesting results in metastatic BC,[25] particularly in the triple-negative (TN) subtype, both as monotherapy[26] and in combination with conventional treatments.[27,28]The FDA recently approved the use of atezolizumab, an anti-PDL1 antibody, combined with nab-paclitaxel for patients with metastatic TNBC. In the neoadjuvant setting, promising results have been obtained in TNBC.[29,30] Anti-CTLA4 ICIs have been more recently assessed in metastatic BC.[31,32] Exploration of additional actionable immune checkpoints is ongoing.

To date, no clinical trial has tested ICIs in IBC, although pre-clinical data suggest that they deserve particular attention. The importance of the tumor stroma in IBC has been underlined.[33,34] Our IBC/non-IBC 79-gene signature was in part characterized by a decreased TGFβ pathway and an altered immune response program.[7] Recent data revealed a central role of stroma-derived TGFβ in inducing immune evasion and resistance to ICIs.[35,36] Inflammatory signaling pathways, such as NFkB, COX2, and JAK2/STAT3, are active in IBC.[37,38] A signature predictive for pathological complete response (pCR) to neoadjuvant chemotherapy in IBC was mainly composed of genes related to T cytotoxic immune response.[5] PDL1 expression is higher in IBC than in non-IBC at the mRNA[39] and protein[40] levels, with positive correlation with pCR to chemotherapy. Finally, IBC samples display higher tumor mutational burden (TMB) than non-IBC,[41–43] which might increase the antigen-based attraction of immune effector cells. To date, the characterization of immune tumor microenvironment (TME) of clinical IBC samples has been limited to the quantification of a few cell types including TILs,[40,44,45] CD8 + T-cells,[45] CD20 + B-cells,[45,46] and analysis of PDL1 expression at the mRNA[39] and protein[40,45,46] levels. Very little is known about the relative abundance of immune cells and expression of numerous actionable immune checkpoints.

To better decipher the immune landscape in IBC, we analyzed our World IBC Consortium's expression dataset including 137 IBC and 252 non-IBC samples. We measured the fractions of 22 immune cell types, expression of gene signatures associated with tertiary lymphoid structures (TLS) and with response to ICIs (T cell-inflamed signature: TIS) and of 18 genes coding for major actionable immune checkpoints.

Materials and methods

Patients and breast cancer samples

We analyzed mRNA expression data of 137 IBC and 252 non-IBC clinical samples (N = 389) collected within the World IBC Consortium. IBC definition was clinical, as defined according to international consensus criteria.[1] Collection criteria and sample characteristics have been previously described.[7] Briefly, all samples were pre-treatment primary tumor samples from patients with invasive breast adenocarcinoma treated at the Institut Paoli-Calmettes (IPC: 71 IBC, 139 non-IBC), the General Hospital Sint-Augustinus (TCRU: 41 IBC, 55 non-IBC), and the University of Texas MD Anderson Cancer Center (MDA: 25 IBC, 58 non-IBC). IBC samples correspond to diagnostic biopsies taken from consecutively treated patients, clinically annotated, and with good-quality tumor RNA. Non-IBC samples were either diagnostic biopsies (advanced stage disease) or surgical specimen (early stage disease). Each patient gave written informed consent and the study was approved by our institutional review boards. We also profiled four normal breast samples that represented a pool of samples from 11 healthy women (4 from reduction mammoplasty, and 3 commercial pools of, respectively, 1, 2 and 4 normal breast RNA (Clontech, Palo Alto, CA)).

Neoadjuvant chemotherapy delivered to IBC patients was anthracycline-based, often including taxane, and associated with trastuzumab in more than 50% of HER2+ cases. Chemotherapy was followed by mastectomy and axillary lymph node dissection for clinically non-progressive and consenting patients, then radiotherapy. The pathological response to chemotherapy was defined on the operative specimen (primary tumor and lymph nodes) using Chevallier grading,[47] grades 1 and 2 (ypT0/Tis ypN0) being considered as pCR, and grades 3 and 4 as no-PCR.

We also collected the gene expression and protein expression data of breast cancer cell lines of the Broad Institute Cancer Cell Line Encyclopedia (CCLE)[48] and hosted on the Cancer Dependency Portal (DepMap).

Gene expression data analysis

Each institution had generated the gene expression profiles of its own samples using Affymetrix platforms (Affymetrix®, Santa Clara, CA, USA: U133 Plus 2.0 human microarrays for the French and Belgium institutions and U133A human microarrays for the US institution) as previously described.[49] Data analysis required pre-analytic processing. We first normalized each data set separately using Robust Multichip Average (RMA).[50] Normalization was done in R using Bioconductor and associated packages. The gene annotation of hybridization probes was updated using NetAffx Annotation files (www.affymetrix.com; release from 01/12/2008). The probes were then mapped based on their EntrezGeneID. When multiple probes mapped to the same GeneID, we retained the one with the highest variance in a particular dataset. We then merged the three data sets by using COMBAT (empirical Bayes)[51] as batch effects removal method, included in the inSilicoMerging

R/Bioconductor package.[52] ER, PR, and ERBB2/HER2 statutes of samples were based on mRNA expression of the 205225_at (*ESR1*), 208305_at (*PGR*), and 216836_s_at (*ERBB2*) Affymetrix probe sets and defined as discrete values (positive/negative) using a 2-component Gaussian mixture distribution model.[53] The molecular subtypes of tumors were defined as HR+/HER2- (ER- and/or PR-positive and HER2-negative), HER2+ (HER2-positive, regardless ER and PR), and TN (ER-, PR-, and HER2-negative).

We tested different immune variables from gene expression data. First, we applied the CIBERSORT algorithm[54] to quantify the absolute amount of 22 infiltrating immune cell types in each sample in the merged data set. CIBERSORT employs deconvolution of bulk gene expression data and a sophisticated algorithm for *in silico* quantification based on a leukocyte gene signature matrix, termed LM22, which contains 547 genes that distinguish 22 human hematopoietic cell phenotypes. The 22 cell types include naïve and memory B-cells, plasma cells, seven T-cell types (CD8, naïve CD4, resting memory CD4, activated memory CD4, follicular helper, regulatory, and γδ), resting and activated natural killer (NK) cells, monocytes, three macrophages types (M0, M1, and M2), resting and activated dendritic cells (DC), resting and activated mast cells, eosinophils, and neutrophils. We also compared the CIBERSORT scores between IBC and 14 solid cancer types previously analyzed by others and profiled using Affymetrix microarrays.[55] In this comparison, the 22 immune cell types were aggregated into 11 cell immune classes as reported.[55] The similarity between samples was assessed using hierarchical clustering (hclust function in R with Euclidean distance and average linkage). Second, we applied two other immune signatures, TIS and TLS. The TLS signature is a 12-chemokine-gene signature[56] associated with the presence of TLS in human cancers. The TIS signature is an 18-gene signature associated with the response to ICIs in different cancer types.[57] Both signatures were applied as metagenes to IBC and non-IBC samples. Finally, we selected 18 genes coding for actionable immune checkpoints targeted by immuno-oncology drugs FDA-approved (*CD274/PDL1, PDCD1*, and *CTLA4*,) and under clinical development (*CD276, BTLA, CD27, CD40, CD70, HAVCR2/TIM3, ICOS, IDO1, LAG3, PVRIG, TIGIT, TNFRSF4, TNFRSF9, TNFRSF18*, and *VSIR*). All those immune variables were analyzed as continuous values. In order to compare the samples with respect to all immune variables pooled, we defined a PanImmune score for each sample. A z-score transformation was first applied to the 42 immune variables tested (22 CIBERSORT estimates, 2 signature scores, and expression of 18 genes) to homogenize them; then for each sample, the PanImmune score was defined as the mean of the 42 transformed variables.

Statistical analysis

Correlations between sample groups and clinicopathological variables were tested using the Student's t-test for continuous variables and Fisher's exact test for discrete variables. Correlation between continuous variables was tested using the Pearson correlation coefficient and the Spearman's rank correlation coefficient. Univariate and multivariate comparative analyses of gene expression data between IBC and non-IBC and between IBC samples with pCR and no-pCR were done using a logistic regression analysis (glm function and significance estimated by specifying a binomial family for model with a logit link). Variables tested in univariate analyses included the immune variables (CIBERSORT modules, immune signatures, and gene expression levels) and the following clinico-pathological variables: patients' age at time of diagnosis (continuous value), pathological type (ductal *vs* lobular *vs* mixed *vs* other) and grade (3 *vs* 1–2), molecular subtypes (HR+/HER2- *vs* HER2+ *vs* TN), and presence of dermal lymphatic emboli (yes *vs* no). Variables with a p-value <0.05 in univariate analysis were tested in multivariate analysis in the IBC *versus* non-IBC comparison. All statistical tests were two-sided at the 5% level of significance. Statistical analysis was done in the R software (version 3.5.2; http://www.cran.r-project.org/).

Results

Patients' population

The clinicopathological characteristics of 137 patients with IBC and 252 with non-IBC are summarized in Table 1. As expected, IBC patients were younger than non-IBC patients, and, compared to non-IBC samples, IBC samples tended to be more frequently ductal type, displayed more frequent dermal lymphatic tumor emboli, and were more frequently pathological grade 3, and HER2+ or TN. In univariate analysis (logistic regression), younger patients' age, presence of dermal lymphatic tumor emboli, grade 3, and HER2+ and TN subtypes were associated with IBC phenotype (data not shown). Such expected differences, as well as the difference in 5-year MFS (79% in non-IBC and 53% in IBC; data not shown), confirmed the coherence of our data set. The pathological response to

Table 1. Clinico-pathological characteristics of IBC and non-IBC samples.

	All (N = 389)	IBC (N = 137)	non-IBC (N = 252)	p-value
Patients' age, years	54 (24–89)	50 (24–82)	55 (24–89)	**2.08E-03**
Pathological type				0.0981
ductal	325	122 (90%)	203 (81%)	
lobular	30	7 (5%)	23 (9%)	
mixed	14	3 (2%)	11 (4%)	
other	18	3 (2%)	15 (6%)	
Dermal lymphatic tumor emboli				**4.43E-16**
absence	123	29 (24%)	94 (76%)	
presence	121	91 (76%)	30 (24%)	
Pathological grade				**5.62E-11**
1–2	174	30 (23%)	144 (58%)	
3	207	101 (77%)	106 (42%)	
Molecular subtype				**1.75E-04**
HR+/HER2-	223	60 (44%)	163 (65%)	
HER2+**	82	42 (31%)	40 (16%)	
TN	84	35 (26%)	49 (19%)	
Pathological complete response				
no	59	59 (68%)	NA*	
yes	28	28 (32%)	NA	

*NA, not available; **; HER2+ positive included 12 HR+ and 30 HR- cases in IBC and 20 HR+ and 20 HR- cases in non-IBC

neoadjuvant chemotherapy was available for 87 IBC samples and included 28 cases with pCR (32%).

Immune cell composition in IBC samples

We first studied the landscape of 22 immune cell types infiltration in the 137 IBC samples. As shown in Figure 1a, the different immune cell types showed great variability, the five most abundant ones being M2 macrophages, CD8 + T-cells, M0 macrophages, M1 macrophages, then plasma cells, while the less abundant was eosinophils. Each immune cell type showed large variability between samples, the most variable types being M0 and M2 macrophages, and the less variable being eosinophils. As expected, the total number of immune cells (total CIBERSORT absolute score) positively correlated with the Rooney's cytolytic activity score of samples,[58] an *in silico* metric of inflammation based on the geometric mean of *GZMA* and *PRF1* expression (r = 0.78, Figure 1a).

We then compared the CIBERSORT profile of IBC samples to that of the 252 non-IBC samples. The respective distributions of immune cell types seemed globally similar between IBC and non-IBC. However, significant differences existed (Figure 1b). In univariate analysis, the γδ T-cells were more numerous (OR = 1.20, CI95% 1.09–1.33; p = 2.65E-03) in IBC than in non-IBC, as were the memory B-cells (OR = 1.16, CI95% 1.06–1.28; p = 1.01E-02), M1 macrophages (Odds Ratio: OR = 1.09, CI95% 1.05–1.15; p = 1.18E-03) and plasma cells (OR = 1.05, CI95% 1.01–1.09; p = 2.10E-02). In multivariate analysis including the variables significant in univariate analysis (patients' age, pathological grade, dermal lymphatic tumor emboli, and molecular subtypes), the M1 macrophages, γδ T-cells, and memory B-cells remain significantly more numerous in IBC (p < .05), whereas plasma cells tended to remain significant (p = .107). Of note, *CD3E, CD20*, and *CD68* mRNA expressions were not different between IBC and non-IBC samples, suggesting similar amounts of T-cells, B-cells, and macrophages, respectively, whereas the Rooney's cytolytic activity score was higher in IBC samples than non-IBC samples, even after adjustment in multivariate analysis (Figure 1b).

Finally, to compare IBC to other solid cancers, we aggregated these 22 immune cell types into 11 immune cell classes and compared their profile with that of 14 solid cancer types publicly available.[55] There was a similarity between IBC and primary melanoma that clustered together, and higher abundance of CD8 + T-cells in IBC and primary and metastatic melanoma than in other types (Figure 1c). Altogether, these

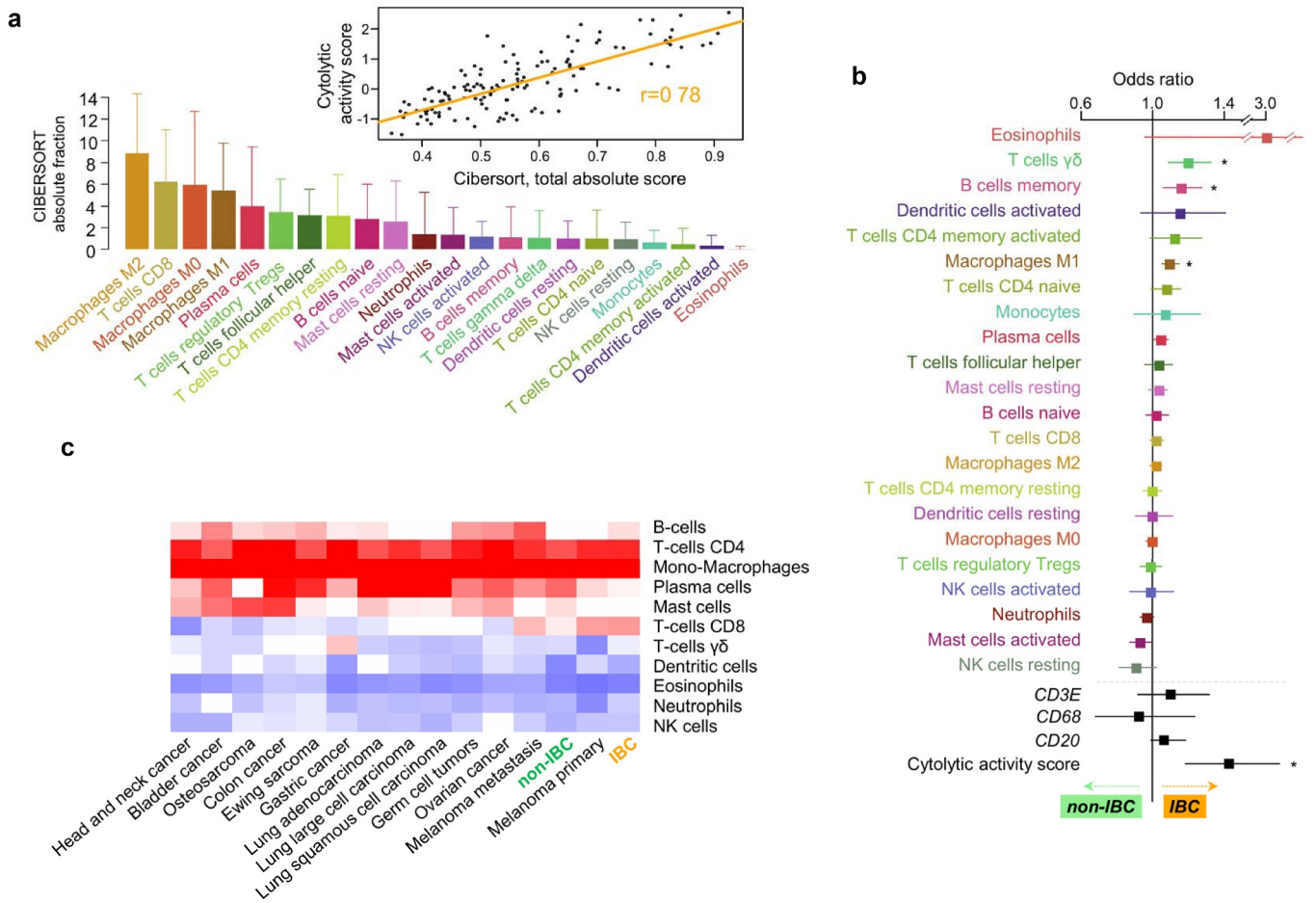

Figure 1. Immune cell composition of IBC samples. *A*/Bar plots showing the absolute percentage of the 22 CIBERSORT immune cell types in the 137 IBC samples. The box shows the correlation between the total number of immune cells (total CIBERSORT absolute score) and the Rooney's cytolytic activity score. *B*/Unadjusted IBC/non-IBC Odds Ratios (boxes and 95%CI) of the estimates of absolute fraction of the 22 CIBERSORT immune cell types, of the mRNA expression levels of *CD3E* (T-cells), *CD20* (B-cells), and *CD68* (macrophages), and of the Rooney's cytolytic activity score for the IBC versus non-IBC comparison. Asterisks denote variables significant in multivariate analysis. *C*/Hierarchical clustering of 14 solid cancer types and our IBC class (N = 137) and 11 immune cell classes.

data suggested that the immune cell microenvironment of IBC is different from non-IBC and associated with higher cytolytic activity. This difference is not due to different amounts of T-cells, B-cells, and macrophages, but results from more subtle differences with respect to more specific immune cell subpopulations.

Tertiary lymphoid structures in IBC

Independently of the amount of each immune cell type, the tissue organization of immune cells in tumors is biologically and clinically relevant, as exemplified by the importance of TLS[59]), which have been associated with response to ICIs in clinical samples.[60–62] We analyzed *in silico* the presence of TLS in IBC samples by applying the 12-gene TLS signature.[56] As shown in Figure 2a, this signature was heterogeneous across all samples, but clearly expressed in a significant subset of cases. The comparison with non-IBC samples showed higher TLS score in IBC (Figure 2b). In univariate analysis, the OR for IBC type was 1.62 (95%CI 1.28–2.03; p = 5.76E-04), which remained significant in multivariate analysis (OR = 1.5 (95% CI 1.12–2.00), p = 2.20E-02). These results suggested higher frequency of TLS in IBC than non-IBC, independently from the clinicopathological variables, notably the molecular subtypes.

Signature predictive for response to ICI in IBC

In addition to the TLS signature, we tested an immune gene signature (TIS) defined as predictive for response to ICIs in different cancers.[57] This signature was heterogeneous across IBC samples and highly expressed in a subset of samples (Figure 3a). The comparison of the TIS scores between IBC and other cancers from TCGA showed that IBC had relatively high median scores, close to that of cancers classically sensitive to ICIs, such as lung and cervical squamous cell carcinomas (Figure 3b). TIS scores were higher in IBC samples than in TCGA breast cancer samples that exclusively included non-IBC (p = 1.25E-04). We confirmed this difference in our IBC *versus* non-IBC samples (Figure 3c). In univariate analysis, the OR for IBC type was 1.47 (95%CI 1.17–1.86; p = 5.99E-03), and higher expression in IBC remained significant in multivariate analysis (p = 3.38E-03), suggesting independency with respect to clinicopathological variables. Of note, the TIS score variability in IBC was similar to that observed in TCGA breast cancer samples and other cancer types, as previously reported.[63] Altogether, these data suggested that IBC might be more vulnerable to ICIs than non-IBC.

Expression of targetable immune checkpoints in IBC

We thus analyzed the expression of 18 genes coding for actionable immune checkpoint regulators. As shown in Figure 4a, expression in IBC samples showed a great variability between the different genes, with higher median expression for *VSIR* and lower expression for *TNFRSF4*. There was also a great heterogeneity in expression of each gene across the different samples, with a range of intensities ranging from eight intervals in $\log_2$ scale for the most variable (*IDO1*) to one interval for the least variable (*TNFRSF4*). Twelve out of 18 genes (67%) were significantly overexpressed (Mann–Whitney test) in IBC samples as compared to normal breast samples (Table 2).

The range of expression levels of these 18 genes in non-IBC samples was close to that observed in IBC samples (data not shown). However, expression levels were significantly different (continuous values) between IBC and non-IBC samples ($p \leq 0.05$, Student's t-test; Figure 4b, Table 3) for 12 out of 18 genes (67%) in univariate analysis, with higher expression in IBC for *BTLA, CD274/PDL1, TIGIT, VSIR, CD27, CD40, CD70, IDO1*, and *PVRIG*, and lower expression in IBC for three co-stimulating checkpoints (*CD276, TNFRSF18*, and *TNFRSF4*). In multivariate analysis adjusted for the clinicopathological variables, six genes remained differentially expressed between IBC and non-IBC, including notably *CD274/PDL1, VSIR*, and *IDO1*. Using the available omics data of CCLE breast cancer cell lines, we measured the correlation between the mRNA and protein expression

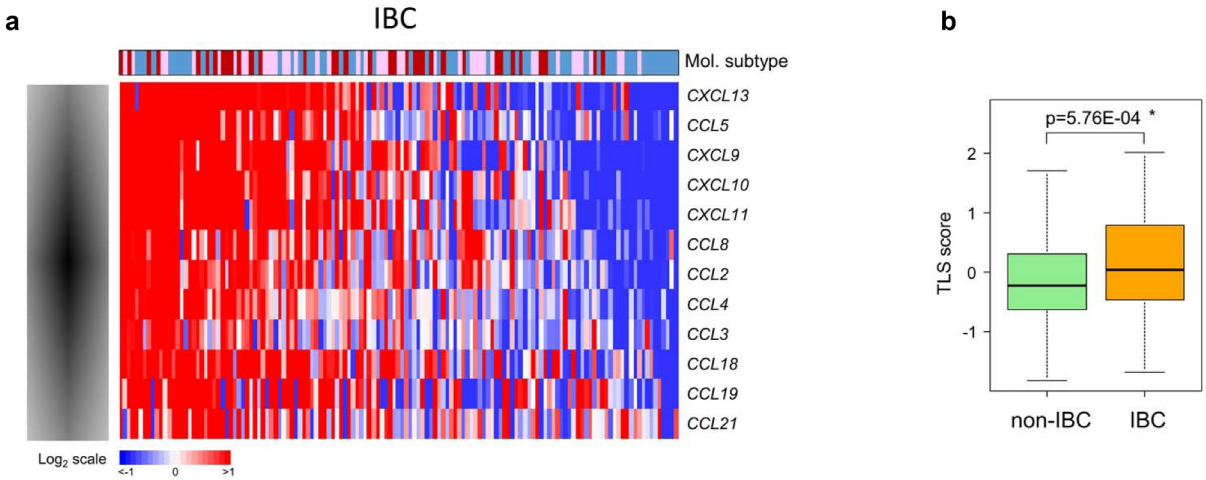

Figure 2. Expression of the TLS signature in IBC samples. ***A***/Hierarchical clustering of the 137 IBC samples and the 12 genes of the TLS signature. The molecular subtype of samples is shown above the color matrix and color-coded as follows: blue: HR+/HER2-, pink: HER2+, and brown: TN. ***B***/Box plot comparing the TLS metagene score in IBC *versus* non-IBC. The *p*-value if for univariate analysis and the asterisk denotes significance in multivariate analysis.

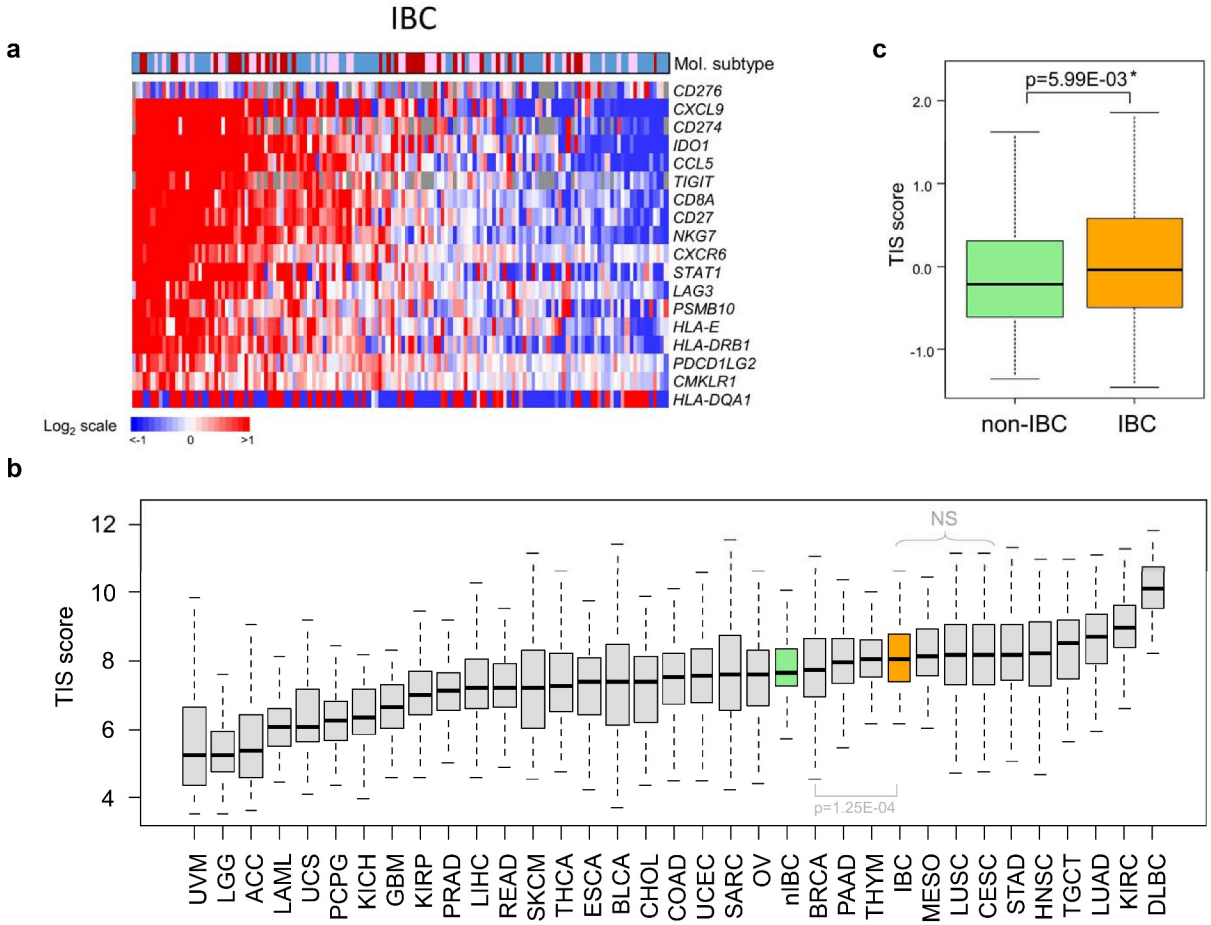

Figure 3. Expression of the TIS signature in IBC samples. ***A***/Hierarchical clustering of the 137 IBC samples and the 18 genes of the TIS signature. The molecular subtype of samples is shown above the color matrix and color-coded as follows: blue: HR+/HER2-, pink: HER2+, and brown: TN. ***B***/Box plots of the TIS scores between IBC (orange box), non-IBC (green box) and 33 cancer types from TCGA (gray boxes). ACC: adrenocortical carcinoma; BLCA: bladder urothelial carcinoma; BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: cholangiocarcinoma; COAD: colon adenocarcinoma; DLBC: lymphoid neoplasm diffuse large B-cell lymphoma; ESCA: esophageal carcinoma, GBM: glioblastoma multiforme; HNSC: head and neck squamous cell carcinoma; KICH: kidney chromophobe; KIRC: kidney renal clear cell carcinoma; KIRP: kidney renal papillary cell carcinoma; LAML: acute myeloid leukemia; LGG: brain lower grade glioma; LIHC: liver hepatocellular carcinoma; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma, MESO: mesothelioma; OV: ovarian serous cystadenocarcinoma; PAAD: pancreatic adenocarcinoma; PCPG: pheochromocytoma and paraganglioma; PRAD: prostate adenocarcinoma; READ: rectum adenocarcinoma; SARC: sarcoma; SKCM: skin cutaneous melanoma; STAD: stomach adenocarcinoma; TGCT: testicular germ cell tumors; THCA: thyroid carcinoma; THYM: thymoma (THYM); UCS: uterine carcinosarcoma; UCEC: uterine corpus endometrial carcinoma; UVM: uveal melanoma. ***C***/Box plot comparing the TIS metagene score in IBC *versus* non-IBC. The *p*-value if for univariate analysis and the asterisk denotes significance in multivariate analysis.

levels of these six genes. No data was available for *CD70*. For the five other genes, the correlation was very good, with Spearman's rank correlation coefficient (rho) ranging from 0.66 to 0.82 and a mean equal to 0.74 (CI95 0.66–-0.82, p = 1.53E-05) (Supplementary Table S1). Such correlation indirectly suggested that the changes in gene expression in tumors likely translate into changes at the protein level for these genes. Of note, all other genes significant in univariate analysis showed higher odds ratios in multivariate analysis than in univariate analysis, but did not keep significance likely because of the too small sample size. *VSIR* was the gene showing the highest odds ratio. Altogether, these data suggested frequent higher expression of genes coding for actionable immune checkpoint regulators in IBC, compared to non-IBC.

Correlations between immune variables in IBC

Figure 5 shows the matrix of pairwise correlations of all immune variables in IBC samples. Expression of most immune actionable genes was positively correlated to different types of T-cells, B-cells and macrophages: the top five cell types included the three types significantly enriched in IBC (M1 macrophages, memory B-cells, and γδ T-cells) and memory activated CD4 + T-cells and CD8 + T-cells also more abundant, although not significantly, in IBC. A positive correlation also existed between the expression of several immune genes, suggesting frequent high co-expression. For example, there was a strong correlation between expression levels of *CD274*/*PDL1*, *CTLA4* and of the four immune genes targeted by major immunotherapies under development (*LAG3*, *HAVCR2*/*TIM3*, *TIGIT*, and *IDO1*) and the 112 IBC samples informative for these four genes, with a mean correlation coefficient equal to 0.61 (95%CI 0.55–0.67; p = 5.37E-12). Interestingly, the TIS signature score was strongly associated with high expression of

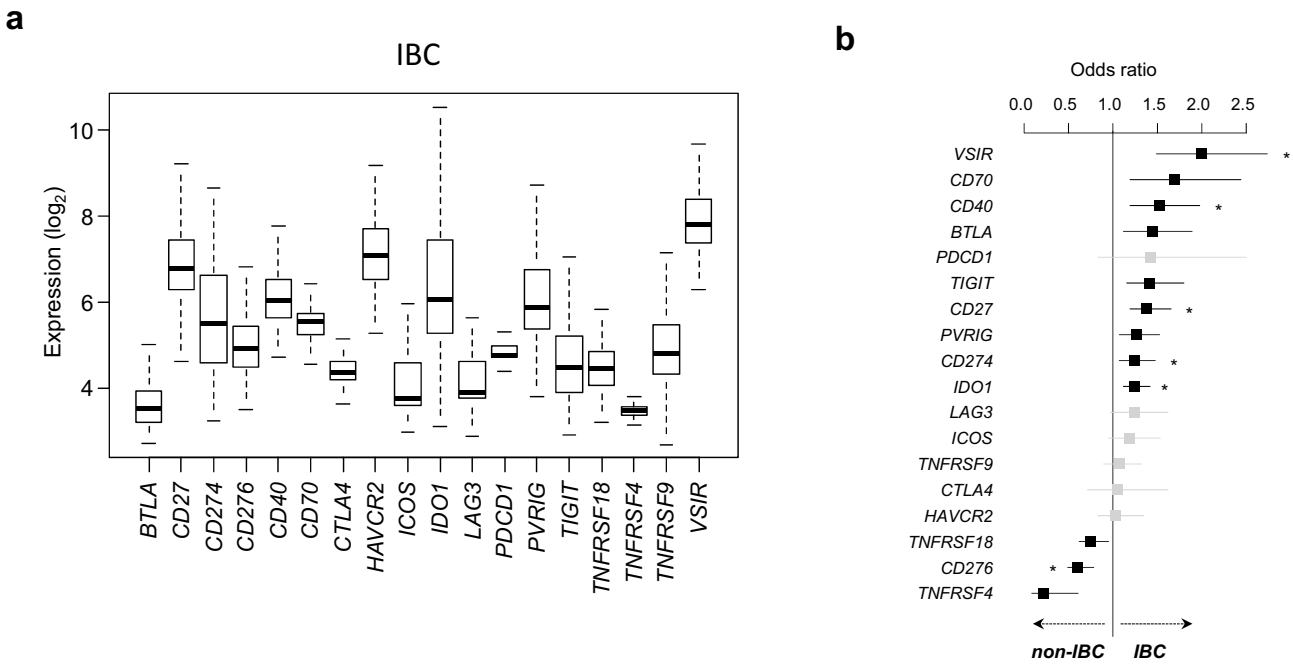

Figure 4. Expression of actionable immune genes in IBC samples. *A*/Box plots of mRNA expression levels (log_2 scale) of 18 actionable immune genes in the 137 IBC samples. The genes are alphabetically ordered. *B*/Unadjusted Odds Ratios (boxes and 95%CI) of expression of the 18 actionable immune genes for the IBC *versus* non-IBC comparison. Asterisks denote genes significant in multivariate analysis.

most of the genes coding for immune checkpoints targeted by current and emerging immuno-oncology drugs.

To what extent are IBC and non-IBC immune portraits different?

We thus assessed the extent of the IBC/non-IBC differences with respect to immune portrait comparatively to the same differences that exist between the molecular subtypes of breast cancer. Analysis concerned the 42 immune variables tested assessed both as individual variables and as a combined PanImmune score. This score corresponded to the mean of all 42 z-transformed variables. In the whole series of 389 samples, more differences were evidenced between the molecular subtypes than between IBC and non-IBC: 28/42 variables were significantly different between the molecular subtypes *versus* 18 between IBC and non-IBC (Supplementary Table S2), and the PanImmune score was also more significantly different (Supplementary Figure S1).

We then assessed and compared the degree of differences between IBC and non-IBC in each molecular subtype. We found more differences (Supplementary Table S2; Supplementary Figure S2) in the HR+/HER2- subtype (17 variables significant, PanImmune score significant) than in the TN subtype (3 variables significant, PanImmune score not significant) and the HER2+ subtype (3 variables significant, PanImmune score not significant).

Conversely, we compared the degree of differences between the molecular subtypes in IBC and in non-IBC. In non-IBC samples, many differences were evidenced: 30/42 variables were significantly different, including 26 higher in TN samples, 4 higher in HER2+ samples, and only 1 in HR+/HER2 samples (Supplementary Table S2). The PanImmune score was also significantly higher in TN samples, followed by HER2+ samples and then HR+/HER2- samples (Supplementary Figure S3). Such result, in agreement with the literature, further validated the coherence of our expression data. By contrast, less differences were observed in IBC samples, with only 10/42 variables significantly different (8 higher in TN samples, 1 higher in HER2+ samples, and 1 in HR+/HER2 samples; Supplementary Table S2), whereas the PanImmune score, although higher in TN *versus* HER2+ *versus* HR+/HER2- samples, was not significantly different (Supplementary Figure S3). For example, regarding the PD1/PDL1 axis, *PDL1 (CD274)* and *PD1 (CD279)* expressions were strongly associated with the molecular subtypes in non-IBC (higher in TN, followed by HER+, then HR+/HER2-; p = 2.96E-07 and p = 1.85E-04, respectively), as reported at the protein level (for PDL1 and PD1) in a series of 1,318 non-IBC[64] and at the mRNA level in a series of 5,454 non-IBC for PDL1[24] and in the UALCAN database for PD1.[65] By contrast, in IBC, *PDL1 (CD274)* expression was not significantly associated with the molecular subtypes (higher in TN, followed by HER2+, then HR+/HER2-; p = .082) as reported at the protein level in a series of 143 patients[40] and *PD1 (CD279)* expression was not associated (p = .912; Supplementary Table S2).

Altogether, these results suggested that the immune differences between IBC and non-IBC are less important than the differences between the molecular subtypes of breast cancer, and that these latter are less important in IBC than in non-IBC.

Table 2. mRNA expression of 18 actionable immune genes in IBC *versus* normal breast.

	IBC *versus* normal breast comparison				
	Univariate				
Genes	N	Normal (median, log2)	IBC (median, log2)	IBC vs. Normal Breast (log2-ratio)	p-value*
BTLA	118	3.5 (3.22–4.18)	3.54	(2.71–6.43)	0.04
0.946					
CD274	118	5.32 (5–5.48)	5.51	(3.23–8.66)	0.19
0.768					
CD276	118	5.55 (5.49–5.91)	4.93	(3.52–6.81)	−0.62
0.052					
HAVCR2	118	5.94 (5.61–6.55)	7.07	(5.27–9.20)	1.13
7.57E-03					
TIGIT	118	3.96 (3.64–4.17)	4.48	(2.91–7.06)	0.52
0.056					
TNFRSF18	118	4.59 (3.94–4.72)	4.45	(3.21–8.17)	−0.14
0.958					
VSIR	118	8.09 (7.85–8.78)	7.80	(5.66–9.68)	−0.29
0.293					
CD27	141	5.42 (4.64–7.06)	6.80	(4.63–12.4)	1.38
2.84E-02					
CD40	141	5.33 (5.05–5.97)	6.05	(4.71–9.06)	0.72
3.53E-02					
CD70	141	3.32 (3–4.05)	5.55	(4.57–8.73)	2.23
6.82E-04					
CTLA4	141	2.26 (2.07–2.32)	4.35	(3.33–6.73)	2.09
6.69E-04					
ICOS	141	2.21 (1.99–2.36)	3.76	(2.97–8.44)	1.55
6.82E-04					
IDO1	141	4.51 (2.75–6.06)	6.08	(3.11–10.8)	1.57
4.49E-02					
LAG3	141	2.14 (1.85–2.31)	3.91	(2.45–7.17)	1.77
6.69E-04					
PDCD1	141	2.48 (1.96–3.04)	4.76	(4.01–6.59)	2.28
6.43E-04					
TNFRSF4	141	2.15 (1.77–2.42)	3.48	(2.92–3.96)	1.33
6.09E-04					
TNFRSF9	141	3.21 (2.17–3.53)	4.80	(2.69–8.43)	1.59
9.35E-04					
PVRIG	141	4.65 (4.21–5.43)	5.87	(3.8–10.27)	1.22
6.41E-03					

*, Mann-Whitney test

Correlations of immune variables with response to chemotherapy in IBC

We then searched for correlations between these immune variables and the pathological response to neoadjuvant chemotherapy in patients with IBC. The CIBERSORT analysis suggested that higher levels of follicular helper T-cells (Tfh cells), naïve B-cells, M1 macrophages, and CD8 + T-cells were associated with higher pCR rate ($p < .05$) (Figure 6a), whereas higher levels of memory B-cells and activated mast cells tended to be associated with higher ($p = .07$) and lower pCR ($p = .08$) rate, respectively. Higher TLS score was associated with more frequent pCR with an OR for pCR equal to 2.52 (95%CI 1.54–4.12; $p = 2.07E-03$, logit link function; Figure 5b), as was higher TIS score (OR = 3.41, 95%CI 1.95–5.86; $p = 3.10E-04$, logit link function; Figure 6b). Twelve out of 18 tested genes (67%) were significantly upregulated in samples with pCR. The three genes with highest OR were *TNFRSF9, BTLA*, and *TIGIT* (Figure 6c). All these immune variables remained significant in multivariate analysis including the pathological type and grade and the molecular subtypes (data not shown).

Discussion

We aimed at documenting on a relatively large scale the immune landscape of IBC to improve our understanding of the nature and diversity of immune response, and eventually to provide further rationale for immunotherapy. We show that the immune profile of IBC is heterogeneous between patients and rich in macrophages and CD8 + T-cells, is enriched, when compared with non-IBC, in M1 macrophages, γδ T-cells, and memory B-cells, in high co-expression of several genes coding for actionable immune checkpoints, and in signatures predictive for response to ICIs (TIS and TLS). To our knowledge, these are novel results for IBC.

Study at the mRNA level allowed the simultaneous analysis of many genes while avoiding the limitations of immunohistochemistry (lack of specificity and reproducibility of antibodies, optimal cutoff definition). It allowed the application of deconvolution algorithm (CIBERSORT) and relevant gene signatures (TIS, TLS, and Rooney's signatures), the coherence of which was confirmed by the expected association of immune variables with the molecular subtypes in the non-IBC samples. Analysis was based upon the largest gene expression dataset reported in the literature we had generated within the World IBC Consortium. Importantly, the number of 137 IBC samples, relatively high given the scarcity of the disease and the difficulty to obtain pre-therapeutic samples, allowed adjusting the IBC/non-IBC comparison upon the clinicopathological variables including the molecular subtypes.

To our knowledge, the quantification of immune cell types of IBC has been limited in the literature to only one to three immune types simultaneously.[40,44–46] One study only compared the stromal TILs infiltration between IBC and non-IBC and showed no significant difference.[40] Such observation was confirmed in our series with similar expression for *CD3* (T-cells), *CD20* (B-cells), and *CD68* (macrophages). However, CIBERSORT analysis revealed more subtle differences. The TME of IBC was rich in macrophages, CD8+ and CD4 + T-cells, with remarkable similarity (as assessed using hierarchical clustering) with that of melanoma, known to harbor a peculiar immunogenic profile and high sensitivity to ICIs. The reasons of such stronger similarity of IBC (*versus* non-IBC) with melanoma are unknown, and of course, it does not automatically mean that IBC will be as sensitive as melanomas to ICIs. The comparison with non-IBC showed significant enrichment in M1 macrophages, γδ T-cells, and memory B-cells. Previous studies in non-IBC revealed the high variability in the composition of immune TME across tumors, partly associated with molecular status of tumors and clinical outcome.[66,67] CD20 + B-cells were observed in 62% of IBC samples and were associated with better survival and higher pCR,[46] as reported in our study. The higher abundance of

Table 3. mRNA expression of 18 actionable immune genes in IBC *versus* non-IBC.

	mRNA expression levels			IBC *versus* non-IBC comparison					
				Univariate			Multivariate		
Genes	N	non-IBC (median, log2)	IBC (median, log2)	N	Odds ratio [95%CI]	p-value*	N	Odds ratio [95%CI]	p-value*
BTLA	306	3.32 (2.45–6.31)	3.54 (2.71–6.43)	306	1.46 [1.12–1.9]	**2.05E-02**	172	1.57 [0.89–2.76]	0.188
CD274	306	5.1 (3.06–8.86)	5.51 (3.23–8.66)	306	1.26 [1.07–1.48]	**2.01E-02**	172	1.68 [1.14–2.47]	**2.86E-02**
CD276	306	5.46 (3.39–7.66)	4.93 (3.52–6.81)	306	0.62 [0.49–0.79]	**8.38E-04**	172	0.53 [0.33–0.84]	**2.50E-02**
HAVCR2	306	7.03 (4.59–9.24)	7.07 (5.27–9.2)	306	1.06 [0.83–1.35]	0.692	172	1.65 [0.97–2.79]	0.120
TIGIT	306	4.06 (2.8–7.41)	4.48 (2.91–7.06)	306	1.44 [1.16–1.80]	**6.64E-03**	172	1.69 [1.05–2.71]	0.069
TNFRSF18	306	4.7 (2.92–8.29)	4.45 (3.21–8.17)	306	0.77 [0.63–0.95]	**4.12E-02**	172	0.75 [0.51–1.09]	0.198
VSIR	306	7.52 (6.03–8.97)	7.8 (5.66–9.68)	306	2.02 [1.49–2.74]	**1.41E-04**	172	2.55 [1.36–4.78]	**1.43E-02**
CD27	389	6.49 (4.78–9.76)	6.8 (4.63–12.41)	389	1.40 [1.19–1.66]	**9.28E-04**	239	1.46 [1.11–1.92]	**2.33E-02**
CD40	389	5.82 (4.19–7.96)	6.05 (4.71–9.06)	389	1.55 [1.2–1.98]	**4.09E-03**	239	2.09 [1.32–3.32]	**8.76E-03**
CD70	389	5.36 (4.19–7.03)	5.55 (4.57–8.73)	389	1.71 [1.2–2.44]	**1.26E-02**	239	1.5 [0.82–2.73]	0.269
CTLA4	389	4.36 (3.09–5.95)	4.35 (3.33–6.73)	389	1.08 [0.72–1.62]	0.757	239	1.23 [0.66–2.29]	0.580
ICOS	389	3.81 (3–6.53)	3.76 (2.97–8.44)	389	1.21 [0.96–1.54]	0.185	239	1.3 [0.88–1.93]	0.274
IDO1	389	5.5 (2.96–10.88)	6.08 (3.11–10.76)	389	1.26 [1.12–1.42]	**1.32E-03**	239	1.43 [1.15–1.79]	**7.76E-03**
LAG3	389	3.9 (2.63–6.43)	3.91 (2.45–7.17)	389	1.26 [0.98–1.62]	0.132	239	1.38 [0.92–2.07]	0.194
PDCD1	389	4.76 (4–5.72)	4.76 (4.01–6.59)	389	1.45 [0.84–2.51]	0.265	239	0.95 [0.41–2.21]	0.926
TNFRSF4	389	3.48 (3.02–4.52)	3.48 (2.92–3.96)	389	0.24 [0.09–0.63]	**1.30E-02**	239	0.30 [0.07–1.29]	0.173
TNFRSF9	389	4.72 (3.28–8.2)	4.8 (2.69–8.43)	389	1.09 [0.90–1.33]	0.469	239	1.14 [0.82–1.57]	0.524
PVRIG	389	5.67 (3.58–9.61)	5.87 (3.8–10.27)	389	1.28 [1.08–1.53]	**1.96E-02**	239	1.31 [0.98–1.74]	0.125

*, glm

memory B-cells in IBC *versus* non-IBC might contribute to enhance efficiently the T-cell anti-tumor response in IBC, notably by organizing the formation of TLS. In patients with melanoma, single-cell studies of TME showed that higher infiltration of memory B-cells was associated with response to PD1/PDL1 blockade.[68] The γδ T-cells also represent a small component of TME dedicated to infectious and intracellular stresses recognition. They are cancer-killing effector T-cells endowed with both innate and adaptive functions, since they harbor both NK receptors (such as NK group 2 member D (NKG2D) or natural cytotoxicity receptors (NCRs)), growth factor receptors, and γδ TCRs, which recognize MHC-like molecules that are upregulated in cancer cells. Consequently, they are often the target for tumor escape mechanisms. In clinical breast cancer samples, some studies showed poor-prognosis value of high tumor γδ T-cells count measured using IHC,[69,70] whereas others showed favorable prognostic value of high fraction estimated using CIBERSORT.[55,67] The explanation and role of their higher amount in IBC remain to be investigated, but may open new promising therapeutic avenues.[71] Regarding macrophages, their role in breast cancer is known since many years.[72] Tumor-associated macrophages (TAMs) are the major inflammatory cells that infiltrate breast cancers,[73] and M2 macrophages are known to contribute to tumor progression and invasion.[74] In our series, CIBERSORT analysis showed that TAMs were the most abundant immune cells in IBC and M2 macrophages were more numerous than M1 macrophages. Previous studies reported that IBCs show high infiltration of macrophages secreting cytokines such as TNFα, CCL2, IL6, IL8 and IL10 to which IBC cells are more sensitive than non-IBC cells, thus likely contributing to higher IBC invasion and motility, high angiogenic nature, and local immune suppression.[75,76] Reciprocally, TME macrophages polarize into heterogeneous subpopulations depending on the type of external stimuli they receive.[77] In IBC, the cytokines secreted by TME cells and tumor cells that induce the M2 polarization include CSF1, TGFβ, IL8, IL10 and IL6.[78] In fact, a complex chemokine network involving IL8 and GRO chemokines exists in IBC in which IBC cells command macrophages to become tumor-promoting (M2), which in turn drive IBC cells to be more cancer stem-like, mesenchymal, and aggressive.[79] Importance of macrophages in IBC was further underlined by enrichment in macrophage markers of the normal mammary tissue adjacent to the tumor.[45]

Another interesting result is the higher expression in IBC than in non-IBC of two gene signatures directly (TIS signature) and indirectly (TLS signature) associated with response to ICIs in different cancers, independently from the clinicopathological variables. The TIS signature reflects an efficient adaptive immune response by quantifying expression of genes associated with cytotoxic cells, antigen presentation, and interferon-gamma activity. The TIS score in IBC was close to that of other cancer types classically sensitive to anti-PD1/PDL1 ICIs, such as lung, cervical, and head and neck squamous cell carcinomas. Because a correlation exists between higher average TIS score and higher sensitivity to anti-PD1/PDL1 ICIs across multiple cancer types,[63] our results suggest higher vulnerability of IBC than non-IBC to ICIs.

This observation is important since many actionable immune checkpoints were overexpressed in IBC. We analyzed the expression of 18 genes coding for therapeutic targets of ICIs, marketed (such as CD274/PDL1 and CTLA4) or under clinical development, the most advanced being those targeting LAG3, TIM3, TIGIT, and IDO1. Expression was heterogeneous between patients and genes, the majority of samples simultaneously expressing several of the tested genes. Two-thirds of the genes were overexpressed in IBC samples as compared to normal breast. The comparison with non-IBC identified nine genes with higher expression in IBC, and three genes coding for co-stimulating checkpoints with lower expression. The most differentially expressed gene was *VSIR*, with an Odds Ratio of expression superior to 2 in IBC *versus* non-IBC. Its expression has never been assessed in IBC, but a recent study in non-IBC using IHC identified expression on

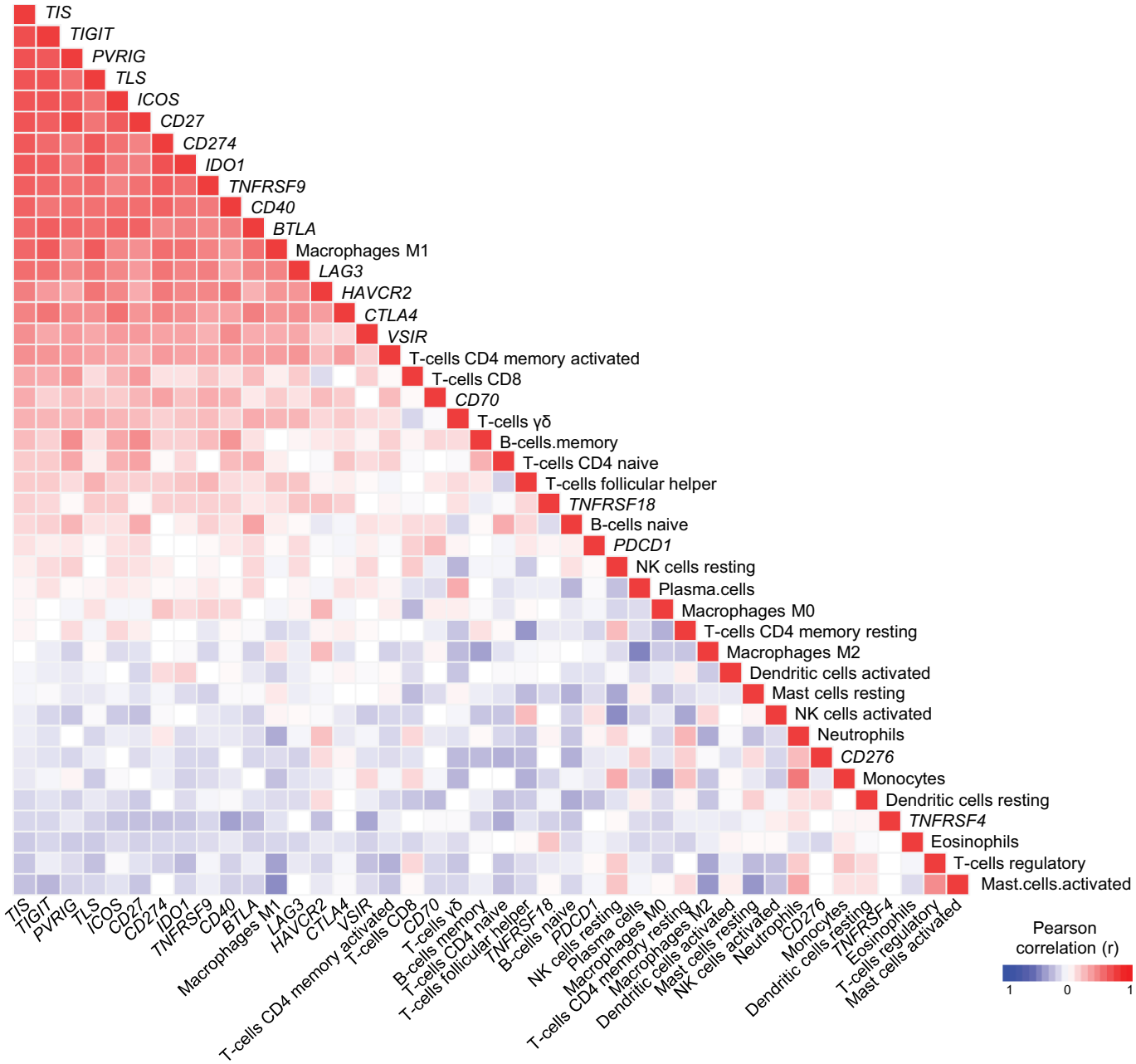

Figure 5. Correlation of immune variables in IBC samples. Matrix of correlation between the TIS metagene score and the absolute percentage of 22 CIBERSORT immune cell types, the TLS metagene score, and the mRNA expression levels of the 18 actionable immune genes in the 137 IBC samples. The correlations coefficients are color-coded according to the color scale below the matrix.

immune cells in ~29% of samples and on tumor cells in ~8% of samples.[80] Its expression correlated with PDL1 expression, as observed in our study, and was associated with longer survival in TN samples. Numerous clinical trials testing combinations of ICIs are ongoing in oncology. In this context, the frequent high co-expression of the actionable immune checkpoints tested in IBC calls for such combinations. Expression of actionable immune checkpoints in IBC was also associated with expression of signatures predictive for response to ICIs such as TIS, and such association has been reported in other cancers sensitive to ICIs such as lung and head and neck cancers and melanoma.[63] In addition to the frequent overexpression of actionable immune checkpoints, these associations clearly support assessment of ICIs in IBC.

We also showed that the extent of immune differences between IBC and non-IBC was inferior to those observed between the molecular subtypes of breast cancer. Such result has already been observed in pan-genomics/transcriptomics studies showing IBC/non-IBC differences much more subtle than the strong differences observed between the molecular subtypes,[81] that result from a combination of numerous and diverse influences including specific molecular alterations, cell-of-origin (stem cell, progenitor or lineage-committed cell), and various regulations such as microRNA expression and stromal

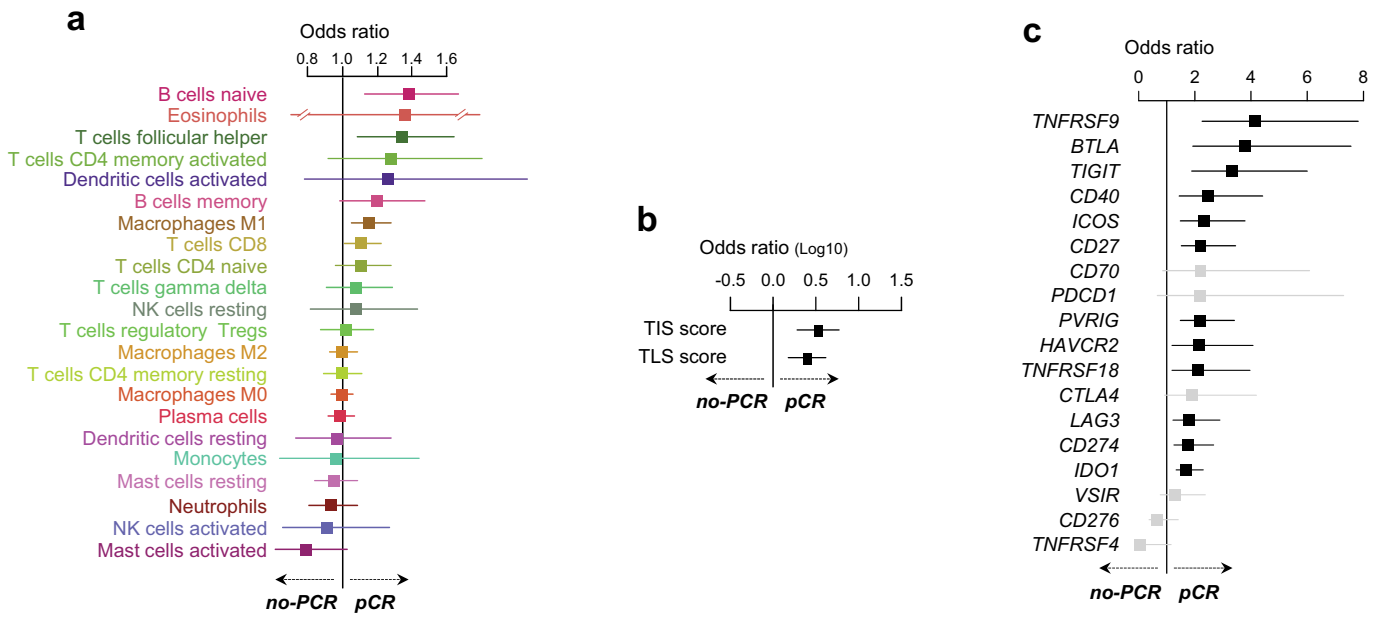

Figure 6. Correlation of immune variables with pathological response to neoadjuvant chemotherapy in IBC samples. *A*/Unadjusted Odds Ratios (boxes and 95%CI) of the absolute percentage of 22 CIBERSORT immune cell types for the pCR *versus* no PCR comparison in the 87 informative IBC samples. *B*/Similar to **A**, but concerns the TIS and TLS metagene scores. *C*/Similar to **A**, but concerns the mRNA expression of the 18 actionable immune genes.

interactions. Interestingly, these IBC/non-IBC immune differences were more numerous within the HR+/HER2- subtype, suggesting more similarities between IBC and non-IBC in the TN subtype, and the differences between the molecular subtypes were less pronounced in IBC than in non-IBC. The reasons for such disparity remain unknown. They may be related to the overall higher immune profile of IBC *versus* non-IBC and of TN *versus* HR+/HER2- subtypes, as if the higher immune response observed in IBC versus non-IBC would make the differences between the molecular subtypes less important in IBC than in non-IBC. Associated with the results of TIS and TLS signatures that might suggest that the differences in response to ICIs should be less marked in IBC than in non-IBC. However, clinical trials only will be able to address this issue.

Finally, we showed that most of the immune variables were associated with a better response to chemotherapy of IBC patients. Regarding the immune cell types, the Tfh cell type was the most strongly associated with higher pCR rate, followed by naïve B-cells, M1 macrophages, and CD8 + T-cells. These results confirm the correlations previously reported in IBC for CD20 + B-cells[45,46] and CD8 + T-cells,[45] and their independent predictive value highlights their biological complementarity for governing the response to chemotherapy. M1 macrophages and naïve B-cells are associated with pCR in ER-positive non-IBC.[65] Tfh cells have been little investigated in solid cancers. They play a role in cooperation with B-cells in organization of TLS and response to chemotherapy:[82] in this context, it is notable that the two cell types most strongly associated with pCR were Tfh and B-cells, as previously reported in ER-negative non-IBC.[66] Because the expression of most of the tested immune checkpoints and high TIL and TLS scores were also associated with a better sensitivity to chemotherapy – based on drugs able to induce the immunogenic cell death[83] -, it is reasonable to hope that IBC patients could be more sensitive to both ICIs and chemotherapy. This observation further feeds the strategy of combining ICIs with chemotherapy in IBC, recently reported as effective in breast cancer.[84]

In conclusion, our study provides the first comprehensive immune portrait of IBC. IBC samples display an immune infiltrate rich in macrophages and CD8 + T-cells, along with frequent high co-expression of genes coding for actionable immune checkpoints and of signatures predictive for response to ICIs (TIS and TLS). Such correlations suggest the existence of a preexisting active immune TME suppressed by the expression of inhibitory checkpoints. Differences with non-IBC include an enrichment in M1 macrophages, γδ T-cells, and memory B-cells, higher expression of predictive signatures, and more frequent overexpression of genes coding for actionable immune checkpoints. IBC displays more similarities than non-IBC with other cancer types classically sensitive to ICIs, such as melanoma or lung cancer. Furthermore, we show more immune differences between IBC and non-IBC in the HR+/HER2_ subtype than in the TN subtype. The strengths of our study include: i) its originality: to our knowledge, it is the first study describing the immune landscape of IBC in terms of specific immune cell types, signatures predictive for response to ICIs and expression of numerous ICIs targets; ii) the relatively high number of samples, allowing us to adjust the IBC/non-IBC comparison upon relevant variables such as the molecular subtypes; and iii) a widely representation of different ethnicity groups from Europe and USA. The two main limitations include the retrospective nature and associated biases, and the use of DNA microarrays that quantify mRNA expression level of both tumor and immune cells; such approach does not provide the protein expression level, nor a spatial information of immune cells that could be obtained by using multicolor immunohistochemistry. Even

if we show good correlation between mRNA and protein expression levels for the immune checkpoint genes tested in breast cancer lines, further validation at the protein level directly on clinical samples remains warranted. But yet, altogether and coupled with higher TMB in IBC, our results suggest higher sensitivity to ICIs in IBC than in non-IBC, and call for assessment in clinical trials. We have recently launched the PELICAN trial, a prospective, multicenter, open-label, randomized, phase II study of pembrolizumab in combination with neoadjuvant chemotherapy in HER2-IBC.[85] To our knowledge, this is the first clinical trial of ICIs in IBC: we focused on the HER2-population because we did not want to include anti-HER2 drugs with pembrolizumab and chemotherapy. But the testing of new ICIs alone or in combination is also warranted and might be prompted by these new data.

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Competing interest

The authors report no conflict of interest.

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