TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Differences in the Immune Response of the Nonmetastatic Axillary Lymph Nodes between Triple-Negative and Luminal A Breast Cancer Surrogate Subtypes

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Breast cancer (BC) comprises four immunohistochemical surrogate subtypes of which triple-negative breast cancer (TNBC) has the highest risk of mortality. Axillary lymph nodes (ALNs) are the regions where BC cells first establish before distant metastasis, and the presence of tumor cells in the ALN causes an immune tolerance profile that contrasts with that of the nonmetastatic ALN (ALN⁻). However, few studies have compared the immune components of the ALNs⁻ in BC subtypes. The present study aimed to determine whether differences between immune populations in the primary tumor and ALNs⁻ were associated with the luminal A or TNBC subtype. We evaluated a retrospective cohort of 144 patients using paraffin-embedded biopsies. The TNBC samples tended to have a higher histologic grade and proliferation index and had higher levels of immune markers compared with luminal A in primary tumors and ALNs⁻. Two methods for validating the multivariate analysis found that histologic grade, intratumoral S100 dendritic cells, and CD8 T lymphocytes and CD57 natural killer cells in the ALNs⁻ were factors associated with TNBC, whereas CD83 dendritic cells in the ALNs⁻ were associated with the luminal A subtype. In conclusion, we found that intratumoral regions and ALNs⁻ of TNBC contained higher concentrations of markers related to immune tolerance than luminal A. This finding partially explains the worse prognosis of patients with TNBC. (*Am J Pathol* 2021, 191: 545–554; <https://doi.org/10.1016/j.ajpath.2020.11.008>)

Cancer is one of the most prominent of all human diseases, and breast cancer (BC) is the most frequent type in women.^{1,2} Four surrogate subtypes of BC with distinct clinical behaviors are recognized: luminal A—like, luminal B—like *HER2*-negative and *HER2*-positive, *HER2*-positive (nonluminal), and triple-negative BC (TNBC).³ These subtypes can be distinguished

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by immunohistochemistry (IHC) by quantifying the expression of estrogen receptor (ER), progesterone receptor (PR), and the *HER2*^{4,5} and by determining tumor proliferation, as measured by Ki-67.⁶ TNBC accounts for 10% to 15% of all BC cases and has a higher risk of mortality and worse prognosis than the other subtypes.^{7,8} In contrast, 60% to 70% of patients with BC present with the luminal A subtype, which has a better prognosis than the other subtypes and tends to relapse later than TNBC (after 5 years versus within 2 to 3 years of first presentation, respectively).⁹

The microenvironment of the tumor, especially that of the immune cell populations, plays a key role in BC progression and patient outcome. Depending on the BC subtype, the immune system may play one of two roles: defending from cancer progression or stimulating tumoral growth, thereby facilitating the establishment and metastasis of cancer.^{10,11} For instance, TNBC in the primary tumor is more immunogenic than the luminal A subtype^{12,13} and expresses a greater variety of cytokine receptors than do luminal BC cells as a consequence of its greater invasion and metastasis rates.¹⁴

In BC, the growing primary tumor can invade nearby tissues and migrate into other regional or distant tissues or organs, where it establishes a new tumor or metastasis, the latter causing most BC deaths. BC tumoral cells are known to escape the primary tumor through blood or lymphatic vessels.¹⁵ In particular, axillary lymph nodes (ALNs) are one of the first regions where BC metastasis becomes established,¹⁶ and infiltration of ALN by tumoral cells is known to be a poor prognosis factor,^{17,18} which has been linked to the BC subtypes in various ways.¹⁹ Although the ALN is particularly important in antitumoral immunity,²⁰ and the presence of tumor in the ALNs causes immune tolerance,^{21–23} little research has been performed on the immune response in the ALNs and less still on nonmetastatic ALNs (ALNs[−]). In a previous study, our group found the immune response of ALNs[−] to be an important factor in ALN metastasis in BC that could be indirectly involved in patient outcome.²⁴ Although the immune response is decisive in tumoral progression,^{10,11} few studies have compared the immune markers in the ALNs[−] from the TNBC and luminal A subtypes.

It is clear that the prognosis, ALN status, and probability of metastatic spread of BC subtypes differ greatly. Moreover, the type of immune response is important in BC development, and the immune response in the ALN may be involved in tumor progression, even in the ALN[−]. Given this evidence, we compared the expression of specific immune populations by IHC in patients with BC diagnosed with luminal A and TNBC in the intratumoral microenvironment and in the ALNs[−] and investigated their association with the BC subtype. To our knowledge, this is the first comparison of the immune response in the ALNs[−] of the two subtypes.

Materials and Methods

Study Design and Participants

A retrospective cohort of patients diagnosed with invasive breast carcinoma of no special type during 2000 to 2008 were

evaluated. Of the 144 patients with BC evaluated, 88 were diagnosed as having luminal A (61%) and 56 as having TNBC (39%) subtypes. Regarding the treatments administered, 12.5% of patients with the luminal A subtype and 9.1% of patients with the TNBC subtype received neoadjuvant therapy, 98.9% of patients with the luminal A subtype and 100% of patients with the TNBC subtype received adjuvant therapy (hormonal therapy and/or chemotherapy), and 80.7% of patients with the luminal A subtype and 85.5% of the patients with TNBC received radiotherapy. Biopsy specimens were obtained from the primary tumors and ALNs[−] to evaluate 11 immune populations. The biopsy specimens were collected from the Tumor Bank of the Pathology Department of the Hospital of Tortosa Verge de la Cinta and the Hospital Joan XXIII of Tarragona (Spain). All the biopsy specimens were reviewed using a new hematoxylin-eosin slide to check tissue availability.

The Ethics Committee of the Hospital Joan XXIII of Tarragona and the Research Committee of Hospital of Tortosa Verge de la Cinta approved this study (reference number 24p/2012). All patients provided written informed consent to participate in the study and for their biopsy specimens and clinical data to be used. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines were followed.

TMA Construction and IHC

Pathologists selected four representative areas to obtain four 2-mm cylinders: two from the primary tumor biopsy specimens (intratumoral region) and two from the central region of ALN[−] biopsy specimens. This gave a total of 576 cylinders (352 from patients with luminal A subtype and 224 from patients with TNBC), which were incorporated into tissue microarrays (TMAs) as previously described.²⁵ A total of 12 TMAs were examined and each TMA block contained 50 cylinders. Eleven slides were sectioned from each TMA, one for each of the 11 immune markers studied, producing a total of 132 slides. TMAs allow the evaluation of a large number of cases, but with the drawback that they are not closely correlated with whole-tissue sections to permit clinical diagnosis. Even so, their use for research purposes is considered adequate,²⁶ and a PubMed search of TMAs in breast cancer yielded >200 articles, which is an indication of their popularity for studying the immune response in BC.^{27,28}

For the immunohistochemical detection in the immune cell populations studied here, the following primary antibodies (Figure 1) were used: T helper lymphocytes (anti-CD4, clone 4B12, Dako, Santa Clara, CA), cytotoxic T lymphocytes (anti-CD8, clone C8/144B, Dako), natural killer (NK) cells (anti-CD57, clone NK1, Zymed, Thermo Fisher Scientific, Waltham, MA), regulatory T cells [anti-forkhead box protein P3 (FOXP3), clone 236A/E7, CNIO, Madrid, Spain], macrophages (anti-CD68, clone KP1, Dako), follicular dendritic cells (DCs) (anti-CD21, clone 1F8, Dako), Langerhans DCs

(anti-CD1a, clone 010, Dako), plasmacytoid DCs (anti-CD123, clone 6H6, eBioscience, San Diego, CA), interdigitant DCs (anti-S100, polyclonal, Leica Microsystems GmbH, Wetzlar, Germany), lysosome-associated membrane glycoprotein 3 (LAMP3) DCs (anti-CD208, polyclonal, Proteintech, Rosemont, IL), and mature DCs (anti-CD83, clone 1H4b, Leica Microsystems GmbH). The final detection of the staining was performed using the ENDAVISION FLEX method (Dako), with the chromogen diaminobenzidine (Dako) as a substrate and hematoxylin as counterstain, in accordance with the manufacturer's protocol.

Slides were scanned at $\times 40$ magnification with the Aperio ScanScope XT scanner (Leica Microsystems GmbH, Nussloch, Germany). TIFF digital images were obtained at a resolution of 0.25 μm per pixel and with an approximate size of 25 GB. This method for obtaining these types of image is known as whole slide imaging and is the most recent imaging modality in the field of pathology.²⁹ To analyze images correctly, each cylinder was individually examined in a single image with automatic tools previously developed by our team.^{30,31} These 6336 individual digital images (11 immune markers $\times$ 576 cylinders) were stored in TIFF format with a size of approximately 500 MB and coded with the case number and name of the corresponding immune marker. Each cylinder area and its respective immune markers were evaluated by digital image analytical procedures, as previously described.^{24,25,32–34}

The following clinical and pathologic data were compiled: age, tumor diameter, number of metastatic lymph nodes, presence of axillar metastasis, lymphovascular invasion, perineural invasion, histologic grade, proliferation index (Ki-67), and menopausal status. These variables were compared in patients with the luminal A and TNBC subtypes.

Statistical Analysis

To evaluate the differences in the mean or median concentrations of the studied immune populations and the differences in the quantitative clinical and pathologic variables between the two BC subtypes, independent-samples *t*-test or the *U*-test were used. Categorical clinical and pathologic variables were examined using the χ^2 or Fisher exact test.

To assess which of the immunologic, clinical, and pathologic variables were associated with each of the studied subtypes (with luminal A as reference), univariate logistic regression models were fitted for each variable to estimate the odds ratio (OR) and 95% CI. These models allowed us to identify the studied variables differentially associated with the TNBC and luminal A subtypes. The variables attaining a level of significance of $P < 0.10$ in the univariate analyses to develop a multivariate logistic regression model. Model goodness of fit was concluded if the Hosmer-Lemeshow test was nonsignificant for all the variables.

Figure 1 Immunohistochemical staining patterns for the immune markers in formalin-fixed, paraffin-embedded sections. Representative examples of membrane (CD4, CD8, CD21), cytoplasmic [CD68, CD123, lysosome-associated membrane glycoprotein (LAMP3)], membrane and/or cytoplasmic (CD57, CD1a, CD83), nuclear and/or cytoplasmic (S100), and nuclear [forkhead box protein P3 (FOXP3)] reactivity of the biomarkers. Original magnification, $\times 40$.

Any quantitative variable that was significant in the univariate analysis that contributed to the goodness of fit of the model was dichotomized according to its distribution: greater than or less than/equal to the mean (normally distributed variable) or median (non-normally distributed variable). As a rule of thumb, a logistic regression should be based on a minimum of 10 events for each variable included in the model.^{35,36} Luminal A was used as the reference group in the analyses, with TNBC as the event. With a final multivariate model that included 4 variables, the sample size of 57 events was therefore considered sufficient. The ability of the model to correctly assign patients to the luminal A or TNBC subtype was evaluated using Nagelkerke's R^2 , the sensitivity and specificity, and the area under the receiver operating characteristic curve (AUC).

The model obtained was validated by two statistical techniques. The first validation was performed with the bootstrapping simulation technique using IBM SPSS Statistics version 23.0 (IBM, Armonk, NY), assuming a 95% CI and using 10,000 samples. The second validation used the Multiple Imputation method of Stata software version 14.0 (StataCorp LLC, College Station, TX). Ten imputed data sets were used to handle missing data, and all the potential predictive variables and outcomes were included in the imputation model. Rubin's rules were used to combine

logistic model estimates and SEs. The AUC was calculated for both validation methods. $P < 0.05$ was considered statistically significant for all analyses.

Results

Patients' Clinical and Pathologic Data

Table 1 summarizes the differences in the clinical and pathologic characteristics between the patients with the luminal A and TNBC subtypes. As expected, patients with TNBC had a significantly greater proportion of higher histologic grades than those in the luminal A group. Similarly, the TNBC group also had a greater percentage of patients with a high level of Ki-67 expression.

Immune Cell Markers

The immune microenvironment of the intratumoral region in the TNBC group had a statistically significantly higher concentration of the CD4, CD8, FOXP3, CD21, CD68, CD1a, CD123, S100, and CD208 markers than the luminal A subtype (Table 2). Nine of the 11 immune populations studied differed between subtypes with respect to concentrations in the intratumoral area. The ALNs[−] had similar

Table 1 Differences in the Clinical and Pathologic Variables between Patients with Luminal A and Triple-Negative Molecular Breast Cancer Profiles

Variable	Luminal A (n = 88)	Triple-negative (n = 56)	P
Age, years	61.1 ± 11.7	58.7 ± 12.0	0.234*
Tumor diameter, mm	18.0 ± 12.0	18.5 ± 19.5	0.959†
Metastatic lymph nodes, n	1.0 (2.0)	0.0 (2.0)	0.625‡
Axillar metastasis			
Positive	46 (52.3)	26 (46.4)	0.608‡
Negative	42 (47.7)	30 (53.6)	
LVI			
Present	42 (47.7)	8 (50.0)	1.000‡
Absent	46 (52.3)	8 (50.0)	
PNI			
Present	28 (31.8)	5 (31.3)	1.000‡
Absent	60 (68.2)	11 (68.8)	
Histologic grade			
1	24 (27.3)	3 (5.4)	<0.001‡
2	45 (51.1)	10 (17.9)	
3	19 (21.6)	43 (76.8)	
Ki-67 grade			
Low	35 (39.8)	26 (47.3)	0.037‡
Medium	35 (39.8)	11 (20.0)	
High	18 (20.5)	18 (32.7)	
Menopausal status			
Premenopausal	13 (15.9)	2 (15.4)	1.000‡
Menopausal	69 (84.1)	11 (84.6)	

Data are expressed as means ± SD for age and tumor diameter, as median (interquartile range) for number of metastatic lymph nodes, and as n (%) for the rest of the data.

*Independent-samples *t*-test.
†*U*-test.
‡The χ^2 or Fisher exact test.
LVI, lymphovascular invasion; PNI, perineural invasion.

results to those of the intratumoral region, with higher concentrations of CD4, CD8, CD57, and CD123 markers in the TNBC group. Conversely, CD83 tended to be present at significantly higher concentrations in the luminal A group than in the TNBC group.

Logistic Regression

The associations of each variable with the tumor subtype estimated in univariate logistic regressions are presented in Table 3. To evaluate which of the variables were independently associated with each subtype, all the variables with an associated $P < 0.10$ in the univariate analyses were considered in the multivariate logistic regression model. The multivariate model (Table 3) indicated that none of the clinical or pathologic factors was independently associated with the TNBC when the immunologic variables were also considered in the analysis. On the other hand, regarding the immune populations, the model found that higher concentrations of intratumoral S100, interdigitant DCs, CD8 cytotoxic T lymphocytes, and CD57 NK cells in the ALNs⁺ were factors independently associated with the TNBC subtype. CD83 mature DCs in the ALNs⁺ were also a protective factor in the TNBC subtype. The Hosmer-Lemeshow test was not significant ($P = 0.839$), indicating a good fit.

Nagelkerke's R^2 for the model was 0.732, which means that approximately three-quarters of the variation between the subtypes may be explained by the immune response of the primary tumor and ALNs⁺. The receiver operating characteristic curve (Figure 2) indicated that the multivariate model had a sensitivity of 81.3% and a specificity of 91.0%. Its AUC was 0.956 (95% CI, 0.916–0.995).

Of the two methods used to validate the multivariate model, the bootstrap simulation produced results similar to those of the unvalidated multivariate model. The S100 immune marker in the intratumoral region had an OR of 2.04 (95% CI, 1.10–3.79; $P = 0.024$). The ORs for the immune markers in the ALNs⁺ were as follows: 1.15 (95% CI, 1.04–1.28; $P = 0.002$) for CD8, 23.23 (95% CI, 4.76–113.30; $P < 0.001$) for CD57, and 0.052 (95% CI, 0.012–0.23; $P < 0.001$) for CD83. The Hosmer-Lemeshow test was not significant ($P = 0.773$), indicating a good fit. The AUC was 0.960 (95% CI, 0.960–0.961) (Figure 2) with a Nagelkerke's R^2 of 0.746. The validation of the multivariate model by multiple imputation confirmed the immune populations of the ALNs⁺ in the unvalidated model: OR = 1.12 (95% CI, 1.04–1.21; $P = 0.004$) for CD8; OR = 15.40 (95% CI, 3.10–76.60; $P = 0.001$) for CD57, fitted as a continuous variable because, after multiple imputation, the Hosmer-Lemeshow test indicated it had a good fit; and 0.19 (95% CI, 0.07–0.51; $P < 0.001$) for CD83. The intratumoral S100 immune population was not significant and was therefore not included in the final model. On the other hand, histologic grade was significant in the validated model: OR = 2.01 (95% CI, 0.21–19.33;

Table 2 Differences in the Concentration of the Immune Populations in the Intratumoral and ALN⁺ Regions between Patients with Luminal A and TNBC Subtypes

Variable	Luminal A (n = 88)	TNBC (n = 56)	P
Intratumoral			
CD4	0.61 (1.11)	3.34 (7.15)	<0.001*
CD8	1.06 (1.53)	1.54 (3.31)	0.050*
CD57	0.10 (0.33)	0.12 (0.24)	0.993*
FOXP3	0.06 (0.13)	0.17 (0.31)	<0.001*
CD21	0.001 (0.027)	0.006 (0.017)	0.021*
CD68	2.63 (2.16)	3.69 (4.46)	<0.001*
CD1a	0.09 (0.20)	0.22 (0.68)	0.009*
CD123	0.000 (0.000)	0.035 (0.099)	<0.001*
S100	0.12 (0.29)	0.32 (0.62)	0.002*
CD208	0.02 (0.08)	0.07 (0.15)	0.015*
CD83	0.11 (0.18)	0.11 (0.26)	0.650*
ALN ⁺			
CD4	56.25 ± 13.30)	63.32 ± 14.24)	0.004 [†]
CD8	13.07 (8.47)	19.45 (8.35)	<0.001*
CD57	0.18 (0.23)	0.76 (0.82)	<0.001*
FOXP3	1.99 (1.42)	2.12 (1.64)	0.927*
CD21	0.70 (1.76)	1.01 (1.17)	0.539*
CD68	9.32 (4.93)	9.91 (6.82)	0.225*
CD1a	1.64 (3.49)	1.76 (3.19)	0.797*
CD123	1.32 (1.84)	2.35 (3.13)	0.001*
S100	4.16 (5.40)	3.95 (4.48)	0.621*
CD208	0.24 (0.41)	0.18 (0.27)	0.112*
CD83	1.07 (1.26)	0.19 (0.59)	<0.001*

Data are expressed as means ± SD or median (interquartile range) of the percentage of positive stained area expressed for each marker.

*U-test.

[†]Independent-samples *t*-test.

ALN⁺, nonmetastatic axillary lymph node; FOXP3, forkhead box protein P3; TNBC, triple-negative breast cancer.

$P = 0.547$) for grade 2 and 12.27 (95% CI, 1.39–108.17; $P = 0.024$) for grade 3. The Hosmer-Lemeshow test indicated a good fit ($P = 0.466$). The model had an AUC of 0.940 (95% CI, 0.898–0.982) (Figure 2) and a Nagelkerke's R^2 of 0.716. Thus, the significant associations in the multivariate model of the three immune populations with TNBC in the ALNs⁺ were confirmed by both validation methods.

Discussion

Most studies in this field have compared metastatic ALNs (ALNs⁺) with primary tumor or with ALNs⁺³⁷; however, not many have compared the immune response in ALNs⁺ of different types of patients with BC.^{24,38} Thus, we evaluated the differences in immune cell markers in the intratumoral region and ALNs⁺ of patients with luminal A and TNBC subtype invasive BCs. We found that the TNBC subtype had more aggressive features, a higher histologic grade, and a higher proliferation index than luminal A as noted in

Table 3 Univariate and Multivariate Analysis of the Differences in Variables in TNBC Compared with Luminal A Subtype

Variable	Univariate OR (95% CI)	P	Multivariate OR (95% CI)	P
Age, years	0.98 (0.95 to 1.01)	0.233		
Tumor diameter, mm	1.00 (0.98 to 1.02)	0.761		
Metastatic lymph nodes, <i>n</i>	1.01 (0.94 to 1.08)	0.837		
Axillar metastasis				
Positive	0.79 (0.40 to 1.55)	0.494		
Negative	1.0			
LVI				
Present	1.10 (0.38 to 3.18)	0.867		
Absent	1.0			
PNI				
Present	0.97 (0.31 to 3.07)	0.964		
Absent	1.0			
Histologic grade				
3	18.11 (4.86 to 67.51)	<0.001		
2	1.78 (0.45 to 7.08)	0.415		
1	1.0			
PI (Ki-67)				
High	1.35 (0.59 to 3.08)	0.481		
Medium	0.42 (0.18 to 0.99)	0.046		
Low	1.0			
Menopausal status				
Postmenopausal	1.04 (0.21 to 5.23)	0.966		
Premenopausal	1.0			
Intratumoral				
CD4	1.26 (1.12 to 1.41)	<0.001		
CD8	1.07 (0.97 to 1.18)	0.181		
CD57	1.00 (0.94 to 1.06)	0.886		
FOXP3	35.60 (4.79 to 264.68)	0.001		
CD21c				
Greater than median	2.50 (1.24 to 5.05)	0.011		
Median or less	1.0			
CD68	1.14 (1.01 to 1.30)	0.034		
CD1a	1.82 (1.06 to 3.14)	0.030		
CD123c		<0.001		
Greater than median	11.55 (5.12 to 26.06)			
Median or less	1.0			
S100	1.93 (1.01 to 3.70)	0.046	2.03 (1.09 to 3.77)	0.026
CD208	3.45 (1.65 to 7.19)	0.001		
Greater than median				
Median or less				
CD83	3.50 (0.97 to 12.63)	0.056		
ALN ⁺				
CD4	1.04 (1.01 to 1.07)	0.005		
CD8	1.09 (1.03 to 1.15)	0.001	1.15 (1.03 to 1.28)	0.011
CD57c		<0.001		<0.001
Greater than median	10.82 (4.69 to 24.92)		22.65 (4.63 to 110.73)	
Median or less	1.0		1.0	
FOXP3	1.06 (0.79 to 1.43)	0.685		
CD21	0.96 (0.80 to 1.15)	0.642		
CD68	1.06 (0.98 to 1.14)	0.151		
CD1a	1.03 (0.93 to 1.14)	0.541		
CD123	1.48 (1.17 to 1.86)	0.001		
S100	0.98 (0.89 to 1.09)	0.752		

(table continues)

Table 3 (continued)

Variable	Univariate OR (95% CI)	P	Multivariate OR (95% CI)	P
CD208	0.42 (0.14 to 1.24)	0.116		
CD83	0.15 (0.07 to 0.32)	<0.001	0.053 (0.012 to 0.239)	<0.001

ALN⁻, nonmetastatic axillary lymph node; FOXP3, forkhead box protein P3; LVI, lymphovascular invasion; OR, odds ratio; PNI, perineural invasion; TNBC, triple-negative breast cancer.

earlier studies.^{7,39–41} Some studies have reported the metastatic status of the ALNs to be more closely associated with the TNBC than the luminal A subtype.^{39,40,42–48} Other authors have noted that TNBC cases have a similar⁴⁹ or a lower⁵⁰ probability of developing ALN metastasis than other subtypes, but we did not find any significant differences in the proportion of ALN⁺ between the two groups. Apart from the histologic differences, the immune response in the tumor is also known to differ according to the BC subtype because the TNBC subtype more strongly activates the immune system than does luminal A.¹² We found clear differences between the subtypes, whereby a significantly higher proportion of stained cells in nine of the 11 immune populations studied were present in the intratumoral region of the TNBC. Similar results were found in the ALNs⁻, where four immune populations had a higher proportion of stained cells in the TNBC group and one was at a higher proportion in the luminal A group. Although fighting cancer is a primary function of the immune cells, cancer promotes immunosuppression; thus, immune cells may shift their action toward tolerance to cancer.^{51,52} Six of the 11 studied markers that were highly expressed in TNBC samples could be directly or indirectly linked to tolerance to cancer. Several studies^{53–57} have suggested that CD123 DCs and FOXP3⁺ cells stimulate tolerance of tumoral cells. Controversies surround the role of CD4 and CD8 in cancer progression, but some studies have reported that they

contribute to a worse outcome.^{58–60} In addition, a high density of CD68⁺ tumor-associated macrophages and S100 expression have been linked to worse outcome.^{61,62} This finding is consistent with our findings and indicates that immune cells in TNBC may become tumor tolerant and promote poor prognosis.

Considering the factors associated with each intrinsic subtype, we found that some immune cell populations in the intratumoral region and in the ALN⁻ were more strongly associated with the subtypes than the well-established pathologic factors. We noted this strong association in prior studies of immune populations associated with the occurrence of metastasis in the ALN.²⁴ Nevertheless, as previously discussed, this association does not mean that immune cell populations are more important than the pathologic factors used for predicting patient outcome, but that they are complementary and could also be associated with patient outcome. This association is evident from the multivariate model in which we found immune populations to be associated only with the BC subtypes, whereas the pathologic factors proved not significant. At the intratumoral level, only higher concentrations of interdigitant S100 DCs were associated with the TNBC subtype. These DCs are derived from bone marrow and located in the T-cell domains of various humans tissues.⁶³ S100 gene expression is more active in the basal than in the nonbasal BC subtype, and although S100 proteins are a diverse group, the expression of most of them is linked to worse outcomes.⁶² Regarding the ALNs⁻, higher concentrations of CD8⁺ T lymphocytes and CD57⁺ NK cells were significantly associated with the TNBC, whereas higher proportions of mature CD83⁺ DCs were associated with the luminal A subtype. In the intratumoral area, tumoral cells directly affect the microenvironment, modulating the immune response, whereas metastasis has not yet been established in the ALNs⁻, so the immune responses are expected to be different. Even so, some studies have provided evidence that the primary tumor alters the immune microenvironment in the ALNs even before establishing metastasis.^{23,38} Hu et al⁶⁴ suggested that NK (CD57) cells are associated with better clinical outcomes in a variety of solid tumors, but they did not include BCs in their study. Although overall survival or relapse-free survival cannot be estimated, as explained earlier, TNBCs are typically more aggressive and have a worse prognosis than luminal A BCs, which makes it difficult to explain the higher proportion of CD57⁺ NK cell; however, it might be related to the increased

Figure 2 Receiver-operating characteristic (ROC) curve for the multivariate logistic regression model prediction.

immunogenicity of TNBCs. Moreover, although the presence of CD57⁺ NK cells indicates a possible better prognosis in solid tumors, the nature of their involvement in the ALNs[−] might not be the same. Indeed, Rezaeifard et al⁶⁵ recently found that the presence of NK lymphocytes in the tumor draining lymphatic nodes was associated with poor prognosis in patients with BC. Similarly, although controversy persists regarding the association of CD8⁺ T lymphocytes with better or worse prognosis,^{58,59,66–68} their presence in TNBC is usually associated with increased patient survival.^{69,70} Some authors have suggested that the differences between subtypes arise because highly proliferative cancers, such as TNBC, develop a stronger immune response.⁷⁰ Accumulation of CD57⁺ and CD8⁺ T cells occurs frequently in individuals with various forms of cancer and has been linked to reduced survival.^{71–75} Our study found an association of CD8⁺ T lymphocytes and CD57⁺ NK cells in the ALNs[−] with the TNBC, possibly because of the greater proliferation of this subtype. This occurrence explains the stronger immunogenicity, which could also have occurred because the ALNs[−] are not sufficiently affected by the immune escape component of the immunoediting process of tumoral growth by that stage. This finding means that neither the immune resistance of tumoral cells nor the down-regulation of effector cells, such as CD8⁺ lymphocytes, that typically occurs in advanced tumors, may have affected the ALNs[−].⁷⁶ In addition, most of the studies of the immune response have focused on primary tumors; therefore, the role of these immune cells in the ALNs, in particular the ALNs[−], might differ.

The expression of mature CD83⁺ DCs is regarded as an independent prognostic marker of survival,⁷⁷ which is consistent with the higher concentration we observed in the ALNs[−] of the luminal A subtype. Overall, our results suggest that the ALN[−] is an active immunogenic region where the immune response to cancer develops. On the other hand, validating our multivariate model by multiple imputation led to the inclusion of histologic grade as an independently significant factor. As previously explained, higher histologic grade may be linked to the greater aggressivity of the TNBC,⁷ although, in our study, the immune markers were closely associated with the BC subtypes. This finding indicates that the BC subtype not only affects these clinical and pathologic parameters but also strongly affects the immune response, particularly in the ALNs[−]. Moreover, S100 (in one of the two validations) in the intratumoral region and the CD8, CD57, and CD83 markers in the ALNs[−] (in both validations) are independent factors associated with the TNBC subtype, which suggests that they may be good predictors of the BC subtype. The ability to predict the BC subtype clearly illustrates how the immune response in the ALNs[−] can be associated with each subtype.

In conclusion, our results indicate that the luminal A and TNBC subtypes clearly differ with respect to their proportions of immune markers, not only in the primary tumor,

as expected, but also in the ALNs[−], an aspect that has not been previously studied. TNBC has a more active immune response, with an emphasis on the markers related to immune tolerance, in the intratumoral region and in the ALNs[−], which might be related to its poorer prognosis and histologic grade. These results are significant because they highlight the importance of the immune response of the ALNs[−] in cancer progression. One of the limitations of IHC is that using single IHC markers for specific immune populations could lead to them being overestimated. Nevertheless, our results clarify the differences in the composition of the immune cells in the ALNs[−]. Further studies are required to fully establish the specific type of the immune cells in the ALN[−] and their role in the survival and relapse of patients with BC.

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Author Contributions

C.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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