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REVIEW ARTICLE

Tumor-infiltrating lymphocytes and ductal carcinoma in situ of the breast: friends or foes?

Marie Colombe Agahozo[1] · Dora Hammerl[2] · Reno Debets[2] · Marleen Kok[3] · Carolien H M van Deurzen[1]

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Abstract

In the past three decades, the detection rate of ductal carcinoma in situ of the breast has dramatically increased due to breast screening programs. As a consequence, about 20% of all breast cancer cases are detected in this early in situ stage. Some ductal carcinoma in situ cases will progress to invasive breast cancer, while other cases are likely to have an indolent biological behavior. The presence of tumor-infiltrating lymphocytes is seen as a promising prognostic and predictive marker in invasive breast cancer, mainly in HER2-positive and triple-negative subtypes. Here, we summarize the current understanding regarding immune infiltrates in invasive breast cancer and highlight recent observations regarding the presence and potential clinical significance of such immune infiltrates in patients with ductal carcinoma in situ. The presence of tumor-infiltrating lymphocytes, their numbers, composition, and potential relationship with genomic status will be discussed. Finally, we propose that a combination of genetic and immune markers may better stratify ductal carcinoma in situ subtypes with respect to tumor evolution.

Introduction

Ductal carcinoma in situ is the most common breast cancer precursor lesion [1]. The implementation of mammography screening in women >40–50 years old has exponentially increased the number of patients diagnosed with ductal carcinoma in situ in the past three decades [1–3]. The majority of patients with ductal carcinoma in situ are treated by surgery, followed by radiotherapy in case of breast conserving surgery [1]. However, it has been estimated that around 40–50% of cases with ductal carcinoma in situ will remain in situ when left untreated [4–6] and some cases even show signs of regression [7, 8]. Consequently, a substantial proportion of patients with ductal carcinoma in situ are currently being overtreated, which results in unnecessary morbidity and costs. Therefore, finding ductal carcinoma in situ progression markers is of clinical importance since this could result in reduction of overtreatment in low-risk patients, while still providing effective treatment for patients with a high progression risk.

In recent years, it has generally been accepted that tumor-infiltrating lymphocytes play a role as prognostic and predictive markers in invasive breast cancer [9–12]. However, with regard to ductal carcinoma in situ, data on the presence, composition, and clinical significance of immune infiltrate is limited. Since ductal carcinoma in situ is an early stage of disease, increased knowledge of the role of the immune response in ductal carcinoma in situ progression could have major clinical consequences, as it might form the basis for future immune-modulation and potential prevention of progression to invasive breast cancer. This review summarizes current knowledge regarding the role of the immune response in invasive breast cancer and highlights current studies concerning the presence and potential clinical significance of the immune infiltrate in patients with ductal carcinoma in situ.

Immune response and cancer

The interplay between cancer and the immune system is seen as one of the most promising areas with respect to the

✉ Marie Colombe Agahozo, MSc
m.agahozo@erasmusmc.nl

[1] Department of Pathology, Erasmus MC Cancer Institute, Rotterdam, The Netherlands

[2] Department of Medical Oncology, Laboratory of Tumor Immunology, Erasmus MC Cancer Institute, Rotterdam, The Netherlands

[3] Departments of Medical Oncology and Immunology, Netherlands Cancer Institute, Amsterdam, The Netherlands

development of novel anti-cancer treatments [13–16]. Emerging therapies, such as immune checkpoint inhibitors have provided encouraging results, especially in the treatment of immunogenic tumor types, such as advanced melanoma and non-small cell lung cancer [17, 18]. For several immune cell subsets, a potential role in tumor evolution has been described. Table 1 provides a summary of these immune cell subpopulations, including their general proposed effect on tumor progression or control. The interplay between cancer and the immune system was summarized by Dunn and colleagues as a process of cancer immune editing, which consists of three stages [14]. In the first stage, immune cells constantly survey the environment and suppress the outgrowth of dysplastic cells. During this process, neoplastic cells are recognized as foreign, which elicits a pro-inflammatory immune response. This antitumor immune response is generally characterized by infiltration of type 1 macrophages, dendritic cells, natural killer cells, $CD8^+$ cytotoxic T cells and CD4+ T helper 1 cells [19–23]. With the help of pro-inflammatory chemokines and cytokines, these immune cells are recruited toward the tumor. In case of $CD8^+$ T cells, they are capable of recognizing tumor antigens presented on the surface of tumor cells, which might result in tumor cell lysis and elimination [22]. In this stage of carcinogenesis, the immune system is able to prevent the outgrowth of neoplastic cells. However, neoplastic cells can persist during the second stage, in which initially immunogenic tumor cells are maintained in a stage of latency. In the final stage of immunoediting, tumor cells are able to escape the immune control as genetic instability and tumor heterogeneity progresses. This stage is characterized by low antigenicity, low numbers of immune effector cells, high numbers of immune suppressor cells and expression of immune checkpoints [24, 25]. In this stage, the immune profile has a more anti-inflammatory profile and predominantly consists of myeloid-derived suppressor cells, CD4+FOXP3+ regulatory T cells and type 2 macrophages [15]. The exact role of myeloid-derived suppressor cells in this context, which includes a heterogeneous cell population, is yet to be established [26]. Nevertheless, there is evidence that myeloid-derived suppressor cells down-tune an effective immune response through the production of several immune suppressive factors such as arginase and indolamina-2,3-dioxygenase [27, 28].

CD4+FOXP3+ regulatory T cells and type 2 macrophages are able to inhibit $CD8^+$ T cells and stimulate apoptosis of $CD8^+$ T cells, partly by upregulating co-inhibitory ligands such as programmed cell death-1/2 ligand (PD-L1/2) that bind programmed cell death-1 [15, 19–21, 23].

Next to the tumor phenotypes described above, there are also tumor phenotypes without a visible cancer–immune interaction [29]. These immune-deserted or immune-excluded phenotypes result from downregulated immunogenicity or a specific chemokine and cytokine profile.

Altogether, tumor-associated immune cells can have both positive and negative effects on cancer progression, which has been observed in numerous types of cancer, including invasive breast cancer [15, 30, 31].

Invasive breast cancer immune response

Breast cancer is a heterogeneous disease that can be divided in several molecular subtypes with distinct biological behavior and prognosis [32]. Each of these subtypes, which are based on gene-expression studies, has an immunohistochemical surrogate: luminal A (ER+ and PR+/HER2-, low Ki67 index), luminal B (ER+, HER2-, PR- or low and/or high Ki67 index or ER+HER2+ with any PR expression and Ki67 index), triple negative (ER-, PR-, and HER2-) and HER2-overexpressed (ER-, PR-, and HER2+) [33]. Several studies reported that these subtypes also differ immunologically; dense immune infiltration for instance has mainly been associated with high histological grade, triple negative and HER2+ subtypes [12, 34–37]. The latter might be explained by the relatively high mutational load that is associated with these subtypes, compared to luminal subtypes [38, 39]. However, within HER2+ and triple negative subtypes, a high mutational load is not associated with high levels of immune gene expression [40, 41].

Immune infiltrates in invasive breast cancer: prognostic markers

There is extensive literature with respect to tumor-infiltrating lymphocytes as a prognostic marker for invasive breast cancer [12, 34]. One of the first large studies investigating tumor-infiltrating lymphocytes in invasive breast cancer included over 1900 patients with a follow-up time of 14 years. The density of tumor-infiltrating lymphocytes was scored based on hematoxylin and eosin staining. These studies reported a strong association between density of tumor-infiltrating lymphocytes and improved breast cancer-specific survival in young patients (<40 years) (p value <0.001) [36]. This effect was consistent with other studies, which reported an association between dense infiltrates of tumor-infiltrating lymphocytes and high grade and HER2+ invasive breast cancer, irrespective of ER status [35, 37]. Studies using adjuvant chemotherapy in cohorts of over 2000 and 935 patients, respectively, reported that an increase in the number of tumor-infiltrating lymphocytes significantly correlated with decreased risk of both local recurrence and overall survival [41, 42]. For every 10% increase in the number of tumor-infiltrating lymphocytes, a 15–17% decreased risks for local

Table 1 Tumor-associated immune cells and their proposed mechanism of action during tumor growth

Immune cell	Immune subsets	Differentiation markers	Effector enzymes, cytokines, and chemokines	Receptor—ligand interactions	Proposed mechanism of action	Effect on tumor growth
T cell	Cytotoxic T cell	CD8	Granzyme, perforin and interferon gamma	FAS ligand - FAS and TNF related apoptosis inducing ligand—TNF-related apoptosis indused receptor	Activates tumor cell lysis	Suppression
	T helper cell 1	CD4 CCR1	Granzyme, perforin, interferon gamma tumor necrosis factor alpha and nitric oxide synthase	Not applicable	Activates cytotoxic T cell	Suppression
	T helper cell 2	CD4 CCR3	Interleukine-4 and 10	Not applicable	Not applicable	Stimulation
	Folical T helper cell	CD4	CXCL13	Not applicable	Mediation of B cell activation	Suppression
	Regulatory T cell	CD4 FOXP3	Adenosine and Interleukine-10	Receptor activator of nuclear factor kappab ligand—receptor activator of nuclear factor kappaB	Stimulates tumor cells, suppresses cytotoxic T cell, T helper 1, type 1 macrophage and dendritic cells	Stimulation
B cell	Not applicable	CD19 CD20	Tumor necrosis factor alpha, interleukin-6, lymphotoxin α1–β2	Antitumor antibodies, development of tertiary lymph node structures	Activation T cells through antigen presentation	Suppression
	Regulatory B cell	CD19 CD20	Interleukin-10 and 35	Complement system	Increased regulatory T cells and decreased inflammation	Stimulation
Natural killer cells	Not applicable	CD16 or CD56	Not applicable	Not applicable	Activate tumor cell lysis and enhanced T helper 1 activation	Suppression
Macrophages	Type 1 macrophage	CD68	Granzyme, perforin, interferon gamma tumor necrosis factor alpha and nitric oxide synthase	Fragment crystallizable receptor	Enhanced T helper 1 activation and antigen presentation	Suppression
	Type 2 macrophage	CD163	Vascular endothelial growth factor, epidermal growth factor, basic fibroblast growth factor, transforming growth factor beta, interleukin-10 and 4, matrix metalloproteinase 2 and 9	Programed cell death ligand 1—programed cell death receptor 1, and B7-1/2—cytotoxic T-lymphocyte-associated antigen	Stimulates tumor cells, Suppresses $CD8^+$ T cells, T helper 1, type 1 macrophage and dendritic cells	Stimulation
Myeloid-derived supressor cells	Not applicable	CD11b CD33	Arginase, indoleamine 2,3-dioxygenase, reactove oxygen species and reactive nitrogen species	Not applicable	Suppresses cytotoxic T cells and natural killer cells	Stimulation
Dendritic cells	Not applicable	B7, CD1a/c	Interleukin-6	Programed cell death ligand 1—programed cell death receptor 1, and B7-1/2—cytotoxic T-lymphocyte-associated antigen	Activation T cells through antigen presentation	Suppression

recurrence and a 17–27% reduced risk of death was reported in ER−HER2− invasive breast cancer. With respect to HER2+ invasive breast cancer, high densities of tumor-infiltrating lymphocytes are associated with increased response to adjuvant trastuzumab [42]. This was later confirmed by a large prospective study of over 1200 patients with HER2+ invasive breast cancer, using transcriptome analysis [43]. However, some other studies did not find an association between tumor-infiltrating lymphocytes and improved outcome, or even reported the opposite effect [12, 34]. A recent meta-analysis, including microarray-based gene-expression data, evaluated the prognostic value of genes associated with tumor-infiltrating lymphocytes in over 1000 patients. They reported an ER-dependent association between tumor-infiltrating lymphocytes and outcome; high numbers of tumor-infiltrating lymphocytes in ER− tumors correlated with improved survival while high numbers of tumor-infiltrating lymphocytes in ER+ tumors were associated with decreased survival [44]. These conflicting results might partly be explained by different definitions and scoring methods of tumor-infiltrating lymphocytes. An international working group therefore defined a standardized methodology to evaluate tumor-infiltrating lymphocytes in invasive breast cancer, in order to improve consistency and reproducibility in the measurement of tumor-infiltrating lymphocytes for future studies [45].

Tertiary lymphocyte structures have also been observed in invasive breast cancer. These structures contain a T cell zone with dendritic cells, a germinal center with proliferating B cells and high endothelial venules [46]. In line with tumor-infiltrating lymphocytes, high numbers of tertiary lymphocyte structures are associated high grade, ER −/HER2+ invasive breast cancer [47]. Furthermore, high numbers of tertiary lymphocyte structures are reported to be associated with improved outcome in invasive breast cancer [48, 49], specifically in triple negative breast cancer [50, 51]. The latter study also found an association between high numbers of tertiary lymphocyte structures and high numbers of tumor-infiltrating lymphocytes [51]. The prognostic effect of tumor-infiltrating lymphocytes correlated with the presence of tertiary lymphocyte structures: patients with high levels of tumor-infiltrating lymphocytes and high levels of tertiary lymphocyte structures had a longer disease-free survival compared to those with high numbers of tumor-infiltrating lymphocytes but low tertiary lymphocyte structures.

Since tumor-infiltrating lymphocytes consist of different cell types, this prognostic role might also depend on the specific immune cell composition.

Generally, $CD8^+$ T cells have been associated with favorable clinical outcome in ER− invasive breast cancer [52–56]. Regarding ER+ invasive breast cancer, the numbers of CD8+ T cells are generally lower, which makes the prognostic effect of these cells less evident [52, 53, 56]. Besides CD8+ T cells, CD4+ follicular helper T cells and B cells have also been reported to have an anti-tumorigenic effect [21]. CD4+ follicular helper T cells have been suggested to function as mediators of B cell activation and were also linked to improved survival in HER2+ invasive breast cancer [21]. Concerning B cells, high numbers have been reported to be associated with improved breast cancer-specific survival [57, 58]. Nevertheless, no significant or the opposite effect has also been reported [34, 59, 60]. This could be explained by the fact that B cells can be differently activated via a T cell dependent or T cell independent pathway [21].

The presence of CD4+FOXP3+ regulatory T cells has generally been associated with poor clinical outcome, possibly by facilitating tumor growth though immune suppressing properties [12, 15, 19–21, 34, 61]. To our knowledge, Bates and colleagues were the first to correlate high CD4+FOXP3+ regulatory T cell infiltration to poor prognosis [62]. This was consistent with other studies, although these studies concluded that this effect was only true for ER+ invasive breast cancer [31, 56, 63, 64]. In ER − invasive breast cancer, high CD4+FOXP3+ regulatory T cell infiltration was associated with improved prognosis [65]. A recent analysis of over 7200 invasive breast cancer samples reported an association between increased gene expression related to CD4+FOXP3+ regulatory T cell infiltration and improved outcome in HER2+ invasive breast cancer, regardless of ER status [31].

Tumor-associated macrophages have also been associated with negative clinical outcome [34, 64]. Tumor-associated macrophages are believed to skew into a more type 2 macrophage phenotype, once they arrive at the tumor site, which in turn is believed to cause this negative prognostic effect [66–69]. This is consistent with the results of Ali et al., who performed gene-expression analysis of over 10.000 invasive breast cancer patients and concluded that gene-expression reflecting type 2 macrophages was associated with negative prognosis in ER− invasive breast cancer [70]. A more recent similar study including over 7200 invasive breast cancer patients reported no association between gene-expression reflecting type 2 macrophages and prognosis, but rather found that undifferentiated macrophages were associated with unfavorable outcome [31].

Additionally, it has been reported that myeloid-derived suppressor cells are associated with poor prognosis in invasive breast cancer [27]. In this study, myeloid-derived suppressor cells were isolated from fresh frozen breast tumor tissue. High numbers of myeloid-derived suppressor cells were associated with increased numbers of FOXP3+ regulatory T cells and lymph node metastases.

Table 2 Overview of studies investigating the presence and/or potential significance of ductal carcinoma in situ-associated immune infiltrates

Year	Author	Number of patients	Cohort	Whole section vs tissue microarray	Immune cells	Immunohistochemical markers	Additional assays	Immune cells and histopathological features	Clinical outcome
1997	Lee	41	Ductal carcinoma in situ	Whole section	Cytotoxic T cells, regulatory T cells, B cells and tumor-associated macrophages	CD3, CD8, FOXP3, CD20, CD68	Not applicable	Clustered immune pattern (high numbers of tumor-infiltrating lymphocytes and low numbers of tumor-associated macrophagess) is associated with high-grade ductal carcinoma in situ and HER2+ subtype	Not applicable
2010	Sharma	285	Ductal carcinoma in situ	Tissue microarray	Tumor-associated macrophages	CD138, CD163, MMP11	Gene-expression analysis	Macrophage response signature is associated with high grade, ER−/PR− ductal carcinoma in situ	Not applicable
2015	Knopfelmacher	46	Ductal carcinoma in situ	Whole section	Tumor infiltrating lymphocytes	Not applicable	Gene-expression analysis	High numbers of tumor-infiltrating lymphocytes is associated with a high oncotype DX ductal carcinoma in situ score	Not applicable
2016	Morita	82	Ductal carcinoma in situ	Whole section and Tissue microarray	Cytotoxic T cells	CD8	Not applicable	High numbers of tumor-infiltrating lymphocytes is associated with high numbers of CD8+ T cells, high grade, presence of comedonecrosis, HER2+, triple negative subtype and spontaneous "healing"	Not applicable
2016	Pruneri	1488	Ductal carcinoma in situ	Whole section	Tumor infiltrating lymphocytes	Not applicable	Not applicable	High numbers of tumor-infiltrating lymphocytes is associated with high grade, presence of comedonecrosis and HER2+ subtype	Tumor infiltrating lymphocytes number is not associated with ipsilateral recurrence
2016	Thompson	27	Ductal carcinoma in situ with invasive breast cancer[a]	Tissue microarray	T helper cells, cytotoxic T cells, regulatory T cells and B cells	CD3, CD4, CD8, FOXP3, CD20, PD-L1	Not applicable	Not applicable	Tumor infiltrating lymphocytes number is not associated with ipsilateral recurrence
2016	Semeraro	199	Ductal carcinoma in situ	Not applicable	Cytotoxic T cells and regulatory T cells	CD8 and FOXP3	Not applicable	High numbers of CD8+ and FOXP3+ cells and high ratio of CD8+/FOXP3+ is associated with large tumor cell diameter	Low numbers of CD8+ cells and low CD8+/FOXP3+ ratio was associated with ipsilateral recurrence
2016	Kim	177		Whole section	Tumor infiltrating lymphocytes and	Not applicable	not applicable	High numbers of tumor-infiltrating lymphocytes and	Not applicable

Table 2 (continued)

Year	Author	Number of patients	Cohort	Whole section vs tissue microarray	Immune cells	Immunohistochemical markers	Additional assays	Immune cells and histopathological features	Clinical outcome
			Ductal carcinoma in situ		tertiary lymphoid structures			tertiary lymphoid structuress is associated with HER2+ and triple negative ductal carcinoma in situ	
2017	Miligy	36	Ductal carcinoma in situ	Whole section	B cells, plasma cells and tertiary lymphoid structures	CD20,CD19 and CD138	not applicable	High numbers of B cells and tertiary lymphoid structuress is associated with larger tumor size, HER2+ and ER−/PR− ductal carcinoma in situ	High numbers of B cells are associated with shorter recurrence free survival
2017	Campbell	117	Ductal carcinoma in situ	Whole section	T helper cells, cytotoxic T cells, regulatory T cells, B cells and tumor-associated macrophages	CD4, CD8, FOXP3, CD20, CD68, CD115, HLA-DR,HLA-DP, HLA-DQ, MRC1 and PCNA	Not applicable	CD68+Mac387+ and CD115 + macrophages are associated with high VNPI, high grade, presence of comedonecrosis and ER−/PR− ductal carcinoma in situ CD8+ or HLADR+ cells are associated with low-grade ductal carcinoma in situ and absence of comedonecrosis	Low number of CD8 +HLADR+ cells is associated with high risk of ipsilateral recurrence. High number of CD8 +HLADR+ cells in combination with low numbers of CD8 +HLADR- or CD115+ cells is associated with low risk of ipsilateral recurrence
2017	Hendry	138	Ductal carcinoma in situ	Tissue microarray	Tumor infiltrating lymphocytes	PD-L1	Copy number variation and mutation analysis	High numbers of tumor-infiltrating lymphocytes and PD-L1+ cells is associated with high grade, HER2+ and ER− ductal carcinoma in situ high numbers of tumor-infiltrating lymphocytes is associated with the presence of comedonecrosis. The number of tumor-infiltrating lymphocytes is higher in cases with high chromosomal copy number variation and TP53 mutation compared to cases with low chromosomal copy number variation, PIK3CA and GATA3 mutation	Not applicable

[a]Majority of cases are pure ductal carcinoma in situ (24), three cases of ductal carcinoma in situ and adjacent invasive breast cancer

Immune infiltrates in invasive breast cancer: predictive markers

Apart from the prognostic associations mentioned above, there is also evidence for a predictive value of quantity and composition of the immune infiltrate [12, 21, 71]. Several studies reported an association between a high density of tumor-infiltrating lymphocytes and favorable therapy response [41–43, 72, 73]. This was demonstrated in a study of over 1000 patients treated with neoadjuvant chemotherapy [72]. Patients with high numbers of tumor-infiltrating lymphocytes (50% or more of the tumor area occupied by tumor-infiltrating lymphocytes) had a higher pathologic complete response rate compared to those with limited infiltration of tumor-infiltrating lymphocytes (p value = 0.001). This effect was only seen in triple negative and HER2+ invasive breast cancer subtypes, and was independent of other markers.

Several studies suggested that the predictive value of tumor-infiltrating lymphocytes depends on the exact composition of the infiltrate. Patients who reached a pathological complete response after neoadjuvant chemotherapy had high baseline levels of CD4+FOXP3+ regulatory T cells and CD8+ T cells in the pre-treatment needle biopsy [74–76]. Both CD8+ T cells and FOXP3+ regulatory T cells numbers were reported to be predictors of a pathological complete response in triple negative or HER2+ invasive breast cancer. Interestingly, the association of CD4+FOXP3+ regulatory T cells with pathological complete response seemed to depend on the presence of CD8+ T cells. This suggests that the previously observed effect of CD4+FOXP3+ regulatory T cells may be caused by CD8+ T cells solely [76]. CD4+ follicular T helper cells have also been reported to be associated with higher levels of pathologic complete response and favorable disease-free survival after neoadjuvant chemotherapy [48]. With regard to tumor-associated macrophages, type 1 macrophages have been associated with higher pathological complete response rates in ER+ invasive breast cancer, irrespective of HER2 status [31].

Ductal carcinoma in situ immune response

A gradual increase in the number of immune cells during the progression from normal breast tissue to invasive breast cancer has been reported [62, 77, 78]. The highest density of immune cells was observed in invasive breast cancer, however the sharpest difference in immune cell density was observed between the adjacent normal breast tissue and ductal carcinoma in situ. In other words, the immune cells are already present in the in situ stage of breast cancer development. In studies exclusively incorporating pure ductal carcinoma in situ (without adjacent invasive breast cancer), the presence of tumor-infiltrating lymphocytes was reported two decades ago [79]. However, this was only based on hematoxylin and eosin stainings; detailed analyses regarding the exact composition of these immune cells has not been investigated until very recently [80–83]. This area of research is growing and studies are shifting from a more descriptive perspective to correlation with clinical outcome. Table 2 provides an overview of studies regarding ductal carcinoma in situ-associated immune infiltrates.

Histopathologic and genetic features associated with ductal carcinoma in situ immune infiltrate

In line with invasive breast cancer, ductal carcinoma in situ can also be subdivided by histological grade or surrogate subtypes based on ER, PR, HER2 status and the expression of Ki67 [84]. Lee et al. reported an association between dense immune infiltrates, poor differentiation, and HER2 amplification [77]. Larger studies confirmed this finding of an association between high grade, generally ER− and/or HER2+ ductal carcinoma in situ with dense immune infiltrates [6, 80, 81, 85–88]. In the largest study published regarding this subject ($n = 1400$ patients with pure ductal carcinoma in situ), Pruneri et al. reported that 6.5% of all ductal carcinoma in situ cases were associated with dense infiltrates of peri-ductal tumor-infiltrating lymphocytes. The ductal carcinoma in situ subtype that was most frequently associated with high number of tumor-infiltrating lymphocytes was ER−HER2+ (23.6% of 254 patients), followed by ER−HER2− (11.1% of 63 patients) and ER+/HER2+ (8.9% of 258 patients) [80]. Figure 1 provides an example of ductal carcinoma in situ with a high and low density of tumor-infiltrating lymphocytes (A and B, respectively).

Some studies also assessed the presence of tertiary lymphoid structures in ductal carcinoma in situ [86, 88]. Both of these studies reported that high levels of tertiary lymphoid structures were significantly associated with high-grade ductal carcinoma in situ and the presence of comedonecrosis. Regarding surrogate ductal carcinoma in situ subtypes, the number of tertiary lymphoid structures was higher in ER−HER2+ and triple negative subtypes compared to ER+HER2+ and ER+HER2− subtypes. This might be explained by the high correlation between the presence of tertiary lymphoid structures and tumor-infiltrating lymphocytes.

The ductal carcinoma in situ-associated immune response has recently also been linked to genetic features of the tumor cells and the expression of PD-L1 [87]. Hendry et al. assessed the presence of tumor-infiltrating lymphocytes and the expression of PD-L1 in a series of 138 patients with ductal carcinoma in situ. The presence of tumor-infiltrating lymphocytes and PD-L1 was then further

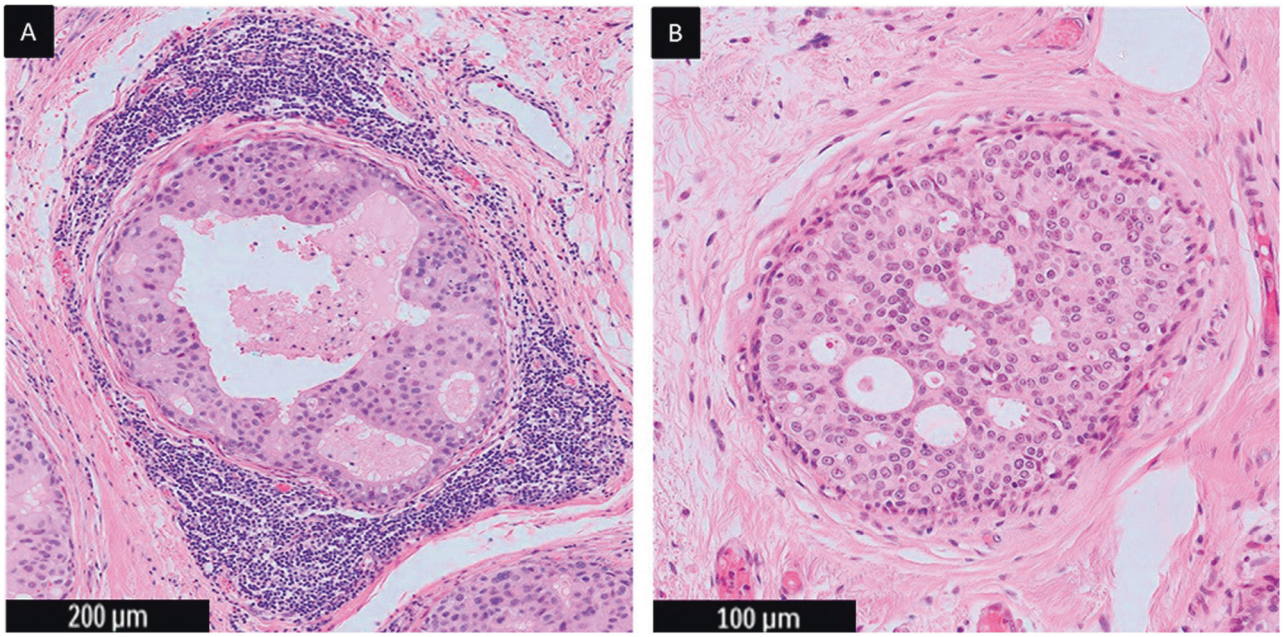

Fig. 1 Example of a ductal carcinoma in situ case with a high (**a**) and low density (**b**) of tumor-infiltrating lymphocytes (hematoxylin and eosin staining; original magnification x5 (**a**) and x10 (**b**))

associated with genetic features including copy number variation and TP53, GATA3, or PIK3CA mutation data. They reported PD-L1 expression on 11% of the tumor cells and 25% of the immune cells, while Thompson et al. [82] reported no PD-L1 expression on the ductal carcinoma in situ cells, but 81% of the ductal carcinoma in situ-associated tumor-infiltrating lymphocytes, in a series of 23 cases. With regard to genetic features, copy number variation was only available for 55 cases of ductal carcinoma in situ [87]. There was a positive correlation between the number of tumor-infiltrating lymphocytes and the fraction of the genome altered by copy number variation and number of telomeric allelic imbalance. Cases with low numbers of tumor-infiltrating lymphocytes had a significantly lower fraction of the genome altered by copy number variation and a lower number of telomeric allelic imbalances. Additionally, patients with a TP53 mutation had significantly higher numbers of tumor-infiltrating lymphocytes compared to patients with a PIK3CA or a GATA3 mutation. In this analysis, ER and HER2 status was not considered. However, since these mutations are associated with ER and HER2 status of invasive breast cancer, this is also likely to be the case in ductal carcinoma in situ [40, 89]. These data suggest that the ductal carcinoma in situ-associated immune response is associated with ductal carcinoma in situ subtype, which is in line with invasive breast cancer studies [35, 37, 61, 90]; high numbers of tumor-infiltrating lymphocytes are mainly seen in ductal carcinoma in situ cases with high grade, ER− and/or HER2+ status and genomic imbalance.

Characterization of the composition of the ductal carcinoma in situ-associated immune infiltrate

The first detailed description of the ductal carcinoma in situ-associated immune infiltrate included a series of 41 patients with ductal carcinoma in situ [79]. The authors reported two architectural patterns of ductal carcinoma in situ-associated inflammation; a clustered (defined as patchy) or a diffuse pattern. The infiltrate density was semi-quantified as absent/minimal, mild, moderate, or marked. The clustered pattern primarily consisted of B cells in the center, surrounded by CD8+ T cells. By contrast, the diffuse pattern consisted of very few numbers of B cells and high(er) numbers of tumor-associated macrophages and T cells.

Sharma et al. examined the presence of a stromal-induced response of tumor-associated macrophages, in a set of 40 cases of ductal carcinoma in situ, using publicly available data of 112 genes [91]. These genes were reported to be involved in a tumor-associated macrophages response to colony-stimulating factor in invasive breast cancer. The majority of these genes, 100 out of 112, were also expressed in ductal carcinoma in situ. The presence of this tumor-associated macrophages signature gene response was associated with high grade and ER−PR− ductal carcinoma in situ.

Additional analysis were performed in a larger series, including 117 patients with pure ductal carcinoma in situ [81]. In this study, the overall number of tumor-infiltrating lymphocytes was significantly higher in high-grade ductal carcinoma in situ compared to non-high-grade ductal

carcinoma in situ. In-depth analyses of the infiltrate showed higher percentages of FOXP3+, CD68+, and CD68 +/−PCNA+ tumor-associated macrophages (proliferating macrophages), HLA−DR+ cells, CD4+ T cells, and CD20 + B cells in high-grade ductal carcinoma in situ. CD68+ and/or Mac387+ tumor-associated macrophages (reactive/ infiltrating macrophages) were associated with a high Van Nuys Prognostic Index, which is an index used to estimate the ductal carcinoma in situ progression risk. These cells were also associated with palpability, high grade, presence of comedonecrosis, ER and PR negativity. On the other hand, CD68+MRC1+ (type 2 macrophages) tumor-associated macrophages did not have any correlation with clinicopathological features. CD8+ or HLA-DR+ cells were associated with low-grade ductal carcinoma in situ, absence of comedonecrosis and low risk of local recurrence [81].

The number of B cells and plasma cells have also been associated to clinicopathological features by Miligy et al. [86]. In this study, whole tissue sections of 36 patients with pure ductal carcinoma in situ were immunostained for CD19, CD20, and CD138. They reported an association of high numbers of tertiary lymphocyte structures and stromal B cells with larger tumor size, ER/PR negativity, and HER2 positivity [86]. There was no association between the number of plasma cells and clinicopathological features.

Recently, Gil Del Alcazar et al. analyzed the composition of immune cells in normal breast tissue, ductal carcinoma in situ, and invasive breast cancer [92], using flow cytometry and RNA sequencing. In this study, ductal carcinoma in situ-associated T cells were enriched for CD8+ and undifferentiated/naïve CD4+Th17 compared to invasive breast cancer, whereas the cases of invasive breast cancer had more activated CD4+ T cells and regulatory T cells compared to ductal carcinoma in situ. The cytotoxic T lymphocyte antigen 4 (checkpoint inhibitor) pathway was upregulated in invasive breast cancer compared to ductal carcinoma in situ, while the interleukin-4 signaling pathway was downregulated in the invasive component. Besides, ductal carcinoma in situ-associated T cells appeared to be in a relatively activated state. In this study, the T cell activation state was based on the expression of granzyme B and Ki67. Overall, these results suggest that there is an immune escape during the progression from ductal carcinoma in situ to invasive breast cancer.

Ductal carcinoma in situ-associated immune infiltrate and ductal carcinoma in situ evolution

One of the potential ways of ductal carcinoma in situ evolution is regression. This concept of regression, also referred to as “spontaneous healing”, is a process in which changes in the stromal structure somewhat indulge the neoplastic cells [8, 85]. This process has been associated with the presence of dense immune infiltrates. In a cohort study of 82 pure ductal carcinoma in situ patients, 32 showed signs of spontaneous healing [85]. The majority of these cases with “healing” were ER+ and HER2− (73.2%), followed by HER2+ (23.2%) and triple negative (3.6%), respectively. The presence of CD8+ T cells was associated with spontaneous healing. These CD8+ T cells were also reported to be predictive for the risk of local recurrence [81, 93]. Semeraro et al. reported an association between low numbers of CD8+ T cells or a low CD8+/FOXP3+ T cell ratio and local recurrence in a cohort of 199 patients with ductal carcinoma in situ [93]. In-depth analysis by Campbell et al. provided a series of immune cell subsets predictive for local recurrence [81]. The number of CD8+HLADR+ (activated), CD8+HLA−DR− T cells (non-activated) and CD115+ cells, expressed on monocytes/macrophages and dendritic cells, predicted the risk of local recurrence, with an accuracy of 87% (sensitivity = 76%, specificity = 89%). Patients with low numbers of CD8+HLA-DR+ cells had the highest risk of local recurrence, regardless of the numbers of CD8+HLA−DR− and CD115+ cells. The lowest risk for local recurrence was observed in cases with high numbers of CD8+HLA−DR+ cells and low numbers of CD8+HLA−DR− and CD115+ cells. Therefore, low risk for local recurrence seems to be catalyzed by CD8+HLA −DR+ T cells, which argues that CD8+ T cells need to be activated in order to be effective.

Knopfelmacher et al. reported an association between high numbers of ductal carcinoma in situ-associated TILs and a high oncotype DX ductal carcinoma in situ recurrence score (a gene expression score which is clinically used to select patients with a high risk of local recurrence) [94]. However, in this study, a high oncotype DX score was also significantly associated with high grade.

Recently, Miligy et al. reported a significant association between the number of stromal B cells and recurrence free survival in ductal carcinoma in situ [86]. The authors defined five locations of B cells and plasma cells in relation to the lesion. There was no significant association between the number of plasma cells and recurrence free survival, regardless of their location. However, they reported a significant association between low numbers of B cells and longer recurrence free survival. This effect was only seen for those B cells that directly surrounded the ductal carcinoma in situ component; the presence of B cells at a somewhat larger distance (>1 mm from the ductal carcinoma in situ component) did not affect ductal carcinoma in situ recurrence [86]. This study suggests that not only the presence of different immune cells is important, but also their exact location.

Other support for the role of ductal carcinoma in situ-associated tumor-infiltrating lymphocytes in tumor

evolution is extracted from the difference in the frequency of HER2+ and triple negative ductal carcinoma in situ versus HER2+ and triple negative invasive breast cancer, which are both frequently associated with high numbers of tumor-infiltrating lymphocytes. In studies restricted to patients with ductal carcinoma in situ, the frequency of HER2+ ductal carcinoma in situ is relatively high (15–34%) [80, 85, 95, 96] compared to invasive breast cancer studies, which report HER2+ invasive breast cancer in about 11–20% of cases [97–99]. In contrast, the frequency of triple negative ductal carcinoma in situ is relatively low in ductal carcinoma in situ studies (4–8%) [80, 85, 95, 96] compared to invasive breast cancer studies, which report that 10–13% of all invasive breast cancer cases are triple negative [97–99]. In line with this, there is a high frequency of extensive ductal carcinoma in situ adjacent to HER2+ invasive breast cancer, whereas the ductal carcinoma in situ component in triple negative ductal carcinoma in situ, if present, is rather limited [99]. Ductal carcinoma in situ-associated tumor-infiltrating lymphocytes might therefore play divergent roles with regard to progression of HER2+ and triple negative ductal carcinoma in situ; they might have an anti-invasion effect in HER2+ ductal carcinoma in situ and a pro-invasion effect in triple negative ductal carcinoma in situ.

Prospects for immunotherapy against ductal carcinoma in situ

In the past decade, HER2 pulsed dendritic cell vaccines have been tested in vivo as a potential treatment of HER2+ ductal carcinoma in situ patients [100–103]. These dendritic cells are able to induce an antitumor immune response by stimulating and activating CD8+ T cells via HER2 antigen presentation [103]. An early clinical study in 27 HER2+ ductal carcinoma in situ patients vaccinated with autologous HER2 peptide pulsed dendritic cells reported a major impact of this vaccine; 5 out of 27 patients showed a pathologic complete response in the surgical specimen [101]. Of the remaining 22 patients with residual ductal carcinoma in situ, 11 had a complete loss of HER2 expression. Overall, HER2 pulsed dendritic cell vaccination appear to induce either destruction of HER2+ cells or loss of HER2 expression. This effect seemed more pronounced in ER−/HER2+ ductal carcinoma in situ compared to ER+/HER2+ ductal carcinoma in situ [101], despite an equivalent immune response after vaccination [100]. More recently, a randomized clinical trial with 42 HER2+ ductal carcinoma in situ patients, reported that anti-HER2 dendritic cell vaccination is a save way to induce an antitumor T cell response in HER2+ patients [102]. Regardless of the administration route, a total number of 12 out of 42 patients achieved pathologic complete response, which is in line with previous studies [100–103]. These studies demonstrate that vaccination with dendritic cells might be an effective way to treat HER2+ ductal carcinoma in situ. However, other immune-modulating strategies might also be worth considering in treating HER2+ and other ductal carcinoma in situ subtypes. Data regarding ductal carcinoma in situ-associated PD-L1 is limited, but PD-L1 expression is reported in a subset of ductal carcinoma in situ cases [82]. Currently, immune-modulating checkpoint inhibitors are expensive and associated with substantial adverse effects. Therefore, checkpoint blockade as treatment for ductal carcinoma in situ patients is not anticipated at this moment. Nevertheless, the rapid development of checkpoint inhibitors with increased effectiveness, combined with decreased costs and side effects, may facilitate its use at earlier disease stages [104].

Summary

Research regarding the role of the immune system in breast cancer progression has primarily been focused on invasive breast cancer. High levels of immune infiltrates, in particular effector immune cells such as CD8 T cells, are more frequently observed in HER2+ and triple negative invasive breast cancer. In line with this, the prognostic and predictive value of these immune infiltrates is linked to these subtypes. Concerning the immune infiltrate composition, there also seems to be an association between the presence of certain cell types and prognosis; the presence of CD8+ T cells has mainly been associated with favorable clinical outcome in ER− invasive breast cancer [12, 31, 34]. A consensus on the observed effects with regard to CD4+FOXP3+ regulatory T cells and subsets of tumor-associated macrophages is yet to be reached.

In the last few years, there is increased interest regarding the presence and potential clinical significance of the immune infiltrate in patients with ductal carcinoma in situ. In line with invasive breast cancer studies, dense immune infiltrates are mainly present in HER2+ and triple negative ductal carcinoma in situ. The presence of high chromosomal copy number variation or a TP53 mutation also seems to initiate more immune response compared to limited copy number variation or a GATA3 and PIK3CA mutation, although it is unknown whether this effect is independent from ER and/or HER2 status [105, 106].

In-depth analysis of ductal carcinoma in situ-associated immune cell subsets reported that specific subsets (e.g., CD8+ cells, CD115+ cells, and CD20+ B cells) were associated with local recurrence [81, 86], which supports the hypothesis of an active role for tumor-infiltrating lymphocytes during the progression of ductal carcinoma in situ. However, based on current data no definite conclusion can

be drawn regarding the exact role of immune cell subsets regarding the progression of ductal carcinoma in situ subtypes. Therefore, in our opinion, there is no indication yet for standard reporting of ER, PR, HER2, and presence of ductal carcinoma in situ-associated tumor-infiltrating lymphocytes in daily clinical practice. However, as data with respect to the clinical significance of the ductal carcinoma in situ-associated immune response is rapidly expanding, pathologists might be challenged to report the presence, density, and composition of the immune cells in the future.

In conclusion, based on current literature, tumor-infiltrating lymphocytes are mainly present in HER2+ and triple negative ductal carcinoma in situ, which is in line with invasive breast cancer studies. This is consistent with studies that report high numbers of tumor-infiltrating lymphocytes in high grade and genetically instable ductal carcinoma in situ. Several studies reported an association between tumor-infiltrating lymphocytes and local recurrence, although the exact role of the immune system during the progression of ductal carcinoma in situ progression has to be elucidated. In-depth analyses of the interaction between ductal carcinoma in situ genetics and the immune cell composition and function are needed for a better understanding of the immune response in ductal carcinoma in situ subtypes. This might provide targets for successful immune intervention at an early disease stage, and therefore prevention of progression to invasive breast cancer.

Compliance with ethical standards

Conflict of interest MK: Unrestricted research grant of BMS to the institute, travel support by Roche to the institute. The remaining authors declare that they have no conflict of interest.

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