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REVIEW

Prognostic role of immune infiltrates in breast ductal carcinoma in situ

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Abstract

Purpose Ductal carcinoma in situ (DCIS) of the breast is often regarded as a non-obligate precursor to invasive breast carcinoma but current diagnostic tools are unable to accurately predict the invasive potential of DCIS. Infiltration of immune cells into the tumour and its microenvironment is often an early event at the site of tumourigenesis. These immune infiltrates may be potential predictive and/or prognostic biomarkers for DCIS. This review aims to discuss recent findings pertaining to the potential prognostic significance of immune infiltrates as well as their evaluation in DCIS.

Methods A literature search on PubMed was conducted up to 28th January 2019. Search terms used were "DCIS", "ductal carcinoma in situ", "immune", "immunology", "TIL", "TIL assessment", and "tumour-infiltrating lymphocyte". Search filters for "Most Recent" and "English" were applied. Information from published papers related to the research topic were synthesised and summarised for this review.

Results Studies have revealed that immune infiltrates play a role in the biology and microenvironment of DCIS, as well as treatment response. There is currently no consensus on the evaluation of TILs in DCIS for clinical application.

Conclusions This review highlights the recent findings on the potential influence and prognostic value of immunological processes on DCIS progression, as well as the evaluation of TILs in DCIS. Further characterisation of the immune milieu of DCIS is recommended to better understand the immune response in DCIS progression and recurrence.

Keywords DCIS · Biomarker · Immune · Prognostic · Progression · Recurrence

Introduction

The global introduction of mammography screening programmes has improved the detection of breast cancer, especially ductal carcinoma in situ (DCIS) [1]. Although initially rare, DCIS now represents 20–25% of all newly detected breast cancers in the mammographic era [2]. DCIS is genetically, morphologically, radiologically, and clinically heterogeneous, consisting of preinvasive neoplastic epithelial cells that proliferate within mammary ducts, without breaching the basement membrane [3].

Ductal carcinoma in situ is regarded as a non-obligate precursor to invasive ductal carcinoma (IDC), following a traditional linear ductal model that progresses from normal epithelium through to flat epithelial atypia, atypical ductal hyperplasia, DCIS, and ultimately invasive carcinoma [4]. Previous studies have supported this claim by demonstrating the genetic similarities between the two diseases [5–11], such as chromosomal abnormalities [12]. Overall, about 50% of all invasive breast carcinomas (IBC) are associated with DCIS, and the majority of these tumours are caused by the progression from in situ to invasive disease; as highlighted by studies that showed similarities in promoter gene hypermethylation [13, 14], global gene expression [15], copy number variation (CNV) [16, 17], DNA ploidy [18], and nuclear morphology [19]. Supporting the linear progression model, atypical ductal hyperplasia, DCIS, and IDC, in particular the low-grade (LG) forms, have been demonstrated to share several genomic alterations [9], especially CNV. However, despite the evidence supporting the role of DCIS as a precursor of IBC, longitudinal studies have revealed that untreated DCIS only progresses to IBC in the

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same quadrant of the ipsilateral breast in 20–50% of patients [20–22]. Although approximately half of IBCs are diagnosed as being associated with preceding DCIS [23], the likelihood of progression varies depending on the molecular subtype of breast cancer [24]. IBC molecular subtype classification can also be applied to DCIS [25–28]. However, due to the limitations of current diagnostic tools, including clinical parameters and histopathological features [11, 29], these classifications are not sufficient to accurately predict the invasive potential of DCIS [30, 31]. Novel parameters including new biomarkers are required to assist in stratifying DCIS into those that remain indolent versus those that are likely to become invasive [29].

In this era of stratified medicine, the development of cancer biotherapies of an immunological nature is of increasing importance [32]. Recent flow cytometric analysis revealed that there are more immune infiltrates, from both the innate and adaptive immunity, in both DCIS and IDC than in the normal breast [33]. Although the number of studies exploring the immune milieu of DCIS has increased, little is known about the influence of the immune response on DCIS progression, or its prognostic and predictive value. Notably, the infiltration of immune cells into tumour and its microenvironment is an early event occurring at the site of tumourigenesis. The interactions between leukocytes of both myeloid and lymphoid lineages, as well as the cytokines and chemokines they secrete, have been demonstrated to serve important functions in tumour progression [34], tumour suppression, and the efficacy of anticancer therapies [35]. Cytotoxic ($CD8^+$) T cells, T helper ($CD4^+$) cells, Natural Killer (NK) cells, and dendritic cells (DCs) are all involved in the effective immune response, while M2 tumour-associated macrophages (TAMs), $FOXP3^+$ regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs) are associated with suppression of the immune response [36]. These immune infiltrates may serve as potential predictive and prognostic biomarkers, shedding light on the relationship between cancer and the immune system (Fig. 1).

This review aims to discuss recent findings concerning immunological processes that may influence the progression of DCIS to IDC, the potential prognostic significance of these immuno-oncological biomarkers, and the evaluation of tumour-infiltrating lymphocytes (TILs) in DCIS (Fig. 2a).

Tumour-infiltrating lymphocytes

TILs, which include both B and T cells, migrate from the bloodstream into the tumour and stroma as part of the adaptive immune response (Fig. 2b, c). The roles and functions of TILs may be altered during cancer progression and in response to anticancer treatments [37, 38]. The number of TILs is often used to evaluate tumour immunogenicity, and the composition of this compartment appears to decide whether the immune response will be effective or suppressed [39, 40]. While guidelines for pathologic evaluation of TILs in DCIS have been recommended [41], universal application remains lacking. In DCIS, T and B cells typically outnumber macrophages, while T cells and macrophages outnumber B cells in IBC [42]. Thompson et al. [43] reported that the TIL compartment in DCIS was primarily composed of $CD3^+$ T cells, followed by $CD4^+$ T cells, $CD8^+$ T cells, $CD20^+$ B cells, with Tregs being present. The TIL phenotype in nuclear grade 2 and 3 DCIS was similar to the ones observed in IBC [43]. Campbell et al. [44] observed more $CD8^+$, $CD4^+$, $FOXP3^+$, and $CD20^+$ infiltrates in pure high-grade (HG) DCIS than non-HG (nHG) cases. Although both studies showed that different profiles of immune infiltrates were associated with high-risk features, none of these cell populations alone were associated with recurrences [43, 44]. Furthermore, Beguinot et al. [45] observed that TIL-rich DCIS, which typically contains large fractions of HG and intermediate grade DCIS, had more $CD8^+$, $CD4^+$, $FOXP3^+$, $CD20^+$, and $CD38^+$ infiltrates than TIL-poor DCIS, which tends to consist of fewer HG lesions. Through flow cytometric and transcriptomic analysis, Gil Del Alcazar et al. [33] found more $CD8^+$ T cells and naïve $CD4^+$ T cells in DCIS than IBC, while there were more activated $CD4^+$ T cells and Tregs in IBC than DCIS. Some studies even showed that the phenomenon of DCIS regression is associated with dense immune infiltrates [46, 47]. These phenomena were observed largely in cases with oestrogen receptor (ER) positivity and human epidermal growth factor receptor 2 (HER2) negativity, followed by HER2 positivity and triple negativity.

TIL density

Increased TIL infiltration is correlated with more aggressive behaviour in DCIS and is associated with a higher risk of progression and recurrence [48, 49]. This can be explained by the influence of inflammatory cells on DCIS progression [43, 50–52]. Increased TIL density is consistently observed in more aggressive breast cancer subtypes, including triple-negative breast cancer (TNBC) and $HER2^+$ breast cancer, but rarely in the luminal subtypes [53]. Hendry et al. [54] found that increased TIL density was associated with HG DCIS and comedo type. Several investigators have reported higher TIL density in ER^- DCIS compared to ER^+ ones [44, 45, 49, 54]. Both Hendry et al. [54] and Pruneri et al. [49] found that TIL density was higher in $HER2^+$ DCIS than any other subtype, unlike reports in IBC where TNBC has highest TIL density [55], suggesting that increased HER2 levels stimulate the immune system. Toss et al. [48] observed that increased TIL density was correlated with younger age, greater frequency of symptomatic presentation, larger tumour size, HG DCIS, comedonecrosis, and shorter

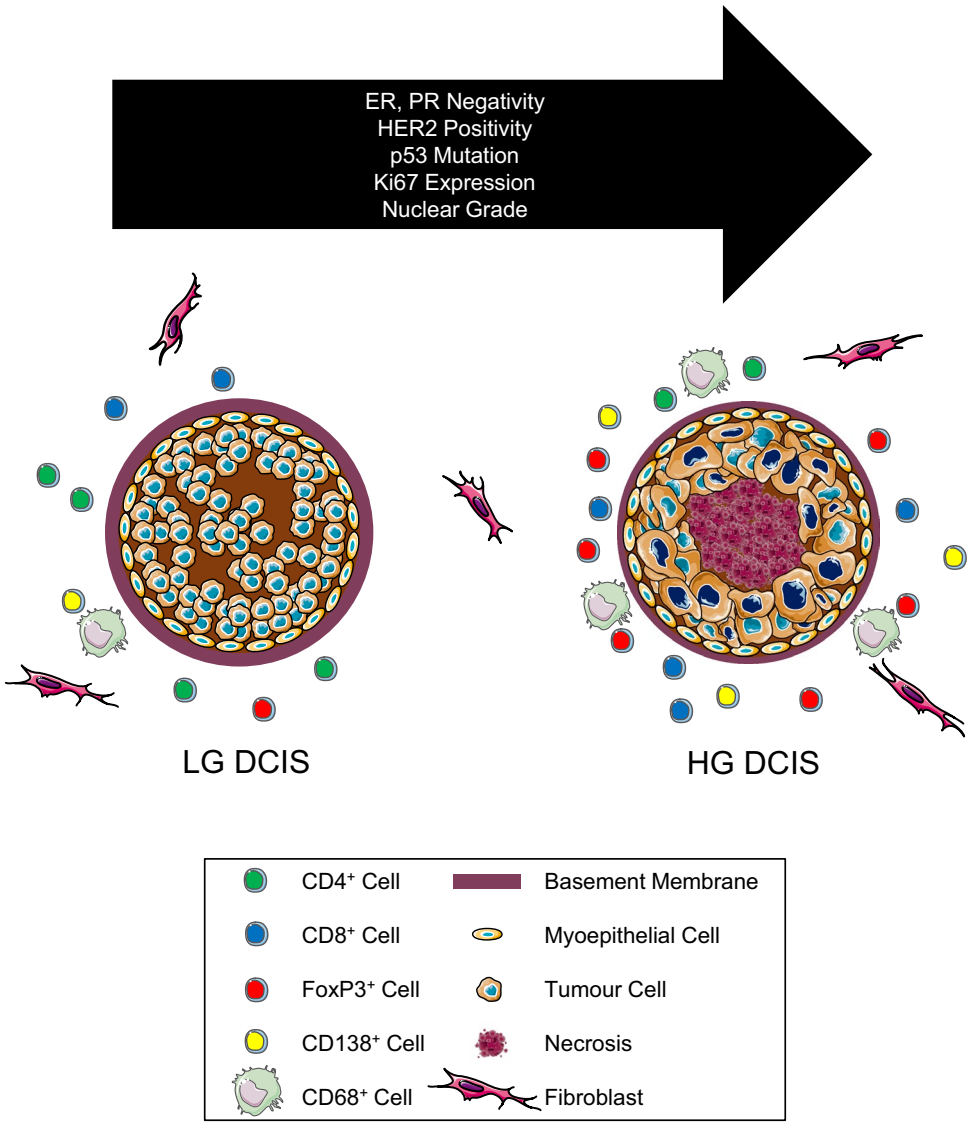

Fig. 1 Hypothetical model of TILs in LG DCIS and HG DCIS. Compared to LG DCIS, HG DCIS tends to display ER and PR negativity, HER2 positivity, p53 mutations, higher Ki67 expression, higher nuclear grade, as well as comedonecrosis. HG DCIS usually has higher TIL density than LG DCIS. There are more $CD4^+$ cells than $CD8^+$ cells initially, but the proportion of $CD8^+$ cells increases with tumour progression. $CD68^+$ and $FoxP3^+$ cells are more commonly observed in HG DCIS than LG DCIS. Increased $CD138^+$ cell levels are also associated with ER and PR negativity

recurrence-free interval (RFI). DCIS TIL density in patients treated with breast conserving surgery was found to be an independent predictive indicator of shorter RFI from a multivariate survival analysis [48]. Denser TILs were observed in DCIS associated with IBC compared to pure DCIS [48]. In addition to pure HG DCIS, microinvasive carcinoma (miCa) is often accompanied by higher TIL density compared with IBC and normal breast tissue [56–59].

The same observations, specifically T cells, were also present in a study utilising various analyses involving gene expression profiling, flow cytometry, and multiplex immunofluorescence [33]. HG DCIS was observed to have more infiltrating T cells than LG ones [33, 41]. Similarly, more T cells were reported in $HER2^+$ DCIS than $HER2^-$ ones [33]. Greater numbers of T cells were also observed in DCIS with recurrent IDC, compared to pure DCIS [33]. DCIS with p53 mutation was detected with higher TIL density [48, 54]. Although the biological reasons for T cell localisation are unknown, T cells have been observed in the stromal area and at the sites of disrupted myoepithelium [33]. More studies have to be done to identify the specific T cell subtypes as well as their activation status.

From these observations, ER^- DCIS, triple negative DCIS, $HER2^+$ DCIS, and HG DCIS may present a more immunogenic milieu, which may potentially allow for a more favourable response towards immunotherapy. Additionally, the implementation of DCIS TIL density assessments in clinical practice may help to predict the invasive potential of these lesions.

Influence of oestrogen on TILs

Despite the association between increased TILs and DCIS progression [48, 49] with more aggressive breast cancer

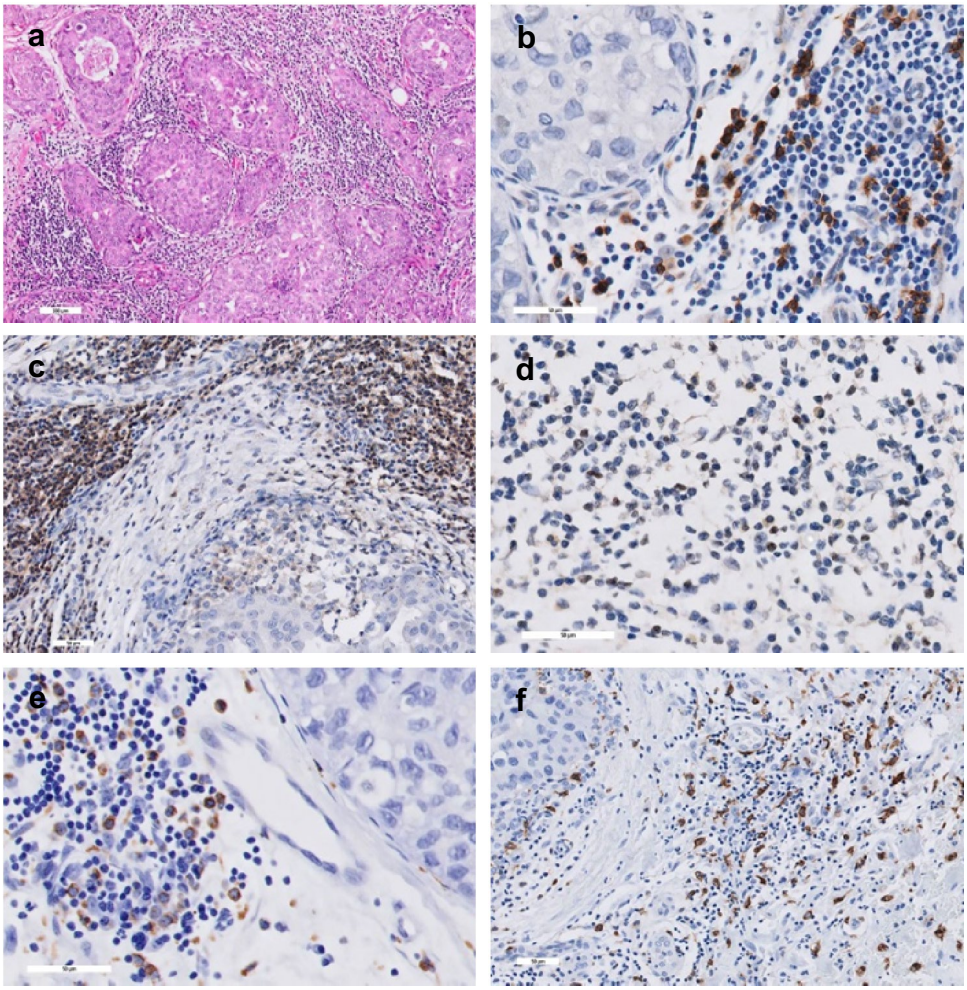

Fig. 2 Histological images of various immune infiltrates in DCIS tissues. **a** H&E staining of DCIS with TILs at ×100 magnification. Immunohistochemical staining of DCIS tissues with **b** $CD8^+$ cells at ×400 magnification, **c** $CD4^+$ cells at ×200 magnification, **d** $FoxP3^+$ cells at ×400 magnification, **e** $CD68^+$ cells at ×400 magnification, and **f** $CD163^+$ cells at ×200 magnification

subtypes [57], the primary role of TILs remains the elimination of cancer cells. Thus, an increased number of TILs is associated with improved prognosis in TNBC [60–62]. One potential reason underlying the increased TIL levels observed in ER^- tumours is that the ER pathway tends to create a more immunosuppressive stroma [61]. Another explanation is higher overall mutational load may lead to an increase in neoantigen formation in the TNBC subtype compared with the luminal subtype, resulting in increased numbers of TILs [62]. However, evidence supporting a direct correlation between immune signatures or TIL levels with mutational load in breast cancer subtypes remains lacking [36, 63].

TILs and copy number variation

A small cohort study found a significant association that had not previously been indicated in IDC, between CNV and TIL infiltration in pure DCIS [54]. From this finding, Hendry et al. [54] developed some hypotheses. One explanation is CNV in breast cancer may drive the generation of antigens and thus increase the number of TILs in the DCIS stroma. Another hypothesis is tumour moderation of the immune milieu or cancer immunoediting based on copy number could be due to changes in the immune compartment when there is direct contact between the tumour cells and TILs. However, these results need to be replicated in other cohorts where DCIS and IDC are scored for both CNV and TILs, following the same procedures, for confirmation.

Davoli et al. [63] identified a correlation between low immune gene expression profiles and high aneuploidy from an analysis of CNVs and TILs. In particular, a reduction in the $CD8^+$ T cell signature in IBC was associated with high aneuploidy. A higher degree of TIL infiltration was also positively correlated with DCIS Oncotype DX score [64]. These results suggest that immunoevasion plays a critical role in the progression of DCIS to IDC due to the need for neoantigen removal by copy number changes, as driven by immunoediting [54, 63].

Cytotoxic T cells

T cell infiltration increases during inflammation, with a normal ratio of $CD4^+$ T cells to $CD8^+$ T cells. In the early stages of DCIS, more $CD4^+$ T cells than $CD8^+$ T cells are present, but the reverse is true during later stages as the proportion of $CD8^+$ T cells increases with tumour progression [65]. $CD8^+$ T cells are observed more frequently in IBC compared with DCIS, and are associated with tumour malignancy [66]. Both $CD4^+$ T cells and $CD8^+$ T cells have been observed to eliminate breast carcinoma cells [67], and the inflammation observed in HG DCIS regression could signify a directed immune response against the tumour [46]. However, increased proportions of $CD8^+$ T cells and nuclear size are correlated with grade and HER2 status [68]. Through recursive partitioning and regression tree analysis, Campbell et al. [44] found that decreased $CD8^+HLADR^+$ or increased $CD8^+HLADR^-$ T cells with increased $CD115^+$ macrophages are associated with poorer prognosis. The increase in unstimulated $CD8^+HLADR^-$ T cells could be due to an immunosuppressive environment created by the increase in $CD115^+$ macrophages [44]. A low ratio of $CD8^+$ T cells to Tregs is correlated with worse prognosis [69, 70] and relapse [68], suggesting that the local immune response is protective against tumour progression.

Regulatory T cells

Tregs are derived from a lineage shared with naïve $CD4^+$ T cells, and are characterised by CD4, CD25, and FOXP3 expression [71] (Fig. 2d). These immune cells are involved in the promotion of tumour immunoevasion [72] through suppressing effector T cell induction and proliferation [73]. Tregs maintain homeostasis of the immune response, but oversuppression of this response to tumours may promote cancer progression [74]. The number of Tregs increases with malignant progression [57, 75, 76] and they are more commonly observed in HG DCIS compared with LG DCIS [44]. More Tregs were found in ER^- DCIS compared to ER^+ ones [43]. Increased Treg infiltration in DCIS is also associated with disease recurrence in both pure DCIS and IDC cases, even after 5 years [76]. It is believed that if the tumour and its stroma become more immunosuppressive through increased infiltration of Tregs, the greater the chance that immunoevasion and malignant progression will occur [54].

Tumour-infiltrating B cells

B cells are lymphocytes that take part in the adaptive immune response, serving important roles in humoral and cellular immunity by secreting antibodies [77]. Antibodies can perform several functions, such as initiating the complement cascade, assisting antibody-dependent cell-mediated cytotoxicity with NK cells, opsonising cancer cells for antigen presentation or cross-presentation by DCs, or simply altering the functions of their target antigens on tumour cells [78]. Although antibodies against tumour antigens are commonly observed in blood samples taken from cancer patients [79], their involvement in the cellular and humoral immune responses against cancer remains controversial [77]. Notably, some of these antibodies target autoantigens which are found on both cancer cells and normal cells. Plasma B cells have been demonstrated to promote anticancer responses, whereas other immunosuppressive subtypes, such as regulatory B cells (Bregs), encourage tumour progression [80]. Activated B cells promote the T cell response, while resting B cells suppress it [81]. While little is currently known about the involvement of B cells in DCIS behaviour and progression [82], increased numbers of infiltrating B cells in DCIS are correlated to poorer prognosis and a higher risk of recurrence [83]. Increased B cell infiltration in pure DCIS is associated with shorter recurrence-free survival (RFS) [84]. The presence of IgG^+ or IgM^+ B cells in the draining axillary lymph nodes of DCIS is also associated with higher histological grading and lymph node metastases [85]. Miligy et al. [83] found an association between the number of stromal B cells and tertiary lymphocyte structures, with HER2 positivity, ER and progesterone receptor (PR) negativity, and tumour size.

$CD19^+$ B cells and $CD20^+$ B cells

All mature B cells, apart from plasma cells, can be identified by the presence of CD20 [86]. CD19 regulates B cell development, activation, and differentiation through signal transduction [87]. However, like CD20, it is only present on B cells from the late pro-B cell stage until maturation of the B cells into plasma cells [88]. Campbell et al. [44] found that higher frequency of $CD20^+$ B cells in patients with HG DCIS is associated with parameters of higher risk, while Thompson et al. [43] reported increased $CD20^+$ B cells in ER^- DCIS compared to ER^+ ones. Miligy et al. [83] observed fewer $CD19^+$ B cells compared with $CD20^+$ B cells in DCIS, and the $CD19^+$ cells present were identified as a subset of $CD20^+$ B cells using an antibody against CD20. This suggests that these infiltrating B cells were at an earlier stage of maturation than those present in the blood, and that an immune response occurs during early DCIS development [83].

Plasma cells

CD138 may be expressed on stromal cells and mature epithelial cells [89], but high expression of this marker can be used to identify plasma cells [90]. Higher plasma cell infiltration has been observed in pure DCIS compared with

DCIS with invasion [83]. Increased plasma cell levels are also associated with ER and PR negativity [83], with the inability of ER^- tumours to respond to oestrogen-dependent proliferation signals thought to activate pathways resulting in increased expression of CD138. This may heighten the tumour response to other growth factors, conferring a growth advantage [91]. Another explanation is that CD138 is known to form a ternary signalling complex by binding to fibroblast growth factors (FGFs) [92] or behave as a potent FGF-2 activator through enzymatic degradation of its extracellular domain and physiological shedding [93], which consequentially serve as mitogenic and angiogenic growth factors to breast carcinomas [92].

Tumour-associated macrophages

TAMs differentiate from circulating monocytes that infiltrate the tumour site from the bloodstream. This differentiation is influenced by the presence of certain growth factors and cytokines present in the stroma. The TAM phenotype is plastic, and can vary and change depending on the composition of the microenvironment [94]. M1 macrophages are classically activated, inducible nitric oxide synthase $(iNOS)^+$ major histocompatibility complex class (MHC)-$II^{hi}CD68^{hi}CD80^{hi}CD86^{hi}$ [95] (Fig. 2e), pro-inflammatory macrophages that secrete tumour necrosis factor-α and interleukin (IL)-6, IL-12 and IL-13 [96], and participate in anticancer activity. M2 macrophages, on the other hand, are alternatively activated, $CD163^+$MHC-$II^{lo}CD200R^+$ macrophage galactose-type lectin $(MGL)1^{hi}MGL2^{hi}$ [97–99] (Fig. 2f), immunosuppressive macrophages that secrete transforming growth factor-β and IL-10 [100], and promote tumour progression by secreting growth factors and cytokines responsible for immunoevasion, angiogenesis, and matrix remodelling [101]. M1 macrophage differentiation is induced by factors associated with Th1 response, like interferon-γ, toll-like receptor ligands, and lipopolysaccharide [96], while M2 macrophage differentiation is stimulated by secreted cytokines from tumours, such as IL-4, IL-10, and IL-13 [102, 103].

The presence of TAMs is positively correlated with vascularity in DCIS [42, 104] and with poor prognosis in IDC [105]. Campbell et al. [44] found that there are more $CD68^+$ as well as $CD68^+$ proliferating cellular nuclear antigen expressing macrophages in HG DCIS, which are also associated with poor prognosis in IBC. $CD115^+$ macrophages were also found to be correlated to HER2 positivity, higher Ki67 expression, as well as greater risk of recurrence [44]. TAMs were also observed to localise around the foci of DCIS [44]. More studies involving the specific activation and localisation status of TAMs may help uncover their biological role in the invasion and progression of DCIS. An interesting study from Bögels et al. [106] found that the supernatant from breast carcinoma cell lines induced an M2 macrophage phenotypic differentiation on monocytes. The possibility of re-educating M2 macrophages to an M1 phenotype can serve as a potential therapeutic intervention in DCIS patients, for instance, targeting nuclear factor (NF) κB which regulates macrophage phenotype [107].

Table 1 Summary of different TILs assessment used for DCIS

References	Objective	Cohort	TILs assessment
Pruneri et al. [49]	Investigate distribution and clinical relevance of TILs in DCIS	1488 consecutive DCIS cases	Percentage of tumour stromal area occupied by TILs
Toss et al. [48]	Evaluate TILs assessment methods in DCIS, and their prognostic significance	684 pure DCIS cases and 132 DCIS mixed with IDC cases	TILs touching basement membrane of duct, or a single lymphocyte away
Thompson et al. [43]	Profile TILs and PDL1 expression in DCIS using tissue microarray	27 DCIS cases with known ER, PR, and HER2 expression	TILs occupying < 5% of tumour area as mild, 5–50% as moderate, and > 50% as diffuse/marked
Campbell et al. [44]	Determine predictive value of TILs in recurrence and metastasis of DCIS	52 HG DCIS and 65 nHG DCIS	Percentage of tumour stromal area occupied by TILs
Hendry et al. [41]	Investigate PDL1 expression and TILs in DCIS and correlate with clinicopathologic features and genomic information	138 pure DCIS cases	Percentage of tumour stromal area occupied by TILs
Knopfelmacher et al. [64]	Determine if pathologic assessment could predict Oncotype DX DCIS Score	46 DCIS cases	> 75% of circumference cuffing of duct by ≥ 3 layers of TILs
Beguinot et al. [45]	Investigate characteristics of stromal TILs in larger DCIS series	96 pure DCIS and 35 miCa cases	Percentage of tumour stromal area occupied by TILs classified into four grades: 0 (minimal, 0–9%), 1 (low, 10–29%), 2 (moderate, 30–49%), and 3 (high, 50–100%)

Assessment of TILs

The degree of immune infiltration in DCIS tissues can be visualised on routine haematoxylin and eosin (H&E) stained sections [108] (Fig. 2a). Early studies did not clearly define the DCIS stromal area for the evaluation of TILs and did not identify cut-off points for DCIS prognostic stratification [48]. Other studies have demonstrated the application of the modified International Working TILs Group guidelines for DCIS [109], assessing the percentage of stromal TILs surrounded by the margins of the whole lesion [43–45, 54, 110] (Table 1). Thompson et al. [43] defined TILs occupying < 5% of the tumour area as mild, 5–50% as moderate, and more than 50% as diffuse/marked. Meanwhile, Campbell et al. [44] visually identified and manually counted the TILs present in DCIS lesions in ten high-power fields (HPF), took the mean value of TILS per HPF for each case, and estimated the stromal TILS as a percentage of the total stroma per section. Knopfelmacher et al. [64] scored TIL density as circumferential or near circumferential (> 75% of the circumference) cuffing of the duct by at least three layers of TILs.

One commonly used method defines only those TILs situated within two HPF from the DCIS ducts as stromal TILS [41, 49, 54, 111]. However, findings from these studies showed no association between DCIS recurrence and TIL density.

Toss et al. [48] suggested that a scoring method using only TILs that were touching the basement membrane of the duct, or that were a single lymphocyte away, would be the optimal method for DCIS. This method was found to have the best concordance rate between observers, with results positively correlated among those from different topographic locations within the tissue. It was also easy and fast to assess, and strongly associated with RFI and prognostic clinicopathological parameters.

Conclusion

Current clinicopathological parameters for DCIS, like age, tumour size, and nuclear grade, are still unable to accurately predict the risk of disease recurrence. The search for a reliable biomarker to predict DCIS recurrence and invasive potential has been going on for decades but not a single robust one has been found. Over the years, studies have discovered that immune infiltrates play a critical role in cancer biology and even treatment response. Although there have been some recent proposals on how to evaluate TILs in DCIS, there is yet to be any consensus on what the best method is for clinical application. Future studies involving the characterisation of the immune milieu of DCIS may help to unravel the role of the immune response in DCIS progression and recurrence, develop robust predictive and prognostic biomarkers, or even illuminate potential targets for immunotherapy.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This is a review article and does not contain any studies with human participants or animals performed by any of the authors.

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