ABSTRACT

Immunotherapy using checkpoint inhibitors is one of the most promising current cancer treatment strategies. However, in breast cancer, its success has been limited to a subset of patients with triple-negative disease, whose durability of observed responses remain unclear. The lack of detailed understanding of breast tumor immune evasion mechanisms and the treatment of patients with highly heterogeneous metastatic disease contribute to these disappointing results. Here we discuss the current knowledge about immune-related changes during breast tumor progression, with special emphasis on the in situ-to-invasive breast carcinoma transition that may represent a key step of immunoediting in breast cancer. Comprehensive characterization of early-stage disease and better understanding of immunologic drivers of disease progression will likely expand the tools available for immunotherapy and improve patient stratification.

Introduction

Immunoediting is a dynamic process by which the immune system shapes the evolution of tumors. It is marked by three phases: elimination, equilibrium, and escape (ref. 1; Fig. 1A). Most malignant cells are eliminated by immunosurveillance before clinical presentation (Fig. 1B). In this elimination phase, antitumor immunity is stimulated through innate and adaptive immune responses. During the equilibrium phase pro- and antitumor immunity fail to fully eradicate tumors, but keep them under control. In the escape phase, cancer cells completely evade immune control as demonstrated in experimental models and in patients with cancer (1). Mechanisms of immune escape include decreased immune detection, downregulation of costimulatory molecules, and/or overexpression of coinhibitory molecules, resulting in reduced CD8+ T-cell activity (Fig. 1C). Immune escape is a requirement for breast tumor progression and a critical step in the transition from preinvasive to potentially lethal invasive disease. In this review, we discuss immune-related changes during breast cancer progression with special emphasis on the preinvasive-to-invasive transition.

Breast Tumor Progression

Histopathologic progression and classification

Ductal carcinoma, the most common histologic subtype of breast cancer, begins as abnormal epithelial proliferation in milk ducts of mammary glands, then progresses to ductal carcinoma in situ (DCIS), followed by invasive ductal carcinoma (IDC), and finally metastatic disease (Fig. 1A; ref. 2). DCIS is characterized by proliferation of cancer cells inside mammary ducts, which are surrounded by an intact layer of myoepithelial cells and basement membrane separating the epithelium from stroma. In contrast, IDC lacks myoepithelium and tumor epithelial cells invade the stroma. The major clinical and molecular breast cancer subtypes, defined by the presence of estrogen (ER) and progesterone (PR) receptors, HER2, and luminal or basal differentiation status, are present in preinvasive and invasive disease (3). Thus, tumors are classified as luminal (ER+ and/or PR+), HER2+, or triple-negative (lacking ER/PR/HER2). However, pure DCIS (no evidence of invasion) is not routinely tested for these classifying markers aside from ER, as most patients with DCIS do not receive systemic adjuvant therapy. On the basis of a comprehensive meta-analysis of all prior publications, African-American race, premenopausal status, detection by palpation, high histologic grade, involved margins, and high p16 expression are all significantly associated with risk of invasive recurrence (4). The Oncotype DCIS Score is a commercial gene signature test predicting the probability of recurrence in women >50 years of age, reducing the need of radiotherapy for low-risk patients (5). However, this score is not routinely used in the clinic to inform treatment decisions in patients with DCIS.

Molecular changes in tumor epithelial cells

Despite significant genetic and gene expression changes during tumor progression, mutations or gene signatures that consistently differentiate DCIS from IDC are unknown (6). Attempts to improve classification by stratification according to intrinsic subtypes and comparing DCIS and IDC within the same subtype did not yield consistent in situ and invasive epithelial gene signatures (7). Similar to IDC, the top mutated genes in high-grade DCIS include PIK3CA, TP53, GATA3, and KMT2C, with TP53 inactivating being a common event at the pathway level (8). High-grade DCIS also has frequent copy number aberrations including gain of chromosomes 1q, 8q, 11q13, 17q12, and 20q13 (9). PIK3CA mutations, more common in ER+ luminal cases, are sometimes discordant between IDC and adjacent DCIS (10).

Comparing genomic copy number profiles of IDC and adjacent synchronous DCIS at single-cell resolution complemented with exome sequencing confirmed known copy number alterations in breast cancer and revealed many shared clones between in situ and invasive regions of the same tumor, suggesting a multiclonal invasion model (11). However, analysis of pure DCIS and subsequent IDC recurrences is required to validate this model.

Gene expression changes in the stroma

Because of inability to define consistent epithelial genetic changes between DCIS and IDC and the role of microenvironment in tumor progression, researchers have profiled various stromal cells to find potential drivers of invasiveness (12). Contrary to the heterogeneity of

doi: 10.1158/2326-6066.CIR-19-0786
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Immune Escape during Breast Tumor Progression

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epithelial changes, stromal cell epigenetic and gene expression profiles show significant and consistent differences between normal breast tissue, DCIS, and IDC (13, 14). For instance, DCIS-associated myoepithelial cells are distinct from normal myoepithelia, with alterations in numerous genes encoding secreted proteins and extracellular matrix components (14). The myoepithelium contracts ducts during lactation for milk expulsion, controls mammary gland function via regulation of epithelial cell polarity, branching, and differentiation (15), and is a natural tumor suppressor by restricting angiogenesis and invasion (16, 17). However, myoepithelial cells lose this function during tumor progression and are absent in IDC. Molecular changes in DCIS-associated myoepithelium reflect perturbed differentiation and upregulation of genes related to angiogenesis and invasion (14, 18). Several genes altered in DCIS-associated myoepithelium have immune-related functions, implying a potential role for myoepithelial cells in immune regulation (14).

### Immune-Related Changes during Breast Cancer Progression

#### Myeloid cells and lymphocytes

Leukocytes, which mount antitumor immune responses, present a barrier and selective pressure in tumor progression (19). Innate immune responses do not rely on antigens for activation, represent the first-line of defense against pathogens and cancer, and are responsible for activating adaptive immunity. In normal breast, CD45

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þ leukocytes are relatively rare, but detectable in both stroma and within mammary ducts (20). In DCIS, leukocytes are abundant in the stroma surrounding the ducts (especially in high-grade and HER2

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þ lesions), but intraepithelial leukocytes are rarely detectable (21). Leukocytes also localize to sites of myoepithelial cell layer disruption/microinvasion (21). This limited interaction between leukocytes and cancer cells in DCIS may underlie a mechanism by which tumors evade immune surveillance. Therefore, in DCIS, tumors could still exist in the equilibrium phase, with immune escape likely occurring during or just prior to invasive transition (Fig. 1A).

Both, myeloid and lymphoid lineage immune cells are recruited at all stages of breast tumor progression. However, leukocyte composition and relative abundances of innate and adaptive immune cells change according to histologic stage, underscoring the importance of both arms of immunity in tumor progression (21). For example, the relative fraction of neutrophils increases as cancers progress from normal to DCIS to IDC (21). Similarly, the relative proportion of macrophages (Mφ) increases in HER2

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þ and triple-negative IDC compared with DCIS, while the fraction of dendritic cells (DC) decreases as tumors progress (21). Besides changes in relative abundance, the role of neutrophils and DCs in the DCIS-to-IDC transition is largely unknown, although they have been extensively studied in invasive disease.

DCs can have pro- or antitumor effects. They are functionally defective in patients with breast cancer potentially because of perturbed metabolism (22). However, HER2-targeting DC vaccines have been tested in patients with HER2

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þ DCIS to prevent invasive progression with some promising results (23). Neutrophil infiltration associates with breast tumor grade and the triple-negative subtype (TNBC; ref. 24). TNBC can be classified into subtypes enriched for either Mφ or neutrophils, with a Mφ-to-neutrophil conversion mediating immune checkpoint blockade resistance (25). Neutrophils modulate local and systemic immune environments and promote breast cancer metastasis (26).

Mφ can also have pro- or antitumor effects. Tumor-associated Mφ (TAM) infiltration associates with poor survival in breast cancer (27). TAMs and their progenitors, monocytes, have distinct antitumor expression profiles in IDC when compared with their homeostatic tissue counterparts, correlating with subtype and tumor aggressiveness (28). In HER2

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þ breast tumor models, Mφ recruited by tumor-derived CCL2 promote progression and early dissemination even from preinvasive lesions (29), which can be prevented by anti-CSF1R or CCR2 inhibition. In DCIS, CD68

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Mφ are detected within ducts near cancer cells with lower E-cadherin levels implying that they may play a similar progression-promoting role (29).

High-grade DCIS has significantly more tumor-infiltrating lymphocytes (TIL) than low-grade DCIS (21, 30–33), particularly CD68

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þ Mφ, CD4

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þ T cells, CD20

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þ B cells, and HLA-DR

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þ and FoxP3

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þ cells (30). High TIL content associates with high-grade, comedo necrosis, spocirone features, high CD8

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þ T cells, and HER2

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þ / triple-negative subtypes (21). DCIS with microinvasion or adjacent to IDC have higher TIL density compared with pure DCIS, with CD8

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þ cells being more common in adjacent DCIS lesions (21, 34).

The spatial distribution of TILs is also highly heterogeneous in DCIS and IDC. In DCIS, some ducts are surrounded by TILs while other regions are devoid of leukocytes; however, the biological mechanism underlying this heterogeneity and its potential clinical relevance are unknown. In IDC, TILs are found in discrete spatial arrangements. For instance, in TNBC there are four distinct topologic patterns correlating with gene signatures and clinical outcomes: infiltrated (TILs are fully intermingled with malignant cells), stroma restricted (TILs are within the tumor, but only detectable in stroma), margin restricted (TILs surround the tumor, but do not enter), and immune desert (few to complete lack of TILs; refs. 35–37).

Distant metastatic tumors appear to have lower TIL densities compared with matched primary tumor sites, with brain metastases having the lowest T-cell infiltration among all metastatic sites (38–40). However, these studies have several limitations, including small cohorts (<10 cases/subtype), varying metastatic sites (e.g., lung, brain, and liver), and the use of post-systemic therapy recurrent patients. Regardless, these findings suggest a decline in antitumor immunity with metastatic progression making immunotherapy less effective. One reason for this decline could be an increase in intratumor heterogeneity because subclonal neoantigens generate less-effective antitumor immune responses (41). Indeed, immunotherapy in adjuvant and neoadjuvant settings has induced robust immune responses. Even in metastatic disease, earlier treatment was more effective (42). One limitation of applying immunotherapies to earlier settings in patients who may have complete responses with standard of care is the high frequency of serious and lasting side effects. Thus, improving patient selection is critical for broader use of immunotherapies in earlier stage disease.

#### Increased immunosuppression leads to immune escape

Paradoxically, whereas leukocyte infiltration increases from normal to DCIS and IDC progression, there is a marked decrease in the frequency of activated immune cells and a progressively suppressive immune microenvironment. The relative fraction of cytotoxic CD8

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þ T cells is also variable based on tumor subtype, with triple-negative and HER2

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þ pure DCIS having a higher proportion compared with IDC adjacent to IDC (21). This decline was also observed in patients diagnosed with pure DCIS who underwent lumpectomy, but years later recurred locally with IDC (21). Gene set enrichment analysis of CD3

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þ T cells from DCIS compared with IDC also demonstrates a
Breast tumor progression in the context of immunoediting. A, Breast tumor progression (top) is accompanied by numerous stromal modifications, particularly immunoediting (bottom). Normal milk ducts, lined by luminal epithelial cells, myoepithelial cells, and basement membrane, progress to atypical hyperplasia through aberrant epithelial proliferation initiated by genetic and/or epigenetic alterations. Further acquisition of hereditary changes in combination with selection (I) promotes progression to DCIS, which is accompanied by an exclusion of leukocytes from the ducts (IV), recruitment and activation of surrounding immune infiltrates such as cytotoxic CDB⁺ T cells (IV), and extensive stromal reprogramming (I). (Continued on the following page.)
switch from cytotoxic T-cell to immunosuppressive regulatory T-cell (Treg) signatures (21).

Multiple mechanisms contribute to the progressively suppressive immune environment during breast tumor evolution. The 9p24 amplicon containing CD274 (encoding for PD-L1) is present in approximately 20% of primary TNBC, increasing in residual tumors after neoadjuvant chemotherapy (43). In triple-negative pure DCIS and IDC comparison, CD274 amplification was only detected in IDC and associated with higher tumor cell expression of PD-L1 (21). Similarly, the 17q12 amplicon in close proximity to ERBB2 (encoding HER2) contains a cluster of chemokine (CC) genes with diverse functions. In HER2+ pure DCIS and IDC, amplification of ERBB2 associates with coamplification of this CC, which inversely correlates with the frequency of intratumoral GZMB+CD8+ T cells (21).

HER2 itself can trigger an antitumor immune response in ERBB2-amplified tumors. Progressive loss of anti-HER2 Th1 function is found when comparing healthy individuals with breast disease. This loss, due to HER2+ DCIS and HER2+ IDC, and this associates with a functional shift in IFNγ:IL10 producing phenotypes, potentially reflecting a mechanism of immune evasion in HER2-driven breast tumors (44). A vaccine against HER2 tested as an invasive breast cancer prevention strategy in patients with HER2+ DCIS yielded promising results (45). However, HER2-targeted immune responses could favor the outgrowth of HER2+ breast tumors with less favorable prognoses.

Changes in TIL composition, such as increased accumulation of Treg cells during tumor progression, contribute to immune suppression. Synchronous DCIS and IDC cases have increased infiltration of Treg cells in DCIS compared with normal breast and a further increase in IDC comparison (46). Higher Treg infiltration associated with high grade but not tumor subtype, size of the invasive tumor, lymph node status, or disease stage (46). Expression of CTLA-4 also significantly increases in T cells from IDC compared with DCIS regardless of subtype (21), potentially contributing to immune exhaustion.

In breast cancer, aberrant maturation and differentiation of DCs (47, 48), downregulation of neoantigen peptide loading genes including MHC class I (49, 50), and upregulation of HLA-G (21, 50), which is highly expressed in placenta and results in a tolerogenic phenotype permissive for embryo development, associate with malignant progression. In other cancer types, such as lung cancer and melanoma, downregulation of neoantigens results in decreased immune recognition (51). Following anti-PD-L1 or anti-CTLA-4 treatment of lung cancer, seven to 18 putative mutation-associated neoantigens that might be only subclonal or not reactive (41). Evolutionary studies like these have not been conducted in breast cancer in part due to difficulties with acquisition of fresh tissue from early-stage tumors and the limited success of immunotherapy.

**Immunotherapy in Breast Cancer**

The first FDA approval for a breast cancer immunotherapy was in April 2019 for atezolizumab (anti–PD-L1) in combination with nab-paclitaxel for triple-negative metastatic disease. This led to a sustained enthusiasm for immunotherapy, with around 300 trials exploring immunotherapies in breast cancer, the vast majority being phase I or II trials for immune checkpoint blockade (45). Vaccination against HER2 is being tested in the clinical setting and elicits tumor-specific T-cell responses (45). In the adjuvant setting, vaccination against HER-2 resulted in no tumor recurrences after a 34-month period (45). In addition, current clinical trials test vaccines in combination with immune checkpoint blockade (NCT03362060, NCT03199040, and NCT02826434), adoptive natural killer cell therapy (NCT02844335 and NCT02843126), and chimeric antigen receptor T cells targeting overexpressed proteins in breast cancers including HER2 (NCT02713984, NCT01837632, NCT02792114, and NCT0285909). A concerted action targeting both types of immunity will lead to stronger immune responses in a wider set of patients (27). For example, targeting TAMs may lift immunosuppression on effector cells and reestablish Mφ antitumor effects (27). Several clinical trials aim at depleting Mφ by targeting CSF1R (NCT01346358, NCT02265536, and NCT03153410) or through specially formulated inorganic biphosphonates, such as zoleodronate (NCT02347163) or cladronate (NCT00127205S). Decreasing TAM recruiting by CCL2-neutralizing antibody treatment and Mφ reprogramming have also been evaluated in preclinical models. For example, inhibition of class IIA HDACs in luminal B breast cancer increased the efficacy and durability of immune checkpoint inhibitor and chemotherapy (54), while neutralization of the macrophage receptor with collagenous structure (MARCO) on a subset of inflammatory TAMs inhibited tumor metastasis (55). MARCO+ TAMs are also present in human breast cancers, particularly in basal/triple-negative tumors. Thus, repolarizing TAMs through MARCO may be therapeutically beneficial for...
patients with TNBC. Toll-like receptor (TLR) activation by bacterial particles polarizes plasmacytoid DCs and Mφ toward a proinflammatory phenotype, leading to antitumor effects in breast cancer (56). The TLR7 agonist, imiquimod, increases lymphoid immune infiltration and tumor regression of skin metastases from breast cancers in patients (57).

Summary and Future Directions

With immune escape marking the DCIS-to-IBC transition, we hypothesize that it is a requirement for invasive progression and tumor dissemination, because only cancer cells evading immune surveillance can contribute to tumor formation (Fig. 1). Therefore, the in situ-to-invasive carcinoma transition represents an evolutionary bottleneck, which may be determined by the host’s immune status. Thus, assessing systemic and local immune environments in patients with DCIS could serve as a risk predictor of invasive progression. Comprehensive characterization of pure DCIS and their local invasive recurrences at the single-cell level while preserving topology could reveal mechanisms underlying immune escape, which can facilitate the design of more effective immunotherapies for the treatment of both early and advanced stage disease.

One limitation of implementing immunotherapies in breast cancer is the scarcity of preclinical models that reproduce the natural progression of human breast cancer. Engineered and spontaneous mouse mammary tumors neither recapitulate the histopathologic progression nor the immune microenvironment of human breast tumors. Carcinogen-induced mammary tumors in Sprague Dawley and Wistar-Furth rats show remarkable similarities to human disease with regards to hormone dependence and histopathologic stages of progression (58), but their immune environments remain to be characterized. However, based on data highlighting the importance of the microbiome in antitumor immunity and success of immunotherapy (59), no preclinical model faithfully reproduces the complexity of the human body, limiting the predictive power of such models. Therefore, improved molecular and cellular understanding of how tumors evade immune surveillance in patients with breast cancer coupled with rationally designed clinical trials with strong correlative studies are necessary to make progress.

Disclosure of Potential Conflicts of Interest

K. Polyak is a scientific advisory board member for Farcast Biosciences and is a consultant/advisory board member for Acrivon Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

The authors would like to thank Drs. Judith Agudo and Jennifer Guerriero, and members of the Polyak laboratory for critical reading of this article and for helpful discussions. This work was supported by the NCI (R35CA197623, to K. Polyak), the Breast Cancer Research Foundation (to K. Polyak), and the Claudia Adams Barr Program (to C.R. Gil Del Alcazar).

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