Immune Infiltrates in Breast Cancer

Subjects: Immunology

Contributor: Maria Vittoria Dieci

In recent decades, the increasing interest in the field of immunotherapy has fostered an intense investigation of the breast cancer (BC) immune microenvironment. In this context, tumor-infiltrating lymphocytes (TILs) have emerged as a clinically relevant and highly reproducible biomarker capable of affecting BC prognosis and response to treatment. Indeed, the evaluation of TILs on primary tumors proved to be strongly prognostic in triple-negative (TN) BC patients treated with either adjuvant or neoadjuvant chemotherapy, as well as in early TNBC patients not receiving any systemic treatment, thus gaining level-1b evidence in this setting. In addition, a strong relationship between TILs and pathologic complete response after neoadjuvant chemotherapy has been reported in all BC subtypes and the prognostic role of higher TILs in early HER2-positive breast cancer patients has also been demonstrated. The interest in BC immune infiltrates has been further fueled by the introduction of the first immune checkpoint inhibitors in the treatment armamentarium of advanced TNBC in patients with PD-L1-positive status by FDA-approved assays. However, despite these advances, a biomarker capable of reliably and exhaustively predicting immunotherapy benefit in BC is still lacking, highlighting the imperative need to further deepen this issue. Finally, more comprehensive evaluation of immune infiltrates integrating both the quantity and quality of tumor-infiltrating immune cells and incorporation of TILs in composite scores encompassing other clinically or biologically relevant biomarkers, as well as the adoption of software-based and/or machine learning platforms for a more comprehensive characterization of BC immune infiltrates, are emerging as promising strategies potentially capable of optimizing patient selection and stratification in the research field.

breast cancer immune infiltrate immune biomarker tumor-infiltrating lymphocytes PD-L1 immunotherapy

1. Introduction

Breast cancer (BC) is not considered a highly immunogenic tumor type, especially if compared with melanoma or lung cancer. However, in recent decades, it has been consistently reported that the BC tumor microenvironment (BC-TME) encompasses a wide range of cell populations of both the innate and adaptive immune systems, which have been reported to be biologically/clinically relevant to varying degrees [1], as summarized in Figure 1. Immune infiltrates in BC encompass immune cells both directly in contact with tumor cells, known as “intratumoral” (int), and within the surrounding stroma, known as “stromal” (str). The evaluation of BC immune infiltrates relies on, among other things, morphological evaluation of immune cells on H&E-stained tumor samples, immunohistochemical staining for specific subsets of immune cells evaluated by classic semiquantitative scoring or by digital pathology, multiplexed fluorescent immunohistochemistry with multispectral imaging that simultaneously identifies and

quantifies multiple immune cell subsets in a single formalin-fixed paraffin-embedded (FFPE) slide and provides information on the distribution of immune infiltrates, flow cytometry on fresh tissue and computational tools for immune cell quantification from transcriptomics data.

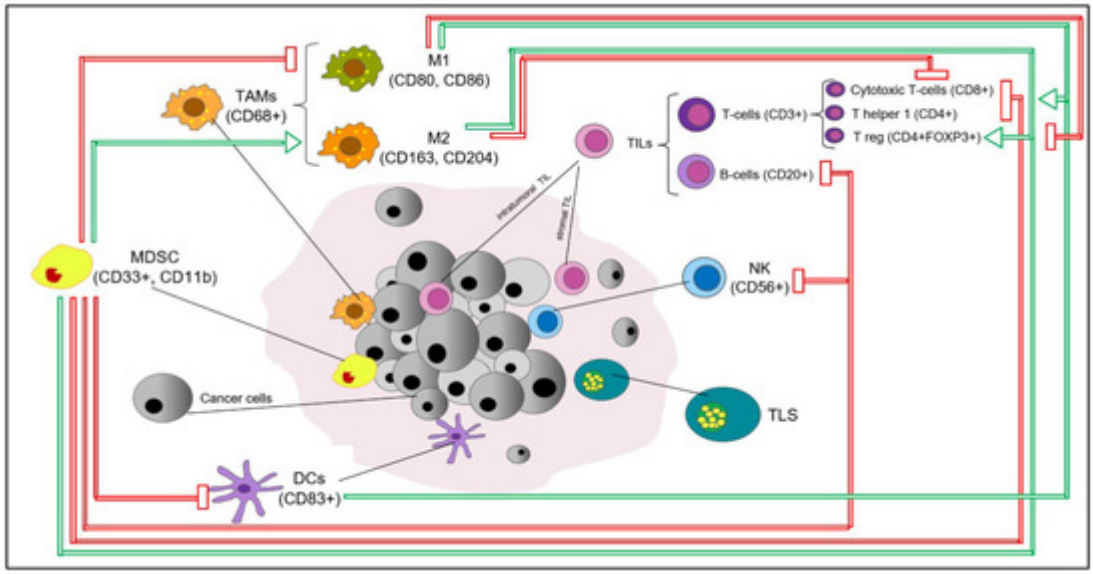

Figure 1. Key immune cell subsets in breast cancer tumor microenvironment. The production of IFNγ by CD4+ Th1 cells mediates the expansion, differentiation and activation of CD8+ tumor-infiltrating lymphocytes (TILs), which subsequently release cytotoxic cytokines and directly kill cancer cells (via recognition of specific tumor-associated antigens on the surface of antigen presentation cells (APCs) or cancer cells); CD4+FOXP3+ TILs represent immunosuppressive mediators through the inhibition of CD8+ T cells, CD4+ Th1 cells, APCs and natural killer cells (NKs); M1 tumor-associated macrophages (M1-TAMs) are associated with Th1 cytotoxic immune response, thus exhibiting antitumor properties; M2-TAMs contribute to the activation of Th2 immune response, thus showing an immunosuppressive role (e.g., suppression of T cell function); NKs are cytotoxic members of the innate immune system (release of cytotoxic cytokines and direct killing of cancer cells); dendritic cells (DCs) are antigen-presenting cells and are crucial players of the adaptive immune system; myeloid-derived suppressor cells (MDSCs) represent immature myeloid cells (possibly originated from bone marrow precursors) with an immunosuppressive function via the inhibition of T cells, B cells, NKs, M1-TAMs and DCs. The recruitment and accumulation of immunosuppressive mediators into the tumor bed is mediated by the secretion of cytokines and chemokines (e.g., IL6, IL1-β, TGF-1β, CCL2) by tumor cells. Red and green arrows reflect inhibitory and stimulatory relationships, respectively. Abbreviations: IFNγ, interferon gamma; TILs, tumor-infiltrating lymphocytes, APCs, antigen presentation cells, NKs, natural killer cells, DCs, dendritic cells, TAMs, tumor-associated macrophages; MDSCs, myeloid-derived suppressor cells; TLS, tertiary lymphoid structure.

BC immunogenicity is highly heterogeneous, with different BC subtypes showing different degrees of immune infiltration. In detail, the most biologically aggressive subtypes, namely, triple-negative BC (TNBC) and HER2-positive (HER2+) BC, are characterized by high genomic instability and tumor mutational burden (TMB), both fueling the generation of neoantigens, ultimately fostering the antitumor immune activity. However, an inverse association between TMB/genomic heterogeneity and levels of immune infiltrates in TNBC subtypes has recently

been suggested, with immune-rich tumors showing lower degrees of clonal genomic heterogeneity, lower neoantigen loads and somatic mutations and fewer somatic copy number alterations [2]. While seemingly counterintuitive, this observation may reflect the elimination of immunogenic clones by an effective antitumor immune surveillance, thus resulting in lower clonal genomic heterogeneity. Conversely, higher clonal heterogeneity may reflect the escape phase of the immunoediting process, where the selection of cancer clones results in reduced immunogenicity.

Both cytotoxic treatments and anti-HER2 agents are known to be capable of further activating the immune system through immunogenic cell death and antibody-dependent cellular cytotoxicity (ADCC), respectively. In addition, in HER2+ BC, oncogene addiction may trigger the immune system, with HER2 itself acting as a tumor-associated neoantigen [3]. Hormone receptor-positive (HR+)/HER2-negative (HER2−) BC, also known as luminal-like BC, is traditionally considered to be less immunogenic than TNBC and HER2+ BC, given the lower genomic instability and mutational load [3][4][5]. However, available evidence suggests that the immunogenicity of this BC subtype may rely on subtler mechanisms reflecting the complex and dynamic relationship between HR+ BC cells, inflammatory mediators, estrogen levels, endocrine treatments and menopausal status [6].

2. Adaptive Immunity

2.1. Tumor infiltrating lymphocytes

In recent decades, the morphological evaluation of immune infiltrates in BC has gained tremendous interest in the light of accumulating high-quality evidence supporting TIL clinical validity in BC. The prevalence of TILs is heterogeneous across different BC subtypes, with TNBC and HER2+ BC typically exhibiting greater TIL infiltration as compared to the luminallike BC subtype [7].

An additional source of heterogeneity is represented by the disease setting. Indeed, it has been suggested that TIL infiltration tends to weaken throughout the natural history of BC from the early to advanced stages. In particular, it has been consistently reported that
overall TIL levels are not only lower in patients with advanced disease as compared to the early setting, but also in heavily treated advanced BC patients as compared to those treated in the first-line setting for their metastatic disease [8][9][10][11]. Moreover, heterogeneity in TIL levels has also been observed within different sites of BC metastases, with the lungs showing the highest degree of TIL infiltration, while the liver and skin show the lowest [10][12][13].

The ever-growing interest towards the evaluation of TILs fostered the development of the International Working Group on Immuno-Oncology Biomarkers, aiming at providing a standardized methodology for TIL assessment in BC samples, in order to improve consistency and reproducibility across studies, in preparation for TIL clinical implementation, also given the endorsement of TIL quantification and reporting in TNBC and HER2+ BC by the St Gallen Consensus Conference (TNBC), WHO (both TNBC and HERBC2+) and ESMO 2019 Guidelines [14][15]. The

clinical validity of TILs as a prognostic marker and potential clinical utility in patients with early breast cancer is summarized in Table IV

Table IV. Summary of clinical validity of TILs as a prognostic marker and potential clinical utility in patients with early breast cancer

BC Subtype	Clinical validity (prognostic)	LoE	Evaluation endorsed by guidelines	Potential clinical utility[b] (to be demonstrated)
TNBC	High TILs are associated with improved outcome [16][17][18][19][20][21][22][23][24]	IB[a]	Yes: Expert Opinion at the 16th St Gallen International Breast Cancer Conference[15]; ESMO 2019 Early Breast Cancer guidelines[16]; WHO classification of Tumors, Breast Tumors, 5th edition;	Integration with other clinicopathological variables to guide treatment de-escalation in low-risk patients (i.e. no anthracyclines or even no treatment; prognostic tool available at www.tilsinbreastcancer.org).
	High TILs are associated with increased pCR rate after neoadjuvant chemotherapy[25]			Risk stratification in post-neoadjuvant setting based on TILs and RCB to guide the decision of further adjuvant treatment.
	In TNBC, high TILs on residual disease after neoadjuvant chemotherapy are associated with improved outcome[26][27]			Stratification factor in clinical trials.
HER2+			Yes: ESMO 2019 Early Breast Cancer guidelines[16]; WHO classification of Tumors,	Integration in multiparametric scores to guide treatment escalation and de-escalation (i.e. HER2DX).

			Breast Tumors, 5th edition.	Stratification factor in clinical trials.	
HR+/HER2-	Not demonstrated: conflicting results from studies in the adjuvant setting; high TILs associated with increased pCR rate after neoadjuvant chemotherapy, but less favorable survival[28]	-	No	Unknown	

The interest in BC immune infiltrates has been further fueled by the introduction of the first immune checkpoint inhibitors in the treatment armamentarium of advanced TNBC in patients with PD-L1-positive status by FDA-approved assays. Indeed, at present, the only established predictive biomarker for immunotherapy efficacy in metastatic BC is represented by PD-L1 expression, as emerged in the context of randomized phase III trials for TNBC BC. Beyond PD-L1 expression, several other biomarkers proved to be potentially capable of improving our ability to select patients for immunotherapy. Focusing on BC immune infiltrates, interestingly, accumulating evidence suggests that the morphologic evaluation of TILs may have a role in this regard. Notably, it has been consistently reported that PD-L1 expression is predominant on immune cells rather than tumor cells[12][29]. In addition, it has been reported that PD-L1 expression and TIL levels are strongly correlated with each other, especially in the TNBC subtype, thus suggesting that the simple morphological evaluation of TILs may serve as a surrogate reflecting a general state of immune activation[12][30]. Table II summarizes results from translational analyses of several trials testing diverse immune checkpoint blockade strategies, which suggested a potentially clinically relevant role of TILs in BC patients treated with immunotherapy either in the early or advanced setting.

Table II. Summary of phase II/III clinical trials investigating immunotherapy in BC with results from TIL analysis available

Trial (design)[38]	Setting, BC subtype	Treatment arms	TIL variable	Outcomes	Results[c]

2.1. Tumor infiltrating Dendritic Cells (DCs)

DCs represent antigen-presenting cells and are crucial players of the adaptive immune system. Interestingly, an

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			(cutoff[a])		[39]
Keynote-086[31] (II)[e]	Advanced, TN [40]	Pembrolizumab (2 cohorts)	Binary (median) [41][42]	ORR DCR	Combined cohort: OR for ORR=1.26 (CI 1.03-1.55), p=0.01; OR for CDR 1.22 (CI 1.02-1.46), p=0.01 [43]
Impassion-130[29] (III)	Advanced, TN [41]	NabP-Atezo NabP-Pbo	Binary (10%)	PFS OS	Predictive for Atezo benefit only in PD-L1+: PD-L1+/strTILs low à PFS: HR 0.74 (0.54-1.03), p=0.07, OS: HR 0.65 (0.41-1.02) p=0.06. PD-L1+/strTILs high à PFS: HR 0.53 (0.38-0.74), p<0.005; OS: HR 0.57 (0.35-0.92), p=0.02. PD-L1-/strTILs high à PFS: HR 0.99 (0.62-1.57), p=0.97; OS: HR 1.53 (0.76-3.08), p=0.24
Keynote-119 (III) [32]	Advanced, TN [44]	Pembro CT PFC[b]	Continuous, Binary (5%)	BOR DCR PFS OS	Positive association with clinical outcomes only in Pembro arm (p<0.05) TILs <5% à Pembro vs CT: median OS 5.9 vs

the BC-TME, results from clinical studies, deepening their role in BC patients, are scattered and broadly inconsistent.

					8.8 months, HR 1.50 (1.14-1.97) [45][46][47] TILs ≥5% à Pembro vs CT: median OS 12.5 vs 11.3 months, HR 0.75 (0.59-0.96)
Panacea[10] **(Ib-II)**	Advanced, HER2+ [47][49]	Trastuzumab-Pembro	Continuous	OR [50][51] DCR	Positive association with ORR (p=0.006) and DCR (p=0.0006)
KATE-2 **(II-R)**[33][34]	Advanced, HER2+	TDM1-Atezo [54][55] TDM1-Pbo	Binary (5%)	OS	Borderline positive association with OS in the Atezolizumab arm [52][53] TILs<5% à Atezo vs Placebo: 1-year OS 84.2% vs 100%, HR 1.43 (0.51-4.01) TILs≥5% à Atezo vs Placebo: 1-year OS 90.8% vs 83.5%, HR 0.55 (0.26-1-12)
GeparNuevo **(II R)**[35]	Neoadjuvant, TN	Durva[e] à Durva-NabPà Durva-EC Pboà Pbo-NabPà Pbo-EC	Continuous Categorical (10%, and 60%)	pCR	strTILs (continuous): positive association with pCR, no predictive for Durva benefit [58] OR 1.23 (1.04-1.6), p=0.019 in Durva arm; OR=1.39 (1.12-1.74), p=0.003 in Placebo arm.

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® [53]

phase II trial of anthracycline–taxane-based treatment (+ bevacizumab), no significant association between M2-TAMs (CD163+) and pCR rates was reported [59].

					intTILs: increase between baseline and end of the window phase associated with Durva benefit. OR 9.36 (1.26-69.65) p=0.029
			[41]		
NeoTRIPaPDL1 (III)[36]	Neoadjuvant, TN	Cb-NabP-Atezo Cb-NabP	Binary (40%)	pCR	Positive association with pCR, no predictive role Atezo arm: strTILs ≥40% vs <40% à pCR 71.43% vs 28.07%, p=0.001 CT arm: strTILs ≥40% vs <40% à pCR 63.16% vs 33.9%,

Mechanism of Action	Target	Drug	Partners	Trials
	Membrane cell receptor activation	Trabectidine	CT, PARP inhibitor	NCT00050427 NCT00580112 NCT03127215
TAM killing	CSF1-CSF1R inhibition	Emactuzumab Pexidartinib Lacnotuzumab Cabiralizumab	CT, immune checkpoint inhibitors	NCT02323191 NCT02760797 NCT01494688 NCT01596751 NCT01525602 NCT01042379 NCT02435680 NCT02807844 NCT03285607 NCT04331067
Inhibition of TAM recruitment	CCL2-CCR2 inhibition	Carlutumab	CT	NCT01204996
Modulation of TAM polarization (from M2 into M1 TAM phenotype)	Macrophage	Zoledronic Acid	NA	Approved in both early and metastatic settings
	CD40 stimulation	Selicrelumab	Other anti-TAM agents	NCT02225002 NCT02157831 NCT02665416 NCT02760797

Mechanism of Action	Target	Drug	Partners	Trials
	CR3 stimulation	1,3–1,6 β-glucan	Immunotherapy	NCT02981303
Induction of cancer cell phagocytosis by TAMs	CD47-SIRPα inhibition	TTI-621, ALX148 Hu5F9-G4	Immunotherapy, anti-VEGF agents, anti-HER2 agents	NCT02890368 NCT03013218 NCT02216409 NCT02953782
Regulation of antigen presentation by TAMs	TLR7 stimulation	Imiquimod 852A	CT, RT	NCT00899574, NCT01421017, NCT00821964, NCT00319748
Vaccination	NA	SV-BR-1-GM H2NVAC	Immunotherapy	NCT04418219, NCT04144023

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expression of NK-activating genes was associated with better RFS in a small series of unselected early BC patients [60]. In addition, in a small retrospective cohort of BC patients with locally advanced disease undergoing neoadjuvant treatment [61], higher levels of NK cells (CD56+) on pre-therapeutic samples were significantly associated with better pCR rates. The involvement of NKs in the ADCC-mediated mechanism of action of anti-HER2 monoclonal antibodies is well recognized. Interestingly, several strategies aiming at enhancing NK cell effector function (via ADCC) by anti-HER2 agents are currently under investigation and results are awaited [62].

Abbreviations: TAMs, tumor-associated macrophages; CT, chemotherapy; PARP, poly-ADP polymerase; M2pep, M2peptide; CSF1 (R), colony stimulating factor 1 (receptor); CCL2, chemokine C-C motif ligand 2; CCR2, CCL2 receptor; mAb, monoclonal antibody; Fbln7, fibulin 7; CXCL1, C-X-C Motif Chemokine Ligand 1 Pathway; ZnPPIX, zinc protoporphyrin 9; SIRPα, signal regulatory protein alpha; TLR7, toll-like receptor 7; GM-CSF, granulocyte-macrophage colony stimulating factor.

Although promising, available evidence is immature and further efforts should be made in order to deepen the role of NKs in BC biology and clinical behavior.

4. Tertiary Lymphoid Structures (TLS)

TLS represent lymph node-like aggregates (with a germinal well-defined B lymphocyte- and follicular DC-rich area surrounded by a parafollicular T lymphocyte-rich area) which can be found in non-lymphoid organs, including cancer.

So far, data on the clinical relevance of TLS in BC remain speculative. However, in various retrospective series of cancer patients including breast tumors, the presence of TLS—along with general TIL assessment—has been associated with improved prognosis and increased pCR rates after neoadjuvant therapy in unselected BC as well as in patients with HER2+BC or TNBC subtypes receiving standard treatments. In addition, a recent retrospective study evaluating TLS in primary BC samples and paired metastases showed lower TLS in metastatic samples as compared to paired primary tumors, thus further confirming that BC metastases are generally characterized by lower immune infiltration as compared to primary BC [63].

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