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The Influence of Host Factors on the Prognosis of Breast Cancer: Stroma and Immune Cell Components as Cancer Biomarkers

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Abstract: In this paper, we will review the data on stromal components and immunological parameters in the cancer microenvironment as prognostic and predictive markers in breast cancer. Host immunological response to cancer has gained importance because of recent breakthroughs in immunotherapy. Currently, molecular and clinical subtyping of breast cancer is solely based on the molecular features of the cancer cells without considering the importance of stromal components. There is now clear evidence that infiltrating immune and inflammatory cells influence the biology and clinical course of breast cancer. However, the prognostic and predictive function of immune cells differs between breast cancer subtypes. Immune parameters are established and validated prognostic and predictive markers in triple negative and for HER2 positive breast cancers and may be ready to be used as stratification parameters in clinical trials and as adjunct variables when making clinical decisions. On the other hand, the prognostic and predictive impact is minimal in low grade, luminal A type breast cancers. The strong association between higher lymphocytic infiltration and better outcome (including greater chemotherapy sensitivity) in TNBC and HER2 positive cancers also raises novel therapeutic options that target immune cells to increase their activity. Immune markers also carry the potential to serve as predictive markers to select patients for immunotherapeutic regimens (*e.g.* checkpoint inhibitors).

Keywords: Breast cancer, host factors, immune cells, immune checkpoints, immune therapy, prognosis, tumor infiltrating lymphocytes.

INTRODUCTION

The contribution of stromal components (*e.g.* immune and inflammatroy cells, fibroblasts, adipocytes and blood vessels) to cancer biology has long been recognized and covered by numerous reviews. In this paper, we will focus on how inflammatory and immune cells influence the clinical behaviour of different breast cancer subtypes and discuss the potential therapeutic implications.

HOST FACTORS IN BREAST CANCER

Breast cancer includes several different molecular subtypes with distinct molecular and clinical features which also guide treatment approaches [1-3]. Currently, molecular subtyping only takes into account the molecular features of the carcinoma cells [4, 5] even though it is increasingly clear that factors in the tumor micro environment have also a major impact on treatment response and clinical course of the disease [6]. The microenvironment includes fibroblasts, endothelial and fat cells and immune and inflammatory cells, frequently referred to as the tumor stoma [6, 7]. Among the stromal cells, the various subpopulations of lymphocytes, dendritic cells and macrophages appear to have profound prognostic value and are also increasingly recognized as mediators of treatment response to a broad range of drugs [8-11]. One and a half centuries ago Rudolf Virchow noted the presence of leukocytes in tumors and proposed a possible link between inflammation and cancer [12]. In 1986 Dvorak suggested that tumors may be regarded as "wounds that do not heal" [13]. The functional relationship between cancer and the immune microenvironment is complex and dynamic because of the multiple opposing signals and feedback loops that coexists between various immune cells and cancer cells. Cells in the tumor microenevironment communicate through a plethora of cytokines and cell surface molecules which regulate the activity status, motility, proliferation and apoptosis in both the neoplastic and host cells[10]. Different immune cell types may have either anti-tumor or tumor-promoting effects and these effects may change over time as the activation status of these cells changes (Table **1**) [10, 14, 15, 7, 11, 16]. Therefore, subtypes of lymphocyte, macrophages, granulocytes, and antigen presenting cells may need to be considered separately when studying the prognostic or predictive value of the immune system [17, 11, 7]. Furthermore, the role of immune cell infiltration also differs by breast cancer subtype [18, 19]. In this paper, we will use the term "inflammation", to refer to the presence of leukocytes in the tissue and not as a clinical entity (characterized by symptoms of pain, heat, redness, swelling) [20, 21, 15]. In recent years, the classical hypothesis on immunosurveillance of cancer has been refined as "immuno-editing theory" describing how the surviving cancer represents the cell populations sculpted by the immune system [14]. According to this theory, the host immune system and the evolving tumor participate in a continuous

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Table 1. Positive and negative effects of immune cells in cancer.

Cell Type /Factors	Tumor Inhibition [7, 10, 11, 15, 25]	Tumor Promotion [7, 10, 11, 15, 25]
Natural killer (NK) cells	Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines	
$CD8^+$ Cytotoxic T cells (CTL)	Direct lysis of cancer cells; production of cytotoxic cytokines	Release of growth promoting cytokines
$CD4^+$ Th1 cells	Help cytotoxic T lymphocytes (CTLs) in tumor rejection; production of pro-apoptotic cytokines (IFNγ)	Release of cytokines
$CD4^+$ Th2 cells		Education of tumor promoting M2 macrophages; B cell activation, suppress CTLs
$CD4^+$ Th17 cells	Activation of CTLs	Production of cytokines
$CD4^+$ Treg cells	Suppression of inflammatory cytokines	Immunosuppression; production of cytokines
γδ-T cells	direct cytotoxic activity and indirect stimulation of DC or CTL	Suppression of DC maturation, immunosuppression by γδ-T-regs
B cells	Production of tumor-specific antibodies, functioning as APCs	Production of cytokines and antibodies; activation of mast cells; immunosuppression by B-reg cells
Macrophages, dendritic cells, myeloid-derived suppressor cells	M1 macrophages; Antigen presentation; production of cytokines (IL-12 and type I IFN)	M2 macrophages; Myeloid-derived suppressor cells; Immunosuppression of CTL and NK cell activity; production of cytokines, chemokines, proteases, growth factors, and angiogenic factors
Mast cells		Production of pro-angiogenic; pro-invasive cytokines; suppress CTL and NK cell activity
Neutrophils	Direct cytotoxicity; regulation of CTL responses	Production of cytokines, proteases, and ROS, mutagenic, mitogenic, pro-angiogenic, pro-invasive, pro-metastatic
Cytokine profiles	Th1 CX3CL1 CXCL9, CXCL10 CXCL13 (tertiary lymphoid structures)	Th2 Th17
Immune cell distribution	Intratumoral, close to cancer cells, in the invasive front	Peritumoral

"arms race" where the immune system constantly attacks cancer cells which escape through constant evolution towards less immunogenic or more immunosuppressive phenotypes. This mechanism results in a dynamic equilibrium of cell loss and cell escape that often ends in growth of the cancer despites active immune surveillance [22, 14]. Another important emerging concept is that the same immune factor (*e.g.* IFNγ) may sometimes function as an anti-tumor signal and in other times it may have a tumor-promoting effect depending on the local immune context which could explain inconsistent results across studies [23, 24].

TUMOR INFILTRATING LYMPHOCYTES (TILs) AS BIOMARKER IN BREAST CANCER

A large and consistent body of evidence demonstrates a positive association between the presence of tumor infiltrating lymphocytes (TILs) and better prognosis in different types of solid tumors. In colorectal and ovarian cancers, the presence of tumour infiltrating T cells is associated with reduced recurrence rates and longer survival [26, 27]. In breast cancer, similar associations are seen but the magnitude of effect and its significance varies by disease subtype. More than 20 year ago, Aaltomaa *et al*. reported improved survival for TIL-rich tumors among rapid proliferating breast cancers (n=489) [28]. Ménard and colleagues observed a positive prognostic effect of TILs in patients less than 40 years of age [29]. In these older studies estrogen receptor status was not available but it is important to note that both, high proliferation rate and young age at diagnosis are frequently associated with ER negative status [30].

IMMUNE GENE SIGNATURES

Gene expression signal originating from immune cells is easy recognized in high throughput transcriptional profiling data and the first microarray analyses of breast cancer tissues already described signatures of TILs [31, 4, 32]. Between 2007 and 2011 several larger microarray studies with clinical follow up showed a positive prognostic value of immune signatures in ER negative and highly proliferative ER positive tumors (Table **2**). Early studies identified single genes from supervised analyses comparing patients with differing prognosis among ER negative [33] and HER2-positive breast cancers [34-36]. Researchers then applied

Table 2. Gene expression based detection of TILs in breast cancer and prognosis.

Gene Expression Signature	Prognostic Value in Subgroup	n=	Study
7 gene module related to immune system	ER negative	186	[33]
651 gene cluster enriched in immune genes	HER2 positive	286	[34]
26 gene stroma derived prognostic predictor (SDPP) including 10 immune genes	Overall	53	[35]
Separate metagenes for B-cells and T-cells	Highly proliferating tumors	200	[37]
95 gene STAT1-correlated immune metagene	ER negative and HER2 positive	2100	[38]
7 metagenes discriminating distinct immune cells	ER negative and HER2 positive	1781	[39]
Immune kinase metagene	ER negative and HER2 positive	684	[45]
5 metagenes identifying immune and stroma cells	ER negative and highly proliferation ER positive	1321	[40]
"HER2-derived prognostic predictor" (HDPP) enriched in immune genes	HER2 positive and ER negative	261	[36]
386 immune related gene signature from medullary BC	Basal-like	466	[46] [36]
28-kinase metagene associated with immune response	Basal-like	591	[47] [36]
High B-cell metagene combined with low IL8-metagene	TNBC	3488	[41, 43]
3 immune metagenes	high proliferating tumors	1954	[42]
8 gene follicular helper T cell (Tfh) signature	All subtypes	794	[48]

Table 3. Predictive value of immune cell gene signatures for response to neoadjuvant chemotherapy in breast cancer.

Gene Expression Signature	Predictive Value for pCR in Subgroup	n=	Study
Immune cell marker genes identified in supervised analysis	Overall cohort	89 + 82	[49]
B-cell and T-cell metagene	Overall cohort	198	[39]
"A-score" including stroma and immune metagenes	Anthracycline based NACT	139	[50]
8 gene TIL signature	ER negative	113	[51]
7 gene module [33] and 95 probes metagene [38]	All subtypes.	996	[52]
8 gene follicular helper T cell (Tfh) signature	All subtypes	996	[48]

gene signatures or metagenes in large sample cohorts. These methods allowed distinguishing subsets of immune cells as B- and T-lymphocytes or macrophages [37-42]. The larger cohorts helped to provide answers to two important questions: First, in which subtype of breast cancer do we frequently observe TILs, and secondly in which breast cancer subtype do TILs mostly contribute to prognosis? Strongest expression of metagenes for all immune cell subtypes was detected in basal-like and HER2-like tumors while low expression was observed in luminal A and B cancers [40, 43]. Thus, TILs are found most frequently in ER negative tumors. Secondly, as shown in Table **2**, the prognostic value of immune signatures was low in luminal A tumors but strong in ER negative (basal-like and HER2-like) as well as highly proliferating luminal-B tumors [37-40]. The results made clear that separate analyses of each subtype are essential when studying TILs. Otherwise obtained data may be biased and could just represent a surrogate of the differences in prognosis between intrinsic subtypes. Examples of such separate analyses were recently published [41, 44]. Lehmann and colleagues identified six different TNBC subtypes [44]. One of them, the "immunomodulatory (IM)" subtype, was enriched for immune signaling and cell-surface antigens, even when the authors presumed that the IM characteristics may be unique to the carcinoma cells and not a reflection of immune cell infiltrates [44]. Rody *et al*. identified TILs as a continuous parameter based on gene expression signatures among all TNBC subtypes (as *e.g.* basal-like, molecular-apocrine, claudin-low) [41].

Several studies suggest that gene expression based detection of TILs is not only associated with prognosis but has also predictive value for response to neoadjuvant chemotherapy (Table **3**). This predictive ability was observed in all subtypes and seems not to be restricted to ER negative cancers. The value regarding specific treatment modalities will be discussed further below.

HISTOLOGICAL DETECTION AND QUANTIFICATION OF TILs

A beneficial influence of histologically detected TILs on prognosis in some subgroups of breast tumors had already been reported in earlier studies [28, 29]. More recently, following the observations from gene expression profiling additional studies validated the value of TILs through histological analyses of samples from large clinical trials [53-55]. They clearly demonstrated the association of an improved prognosis with increasing amounts of TILs especially in ER negative breast cancer (Table **4**). Moreover, a predictive value of TILs for response to chemotherapy was detected in the neoadjuvant GeparDuo and GeparTrio trials [56]. The predictive value was mainly seen in ER-negative cases but also in ER-positive samples to some extent [56] and has been validated in the GeparQuinto trial [57]. Data from the BIG 02-98 trial showed an excellent prognosis of the group of tumors with very high TILs (designated Lympocyte Predominant Breast Cancer, LPBC) in node-positive TNBC [53]. The effect was independent of the type of applied chemotherapy but not significant in ER-positive/HER2-negative cancers [53]. Analysis of samples from the FinHER trial confirmed these results but also suggested an association of TILs with benefit from trastuzumab in HER2 positive disease [53]. The most recent combined analysis of TNBC patients from two trials (ECOG 2197 and ECOG 1199) again reconfirmed the value of TILs as robust independent prognostic marker [55]. This "prognostic" value may result from "pure prognostic" or "pure predictive" effects or a mixture of both.

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF INFILTRATING IMMUNE CELLS IN BREAST CANCER

A considerable number of studies focused on the prognostic value of specific immunohistochemically defined subtypes of immune cells in breast cancer (Table **5**). Several studies on CD8+ cytotoxic T lymphocytes (CTLs) showed highly significant associations with prognosis. Some reported a beneficial effect for the overall cohort [59, 60] while most found it restricted to ER negative and HER2 positive and not to the ER positive/HER2 negative subtype [60-63]. Moreover CD8+ CTLs have been shown to predict the response to neoadjuvant chemotherapy [51, 64] as has also been reported for CD3+ T- and CD20+ B-lymphocytes [56]. In addition CD20+ B-lymphocytes which are mostly found in the tumor stroma have been associated with an independent positive prognostic value in multivariate analysis including CD8+ CTLs [65]. A common assumption suggests that tumors may hijack the immunosuppressive function of regulatory T cells (Tregs) which are characterized by FOXP3 positivity [11]. Consequently, the presence of FOXP3+ cells should correlate to poor prognosis. In breast cancer FOXP3+ T lymphocytes have been associated with reduced survival [66-69] but also with improved survival in the ER negative subgroup [70]. Obtained discrepancies may be explained by the use of different cutoffs, different subgroups (ER negative vs. all subtypes) and the high co-infiltration of CD8+ CTLs and FOXP3+ Tregs in tumors [70]. Moreover, expression of FOXP3 seems not to be restricted to Tregs [11, 71]. A recent study of FOXP3 in more than 5000 samples observed no significant association with prognosis [63]. In conclusion, current results allow monitoring potential antitumor immunity in breast cancer by evaluation of CD8, but we are not yet able to reliably monitor the immunosuppressive activity in the tumor immune infiltrate [72].

CD68+ macrophages were associated with poor survival but had no independent prognostic value in multivariate analysis [73]. However, DeNardo *et al*. [74] developed a combined score of high amounts of CD68+ macrophages and CD4+ T helper cells in combination with low amounts of CD8+ CTLs. This classifyer was correlated to poor prognosis. Another recent study from that group analyzed leukocyte composition of tumor biopsies and adjacent normal tissue pre and post neoadjuvant chemotherapy [75]. Results suggest that TILs mainly consist of CD4+ and CD8+ T cells, while NK cells and B lymphocytes are the minority. CD8+ T cells did not express Granzyme B, suggesting no activation at baseline. Interestingly however, in one third of the patients CD8+ cells turned to express Granzyme B after

Table 4. Histological detection of TILs in breast cancer and prognostic or predictive value.

Biomarker	Outcome Variable	Prognostic Value	Predictive Value	n=	Study
Total TILs	survival	in high proliferation tumors	n.a.	489	[28]
sTILs	survival	in patients <40 years	n.a.	1919	[29]
Continous sTILs, iTILs	survival	in TNBC	n.a.	2009	[53].
Continous sTILs	survival	in TNBC	Trastuzumab	935	[54]
Continous sTILs, iTILs	survival	in TNBC	n.a.	481 TNBC	[55]
Continous sTILs, iTILs, categorical LPBC	pCR	n.a.	Chemotherapy	1058	[56]
Continous sTILs, iTILs, categorical LPBC	pCR	n.a.	Chemotherapy	313 TNBC	[57]
Continous sTILs, categorical LPBC	pCR	n.a.	Chemotherapy	580 TNBC or HER2	[58]

TILs: Tumor infiltrating lymphocytes; sTILs: stromal TILs; iTILs: intratumoral TILs; LPBC: Lymphocyte predominant breast cancer.

Table 5. Immunohistochemical detection of specific subgroups of immune cells in breast cancer.

Cell Type	Finding	n=	Study
CD8+	Good prognosis	1334	[59]
CD8+	Good prognosis in ER-negative subgroup	1953	[61]
CD8+	Good prognosis in core-basal-like group	3403	[62]
CD8+	Good prognosis overall and in ER-negative subgroup	332	[60]
CD8+	Good progn in ER-negative and in ER-positive/HER2-positive subgroups	8978	[63]
CD8+	pCR prediction after NACT	153	[64]
CD3+, CD20+	pCR prediction after NACT	840	[56]
CD3+, CD8+	Good prognosis after Anthracycline treatment	255	[51]
CD20+	Good prognosis	1470	[65]
FOXP3+	Poor prognosis in ER positive	237	[66]
FOXP3+	Poor prognosis in ER positive and ER negative	1270	[67]
FOXP3+	No independent prognostic value	1445	[68]
FOXP3+	Good prognosis in ER negative cohort	253	[70]
FOXP3+	Poor prognosis	143	[69]
FOXP3+	No prognostic value in neither ER negative nor ER positive group	5239	[63]
CD68+	No independent prognostic value	1322	[73]
CD68↑/CD4↑/CD8↓	Poor prognosis	677	[74]
Leukocyte panel	T_H2-type response reversed by NACT	20	[75]
CXCL13+ Tfh	Good prognosis CXCL13+ Tfh cells in TLS	70	[48]

exposure to neoadjuvant chemotherapy. A recent seminal study by Gu-Trantien and colleagues [48] also showed that comprehensive profiling of the immune component is achievable. The authors profiled infiltrating CD4+ T cells from invasive tumors and compared them to those from peripheral blood. The infiltrating subpopulations included follicular helper T (Tfh) cells as well as Th1, Th2, and Th17 effector memory cells and Tregs. Presence of peritumoral tertiary lymphoid structures (TLS) with germinal centers were validated by immunohistochemistry. The TLS may represent an important site for antitumor immune responses in line with earlier studies [76]. T cell signaling pathway alterations included a mixture of activation and suppression and were reproduced with primary tumor supernatant. Extensively versus minimally infiltrated tumors were distinguishable by CXCL13-producing CD4+ Tfh cells associated with survival and pCR [48]. The results may raise the possibility that some patients might derive specific benefit from therapies designed to boost their immunity before removing the tumor, as a source of antigen.

IS IT POSSIBLE TO IDENTIFY WHICH SPECIFIC IMMUNE CELL TYPE IS RESPONSIBLE FOR OR MOST PREDICTIVE OF PROGNOSIS?

Gene expression signatures can discriminate different immune cell types within the tumor. Several reports identified specific metagenes representing signatures for either T-lymphocytes, B-lymphocytes, or dendritic cells and those of the macrophage/monocyte lineage [37, 39-41]. However, there seem to be no distinct subgroups of tumors infiltrated by only one specific immune cell type. In contrast, one usually observes co-infiltration of the tumor by several different types of immune cells. Scatter plots of metagenes measuring expression of markers for T- and B-lymphocytes within the tumor tissue demonstrate this as shown in Fig. **1**. Results were also confirmed by immunohistochemical verification of specific immune cell types [39, 56, 75]. Therefore any marker for a subtype of TILs is at the same time a surrogate for co-infiltration with other types of immune cells [77]. Further complexity comes by the fact that there are no clear cutoff values for the number of TILs or expression of metagenes regarding their prognostic and predictive value. In contrast, a continuous relationship between number of TILs and prognosis exists in several studies [41, 50, 52, 53, 56]. Consequently, superiority of any specific marker (*e.g.* for T- or B-lymphocytes or subclasses of them) in a prognostic model mostly depends on applied cutoff values and heterogeneity between different cohorts [37-39, 41, 56]. The level of gene expression per cell (as *e.g.* strong immunoglobulin expression) may even confound results of head to head comparisons of continuous scores in multivariate models [41]. Overall, the level of lymphocytic infiltration seems to have a greater effect on outcome than

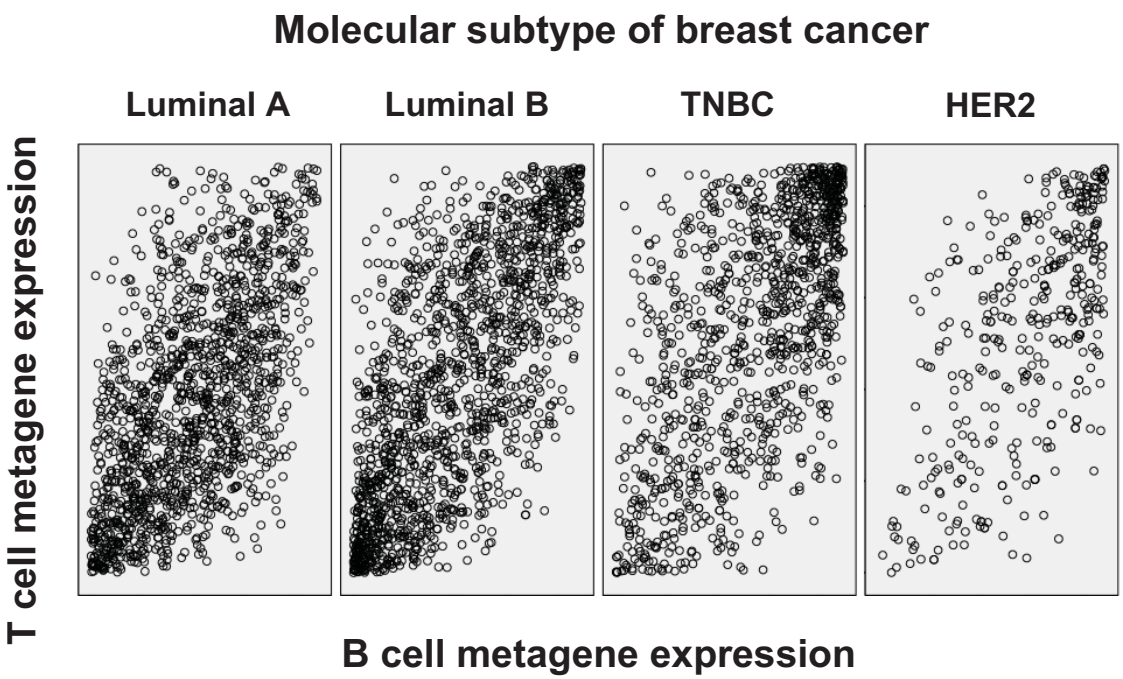

Fig. (1). Coinfiltration of different types of immune cells in breast cancer. Two metagenes specifically distinguishing T- and B-lymphocytes, respectively, in tumor biopsies were used to determine the relative amount of these TILs in 4467 breast cancer samples with available Affymetrix gene expression data [43]. Separate scatter plots of the two metagene expression values are shown for the four principal molecular subtypes of breast cancer. High expression of both metagenes is more frequent in the TNBC and HER2 groups. But generally coinfiltration of both T- and B-lymphocytes is detected as suggested by the observed high correlation of the two metagenes in all subtypes.

differences in the mixture of immune cell types. Although gene signatures are highly sensitive to the composition of the infiltrate it may be difficult to detect this in a background of differential infiltration levels between tumor samples [39]. Hence, hypotheses on causal relationships from the correlative science should use great caution. In this regard, immune signatures in ER negative and HER2 positive breast cancer face similar problems as proliferation associated markers in the ER positive subtypes. In either case there is a strong continuous relationship with outcome and a multiplicity of highly correlated biomarkers. The observations seem also to extend to analyses of immune checkpoints. E.g. in a recent paper unexpectedly both CTLA4 and PD-L2 expression were almost as good predictors of benefit from anti-PD-L1 antibody as the expression of PD-L1 [78]. A large immune gene mRNA expression panel did also not contribute much beyond the IHC based PD-L1 expression. Similarly, in a recent analysis of the GeparSixto trial all mRNA immune markers were highly correlated with one another and with TILs. Even those markers linked to immunosuppression (PD-1, PD-L1, CTLA4, IDO1) had a significant positive correlation with other immune markers and with TILs [58]. These striking findings fit with the inter-correlated nature of local immune biomarkers which may result from feedback loops between immune activation and suppression.

OPPOSING EFFECTS OF DISTINCT IMMUNE PHENOMENA - THE THERAPEUTIC POTENTIAL OF IMMUNOMODULATION AND IMMUNOTHERAPY

While evidence suggests that antitumor immunity can at least partially control progression and patient outcome in TNBC, obviously, the antitumor immunity is not efficient enough to eliminate the cancer. Tumors persist despite infiltration with tumor-specific CD8+ T cells. This apparent paradox may be partly due to the exhausted nature of tumor-infiltrating T cells and the presence of immunosuppressive factors in the tumor microenvironment [79]. Reciprocal effects on prognosis were found for some types of immune cells *e.g.* CD68+ and CD4+ cells [75] allowing their use as a combined prognostic score. Similarly, the combination of a B-cell metagene predicting good prognosis with the opposing effect of an IL-8 metagene resulted in a clinically relevant gene signature for triple negative and basal-like breast cancer [19, 41, 43]. The dynamic plasticity of the tumor microenvironment itself adds further complexity to the task of translating immune cancer genomics into cancer therapies. Shifts in T-cells *e.g.* from a T_H17 commitment to a T_H1-, T_H2- or T-reg-specific pattern ultimately determine their functional role [80]. On the other hand, modulation of T-cell response has demonstrated clinical efficacy in solid tumors [81]. Examples include new therapeutic antibodies that unleash the antitumour properties of the immune system effectively as ipilimumab, or antibodies that block PD1 (programmed cell death 1) and PD-L1 (programmed cell death 1 ligand 1) [78, 82-86]. Thus, it might be worth to evaluate a modulation of the immune system in TNBC or HER2-subtype breast cancers with TILs to enhance antitumor activity, for example by clinical evaluation of immune checkpoint inhibitors [72, 78, 79]. As TILs in breast cancers predominantly reflect a T_H1 immune response, targeting immunosuppressive mechanisms may be a promising approach and preclinical data of the HER2-subtype support this notion [87, 88]. Especially for the

HER2 subtype it has been speculated that combination of HER2-directed drugs and immune modulation *e.g.* by inhibiting immune checkpoints may result in augmented response and clinical outcome [89]. Further questions would be how to transform a TIL-negative tumor into a TIL-positive tumor or the potential use of tumor vaccines. Different approaches of cancer immunotherapy using tumor antigens in TNBC have been recently reviewed [79]. CTLs must recognize specific antigens on tumor cells presented by MHC-I. Antigens preferentially expressed in tumors such as cancer testis (CT) antigens represent attractive candidates for cancer immunotherapy. High frequency of CT-X antigen expression has been observed especially in TNBC [90-92].

MECHANISTICAL HYPOTHESES TO EXPLAIN HOW IMMUNE INFILTRATION CAN SERVE AS PROGNOSTIC AND PREDICTIVE BIOMARKER

Both TNBC and HER2 subtypes of breast cancers display high proliferation and genomic instability. Since these subtypes are also characterized by higher TIL levels it has been suggested that genomic instability may lead to neo-antigen generation serving as targets and attractors for immune cells [93]. However, genomic instability is also associated with clonal heterogeneity and may facilitate tumor escape from immunosurveillance. Indeed a cancer exome analysis study suggested a T-cell-dependent mechanism of cancer immunoediting [94]. It is clinically apparent that even if TILs contribute to, and partially mediate, the better prognosis seen in TIL rich TNBC, they only partially control micro-metastasis since the prognostic effect is significant but modest. This raises the possibility that further activating the local immune system with checkpoint inhibitors as adjuvant therapy could improve the survival of TNBC or HER2+ breast cancers [93].

Why do only a subset of tumors have TILs and why is TIL count variable over a broad range? Genomic data suggest that it is not the mutational load *per se* but rather those mutations which are immunogenic that determine the presence of TILs [95]. One has to bear in mind that immunogenic mutations may or may not have oncogenic functions [72]. Recent data also suggest that the presence of Tfh cells in tertiary lymphoid structures can serve as a morphologic surrogate marker indicative of effective local anti-tumor immune response. However, correlation between immunogenic mutation load and tertiary lymphoid structures has not yet been studied. There is interest in learning how the antigenicity of cancer could be augmented in addition to boosting the existing, although often attenuated, local anti-tumor immune response [48].

TILs are also implicated in mediating cytotoxic response to chemotherapies which could explain its predictive function. The quantity of TILs present in the residual disease after neoadjuvant chemotherapy is significantly associated with a better prognosis, and increasing lymphocytic infiltration during neoadjuvant chemotherapy has been shown to correlate with smaller residual cancer burden [96-98]. This supports other data that chemotherapy can favorably modify the tumor immune microenvironment, perhaps by altering T effector / T regulatory cell fraction, removing myeloid-derived suppressor cells and/or creating new tumor antigens [97, 99]. Moreover, the nature of cell death induced by different chemotherapy drugs can be either non-immunogenic (*e.g.* etoposide or mitomycin) or immunogenic (*e.g.* doxorubicin, oxaliplatin, or cyclophosphamide) [100]. It has been shown that immunogenic cell death leads to effective activation of adaptive immunity that significantly contributes to overall antitumour effects of these agents [101-103]. Anthracycline-based chemotherapies have been shown to induce a vigorous infiltration of anticancer immune effectors in mice [104] and to require priming of IFNγ-producing CD8+ T cells [105]. Calreticulin, HMGB1, and ATP act in concert to promote tumor antigen presentation by dendritic cells *via* activation of TLR4 and P2RX7 [100].

Immune markers and TILs also play an important predictive role in HER2 positive breast cancers. Anthracyclines may somehow relieve or modulate immunosuppression in HER2 positive cancer [53]. Also results from FinHER trial suggest that trastuzumab is most efficacious in the presence of TILs [54]. Trastuzumab can interfere with HER2 signalling and also kill HER2-expressing tumor cells *via* antibody-dependent cellular cytotoxicity (ADCC) directed by the Fc receptor present on natural killer cells as well as macrophages, neutrophils, and eosinophils [106]. The combination of trastuzumab with the HER2-kinase inhibitor lapatinib increases ADCC because of accumulation of HER2 at the cell surface [89]. Nevertheless, the innate immune response alone seems to be insufficient for a therapeutic effect of HER2-directed antibodies, with the adaptive immune response also needed [87, 89, 100, 107].

CURRENT CLINICAL UTILITY AND FUTURE POTENTIAL OF IMMUNE CELL INFILTRATES AS BIOMARKER IN BREAST CANCER

Traditionally, breast cancer has not been deemed as an immune system related cancer and shows no significantly increased or worse prognosis in transplanted patients or patients with HIV. However, these older studies did not examine the incidence or outcome of different subtypes separately and may have had limited power to inform on the importance of immune surveillance since breast cancer tends to develop in older patients and includes a large subset that has indolent course (luminal A cancers). In contrast to these epidemiological studies, a large body of literature suggests that lymphocytes in the tumor microenvironment are prognostic, which raises the hypothesis that they mediate the better survival. Technically, a histological assessment of TILs is relatively straight forward and the inclusion of such methods in standard pathology reports has been proposed [93, 108-110]. Histological assessment allows the simultaneous quantification of stromal, peri-, and intra-tumoral lymphocytes. However, it remains somewhat subjective and semi-quantitative. Digital imaging has been proposed to improve reproducibility and quantification [111]. Consensus efforts to develop an “immunoscore” for inclusion into traditional classification of breast cancer are ongoing [110]. An important observation that has emerged from these studies is that TILs do not represent a dichotomous variable but rather a continous variable both with respect to measurement and their prognostic value [53, 56]. The situation is similar to what is seen with Ki67 or

proliferation associated multi gene assays in luminal subtypes of breast cancer. The optimal cutoff value for these biomarkers will depend on the clinical question and different cutoffs may be required for distinct breast cancer subtypes. The most consistent prognostic function for TILs is observed in TNBC and has been observed in patients treated with surgery alone, as well as in patients who received state of the art adjuvant or neoadjuvant chemotherapies [53-55, 98]. Adams *et al*. performed a prospectively validation of the prognostic value of TILs in TNBC using a previously reported method and thresholds [55]. Hazard ratios for DFS and OS were remarkably similar to the original report. Thus, there is now high level of evidence for better outcome in TNBC treated with adjuvant anthracycline based chemotherapy. Cancers classified as lymphocyte predominant (LPBC) corresponding to 6-12% of all TNBC have a particularly favorable prognosis [109]. In the BIG 02-98 trial, the 9-year overall survival was 92% for LPBC TNBC compared to less than 70% overall survival in the remaining 229 TNBC with no or lesser lymphocytic infiltration [53]. Medullary cancers also represent a special, rare (1%) histologic subtype of TNBC with very high lymphocytic infiltration and excellent prognosis [112]. In addition, the data from neoadjuvant studies also indicate that TNBC with TILs experience higher pathologic complete response rates [52, 112]. Because TILs are not only associated with better (although not perfect!) prognosis in the absence of adjuvant chemotherapy but are also predictive of greater chemotherapy benefit, TILs cannot be used to identify TNBC patients for whom chemotherapy would not be recommended. Thus at present, the clinical utility of TILs as prognostic marker in daily management of TNBC is clearly limited. But TILs could be of high value for stratification in future clinical trials enrolling TNBC patients once the evaluation has been standardized [109, 110]. Presently it is not clear whether subtyping of immune cells or more complex immune gene signatures add any significantly more predictive or prognostic information to the TIL quantification.

In contrast to TNBC, TILs have less pure prognostic value in the HER2 subtype but continue to show predictive function to chemotherapy and trastuzumab [88]. Gene expression immune scores were also predictive for response to different neoadjuvant chemotherapies in the HER2 group [52, 113]. Moreover, six studies analyzing a total of more than 5500 HER2 positive samples from clinical trials (one peer reviewed [54] and five presented as meeting abstracts [114-118, 113]) suggest that TILs are also associated with increased benefit from anti HER2 targeted therapy [111]. The low toxicity of trastuzumab makes it unlikely that any immune biomarker will have enough specificity to warrant withholding trastuzumab, but a potential clinical utility might be selection of patients suitable for dual anti-HER2 blockade. So TILs may not be used to decide whether a patient should receive trastuzumab but may define those with good prognosis that might not require further addition to trastuzumab [111]. In the NeoSphere trial increased expression of immune metagenes linked to the adaptive immune system were associated with a higher likelihood of achieving a pCR while high expression of PD-L1 was associated with a lower pCR rate [89, 114].

CONCLUSION

Breast cancer is a heterogeneous disease and variations in clinical outcome extend beyond the molecular subtypes of the neoplastic cells. Even within the same molecular subtype, substantial case-to-case cellular and molecular heterogeneity exists in the stromal components of the cancer. Variations in immune cell composition in the tumor microenvironment show a strong association with prognosis and response to chemotherapy in TNBC and HER2 subtypes. More tumor infiltrating lymphocytes at the time of diagnosis predict for better outcome with or without systemic chemotherapy and also for higher rates of response to neoadjuvant chemotherapy. These prognostic and predictive functions are independent of proliferation rate and anatomical risk factors in these cancer subtypes. Inclusion of immune parameters in future multivariate prognostic and predictive models could result in more accurate prognostic estimates. Quantification of immune parameters may also help identifying patients who may benefit the most from immune-checkpoint therapies which are now tested in clinical trials.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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