

Clinical relevance of host immunity in breast cancer: from TILs to the clinic

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Abstract | The clinical relevance of the host immune system in breast cancer has long been unexplored. Studies developed over the past decade have highlighted the biological heterogeneity of breast cancer, prompting researchers to investigate whether the role of the immune system in this malignancy is similar across different molecular subtypes of the disease. The presence of high levels of lymphocytic infiltration has been consistently associated with a more-favourable prognosis in patients with early stage triple-negative and HER2-positive breast cancer. These infiltrates seem to reflect favourable host antitumour immune responses, suggesting that immune activation is important for improving survival outcomes. In this Review, we discuss the composition of the immune infiltrates observed in breast cancers, as well as data supporting the clinical relevance of host antitumour immunity, as represented by lymphocytic infiltration, and how this biomarker could be used in the clinical setting. We also discuss the rationale for enhancing immunity in breast cancer, including early data on the efficacy of T-cell checkpoint inhibition in this setting.

Tumour-infiltrating lymphocytes (TILs) are mononuclear immune cells that infiltrate tumour tissue, and have been described in most types of solid tumours, including breast cancer, colon cancer, cervical cancer, melanoma and lung cancer^{1,2}. The presence of TILs in breast tumours and a positive association between higher numbers of TILs and more-favourable clinical outcome was documented in a clinical cohort as early as 1922 (REF. 3). The relationship between higher levels of TILs and improved prognosis in patients with early stage breast cancer has now been confirmed in thousands of patients. A number of studies have also contributed to understanding the function and origins of TILs^{4–11}. An early report associated high levels of lymphocytic infiltrates in breast cancers with *HER2* amplification, which presaged important clinical observations reported many years later⁴. These early studies identified TILs in breast cancer as a lymphocyte population that mainly comprise cytotoxic (CD8⁺) T cells, together with varying proportions of helper (CD4⁺) T cells and CD19⁺ B cells, and rare NK cells^{5,6}. *In vitro* studies demonstrated the tumour killing capacity of TILs isolated from breast-tumour tissues, showing they were capable of cytolytic activity and cytokine secretion (Glossary BOX 1) on admixture with the autologous tumour tissue from which they were derived^{7–9}. The notion that tumours develop mechanisms to avoid recognition by the immune system was also heralded by

the observation that tumours with low human leukocyte antigen (HLA) class I expression showed reduced infiltration with CD8⁺ T cells⁵. The role of CD8⁺ and CD4⁺ T cells in recognizing unique tumour antigens was also noted^{10,11}. Since these early studies, modern techniques have dissected the tumour immune microenvironment in great detail, providing a strong foundation for exploiting antitumour immunity in the management of breast cancer. This approach is particularly relevant given the advent of effective immunotherapies that have become the standard of care in treating other malignancies, such as melanoma and non-small-cell lung cancer.

In this Review, we focus on the importance of TIL populations in breast cancer, introduce how they are measured in clinical practice, and discuss their composition. We review data supporting the use of TILs as a prognostic and predictive biomarker, and explore how this application relates to our understanding of the tumour immune microenvironment. Studies of immune-checkpoint inhibitors in patients with triple-negative breast cancer (TNBC) are discussed, as are upcoming immunotherapy trials and future directions for research.

Defining TILs in breast cancer

In contrast to mucosal tissues, such as the intestine, normal breast tissue does not contain large aggregates of immune cells¹². Some immune-cell populations exist in

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doi:10.1038/nrclinonc.2015.215
Published online 15 Dec 2015

Key points

- The detection of tumour-infiltrating lymphocytes (TILs) on routine histology constitutes a robust prognostic and predictive biomarker in patients with early stage breast cancer, despite the complexity of host antitumour immunity
- Ongoing efforts to ensure reliable and reproducible reporting of TILs will facilitate their use in the routine management of breast cancer
- Exploiting the antitumour immune response in breast cancer for therapeutic benefit is currently an area of active research
- Early phase trials of antibodies that target programmed cell-death protein 1 (PD-1) and PD1 ligand 1 in patients with metastatic triple-negative breast cancer have shown promising and durable responses
- Useful biomarkers to predict benefit from immunotherapy are urgently needed — TILs might fulfil this role

Box 1 | Glossary

- Adjuvant therapy: systemic therapy after surgery
- Adoptive T-cell therapy: a class of therapies in which modified T-cells, usually autologous, are transferred to a patient
- Biomarker: a particular feature of a tumour that can be assayed. Prognostic biomarkers predict survival; predictive biomarkers predict benefit from therapy
- Cytolytic activity: the mechanism by which immune cells cause lysis of target cells, commonly through the delivery of the pore-forming protein perforin and the protease granzyme
- Flow cytometry: analysis of single cells using a variety of cell surface and intracellular fluorescent markers
- Immunohistochemistry: labelling of protein structures on histological slides with antibodies and a chromogenic or fluorescent reporter that renders the feature visible
- Modified immune-response-related criteria: a modification of the traditional criteria for determining disease progression or response, that takes into account that immunotherapy can cause transient increases in size of cancer lesions before a response manifests
- Neoadjuvant therapy: systemic therapy before surgery
- Nonsynonymous mutation: alterations in the DNA sequence of a gene that alters the amino acid sequence of the translated protein
- pCR: pathological complete response of a tumour. Refers to the absence of residual tumour when a surgical specimen is examined with light microscopy. Certain definitions also include complete response in draining lymph nodes
- Peyer's patches: specialised lymphoid aggregations found in the ileum
- Pseudoprogression: the phenomenon of temporary enlargement of a tumour lesion due to immune infiltration and inflammation
- Type I interferon immune response: a battery of interferons secreted in response to bacterial or viral infection
- Tumour-cell nest: an aggregation of tumour cells, without intervening stroma or nonmalignant cells
- Whole-exome sequencing: sequencing the DNA of the coding regions in the genome, which account for approximately 1% of the whole genome

nonmalignant breast tissue. For example, macrophages have an important role in the development of the normal breast lobular anatomy¹² and, during lactation, mucosa-associated B cells produce immunoglobulins that are secreted in breast milk¹³. Low numbers of CD8⁺ and CD4⁺ T cells can be purified from bulk normal adult breast tissue, but they are rarely detected on histological examination and their functional significance is uncertain^{14,15}. In contrast to normal breast tissue, breast tumours and their adjacent stroma display higher levels of immune-cell infiltrates^{14,16}. TILs are more commonly found in TNBC

and HER2-positive breast cancers^{17–19}. In one of the largest studied cohorts of women with lymph-node-positive disease, the median percentage of stromal tissue infiltrated with TILs was 10% in oestrogen receptor (ER)-positive and HER2-negative samples, 15% in HER2-positive samples, and 20% in ER-negative and HER2-negative disease¹⁸. Although a prognostic association between stromal TILs and ER-positive and HER2-negative early stage breast cancer has not been found, expression of immune-related genes in this subtype is associated with a better prognosis²⁰, and a higher chance of pathological complete response (pCR) to chemotherapy²¹.

Detecting and quantifying TILs

TILs are commonly detected in haematoxylin and eosin (H&E) stained histological slides via light microscopy, wherein they are distinguished by the typical features of lymphocytes or by additional immunohistochemical (IHC) staining for lymphocyte markers (BOX 2). When evaluating H&E-stained slides, TILs are divided into stromal-compartment TILs and intratumoural-compartment TILs²². Stromal TILs infiltrate the stromal tissue adjacent to the tumour cells — as this fibrous stroma is part of the malignant tumour, stromal immune cells are considered to be true tumour-infiltrating cells. Intratumoural TILs actively infiltrate tumour-cell 'nests' where they have direct contact with tumour cells²². At any given point in time, the majority of TILs in breast cancers are typically located in the tumour stroma²². Histological specimens are not able to reveal the spatio-temporal dynamics of the immune microenvironment, although the simple classification of TILs into stromal compartment-TILs and intratumoural compartment-TILs in H&E-stained sections remains useful (FIG. 1). Both stromal and intratumoural TILs comprise a complex mixture of different lymphocyte subtypes, dominated by T cells, with lesser proportions of B cells, NK cells, and macrophages^{14,23}.

Once identified, TILs populations are quantified by determining how much of a demarcated area of stroma or tumour present on a slide is infiltrated by immune cells²². Intratumoural and stromal TILs populations are highly correlated, although stromal TILs are typically observed in higher numbers and display much larger variability between different tumours²⁴. Whereas some tumours have no stromal TILs, others have more than 10,000 immune cells per square millimetre of stromal tissue. Stromal TILs are thus more reliable biomarkers than intratumoural TILs in predicting response to therapy and patient outcome, when assessed via light microscopy of H&E-stained tissue slides^{22,24}. The use of IHC staining permits more-accurate identification of intratumoural TILs, as they can be more easily distinguished from epithelial tumour cells by this method²².

Difficulties in evaluating TILs

The distribution of TILs in a tumour can cause difficulties in consistent evaluation on H&E-stained sections. These issues are summarized in this section, and have been addressed in more detail elsewhere²². Stromal TILs occur in a variety of patterns, including a diffuse but even

Box 2 | Methods for TIL evaluation

TILs may be assessed in a variety of ways that have different advantages and disadvantages.

Haematoxylin and eosin (H&E) staining

Advantages

- Low cost
- Aligned with routine clinical pathology
- Accounts for intratumoural heterogeneity of lymphocyte infiltrates

Disadvantages

- Nonspecific
- Does not delineate different lymphocyte subsets that might differ greatly in their roles in antitumour immunity

Staining for immunohistochemical markers

Advantages

- Permits a more-functional assessment of the tumour microenvironment
- More-accurate distinction of lymphocytes admixed with tumour cells (intratumoural lymphocytes)
- Immunohistochemical markers of different lymphocyte subsets can be utilized; useful immunohistochemical markers include CD3 and CD8 (T cells), CD20 (B cells) and CD103 (intraepithelial T cells and dendritic cells)
- Can be performed on large numbers of samples by utilizing tissue microarrays (TMAs), whereby a core of tissue from the larger primary specimen is combined with many other samples on the same slide

Disadvantages

- The small portion of tissue examined by TMAs might introduce sampling bias in heterogeneous samples
- Might exclude important peripheral features, such as tertiary lymphoid structures
- Additional tissue processing over routine diagnostic pathology
- Problems with unreliable and unstandardized immunohistochemical markers, as is evident with PD-L1 staining antibodies¹⁰¹

distribution, a focal infiltrate, or multifocal 'sprinkling' across the tumour bed. Sometimes a gradient exists between zones of high and low TILs numbers within the stroma of a single tumour, and confluence with lymphocytes adjacent to normal lobules or around foci of ductal carcinoma *in situ* (DCIS). In addition, specific tumour-growth patterns can influence the disposition of TILs in a manner that might compromise comparison across different tumours. A diffuse and solid tumour-growth pattern, consisting of dense tumour-cell nests with only limited intervening stroma, is rapidly evaluated as having a high level of TILs because the stromal compartment is small. On the other hand, tumours with a more infiltrative and dissociative growth pattern and larger areas of stroma will generally be scored as having lower TILs²². Furthermore, the scoring of TILs can also be complicated by the existence of different histological subtypes of breast cancer. In the classic subtype of invasive lobular adenocarcinoma, distinguishing infiltrating tumour cells from TILs in the stroma is not always straightforward. Further difficulties can arise from interpreting the remarkable perivascular abundance of TILs in some tumours with minimal stromal infiltration, and distinguishing clearly intratumoural TILs from tumour cells when IHC staining is not used. Finally, the clinical significance of the spatial heterogeneity in TILs is undetermined and the best approach to scoring tumours in this scenario remains uncertain²⁵.

All the above-mentioned factors can potentially increase the interobserver and intraobserver variability of manual TILs assessment. Despite the challenges pathologists face in scoring TILs, the reported prognostic and predictive value of TILs has been very consistent across multiple datasets analysed by independent investigators²⁶. As with any biomarker, the utility of TIL assessment relies on the development of a standardized and reproducible scoring methodology. International guidelines for the evaluation of TILs were published in 2014²², are an important step in this process (TABLE 1). The proliferation marker Ki67 is an example of a useful biomarker that has failed to enter routine practice due to inconsistent results between different laboratories and pathologists²⁷, and care is needed to prevent TILs from becoming another example.

Tertiary-lymphoid structures (TLS) are not a common feature of the immune microenvironment in breast cancer²². TLS resemble secondary lymphoid organs, such as lymph nodes or Peyer's patches, and consist of a follicular aggregate containing predominantly B lymphocytes with a germinal centre containing follicular dendritic cells^{23,28}. TLS are thought to be important in the generation of antigen-specific memory T cells and B cells²³. TLS typically form an incomplete ring around the outer perimeter of the tumour in the adjacent normal tissue. Owing to the small and discontinuous nature of TLS, they are more difficult to detect and might be entirely excluded when only small areas of tissue from core biopsies or tissue microarrays are examined. The body of evidence describing the clinical significance of TLS is, therefore, more limited than that for TILs; however, their presence has been associated with favourable outcomes in patients with breast cancer in the small studies that have been conducted to date^{16,23}.

The richness and diversity of the antitumour immune response is, of course, severely underestimated by assessment with H&E or a few IHC markers. More-comprehensive techniques, such as flow cytometry²⁹ and multicolour IHC staining with multispectral imaging³⁰, enable the quantification and localization of different lymphocyte subpopulations. In a complementary fashion, gene-expression analysis (measured using quantitative polymerase chain reaction (qPCR³¹), RNA microarrays²³ or RNA sequencing (RNASeq³²), also provide information about relevant lymphocyte subpopulations. As a result of the aforementioned techniques, it has now been established that TILs in breast tumours comprise lymphocyte subpopulations with differing functional significance (TABLE 2). This detailed decomposition of TIL subpopulations is desirable, but such an analysis is not generally feasible in studies involving large cohorts of patients, because tissue samples collected in clinical trials are not routinely suitable for flow cytometry, and the amount of archived tissue available is usually quite limited. In this situation, the interpretation of the immune microenvironment often relies on the simple evaluation of standard H&E-stained sections, as discussed earlier. Indeed, in a patient cohort of a randomized trial of neoadjuvant chemotherapy, the predictive value of evaluating TILs in H&E-stained samples has been shown to be very similar to that derived from the analysis of immune gene-expression profiles³¹.

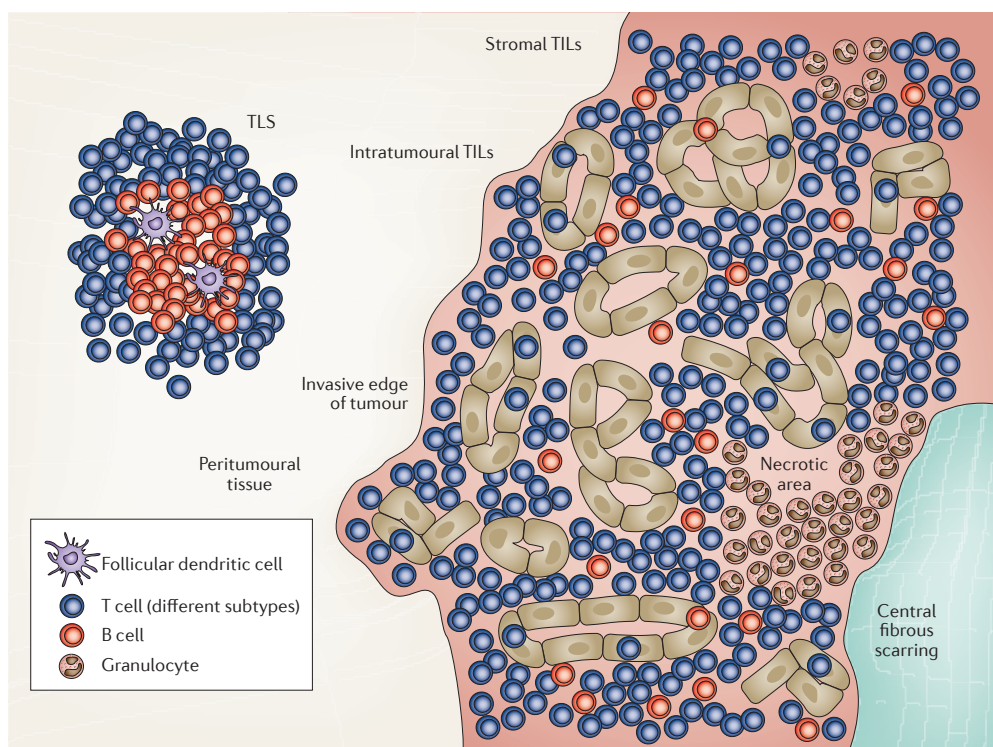


Figure 1 | Diagram of the immune microenvironment in breast cancer. Tumour-infiltrating lymphocytes (TILs) can be detected in breast tumours in the stromal tissue adjacent to the tumour (stromal TILs), or actively infiltrating tumour areas in direct contact with tumour cells (intratumoural TILs). The TIL population consists of cytotoxic (CD8⁺) and helper (CD4⁺) T cells, B cells, follicular dendritic cells and granulocytes. TLS, tertiary lymphoid structure.

Clinical significance of TILs

In patients with early stage breast cancer, no well-validated biomarkers contribute additional information to determining prognosis beyond that provided by analysis of well-characterized clinicopathological factors, such as tumour size and grade, nodal status, hormone-receptor and HER2 status, and more recently, gene-expression-based recurrence scores. In the neoadjuvant setting, a high priority has been assigned to achieving a pCR with neoadjuvant chemotherapy for HER2-positive and TNBC, as this is associated with excellent long-term survival³³. The clinicopathological factors mentioned, as well as the use of proliferation markers, such as Ki67, can help identify the patients who are more likely to respond to neoadjuvant chemotherapy^{31,34–36}. However, these factors are not sufficiently accurate to distinguish the patients who have virtually no chance of a pCR and would, therefore, be candidates for alternative therapy, or the patients who have a very high chance of pCR, such that more-intensive therapy is definitely not required³¹. TILs might fulfil the need for a better prognostic and predictive biomarker that could be incorporated into routine care. Many retrospective case studies have produced results showing a positive association between higher TIL numbers and prognosis or response to treatment, but more-robust evidence arises from so-called retrospective–prospective studies³⁷. In these studies, TILs are assessed in tumours samples

from patients enrolled in clinical trials, which offers the advantage of randomization, consistent and well-documented treatment, large sample sizes, and complete clinical follow up³⁷. Several clinical trials have examined the prognostic value of TILs, or surrogates thereof (TABLE 3), as well as the association of TILs with pCR (TABLE 4).

TILs as a prognostic biomarker

In the past 5 years, TILs have been evaluated in nearly 16,000 patients in prospective studies with clinical follow-up data available, which highlights a remarkably rapid accumulation of evidence^{18,19,38–40}. The conclusions regarding prognosis have been relatively consistent. Patients with TNBC display a robust linear relationship between an increase in TILs numbers over time and improved recurrence-free survival (RFS) end points, as noted in retrospective–prospective analyses performed on the Breast International Group (BIG) 2–98 trial¹⁸, Finland Herceptin Trial (FinHER³⁸), Eastern Cooperative Oncology Group (ECOG) 2197 and ECOG 1199 trials³⁹, and the National Epirubicin Adjuvant Trial (NEAT)/BR9601 trial¹⁹. A similar relationship between RFS and TIL population was seen for patients with HER2-positive tumours in the NeoALLTO trial⁴⁰ and the HER2-positive patient cohorts reported by Ali *et al.*¹⁹. Interpretation of the data for HER2-positive tumours is complicated by the varied use of adjuvant trastuzumab

Table 1 | **Standardized evaluation of TILs as a biomarker in H&E sections**²²

Parameter	Recommendation
Selection of tumour area	Evaluate only immune cells within the tumour margin, do not include immune infiltrate around DCIS or normal tissue next to the tumour Do not focus on the invasive edge, but include invasive edge in overall assessment Do not focus on hot spots, but provide overall assessment of TILs Do not include necrosis or large fibrous areas in the tumour centre
Type of immune cell	Evaluate all mononuclear lymphocytes and plasma cells Do not include granulocytes in necrotic areas
Localization of immune cells	Stromal TILs: in the fibrotic tissue between tumour cell nests Intratumoural TILs: directly infiltrating the tumour cell nest, direct contact to tumour cells Stromal and intratumoural TILs should be evaluated separately and reported semi-quantitatively as a percentage of the total area occupied by the infiltrate Example images for standardized reporting of percent stromal infiltration have been published ²² ; software-based systems are in preparation
Analysis of immune biomarkers	Stromal TILs: it is currently recommended that this is used as the primary end point as a continuous variable Intratumoural TILs: can be reported as a secondary end point Use of the LPBC binary variable as previously defined (>50–60% of the tissue comprises stromal and/or intratumoural TILs) is not encouraged as the intratumoural TILs variable is not highly reproducible between pathologists ^{24,31}

DCIS, ductal carcinoma *in situ*; H&E, haematoxylin and eosin; LPBC, lymphocyte-predominant breast cancer; TIL, tumour-infiltrating lymphocyte.

in early study cohorts and the fact that trastuzumab benefit seems to be greater in patients with higher TILs numbers, as seen in the FinHER data³⁸. Finally, no significant prognostic value has been found for TILs in ER-positive HER2-negative disease¹⁸.

TILs as a predictive biomarker

Results from several studies using adjuvant therapy suggest that TILs might also serve as a predictive biomarker of benefit from cytotoxic chemotherapy (BOX 3). In the BIG 02–98 trial¹⁸, evidence suggested that increased levels of TILs were associated with greater benefit from anthracycline chemotherapy in patients with HER2-positive disease. This association was only noted for anthracycline alone and not for the combination of anthracycline and docetaxel. In the randomized NEAT/BR9601 study¹⁹, it was observed that the presence of CD8⁺ T-cells was associated with greater benefit from epirubicin, with no differences between histological subgroups. This finding is based in preclinical studies, which have shown that anthracycline-induced tumour-cell death is particularly immunogenic^{41,42}.

The presence of TILs is also associated with increased rates of pCR. Initial evidence from a randomized controlled trial of the association between increased TILs and pCR rates was reported by Denkert and coauthors²⁴. The GeparDuo and GeparTrio clinical trials of neoadjuvant anthracycline and taxane chemotherapy enrolled a total of 1,058 patients with samples that could be assessed for TILs²⁴. All histological subtypes were included, and

trastuzumab was not used. TILs were analysed as a continuous variable, and also as a dichotomous variable with a cutoff of 60% (with lymphocyte predominant breast cancer (LPBC) defined as the presence of ≥60% TILs). The pCR rate was roughly 30% higher in tumours with TILs ≥60% compared with tumours with ≤60% in GeparDuo, and this finding was also observed in the GeparTrio trial²⁴. The data from more than 2,000 patients in prospective studies have now been analysed, with the consistent result that increased TILs are associated with higher rates of pCR after neoadjuvant chemotherapy and targeted therapy^{24,31,43–46}. In the most-recent study to be published, an analysis of GeparSixto³¹, patients with LPBC who were treated with carboplatin in addition to anthracycline and taxane had impressive pCR rates (74% and 78% in TNBC and HER2-positive disease, respectively) compared with the pCR rate of patients with LPBC not treated with carboplatin (43% and 50% in TNBC and HER2-positive disease, respectively). This difference was highly significant ($P < 0.005$). Most of the patients in the neoadjuvant studies mentioned above had TNBC or HER2-positive disease, although GeparDuo, GeparTrio, and GeparQuinto included small numbers of patients with ER-positive tumours. Exploratory analyses in this subgroup revealed that patients with LPBC had a statistically significant higher rate of pCR compared with patients with non-LPBC^{24,43}.

TILs as biomarkers of residual disease

Residual disease following neoadjuvant chemotherapy in TNBC is considered a very poor prognostic marker³³. Neoadjuvant chemotherapy (NACT) can result in the accumulation of TILs in the residual tumour, and depletion of immunosuppressive regulatory T (T_{REG}) cells, which is expected to portend a favourable outcome^{47–49}. However, NACT might also promote tumour infiltration by macrophages, which are associated with an increased chance of recurrent disease^{14,47,49}. Several studies have shown that the presence of TILs in patients with residual disease, nevertheless, confers a good prognosis in an otherwise poor prognostic group^{50–52}. The attainment of a pCR, while strongly associated with a good prognosis, is not a validated surrogate for improvement in more-robust survival end points, such as event-free survival and overall survival³³. In the NeoALTTO study, in which both pCR and event-free survival end points were assessed, TILs provided prognostic information independent of pCR rates⁴⁰. In particular, those patients with high TILs had the most-favourable outcomes, even if they did not achieve a pCR⁴⁰. By contrast, those patients who did not achieve a pCR and had low levels of TILs had a poor event-free survival (EFS) at 3 years (67%)⁴⁰. These results suggest the possibility that a group of HER2-positive patients with high TILs have an excellent outcome independent of achieving a pCR and only require trastuzumab as single anti-HER2 therapy with their chemotherapy, rather than more expensive or toxic combination therapies.

The immune response in breast cancer

The data discussed in previous sections are in agreement with the considerable literature supporting the substantial

immunomodulatory effects of chemotherapy. Broadly speaking, both in the adjuvant and neoadjuvant settings, chemotherapy might relieve immunosuppression and 'reset' attenuated functional immunity. An immune response could also be triggered by chemotherapy-induced cell death; this response might contribute to eliminating the remaining viable tumour in the breast and distant micrometastatic disease. These concepts will be discussed in the following sections.

Origin of the breast-cancer immune response

Studies performed in melanoma show that CD4⁺ and CD8⁺ T cells that are reactive to peptides arising from cancer-specific mutations (termed 'neoantigens') can be identified in tumour infiltrates^{53,54}. Furthermore, in a patient with advanced-stage cholangiocarcinoma, regression of metastatic lesions was induced by purifying, expanding, and re-infusing clonal T cells specific to an epitope arising from a single mutation found in the tumour⁵⁵. In patients with breast cancer, it has been reported that cytotoxic T cells extracted from tumours are specific to antigens present in the tumour from

which they were derived, although these antigens were not defined⁷. In addition, in a study across multiple tumour types using The Cancer Genome Atlas (TCGA) data, investigators found a variable correlation between increasing tumour mutational burden and expression of T-cell effector function, although this correlation was only weakly positive in breast cancers³². Given these findings, it seems likely that an important stimulus for immune recognition of breast tumours is the repertoire of mutant peptides produced by the tumour. Extensive studies of this process in model systems have refined the general concept of 'immunoediting'⁵⁶. Initially, the immune system efficiently removes immune-naïve tumour cells, but some tumour-cell clones might survive. This is followed by an equilibrium or dormant phase, during which the host immunity controls the cancer but does not eliminate it completely, permitting eventual evolution and outgrowth of tumour-cell clones that eventually escape immune control through various mechanisms, including the diminution of antigenic stimuli and the generation of an immunosuppressive tumour milieu. In the breast-cancer setting, some questions about

Table 2 | Tumour-infiltrating lymphocyte subpopulations and molecular markers

Cell	Markers	Subtypes	Normal function	Checkpoint markers	Known/suspected function in tumour microenvironment	Proportion of TILs
Cytotoxic T cells	CD3, CD8	NA	Cytolysis of virally infected cells	PD-1, PD-L1, CTLA-4, TIM3, OX40	Cytolysis of tumour cells	~20%
Helper T cells	CD3, CD4	T _H 1	Response to intracellular pathogens	OX40	Support CD8 ⁺ T cells by secreting IFN γ	~40%
		T _H 2	Response to parasites	NA	Associated with ineffective antitumour immunity	
		T _H 17	Response to fungi and extracellular bacteria	NA	Antitumour and protumour effects	
		T _{FH}	B-cell help	NA	Role in tertiary lymphoid structures	
		T _{REG}	Self-tolerance	CTLA-4	Tolerance of tumour antigens	
B cells	CD19, CD20	NA	Antibody production	NA	Antitumour antibodies Possible direct cytotoxic action	<20%
Macrophages	CD14, CD11b, HLA-DR	M1	Inflammation and response to intracellular pathogens	IDO	Secretion of antitumour cytokines	<5%
		M2	Wound response, angiogenesis	NA	Immunosuppressive cytokines, angiogenesis	
NK cells	CD16, CD56, NKG2D	NA	Innate immunity	IDO	Killing of tumour cells	<5%
Dendritic cells	CD135, Flt3, CD117, CD26, CD103	NA	Antigen presentation Co-stimulation	IDO	Cross presentation of tumour peptide antigens	1%

CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IDO, indoleamine 2,3-dioxygenase; NA, not applicable; PD-1, programmed cell-death protein 1; PD-L1, PD-1 ligand 1; TIL, tumour-infiltrating lymphocyte; TIM3, T-cell membrane protein 3.

Table 3 | Adjuvant trials in which TILs have been assessed

Trial analysed	Trial type	Treatment	TILs assessment	Population	n	Recurrence end points		
BIG 2-98 (REF. 18)	Adjuvant	Doxorubicin	Stromal on H&E	ER ⁺ /HER2 ⁻	1,079	Not significant		
	Prospective	Cyclophosphamide		HER2 ⁺	297	Not significant		
	RCT	CMF		TNBC	256	For each 10% increment of sTILs: DFS, HR = 0.84 (95% CI 0.74–0.98, <i>P</i> = 0.025)		
		Docetaxel						
FinHER ³⁸	Adjuvant	Docetaxel	Stromal on H&E	ER ⁺ /HER2 ⁻	591	Not significant		
	Prospective	Vinorelbine		HER2 ⁺	209	Not significant		
	RCT	FEC		TNBC	134	For each 10% increment of sTILs: DDFS, HR = 0.79 (95% CI 0.64–0.98, <i>P</i> = 0.032)		
		Trastuzumab						
E2197 and E1199 (REF. 39)	Adjuvant	Doxorubicin	Stromal on H&E	TNBC	481	For each 10% increment of sTILs: DFS, HR = 0.84 (95% CI 0.74–0.95, <i>P</i> = 0.005)		
Prospective RCT	Cyclophosphamide Docetaxel							
SEARCH, BCCA, NBCS, NEAT ¹⁹	Prospective	Various, not standardised No trastuzumab	IHC for CD8 in stroma (sCD8) IHC for CD8 in tumour (iCD8)	ER ⁺ (including HER2 ⁺)	8,775	Presence versus absence of iCD8: Breast cancer-specific survival, HR = 0.95 (95% CI 0.85–1.07, <i>P</i> = 0.43)		
	Observational RCT (NEAT)			ER ⁻ /HER2 ⁺			3,591	Presence versus absence of sCD8: Breast cancer-specific survival, HR = 0.79 (95% CI 0.67–0.93, <i>P</i> = 0.004)
NeoALTO ⁴⁰	Neoadjuvant	Trastuzumab	Stromal on H&E	HER2 ⁺	387	3% decrease in rate of recurrence (event free survival) for every 1% increase in TILs <i>P</i> = 0.002		
	Prospective	Lapatinib						
	RCT	Paclitaxel						
		FEC						

Trials overall include a total of 15,800 patients. BIG, Breast International Group; CMF, cyclophosphamide, methotrexate, 5-fluorouracil; DDFS, distant disease-free survival; DFS, disease-free survival; ER, oestrogen receptor; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; H&E, haematoxylin and eosin; HR, hazard ratio; IHC, immunohistochemistry; PR, progesterone receptor; RCT, randomized controlled trial; sTIL, stromal TIL; TIL, tumour-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

this concept remain unanswered. Compared with other solid tumours, breast tumours have a low mutation rate, but are nevertheless predicted to overexpress a median of around 50 'private' potentially immunogenic peptides⁵⁷. The mutation rate varies considerably between disease subtypes, with TNBCs reported to accumulate mutations 13 times faster than ER-positive tumours⁵⁸. The accurate prediction of which mutations give rise to immunogenic peptides recognized by effector T cells, is currently impossible without highly involved experimental validation^{59,60}. In melanoma, the characterization of mutations that give rise to reactive T-cell clones in tumours of patients has revealed that only relatively few mutations seem to be immunogenic^{53,54}. Nevertheless, considerable technical limitations are present in purifying rare T-cell clones and detecting mutations that might be present in a small fraction of tumour cells, such that the neoantigen repertoire of a tumour is likely to be underestimated by these methods^{53,54}. Of note, the most-commonly mutated gene in breast cancer, *PIK3CA*⁶¹, does not seem to affect the level of TILs in the HER2-positive subtype⁴⁰.

The T-cell repertoire found in a tumour is presumed to arise from the clonal expansion of suitably stimulated naive T-cell clones, as is the case for the immune response to infection^{62,63}. In addition to antigen affinity, the proliferative capacity of each T cell broadly depends on co-stimulatory signals, such as CD28 expressed on antigen-presenting cells, and proinflammatory cytokines, such as IL-2 and IL-12 (REF. 64). Colorectal cancers with deletions of genes encoding proinflammatory cytokines display a reduced density of tumour infiltrating T cells and B cells⁶⁵. Some cytokines can promote an immunosuppressive microenvironment, and this capacity has been demonstrated in animal models of breast cancer, whereby recruitment of T_{REG} cells to the tumour was driven by tumour-derived CXCL10 (REF. 66). Priming of T cells by dendritic cells is another crucial step in generating CD8⁺-T-cell-mediated antitumour immunity, which also requires type I interferons^{67,68}. Interferon production and recruitment of dendritic cells forms part of innate antitumour immunity, with work suggesting that a key mediator of T-cell priming by dendritic

cells is the stimulator of interferon genes complex (STING), which detects tumour DNA taken-up by antigen-presenting cells⁶⁹.

Aside from mutant peptides, the presentation of damaged intracellular proteins in an irregular context can generate tumour antigens⁷⁰. Different modes of tumour cell death, either spontaneous or induced by chemotherapy or radiotherapy, are also known to lead to immunogenic or immunosuppressive signals in the immune microenvironment⁷¹. Anthracyclines are notable for

causing 'immunogenic' tumour-cell death by promoting phagocytosis of tumour cells and co-stimulatory responses by dendritic cells⁴¹. Indeed, the innate and adaptive immune systems in the tumour milieu are co-dependent, as illustrated by the requirement for functional cytotoxic T-cells to achieve more-efficient tumour elimination in response to anthracycline treatment⁴². Of interest, germline loss of function of alleles, such as the toll-like receptor 4 (TLR4), impair the innate immune response to tumour-cell death, and are associated with

Table 4 | Neoadjuvant trials that have assessed TILs

Trial and treatments	Subtype	n	TILs assessment	Outcome	Multivariate analysis
GeparDuo ²⁴ Doxorubicin Docetaxel Cyclophosphamide	All	218	sTILs and iTILs on H&E	>60% sTILs: pCR 41.7% <60% sTILs: pCR 9.3%	OR 1.38 of pCR per 10% iTILs (95% CI 1.08–1.78, <i>P</i> = 0.012)
GeparTrio ²⁴ Doxorubicin Docetaxel Cyclophosphamide Vinorelbine Capecitabine	All	840	sTILs and iTILs on H&E	>60% sTILs: pCR 40% <60% sTILs: pCR 13.9%	OR 1.21 of pCR per 10% iTILs (95% CI 1.08–1.35, <i>P</i> = 0.001)
GeparQuattro ⁴⁶ Epirubicin Cyclophosphamide Docetaxel Capecitabine Trastuzumab	HER2 ⁺	156	sTILs on H&E	>50% sTILs: pCR 47.4% <50% sTILs: pCR 31.7%	OR 1.16 of pCR per 10% sTILs (95% CI 1.01–1.32, <i>P</i> = 0.038)
GeparQuinto ⁴³ Epirubicin Cyclophosphamide Taxane Everolimus	ER ⁺ and TNBC	313	sTILs and iTILs on H&E	>60% sTILs: pCR 36.6% <60% sTILs: pCR 14.3% (<i>P</i> < 0.001)	OR 1.2 of pCR per 10% sTILs (95% CI 1.0–1.3, <i>P</i> = 0.01)
GeparSixto ³¹ Paclitaxel Liposomal Doxorubicin Carboplatin Bevacizumab Trastuzumab	HER2 ⁺ and TNBC	580	sTILs and iTILs on H&E	>60% sTILs: pCR 59.9% <60% sTILs: pCR 33.8% (<i>P</i> < 0.001) Significant test for interaction between increased TILs and response to carboplatin therapy	OR 1.2 of pCR per 10% sTILs (95% CI 1.11–1.29, <i>P</i> < 0.001) OR 2.66 of pCR for >60% versus <60% sTILs (95% CI 1.76–4.02, <i>P</i> < 0.001)
EORTC 10994 and BIG 00-01 (REF. 44) FEC Docetaxel	ER ⁺	111	gTILs	High gTILs: pCR 74.2% Low gTILs: pCR 31.3%	OR 6.42 of pCR for high versus low gTILs (95% CI 2.08–19.83, <i>P</i> = 0.001)
CHER-LOB ⁵⁰ Trastuzumab Paclitaxel FEC	HER2 ⁺	105	sTILs and iTILs on H&E	>60% sTILs: pCR 59% <60% sTILs: pCR 27% (<i>P</i> < 0.015)	Not reported

Trials overall include a total of 2,323 patients. ER, oestrogen receptor; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; gTIL, gene-expression surrogate TIL; H&E, haematoxylin and eosin; iTIL, intratumoural TIL; OR, odds ratio; pCR, pathological complete response; sTIL, stromal TIL; TIL, tumour-infiltrating lymphocyte.

Box 3 | Describing relationships between TILs and outcome

Lymphocyte-predominant breast cancer (LPBC) refers to tumours where the percentage of the stromal and intratumoural tumour-infiltrating lymphocytes (TILs) is greater than a cutoff value, typically set to be 50% or 60% of the tumour tissue^{18,24}. However, it should be noted that TILs are linked with survival end points and neoadjuvant response (pCR) in a continuous fashion; it is therefore appropriate to perform analyses with TILs as a continuous variable. Arbitrarily dichotomising the measured TILs into LPBC and non-LPBC might reduce statistical power. LPBC should preferably be used only as a descriptive term for those tumours that contain more lymphocytes than tumour cells.

higher rates of relapse after chemotherapy and radiotherapy given with curative intent in patient with breast cancer^{72,73}.

Lymphocyte subpopulations—functional impact

The presence of TILs confers a favourable prognosis, but it ultimately represents an inadequate immune response when viable tumour deposits remain. As discussed earlier, constant interplay exists between tumours and the immune system via the process of immunoeediting, which begins long before a tumour is clinically evident³⁰. Another relevant feature is that the immune micro-environment is shaped by competing co-stimulatory and co-inhibitory signals. For example, although the presence of activated CD8⁺ T-cells in the tumour microenvironment is associated with favourable outcomes, they also induce counter-immune mechanisms, such as expression of PD-L1 on tumours, as part of the physiological mechanisms to restrain T-cell activity⁷⁴. Similarly, the tumour-specific CD8⁺ T cells infiltrating melanomas have been shown to express receptors that mediate immunosuppression⁷⁵. Thus, both stimulatory and suppressive signalling in the immune microenvironment are correlated with the presence of an antitumour immune response. Results from several groups, including ours, indicate that TILs predominantly represent a type I interferon immune response^{23,31}. Strong correlations at the mRNA level between TILs and many genes representing T-cell activation have been observed, but cases with the highest TILs also show the highest expression of T-cell checkpoint receptors, indoleamine 2,3-dioxygenase 1 (IDO1), and T_{REG}-cell markers^{23,31}. These data indicate that the TILs present in breast tumours consist of tumour-specific T cells that are chronically exposed to antigen, rather than merely being nonspecific ‘passengers’ or resident memory lymphocytes that have persisted after the elimination of antigenic tumour cells.

Immune-checkpoint inhibitors in breast cancer

Preclinical studies carried out over many years have defined a number of immunosuppressive mechanisms that function to prevent autoimmunity, but that can be repurposed in malignant disease states to impair antitumour immunity. These mechanisms, in turn, constitute prime targets for immunotherapeutics. The prototypical example is cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an inhibitory receptor found on CD8⁺ and CD4⁺ T cells, and constitutively expressed on T_{REG} cells⁷⁶. CTLA-4 functions to attenuate

co-stimulatory signalling by competing with the stimulatory receptor CD28 expressed by T cells for the ligands CD80 and CD86 presented by antigen-presenting cells⁷⁷. The blockade of CTLA-4 with monoclonal antibodies enhances antitumour immunity in mouse models⁷⁸. The anti-CTLA-4 antibody ipilimumab is now a standard-of-care therapy in metastatic melanoma, having been shown to significantly prolong the overall survival of patients with this disease⁷⁹. The programmed cell-death protein 1 and programmed cell death 1 ligand 1 (PD-1/PD-L1) axis is another mechanism for eliminating activated T cells that has been co-opted by tumours⁸⁰. The key event in this process is that tumour expression of PD-L1 activates PD-1 receptors on T cells to suppress their proliferative capacity⁸⁰. Monoclonal antibodies that block PD-1 or PD-L1 are among the most-promising immunotherapies available⁸¹, and early studies with these agents in patients with breast cancer are discussed in the next section. It should be noted that combination therapy with anti-CTLA-4 and anti-PD-1 therapy has shown particularly impressive outcomes in patients with melanoma⁸². Importantly, the expression of immunosuppressive markers increases with TIL infiltration; thus, their presence does not indicate ineffective antitumour immunity and poor clinical outcome when compared with tumours that do not have TILs, but rather the opposite^{31,83}.

Other modulators of host–tumour immunity are under investigation for their therapeutic potential. An anergic endothelial phenotype capable of killing effector CD8⁺ T cells before they can infiltrate is induced in some tumours (including breast cancers) by the secretion of VEGF, IL-10 and prostaglandin E2 (REF. 84). This mechanism might have a role in limiting lymphocyte infiltration in some tumours; studies in animal models have shown that antiangiogenic therapy permits T cells to enter the tumour microenvironment more readily⁸⁵. OX40 is a co-stimulatory receptor found on the surface of T cells that promotes their clonal expansion and also prevents the differentiation of CD4⁺ T cells into immunosuppressive T_{REG} cells^{86,87}; agonists of OX40 enhance antitumour T-cell and B-cell responses⁸⁸. Regression of metastatic lesions was observed in 12 out of 30 patients in an early phase trial using an agonistic OX40 antibody⁸⁹. TIM3 is a T-cell inhibitory receptor expressed on CD8⁺ and CD4⁺ T cells, in which it acts as an immune checkpoint that restricts autoimmunity⁹⁰. In solid tumours, TIM3 is found on so-called exhausted T-cells, which exhibit a dysfunctional phenotype characterized by failure to proliferate or produce cytokines despite antigen stimulation⁹¹. Some data in mouse models suggest that major populations of CD8⁺ T-cells infiltrating breast tumours express TIM3 (REF. 91). In addition to inhibiting CD8⁺ and CD4⁺ T-cell responses⁹², TIM3 is also found on T_{REG} cells⁹³. Inhibition of TIM3 ligand–receptor interaction promotes antitumour immunity in animal models, and might be a promising therapeutic approach^{91,94}. IDO, an immunosuppressive enzyme expressed on tumour cells, NK cells, dendritic cells and macrophages, depletes amino acids critical to T-cell clonal expansion and proliferation⁹⁵. In ovarian

cancer models, inhibition of IDO can facilitate proliferation of suppressed T cells⁹⁶. Clinical trials of IDO inhibitors alone or in combination with chemotherapy in patients with breast cancer are underway^{97,98}. The adenosine molecule is often enriched in the tumour immune microenvironment, where it acts as a danger signal of tissue damage⁹⁹. Tumour cells produce adenosine via the membrane-bound enzyme CD73, and expression of this marker is associated with a poor prognosis in patients with TNBC¹⁰⁰. Treatment with antibodies directed against CD73 inhibit breast-cancer growth and metastasis in immunocompetent mouse models, and the efficacy of anthracycline chemotherapy is lower in tumours positive for CD73 expression, suggesting that adenosine signalling is a valid therapeutic target^{100,101}. In mouse models of breast cancer, pharmacological blockade of the adenosine receptor reduces the metastatic potential of tumours that express CD73, and enhances T-cell effector function in combination with anti-PD-1-antibody therapy^{102,103}. Of note, adenosine-receptor antagonists already exist as small-molecule inhibitors with a favourable safety profile, and have been investigated as a treatment for Parkinson disease¹⁰⁴.

The tumour-intrinsic signalling pathways that contribute to suppression of host immunity remains poorly understood. A study of residual disease in TNBC following NACT found that transcriptional activity in the RAS/MAPK pathway was correlated with reduced infiltration by TILs⁵¹. Further evidence for the immunosuppressive effects of this pathway was observed when breast cancer cell-lines treated with a MEK inhibitor showed upregulation of HLA class I and II, as well as increased tumour PD-L1 expression on the tumour-cell surface⁵¹. The mechanism for increased PD-L1 levels is unknown, but combination therapy with a MEK inhibitor and with antibodies directed at PD-1/PD-L1 produced synergistic effects in immunocompetent syngeneic mouse models of TNBC⁵¹. This combination of immunotherapy and targeted therapy might be useful for promoting a more-functional immune microenvironment in patients with TNBC with residual disease after NACT that lacks TILs.

Immune-checkpoint inhibitors — clinical trials

Two recent trials have evaluated the efficacy of antibodies directed at PD-1/PD-L1 in patients with TNBC, with promising results. The first was a phase Ia study of the anti-PD-L1 antibody MPDL3280A in patients with PD-L1-positive TNBC¹⁰⁵. The expression of PD-L1 on tumour-infiltrating immune cells was assessed using a proprietary IHC assay (SP142 antibody, Spring Bioscience)¹⁰⁵. The presence of PD-L1 on at least 5% of tumour-infiltrating immune cells was considered a positive result. In 21 patients assessed for response, the overall response rate was 19%. This finding might be an underestimate, as the radiological response was assessed by RECIST 1.1 rather than by the modified immune response-related criteria¹⁰⁶, with at least one patient showing evidence of 'pseudoprogression' in axillary nodes that subsequently resolved. At 24 weeks, 27% of patients remained free from disease progression. Among

the screened patient population, the proportion of PD-L1-positive patients was 23%¹⁰⁵. A cohort of patients with less than 5% PD-L1-positive tumour-infiltrating immune cells is also being treated in this trial, but the efficacy results are currently unavailable. In an ongoing phase III study¹⁰⁷, investigators are randomly assigning newly diagnosed patients with advanced-stage TNBC to abraxane with or without the anti-PD-1 immunotherapy with MPDL3280A; expression of PD-L1 is not a selection criterion for patient enrolment in this trial.

The second trial was KEYNOTE-012, a phase Ib study of pembrolizumab (MK-3475) in patients with advanced-stage TNBC¹⁰⁸. The anti-PD-1 antibody pembrolizumab has been approved for the treatment of advanced-stage melanoma by the FDA and the European Medicines Agency. In this phase Ib study, 32 patients with metastatic TNBC were treated with 10 mg/kg pembrolizumab intravenously every 2 weeks. Patients were required to have a PD-L1-positive tumour, which was defined as any IHC staining in the tumour stroma or on at least 1% of tumour cells. The rate of PD-L1 positivity was 58% in the screened patient population. Over 90% of the enrolled patients had been previously treated with a taxane, anthracycline, capecitabine, or platinum-based chemotherapy, with 21 out of 32 patients having had more than one line of therapy. In total, 56.3% patients experienced treatment-related adverse events (all grades). Grade 3 treatment-related adverse events consisted of anaemia, headache, aseptic meningitis and pyrexia. One patient developed disseminated intravascular coagulation that resulted in death, which was attributed to the treatment. The median time on therapy with pembrolizumab was 59.5 days. The overall response rate by RECIST 1.1 was 18.5% (with 5 out of 27 patients evaluable), with one complete response and four partial responses noted¹⁰⁸. Out of the five responding patients, four had received greater than two lines of prior chemotherapy. Three patients did not receive a post-baseline response evaluation due to rapid disease progression. The patients that had a response continued on therapy for 40 weeks or more, with the median duration of response not reached at the time of reporting. The authors noted that they were unable to establish whether higher levels of PD-L1 positivity were associated with improved response because of the small number of patients enrolled¹⁰⁸. A phase II study, KEYNOTE-086, is planned for 2015 (REF. 109).

The results of these two studies highlight the potential of immune-checkpoint inhibitors targeting the PD-1/PD-L1 axis in patients with TNBC, demonstrating durable responses in heavily pretreated patients. This observation is in contrast to the results seen in a phase I trial with the anti-CTLA-4 antibody tremelimumab in combination with exemestane in patients with metastatic ER-positive breast cancer, wherein no responses were seen in the 26 patients treated¹¹⁰. These experiences and the fact that some patients on the aforementioned studies showed rapid disease progression after initiation of immunotherapy emphasizes the importance of careful patient selection for appropriate use of immunotherapies for the treatment of breast cancer. Another significant challenge is that a consistent biomarker of benefit from

therapy with T-cell checkpoint inhibitors remains elusive. This type of biomarker has proven difficult to establish even in melanoma, a setting in which many more patients have been treated with immune-checkpoint inhibitors in phase I, II and III trials¹¹¹.

Useful synergies between existing therapies for breast cancer and new immunotherapies remain to be discovered. An example is the evidence in mouse models that trastuzumab is synergistic with anti-PD1 therapy for HER2-positive breast cancers¹¹². This result formed the basis for the design of PANACEA, an international phase Ib/II trial in which patients who have disease that progressed on trastuzumab will receive trastuzumab plus the anti-PD-1 antibody MK-3475 (REF. 113). Comparable results from studies in mouse models have shown that breast tumours can be eradicated by combining radiotherapy with immune-checkpoint inhibitors, such as anti-CTLA-4 and anti-PD-1 antibodies^{114,115}. This synergy has also been observed in a clinical trial combining radiotherapy with ipilimumab in patients with advanced-stage melanoma¹¹⁶. In this trial, patients with multiple metastatic lesions underwent irradiation of a single lesion followed by administration of ipilimumab. The rate of response in the non-irradiated lesions was 18%, much higher than would otherwise be expected with ipilimumab alone, suggesting that the effect of ipilimumab was potentiated by focal radiotherapy. Results from parallel studies using mouse models have found that expression of PD-L1 on tumours after radiation could predict treatment resistance¹¹⁶. In light of these reported synergies, the BOSTON II pilot study of the combination of MK-3475 with stereotactic ablative radiotherapy in patients with oligometastatic breast cancer will explore the efficacy of combination radiation and immunotherapy¹¹⁷. Finally, the use of immunotherapy in the adjuvant and neoadjuvant settings in breast cancer should be considered as a therapeutic option in the future, in light of data from patients with advanced-stage melanoma, which indicate that ipilimumab is an effective adjuvant therapy¹¹⁸.

Biomarkers for immune-checkpoint inhibitors

Currently, no proven biomarkers are available to predict accurately whether a patient will benefit from immune-checkpoint inhibitors. The investigation of potential biomarkers from the immune microenvironment in multiple tumour types treated with the anti-PD-L1 antibody MPDL3280A has shown that a favourable response to treatment is usually correlated with high PD-L1 expression measured via IHC staining, particularly on infiltrating immune cells, but also on tumour cells¹¹⁹. Only three patients with breast cancer were included in this analysis, and results are available only for one patient who had no immune infiltrate at baseline, did not respond to therapy with MPDL3280A, and did not have any immune infiltrate in a biopsy taken at disease progression. Data for the other solid tumours assessed in this study indicate that the expression of PD-L1 on the intratumoural TILs seems to predict better responses to the anti-PD-L1 agent, suggesting that the primary mechanism of action for checkpoint blockade is the reactivation of pre-existing antitumour immunity¹¹⁹.

A second study of the immune microenvironment in metastatic melanoma treated with pembrolizumab determined that the best predictor of treatment response was the density of CD8⁺ T cells at the invasive tumour margin¹²⁰. Intratumour CD8⁺ T-cell density, and intratumour and invasive margin PD-1/PD-L1 density were also predictors of response. As discussed previously, CD8⁺ T-cell density is highly correlated with TILs determined using light microscopy of samples from patients with breast cancer³¹.

One important caveat is that these analyses were conducted in small single-arm phase I trials. PD-L1 expression, measured by IHC analysis, was determined prospectively in the phase III trial of the anti-PD-1 antibody nivolumab in patients with metastatic *BRAF*-wild-type melanoma⁸¹. In this study, PD-L1 positivity was defined as at least 5% of tumour cells showing IHC evidence of PD-L1 expression. No clear difference in outcome in patients with PD-L1-positive versus PD-L1-negative tumours treated with nivolumab was observed, although the median progression-free survival (PFS) and response rate were numerically higher in the PD-L1-positive group. The overall response rate was 33% in the PD-L1-negative group and 53% in the PD-L1-positive group⁸¹. These findings suggest that patients with PD-L1-negative tumours should not be excluded from treatment with PD-1/PD-L1 inhibitors. Concerns over the technical validity of anti-PD-L1 antibodies used in IHC analyses also remain¹²¹. The recently published phase III trial of nivolumab alone versus nivolumab plus ipilimumab versus ipilimumab alone in patients with advanced-stage melanoma adds further complexity to the picture¹²². In this trial, PD-L1 positivity was defined as in the aforementioned phase III study⁸¹. In PD-L1-positive patients, PFS was equivalent between nivolumab and nivolumab plus ipilimumab arms, whereas in PD-L1-negative patients, nivolumab plus ipilimumab was superior in this regard. It has been hypothesised that PD-1/PD-L1 inhibition is only a relevant therapeutic target if substantial numbers of activated T cells are in direct contact with tumour cells¹²³. In tumours in which T cells are mostly sequestered in the adjacent stroma, additional strategies might be required to induce transit of the T cells into tumour-cell nests. CTLA-4 inhibition seems to induce this transit through unknown mechanisms, and thus facilitates the efficacy of PD-L1 inhibition¹²⁴. This additive and possibly synergistic mechanism, which requires further experimental validation, might prove important in the setting of breast cancer, wherein large intratumoural TIL populations are generally rare in comparison to melanoma.

One of the more-promising predictors of benefit from T-cell checkpoint inhibitors is the mutation burden of the tumour. Mutation burden is most-accurately derived from whole-exome sequencing of the tumour and a paired germline sample from the patient to determine the true number of somatic mutations present in the tumour¹²⁵. As discussed previously, a larger number of nonsynonymous mutations is expected to give rise to a larger number of tumour neoantigens that could potentially be recognized by T cells. In one study that used

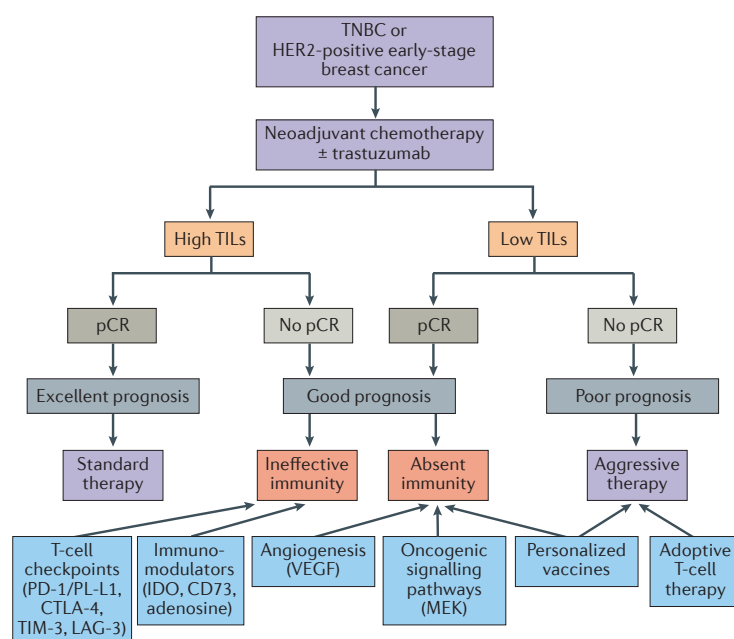


Figure 2 | Possible trial design using tumour-infiltrating lymphocytes as a biomarker. The combination of tumour-infiltrating lymphocytes (TILs) and pathological complete response (pCR) can enable patient stratification after neoadjuvant chemotherapy, and provides an efficient strategy for developing improved therapies. Patients with 'high TIL' levels who achieve a pCR have an excellent outcome, and might avoid the potential toxicity of immunotherapy. Patients with 'high TIL' levels who do not achieve pCR also have good outcomes, but might require further enhancement of immunity by targeting counter-regulatory mechanisms that prevent tumour elimination, such as the T-cell checkpoints cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and/or programme cell death 1 ligand 1 (PD-L1). Patients with 'low TIL' levels who achieve a pCR might require therapies aimed at establishing immune infiltration by tumour-specific T cells, which could be achieved through creating a more-permissive immune microenvironment by modulating VEGF or MEK signalling, or using personalized vaccines to induce an otherwise absent T-cell response to tumour antigens. Patients with 'low TIL' levels who do not achieve pCR have the worst prognosis of all, and might benefit from more complex, expensive and potentially toxic approaches, such as adoptive T-cell therapy, which might also be combined with the above-mentioned approaches. IDO, indoleamine 2,3-dioxygenase; LAG-3, lymphocyte activation gene-3; PD-1, programmed cell death 1; TIM-3, T-cell membrane protein 3; TNBC, triple-negative breast cancer.

whole-exome sequencing of non-small-cell lung cancers treated with anti-PD-1/PD-L1 antibodies, patients harbouring tumours with a higher number of nonsynonymous mutations had greater response rates and longer PFS¹²⁵. The differences were statistically significant in this small cohort ($n = 30$), and a cutoff of 177–209 nonsynonymous mutations defined the threshold for benefit¹²⁵. In another study of patients with metastatic melanoma treated with ipilimumab ($n = 110$), those who had tumours with >100 nonsynonymous mutations achieved greater clinical benefit, which was defined by a composite end point of response rate and overall survival¹²⁶.

TILs can fundamentally be used to quantify a host–tumour interaction phenotype, which incorporates pre-existing antitumour immunity and expression of co-inhibitory markers such as PD-L1. If used as biomarkers for immunotherapeutics, TILs might have advantages

over single markers, such as PD-L1, in that they represent a biologically valid and meaningful measure of immune activity. Investigations are ongoing to establish the clinical utility of TILs for this purpose. Studies in other tumour types suggest that a more-comprehensive evaluation of different lymphocyte subsets in the tumour microenvironment, using methods such as immunohistochemistry³⁰ or flow cytometry²⁹, is likely to have a future role in predicting benefit from immunotherapy, but no clear lymphocyte-subset biomarker currently exists. The relationship between mutation burden and TILs also remains to be explored, with preliminary evidence suggesting that there is no correlation between these parameters¹²⁷.

Conclusions

In summary, several lines of evidence indicate that a greater number of TILs in the tumour stroma is associated with a higher probability of cure in patients with early stage TNBC and HER2-positive breast cancer. An increase in TILs is also associated with a higher probability of pCR, and improved survival with anthracycline-based chemotherapy and trastuzumab in patients with the relevant disease subtypes (TNBC and HER2-positive, respectively). The ability of TILs to act as a prognostic and a potential predictive biomarker provides many opportunities in the future for routine use in clinical care and clinical trial design. First, TILs might be incorporated into the risk-prediction models used by clinicians to decide on the proposed benefit of adjuvant chemotherapy. Second, TILs might permit a more-accurate prediction of the chance of pCR with NACT. Third, TILs might carry predictive and prognostic associations in metastatic disease, which remain unexplored at this time^{128,129}. Fourth, TILs can be used in designing novel and efficient clinical trials by selecting those patients most in need of novel strategies (FIG. 2). TILs could also be used in the foreseeable future to de-escalate therapy in patients with high numbers of TILs, for example using a taxane and trastuzumab combination rather than maximal anthracycline, taxane, trastuzumab and pertuzumab therapy in those with HER2-positive disease. Such an eventuality would require appropriate models to predict the risk of recurrence that incorporate TIL-related parameters. We believe that such models should be derived from the analysis of large adjuvant or neoadjuvant studies.

TILs might also have the potential to act as a biomarker for immune-checkpoint inhibitors. This role could be most pertinent in the adjuvant setting, in which immunotherapy might improve response rates in appropriately selected patients. Another key question is how to promote infiltration of TILs in cases with low or absent levels. The reasons for the variability in TILs between different tumours remain under active investigation, with accumulating evidence for the importance of the aberrant peritumoural inflammatory milieu¹³⁰. By modulating the core elements of tumour-associated inflammation, such as stimulation of angiogenesis and recruitment of myeloid-derived suppressor cells, macrophages and stromal fibroblasts, combinations of immune-checkpoint inhibitors with other therapies might be required to

transition from poorly infiltrated to highly infiltrated tumours. Vaccines and adoptive T-cell therapies might also have a role in this process.

Considerable challenges remain in understanding and exploiting the immunobiology of breast cancer, particularly in developing useful and reliable biomarkers and positioning immunotherapies among the standard

therapeutic options already available. Nevertheless, the large amount of clinical and preclinical data in support of the importance of the breast cancer immune micro-environment comes just as we are beginning to appreciate the full potential of immunotherapy in the clinic. This is a happy coincidence that should greatly benefit patients with breast cancer.

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Acknowledgements

The work of S.L. is supported by the Cancer Council Victoria, the National Breast Cancer Foundation (NBCF) Australia, the Breast Cancer Research Foundation (BCRF), New York, and the National Health and Medical Council of Australia (NHMRC). The work of P.S. is supported by the NHMRC. The work of C.D. is supported by the German Cancer Consortium (DKTK), as well as the European Commission (FP7, RESPONSIFY project and TRANSCAN UG1 project). The work of M.J.S. is supported by the NHMRC and a Susan Gomen for the Cure Grant. The work of P.K.D. is supported by an NHMRC Senior Research Fellowship.

Author contributions

P.S., R.S., C.D. and S.L. researched data for the article. P.S., R.S., C.D., P.K.D., M.J.S. and S.L. contributed to discussing the article's content. All authors wrote, reviewed and edited the manuscript before submission.

Competing interests

S.L. receives research support from Merck, Novartis, Pfizer and Roche-Genentech, and co-chairs the PANACEA and BOSTON-II studies. M.J.S. has received research support from Bristol-Myers Squibb, and is a consultant for Amgen, F-star and Kymab. C.D. is the cofounder and shareholder of Sividon Diagnostics, Cologne, Germany. The other authors declare no competing interests.