reviews

The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014

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Background: The morphological evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer (BC) is gaining momentum as evidence strengthens for the clinical relevance of this immunological biomarker. Accumulating evidence suggests that the extent of lymphocytic infiltration in tumor tissue can be assessed as a major parameter by evaluation of hematoxylin and eosin (H&E)-stained tumor sections. TILs have been shown to provide prognostic and potentially predictive value, particularly in triple-negative and human epidermal growth factor receptor 2-overexpressing BC.

Design: A standardized methodology for evaluating TILs is now needed as a prerequisite for integrating this parameter in standard histopathological practice, in a research setting as well as in clinical trials. This article reviews current data on the clinical validity and utility of TILs in BC in an effort to foster better knowledge and insight in this rapidly evolving field, and to develop a standardized methodology for visual assessment on H&E sections, acknowledging the future potential of molecular/multiplexed approaches.

Conclusions: The methodology provided is sufficiently detailed to offer a uniformly applied, pragmatic starting point and improve consistency and reproducibility in the measurement of TILs for future studies.

Key words: tumor-infiltrating lymphocytes (TILs), breast cancer (BC), prognosis, prediction, clinical validity

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introduction

A fundamental role of the immune system is maintenance of tissue homeostasis by continuous immunosurveillance and initiation of inflammatory reactions that involve the coordinated activation of innate and adaptive immune cells [1]. Neoplastic

transformation alters the orderly structure of tissues and induces immune responses that can eliminate incipient tumors. In situations where elimination is incomplete, neoplastic transformation of cells is able to escape immune control. This process has been best conceptualized by the cancer immunoediting theory, which is supported by a large body of experimental data and clinical evidence [2]. Immunoediting defines malignant progression on the basis of tumor and immune cell interactions in three phases: elimination, equilibrium and escape. While patients are most frequently diagnosed in the escape phase, this relationship between the tumor and host immunity continues to evolve and sometimes with it the magnitude of the antitumor immune response. Even at advanced disease stages, immune parameters have now been recognized as directly or indirectly influencing patient survival [3].

Recently, new therapies that reactivate anticancer immune responses to cancer, for example in melanomas and lung cancer, have entered clinical practice and have improved outcome [4, 5]. Several recent clinical studies have evaluated the prognostic and predictive importance of tumor-infiltrating lymphocytes (TILs) in breast cancer (BC). Some of these studies used similar methodological approaches for evaluating TILs, which allows for comparison of the results. The foreseen inclusion of TILs assessment in current and future clinical studies and diagnostic assessments necessitates a detailed description of a standardized methodology.

In December 2013, a group of investigators from around the world representing major BC research and clinical teams convened to candidly discuss the important parameters to consider as well as methodological obstacles in evaluating TILs in BC. The group recognized the need to provide the BC community with consensus recommendations for TILs evaluation to foster their integration into future clinical trials, translational research and diagnostic practice. These efforts may evolve into the establishment of a BC 'immunological grade', reflecting the strength of an individual patient's antitumor immune response [6]. Here, we outline the current fundamental concepts for TILs evaluation by pathologists to facilitate its widespread use at this stage in our understanding of its relevance for BC. The recommendations focus on: (i) 'what' areas to examine in the tumor, (ii) 'how' to score the TILs and (iii) 'why' TILs are clinically important.

what is the composition and role of the immune infiltrate in human breast cancer?

Immune cells-infiltrating tumors are frequently observed, but the composition of cells involved in innate and adaptive immunity varies between tumor types or organ sites [7]. Cumulative data from murine and human studies have associated most leukocyte subsets with a predominant contribution to either pro- or antitumor activities (illustrated in Figure 1). Murine models have identified myeloid lineage leukocytes, including tumor-associated macrophages, dendritic cells and myeloid-derived suppressor cells as playing a central role in shaping the microenvironment via the factors they produce, towards either an immunostimulatory antitumor milieu or a wound healing tumor-promoting microenvironment. Antitumor T cells migrating into these contrasting settings can therefore either be activated or suppressed [8]. In turn, macrophage polarization toward protumorigenic M2 or antitumor M1 functional phenotypes are regulated by T lymphocytes [9], highlighting the importance of cellular cross-talk in shaping the tumor microenvironment.

Studies in humans have demonstrated a significant association between the presence of specific subsets of immune cells and clinical response in patients with a variety of solid tumors [7]. Furthermore, accumulating evidence suggests that adaptive immunity mediated by T and B lymphocytes provides the critical foundation for effective and sustained antitumor responses. In BC, extensive tumor infiltration by cytotoxic CD8+ T cells was strongly associated with patient survival [10, 11] and response to therapy [12]. The presence of CD4+ regulatory T cells (Treg) has been associated with both good and bad [13–15]. Among the other CD4+ T-cell subpopulations, Th1 cells (the principal cellular source of interferon-γ) have been associated with favorable clinical outcomes [16], whereas Th2 cells have been reported to be associated with dampening of the antitumor response [17]. Th17 cells, producers of the proinflammatory interleukin 17 cytokine family, appear to have variable effects depending on the surrounding cytokine milieu, which may in part be linked with the organ site and tumor type [18]. The presence of follicular helper (Tfh) cells, the newest CD4+ subset, was recently positively associated with patient outcome both in the adjuvant and neoadjuvant settings [16]. The precise role of tumor-infiltrating B cells is currently not well defined and remains controversial [19, 20].

Given the functional heterogeneity of intratumoral lymphocytes it is intriguing that the degree of lymphocytic infiltration assessed by simple evaluation of hematoxylin and eosin (H&E)-stained tumor sections has been shown to have predictive and prognostic value in triple-negative (TNBC) and human epidermal growth factor receptor 2 (HER2+) BC despite a lack of detailed information on the immune subpopulations of the infiltrate [21–24]. A possible explanation is that negative immune regulators are present as part of a normal feedback loop reacting to an active and ongoing antitumor immune response, which therefore potentially defines tumors that are more immunogenic [25]. This consideration has several important implications. The first is that a focused evaluation of individual subsets may have limited value. For example low or absent Treg infiltration may reflect tumors that are disregarded by the immune system while high Treg in tumors may signal an active, albeit unsuccessful, attempt at tumor rejection. Second, TIL-rich and TIL-poor BCs may each reflect a distinct tumor cell biology that likely has markedly different susceptibility to immunotherapy. Finally, in moderate to extensively infiltrated tumors, the presence of peritumoral or stromal TLS can be seen in some patients [16]. Thus, despite the inability of the immune system to reject a clinically detectable tumor, an organized immune response at the tumor site may signal the generation of immunological memory with the potential to effectively control residual disease. Variability has also been detected within individual tumors [26], suggesting that the nature of tumor–immune interactions may parallel tumor heterogeneity.

Cytotoxic treatments such as chemotherapy and radiotherapy may sometimes act to jump start the system [27–29]. In this context, a stronger antitumor immune response directed to a broader range of BC antigens would potentially have a higher
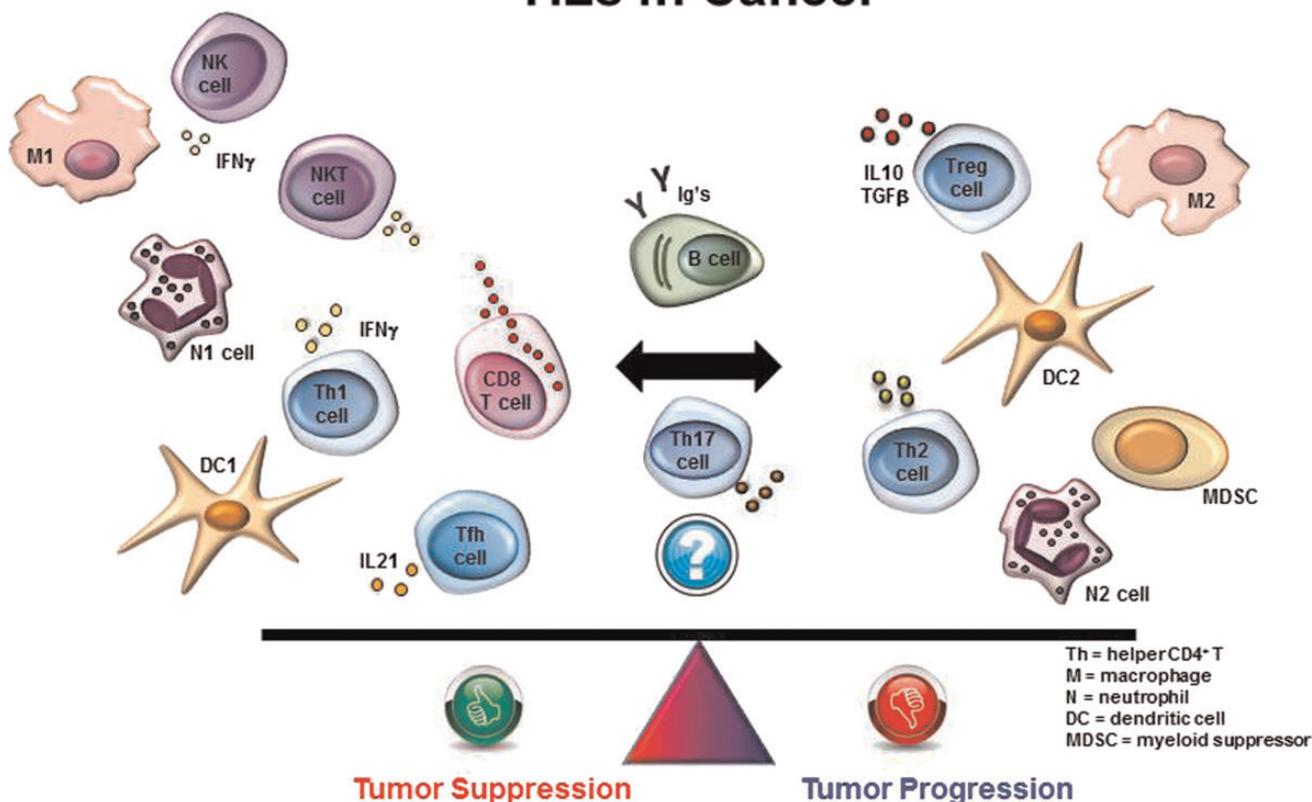
Figure 1. The cellular cross-talk between different leukocyte subsets and their predominant contribution to either pro- or antitumor activities, including myeloid lineage leukocytes, tumor-associated macrophages with either protumorigenic (M2) or antitumorigenic (M1) properties, helper T-cell subsets, cytotoxic T cells, regulatory T cells, B cells, dendritic cells and myeloid-derived suppressor cells are shown. These cells play central roles in shaping the microenvironment via the factors they produce thereby driving either an immune-mediated anti- or protumor activities in the microenvironment.

likelihood of controlling the heterogeneous malignant cell population present in large primary tumors and emerging metastases [26]. This hypothesis is supported by studies showing that the degree of lymphocyte infiltration is predictive of a better local response to neoadjuvant treatment and prognostic of long-term disease control [21, 22, 24].

current data on clinical validity and utility of TILs in breast cancer

Examples of notable adjuvant and neoadjuvant studies that have assessed infiltrating lymphocytes are included in Table 1. In the majority of these studies, both intratumoral and stromal TILs have been assessed, with evaluation of the stromal compartment shown to be more reproducible between studies. Some studies focused on TILs using immunohistochemistry, while others evaluated molecular markers using immunohistochemistry and gene expression analysis.

adjuvant studies

triple-negative breast cancer

TILs have been assessed in full face sections of >1300 TNBC and >3500 hormone-receptor positive BCs at diagnosis. TILs were found to be a positive prognostic biomarker in 297 TNBC

but not in the luminal subtypes. This correlation was first reported using baseline samples from the BIG 2–98 trial [22] and subsequently independently confirmed in 481 TNBC sample prospectively collected during two phase III adjuvant randomized BC trials [United States Eastern Cooperative Oncology Group (ECOG) trials 2197 and 1199 [24]]. Therefore, in TNBC, the more stromal TILs a patient has at diagnosis, the better their outcome after adjuvant anthracycline-based chemotherapy. Hence, according to Simon et al. [30], the results for the prognostic value of TILs in TNBC could be considered Level I evidence. However, given the lack of prognostic information for patients with primary TNBC not treated with chemotherapy, TILs should not be used as a biomarker for withholding chemotherapy.

HER2+ disease

Recent data from randomized clinical trials, evaluating TILs on full face sections suggest the importance of immunity in HER2+ disease [22, 23]. The FINHER study, where patients were randomized to receive received trastuzumab or no trastuzumab, reported that higher TILs in baseline samples resulted in higher responses to trastuzumab treatment. Recent data from the N9831 study [44] suggested that tumors that were 'immune enriched', as defined using gene expression, had better outcomes if they received trastuzumab. While these findings will not affect the

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Table 1. Adjuvant and neoadjuvant studies that have assessed TILs and prognosis are included

Reference	Study (level of evidence if applicable according to Simon et al. [30])	Regimen	Tumor tissue assay	Sample size	Correlation with outcome
Adjuvant studies					
[22]	BIG 02-98 (category B)	A → CMF or AC → CMF	Full section H&E	2009 total 256 TNBC	None Stromal TILs (sTIL) (continuous, per 10% increase) univariate: HR 0.84 ($P = 0.02$, DFS) HR 0.82 ($P = 0.02$, OS) sTIL multivariate: HR 0.85 ($P = 0.02$, DFS) HR 0.83 ($P = 0.02$, OS)
				297 HER2+ 1078 HR+	None None
[24]	E2197 E1199 (category B)	AC versus AC AC → Docetaxel or paclitaxel	Full section H&E	481 TNBC	sTIL (continuous, per 10% increase) Univariate: HR 0.86 ($P = 0.02$, DFS) HR 0.81 ($P = 0.01$, OS) Multivariate: HR 0.84 ($P = 0.005$, DFS) HR 0.79 ($P = 0.003$, OS)
[23]	FINHER (category B)	Docetaxel or Vinorelbine → FEC With trastuzumab if HER2+)	Full section H&E	934 total 134 TNBC	None sTIL (continuous, per 10% increase) Univariate: HR 0.79 ($P = 0.03$, DDFS) HR 0.80 ($P = 0.08$, OS) Multivariate: HR 0.77 ($P = 0.02$, DDFS) HR 0.81 ($P = 0.14$, OS)
				209 HER2+	sTIL (continuous, per 10% increase) correlate with DDFS (HR 0.82, $P = 0.025$ univariate) only with trastuzumab, not OS.
				591 HR+	None
[31]	Four studies Including NEAT clinical trial (category B)		TMA CD8, FOXP3 immunohistochemistry	12 439	CD8+ T cells in tumor and stroma was associated with 28% and 21% reduced risk of BCSS. Greater benefit in ER-negative disease and ER=/HER2
[10]	Consecutive	CMF	TMA CD8-immunohistochemistry	1334	Binary high versus low: total CD8 correlates with BCSS (HR 0.55, $P = 0.001$ multivariate training set; HR: 0.58, $P < 0.002$ multivariate validation set)
[32]	Consecutive	MF, AC, FAC or no chemotherapy	TMA CD8-immunohistochemistry	1985 HR+ 216 HER2+ 496 TNBC	None None Binary any versus none: CD8 correlates with BCSS, multivariate iTIL (intratumoral TILs) HR 0.48, $P < 0.001$

[33]	Institutional	Varied—chemotherapy not specified	PD-L1 mRNA TILs	636	Higher PD-L1 mRNA associated with better recurrence-free survival. PD-L1 mRNA correlated with TILs
[34]	Consecutive	CMF, AC, CEF or CAF	TMA CD3-immunohistochemistry	255	Binary high versus low total CD3 correlates with DFS in anthracycline group (HR 0.25, $P = 0.0056$)
Neoadjuvant studies					
[35]	Institutional cohort	Anthracycline–taxane-based regimens	CD3-immunohistochemistry	73	CD3 positively correlated with pCR
[21]	GeparDuo GeparTrio (category B)	EC-Doc (GeparDuo) TAC ± Vinorelbine/Capecitabine (GeparTrio)	TILs in H&E core biopsy	1058	Stromal TILs and LPBC associated ($P = 0.001$) with pCR TILs significant in subgroups (HR±; HER2±)
[34]	Publicly available gene expression data from EORTC 10994/BIG 00-01	FEC versus TET	Gene expression data	113 ER-	TILS correlate with pCR ($P = 0.001$)
[36]	Institutional cohort	Neoadjuvant anthracycline-based; cyclophosphamide-based or taxane-based regimens	TILs in H&E core biopsy	474 total 92 TNBC	TILs correlate with pCR in TNBC ($P = 0.004$)
[37]	Institutional cohort	Neoadjuvant anthracycline–taxane-based regimens	TILs in H&E core biopsy	68	TILs correlate with pCR ($P < 0.0001$)
[38]	Institutional cohort	Neoadjuvant paclitaxel FEC	CD8, FOXP3, IL17F immunohistochemistry	180	CD8, FOXP3 positively correlated with pCR ($P < 0.001$)
[39]	Publicly available gene expression data (7 cohorts)	Anthracycline-based neoadjuvant therapy	IGKC gene expression	845	IGKC predicted response to NACT ($P < 0.001$)
[40]	GeparQuinto Predict clinical study (category B)	EC-Doc	Core biopsy H&E	313 HER2 negative	Stromal TILs and LPBC associated with pCR ($P < 0.001$)
[12]	Institutional cohort	Neoadjuvant anthracycline–taxane-based regimens	CD8, CD4, FOXP3 immunohistochemistry	153	CD8, CD4, FOXP3 positively correlated with pCR ($P = 0.003$, $P < 0.001$ and $P = 0.001$)
[41]	Institutional cohort	Neoadjuvant anthracycline–taxane-based regimens	TILs by H&E; CD3, CD8, FOXP3 immunohistochemistry	175	TILs, CD3, CD8, FOXP3 positively correlated with pCR
[42]	TVA neoadjuvant phase 2 study	4FEC100 ± 4 docetaxel + panitumumab	CD8-immunohistochemistry	47 TNBC	High CD8/FoxP3 were predictive of pCR
[43]	Pooled analysis of publicly available gene expression data (8 cohorts)	Neoadjuvant anthracycline/anthracycline + taxane-based chemotherapy	STAT1 and immune response gene modules	996	High score of STAT1 and immune response gene modules is associated with increased pCR rates in all breast cancer subtypes based on ER and HER2 status

AC, doxorubin/cyclophosphamide; BCSS, breast cancer specific survival; CAF, cyclophosphamide, doxorubicin, 5-Flourouracil; CEF, Canadian cyclophosphamide, epirubicin, 5-Flourouracil; CMF, cyclophosphamide, methotrexate, 5-Flourouracil; DDFS, distant disease free survival; DFS, disease free survival; ER, estrogen receptor; FAC, 5-Floururacil, doxorubin, cyclophosphamide; HR, hazard ratio; IGKC, gene encoding for immunoglobulin kappa constant; NACT, neoadjuvant chemotherapy; OS, Overall survival; pCR, pathological complete remission; TET, docetaxel, epirubin and docetaxel.

use of trastuzumab in newly diagnosed HER2+ BC, they suggest a potential mechanism of action for trastuzumab-based therapy. Based on current knowledge, however, TILs should not be used to either withhold or prescribe trastuzumab therapy. Given that trastuzumab with chemotherapy is the standard of care today, attention has turned to TILs as a prognostic factor in HER2+ disease treated with anti-HER2 therapy [45]. Data suggest that high levels of TILs are also associated with excellent outcomes in HER2 disease treated with lapatinib as well as dual trastuzumab and lapatinib with chemotherapy (unpublished data).

neoadjuvant studies

To date, core biopsies from more than 3000 patients have been assessed for correlation between immune markers and response to neoadjuvant chemotherapy, including institutional cohorts, but also biomaterials from clinical trials. An overview on the studies is given in Table 1. In summary, histological as well as molecular data indicate that immunological parameters, including stromal TILs are associated with higher rates of pathological complete remission (pCR), independent of other clinico-pathological prognostic factors or the chemotherapy regimen. Intriguingly, an interaction between stromal TILs and a benefit to carboplatin added in the neoadjuvant setting has been reported [46], although the biological mechanism remains unclear.

methodological recommendations for evaluating TILs in breast cancer

Before attending the December 2013 meeting, the participants with experience in evaluating TILs for phase III studies were asked to complete a questionnaire covering topics pertinent to their assessment in BC. These questions are detailed in supplementary Material S1, available at *Annals of Oncology* online. The goal of this approach was to derive a consensus based on current experience within the group and use it as the foundation for this guideline. Based on these discussions, the working group participants made recommendations for harmonizing TILs evaluation, which are summarized in Table 2. Additionally, a tutorial has been prepared and is included as a tutorial [supplementary Material S2, available at *Annals of Oncology* online, 'Standardized evaluation of Tumor-Infiltrating Lymphocytes (TILs) in Breast Cancer for daily clinical and research practice or clinical trial setting'].

technical issues for evaluation of TILs in breast cancer

1) Microscope magnification does not really make a difference, but usually a magnification of ×200–400 (ocular ×10, with an objective of ×20–×40) is recommended.
2) Slide thickness is not critical, with a standard thickness of 4–5 μm considered optimal. The majority of existing experience is based on scoring 4–5 μm sections of formalin fixed and paraffin embedded (FFPE) tissues, while the feasibility of TILs evaluation on frozen sections is undocumented outside of a research setting and thus cannot be recommended for routine use at the present time.
3) TILs can be evaluated using core biopsies in the neoadjuvant setting as well as surgical specimens in the adjuvant setting. Considering all of the above, the scoring of one FFPE-block/patient is sufficient in the neoadjuvant and adjuvant setting.

Table 2. Recommendations for assessing tumor-infiltrating lymphocytes (TILs) in breast cancer

1) TILs should be reported for the stromal compartment (=% stromal TILs). The denominator used to determine the % stromal TILs is the area of stromal tissue (i.e. area occupied by mononuclear inflammatory cells over total intratumoral stromal area), not the number of stromal cells (i.e. fraction of total stromal nuclei that represent mononuclear inflammatory cell nuclei).
2) TILs should be evaluated within the borders of the invasive tumor.
3) Exclude TILs outside of the tumor border and around DCIS and normal lobules.
4) Exclude TILs in tumor zones with crush artifacts, necrosis, regressive hyalinization as well as in the previous core biopsy site.
5) All mononuclear cells (including lymphocytes and plasma cells) should be scored, but polymorphonuclear leukocytes are excluded.
6) One section (4–5 μm, magnification ×200–400) per patient is currently considered to be sufficient.
7) Full sections are preferred over biopsies whenever possible. Cores can be used in the pretherapeutic neoadjuvant setting; currently no validated methodology has been developed to score TILs after neoadjuvant treatment.
8) A full assessment of average TILs in the tumor area by the pathologist should be used. Do not focus on hotspots.
9) The working group's consensus is that TILs may provide more biological relevant information when scored as a continuous variable, since this will allow more accurate statistical analyses, which can later be categorized around different thresholds. However, in daily practice, most pathologists will rarely report for example 13.5% and will round up to the nearest 5%–10%, in this example thus 15%. Pathologist should report their scores in as much detail as the pathologist feels comfortable with.
10) TILs should be assessed as a continuous parameter. The percentage of stromal TILs is a semiquantitative parameter for this assessment, for example, 80% stromal TILs means that 80% of the stromal area shows a dense mononuclear infiltrate. For assessment of percentage values, the dissociated growth pattern of lymphocytes needs to be taken into account. Lymphocytes typically do not form solid cellular aggregates; therefore, the designation '100% stromal TILs' would still allow some empty tissue space between the individual lymphocytes.
11) No formal recommendation for a clinically relevant TIL threshold(s) can be given at this stage. The consensus was that a valid methodology is currently more important than issues of thresholds for clinical use, which will be determined once a solid methodology is in place. lymphocyte-predominant breast cancer can be used as a descriptive term for tumors that contain 'more lymphocytes than tumor cells'. However, the thresholds vary between 50% and 60% stromal lymphocytes.

Some studies have assessed the prognostic or predictive importance of TILs on post-treatment tissues, but more studies are needed before formal recommendations can be made on the methodology of scoring TILs after neoadjuvant treatment [47].

4) Originally, tissue microarrays (TMAs) were not recommended for evaluating TILs, since there was no published evidence that TMAs mirror the potential heterogeneity of TILs, and the number of cores needed and the defined core diameter, accurately reflecting TIL composition in a full section are unknown. However, recently published studies [11, 31, 33] using TMAs and well-annotated clinical datasets show that results are concordant with other studies in the field using mostly biomarker-based determinations of TILs subsets, not H&E determinations of general TILs. TMAs may be a good option for future studies, particularly for the rapid evaluation of large clinical cohorts. More investigation is needed before firm methodological recommendations can be offered.

5) All mononuclear cells including lymphocytes and plasma cells should be scored (granulocytes and other polymorphonuclear leukocytes are excluded). The quantitative assessment of other mononuclear cells such as dendritic cells and macrophages is currently not recommended, although there is increasing evidence that they may be functionally important since they are observed in TLS.

6) Several studies used immunohistochemistry to assess the clinical importance of subtyping lymphocytes. CD45, CD8, CD3 and various other markers expressed on lymphoid cells have been tested and while immunohistochemistry may improve accuracy, at the present time any added value from these markers is unclear. The TILs working group does not currently recommend that immunohistochemistry be used to detect specific subpopulations outside of the research setting, until further evidence is available.

7) Machine scoring approaches, while promising; have not been published in large series with consistent methodology. These approaches represent an important area for further study.

8) It is unknown if either RNA or protein classification of TILs by will reveal prognostic and predictive value beyond that achievable by simple morphology. New techniques, like CyTOF [48], can review protein-based signatures of inflammatory infiltrates. While these are all still in the domain of research, pathologists should be aware of this potential. Clinical utility will drive the development of specific immune markers.

the concept of stromal versus intratumoral TILs—focus on the stromal compartment

The initial studies of BC TILs have evaluated stromal and intratumoral lymphocytes separately. Intratumoral TILs are defined as lymphocytes in tumor nests having cell-to-cell contact with no intervening stroma and directly interacting with carcinoma cells, while stromal TILs are located dispersed in the stroma between the carcinoma cells and do not directly contact carcinoma cells. Since both are localized in the region defined as tumor tissue, it should be emphasized that both categories represent true TILs. Furthermore, as TILs are able to move within a living tissue microenvironment, the distinction may be somewhat artificial and related to the static situation in histological slides that

are used for diagnostic assessment. The original hypothesis was that lymphocytes directly interacting with carcinoma cells might be more relevant and therefore more useful for diagnostic assessment. While this hypothesis may still be biologically and/or clinically relevant, for diagnostic purposes on H&E-stained sections, most current studies have found stromal TILs to be a superior and more reproducible parameter. The main reasons are that intratumoral TILs are typically present in lower numbers and detected in fewer cases, they are more heterogeneous and are difficult to observe on H&E-stained slides (i.e., without using immunohistochemistry or immunofluorescence). Scoring intratumoral TILs does not add to the information provided by stromal TILs since they usually parallel stromal TILs. However, focusing on the stromal compartment (instead of the tumor as a whole) has a clear advantage because the density and growth pattern of carcinoma nests will not affect the TIL count because stromal TILs are measured only in the spaces between the carcinoma nests. Nevertheless, recent evidence indicates that, in the neoadjuvant TNBC setting, and despite the methodological reasoning mentioned above, both stromal as well as intratumoral TILs are predictive of pathological response to neoadjuvant platinum-based chemotherapy [49]. Also, using CD3 or CD8-immunohistochemistry intratumoral TILs may potentially become as easy to detect as stromal TILs. Nevertheless, the TILs working group's current recommendation is to evaluate stromal TILs as the principal parameter in future studies, allowing the straightforward evaluation of a single parameter. Additional parameters, including TLS in the peritumoral region, TILs at the invasive edge or intratumoral TILs can still be included for research purposes to further determine and/or confirm their potential clinical relevance.

In the tutorial, evaluations are based on stromal TILs, which are reported in an approximately manner semiquantitatively on a continuous scale as a percentage of stromal TILs. The working group's consensus is that TILs may provide more biological relevant information when scored as a continuous variable, since this will allow more accurate statistical analyses, which can later be categorized around different thresholds. However, in daily practice, most pathologists will rarely report for example 13.5% of TILs and will round up to the nearest 5%–10%, in this example thus 15%. Pathologist should report their scores in as much detail as the pathologist feels comfortable with.

The original methodology for scoring TILs described by Denkert et al. in 2010 [21] was used in the majority of subsequently published studies, thereby providing sufficient data for this initial stage in developing a uniform methodology (based on the definitions in Figure 2). Interpersonal discussions among pathologists applying this approach since 2010 has slightly modified the original version [21] as shown in Figures 3 and 4). While some studies have scored TILs using other semiquantitative approaches [16, 27, 33], the SABCS TILs working group considers the clinical validity of the modified Denkert et al. [21] approach described here to be superior at this time. This alternative assessment of TILs does of course not invalidate previously published findings using other methods of TILs assessment, but provides a framework for future standardization.

Using the recommendations in Denkert et al. [21], stromal TILs should be scored uniquely as a percentage of the stromal areas alone and areas occupied by carcinoma cells should not be
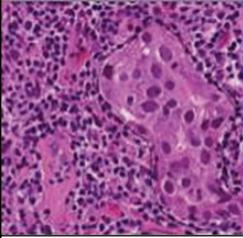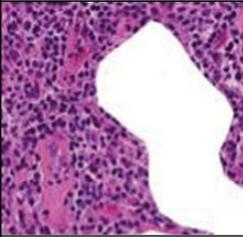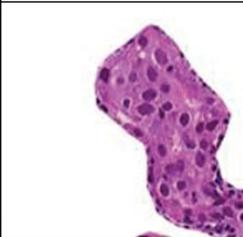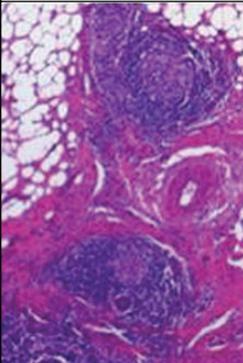
| Morphology	Definition and biological relevance	Diagnostic relevance
Lymphocyte-predominant breast cancer (LPBC)		
	Working category to describe tumors with "more lymphocytes than tymor cells".	Definitions vary across studies with stromal TILs of 50–60% used as a threshold. LPBC can be used for predefined subgroup analyses and for description of tumors with a particularly high immune infiltrate, however, keep in mind that TILs are a continuous parameter and the threshold for LPBC is still arbitrary.
Stromal TILs		
	Indicator of increased accumulation of immune-cells in tumor tissue	Stromal TILs have been shown to be predictive for increased response to neoadjuvant chemotherapy as well as improved outcome after adjuvant chemotherapy. Based on current data, this parameter is the best parameter for characterization of TILs.
Intratumoral TILs		
	TILs with direct cell-cell contact with carcinoma cells, might be an indicator of direct cell-based anti-tumor effects.	Several studies have shown that intratumoral TILs and more difficult to evaluate and do not provide additional predictive/prognostic information compared to stromal TILs.
TILs at the invasive margin		
	The localization of TILs are the invasive edge is included in the evaluation approach presented in this guideline.	For breast cancer there are no studies with a separate evaluation of TILs at the invasive edge. For practical purposes, the reliable evaluation of the invasive edge might be difficult when using core biopsies in the neoadjuvant setting.
Tertiary lymphoid structures (TLS)		
	Typically localized in the surrounding area of the tumor, TLS might be localized in normal tissue directly adjacent to the tumor, consisting of a T cell zone next to a B cell follicle, often with germinal centers.	While these structures may be important for the biology of tumor-immune reactions, they are not yet optimized for non-research based assessments, The main problem is that TILS have a spatial heterogeneity and are principally localized in areas surrounding the tumor. They might not be in the plane of the tissue section that is being evaluated, in paricular when using core biopsies. Furthermore, it might be difficult to distinguish lymphoid aggregates from true TILS, in particular when the germinal center is not in the plane of the section.

Figure 2. Morphology, definitions, biological and diagnostic relevance of the different immune infiltrates found in breast cancer.

included in the total assessed surface area. This is an important point because otherwise the size of the epithelial cell nests as well as the tumor growth pattern could influence the stromal TILs value. For example a score of 50% stromal TILs means that 50% of the stromal surface area and thus not stromal nuclei is occupied by TILs and also not 50% of the stroma plus epithelial cell area. For semiquantitative assessment of percentage values, the dissociated growth pattern of lymphocytes needs to be taken into account. Lymphocytes typically do not form solid cellular aggregates; therefore, the designation '100% stromal TILs' would still allow some empty tissue space between the individual lymphocytes.

This recommendation is based on the methodology used in published phase III studies, implying that there is room for future refinement as evidence accumulates to show the validity
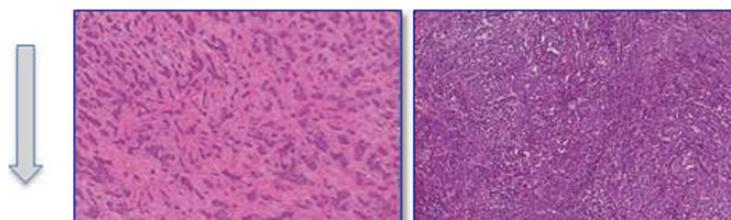
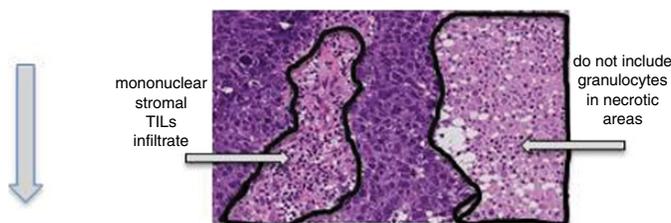Step 1: Select tumor area
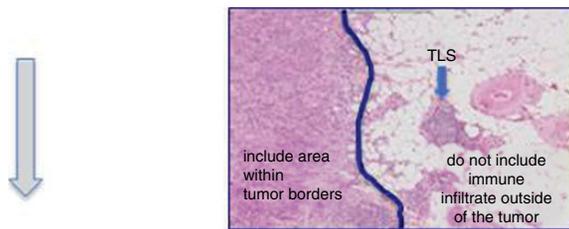
Step 2: Define stromal area
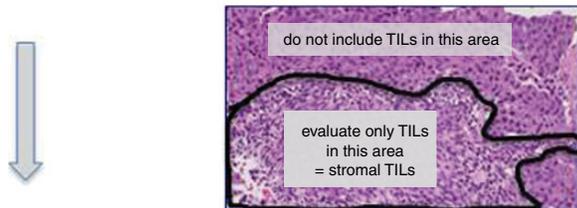
Step 3: Scan at low magnification

Step 4: Determine type of inflammatory infiltrate

Step 5: Assess the percentage of stromal TILs
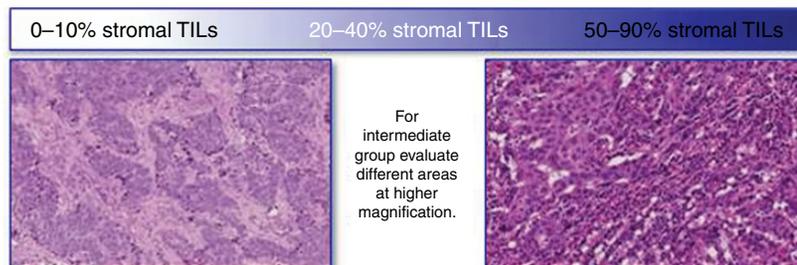(examples of percentages shown in figure 4)

Figure 3. Standardized approach for TILs evaluation in breast cancer.

of alternative parameters and/or methodologies that improve upon this practice. The current recommendations are illustrated in Figures 3 and 4 with the methodology fully explained in the supplementary Material S2, available at *Annals of Oncology* online.

The statistical analysis as a noncontinuous variable can also be considered as a secondary option. For this analysis, the term lymphocyte-predominant breast cancer (LPBC) has been coined, which can be used as a descriptive term for tumors that contain 'more lymphocytes than carcinoma cells'. Typically, the threshold of stromal lymphocytes for LPBC is around 50%–60% of the stromal surface area. Note however that this term should not be used as a definition for a specific tumor type, but just as a descriptive term to facilitate discussions about lymphocyte-rich tumors. LPBC is not the same as medullary BC, which has additional histological features. It is unclear if this cutoff will be
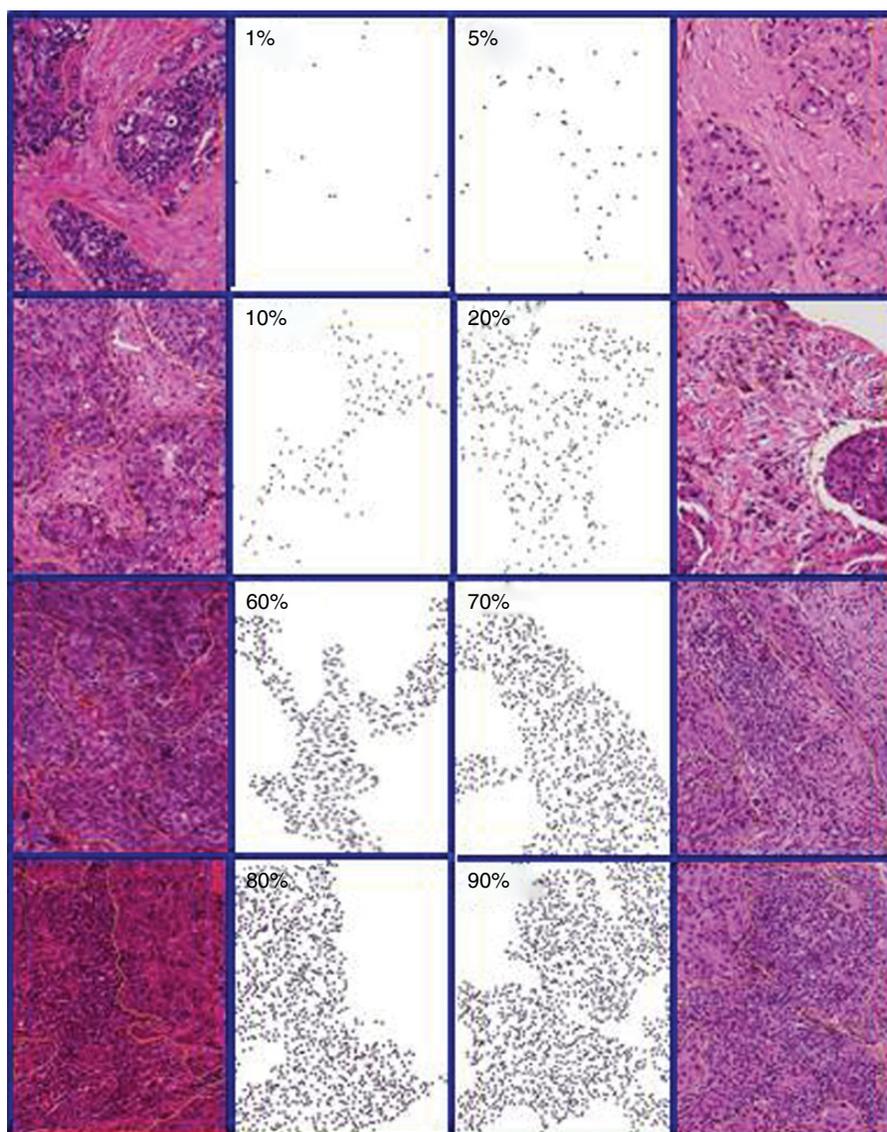
Figure 4. Standardization and guidelines for TILs assessment. Stromal TILs should be reported as a percentage (the schematic images might provide some guidance). If the percentage of TILs is questionable, discuss the case with a second pathologist. In heterogenous tumors, evaluate different regions and report the average. For this standardized graphic, images were selected that are representative of different TILs levels, based on the results of three pathologists as well as image analysis. The stromal area was marked in each image. The central images are digitally generated graphics showing the same region of interest (ROI) and a similar density of TILs as the corresponding histological image. Please note that the central images contain idealized TILs generated graphically with comparably density, but not with the exact configuration and distribution as the TILs in the histological images.

used in the future as such dominant TILs infiltration in tumors has been found to be infrequent (∼10%).

selection of tumor areas for evaluation

For evaluating TILs, the boundaries of the invasive tumor should be identified with only TILs inside them evaluated. TILs in areas with crush artifacts, necrosis, and inflammation around biopsy sites or extensive central regressive hyalinization should not be scored. A necrotic biopsy is considered unscorable.

Most pathologists recognize that immune infiltrates can also be observed at some distance from the main tumor bed, surrounding extra- and intratumoral DCIS and also in adjacent normal lobules. These infiltrates outside of the tumor borders and around DCIS and normal lobules should not be included in the standardized stromal TILs assessment, but they can be recorded as separate parameters for research purposes.

In areas surrounding the tumor, follicular aggregates, although rare can be observed, including TLS with germinal centers indicative of an active immune response. These aggregates should also not be included in the stromal TILs assessment; however, they can be evaluated separately as a research parameter, as they represent areas of T- and B-cell activation. TLS may become important in the future as more evidence emerges on their clinical relevance [16]. In the meantime, no formal recommendations for scoring TLS in daily practice can

be put forward. TLS generally form in the peritumoral regions and therefore may be absent or underrepresented in cores or TMAs and heterogeneous in full sections. Furthermore, the criteria for unequivocal identification of TLS are currently unclear and the use of IHC may be required. Therefore, at the present time, TLS should only be assessed in a research setting.

intratumoral heterogeneity and evaluation of the invasive edge

Based on the collective experience within the TILs working group most tumors are not heterogeneous at the morphological level in their TILs content between FFPE blocks of the same tumor, although there is currently no published evidence to support this statement. Nevertheless, heterogeneity in a single tissue section can be encountered. The most used methodology is global assessment of the slide by a trained pathologist, with a mean infiltrate score based on all available tissue being reported. Stromal TILs should be reported as a percentage. If the percentage of TILs is questionable, discuss the case with a second pathologist. In heterogenous tumors, evaluate different regions and report the average. The working group does not recommend focusing on 'hot spots', defined as small areas with increased TILs. These small areas are often observed, and they should be included in the average TILs assessment. There is no current evidence demonstrating whether the extent of heterogeneity might be clinically important. Since heterogeneity has not yet been investigated neither formally characterized either at the morphological nor functional level, the TILs working group's current recommendation, although not formally supported by data is that if there is a choice between full sections and core biopsies, whole tissue sections are preferred over core biopsies.

There is no current evidence demonstrating that TILs at the invasive edge are functionally different from TILs in the center of the tumor. Based on this lack of knowledge, it was suggested that scoring TILs at the invasive edge as a separate parameter from TILs located in the inner stroma should at the present time merely be done in a research setting. In daily practice, a distinction should not be made and all TILs within the tumor boundaries, including the invasive edge should be scored together as stromal TILs.

inter- and intralaboratory assessment, thresholds and machine learning algorithms

Most published or ongoing unpublished studies have not formally assessed intra- and interpersonal differences in scoring slides by pathologists. The accepted intra- and interpersonal discordance between pathologists depends on the clinical use/consequences of the measurements. The total allowable margin of error between pathologists will thus need to be determined in accordance with the clinical validity and utility of this methodology. It should be emphasized that if the primary purpose is to find an approach for daily practice, the impact on daily routine should be minimal without a significant increase in the pathologist's time. A tutorial to help pathologists evaluate TILs accompanies this paper.

At present, there are no established thresholds for TILs. The consensus of the group was that a valid methodology was top priority and that thresholds for clinical decision can be

determined once a solid methodology with clinical utility is in place. Therefore, there are currently no recommendations for the best threshold in clinical practice. We recommend that TILs be analyzed as a continuous variable unless it is clear that the prognostic information is not linearly associated with increasing levels of TILs. Further research will determine whether a threshold is required. We again emphasize that the level of TILs should not be used to withhold chemotherapy or trastuzumab therapy in TN and HER2+ BC, respectively.

The assessment of TILs by digital image analysis might be useful for standardization in the future, since this approach has the potential, for example, to determine the number of TILs per mm^2 stromal tissue as an exact measurement contrary to the approximate semiquantitative evaluation suggested at this moment. Based on the standardized methodology recommended here (summarized in Table 2), an interlaboratory Ring study will be initiated to assess the reproducibility and clinical validity of TILs assessment, including machine learning algorithms.

future directions

This article has focused on a standardized approach for measuring the percentage of stromal TILs in primary tumor specimens before therapy, using visual assessment of standard H&E-stained sections. Our goal is to facilitate the use of TILs as a biomarker in research and clinical trial settings (i.e. as a stratification or adjustment factor). This should also provide a platform for pathologists to further engage in an effort for the harmonization of the assay.

While it may be argued that stromal TILs are a robust prognostic factor in TNBC treated with standard adjuvant anthracycline-based chemotherapy, with three published prospective validation studies currently provide level I evidence for its clinical validity, we do not yet advocate that adjuvant treatment decisions be based on the level of TILs in the baseline TNBC neither on HER2+ cancer samples because the analytical validity and clinical utility of TILs in these subtypes remains to be firmly determined. We are yet to determine whether TILs will be predictive of response to immunotherapeutic regimens, in particular T-cell checkpoint inhibition, which ultimately may be its clinical utility.

While TILs have been measured morphologically and have been shown to add predominantly prognostic information, methodological open questions in the morphological evaluation of TILs still remain (supplementary Material S3 and supplementary Figure S1, available at *Annals of Oncology* online). The measurement on H&E-stained slides most likely represents the beginning of the efforts to use infiltrating cell properties as companion diagnostic tests. The huge complexity of lymphocytes, both from the standpoint of cell type and activation suggests that molecular characterization of this infiltrate may add both sensitivity and specificity to the predictive value of morphologically defined TILs [50, 51]. Thus, as a field, we should be open to the introduction of molecular methods, most likely *in situ*, that can classify the TILs component. However, at this time, these molecular methods are still experimental and not sufficiently documented for introduction into standard practice.

Further scientific questions concerning the underlying BC pathology associated with higher levels of TILs at diagnosis, the

relevance of TILs subtyping and what can be done clinically to enhance host antitumor immune responses have not been addressed at present. As our understanding evolves and clinical evidence accumulates, some of the methodological statements provided in these 2014 guidelines will be updated in subsequent articles.

context of research

A panel of pathology, clinical oncology, biostatisticians and translational research experts conducted a systematic review of the literature. Panel members invited have had experience in TIL assessment in phase III trials or are involved in breast cancer translational research focused on the interactions between immunology and breast cancer. There are no existing guidelines on TIL assessment in breast cancer available for comparison; neither is there proficiency testing data available from international organizations. No specific funding was obtained for this project. For details on prognosis, prospective–retrospective phase III trials were the main basis for these recommendations. However, we searched PubMed from 1 Jan 2009 to 30 April 2014 for full reports of studies involving clinical trial datasets or large institutional cohorts and evaluation of TILs, CD8+, CD3+ or immune gene signatures in primary breast cancer. Studies were not limited to randomized trials, but included also large consecutive and retrospective series. In-press publications were also taken into consideration. With regards to specific TILs pathological assessment, the panel undertook a formal expert consensus-based process by regular mail, teleconferences as well as two F2F meetings by the writing committee (RS, SL, SdM, KWG, SA) to produce these recommendations. Draft manuscripts were circulated by email to all coauthors and the writing committee had responsibility for approving the final manuscript.

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disclosure

SL , SiL and CS are also named co-inventors of the same patent. All other authors have declared no conflicts of interest.

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