





**Abstract:** Assessment of tumor-infiltrating lymphocytes (TILs) in histopathologic specimens can provide important prognostic information in diverse solid tumor types, and may also be of value in predicting response to treatments. However, implementation as a routine clinical biomarker has not yet been achieved. As successful use of immune checkpoint inhibitors and other forms of immunotherapy become a clinical reality, the need for widely applicable, accessible, and reliable immunooncology biomarkers is clear. In part 1 of this review we briefly discuss the host immune response to tumors and different approaches to TIL assessment. We propose a standardized methodology to assess TILs in solid tumors on hematoxylin and eosin sections, in both primary and metastatic settings, based on the International Immuno-Oncology Biomarker Working Group guidelines for TIL assessment in invasive breast carcinoma. A review of the literature regarding the value of TIL assessment in different solid tumor types follows in part 2. The method we propose is reproducible, affordable, easily applied, and has demonstrated prognostic and predictive significance in invasive breast carcinoma. This standardized methodology may be used as a reference against which other methods are compared, and should be evaluated for clinical validity and utility. Standardization of TIL assessment will help to improve consistency and reproducibility in this field, enrich both the quality and quantity of comparable evidence, and help to thoroughly evaluate the utility of TILs assessment in this era of immunotherapy.

**Key Words:** lymphocytes, tumor-infiltrating, biomarkers, cancer, immunotherapy, pathology

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Pathologists have long recognized the stroma, immune infiltrate, nerves, and vasculature as integral parts of the tumor microenvironment, which often provide important information regarding tumor behavior, prognosis, and response to treatment. It is well established that tumors are antigenic and can induce an immune response, due in part to altered protein products that may be recognized as foreign by the host immune system.<sup>1,2</sup> A growing body of research has shown that the extent and composition of the host immune response to the tumor has prognostic and predictive significance in many solid malignancies (reviewed in<sup>3</sup>). The assessment of immune infiltrate in tumors, most commonly referred to as tumor-infiltrating lymphocytes (TILs), is also gaining importance in the current quest for optimal biomarkers to select patients with the highest likelihood of responding to immunotherapeutic agents. Therefore, TIL assessment has been proposed as a biomarker for inclusion in routine histopathologic reporting.<sup>4,5</sup> Current TIL scoring systems used in research and proposed for different tumor types vary widely in detail, scope, accuracy, and time and resource requirements.

Development of prognostic and predictive biomarkers in oncology requires robust assessment of the test's analytical validity, clinical validity, and clinical utility.<sup>6,7</sup> Evidence is accumulating to support the use of TILs scoring as a prognostic biomarker in various solid tumors and evidence for the predictive benefit of TILs is being investigated at present. Different methods of assessing TILs will have different preanalytical, analytical, and postanalytical challenges. For example, semiquantitative hematoxylin and eosin (H&E)-based scores may suffer from low precision and poor interobserver reproducibility if no clear guidance exists, whereas digital quantification of immunohistochemistry (IHC)-stained sections may produce different

results due to inaccurate measurement of the test variable without controlled calibration. Testing of the clinical validity of biomarkers involves determining the extent to which the biomarker predicts the clinical outcome of interest, that is, patient prognosis or response to treatment.<sup>7</sup> Assessment of the clinical validity of TILs scoring requires a standardized, reproducible method, which can be validated preferably in several independent populations. Many biomarker studies are observational, retrospective studies in which the study population is selected solely by the availability of samples.<sup>8,9</sup> Although prospective controlled studies designed to test biomarkers are rare and unlikely to be performed on a large scale, prospective-retrospective studies may offer a comparable level of evidence.<sup>8</sup> These prospective-retrospective studies involve use of samples collected during a prospective randomized clinical trial, and allow high-quality evaluation of the biomarker of interest provided the study design meets certain criteria and results can be replicated in an independent population.<sup>8</sup> Guidelines for the reporting of biomarker studies are available<sup>9,10</sup> and should be considered when evaluating the TILs literature.

In part 1 of this review, we aim to briefly describe the host immune response to tumors and approaches used to assess this in the current context of immunotherapy. We propose a standardized methodology for TIL assessment in solid tumors, based on the International Immuno-Oncology Biomarkers Working Group guidelines for TIL assessment in invasive breast carcinoma, which may be adapted to different tumor types. We then discuss the literature and our experiences in the areas of invasive breast cancer, ductal carcinoma in situ (DCIS), and metastatic tumor deposits, then conclude with a discussion of open questions and areas for further research. In part 2 of this review, the literature surrounding methods of TIL assessment and the prognostic and potentially predictive significance of TILs in different solid tumors is discussed, including carcinomas of the lung, colon, upper gastrointestinal tract, head and neck, genitourinary tract, and gynecological organs, as well as mesothelioma, melanoma, and primary brain tumors. Ways in which the proposed methodology can be adapted to different tumors are suggested, based on available evidence and expert opinion. Standardization of TIL assessment will allow direct comparison of different studies, highlight areas for further research, and form the basis of TIL assessment in routine histologic practice.

## THE HOST IMMUNE RESPONSE

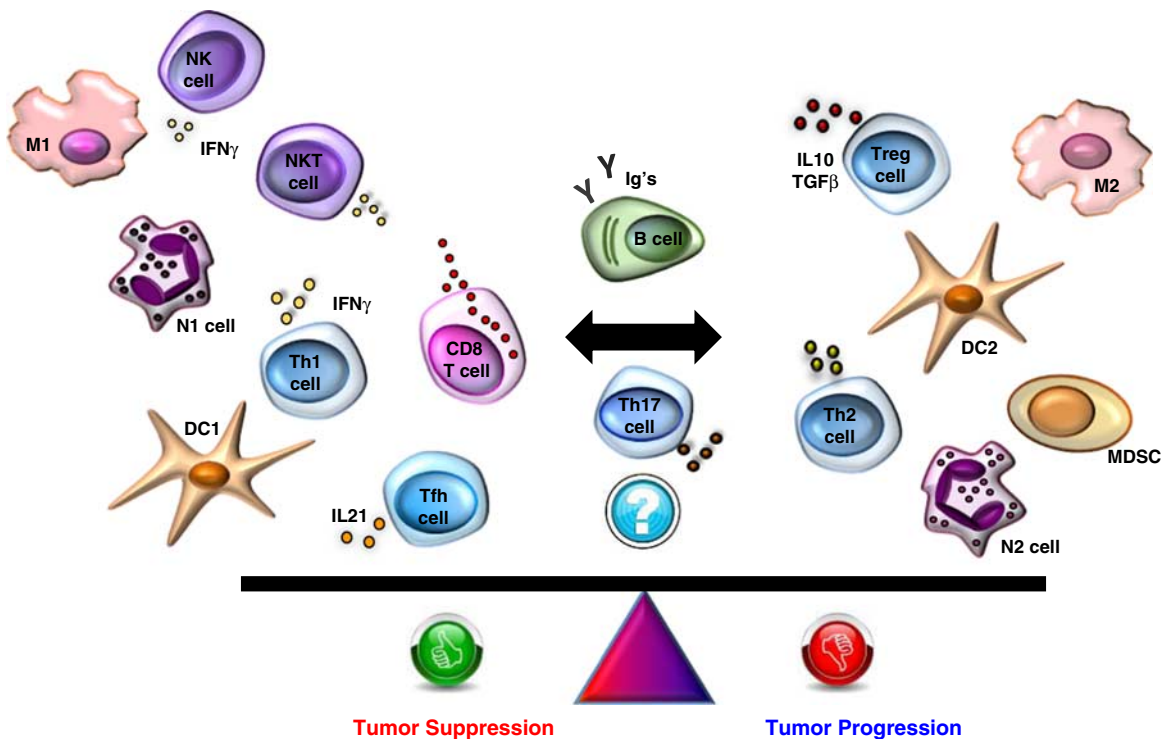
Altered protein products caused by the genetic mutations in cancer cells can function as neoantigens, eliciting an immune response against a perceived “foreign” cell.<sup>2</sup> In addition, the inflammatory, hypoxic, and often necrotic microenvironment of tumors sends concomitant danger signals to the host immune system.<sup>11</sup> Infiltrating immune cells can function to control tumor growth and progression, but can also help to create an immunosuppressive environment in which the tumor can thrive.<sup>12</sup> CD8<sup>+</sup> cytotoxic T cells, T-helper 1 (Th1) cells producing interferon- $\gamma$ , and natural killer cells are generally associated with favorable antitumor immune responses, along with macrophages polarized to an M1 phenotype and dendritic cells showing a DC1 phenotype. Immunosuppressive effects are seen with Th2 cells, M2 macrophages, DC2 dendritic cells, myeloid-derived suppressor cells, and FOXP3<sup>+</sup> regulatory T (T<sub>reg</sub>)

cells producing interleukin-10 and transforming growth factor- $\beta$ . B cells and plasma cells can also adopt either effector or regulatory phenotypes, and hence can carry positive or negative antitumor associations depending on contextual factors. This balance of the cellular constituents of the immune response is illustrated in Figure 1. The presence of tertiary lymphoid structures, aggregates which recapitulate the components and architecture of a lymph node, in the tumor microenvironment is correlated with better prognosis in different types of solid tumors (reviewed in<sup>15</sup>). The exact composition of the immune infiltrate can vary widely within and between tumors and clearly modulate the effectiveness of the antitumor response.

In research settings, many different methods are being used to investigate the host immune response to tumors. Many clinical studies have found significant results using an assessment of H&E-stained sections by trained pathologists, with qualitative or semiquantitative scoring systems which vary according to tumor type.<sup>14-16</sup> IHC allows definition of the majority of immune cell subsets that can be refined by combinations of markers, including CD8<sup>+</sup> cytotoxic T cells, CD4<sup>+</sup> T-helper cells, FOXP3<sup>+</sup> T<sub>regs</sub>, B cells, macrophages, and dendritic cells. Other cell types such as myeloid-derived suppressor cells require multiple cell surface markers for definition and are challenging to identify on serial IHC sections. Digital image analysis has been validated in multiple studies and can provide accurate quantitation of immune cell infiltrates in IHC-stained sections.<sup>17,18</sup> Multiplexed fluorescent IHC with multi-spectral imaging is a recent development that allows in situ

identification of different immune cell subsets on the same section, providing quantitative information on the distribution and composition of the immune infiltrate on formalin fixed, paraffin embedded (FFPE) tissue.<sup>19-21</sup> This technology requires a significant investment in initial optimization, has complex data analysis and storage requirements, and is yet to become routine. Recently, a novel approach to multiplexed IHC was described utilizing NanoString NCounter fluorescent barcodes to identify bound antibodies, allowing quantitation of multiple proteins in situ on an FFPE slide.<sup>22</sup> FFPE tissue can also be used for matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS), a proteomic technique that can identify hundreds proteins in situ without the need for specific antibodies.<sup>23,24</sup> These exciting new technologies, termed “molecular histology” combine the spatial and architectural information from traditional histology approaches with detailed molecular profiling, which is likely to be particularly relevant in describing the immune microenvironment of tumors.

Flow cytometry is a common approach to immune cell profiling and has many benefits including the characterization of immune cell subsets by multiple markers, quantitative data acquisition, wide availability, and the ability to examine small subpopulations of interest.<sup>25</sup> However, fresh tissue is required and no information is provided on the distribution or organization of the immune infiltrate or relationship to other microenvironmental structures. Nevertheless, recent study of TILs in invasive breast carcinoma found a significant positive correlation between fresh tumor tissue analyzed by flow cytometry and IHC-stained sections



**FIGURE 1.** The cellular constituents of the host immune response to tumors can control tumor growth or contribute to an immunosuppressive environment that promotes tumor progression. Breg indicates regulatory B cell; DC, dendritic cell; GC B cell, germinal center B cell; IFN $\gamma$ , interferon  $\gamma$ ; Ig's, immunoglobulins; IL10, interleukin-10; IL21, interleukin-21; M, macrophage; MDSC, myeloid-derived suppressor cell; N, neutrophil; NK, natural killer cell; NKT, natural killer T cell; Tfh, follicular B helper T cells; Th, helper CD4<sup>+</sup> T cell; TGF $\beta$ , transforming growth factor  $\beta$ ; Treg, regulatory T cell.

scored by a pathologist.<sup>26</sup> Messenger RNA (mRNA) profiling of tumor tissue can detect “immune gene signatures,” using the level of expression of immune-related genes to describe the composition and functional status of the immune infiltrate.<sup>27</sup> Again, no information is provided on the distribution of the infiltrate and this resource-intensive technology is currently largely restricted to a research setting. These more complex methods are by their nature more difficult to implement in large, multicenter clinical trials, which ultimately are required for the validation of potential biomarkers. The costs of such detailed techniques must also be weighed against the additional information that may be derived regarding the composition and functional status of the immune infiltrate.

The host immune response to tumors is currently of great interest to oncologists and researchers following impressive early results of immune checkpoint inhibitor therapy. An important mechanism of tumor immune evasion is the expression of immune checkpoint molecules such as cytotoxic T lymphocyte antigen-4 and programmed death ligand-1 (PD-L1), both on tumor cells and on infiltrating immune cells.<sup>28</sup> By blocking these signaling pathways, immune checkpoint inhibitors can reactivate the host immune system to recognize and control the tumor.<sup>28</sup> Clinical trials have demonstrated often durable responses in different tumor types including melanoma,<sup>29,30</sup> urothelial carcinoma,<sup>31</sup> Hodgkin lymphoma,<sup>32</sup> non-small cell lung carcinoma,<sup>33–35</sup> renal cell carcinoma,<sup>36</sup> and squamous cell carcinoma of the head and neck.<sup>37</sup> However, responses within tumor types vary widely and the selection of patients likely to respond remains problematic.<sup>38</sup> Despite Federal Drug Administration approval as companion and complementary diagnostics for the use of anti-PD-1 therapy in non-small cell lung carcinoma,<sup>39,40</sup> immunohistochemical identification of PD-L1 expression on tumor cells and/or immune cells is an imperfect biomarker<sup>40</sup> and a significant research effort is ongoing to identify reliable, broadly applicable, and clinically valid biomarkers. T-cell infiltration into tumors is critical to the success of immune checkpoint blockade,<sup>41</sup> and tumors with high levels of infiltrating effector T cells, as measured by gene expression profiling, appear to show improved responses.<sup>42–44</sup> Assessment of TILs in this context is a highly active area of research and guidance for a standardized methodology is therefore timely.

### PROPOSAL FOR A STANDARDIZED METHODOLOGY FOR SCORING TUMOR-INFILTRATING LYMPHOCYTES IN SOLID TUMORS, IN BOTH PRIMARY AND METASTATIC SETTINGS

Much research has been performed to establish the prognostic and predictive significance of TILs in different solid tumors. However, further work is needed to ensure that the valuable information that could be obtained from TILs assessment is not lost due to issues of resource commitments, methodology, or lack of standardization. It is our view that a semiquantitative H&E-based TILs assessment provides clinically relevant information in a format that is applicable to large-scale randomized clinical trials, to preclinical and clinical research projects, and to everyday pathology practice, whether it be in high, middle, or low-income countries. It needs to be emphasized that the

sophisticated tools mentioned above are resource-intensive and may be difficult to implement in middle or low-income settings. A biomarker based on a plain H&E-stained section is affordable and accessible, and will not add to the often-restrictive costs of accessing therapy. This is an important consideration in the current era of immunotherapeutics.

Over the past few years, Roberto Salgado, Sherene Loi, and Carsten Denkert have developed the International Immuno-Oncology Biomarker Working Group on Breast Cancer, with members including important clinical research groups, pathologists, clinicians, and statisticians worldwide currently knowledgeable in the field of immunooncology biomarkers. The purpose of this Working Group is to develop, in a timely manner, standards on the assessment of immunooncology biomarkers to aid pathologists, clinicians, and researchers in their research and daily practice. The group has, for example, developed the first International Guidelines on TIL Assessment in Breast Cancer<sup>14</sup> and other guideline papers are in development. In addition to the breast cancer experts already included, academic expert groups from other fields and biomarker expert groups from industry were contacted and enthusiastically agreed to be a part of this initiative. We include worldwide representatives of known clinical research groups from expert centers across all continents (United States, Europe, Australia, the Middle East, and Japan) and the member list is growing.

For these papers, a panel of pathologists, medical oncologists, biostatisticians, and translational researchers from different expert groups conducted a systematic review of the literature. Panel members have had experience in TIL assessment in preclinical research, clinical trials, or are involved in translational research focused on the interactions between immunology and cancer. There are no existing guidelines on TIL assessment in solid tumors available for comparison; neither are there proficiency testing data available from international organizations. No specific funding was obtained for this project. The methodology we propose is based on the International Guidelines on TIL Assessment in Breast Cancer,<sup>14</sup> following robust evidence from prospective-retrospective phase III trials in breast cancer. Reviewed studies were not limited to randomized trials, but also included consecutive and retrospective series and in-press publications. Ways in which this methodology could be adapted to different tumor types, while remaining as standardized as possible, were suggested, reviewed, and discussed by members of the author group expert in the particular field, to reach the consensus opinions presented here. Where strong published evidence was lacking, the panel undertook a formal expert consensus-based process by regular mail and face-to-face meetings at the San Antonio Breast Cancer Symposium 2016. Further updates and revisions are planned as more evidence becomes available and the field progresses.

We propose a method for TIL assessment that would be applicable to all solid tumors (Table 1 and Fig. 2). As for any proposed scoring system, this method should be evaluated for clinical validity and utility, as well as inter-observer reproducibility. This method may be used as a reference against which other systems, such as IHC-based digital quantification or mRNA profiling, are compared, to establish a sensible balance between simplicity and information yield. Individual tumor types have particular issues surrounding TILs that may require further investigation to resolve, and this method may be adapted to incorporate

**TABLE 1.** Proposed Guidelines for the Assessment of TILs in Solid Tumors: Recommendations by an International Immuno-Oncology Biomarker Working Group

- (1) TILs should be reported separately for the stromal compartment (= % stromal TILs) and the tumor cell compartment (= % intratumoral TILs). The reasons are (1) in many tumors the TIL density in both compartments is different, and (2) if the TILs are evaluated simply per tumor area, the density and growth pattern of tumor cells (= a nonimmune parameter) will affect the TIL count. The denominator used to determine the % stromal TILs is the area of stromal tissue (ie, area occupied by mononuclear inflammatory cells over total stromal area), not the number of stromal cells (ie, fraction of total stromal nuclei that represent mononuclear inflammatory cell nuclei). Similarly, for intratumoral TILs the tumor cell area is the denominator. In some tumor types, for example, breast cancer, it might be decided to evaluate only the stromal TILs.
- (2) TILs should be evaluated within the borders of the invasive tumor, including both “central tumor” and “invasive margin.” These areas may be reported separately when required.
- (3) The “invasive margin” is defined as a 1-mm region centered on the border separating the malignant cell nests from the host tissue. The “central tumor” represents the remaining tumor area.
- (4) Exclude TILs at a distance outside of the tumor borders. TILs immediately adjacent to the invasive margin, that is, “peritumoral TILs,” may be evaluated when required.
- (5) Exclude TILs in tumor zones with crush artifacts, necrosis, and regressive hyalinization, as well as in previous biopsy sites.
- (6) All mononuclear cells (including lymphocytes and plasma cells) should be scored, but polymorphonuclear leukocytes (neutrophils) should be excluded.
- (7) One section (4–5  $\mu$ m, magnification  $\times$  200 to 400) per patient can be considered to be sufficient for practical purposes. However, assessing additional sections for each case whenever possible and reporting the number of sections reviewed per case specifically in the manuscript is recommended as the extent of heterogeneity for different tumor types is unknown.
- (8) 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.
- (9) A full assessment of average TILs in the tumor area (central tumor and invasive margin) should be used. Do not focus on hotspots.
- (10) TILs should be assessed as a continuous variable, as this may provide more biologically relevant information and allow more accurate statistical analyses. However, in daily practice most pathologists will report discrete estimates, for example, 13.5% will be rounded to 15%. Pathologists should report their scores in as much detail as the pathologist feels comfortable with.
- (11) For assessment of percentage values, the dissociated growth pattern of lymphocytes needs to be taken into account. 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. 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.
- (12) No formal recommendation for a clinically relevant TIL threshold(s) can be given at this stage. 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.

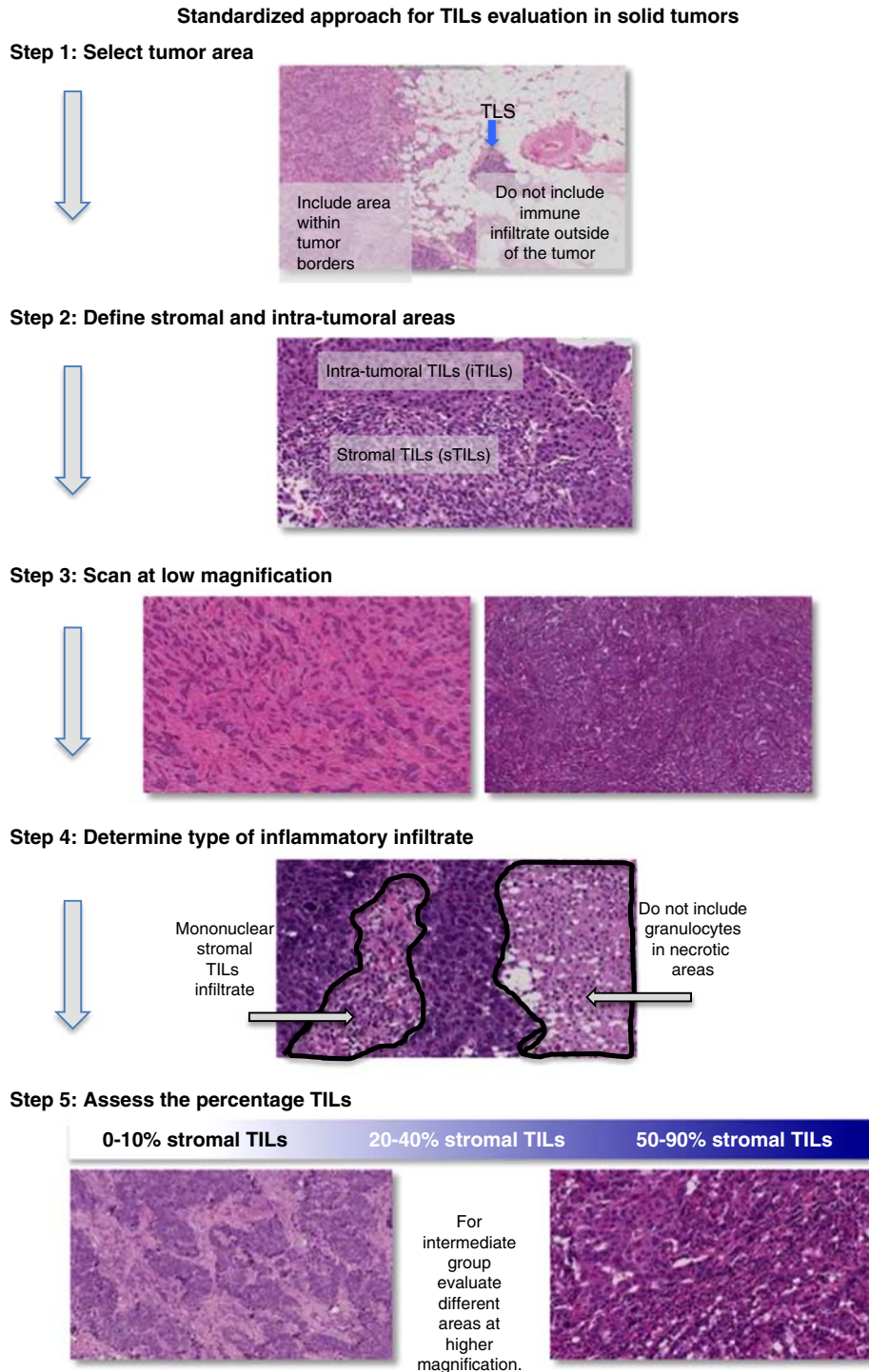
TIL indicates tumor-infiltrating lymphocyte.

those particularities (Table 2). However, most of the issues raised are common to many solid tumor types, such as the relative importance of stromal TILs (sTILs) and intratumoral TILs (iTILs) or the delineation of the central tumor and the invasive margin, and we feel that the method outlined in Table 1 should be able to be applied in most research and clinical settings. Pragmatic definitions of the invasive margin and central tumor areas are discussed further in part 2 of this review, but are illustrated in Figure 3 and summarized as follows: the invasive margin is defined as a 1-mm-wide zone centered on the border of the malignant cells with the host tissue, and the central tumor is defined as the central tumor tissue surrounded by this zone. Tutorials illustrating TIL assessment in different tumor types and reference scoring sheets illustrating densities of sTILs are available as supplementary material online, Supplemental Digital Content 1 <http://links.lww.com/PAP/A13>, Supplemental Digital Content 2, <http://links.lww.com/PAP/A14>, Supplemental Digital Content 3, <http://links.lww.com/PAP/A15>, Supplemental Digital Content 4, <http://links.lww.com/PAP/A16>. Having a reference method to score TILs will help to ensure that potential future studies investigating TILs in each tumor type can be compared. Many potentially useful biomarkers such as the Ki67 proliferative index in breast cancer suffer from a plethora of noncomparable methods, which affects the level of evidence that can be obtained and prevents uniform clinical implementation. International efforts at standardization are also being explored for this important biomarker.<sup>46</sup>

## TUMOR-INFILTRATING LYMPHOCYTES IN INVASIVE BREAST CARCINOMA

The wide variation in the immune infiltrate seen in breast carcinomas is well known to pathologists, with an early description of medullary breast carcinoma by Moore and Foote in 1949<sup>47</sup> highlighting the lymphocytic infiltrate as a key diagnostic criterion and linking it to good prognosis. This lymphocytic infiltrate was suggested to represent a form of host resistance to the tumor; however, conflicting results regarding the prognostic significance of TILs were found in subsequent studies.<sup>48–51</sup> In addition, it was recognized that a prominent lymphocytic infiltrate may be found in tumors not meeting the other strict criteria for medullary breast carcinoma.<sup>51</sup> Most breast carcinomas show some degree of lymphocytic infiltrate, with higher rates of “lymphocyte predominant breast carcinoma,” variably defined as > 50% or > 60% of tumor area occupied by TILs, seen in triple negative breast carcinoma (20%) and HER2-positive breast carcinoma (16%) compared with estrogen receptor positive breast carcinoma (6%).<sup>52</sup> Recently, through large-scale international efforts, evidence has accumulated to support the prognostic and potential predictive impact of TILs, both in triple negative and HER2-positive cancers. The clinical utility of assessing TILs in breast cancer relates to risk prediction models, adjuvant, and neoadjuvant chemotherapy decisions, and the growing potential of immunotherapy.<sup>53</sup>

Several large systematic reviews and meta-analyses have confirmed that high levels of TILs are associated with better disease-free survival and overall survival only in



**FIGURE 2.** Guidelines for a standardized approach to TILs evaluation in solid tumors. Modified from Salgado et al<sup>14</sup> with permission from Oxford University Press on behalf of the European Society for Medical Oncology. TIL indicates tumor-infiltrating lymphocyte. Adaptations are themselves works protected by copyright. So in order to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation. Please see this image in color online.

triple negative and HER2-positive subtypes, with no significant benefit seen in estrogen receptor positive breast carcinoma.<sup>54-56</sup> Pooled hazard ratios for overall survival with variably defined “high TILs” ranged from 0.71 to

0.88.<sup>54-56</sup> A recent pooled analysis of 5 large clinical trials of anthracycline chemotherapy in triple negative breast carcinoma presented similar results, with an adjusted relative risk reduction of 12% for disease-free and overall

**TABLE 2.** Additional Points for Attention When Assessing TILs in Different Tumor Types

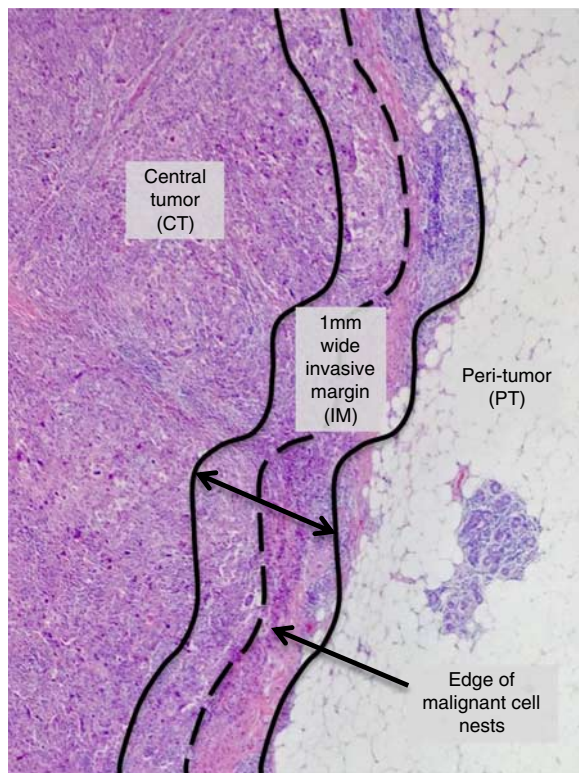
Invasive breast carcinoma	Refer to Salgado et al. <sup>14</sup> Consensus guidelines are reproduced in Table 3.
Ductal carcinoma in situ and other premalignant lesions	Refer to Pruneri et al. <sup>45</sup>
	Stromal area is defined as the specialized stroma surrounding the ducts involved by in situ carcinoma, or when this is not clear, an area surrounding the ducts within 2 high-power fields (~1 mm).
	Any type of circumferential infiltrate should be taken into account, including minimal, partial, subtotal, and total circumferential TILs.
	Exclude TILs that are in continuity between the invasive tumor and the in situ lesions with no clear distinction as to whether these are TILs associated with the invasive or in situ component.
Melanoma	Currently only iTILs are scored in the clinical setting. sTILs and/or peritumoral TILs may be reported separately in research settings.
	Only the vertical growth phase of the primary tumor is evaluated.
	Further research may determine what % iTILs corresponds to the traditional categorization of brisk/nonbrisk/absent.
Colorectal carcinoma	Separately reporting invasive margin and central tumor TILs is recommended. Invasive margin TILs seem to have the most prognostic significance in this setting.
Upper gastrointestinal tract carcinomas	Early evidence supports evaluating only sTILs in gastric carcinoma, due to a lack of prognostic significance of iTILs. This finding requires further validation.
	There are insufficient data on pancreatic ductal adenocarcinoma and hepatocellular carcinoma to make specific recommendations.
Non-small cell lung carcinoma	sTILs and iTILs should be separately reported in the research setting at present. Insufficient evidence is available to support evaluating only sTILs over a combined assessment.
	Do not include areas with pure intra-alveolar tumor spread (aerogenic spread) or with pure lepidic growth (no desmoplastic reaction).
	Do not include alveolar macrophages.
Ovarian carcinoma	Both the central tumor and invasive margin should be included, but it is not currently recommended to report these regions separately.
	iTILs and sTILs should be separately reported at present, as both compartments seem to have prognostic relevance. Further research may determine the relative importance of the different compartments.
	Include TILs in the stroma pertaining to fibrovascular cores of papillary structures.
	Include the invasive margin of superficial peritoneal or ovarian deposits.
Head and neck squamous cell carcinoma	Care should be taken to exclude preexisting lymphoid stroma from the assessment in oropharyngeal (ie, tonsillar and base of tongue) tumors. If a desmoplastic stroma is present, sTILs can be scored in this reactive stroma (as for lymph node metastases).
Genitourinary carcinomas	Separate reporting of iTILs and sTILs is recommended—this is important in the context of immune checkpoint inhibitor therapy with atezolizumab in urothelial carcinoma, where the PD-L1 “immune cell” score is derived from the sTILs score.
	Care should be taken to avoid areas of diathermy/coagulation artifact, a common finding in bladder tumor specimens.
	Noninvasive papillary structures are not currently included in the assessment.
	There are insufficient data on prostate carcinoma and renal cell carcinoma to make specific recommendations.
Primary brain tumors	No evidence-based recommendations on the optimal method for TIL quantification can be made at present.
	Immunohistochemistry may be required to clearly identify TILs and distinguish immune cells from preexisting or neoplastic glial and neuronal cells.
	Consideration should be given to separately reporting TILs in the central tumor, perivascular areas, perinecrotic areas, and the invasive margin.
Metastatic tumor deposits	In metastatic deposits within lymph nodes, if a desmoplastic stroma is present, sTILs and iTILs can be scored as for the primary lesion. If a desmoplastic stroma is not present, focus only on iTILs. Exclude the preexisting lymphoid stroma.
	Other sites are evaluated as for the primary lesion.
	Future research should focus on the relative clinical utility of evaluating TILs in the primary tumor or in the most recent tumor tissue available.

iTIL indicates intratumoral TIL; PD-L1, programmed death ligand-1; sTILs, stromal TILs; TILs, tumor-infiltrating lymphocytes.

survival for every 10% increment in sTILs.<sup>57</sup> Both iTILs and sTILs gave similar results when separately assessed<sup>54,56–58</sup>; however, iTILs did not appear to add prognostic information to sTILs in a multivariate model.<sup>57</sup> The majority of these data come from large prospective-retrospective studies, associated with randomized clinical trials<sup>14,57,59–66</sup>; however, smaller retrospective studies were also included in the meta-analyses. Although many of the included studies follow the TIL scoring method used by Denkert et al in 2010,<sup>61</sup> there is variation with some studies using H&E assessment,<sup>14,59,60,62,63,66</sup> some using IHC,<sup>64,65</sup> and others using mRNA expression profiling.<sup>64</sup> Conclusions from some recent meta-analyses in this field<sup>67,68</sup>

indicate that care must be taken when interpreting the results of studies of prognostic biomarkers. For example, it is suggested that FOXP3<sup>+</sup> T<sub>regs</sub> correlate with poor prognosis in breast cancer<sup>67,68</sup>; however, T<sub>reg</sub> levels also correlate with other poor prognostic factors such as estrogen receptor-negativity, HER2-positivity, lymph node metastasis, and high histologic grade.<sup>69–72</sup> Within the estrogen receptor-negative subgroup, high levels of FOXP3<sup>+</sup> T<sub>regs</sub> may actually correlate with improved prognosis as markers of an active immune response.<sup>69,71</sup> Both primary studies and meta-analyses should report the results of multivariate analyses to confirm the prognostic value of the biomarker in question, independent of other known prognostic factors.<sup>10</sup> Despite these





**FIGURE 3.** The “invasive margin” (IM) is defined as the region centered on the border separating the host tissue from the malignant nests, with an extent of 1 mm. “Central tumor” (CT) corresponds to all the tissue inside the IM, and “peritumor” (PT) to tissue outside of the IM. Please see this image in color online.

inconsistencies, there seems to be a robust prognostic benefit of a high lymphocytic infiltrate in triple negative and HER2-positive breast cancer, irrespective of the exact methodology or scoring system used.

TILs may also have value in predicting benefit from neoadjuvant chemotherapy in subsets of breast cancer patients. Both cytotoxic chemotherapy and endocrine therapy may partly function through immunomodulatory effects. Anthracyclines such as doxorubicin induce immunogenic cell death and depend on adaptive and innate immunity for therapeutic efficacy,<sup>73,74</sup> and it has been suggested that treatment with the aromatase inhibitor letrozole, with or without cytotoxic chemotherapy, may counteract some of the immunosuppressive effects of estrogen signaling through reduction in tumoral  $T_{regs}$ .<sup>75</sup> Higher levels of TILs, both  $CD8^+$  T cells and  $FOXP3^+$   $T_{regs}$ , in pretreatment biopsies correlated with higher rates of pathologic complete response to neoadjuvant chemotherapy in triple negative and HER2-positive breast carcinoma.<sup>14,61–64,76</sup> A meta-analysis of 6 large clinical trials found high sTILs were a strong predictive maker for response to neoadjuvant chemotherapy in triple negative, HER2-positive and luminal subtypes; however, an overall survival benefit was not seen in the luminal cancers.<sup>77</sup> This association is strongest for lymphocyte predominant breast carcinoma<sup>61,63,77</sup> and seems to be independent of the type of chemotherapy used.<sup>53,76</sup> Indeed, in the NeoALLTO trial of neoadjuvant chemotherapy in HER2-positive breast carcinoma, high levels of TILs at diagnosis provided prognostic information

independent of pathologic complete response.<sup>14</sup> Early data suggest that iTILs may be of particular importance in this setting, with iTILs but not sTILs significantly associated with pathologic complete response in triple negative breast carcinoma.<sup>78</sup> In addition, the presence of TILs in post-treatment specimens with residual disease appears to confer a better prognosis than those showing an absence of TILs.<sup>79,80</sup> Guidelines from the International Immunology Biomarker Working Group for assessing TILs in the residual disease setting will soon be available.

Recently published consensus guidelines provide detailed instructions and illustrations to aid pathologists in the assessment of TILs in breast cancer.<sup>14</sup> These guidelines were developed at a meeting of major international breast cancer research teams (the International TILs Working Group, now known as the International Immunology Biomarker Working Group) in 2013, where it was recognized that standardized methodology and scoring systems are critical to the integration of information about TILs into future research and eventually into routine diagnostic practice.<sup>14</sup> The proposed methodology is based on that used by Denkert et al in 2010,<sup>61</sup> and requires assessment of only 1 H&E slide per specimen, as detailed in Table 3. Briefly, TILs are reported as an overall percentage of the stromal area within the borders of the invasive tumor that is covered by mononuclear immune cells. Care is taken to exclude any lymphocytic infiltrate around normal lobules, in the previous biopsy site or in areas of diathermy or crush artifact. It is stressed that no recommendation can currently be made for a clinically relevant cut off, and that TILs should be reported as a continuous variable, in as much detail as is comfortable to the reporting pathologist.<sup>14</sup> A tutorial outlining this methodology to score TILs in invasive breast cancer is available online in Supplementary File 1, Supplemental Digital Content 1 <http://links.lww.com/PAP/A13>, and a reference scoring sheet to illustrate different levels of sTILs in available in Supplementary File 2, Supplemental Digital Content 2, <http://links.lww.com/PAP/A14>.

In this method, only sTILs are assessed. Similar prognostic associations have been identified with both sTILs and iTILs, defined as TILs directly interacting with carcinoma cells with no intervening stroma.<sup>66,79</sup> The tumor stroma and the immune cells within it are integral parts of the tumor microenvironment, thus “stromal TILs” within the borders of the invasive tumor can be considered true TILs. It was felt that the assessment of iTILs in addition to sTILs would be unlikely to add value currently as they can be challenging to detect on H&E, are present in fewer cases and are dependent on the size and distribution of tumor nests.<sup>14</sup> However, due to the difficulties recognizing iTILs on an H&E, their importance may well be underestimated. Efforts are ongoing to identify and characterize iTILs using IHC and/or digital analysis tools, enabling a more accurate assessment of the potential clinical significance of TILs in this compartment. For example, CD103 is a marker of a subset of  $CD8^+$  TILs that are most prevalent amongst iTILs and may reflect  $CD8^+$  T cells that have been engaged in an adaptive immune response.<sup>81</sup> Whereas TIL assessment on H&E was of borderline significance in a small cohort of basal-like invasive breast tumors, dual  $CD103^+$   $CD8^+$  TIL status was strongly prognostic for relapse-free and overall survival.<sup>82</sup> The intensity and distribution of TILs in breast cancers is often heterogenous<sup>83,84</sup> and it is recommended to give an overall assessment rather than focusing on

**TABLE 3.** The Evaluation of TILs in Breast Cancer: Recommendations by an International TILs Working Group 2014

- (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 (ie, area occupied by mononuclear inflammatory cells over total intratumoral stromal area), not the number of stromal cells (ie, 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  $\mu$ m, magnification  $\times$  200 to 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, as 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% to 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.

Reproduced from Salgado et al<sup>14</sup> with permission from Oxford University Press on behalf of the European Society for Medical Oncology. TIL indicates tumor-infiltrating lymphocyte.

hotspots of intense infiltration, based on a lack of data demonstrating any clinical significance of this heterogeneity.<sup>14</sup> Recently, Mani et al<sup>83</sup> demonstrated more heterogeneity within a single biopsy than between biopsies or regions from a given breast cancer, suggesting that the average lymphocyte score from a single biopsy may be representative of the whole tumor, supported by another recent study showing high correlation between TILs assessed on a preoperative core biopsy and those assessed on the resection specimen.<sup>85</sup> Interestingly, despite the lack of functional information gained from assessing lymphocytes on H&E only, studies using this method have shown similar prognostic results to those separating lymphocyte subsets by IHC.<sup>14</sup> In addition, assessment of TILs on H&E-stained sections gave similar predictive information to mRNA expression profiling.<sup>62</sup> It is emphasized that the method proposed leaves a number of open questions requiring further investigation, discussed further below. Refinement of the method is ongoing and updated guidelines will be issued as new evidence becomes available.

Large international reproducibility ring studies by the International Immuno-Oncology Biomarker Working Group have recently been reported.<sup>53</sup> On the basis of the 2014 guidelines, an initial web-based assessment of 120 slides by 32 pathologists yielded good agreement with an intraclass correlation coefficient (ICC) of 0.70 [95% confidence interval (CI), 0.62–0.78]. Although high, this ICC did not meet the prespecified endpoint, and therefore a second round was undertaken, using specifically developed software, which provided visual reference ranges and integrated feedback. ICC improved to 0.89 (95% CI, 0.85–0.92) and it was suggested that visual reference tools such as the software used in this study might be of value both in research and in clinical practice.<sup>14,53</sup> A recent study has shown similarly high interobserver and intraobserver ICC for scoring TILs in invasive breast carcinoma; however, it was noted that agreement for the iTILs scores was lower than for the sTILs scores.<sup>85</sup> There

were no significant differences between the interobserver and intraobserver ICC values when scoring H&E sections or IHC-stained sections, and concordance between H&E and IHC scores was high (ICC, 0.75 for iTIL and ICC, 0.84 for sTIL).<sup>85</sup> Further reproducibility studies such as these are important to thoroughly validate TILs as a biomarker, compare different methodologies, and to work toward a consensus on the degree of reproducibility required for TIL assessment and for biomarkers more generally.

### TUMOR-INFILTRATING LYMPHOCYTES IN DUCTAL CARCINOMA IN SITU

In comparison with invasive breast carcinoma, less is known about the significance of the immune infiltrate associated with DCIS. Although features of the stromal microenvironment of DCIS, such as periductal myxoid change, angiogenesis and stromal proteins, are acknowledged as being important in determining disease outcome,<sup>86,87</sup> the immune infiltrate surrounding ducts involved by DCIS has received relatively little attention. Early studies do, however, suggest that TILs are associated with certain clinicopathologic features and disease progression, but may not have prognostic significance in this setting.

Like invasive breast carcinoma, some degree of lymphocytic infiltration is often present within or surrounding ducts involved by DCIS.<sup>45,88</sup> Pruneri et al<sup>45</sup> have recently reported on the prevalence and clinical relevance of TILs in DCIS, in a large series with substantial length of follow up. sTILs were significantly associated with DCIS grade, patient age, and the presence of comedonecrosis, and were most common in the HER2-positive subtype.<sup>45</sup> No association was observed with ipsilateral recurrence, whether in situ or invasive.<sup>45</sup> In this study, sTILs were scored using a method based on the International Immuno-Oncology Biomarker Working Group guidelines for invasive breast carcinoma,<sup>14</sup> with some modifications specific to the DCIS

setting (Table 2). A reference scoring sheet for DCIS developed by Pruneri et al<sup>45</sup> is available in Supplementary File 3, Supplemental Digital Content 3, <http://links.lww.com/PAP/A15>. This method was highly reproducible with an ICC of 0.96 (95% CI, 0.95-0.97). Thompson et al<sup>88</sup> performed a semiquantitative H&E assessment of TILs on tissue microarray sections, followed by a comprehensive immunohistochemical examination of the lymphocyte subsets involved. No significant associations were found between the H&E-based TILs score and other clinicopathologic features such as hormonal status, age, or concurrent invasive breast carcinoma; however, small numbers precluded complete assessment.<sup>88</sup> Campbell et al<sup>89</sup> used both manual H&E TIL counts and digital image analysis of IHC-stained sections to comprehensively study the immune microenvironment of DCIS. High-grade DCIS cases showed higher overall TILs, CD4<sup>+</sup> T cells, CD20<sup>+</sup> B cells, FOXP3<sup>+</sup> T<sub>regs</sub>, and CD68<sup>+</sup> macrophages than low or intermediate-grade DCIS cases.<sup>89</sup> An exploratory classification system identified CD8<sup>+</sup>HLADR<sup>+</sup> T cells, CD8<sup>+</sup>HLADR<sup>-</sup> T cells, and CD115<sup>+</sup> macrophages to be associated with recurrence.<sup>89</sup> These findings suggest that specific TIL subsets may be of importance in DCIS, and these subsets cannot be differentiated on H&E-stained sections. Further research is required to evaluate this potential effect of TIL subsets on recurrence in DCIS.

The host immune response to tumors impacts disease progression,<sup>12</sup> and this may be identifiable in preinvasive lesions such as DCIS. On the basis of immunohistochemical quantitation, Hussein and Hassan<sup>90</sup> described an increase in stromal lymphocyte subsets through progression from proliferative breast disease and DCIS to invasive carcinoma. Bates et al reported that the density of FOXP3<sup>+</sup> T<sub>regs</sub> in DCIS was higher than that seen in normal breast but less than in invasive carcinoma,<sup>72</sup> with similar findings reported in 2013 by Lal et al.<sup>91</sup> DCIS with higher T<sub>reg</sub> levels had a higher risk of recurrence.<sup>72</sup> Interestingly, similar findings have also been demonstrated in pancreatic lesions, with higher numbers of T<sub>regs</sub> found in the stroma of invasive adenocarcinomas than around premalignant lesions, which showed a higher infiltrate than non-neoplastic lesions.<sup>92</sup> Opposite findings were noted for CD8<sup>+</sup> cytotoxic T cells, which decreased with malignant progression.<sup>92</sup> Higher levels of T<sub>regs</sub> may reflect evasion of the host immune response as the tumor progresses. Challenges remain in assessing TILs in preinvasive lesions, including definition of the tumor area and delineation of reactive and “bystander” lymphocytes. Pruneri et al<sup>45</sup> defined the tumor area as the specialized intralobular stroma surrounding the ducts involved by DCIS (within two high-power microscopic fields). The stroma surrounding ducts involved by DCIS is known to be altered relative to normal breast intralobular stroma<sup>86,87</sup>; however, the functional significance of reactive TILs may be diluted by preexisting lymphocytes in this region. Further study of preinvasive lesions such as DCIS may provide insights into the process of immunoediting and immune evasion as invasive malignancies develop and progress.

### TUMOR-INFILTRATING LYMPHOCYTES IN THE METASTATIC SETTING

The immune response to metastatic tumor deposits as well as the primary tumor is of clear importance to immunotherapies, which are largely being studied and approved in

advanced settings. Important differences in the immune infiltrate may exist between the primary tumor and metastatic deposits, which may reflect changes in the immune response to the tumor over time or in specific anatomic locations. In small studies of paired primary and metastatic breast carcinomas, lower levels of TILs were observed in the metastasis,<sup>93-95</sup> a finding also demonstrated in renal cell carcinoma<sup>96</sup> and non-small cell lung carcinoma,<sup>97</sup> supporting the concepts of immunoediting and immune evasion. In addition, Baine et al<sup>96</sup> demonstrated a lower CD8/FOXP3 ratio in metastatic deposits of renal cell carcinoma than in the primary tumor, suggesting that the TILs in the metastasis may be more polarized toward an immunosuppressive phenotype. The immune response to metastases may be site dependent; both in melanoma<sup>98</sup> and in HER2-positive breast carcinoma,<sup>99</sup> metastases in the lung contained the highest numbers of TILs, although an even distribution of TILs between metastatic sites was found in renal cell carcinoma.<sup>96</sup> Despite the immune-privileged status of the central nervous system,<sup>100</sup> brain metastases often demonstrate an immune infiltrate, which may be associated with improved overall survival.<sup>101,102</sup> As discussed in part 2 of this review, TILs in melanoma metastases have been shown to have similar prognostic importance to those in the primary lesion.<sup>103-106</sup> TILs in liver and lung metastases of colorectal carcinoma also show prognostic significance.<sup>107-109</sup> An interesting observation comparing primary breast tumors and later in-breast relapses, based on a very small cohort, is that higher levels of TILs may be associated with true recurrence type relapses while new primary type relapses show lower TIL levels.<sup>110</sup> This is consistent with the notion that new primaries can only develop through immune evasion in the face of a primed immune system, whereas true recurrences develop despite the immune response. Early data suggest an interesting interaction between TILs in metastatic breast cancer and tumor phenotype, namely high TILs in the metastatic or recurrent lesion were associated with improved prognosis in triple negative breast carcinoma but with worse prognosis in HER2-positive breast carcinoma.<sup>111</sup> Importantly, higher numbers of TILs at the invasive margin of melanoma metastases correlated with improved response to immune checkpoint inhibition.<sup>112</sup>

Evaluating TILs in the most recent tumor sample available, including metastatic deposits, may be more relevant in describing the current immunologic status of the patient than relying solely on the primary tumor. This practice is advocated in the assessment of other oncological biomarkers such as epidermal growth factor receptor mutation status in non-small cell lung carcinoma.<sup>113</sup> The standardized methodology we propose (Table 1) should be able to be easily adapted to the metastatic setting, as metastatic tumor deposits share many histopathologic features with primary tumors. A tutorial illustrating application of this method to breast carcinoma metastasis is available online in Supplementary File 4, Supplemental Digital Content 4, <http://links.lww.com/PAP/A16>. The consensus method previously reported for invasive breast carcinoma has been successfully used to investigate the clinical relevance of TILs in breast cancer metastases in a subset of a large prospective-retrospective study.<sup>99</sup> Evaluating TILs in metastatic deposits in lymph nodes is complicated by the presence of a preexisting lymphoid stroma; this issue is discussed further below. As in primary tumors, our proposed method should be thoroughly validated and shown to be reproducible in metastatic lesions. This will

allow for valid comparison between primary and metastatic tumors, between different tumor types or between different metastatic sites, which may be of particular relevance in the current era of immunotherapy.

### AREAS OF UNCERTAINTY AND OPEN QUESTIONS FOR FURTHER INVESTIGATION

Despite the evidence that is accumulating to support the prognostic and potential predictive value of TILs assessment, a number of areas of uncertainty remain. These open questions encompass a range of practical issues encountered by pathologists when scoring TILs, as well as areas of incomplete understanding in the fundamentals of tumor immunology. Assessing TILs in metastatic tumor deposits is clearly an important area for future study and early evidence suggests that assessing TILs in lymph nodes can demonstrate prognostic value,<sup>99,103,114</sup> despite the obvious difficulties in distinguishing lymphocytes truly related to the tumor from those preexisting “bystander” lymphocytes populating the node. Tumor draining lymph nodes have important immunologic and structural differences from nodes unrelated to the tumor, even before the arrival of metastatic tumor cells.<sup>115</sup> In addition, preclinical evidence suggests that systemic immunity is required for tumor control and eradication,<sup>116</sup> not only an effective local immune microenvironment. To further investigate this question and acknowledging the lack of supporting evidence, we propose a pragmatic method for scoring TILs in lymph node metastases on H&E sections illustrated in Figure 4. Metastatic tumor deposits often induce a desmoplastic stroma within the lymph node, in which case the sTILs may be scored within this reactive stroma (Figs. 4 A–D), and the iTILs scored as those lymphocytes within the tumor nests, analogous to the primary lesion. In cases where the metastatic tumor deposit does not induce a desmoplastic stroma (Figs. 4E, F), we suggest limiting the evaluation to iTILs, that is, lymphocytes infiltrating and disrupting tumor cells nests. The preexisting background lymphoid stroma should be excluded from the assessment. This method would be most applicable where the full lymph node is available for assessment. TILs may not be able to be reliably scored using this proposed method in core biopsies of involved nodes, particularly in the absence of a desmoplastic stroma. Scoring iTILs on H&E sections is admittedly challenging, and this may be an area in which interobserver reproducibility requires particular attention. These methods are suggested as preliminary guidelines that should be validated for reproducibility and clinical validity.

How best to describe regional heterogeneity, variation between multifocal tumors, or biphasic lesions with clear morphologic differences is also not yet well defined. An average whole section assessment may better capture any heterogeneity than a hotspot or random sampling methods; however, further evidence is required to rigorously compare these approaches. Although core biopsies may be used in the neoadjuvant setting, whole sections are preferred. Evidence to support any recommendations on the minimum amount of tumor tissue that reliably represents the immune microenvironment of the whole tumor is not yet available. Whether TILs could potentially be assessed on cytology specimens is also a controversial but important area, particularly in the setting of lung carcinoma in which these are often the only tumor samples available. It is also uncertain how to assess mixed inflammatory infiltrates, for example neutrophilic, eosinophilic, and histiocytic infiltrates not related to necrosis, or how to approach lymphoid infiltrates in

adjacent normal tissue or adjacent noninvasive neoplastic lesions.

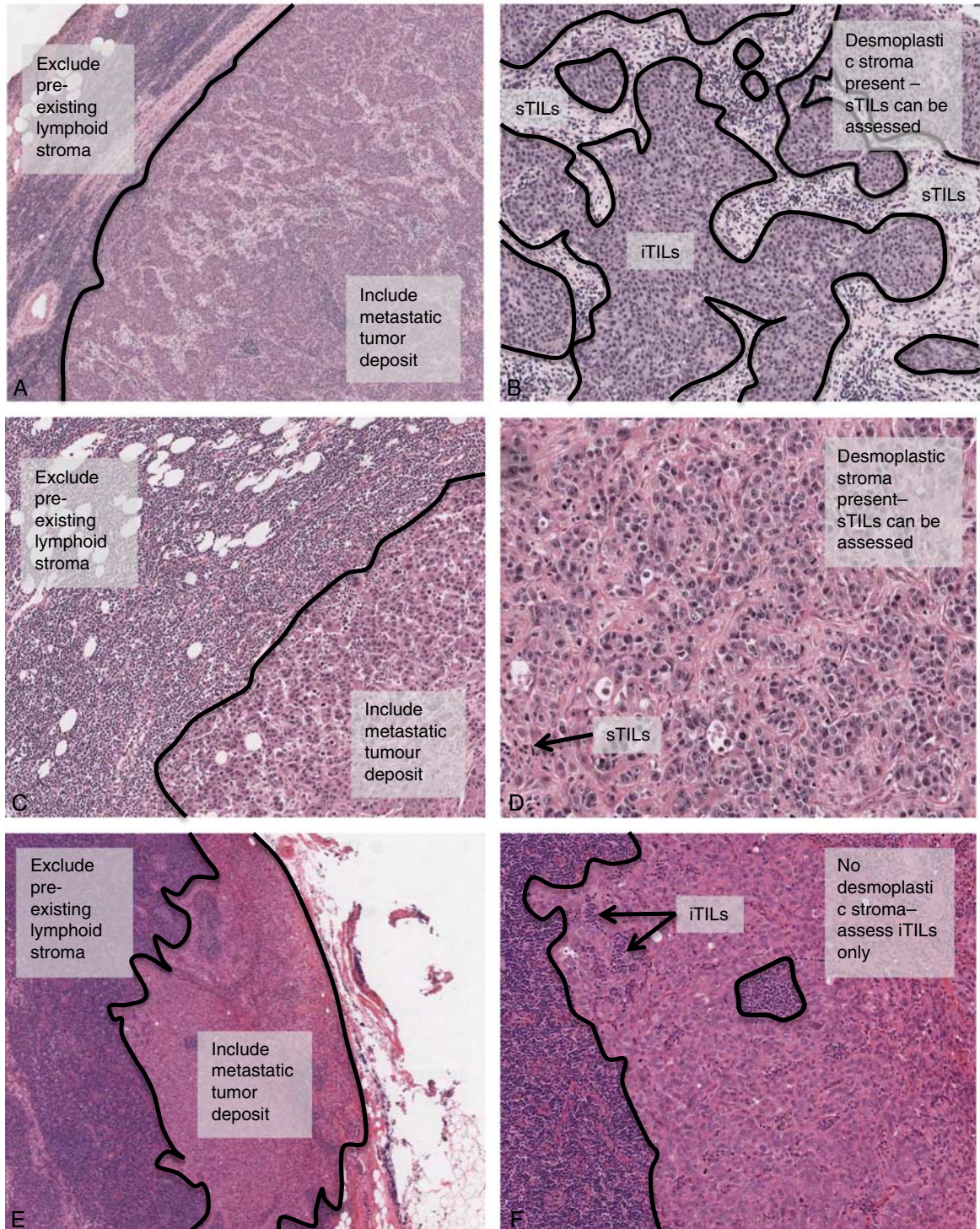
It must be emphasized that the margin between tumor and normal tissue, the “invasive margin,” as we have defined it, may represent different growth patterns and biologies in both primary and metastatic settings. This margin may represent an infiltrative front, but could also represent a pushing front, or a mixture of the two. Currently, research is ongoing to determine whether the general immunologic and biological context, and TIL infiltration patterns in particular, have different clinical meanings associated with specific growth patterns at the tumor margin. Our definition of the invasive margin, based on that proposed by the research group of Jerome Galon,<sup>117</sup> is a pragmatic one, which we hope will be of value in standardizing future research in this clearly important area of the interface between the tumor and the host.

As further study is undertaken and evidence becomes available, these (and other) issues may be resolved and incorporated into future guidelines. The methods we propose should be considered a tool for further investigation, to be adopted, rejected, or improved upon as the field progresses.

### DISCUSSION

It is clear that the host immune response to cancer can alter tumor biology and response to treatment, a feature common to many different solid tumors, as discussed further in part 2 of this review. Many questions regarding the origin, determinants, and clinical relevance of the immune response in cancer remain, and limitations to our proposed method are acknowledged. Importantly, no information regarding the immune cell subsets or functional status is obtained from assessment of an H&E section. The balance of immune cells and soluble factors providing effective antitumor responses to those with immunosuppressive effects may be better described with more sophisticated techniques such as gene expression profiling.<sup>27</sup> Clearly not all mononuclear immune cells are T cells, and the relative proportions of macrophages, dendritic cells, myeloid-derived suppressor cells and plasma cells in the immune infiltrate may also provide important information.<sup>3</sup> TILs may be exhausted or rendered inactive through immune checkpoint pathways such as PD-1:PD-L1 signaling, or lack of immune stimulatory pathways such as OX-40:OX-40L signaling. Expression of these checkpoint molecules is induced by a once-active immune response,<sup>28,118</sup> which may be reactivated through immunotherapy, chemotherapy or radiotherapy. High levels of TILs as defined on H&E may not correspond to high levels of active antitumor TILs and this functional suppression may explain, at least in part, why some patients with high levels of TILs do not show improved prognosis. It is not yet established whether an H&E-based assessment can provide sufficient information for clinical decision making in the context of immunotherapy. However, we feel that this prospect should be thoroughly investigated and not discarded prematurely due to the perceived simplicity of this approach.

The organization and distribution of the immune response may not be fully described by obtaining an average TILs score across the section. The importance of tertiary lymphoid structures, which represent an organized immune infiltrate with all the features of a lymph node to facilitate antigen presentation, lymphocyte education, and effector cell proliferation, has been described in different tumor types.<sup>13</sup> A recent study suggests that IHC is required



**FIGURE 4.** Assessing TILs in metastatic tumor deposits in lymph nodes. Cases showing a desmoplastic stroma can be scored as for the primary lesion, that is, sTILs may be scored within this reactive stroma (A–D). A, B, An example of a metastatic tumor deposit with clearly distinguishable border between preexisting lymphoid tissue and tumoral sTILs. C, D, An example with very few sTILs in the stroma of the tumor deposit. E, F, In cases without a desmoplastic stroma, TILs are scored only within tumor nests, that is, iTILs only. The preexisting lymphoid stroma is excluded from the evaluation. iTIL indicates intratumoral TIL; sTILs, stromal TILs; TILs, tumor-infiltrating lymphocytes. Please see this image in color online.

to reliably identify tertiary lymphoid structures<sup>85</sup>; however, consensus on the most appropriate marker or combination of markers for this task is not yet apparent. Whether further descriptive analysis of the immune infiltrate in tumors, incorporating features such as the density and distribution of TILs, organization into tertiary lymphoid structures, or the presence of hotspots, adds prognostic information to an overall TILs assessment remains to be determined.

Why some tumors have high levels of TILs and others do not is an ongoing area of fascinating research. It has been hypothesized that a higher mutational burden will correspond to a greater potential for neoantigen formation and a higher immune infiltrate.<sup>119</sup> The tumor types with high average mutation load such as melanoma and non-small cell lung carcinoma, as well as the most highly mutated individual tumors within these types, seem to respond better to immunotherapy<sup>120,121</sup>; however, the mutational load does not necessarily correlate with the expression of immune-related genes in the tumor or the effector T-cell infiltrate.<sup>122–124</sup> Specific mutational signatures such as homologous recombination pathway defects,<sup>125</sup> *POLE* mutations, or microsatellite instability may prove to be important. Alternatively, activation of specific oncogenic pathways in tumor cells, such as *WNT*/ $\beta$ -catenin, has been implicated in preventing immune cell infiltration.<sup>123,126</sup> Access of the immune cells to the tumor via the blood vessels and tumor stroma is also a critical consideration.<sup>127</sup> It is clear that a standardized approach to scoring TILs will be of benefit when investigating the origin and determinants of the immune infiltrate.

It is hoped that use of a standardized methodology for TILs assessment as a reference standard will help to resolve many issues associated with interpreting future studies. Comparison of, for example, the value of a Th1 gene expression profile with that of a simple H&E assessment, or the effect of mutational load versus mutational signature, will be informative to preclinical and clinical research. As evidence accumulates for the clinical utility of TILs as a prognostic and predictive biomarker, and as the search for reliable immunotherapy biomarkers continues, pathologists' role in assessing and quantifying immune infiltrates will increase. Technologies will advance, and detailed in situ assessment of the functional status of the immune infiltrate may soon be possible in an inexpensive and widely available manner. However, it is imperative that we develop a pragmatic methodology of generating reliable data on immune infiltrates, that can be applied in large-scale clinical trials and in daily histopathology practice, even in resource poor settings. The method we propose (Table 1) has shown high reproducibility in the context of invasive breast carcinoma,<sup>53</sup> and should be able to be applied, with modifications as required, to all solid tumor types. These pragmatic recommendations may form the basis for further definitive guidelines for different solid tumors as more evidence becomes available. It is hoped that a standardized methodology will be of value to clinicians, researchers, and practicing pathologists, to develop relevant cut-offs and conclusively demonstrate the clinical utility of this simple biomarker.

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