A review of the importance of immune responses in luminal B breast cancer

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ABSTRACT
Historically, the immune environment was not considered an important target for breast cancer treatment. However, the association of lymphocytic infiltrates in triple negative and HER-2 over-amplified breast cancer subtypes with better outcomes, has provoked interest in evaluating the role of the immune system in the luminal B subtype that accounts for 39% of breast cancers and has a poor patient prognosis. It is unknown which immunosuppressive cell types or molecules (e.g., checkpoint molecules) are relevant, or where measurement is most informative. We hypothesize that a profound immunosuppressive tumor and/or lymph node milieu is prognostic and impacts on responses to therapies.

The anticancer immune response

The immune system protects us from pathogens and cancerous cells by differentiating between healthy and damaged self, as well as between safe and pathogenic non-self, through surface and intra-cellular molecules termed pattern recognition receptors (PPRs). These receptors recognize endogenous damage (or danger)-associated molecular pattern molecules (DAMPs) and pathogen-associated molecular pattern molecules (PAMPs). Cells of the innate system, including macrophages and dendritic cells (DCs), use PPR-DAMP/PAMP interactions to prime the highly specific adaptive immune system. DCs play a pivotal role in priming antigen-specific T cells, including tumor-antigen-specific T cells; this process occurs in tumor-draining lymph nodes once DCs have trafficked through tumors and taken up tumor antigens (Fig. 1). Macrophages mostly influence T cell function in peripheral tissues and tumors, although they may exert local effects in lymphoid tissues as well. T cells can be differentiated into CD8+ cytotoxic T lymphocytes (CTLs) that lyse tumor cells, or CD4+ helper T (Th) cells, with the CD4+ Th-1 subset releasing cytokines that enable CTLs to kill tumor cells. As cancer cells are altered-self they express surface molecules similar to healthy cells making it difficult for the immune system to identify them as pathological. Nonetheless, as a result of their genetic instability, and increasing mutational load with disease progression, tumour cells may also express new molecules (neo antigens) that render them visible to the immune system, providing a window of opportunity for effective immune responses, including those driven by therapeutic intervention. However, cancerous cells can...
escape immune-mediated killing through evasion tactics, a major pathway being suppression of effector CTLs, whose cytolytic activity against tumor cells is essential to anticancer immunity.\textsuperscript{11}

**Breast cancer and immunity**

There is an increasing awareness of the relationship between the immune system and BC evolution, with the tumor microenvironment comprising of tumor and stromal cells. The latter includes immune cells, some of which inhibit or promote disease progression. Moreover, there is increasing recognition of a correlation between genetic instability and the BC immune landscape, with mutational load and heterogeneity leading to novel BC antigens. TNBC, luminal B and HER-2\textsuperscript{+} BC are reported to have a high mutational burden rendering them immunogenic, suggesting that immunotherapeutic approaches may be effective in these BC subtypes.\textsuperscript{12} This is supported by database studies that describe immune benefit-enabled or disabled BC subtypes.\textsuperscript{13} The former includes luminal B BC, which could be stratified by immune profile analysis into different prognostic groups, suggesting that the immune signature could be a useful prognostic indicator in luminal B BC. Gene network analysis predicted activation of TNF-\alpha/IFN\gamma signaling pathways in immune-enabled tumors and activation of the transforming growth factor-\beta (TGF-\beta) pathway in immune-disabled tumors.\textsuperscript{13} Gene expression studies of BC-adjacent tissues showed that the TNBC microenvironment had increased expression of genes involved in inflammation\textsuperscript{14} and revealed further subtypes, some with a low immune response defined by a minimal T cell infiltrate and effector function, and high frequency of suppressive M2-like macrophages, and others with a high immune response and low frequency of M2-like macrophages.\textsuperscript{15} These data imply that communication between tumor cells and other cells in cancer-adjacent tissue contributes to BC progression and/or metastasizing potential; the same is likely to be true for Luminal B BC (Fig. 1).

Several cell-intrinsic alterations are reported to be markers of poor prognosis in BC including reduced expression of the DAMP, high-mobility group protein (HMG)B1 by malignant cells, minimal CTL tumor infiltration, significant presence of immunosuppressive cells such as FOXP3\textsuperscript{+} regulatory T cells (Tregs) or CD68\textsuperscript{+} tumor-associated macrophages (TAMS).\textsuperscript{16} Tumor-infiltrating T cells (TILs) are reported to be a useful positive biomarker in early BC in some, but not all, BC subtypes. A review of 15 studies showed that the magnitude of TIL varies within and between BC subtypes; TNBC, HER-2\textsuperscript{+} and HR\textsuperscript{−} BC contained the highest TIL levels, HER-2\textsuperscript{+} BC had the lowest. The highest levels of FOXP3\textsuperscript{+} Tregs were in TNBC and HER-2\textsuperscript{+} BC, only a minority of HR\textsuperscript{+} BC demonstrated high levels of tumor-infiltrating FOXP3\textsuperscript{+} cells.\textsuperscript{17}

**Breast cancer and chemotherapy**

The realization that cancer progression depends on immune suppression suggests that the pre-treatment immune profile in
tumors and lymph nodes may not only be prognostic, but also predictive of responses to therapies that engage the immune system, including standard chemotherapy. Once thought to be deleterious to immune cells, we now know that some chemotherapies stimulate beneficial changes in innate and adaptive antitumor immunity and render tumor cells accessible for immune destruction. Chemotherapeutic and radiotherapeutic regimens commonly employed for the treatment of BC may induce immunogenic cell death in tumor cells, and affect TILs, however, clinical responses vary. Standard neoadjuvant chemotherapy for BC achieves complete pathologic responses in 10 to 20% of cases. While the biological factors that determine these responses are not well understood, the presence of TILs in pre-treatment tumors is believed to play a role.

Chemotherapy and the anticancer immune response

For BC, there is evidence that neoadjuvant chemotherapy is more efficient if tumors show a pre-existing or therapy-induced anticancer immune response, and it is now accepted that the success of anthracycline-based adjuvant chemotherapy is due to enhanced immunogenic tumor cell death, a cell death modality preceded by autophagy and followed by HMGB1 release. Some PPRs, such as Toll-like receptors (TLR) 3 and 4, sense HMGB1. Interaction of HMGB1 with TLR-4 on DCs upregulates tumor antigen cross-presentation by DCs and promotes induction of tumor-specific CD8+ CTLs in lymph nodes. BC patients that have lost HMGB1 expression and/or harbor a loss-of-function mutation of TLR3 and TLR4 exhibit reduced metastasis-free and overall survival after treatment with anthracycline-based adjuvant chemotherapy. These data support the concept that conventional anticancer treatments are only fully efficient once they have stimulated anticancer immune responses.

Cisplatin has been shown to induce necrotic cell death and inflammation, an environment known to stimulate DCs. This may be via expression of HMG proteins that bind platinum complexes to cause repair shielding and initiate apoptosis. We have shown that while cisplatin only slightly enhances in vivo presentation of dominant tumor antigens to T cells in draining lymph nodes, both cisplatin and gemcitabine expand the CTL response to weaker subdominant tumor epitopes; a feature that may be key to tumor destruction as immune tolerance mechanisms are likely to delete responses to dominant epitopes. Moreover, immune-relevant metagene analyses have shown a significant positive correlation with response rates for BC to chemotherapy, in particular to a CXCL13-centered metagene signature reflecting the intratumoral presence of activated IFNγ-producing T cells.

Immune cells as prognostic and predictive markers of responses to therapy in BC

There is increasing evidence that pre-existing TILs and/or immune gene expression signatures indicate the magnitude of immune-suppression that confounds or supports different treatment strategies. Therefore, they may be predictive for responses to chemotherapies and other treatment modalities, including immunotherapy. This has been clearly shown for TNBC and HER-2+ BC wherein a strong association between higher lymphocytic infiltrations predicts a better outcome. Histological studies have shown that co-localization of immune cells with cancer cells is significantly associated with a survival benefit for all BC subtypes. It is not yet clear which immune cell types are useful as prognostic markers, and importantly whether they differ between BC subtypes. Gene expression studies revealed that differing levels of tumor-associated plasma B cells and myeloid-derived antigen-presenting cells (APCs), such as DCs and macrophages, contribute to varying pathologic responses to neoadjuvant chemotherapy. Mouse studies of breast adenocarcinomas showed that doxorubicin treatment efficacy is dependent on CD8+ T cells and IFNγ production. Doxorubicin enhanced tumor antigen-specific CD8+ T cell proliferation in draining lymph nodes and promoted tumor infiltration of activated IFNγ+/CD8+ T cells. A correlation between pre-treatment CD8+ and IFNγ gene expression levels in tumor samples from BC patients with clinical responses to anthracycline chemotherapy supported the pivotal contribution of innate and adaptive immunity in anthracycline treatment outcomes.

Targeted anticancer therapeutics, such as the anti-erb-b2 receptor monoclonal antibody, trastuzumab, also involved innate and adaptive immunity. These data suggest that better prognostic markers, particularly immune prognostic markers, could assist management of BC. This area requires further study in BC in general, and particularly for luminal B BC.

An area that is poorly studied is whether immune responses occurring in tumor-draining lymph nodes affect patient outcomes. We hypothesize that tumor-draining lymph nodes will be impacted during disease progression and treatment, and that some intra-nodal changes will represent potential prognostic markers, as evidenced by the mouse studies in BC described above and our studies in lung cancer and mesothelioma.

Negative immune regulators and breast cancer

Cancer cells modify their tissue environment by secreting factors and expressing molecules that suppress the function of effector immune cells, such as CD8+ CTLs and CD4+ Th-1 cells, and promote expansion of suppressive cells such as CD4+CD25+Foxp3+ Tregs, myeloid-derived suppressor cells (MDSCs) and macrophages skewed toward an anti-inflammatory, pro-wound healing phenotype, termed alternatively-activated or M2 macrophages. Production of immuno-suppressive molecules and overexpression of negative regulatory molecules by tumor cells, M2 TAMs, MDSCs and Tregs stimulate immune and other tissue cells to increase expression of inhibitory surface receptors such as, but not limited to, checkpoint molecules. Using mesothelioma as a model, we have shown that tumor-conditioned media generates M2-like macrophages that suppress T cell function. Our murine studies revealed that M2 macrophages dominate tumor-draining lymph nodes and that a mixed M1/M2 (termed M3) macrophage subset with suppressive function dominates the tumor microenvironment. Thus, ultimately, tumors create a local and regional environment that is not permissive to effector T cell function (Fig. 1).
Negative immune regulatory molecules include a wide range of soluble factors, cell surface and intracellular molecules that in healthy people function to prevent excessive inflammation and dampen responses that could induce autoimmunity. Tumor cells can take advantage of these pathways thereby inactivating antitumor effector immune cells and allowing disease progression. Recently, a family of molecules referred to as immune checkpoint molecules has been described, with new members being reported. In cancer patients, some checkpoint molecules may be over-expressed on immune cells, tumor cells and other tissue cells. Clinical understanding and application is greatest for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and programmed cell death (PD)-1 and its ligands, PD-L1 and PD-L2. Successful clinical trials using antagonist antibodies targeting these molecules has invigorated interest in the role of the immune system in cancer and in anticancer immunotherapies.

CTLA-4 on T cells ligates CD28, CD80 and/or CD86 on APCs such as DCs. Ligating CD28 stimulates effector T cells, which is crucial for eliminating cancerous cells. However, CTLA-4 has a higher affinity for CD80 and CD86, which outcompete CD28 thereby limiting T cell responses that could destroy cancerous tissue (Table 1). Use of CTLA-4 checkpoint inhibitors, i.e., monoclonal antibodies that block CTLA-4 signaling (e.g., Ipilimumab) resulted in response rates of 11.1% to 28.5% in phase III clinical trials. While tumor responses were exciting, with some patients showing complete tumor resolution for over 5 y, a significant number failed to respond, suggesting other immunosuppressive molecules may confound treatment outcomes.

Ipilimumab has not yet been trialed in BC, however, CTLA-4 can be found at the protein and mRNA level in BC tumors, with weakly positive or negative expression in normal breast tissue. Higher mRNA levels were seen in patients with axillary lymph node metastases and higher clinical stage disease, moreover CTLA-4+ T cells from these patients showed blunted responses to stimulation. Responses in BC patients to anti-CTLA-4 antibody therapy may be determined by their pre-existing immunity, as mouse studies using the poorly immunogenic mouse 4T1 BC model, showed that anti-CTLA-4 alone had no effect on tumor growth or survival. CTLA-4 expression may reflect significant immune suppression and therefore has potential as a marker of prognosis in luminal B BC for anti-CTLA-4 treatment and other therapies that rely on a tumor-specific immune response for efficacy, including chemotherapy and newer targeted therapies.

PD-1 on T cells binds PD-L1 and PD-L2 on tumor and APCs causing increased apoptosis of effector CTLs and decreased apoptosis of Tregs resulting in a diminished antitumor T cell response. PD-1 inhibitors block PD-L1/L2-PD-1 interactions, restoring T cell effector function and increasing Treg apoptosis. Response rates of 25–43% for the FDA-approved anti-PD-1 monoclonal antibodies have been seen in metastatic melanoma (Table 1).

PD-1, PD-L1 and PD-L2 RNA and protein have been detected in BC tumors. PD-1 expression is associated with poor prognosis in breast and epithelial-derived cancers. However, likely due to differing techniques the data are inconsistent. PD-1+ TILs have been shown in 15.8% of tested BC cases with subset analyses showing PD-1+ TILs were associated with significantly worse overall survival in luminal B HER-2− and HER-2+ BC. Others have shown that the concomitant presence of FOXP3+ Tregs, CD80+ and PD-1+ TILs correlates with high histological grade, ER− status and a severe lymphocytic infiltration. Higher PD-1 and PD-L1 RNA levels were seen in TNBC and HER-2+ tumors compared with luminal A and luminal BRE H R-2 − tumors; expression levels increased with tumor grade suggesting increasing immune suppression with disease progression. Studies are currently ongoing to evaluate anti-PD-1 and anti-PD-L1 antibodies in metastatic BC.

Interestingly, PD-1− tumors have responded to PD-1/PD-L1 blockade implying that tumors may not be the key sites of PD-1/PD-L1 interactions, or that an unidentified mechanism is operating. PD-1/PD-L1 interactions are also likely to occur in tumor-

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**Table 1.** Checkpoint molecules and their ligands.

| Expressed on | Ligates | Effect | Checkpoint inhibitors | References |
|--------------|---------|--------|-----------------------|------------|
| **Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4 or CD152)** | T Cells | CD28, CD80 (B7-1) and CD86 (B7-2) on APCs | T cells secrete less IL-2, preventing T cell expansion and limiting T cell responses | Ipilimumab (Yervoy), Response rates 11.1% to 28.5% in late stage melanoma | 37-39 |
| **Programmed cell death (PD-1)** | T cells and pro-B cells | PD-L1 (CD274 or B7 homolog 1, B7-H1) and PD-L2 (B7-DC) on tumor cells and APCs | Increased apoptosis of effector CTLs | Pembrolizumab (Keytruda) and nivolumab (Opdivo), Response rates in metastatic melanoma of 25–41% for nivolumab, and 26–43% for pembrolizumab | 43-47 |
| **T cell immunoglobulin and mucin protein-3 (TIM-3, also known as Hepatitis A virus cellular receptor 2 or HAVCR2, and CD366)** | Activated Th-1 cells | Unknown | Th-1 cell apoptosis to prevent autoimmune responses | Gal-9, expressed in many tissues and on many cell types including immune, epithelial and endothelial cells | 48-62 |
| | | | | The Gal-9-TIM-3 pathway suppresses Th-1 and Th-17 cells, leading to decreased production of IFNγ, IL-2, IL-6 and IL-17 | |
| | | | | In pre-clinical development | |

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draining lymph nodes that have not yet been extensively assessed in many cancers, including BC subtypes. Moreover, a threshold level has not been set as inclusion criteria for treatment with PD-1 or PD-L1 in any cancer, and even low levels as reported in luminal B BC might be sufficient justification for a clinical trial. Thus, expression of PD-1 and its ligands in tumor or lymph nodes may be useful prognostic/predictive markers in luminal B BC for PD-1/PD-L1/L2 treatment studies, and may reveal a suppressive factor that confounds the success of other therapies.

T cell immunoglobulin and mucin protein-3 (TIM-3) is found mainly on activated Th-1 cells that assist CTL activation. TIM-3s controversial putative ligand, Gal-9, is expressed on many cell types, including immune cells (Table 1). TIM-3/Gal-9 interactions, or TIM-3 interactions with other as yet unknown ligands, lead to Th-1 cell apoptosis preventing autoimmune responses in healthy people. However, in cancer patients Gal-9 and TIM-3 expression is increased, causing excessive Th-1 cell death and immunosuppression that prevents tumor cell elimination. Gal-9 receptors can also mediate direct apoptotic activity as TIM-3 knockout mice exhibited Th-1 cell apoptosis, and no other ligand has been identified. The Gal-9-TIM-3 pathway also suppresses Th-17 cells, leading to decreased production of pro-inflammatory cytokines (Table 1). Gal-9 expression by endothelial cells enables cytolsis of immune cells before they infiltrate tumors. Moreover, Gal-9 promotes Treg proliferation and tumor metastasis by facilitating tumor-endothelial cell interactions. The role of members of the galectin family including Gal-9 in BC subtypes is unclear although, its expression on stromal cells has been reported in TNBC and HER-2+ subtypes.

Blocking TIM-3 and Gal-9 with antagonistic monoclonal antibodies may yield clinical outcomes similar or better than the anti-CTLA-4 or PD-1 antibodies, and the success of TIM-3 inhibitors in pre-clinical models is promising. For example, TIM-3 inhibitors induced tumor regression in a BC mouse model with improved responses when combined with PD-1 and CTLA-4 inhibitors. No studies using Gal-9 antagonists have been yet been reported. Nonetheless, TIM-3 and Gal-9s immunosuppressive activity suggest their presence may indicate significant immune suppression in association with increased metastatic risk, and that expression of either or both molecules may be associated with a poorer antitumor immune response and poorer long-term prognosis in luminal B BC independent of any treatment given.

There are numerous other molecules including those secreted by tumor or myeloid cells that confound antitumor immunity and increase the risk of metastasis. TGF-β, IL-10 and VEGF are examples of molecules produced by tumor cells and TAMs in BC that contribute to immune suppression. They stimulate MDSCs that promote Treg development and tumor angiogenesis, suppress APCs and produce reactive oxygen species that cause T cell apoptosis. IL-10 downregulates macrophage production of IL-12 an effector T cell-stimulating molecule, and decreases myeloid cell MHC class II expression leading to decreased antigen presentation that decreases T cell activity. Expansion of MDSCs and/or molecules responsible for MDSC expansion may be of prognostic value (Fig. 1).

The way ahead

Reliable prognostic markers for the luminal B subtype, which accounts for up to 39% of BC cases, are lacking. Gene expression profiles such as Oncotype DX and Mammaprint do not provide prognostic evaluation of the luminal B subtype. Further, there are no biomarkers available to predict treatment outcomes in luminal B patients. Thus, it is currently not possible to individualize choice of systemic therapy (chemotherapy and/or endocrine treatment) for these patients in the adjuvant setting. Many luminal B patients do not respond well to traditional chemotherapies and may have a higher 10-y mortality rate despite adjuvant treatment than luminal A or TNBC patients. It is possible that immune suppression accounts for these outcomes and that suppressive immune cells and molecules represent new prognostic markers for this BC subtype.

Thus far, no checkpoint inhibitor clinical trial has been conducted for luminal B BC. Moreover, even in melanoma, believed to be a highly immunogenic cancer, only a proportion (20–40%) of melanoma patients responded to anti-CTLA-4 and/or anti-PD-1 therapy, indicating that a significant percentage of patients do not respond to immune therapies, or conversely, rapidly they acquire resistance. These data suggests that there are more immunosuppressive molecules with potential as prognostic markers for melanoma, and other cancers. Prognostic markers enabling selection of patients who experience durable immune responses to these and other therapies will likely improve therapeutic utility. CTLA-4, PD-1 and other inhibitory checkpoint molecules are therefore attractive study targets for BC. The cell types on which checkpoint molecules are expressed may be important when determining prognosis. For example, tumor-infiltrating CD4+ T cells express CTLA-4 and TIM-3, while CD8+ T cells express PD-1. Tumor-infiltrating CD68+ macrophages may express PD-L1 and Gal-9, and TAMs have been associated with poor outcomes in TNBC, this is likely to be true for other BC subtypes. In contrast, the level of TILs, in particular CD8+ CTLs, has been shown to have prognostic value and potential for predictive value in TNBC and HER-2+ BC patients undergoing neoadjuvant chemotherapy. The localization of these markers can be examined through histochemical assessment. In the future, as costs reduce, molecular/genetic/multiplexed approaches appear promising because reproducibility of correlations among immune-relevant metagenes was highest in BC followed by colorectal cancer, non-small cell lung cancer and melanoma.

It is not yet clear if expression of immune suppressive markers is best measured in tumors and/or in regional lymph nodes for use as prognostic markers. Haematoxylin and eosin (H&E) and immunohistochemical analyses of tumor and lymph node samples can be used to determine which immune cells and their secreted factors are important in BC subtypes. However, to be meaningful, an internationally standardized methodology for examining immune cells in tumors and lymph
Figure 2. Immunohistochemical staining on luminal B breast cancer tissue for PD-1 (a), PD-L1 (b), TIM-3 (c), GAL-9 (d), CTLA-4 (e), CD4⁺ (f), CD8⁺ (g) and CD68 (h). After antigen retrieval on deparaffinized rehydrated sections, endogenous peroxidases were blocked using hydrogen peroxide (Riedel-de Haën, Seelze, Germany) while endogenous avidin and biotin were blocked using an avidin/biotin blocking kit (Invitrogen, Camarillo, USA). Sections were sequentially incubated with a protein block containing fetal calf serum, bovine serum albumin and human plasma to prevent background staining, then incubated with unconjugated primary antibodies or isotype controls; washed; linked first to biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, USA) or biotinylated goat anti-mouse IgG1 (Biolegend, San Diego, USA) secondary antibodies, then to streptavidin-conjugated horseradish peroxidase. Staining was visualized using hydrogen peroxide in 3,3-diaminobenzidine (Sigma Aldrich, St Louis, USA) counterstained with Harris’ haematoxylin. Sections were dehydrated before mounting with Entellan mounting media (Merck Millipore, Darmstadt, Germany). In place of isotype controls for the polyclonal rabbit primary antibodies, TIM-3 (c), GAL-9 (d) and CTLA-4 (e), a secondary antibody-only stain was prepared. Curtin University (Perth Western Australia) and Bellberry (South Australia) Human Ethics Committees approved this study (approval numbers HR 107/2015 and 2015–03–151, respectively).
nodes is required, although considerable efforts are underway.\textsuperscript{76} Pitfalls include the following: subjectivity when selecting areas for analysis, a lack of standardization of the number of cells that should be counted per sample and even the thickness of sections, all of which could mean important areas are overlooked. Strict criteria are all required to address these issues, as well as for selecting markers, determining threshold levels and standardizing staining protocols to determine which specific monoclonal antibodies and controls should be used. We are evaluating our staining protocol on Luminal B BC tissue and show detectable PD-1, PD-L1, TIM-3, Gal-9, CTLA-4, CD4\textsuperscript{+}, CD8\textsuperscript{+} and CD68 (Fig. 2). Preliminary data in a small Luminal B patient cohort suggest PD-L1, Gal-9 and CD68 expression are associated with faster time to relapse, regardless of treatment (data not shown); further studies are required.

We found a lack of standardized staining and analysis for protocols, such as those for Ki67 used to distinguish luminal A and B BC; there is no standardized staining protocol or threshold point and no consensus regarding use of automated image analysis for diagnosis.\textsuperscript{5,77,78} A similar situation exists for staining TILs in BC.\textsuperscript{76} Even TIL terminology needs to be standardized, and possibly re-evaluated, as intratumoral TILs are defined as lymphocytes in tumor nests that are in contact with each other and tumor cells without intervening stroma, while stromal TILs are dispersed between tumor cells that they do not directly contact.\textsuperscript{76} Stromal TILs are reported to be of more and more reproducible parameter despite recognition that stromal and intratumoral TILs are predictive of pathological response to neoadjuvant platinum-based chemotherapy.\textsuperscript{79}

Studies have scored TILs using a variety of semi-quantitative approaches, however, an international TILs working group for BC\textsuperscript{76} reported that the validity of the modified approach based on a method by Denkert et al.\textsuperscript{80} provides a superior framework for future standardization. Key points include

1. TILs should be reported for the stromal compartment.
2. All mononuclear cells should be scored with exception of polymorphonuclear leukocytes.
3. Full sections are preferred over biopsies whenever possible.
4. A full assessment of average TILs in the tumor area should be used. Do not focus on hotspots.

Note that no formal recommendation for a clinically relevant TIL threshold could be given.\textsuperscript{76}

In summary, we suggest that evaluation of markers of immune suppression has prognostic value in luminal B BC. To avoid confounding factors in assessment of the role of the immune system it is vital that the study cohort be carefully selected to minimize variation in Luminal B BC patients’ immune systems, e.g., restricted age range\textsuperscript{15} and absence of comorbid conditions which may affect immune status. Further, utilization of international standardized criteria for histological analysis is vital for interpretation of the results.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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