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Tumor-infiltrating lymphocytes and breast cancer: Beyond the prognostic and predictive utility

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Abstract

The importance of the immune system as a potent anti-tumor defense has been consolidated in recent times, and novel immune-related therapies are today demonstrating a strong clinical benefit in the setting of several solid neoplasms. Tumor-infiltrating lymphocytes reflect the attempt of the host to eradicate malignancies, and during the last decades, they have been shown to possess an interesting prognostic utility for breast cancer, especially in case of HER2 positive and triple-negative molecular subtypes. In parallel, the clinical evaluation of tumor-infiltrating lymphocytes has been shown to effectively predict treatment outcomes in both neoadjuvant and adjuvant settings. Currently, tumor-infiltrating lymphocytes are promising further predictive utility in view of novel immune-related therapeutic strategies which are coming into the clinical setting launching a solid rationale for the future next-generation treatment options. In this scenario, tumor-infiltrating lymphocytes might represent an important resource for the selection of the most appropriate therapeutic strategy, as well as further evaluations of the molecular mechanisms underlying tumor-infiltrating lymphocytes and the immunoediting process would eventually provide new insights to augment therapeutic success. Considering these perspectives, we review the potential utility of tumor-infiltrating lymphocytes in the definition of breast cancer prognosis and in the prediction of treatment outcomes, along with the new promising molecular-based therapeutic discoveries.

Keywords

Tumor-infiltrating lymphocytes, breast cancer, cancer immunotherapy

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Introduction

Breast cancer (BC) is the second leading cause of female cancer-related death worldwide, and the role of the immune system in the context of this type of solid tumor has been at the center of a large debate during the last decades.[1,2] Initially, the early observation of immune infiltrates within tumor niches and stromas introduced a novel topic of research, suggesting that lymphocytes, especially those belonging to the adaptive arm, were at least likely to recognize and potentially eliminate malignant cells. On the contrary, a mechanism allowing tumor cells to evade immune defenses and to proliferate was supposed to exist, in order to explain the anergy associated with immune

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infilatrates. The process which renders tumor cells able to escape from immune recognition and eradication is known today as immunoediting, a multistep mechanism described for the first time by Dunn et al. in 2004.[2] During the early stages of this process, immunosurveillance-related lymphocytes (CD8+ T cells, CD4+ type-1 T-helper (Th-1) cells, and natural killer (NK) cells) are able to recognize and eliminate malignant cells, thus efficiently counteracting cancer proliferation (*elimination* phase). However, tumor cells also begin to undergo a Darwinian selection which progressively favors the proliferation of the malignant clones which are more likely to evade immune recognition and elimination through the adoption of several biological strategies: tumor cells become "invisible" to host recognition by reducing the expression of surface cancer antigens, therefore drastically decreasing the immunogenicity of the lesion (*equilibrium* phase). Also, cancer cells become increasingly able to directly inhibit the activity of immunosurveillance-related lymphocytes by overexpressing immune-checkpoint molecules, which also promote the proliferation and survival of immunosuppressive cells (FOXP3+ regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs)) as an important effect (*escape* phase). The immune checkpoints, which normally serve to avoid host self-reactivity, then become a deleterious mechanism, leading to an extended immunosuppressive environment: after an *equilibrium* phase between eliminated and escaping cancer cells, the tumor lesion becomes characterized by the presence of mostly ineffective lymphocytes and immunosuppressive cells, thus establishing the *escape* phase (Figure 1).[2,3] At this level, the malignancy is largely composed of transformed cells expressing very low levels of cancer antigens or even by a total absence of immunogenic stimuli. In addition, the overexpression of immune-checkpoint molecules, such as programmed cell death ligand-1/2 (PD-L1/PD-L2), vanishes the activity of those immune cells which eventually still recognize transformed cells. The uncontrolled proliferation of Tregs and MDSCs as a consequence of the release of interleukin (IL)-10, IL-35, and transforming growth factor (TGF)-β operated by cancer cells further contributes to the development of a strongly immunosuppressive microenvironment.[4] In this scenario, malignancies are often characterized by the presence of ineffective tumor-infiltrating lymphocytes (TILs), which confirm the existence of an immunosurveillance mechanism.

Immune checkpoints: a new therapeutic opportunity

The described immunoediting process can be seen as an elegant but deleterious mechanism of natural adaptation, and explains the presence of immune cells in proximity of cancer lesions as a reflection of the attempt of the host to eradicate malignancies, especially at their early stages. From a scientific and therapeutic point of view, the implications of this phenomenon have been explored during the past decades, leading to the setting up of novel smart compounds that can restore the activity of immunosurveillance-related cells. The immune checkpoints can be seen, in physiological conditions, as an essential mechanism which regulates the proliferation, survival, and activity of cytotoxic cells, therefore avoiding host self-damage. The overexploitation of this "braking system" adopted by transformed cells during immunoediting phases II and III ultimately renders lymphocytes totally ineffective or anergic. However, in recent years a few drugs have been developed in order to target some known immune checkpoints, such as the programmed cell death protein-1 (PD-1)/PD-L1/PD-L2 axis or cytotoxic T-lymphocyte-associated antigen 4 (CTLA4). Nivolumab and pembrolizumab (two anti PD-1 agents), MEDI4736, MPDL3280A, atezolizumab and avelumab (anti PD-L1 agents), and ipilimumab (an anti CTLA4 agent) are currently under clinical evaluation, showing a certain anti-tumor activity with regard to different solid tumors such as renal-cell carcinoma, non–small cell lung cancer, and melanoma.[5–7] Although another anti PD-1 agent (namely, pidilizumab or MDV9300) has been developed, recent controversies regarding its mechanism of action forced to put a partial halt to its phase II testing, in order to better define its pharmacodynamic profile and revise the whole documentation.

One of the key problems related to immunotherapy is the high level of dynamism associated with anti-cancer responses due to the constant adoption of novel mechanisms of immune escape and survival by tumor cells. In order to overcome this major issue, the latest experimental therapeutic strategies involve the combination of chemotherapy and immunotherapy to potentially attack tumors more efficiently. Moreover, the definition of reliable surrogate markers of treatment strategy success is of paramount importance in the clinical setting. TILs have been demonstrated to possess a strong prognostic value in the clinical setting, as they can be seen as a sort of "snapshot" of the attempt of the immune system to eradicate the neoplasm. Moreover, their evaluation during treatment may reflect the eventual success of the administered therapy. In this study, we will examine the latest findings regarding the role of TILs in the prognosis and in the prediction of clinical outcomes in both neoadjuvant and adjuvant-chemotherapy settings. We will also report the molecular causes which seem to correlate TILs with prognosis to better address the therapeutic choices in light of the recently developed immunotherapy approaches becoming available for BC.[8]

Focus on TIL subpopulations

The composition of BC-infiltrating lymphocytes has been widely investigated so far; however, while it is commonly

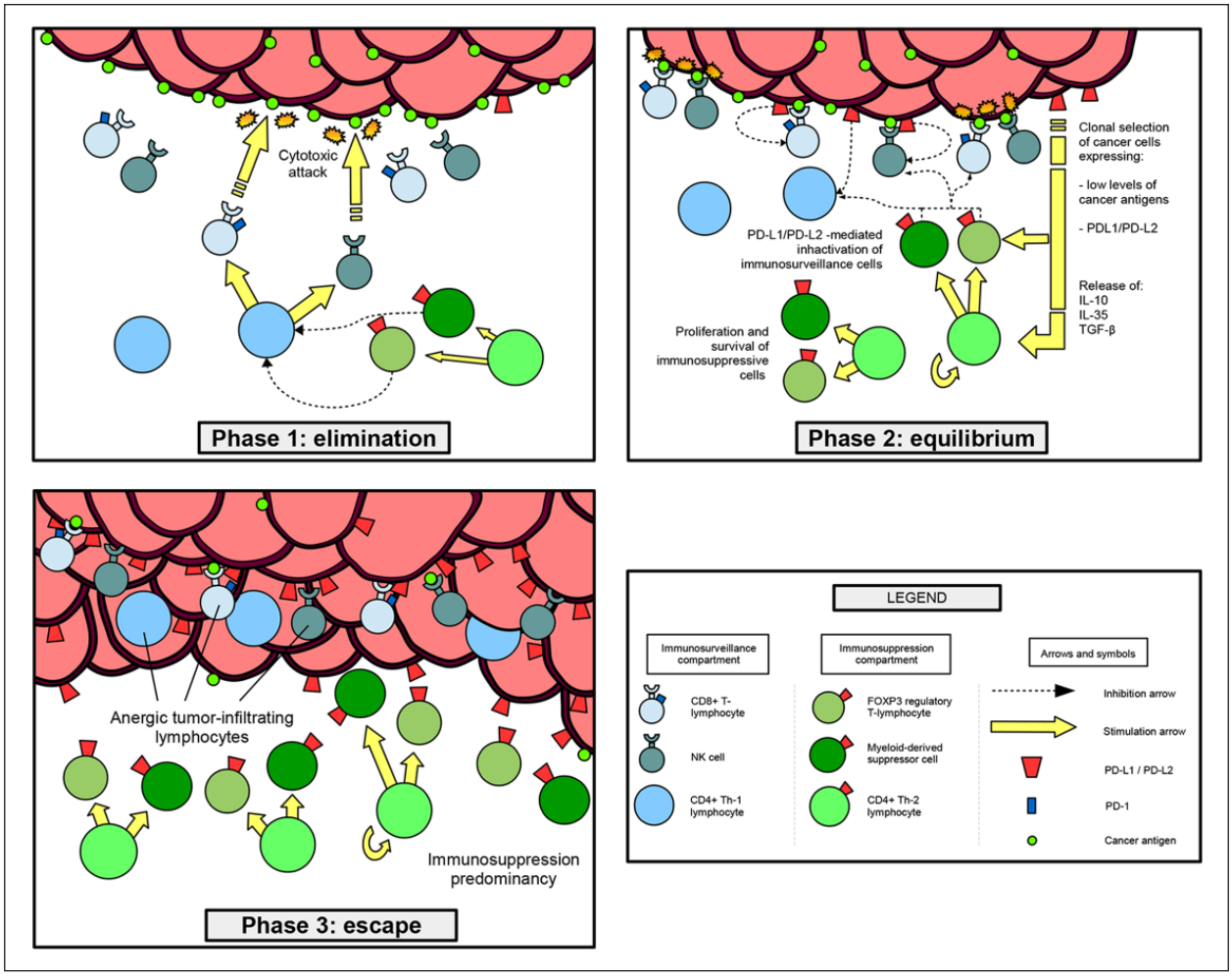

Figure 1. Representation of the immunoediting theory: initially, the immunosurveillance compartment (CD8+ T lymphocytes, natural killer cells (NKs) and CD4+ type-1 T-helper lymphocytes(Th-1)) effectively counteracts the proliferation of cancer cells (*elimination phase*). However, the malignant cell clones characterized by a reduced expression of cancer antigens and by an augmented expression of immune-checkpoint molecules (PD-L1/PD-L2) are Darwinianly selected as escaping cells and proliferate over other cell clones. During this phase, the rate of eliminated cells is equal to the amount of escaping cells (*equilibrium phase*). Ultimately, the tumor microenvironment becomes rich in escaping cells and immunosuppressive lymphocytes (FOXP3+ T-regulatory lymphocytes, myeloid-derived suppressor cells (MDSCs), and CD4+ type-2 T-helper lymphocytes), while the immunosurveillance compartment is mostly anergic (*escape phase*).

accepted that high TIL presence is associated with improved prognoses, the correlation between diverse TIL subpopulations and responses observed experimentally is still characterized by a certain grade of controversy.[9] Seventy-five percent of TILs are found to be T cells. Among these, CD8+ T cells represent the class of lymphocytes that correlate better with overall favorable clinical outcomes, usually infiltrating breast lesions in the largest proportion.[10] It has been widely documented that the presence of high rates of infiltrating CD8+ T cells is associated with overall longer survival rates.[11] NK cells serve as the first-line defense in association with CD8+ T lymphocytes. NKs are found to infiltrate breast lesions in a proportion ranging around 5% of the total lymphocytic population.[12] Recently, it has been observed that low levels of NK cells can be apparently related to more unfavorable clinical outcomes: in 175 formalin-fixed and paraffin-embedded tissue sections, the correlation of NK-based infiltrates with patients' clinical outcome in the invasive ductal BC setting has been investigated, showing a correlation between low NK counts and worse clinical outcomes, even if the presence of a cross-communication between NKs and other cells needs to be taken into account when the outcome data are considered.[13] CD4+ lymphocytes, and specifically Th-1 cells, represent a good prognostic factor as well, being usually associated with overall better clinical outcomes.[10,14] Regarding the immunosuppressive cells, the prognostic utility of infiltrating Tregs has been analyzed in several studies, originating a certain grade of controversy. In general, it appears that higher amounts of infiltrating

Tregs are correlated with worse tumor grade and absence of receptors, along with prognosis of high-risk BC and late relapse.[15,16] On the contrary, a positive correlation between high rates of infiltrating Tregs and improved clinical outcomes has been reported for the most aggressive forms of BC, especially for triple-negative breast cancer (TNBC) patients.[17] Interestingly, a positive correlation between high rates of infiltrating Tregs and higher amounts of CD8+ T cells has often been observed, reflecting the attempt of the host to eliminate malignant cells (immunosurveillance) in an immunosuppressive microenvironment (immunoediting). Nonetheless, the correlation between infiltrating Tregs and CD8+ T lymphocytes has been shown to possess an improved prognostic and predictive significance, according to the location and density of the two diverse subpopulations.[9,17,18] Infiltrating CD19+ B lymphocytes have been much less evaluated as prognostic/predictive factors. Their presence within breast lesions is documented as 20% of total BC TILs, and has been associated with more favorable prognosis due to the ability of these lymphocytes to differentiate into granzyme B-secreting cells upon IL-21-mediated stimulation.[12,19]

TILs as predictive factors in the neoadjuvant setting

The neoadjuvant setting is commonly considered as the best model for the evaluation of the interactions between anti-cancer drugs, the tumor microenvironment, and patient's response. For BC, the first observation of a positive connection between presence of TILs and more favorable clinical outcomes has been reported by Denkert et al.[20] in 2010, documenting a linear correlation between high levels of TILs, especially T cells, and clinical/radiological responses to anthracycline-based neoadjuvant-chemotherapy (NAC) regimens. Since this pivotal study, the predictive role of TILs with regard to the success of NAC has been highlighted by a considerable number of studies, often focusing on HER2 positive and TNBC molecular subtypes.[21] The correlation between CD8+ T lymphocytes and Tregs as a valid indicator of success of NAC has been recently reported in the TNBC setting. Specifically, the CD8+/FOXP3+ ratio represents a reliable parameter, as it considers the relationship between immunosurveillance and immunosuppression cell compartments within the tumor bed. Higher ratios are, in fact, associated with increased probabilities of obtaining pathological complete response (pCR) in TNBC patients undergoing NAC whereas lower values correlate with reduced response ratios.[22] While higher rates of TILs are typically associated with more favorable clinical outcomes, the molecular causes of this observation are still under evaluation.[21] The link between immune infiltrates and therapeutic success seems to be related to two main mechanisms: the first one is based on the assumption that some chemotherapy agents, especially anthracyclines, elicit their effect on highly proliferating cells, leading to an augmented expression of cancer antigens and immunogenic molecules (such as CCL2/CCR2) with a consequent release of immunostimulating chemokines (such as IL-2 and interferons (IFNs)).[23,24] These phenomena lead to an induced recruitment and differentiation of antigen-presenting cells (APCs), to an enhancement of the anti-tumor immune response and to a sensitization of tumor cells to cytotoxic attack, ultimately increasing the effectiveness of a NAC treatment.[25,26] The second major mechanism underlying the success of NAC is related to targeting the immunosuppressive compartment of the immune response, which comprises mainly Tregs and MDSCs.[27] This finding, which is particularly valid for chemotherapy agents, such as cyclophosphamide, taxanes, and 5-fluor-pyrimidynes, is related to an increased expression of apoptotic-related pathways in the aforementioned cell populations, in addition to a downregulation of the anti-apoptotic pathways and an augmented release of immunosuppressive cytokines, such as IL-4, IL-10, and IL-13.[25,28]

The presence of TILs in the residual disease after NAC is considered a positive prognostic factor, with a linear association with improved survival rates. The relationship between the presence of TILs after NAC and the underlying molecular changes has been investigated in the setting of TNBC patients who underwent previous NAC, finding that the presence of TILs was associated with improved outcomes and, interestingly, that it was inversely related to the activation of the RAS/MAPK pathway, a mechanism typically adopted by cancer cells to reach uncontrolled proliferation and possibly to promote immune evasion. As stated by the Authors, this observation provides the rationale of adopting future therapeutic strategies based on the combination between MEK inhibitors and anti PD-L1 agents. Moreover, the activation of the RAS/MAPK pathway might be exploited as a predictive marker of response to immune-checkpoint abrogating agents, along with major histocompatibility complex (MHC) expression levels.[29]

Accordingly, the evaluation of PD-1/PD-L1 expression, in parallel to TIL count and characterization, may putatively be a valid parameter allowing the selection of those patients who might potentially benefit from immune-checkpoint inhibitors-based treatment approaches for better outcomes. Recently, Park et al.[30] investigated the correlation between TIL presence and PD-L1 expression in 333 early-stage BC patients. Specifically, the Authors focused on CD8+ T cells and Tregs and analyzed the eventual correlation between the two populations with regard to PD-L1 expression levels on tumor cells. While no correlation has been documented between Tregs and PD-L1, CD8+ T cells have been found to be associated with low expression levels of PD-L1. Moreover, the amount of CD8+ T cells has been linearly related to higher infiltration of Tregs; thus, increased amounts of infiltrating CD8+

T cells have been positively associated with higher rates of Tregs and, interestingly, with lower expression levels of PD-L1 on tumor cells.[30] Therefore, these data suggest that future neoadjuvant clinical trials aiming to evaluate the anti-tumor activity of anti PD-L1 agents should be performed on patients with low TIL scores and higher tumor PD-L1 expression levels. As one of the latest frontiers of immunotherapy research is to address whether immune-checkpoint abrogating agents elicit their action directly on in situ lymphocytes or as a consequence of the attraction of distant lymphocytes toward the lesion, future neoadjuvant trials might be the proper clinical approach to elucidate this important issue with particular regard to the residual tumor after treatment.[31]

TILs as predictive factors in the adjuvant setting

The utility of TILs as predictive factors in the adjuvant setting is supported by several evidences. In 2013, Loi et al.[32] documented a clear association between the presence of TILs at diagnosis and significantly better clinical outcomes for TNBC patients. The study, conducted on a total of 2009 samples from the BIG 02-98 adjuvant phase III trial, highlighted a linear correlation between higher amounts of TILs and reduced risk of relapse and death regardless of the type of chemotherapy scheme (anthracyclines vs doxorubicin plus docetaxel). In HER2 positive BC population, TILs were also associated with improved outcomes with respect to anthracycline-based treatment.[32] A subsequent prospective–retrospective study was performed on a total of 1010 early-stage BC samples retrieved from the FinHER phase III adjuvant trial based on 778 HER2 negative and 232 HER2 positive patients (who received trastuzumab), revealing a significant association between higher TIL amounts at diagnosis and more favorable clinical outcomes in terms of distant disease-free survival (DDFS) in HER2 positive BC patients.[33] Moreover, a recent pooled analysis evaluated the prognostic value of TILs on a total of 2613 BC patients who underwent an anthracycline-based adjuvant treatment in the pre- and post-trastuzumab era. The analysis involved four prospective clinical trials showing that high TIL rates were associated with longer disease-free survival (DFS) values in 100% of cases. In the HER2 positive setting, lymphocyte-predominant breast cancer (LPBC) subjects, characterized by an immune-infiltration higher than 50%, exhibited a decreased risk of relapse after 3 years from diagnosis compared with non-LPBC patients: recurrence occurred in 8.10% of LPBC patients (3/37) and none of them died, while 14.7% of non-LPBC patients relapsed and 4.37% died. Interestingly, similar results were obtained in the TNBC setting, in which only 3.7% of LPBC patients relapsed and none died, whereas for non-LPBC patients, relapse and death rates have been documented as 28.3% and 23.66%, respectively. Ultimately, after an adjustment for standard clinic-pathological parameters and treatment, the Authors found that high TIL rates were an independent predictive tool for the entire cohort.[34]

The results of a comparison between chemotherapy and chemotherapy plus trastuzumab from the N9831 adjuvant trial showed that TILs, in particular stromal TILs, were related to more favorable outcomes for adjuvant chemotherapy only, whereas their presence was significantly associated with a lack of efficacy of trastuzumab on the sample of patients (total number: 945).[35] Based on these data, a subsequent recently published article analyzed the eventual cause of this controversial observation, finding a possible role of IL-21 and its receptor IL-21R in the modulation of the efficacy of trastuzumab on HER2 positive BC mice models. The Authors found that the expression of IL-21R on CD8+ T cells was related to optimal therapeutic efficacy and that the administration of recombinant IL-21 in association to trastuzumab elicited clear anti-tumor effects on primary lesions and metastases.[36]

The prognostic value of TILs: current status

In spite of the accruing evidences regarding the potential clinical utility of TILs as a valid prognostic factor, especially in case of TNBC, currently TILs determination is not officially recommended. Indeed, a 2015 consensus (the 2015 St Gallen Consensus Conference) clearly denies the recommendation of TIL evaluation, mainly because of the lack of standardized procedures for their isolation and characterization and because no clinical validation and data reproducibility have been documented so far.[37] In parallel to the publication of the consensus, an international expert team with proven experience in TIL analyses (the International TIL Working Group) developed and published a standardized method which might definitively pose the bases for harmonized and clear TIL determination in the clinical setting for diagnostic purposes. The guidelines stated by the Working Group also take into consideration the standardized guidelines for TIL counts, therefore allowing a better reproducibility of the data, which are being increasingly accrued (Table 1).[38] Interestingly, the consensus indicates stromal TILs as the most appropriate population for diagnostic evaluations, in light of a clear advantage, over intratumoral TILs, in terms of isolation and observation difficulty. Besides, intratumoral TILs reflect a higher biological importance which renders them especially indicated for research purposes (Table 2). For diagnostic utility, the amount of stromal TILs is thus referred to as a percentage over the evaluated stromal area (and not over the number of stromal nuclei), calculated excluding malignant cells in the total area.[38]

In order to validate such guidelines, a large retrospective study involving a total of 897 TNBC patients has been

Table 1. Current recommendations for the evaluation of TILs from the 2014 International TILs Working Group.

Step	Recommendations
Step 1. Selection of tumor area	TILs to be analyzed must be located: • Inside the borders of invasive tumors, including invasive edges TIL evaluation must NOT include • Adjoining normal tissue or DCIS: • Large areas of necrosis/fibrosis
Step 2. Definition of stromal TILs	For diagnostic purposes, only stromal TILs must be considered after careful observation.
Step 3. Microscopic observation at low magnification	A magnification of ×200/×400 is considered as optimal.
Step 4. Determination of type of inflammatory infiltrate	• Only infiltrating mononuclear cells must be considered (lymphocytes and plasma cells); • Granulocytes located in necrotic areas must NOT be considered.
Step 5. Assessment of the amount of TILs	Based on low magnification observation, the amount of stromal TILs must be calculated as a percentage over the analyzed stromal area. The lesion must be then included in the following three groups: • group A (0%–10% stromal TILs); • group B (10%–40% stromal TILs); • group C (40%–90% stromal TILs).

TIL: tumor-infiltrating lymphocytes; DCIS: ductal carcinoma in situ.

Table 2. Differences between intratumoral and stromal TILs as indicated by the 2014 International TILs Working Group.

Intratumoral TILs	Stromal TILs
• Direct interaction with cancer cells, reflecting a biologically higher relevance for research purposes.	• No contact with cancer cells, reflecting a reduced biological importance for research purposes.
• Lower presence.	• TIL count is not affected by density and proliferation of cancer lesion.
• Difficult detection and awkward observation through hematoxylin and eosin staining.	• Easier detection, isolation, and observation.
• High heterogeneity.	• Reduced heterogeneity.

TIL: tumor-infiltrating lymphocytes; DCIS: ductal carcinoma in situ.

performed, confirming a clear association between high TIL amounts and better clinical outcomes, especially in terms of DFS, DDFS, and overall survival (OS). A 10-year survival rate of 71%, 84%, and 96% for DFS, DDFS, and OS, respectively, has been documented for LPBC patients, and a positive link between TILs and OS across all the subgroups has been highlighted by stratified analysis. Interestingly, the Authors documented an increased survival rate for each 10% augmentation of TIL amounts, with no correlation with patient's age, Ki67 score, lymph-node involvement, and tumor size and grade.[39]

TILs and BC molecular subtypes

The immunogenicity of BC, a highly heterogeneous disease, has been debated extensively: while the less aggressive variants do not exhibit high rates of infiltrating lymphocytes, the most lethal conditions, meaning in absence of hormones receptors, are highly immunogenic.[40] This concept finds its proof-of-concept in the triple-negative and HER2 positive molecular subsets, which are documented as the most immunogenic conditions.[40,41] The concentration of TILs in these cases is indeed particularly high. In consideration of the lack of targeted therapies which still affects TNBC treatment, the evaluation of TILs can concretely represent a benefit for future clinical trials involving the administration of novel immunotherapeutic agents.[42,43] Infiltrating CD8+ lymphocytes are independently related to more favorable clinical outcomes in the triple-negative subset;[44] instead, lymphocytic infiltration does not exhibit any correlation with clinical outcomes in the setting of estrogen positive cases.[45] Recently, it has been clearly shown that the presence of TILs in TNBC is a robust and independent indicator of better clinical outcomes in terms of DFS, distant recurrence-free interval (DRFI), and OS. The study, conducted on a total of 482 patients in an adjuvant-therapy setting, validates the role of TILs as a strong indicator of prognosis.[46] The relationship between high TIL rates and improved clinical outcomes is also confirmed by the observation of higher expression levels of PD-L1 by TNBC cells, which highlights the importance of the evaluation of immune-checkpoint tissue expression for a more comprehensive characterization of the specific lesion.[47]

Conclusion

Since the first hypotheses regarding the possible existence of an immunosurveillance mechanism against several types of cancer,[48,49] the involvement of the immune system in the early recognition and elimination of malignant cells has been strongly consolidated in the immunoediting theory, becoming one of the cancer hallmarks and the new frontier of next-generation therapies.[50] TILs are one of the best examples of the strict relationship that exists between natural defenses and carcinogenesis and represent a snapshot of the tumor scenario. TILs can be accordingly seen as an unloaded weapon, whose drug-induced reactivation can lead to a restoration of natural anti-cancer defenses, which were once fully operative. Novel immunomodulating therapies, with special regard to immune-checkpoint inhibitors, can concretely represent a valuable chance for the treatment of the most aggressive variants of BC. However, tumors are anyways able to overcome the efficacy of immune-related therapies by overexploiting different mechanisms, which are not the actual target of the selected drug. Although this may initially be seen as a dramatic obstacle, the exploitation of different therapeutic approaches based on the combination between different agents might represent a concrete strategy against solid tumors. In this scenario, the evaluation of TILs as novel prognostic and therapy-predicting factors should become a routinely performed analysis, with particular regard to the most aggressive breast lesions, such as the triple-negative and HER2 positive molecular subvariants. Moreover, the molecular evaluation based on the detection of TILs along with the PD-L1 expression would guide clinicians into the choice of the most appropriate therapy. In the near future, there will be the need for validating evidence-based criteria that might better qualify and quantify TIL expression. In addition, future researches are awaited to evaluate the role of novel immunotherapy drugs with conventional therapeutic modalities based on biomarker selection.[38,51]

Declaration of conflicting interests

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