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# Intra-Tumoral Lymphocytes in Breast Cancer: Real Perspectives?

**Mona Mlika*** Department of Pathology. Abderrahman Mami Hospital.Université de Médecine. Tunis El Manar. Research Unit: 12/SP/18. Tunisia

**Soumeya Laabidi** Department of Medical Oncology. Abderrahman Mami Hospital. Université de Médecine. Tunis El Manar. Research Unit: 12/SP/18. Tunisia

**Ali Mrabet** Université de Médecine. Tunis El Manar. Tunisia

**Hamouda Boussen** Department of Medical Oncology. Abderrahman Mami Hospital. Université de Médecine. Tunis El Manar. Tunisia

**Faouzi El Mezni** Department of Pathology. Abderrahman Mami Hospital.Université de Médecine. Tunis El Manar Research Unit: 12/SP/18. Tunisia

**Abstract:** Background : During the conference of ASCO 2015, there was no consensus about TIL role in beast cancer and no recommendations concerning the microscopic assessment of TIL. In fact, the patients could be put on anti-PD1 drugs without the necessity of highlighting PD1 positive cells by using immunohistochemistry. Nevertheless, many questions about the prognostic impact of TIL, the methods of assessment, the type of TIL to count remain unresolved. Material and Methods : We performed a review of the literature on the sites : Pubmed and Cochrane. We used the key-words : "TIL in cancer"; "TIL in breast cancer" and "prognostic impact of TIL in breast cancer". Results : According to our inclusion and exclusion criteria, thirty eight articles were retained. Our review of the literature showed that assessing TIL in high grade tumors seem unnecessary. They seem available in intermediate graded tumors. In neo-adjuvant and adjuvant conditions, CD3+ lymphocytes seem to be correlated to a good response to chemotherapy. After a chemotherapy, quantification T reg lymphocytes CD4 + FOXP3+ seems helpful because the decrease of their number is correlated to a good prognosis. Conclusion : The role of TIL in breast cancer is clearly established. The mechanisms of immune escape induced to discovery of immune therapy. The role of the microscopic examination and the subtyping of TIL using immunohistochemistry hasn't been clearly established. Through this review of the literature, we tried to establish a diagram highlighting the different subtypes of TIL to evaluate and their prognostic impact.

**Keywords:** Breast cancer, Intratumoral lymphocytes, Immunohistochemistry, Prognosis, Microscopy.

## 1. Introduction

Breast cancer is the most frequent cancer in women with a prevalence of 99, 7 cases per 100.000 women. The World Health Organization of 2012 of breast cancer identified 38 different types of infiltrating carcinomas of the breast. In addition to this morphologic classification, a molecular classification was established. The latter enabled to determine the prognosis of each patient by furnishing predictive features of response to treatment. This classification aimed to improve the efficacy of the treatment and to reduce the secondary effects of the treatment [1-3]. Four molecular subtypes have been identified: luminal A sub-type, luminal B sub-type, Her2 sub-type and basal sub-type [4]. Recently, the tumors «Claudin Low» have been defined as tumors with a low level expression of molecules interacting in tighted junctures and other adhesion molecules such as Claudins 3, 4, 7, occludin and E-cadherin [5] and are associated to a high resistance to chemotherapy and radiation therapy [6]. Treatment modalities are established according to the profile of the tumors [7]. The mainstay treatment of breast cancer is based on the surgical resection especially in localized forms. Classical chemotherapies used are intercaling agents, anti-metabolites, alkylants and antimicrotubules. In case of expression of the hormone receptors by the tumor cells, a hormonotherapy is prescribed. In cases with HER2/Neu amplification, a treatment targetting HER2/Neu (trastuzumab) or inhibiting the tyrosine kinase activity is prescribed (TKI, Lapatinib). In addition to the advances made in morphological and molecular profile, a progress has been made in the domain of immunology. In fact, the tumor cell is confronted to the immune system. In 2000, Hanahan and Weinberg published 6 competences of the

*Corresponding Author

tumor cells including autoproduction of growth signals, escape to apoptosis, no replicative limit, inducing angiogenesis and inducing metastases. This review was recently reviewed by the same authors who added 4 properties including exploitation of metabolic pathways, genetic instablity of tumoral clones, induction of proinflammatory response and the capacity of tumor cells to escape to the immune system [8]. It is now proved that tumoral growth is dependant on mechanisms of escape to the immune system. This fact highlights new therapeutic strategies targetting the increase of the anti-tumoral immune response. Nowadays, we are facing 2 challenges : the comprehension of the interaction between tumor cells and the antitumoral immunity and the identification of therapeutic targets.

We aim to emphasize on the role of intratumoral lymphocytes in breast cancer and to highlight the role of the pathologist in quantifying and identifying the phenotype of the lymphocytes.

# 2. Methods

We performed a review of the literature on the sites : Pubmed and Cochrane. We used the key-words : "TIL in cancer"; "TIL in breast cancer" and "prognostic impact of TIL in breast cancer".

- Inclusion criteria: we included articles written in French or in English that were available freely or in the University of Medicine of Tunis. English or french abstracts with complete data were also used. Concerning the pathological study, we retained only article that speicified clearly in the method section, the techniques used and the cut-off of positivity.

- Exclusion criteria: the articles that didn't meet these criteria were not retained in addition to those with incomplete results.

# 3. Results and Discussion

Thirty eight articles were retained and are represented in Table 1.

## 3.1. The Different Actors in the Anti-Tumoral Immune Reaction

### 3.1.1. Lymphocytes T regulators

Infiltration of the tumor by lymphocytes is conidered as a prognostic factor in solid cancers [9]. Intra-tumoral lymphocytes represent the antitumoral response of the host (TIL). Many authors report that the phenotype of the lymphocytes is more important than their number. The majority of TIL are of CD3 phenotype. CD3+ T lymphocytes are subdivided into CD4 helpers T cells, CD4+ regulators (reg) and CD8+ cytotoxic lymphocytes. Lymphocytes T reg derive from thymic T lymphocytes CD4+ CD25+ expressing lymphocyte antigen (CTLA-4) and secreting immunosuppressive cytokins such as IL-10 and TGF-beta [10]. Reg T lymphocytes account for 10% of T CD4+ lymphocytes and express FOXP3 (the forkhead box P3 transcription factor). This factor of transcription acts in immunotolerance with other genes coding for proteins CD25, GITR, CTLA4. FOXP3 inhibits the cytokines IL-2 production and inhibits directly antigen presenting cells (cell-to-cell contact). [11]. In breast cancer, the important number of lymphocytes T reg CD4+CD25+FOXP3+ and the low ratio CD8/FOXP3 are correlated to a bad prognosis [12].

### 3.1.2. Macrophages Associated To Solid Tumors, Myeloid Suppressive Cells and Relative Cytokins

The tumor associated macrophages (TAM) and the myeloid suppressive cells (MSC) seem to play a key role in the immunotolerance.

TAM promote the development of Treg lymphocytes inducing an immunosuppression [13]. B7-H1 is an antigen expressed by TAM inducing anergy of T lymphocytes and apoptosis after ligation to PD1, which is situated on TAM [14]. B7-H1 is directly implicated in the protection of tumor cells and activated T lymphocytes [15]. It is expressed in 50% of tumor cells and seems to be correlated with a bad prognosis [16]. These interactions between the lymphocytes and the TAM are defined as checkpoints that are able to activate or inhibit the lymphocytes. In breast cancer, macrophages are associated to high tumor grade and an important microvascular density [17]. MSC represent a heterogeneous population of immature myeloid cells [18]. These cells induce immunosuppression. Recently, Cole et al, reported the correlation of MSC to a bad prognosis. These cells may represent further important biomarker and a possible therapeutic target [19].

### 3.1.3. Natural Killer Cells (NK Cell)

NK cells seem to play an important role in the antitumoral immunity [20]. NK cells inhibit the tumoral growth. CD56 antigen is a well known marker of these cells. They are present in blood and also in numerous tissues. The prognostic impact of these cells has been described in many tumors. In a study about 175 breast infiltrating carcinomas, Rathore, *et al*. [20] reported that a low number of NK cells was correlated to a high tumoral grade, advanced stage and lymph node metastases. On the other hand, they seem correlated to a good therapeutic response and a prolonged survival in comparison with tumors presenting high level of NK cells.

## 3.2. TIL: Prognostic Factors In Breast Cancer:

Consensual prognostic factors in breast cancer are represented by the age, the tumor size, the high histologic grade, lymph node metastases, negative hormonal status and the mitotic index [21]. TIL represent emerging prognostic factors. Many studies reported that TIL and reg T lymphocytes have an important impact on the prognosis of breast cancer. The prognostic impact of TIL has been reported mainly in high grade tumors that don 't express hormone receptors and triple negative tumors [22]. Recently, it has been reported that the prognostic impact of TIL depends on their phenotype. Nontheless, the results seem non consensual. Mahmoud et al, reported in a study about 1334 breast cancers that the level of CD8+ T lymphocytes (intra-tumoral, adjacent or distant) was correlated to a better prognosis [23]. T CD4+ lymphocytes expressing FOXP3 were correlated to a poor prognosis [24]. The expression of PD-1 by TIL and the co-expression of Treg FOXP3 and B7-H1+ is associated to a high histological grade and to the negativity of the hormonal receptors [21]. In opposition to these results, West Nr et al, reported that TIL FOXP 3+ were associated to a good prognosis in triple negative tumors [22]. The prognostic impact of TIL seems to be more relevant in infiltrating canalar carcinomas [24]. In lobular carcinomas, the infiltration by TIL seems to have a lower prognostic impact.

## 3.3. TIL : Predictive Factors of the Response to Treatment

Cytotoxic drugs destroy the tumor cells by apoptosis [11]. Recent studies reported that chemotherapy can induce a tumoral destruction by improving the recognition of the tumor cells by the immune system [11]. Drugs like anthracyclins, cyclophosphamids and gemcitabin could promote cellular apoptosis inactivating different immunologic pathways [11]. Anthracyclins were usually designated as immunity interacting drugs. Four major hypotheses have been formulated concerning their mode of functionning :

- the suppression of T reg lymphocytes from the tumoral micro-environment [22].
- lymphopenia induced by these drugs may induce the amplification of the immunotherapy effect [22]
- Anthracyclins may induce a sensitivity of the tumoral cells to the T cell mediated immunity [22].
- Anthracyclins may induce the programmed cell death by the immune system. Her2 and TOP2A mutations were considered as predictive factors to the response to anthracyclins [22]. These markers seem to be of little utility. In a study about 255 triple negative breast carcinomas, West et al, reported that the increased number of TIL was correlated to a good clinical and histological response to a neo-adjuvant chemotherapy based on anthracyclins. In adjuvant condition, anthracyclin was associated to a prolonged survival in patients with tumors CD3+-lymphocytes rich. Cyclophosphamid, methotrexate and fluorouracile based treatments don't seem to be associated to the number of CD3 lymphocytes [22]. Demaria et al, demonstrated that in neo-adjuvant condition, the number of TIL varied after Paclitaxel treatment. These results suggested the role played by the apoptosis induced by the taxanes in the recruitment of TIL [11]. Denkert et al, reported that the density of TIL was predictive of the response to the neo-adjuvant chemotherapy. They described in a study about 1058 patients, that breast carcinomas with an important infiltration by TIL were correlated to a complete histological response in 31 to 41% of the cases [25]. Patients with tumors lacking TIL presented a histological response in 2% of the cases. In a multi-variate analysis, TIL, the age and the hormonal status were predictive of a histological response [21]. Other studies put emphasis on the importance of phenotyping the TIL. In fact, after a neo-adjuvant chemotherapy, CD3 and CD8 lymphocytes remain invariable and reg T lymphocytes FOXP3 decreased significantly. That's why the low number of FOXP3 lymphocytes was correlated to a good therapeutic response [11]. In another study, Perez et al, demonstrated that in advanced breast cancers with HER2 amplification, the tumors presented an important density of T FOXP3 lymphocytes. The different data about the impact of TIL on clinical results are represented in table 2 [11].

## 3.4. The Role of the Pathologic Examination in Quantifying and Phenotyping TIL

TIL can be assessed using 3 techniques: immunohistochemistry, in flux cytometry and transcriptomic studies. The majority of the studies used immunohistochemistry in order to highlight TIL. The main antibodies used are CD3, CD4, CD8, CD20, CD35 and CD56 [16, 24].

Many methods of quantification have been described in the literature with different degrees of accuracy and reproducibility. Loughlin et al described a method of quantification based on the photoshop [16]. In fact, in a study about 16 breast carcinomas, 10 adjacent fields were analyzed using a digital camera. Immuno-phenotyping signals were analyzed using the version 7 of Adobe Photoshop. This method seems to be reproducible but necessitates expensive material especially in low-income countries. The advantage of photoshop is the conversion of pixels in number of lymphocytes/mm2. The other challenge is the quantification of intra tumoral TIL, stromal TIL, epithelial or peri-vascular TIL [16].

Rathore et al, used the manual quantification in a study about 175 cases. They focused on rich fields in intratumoral and stromal TIL. Five fields were selected using the power 200 corresponding to a 0.56 mm2 surface. The number of intra tumoral TIL and stromal ones was quantfied manually [26]. Zhang XD, et al choose a manual method by quantifying 50 fields (x400) and realizing the average of intra-tumoral lymphocytes [27].

Cut-offs used in the literature were variable in the different studies. Rathore et al, used a semi-quantitative method : + (1-25 cells), ++ (26-50 cells) and +++ (> 51 cells). Other authors used quantitative methods using different cut-offs : 5 cells/mm2 for Mahmoud et al and 60% for Dieci, *et al.* [28] Seo, *et al.* [29]. Other studies were based on molecular studies by quantifying the ARNm. The extraction of the tumoral RNA and the study of the differet genomic signatures inabled to discover 3 transcriptomic signatures : antigene IgG genes which is the signature of B lymphocytes and plasmocytes, the genes of lymphocytes T/NK reflecting TH1 type response and the

myeloid specific markers characteristic of monocytes/ dendritic cells and presenting molecules of antigens of classe II [30].

There is a significant variability between morphological analysis and molecular one. This fact could be explained by the tumoral heterogeneity and the inequality of distribution of TIL and the lack of reproducibility of the scoring system and the different cut-offs.

### 3.5. Strategies of Targetting Immuno-Tolerance in Breast Cancer

Many strategies are explored in order to target the immune tolerance in breast cancer. Some strategies are based on immunotherapy. They have two major objectives: increasing/ activating immune cells towards tumoral antigens and inhibiting the response of the microenvironmental tumor. Many efforts have been done in developping drugs enabling the modification of the dendritic cells properties, to develp vaccination strategies against tumoral antigens and the use of antibodies targetting specific cells or molecules implicated in the inhibition of the immune response. An efficient immunotherapy should eradicate the tumor but also induces a protection from recurrences and metastases [31].

## 4. Conclusions

During the conference of ASCO 2015, there was no consensus about TIL role in beast cancer and no recommendations concerning microscopic examination. In fact, patients could be put on anti-PD1 drugs without the necessity of highlighting PD1 positive cells by using immunohistochemistry. Neverthless, many questions about the prognostic impact of TIL, the method of assessment, the type of TIL to count remain unresolved. According to our review of the literature, assessing TIL in high grade tumors seem unnecessary. They seem available in intermediate graded tumors. In neo-adjuvant condition, CD3+ lymphocytes seem to be correlated to a good response to chemotherapy. In adjuvant situtation, CD3+ lymphocytes seem also correlated to the therapeutic response. After a chemotherapy, quantification T reg lymphocytes CD4 + FOXP3+ seems helpful because the decrease of their number is correlated to a good prognosis. Based on these findings, we performed the diagram represented in figure 1.

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**Figure-1.** A diagram representing the utility of assessing TIL in neo-adjuvant, adjuvant and post-chemotherapy

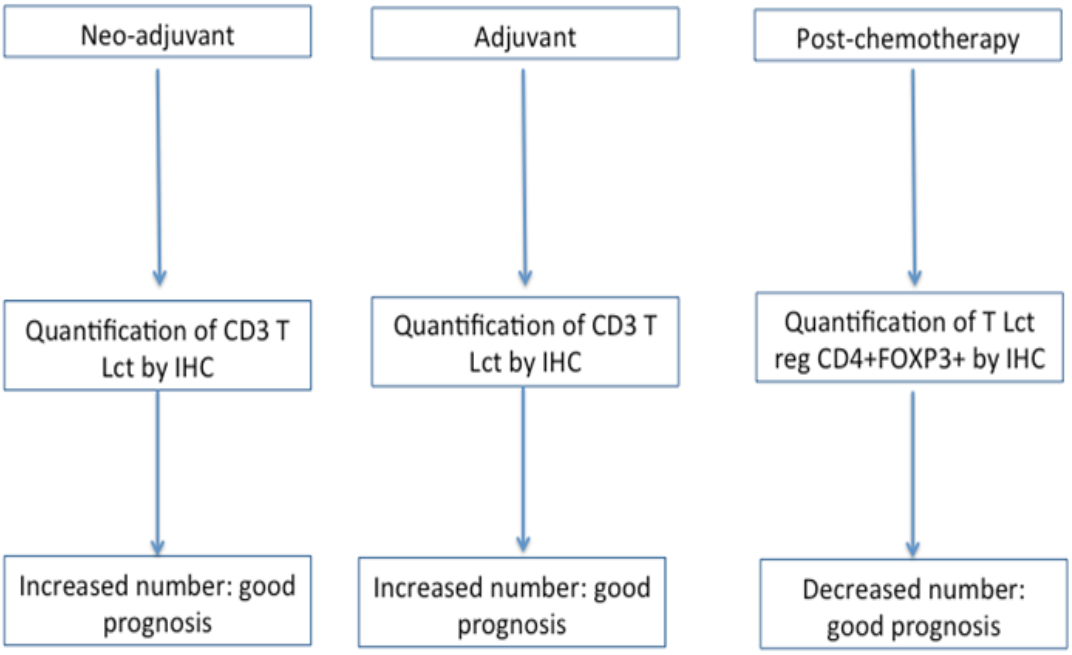


**Table-1.** The different articles included in the study.

| Authors | Year | Title | Journal |
|---|---|---|---|
| Massink MP, Kooi IE, van Mil SE, Jordanova ES, Ameziane N, Dorsman JC [25] | 2015 | Proper genomic profiling of (BRCA1-mutated) basal-like breast carcinomas requires prior removal of tumor infiltrating lymphocytes. | Mol Oncol. 2015 Jan 13. pii: S1574-7891(15)00003-4. |
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| Schalper KA, Velcheti V, | 2014 | In situ tumor PD-L1 mRNA expression is | Clin Cancer Res. 2014 May |

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**Table-2.** Impact of TIL in the different studies of the literature

| Study | Number | Biomarkers | Results |
|---|---|---|---|
| Balsari et al, | -62 In situ carcinomas<br>- 257 infiltrating carcinomas<br>- 10 : normal breast parenchyma | FOXP3 | - FOXP3 increased in in-situ and infiltrating carcinoma<br>- FOXP3 correlated to a short survival. |
| Ladoire et al, | 56 | CD3-CD8-FOXP3 | - FOXP3 before CT correlated to poor prognostic factors (Hormone receptors, grade and lymph node metastases)<br>- Good histologic response to neo-adjuvant chemotherapy correlated to negativity of FOXP3 and increased number of CD8+ lct. |
| Bates et al, | 183 | FOXP3 | - Expression FOXP3 : short survival |
| Demaria et al | 25 | TIL | - Increased number of TT correlated to clinical response to neo-adjuvant CT. |
| Denkert et al | 1058 | TIL | Increased TIL: histologic response in 40 to 42% of cases |
| Perez et al | 24 normal breast<br>74 infiltrating duct carcinoma | Tregs | - Increased Treg in HER2+ cancer<br>- Trastuzumab : decreased number of T reg |
| Mahmoud et al | 1334 | CD8+T | TIL CD8+ increased correlated to good clinical response. |

CT : chemotherapy, TIL : tumor infiltrating lymphocytes, Treg : regulators T lymphocytes