Histochemistry and Cell Biology

Immune response profile of primary tumour, sentinel and non-sentinel axillary lymph nodes related to metastasis in breast cancer: an immunohistochemical point of view.

--Manuscript Draft--

Manuscript Number:	HACB-D-19-00070R2
Full Title:	Immune response profile of primary tumour, sentinel and non-sentinel axillary lymph nodes related to metastasis in breast cancer: an immunohistochemical point of view.
Article Type:	Review
Funding Information:	Instituto de Salud Carlos III (PI11/0488) — Dr Carlos Lopez Instituto de Salud Carlos III (PI13/02501) — Dr. Marylène Lejeune FP7 People: Marie-Curie Actions (612471) — Dr. Gloria Bueno
Abstract:	Approximately 1.67 million new cases of breast cancer (BC) are diagnosed annually, and patient survival significantly decreases when the disease metastases. The axillary lymph nodes (ALNs) are the main doorway for BC tumoral cell escape, through which cells can disseminate to distant organs. The immune response, which principally develops in the lymph nodes, is linked to cancer progression, and its efficacy at controlling tumoral growth is compromised during the disease. Immunohistochemistry (IHC) is one of the most widely used research techniques for studying the immune response. It allows the measurement of the expression of particular markers related to the immune populations. This review focuses on the role of the immune populations in the primary tumour in the locoregional metastasis of the ALN, and the relationship of the immune response in these regions to distant metastasis. We considered only studies of immune cells using IHC techniques. In particular, lymphocytes, macrophages and dendritic cells all play important roles in BC and have been extensively studied. Although further research is needed, there is much evidence of their role in the invasion of the ALN and distant organs. Their association with tumoral growth or protection has not yet been demonstrated decisively and is very likely to be determined by a combination of factors. Moreover, even though IHC is a widely used technique in cancer diagnosis and research, there is still room for improvement, since its quantification needs to be properly standardized.
Corresponding Author:	Carlos Lopez, Ph.D. Hospital Verge de la Cinta Tortosa, Tarragona SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Hospital Verge de la Cinta
Corresponding Author's Secondary Institution:	
First Author:	Albert Gibert-Ramos, Ph.D
First Author Secondary Information:	
Order of Authors:	Albert Gibert-Ramos, Ph.D Carlos Lopez, Ph.D. Ramon Bosch Laia Fontoura Gloria Bueno, Ph.D Marcial García-Rojo, Ph.D Marta Berenguer, Ph.D

	Marylène Lejeune, Ph.D
Order of Authors Secondary Information:	
Author Comments:	
Response to Reviewers:	Hospital Verge de la Cinta Carlos López Molecular Biology and Research Section, Hospital de Tortosa Verge de la Cinta, IISPV, URV, Tortosa, Spain Tel: +34 977 519104Email: clpclp3@gmail.com Tortosa, 28 June 2019 Dear Reviewer, Thank you for your clear revisions and valuable comments, which have enabled us to improve our manuscript. We have modified the 3 small corrections. 1. Page 3, line 14: "other LNs" 2. Page 3, line 33: considered "as" a highly 3. Page 6, line 6: biomarker "for" Yours faithfully, Carlos López.

Immune response profile of primary tumour, sentinel and non-sentinel axillary lymph nodes related to metastasis in breast cancer: an immunohistochemical point of view

Albert Gibert-Ramos[1#], Carlos López[1#*], Ramon Bosch[1], Laia Fontoura[1], Gloria Bueno[2], Marcial García-Rojo[3], Marta Berenguer[4], Marylène Lejeune[1].

[1] Grup de Recerca en Patologia Oncològica i Bioinformàtica, Molecular Biology and Research Section, Hospital de Tortosa Verge de la Cinta, IISPV, URV, Tortosa, Spain

[2] VISILAB, Universidad de Castilla-La Mancha, Ciudad Real, Spain.

[3] Department of Pathology, Hospital Universitario Puerta del Mar, Cádiz, Spain.

[4] Knowledge Management Department, Hospital de Tortosa Verge de la Cinta, ICS, IISPV, Tortosa, Spain.

[#]**Both authors contributed equally**

***Corresponding author:**

Carlos López, PhD

Email: clpclp3@gmail.com / clopezp.ebre.ics@gencat.cat

Phone: +34 977 519104. Fax: +34 977 519104.

ORCID:

AG-R: 0000-0002-5987-1164

CL: 0000-0003-1248-3065

RB: 0003-4104-5515

ML: 0000-0001-8441-9404

Abstract

Approximately 1.67 million new cases of breast cancer (BC) are diagnosed annually, and patient survival significantly decreases when the disease metastases. The axillary lymph nodes (ALNs) are the main doorway for BC tumoral cell escape, through which cells can disseminate to distant organs. The immune response, which principally develops in the lymph nodes, is linked to cancer progression, and its efficacy at controlling tumoral growth is compromised during the disease. Immunohistochemistry (IHC) is one of the most widely used research techniques for studying the immune response. It allows the measurement of the expression of particular markers related to the immune populations. This review focuses on the role of the immune populations in the primary tumour in the locoregional metastasis of the ALN, and the relationship of the immune response in these regions to distant metastasis. We considered only studies of immune cells using IHC techniques. In particular, lymphocytes, macrophages and dendritic cells all play important roles in BC and have been extensively studied. Although further research is needed, there is much evidence of their role in the invasion of the ALN and distant organs. Their association with tumoral growth or protection has not yet been demonstrated decisively and is very likely to be determined by a combination of factors. Moreover, even though IHC is a widely used technique in cancer diagnosis and research, there is still room for improvement, since its quantification needs to be properly standardized.

Keywords: Breast cancer; immune response; tumoral microenvironment; immunohistochemistry; lymphocytes; macrophages; dendritic cells

Introduction

Cancer is the second leading cause of death worldwide (WHO 2018). In 2012, 14.1 million new cases and 8.2 million deaths were estimated to have happened globally (Ferlay et al. 2015), while in 2018, cancer mortality increased to 9.6 million deaths (WHO 2018). The most frequent cancer in women is breast cancer (BC), which accounted for approximately 15% of all cancer deaths in 2018 (WHO 2018). It has been estimated that approximately 12.4% of women will be diagnosed with BC during their lifetime (SEER Cancer Stat Facts: Female Breast Cancer 2018).

Distant metastasis is the main cause of death in BC patients, and lymph nodes (LNs) are the main doorway for tumoral cell escape from the primary site to other regions of the body (Lorusso and Ruegg 2012). In particular, the sentinel lymph nodes (SLNs) are the type of lymph node to which cancer is most likely to spread from the primary tumour, and become part of the locoregional metastasis of the tumour, since the cancer cells appear first in the SLN before spreading to other LNs, mainly in the axilla (Maguire and Brogi 2016). The presence of tumoral cells in the axillary lymph nodes (ALNs) is a bad prognosis factor (Zhang et al. 2017). Two-thirds of the patients diagnosed with ALN metastasis will develop distant metastases, and approximately 6% of BC patients present distant metastases at diagnosis (Lorusso and Ruegg 2012). By then, distant metastatic BC is usually incurable (Nienhuis et al. 2015) and, according to data from the National Cancer Institute, 73% of women with distant BC metastasis succumb to the disease within 5 years of their diagnosis (SEER Cancer Stat Facts: Female Breast Cancer 2018).

The low survival rate of patients with metastatic cancer highlights the utmost importance of understanding the mechanisms that lead to the spread of BC, first to the ALNs and later to distant sites. The immune system plays an important role in the cancer progression, detecting and eliminating tumour cells, and thereby protecting against tumour outgrowth (Gardner and Ruffell 2016; Hanahan and Coussens 2012; Zitvogel et al. 2008). Nevertheless, cancer development stimulates a particular microenvironment in the affected area that suppresses the immune response of the host, either by activating specific regulatory pathways related to the inhibition of the immune system, or by developing characteristics to prevent it being recognised by the immune cells (Chen and Mellman 2017; Gajewski et al. 2006; Mittal et al. 2014). Scientists have paid particular attention to this immune microenvironment because of its potential role in the treatment of cancer (Spranger et al. 2013), although further research is needed. Even though BC is not considered as a highly immunogenic tumour (Gingras et al. 2015), several studies have highlighted the important role played by the immune response in prognosis (Denkert et al. 2010; Gu-Trantien et al. 2013; Loi 2013; Loi et al. 2013), tumour progression, pathological complete response to neoadjuvant therapy, and the relapse and survival of BC patients (de la Cruz-Merino et al. 2013).

A range of techniques are being used to study BC tissues (Song et al. 2016). In particular, immunohistochemistry (IHC), one of the most widely applied techniques in the field of pathology (Peng et al. 2017; Van Bockstal et al. 2018), is used as a biomarker diagnostic tool. As well as providing differential diagnostics, IHC can be used to identify prognostic and predictive markers and to decide specific treatments for cancer patients (Slodkowska and Rojo 2011). In the last twenty years, the number of studies using IHC has tripled (Kalyuzhny 2009). Screening for biomarkers related to patients' outcomes places more responsibility on the pathologist in making decisions about therapy (Kalyuzhny 2009; Slodkowska and Rojo 2011). The uses of IHC in BC patients include the classification of their molecular profile, the determination of the best therapy or approach to the disease, and the characterization of specific cell populations (Burugu et al. 2017). In particular, many scientists use IHC to study the immune response and microenvironment in cancer in order to predict its behavior and outcome (Burugu et al. 2017; Fridman et al. 2011; Goto et al. 2018).

Taking into account the high percentage of deaths in BC patients with distant metastasis, the implications of the immune response in BC development, and the widespread use of IHC in pathology departments, we decided to collect information related to studies that have evaluated the immune infiltrates in BC patients using IHC. Thus, the scope of this review was to produce a comprehensive summary and to consider the most recent updates about the immune microenvironment in the primary tumor, ALNs and distant metastases, and the role played by the immune response in the SLN and ALNs in the development of distant metastases (Fig 1.).

Locoregional nodal metastasis: involvement of the immune response in the primary tumour and axillary lymph nodes

Several types of immune cell are involved in the inflammatory and immune response in tumours, making up part of what is known as the tumoral microenvironment (Soysal et al. 2015). Some of these immune cells can increase the likelihood of tumoral distant metastasis, particularly by stimulating lymphangiogenesis and lymphatic vessel enlargement through the secretion of factors such as vascular endothelial growth factors C and D (VEGF-C, VEGF-D) (Kerjaschki 2005; Schoppmann et al. 2002; Stacker et al. 2014). Nevertheless, before the spread of the tumour to the distant sites, the new lymphatic vessels in the primary tumour are used by tumoral cells to reach the LNs and promote tumour cell dissemination and distant metastasis (Padera et al. 2016; Zhang et al. 2016). This process is promoted by the immune population, which, besides stimulating angiogenesis and lymphangiogenesis, can activate an inflammatory response in the tumour that promotes cancer dissemination into the LNs (Nathanson et al. 2015).

Metastasis in the ALN plays a decisive role in spreading metastatic cells to distal LNs and organs (Ran et al. 2010), and the distinct immune response in these regions determines antitumoral immunity (Kim et al. 2006). This evidence further highlights the role of the immune response in BC outcome. In fact, some studies have shown that, in BC, VEGF-C expressed by

the tumour strongly activates lymphangiogenesis in the SLN, even before the metastasis in this region takes place, and thereby prepares the node for future tumoral spread (Zhao et al. 2012). However, neither the precise mechanism and function of the immune response in the metastasis from the primary tumour to the axillary node, nor the relationship between the immune response in the axillary node and distant metastases is fully understood.

Lymphocytes

Lymphocytes, which are determinants of the innate and adaptive immune system, have been categorized as part of the anti-cancer immune response, correlating positively with cancer survival (de Melo Gagliato et al. 2017; Pruneri et al. 2018). For this reason, tumour-infiltrated lymphocytes (TILs), including their subtypes, are now of particular interest to researchers and pathologists as promising biomarkers for BC prognosis and treatment (de Melo Gagliato et al. 2017; Pruneri et al. 2018). As previously mentioned, the presence of ALN metastasis is a bad prognostic factor, since it means that the tumoral cells have started to spread to other regions of the body. The role of lymphocytes in this initial spread to the LNs has not been widely studied. Nevertheless, some studies have evaluated both the primary tumour and the ALN, and analysed how the lymphocytes could be involved in this first stage of BC dissemination (Table 1).

In particular, *Matkowski et al*. found a strong positive correlation of CD4 and CD8 lymphocytes in the primary tumour with the appearance of ALN metastasis (Matkowski et al. 2009). Other studies have yielded contradictory results, whereby both markers have been found in higher proportions in patients with non-metastatic ALNs compared with those with metastatic ALNs (La Rocca et al. 2008). These differences might be attributed to the differences in sample sizes, since La Rocca and colleagues evaluated a cohort of only 35 patients (La Rocca et al. 2008).

On the other hand, there is evidence that T reg ($FOXP3^+$) lymphocytes are associated with a weaker immune response to the cancer and a higher probability of metastasis to the SLN. *Miyan et al.* showed in an IHC study of human BC that high densities of $FOXP3^+$ cells, cytotoxic ($CD8^+$), functional (ζ-$chain^+$), and CD3 T lymphocytes in the primary tumours and their SLNs, are associated with more aggressive tumours, hormone receptor negativity and higher levels of proliferation, histological grade and lymphovascular invasion (LVI) (Miyan et al. 2016). These results are in accordance with the report of a higher LVI increasing the likelihood of metastasis in the SLN (Karahalli et al. 2017; Malter et al. 2018). The latter study also showed that the variability in the cited immune markers between the different BC subtypes was less than that between primary tumours when only the metastatic SLNs were analysed (Miyan et al. 2016). These results are relevant because although a higher frequency of immune markers was found in more aggressive tumours, $FOXP3^+$ T reg lymphocytes are known to inhibit the immune response against cancer by suppressing effector T cells or secreting cytokines that inhibit antigen presentation (Shou et al. 2016).

Related to the metastasis in the ALN, *Nakamura et al.,* showed that Foxp3⁺ T reg lymphocytes in the SLN clearly distinguished between SLN molecular metastasis-negative and -positive BC patients, and reported that a high Foxp3⁺ T reg lymphocyte content in SLNs is associated with the presence of pathologically undetectable micrometastasis in the SLN (Nakamura et al. 2009). These authors suggested that the presence of Foxp3⁺ T reg lymphocytes in the SLN could be used as an effective IHC biomarker for BC prognosis (Nakamura et al. 2009). This is further explained by *Mansfield et al.*, who showed that levels of Foxp3⁺ T reg lymphocytes in the metastasis-positive SLNs were higher than in metastasis-negative SLNs of BC patients, and that these might be attracted or induced by indoleamine-2,3-dioxygenase (IDO), even before the SLN metastasis starts (Mansfield et al. 2009). Similarly, a meta-analysis of IHC studies found a positive correlation between the level of infiltrated Foxp3⁺ T reg lymphocytes in the primary tumour and LN positivity (Shou et al. 2016). Contradictory results have been reported by *Gökmen-Polar et al.,* who found the concentration of Foxp3⁺ T reg lymphocytes in the SLN to be similar when comparing metastatic and metastasis-free SLN, suggesting that these cells might not affect tumoral growth in the SLN (Gokmen-Polar et al. 2013). However, they suggested that there might be an increase in the T helper 2 lymphocyte (Th2) response in the metastatic SLN (Gokmen-Polar et al. 2013). The variation in the published results might arise from differences in the total pool of patients, the number of cases or the sampling; also, as previously mentioned, tumour malignancy and differences between BC molecular subtypes can affect the immune response. These studies demonstrate that we are still far from reaching a clear conclusion about the specific role of T reg cells in lymph nodes containing metastasized BC cells and their value as a predictive marker in IHC studies.

Considering in greater detail the important role of T reg, only in invasive ductal BC, an association was observed between the number of T reg lymphocytes in SLN and the primary tumour size. However, this correlation was not seen when there was metastasis in the SLN, suggesting that the presence of T reg lymphocytes in the SLN might be caused by factors produced by the primary tumour that travel through the LNs (Gupta et al. 2011). All these studies support the idea that there is a direct communication of signalling factors between the primary tumour and the SLN, even before tumoral invasion starts. Other authors have already proposed that changes in the LNs can precede metastasis, and that the primary tumour influences the metastatic niche of the SLN or LNs, but further research is needed to address these aspects (Kohrt et al. 2005).

Regarding the inhibition of the immune response of lymphocytes, some studies focused on novel biomarkers that can be analysed by IHC. In this context, molecules such as the Programmed Death-Ligand 1 (PD-L1), secreted by the tumour, can interact with the Programmed Death 1 (PD-1) receptors in T lymphocytes, which inhibit the immune response (Kim and Chen 2016). *Baptista et al.* found that PD-L1 expression in BC was related to better overall survival (OS), but not to disease-free survival (DFS), while PD-L2 was not associated with either (Baptista et al. 2016). Additionally, the authors reported that 50% of BC patients in

their study expressed PD-L1, and that it was more likely to be expressed in the primary tumour of patients with positive SLN (Baptista et al. 2016). *Hou* and co-workers studied HER-2 BC patients and also found that higher PD-L1 levels were linked to longer OS, although, unlike other studies, they reported a correlation between PD-L1 and the absence of LN metastasis (Hou et al. 2018), which might be explained by the particular molecular subtype involved. This aspect has been studied further by *Tatara et al.,* who found that almost all metastatic positive and negative SLN of BC patients expressed PD-1^{+} lymphocytes, although metastasis-positive SLNs featured more PD-1^{+} lymphocytes than the downstream SLNs without metastasis (Tatara et al. 2018). At the same time, *Li et al.* published similar results, but from triple-negative BC patients. Since the primary tumour is negative for PD-L1 in a high proportion of cases, the authors proposed that it might be more appropriate to evaluate the LNs, in which PD-L1 is more strongly represented and positively expressed (Li et al. 2018). They also noted a link between the presence of PD-L1 in the LN and a poorer prognosis, independently of primary tumour PD-L1 expression, which is also consistent with the higher frequency of TILs they found in the node and with the results obtained by other authors (Muenst et al. 2014; Qin et al. 2015). Although these are the first studies to relate PD-1 expression in the SLN or LNs of BC patients to metastasis, studies of melanoma have also found that a high percentage of PD-1 lymphocytes is associated with poor prognosis (Kakavand et al. 2015). These results imply that this molecule could be a good biomarker of BC outcome and that the tumour is capable of modulating the immune response. Together, these lines of evidence suggest that PD-L1 is a promising molecule for studying BC prognosis, since there are many results showing its association with OS or metastasis in the ALNs. Even so, PD-L1 research is still relatively new and further evidence is needed to understand its role in BC progression and ALN metastasis.

Macrophages

Macrophages are one of the major populations of immune cells present in BC (Solinas et al. 2009). When found in a tumour, they are identified as tumour-associated macrophages (TAMs), which accumulate in the tissue, stimulated by chemotactic molecules secreted by the tumoral cells (Komohara et al. 2016). Macrophages can usually be divided into M1 and M2 phenotypes. M1 macrophages are capable of activating the type I T lymphocyte response; they also exhibit cytotoxic activity against neoplastic cells and are favourable antigen-presenting cells. By contrast, M2 macrophages have a lower antigen-presenting capacity, can promote angiogenesis and are capable of inhibiting the immune response of M1 macrophages (Solinas et al. 2009). For these reasons, M2 macrophages are believed to be detrimental in cancer (Mantovani et al. 2017). Even so, this established distribution of TAMs does not fully represent all subtypes, and a spectrum model would more accurately describe the array of phenotypes, many of which still need to be characterised (Aras and Zaidi 2017).

Several studies have shown the role of TAMs in the prognosis and treatment of cancer (Table 2). In particular, it has been shown that monocytes develop into different mature macrophages depending on the tumour environment to which they are exposed (Bogels et al. 2012).

Furthermore, in BC, TAM infiltration of the primary tumour, which is generally measured with the $CD68^+$ marker, is associated with LN invasion (Jubb et al. 2010) and is known to stimulate lymphatic metastasis, and thereby to be negatively correlated with BC prognosis (Yang et al. 2015). $CD68^+$ cells in the tumour are associated with the mindbomb E3 ubiquitin protein ligase 1 (MIB1), an enzyme involved in regulating apoptosis and associated with tumour size and proliferation, linking the CD68 cell marker to a more aggressive tumour phenotype (Heiskala et al. 2018).

Regarding LN infiltration, the density of $CD169^+$ macrophages, also known as M1 macrophages, and the $CD169^+/CD68^+$ macrophage ratio in the regional LNs of BC patients are positively correlated with better disease prognosis, including the absence of LN metastasis (Shiota et al. 2016). In fact, CD169 is thought to be decisive for the activation of CD8 lymphocytes in the LNs during BC, since it is involved in the antigen-presentation process (Asano et al. 2011). Another study reported that low concentrations of $CD163^+$ macrophages (M2 phenotype) in SLN are associated with a higher risk of metastatic invasion in the node, and that, in already metastatic SLNs, high levels of $CD163^+$ cells are linked to a low level of $CD8^+$ cell infiltration (Mansfield et al. 2012), which is considered a negative factor in BC (Burugu et al. 2017). In effect, primary tumour might stimulate macrophages to secrete indoleamine-pyrrole 2,3-diogenase (IDO), an enzyme able to induce immune tolerance by inhibiting T-cell function. Under these conditions, even though the normal function of $CD163^+$ macrophages is to offer protection against metastasis in the SLN, their function can be reprogrammed by factors secreted from the primary tumour, with the consequence that IDO^+ macrophages might be deleterious to metastasis protection (Mansfield et al. 2012). In accordance with this, a recent study of luminal B BC, one of the most aggressive BC subtypes, found that $CD163^+$ macrophages in the primary tumour and the SLN, in conjunction with the C-X-C chemokine receptor type 4 and the chemokine (C-X-C motif) ligand 1 axis (CXCR4/CXCL12), stimulate cell migration and metastasis (Raschioni et al. 2018).

Contrary to what was detected in other parts of the tumour, *Buldakov et al.* found that a high content of $CD68^+$ TAMs in the gaps (stromal tissue) between the different foci of the ductal tumour structures of BC patients was negatively associated with the appearance of metastases in regional LNs (Buldakov et al. 2017; Pinder 2010). They attributed this to the lack of stabilin-1, which marks M2 macrophages, and proposed that these macrophages had not been affected by the tumour, but maintained their capacity to reject the metastatic invasion, thus, protecting against cancer progression (Buldakov et al. 2017).

Macrophages are being extensively studied in BC progression, and several studies relate their function to the metastatic spread in the ALNs (Mansfield et al. 2012; Raschioni et al. 2018; Shiota et al. 2016; Yang et al. 2015). The proinflammatory role of macrophages could increase the presence of metastatic ALNs, as observed in some studies of the CD68 marker (Heiskala et al. 2018; Yang et al. 2015). Nevertheless, there are also specific macrophage phenotypes that offer protection against metastatic spread into the ALN (Buldakov et al. 2017; Mansfield et al.

2012; Shiota et al. 2016), which further supports the idea of the extensive array of macrophage subtypes and their different roles in BC progression.

Dendritic cells

Dendritic cells (DCs) are essential to the immune response; they are involved in antigen presentation and the secretion of co-stimulatory signals and cytokines (Waisman et al. 2017). In cancer, DCs exhibit both pro- and anti-tumorigenic activities, depending on the microenvironment to which they are exposed. For this reason, their functions in cancer as an indicator of tumour development and prognosis (Hansen and Andersen 2017), and as a mechanism to activate the T lymphocyte response against the tumour (Gardner and Ruffell 2016), are being closely studied (Table 3).

Plasmacytoid DC (pDC) infiltration of the primary tumour is closely correlated with poor outcome of patients with BC (Mansfield et al. 2011; Treilleux et al. 2004). Higher percentages of pDC are found in BC tissues of patients with metastatic LN than in those with non-metastatic LN. This is believed to stimulate pathways involved in cell migration from the primary tumour to the LNs (Gadalla et al. 2019)

More in relation to the LNs and their invasion by metastatic cells, SLNs with metastasis present fewer mature DCs than do SLNs without metastasis. Even so, the total number of DCs, irrespective of the maturation state, is similar in metastatic and non-metastatic SLNs (Mansfield et al. 2011; Poindexter et al. 2004), suggesting that there is inefficient antigen presentation in the former group of patients, leading to a weaker immune response to the tumoral cells (Mansfield et al. 2011; Poindexter et al. 2004). This is further explained by *Ammar et al.*, who reported that a higher frequency of immature DCs in BC helps to differentiate patients at higher risk of developing local, regional or distant recurrences within 60 months (Ammar et al. 2011), while the presence of DC-lamp, a marker of mature DCs in the SLN of BC patients, is associated with a lower or null risk of LN metastases (Bembenek et al. 2008). Accordingly, a study that analysed CD1a, a marker of DCs such as Langerhans or LN-interdigitating cells, detected more DCs in the stroma of the primary tumour and the LNs of patients with negative LNs than in patients with positive LNs (La Rocca et al. 2008).

In addition to the quantity and maturation level of the DCs, factors such as clustering are also related to the immune response to cancer. The study by Chang *et al*. compared intra-mammary helathy lymph nodes (HLNs) of healthy subjects obtained from prophylactic mastectomy or breast reductions with metastatic and non-metastatic ALN (non-SLN) of BC patients. The results showed that $CD83^+$ DCs tend to aggregate in large clusters in healthy HLNs, while fewer small clusters of mature DCs, or no clusters at all, were detected in the ALN of BC patients. Moreover, greater clustering in metastatic ALNs of BC patients was associated with longer DFS (Chang et al. 2013).

Distant/systemic metastasis: involvement of the immune response present in the primary tumour and axillary lymph nodes

Distant or systemic metastasis require the completion of different steps in which the malignant cells spread from the primary tumour to neighbouring or distant tissues. Tumoral cells travel in the blood or lymph, extravasate the vessels in a different organ or tissue, stimulate angiogenesis and start proliferation, giving rise to the metastasis (Scully et al. 2012). It is thought that primary tumours prepare the soon-to-be metastatic tissues through various cells or by the secretion of factors (Liu and Cao 2016). In particular, immune cells play an important role in metastatic progression, participating in the epithelial-to-mesenchymal transition, facilitating the migration and intravasation of tumoral cells and, among other functions, preparing the microenvironment of the target tissue to establish the metastasis (Smith and Kang 2013). Few studies have so far compared or related the immune response in the distant metastasis with that in the primary tumour using IHC, but we summarise the most relevant of these in this section (Table 4).

Researchers have found that TILs are less numerous in metastatic than in primary tumours. Specifically, *Ogiya et al.* noted that in $HER2^+$ and triple-negative breast cancer (TNBC), metastatic tumours contained fewer TILs and $CD8^+$ and $CD4^+$ cells than did the corresponding primary tumours. They also showed that metastatic tumours with a lower frequency of TILs are associated with patients with shorter OS, compared to patients with higher percentages of TILs (Ogiya et al. 2016). This was also reported by other authors in various BC subtypes (Cimino-Mathews et al. 2013; Sobottka et al. 2016). *Sabottka et al.* found that the proportion of TILs in the metastasis reflected those of the primary tumour, indicating that the primary tumour might influence the immune response at the metastatic site (Sobottka et al. 2016). Furthermore, as *Ogiya et al.* pointed out, the fact that the metastasis shows a weaker immune response to the tumoral cells, draws further attention to the importance of immune escape, as favoured by the primary tumour, in tumour metastasis (Ogiya et al. 2016). A murine study found that a relatively small subset of “unconventional” T cells defined by expression of heterodimeric T-cell receptors composed of γ and δ chains, the IL-17-producing γδ T lymphocytes, might be involved in distant metastasis in BC. These IL-17-producing γδ T lymphocytes appear to up-regulate the production of neutrophils and the alteration of neutrophil phenotype. These phenotypically altered neutrophils produce iNOS, which suppresses the activity of anti-tumour $CD8^+$ T cells, promoting a pre-metastatic niche that helps metastatic cells become established in peripheral organs (Coffelt et al. 2015).

Apart from the interaction between the primary tumour and the distant metastasis, there is also evidence suggesting that ALNs influence the metastatic spread of the BC. In fact, the LNs are believed to be one of the principal paths for metastatic spread in BC (Lorusso and Ruegg 2012; Nathanson et al. 2015; Ran et al. 2010), and its immune populations may have a decisive role in cancer progression (Kim et al. 2006). The content of B ($CD20^+$) and T lymphocytes ($CD3^+$) in

SLNs is associated with a higher DFS in the patient (Blenman et al. 2018). Similar results have been observed with CD8 and cytotoxic lymphocytes, since their intratumoral infiltration in node-negative BC patients has been linked to an overall better prognosis, such as cancer-specific OS and DFS, independent of other relevant parameters such as age at diagnosis and histological grade (Chen et al. 2014). There have been few studies using IHC to predict systemic metastasis using the immune populations in the ALNs, but they have yielded promising results.

Macrophages, which are present at all stages of tumour progression, have been demonstrated to be a key driver of cancer progression and metastasis (Nielsen and Schmid 2017). *Heiskala et al.* showed that macrophages are good indicators of the outcome of a tumour, whereby $CD14^+$ TAMs, in the presence of CCL2-positive tumoral cells, are associated with tumour recurrence (Heiskala et al. 2018). Similarly, $CD163^+$ macrophages have been linked to metastatic spread, since patients with this marker in the primary tumour develop metastasis early (Shabo et al. 2008). The authors argue that the migration capacity of these cells might facilitate the dissemination of tumoral cells.

There is a lack of studies of the role of DC in BC distant metastasis, which further emphasises the need for this kind of research in BC. Considering the importance of DC in locoregional metastasis, we hope this review will inspire readers to undertake research into this crucial field.

Challenges of immunohistochemistry as a diagnostic and predictive tool

IHC is a demonstrably good technique for diagnosing BC in conjunction with other diagnostic tools, such as gene expression analysis (Van Bockstal et al. 2018). IHC techniques are also used for the study of the tumoral microenvironment and the immune response to cancer. Even so, the use of IHC can pose particular problems and challenges, such as choosing the appropriate marker of study, or the correct antibody (Walker 2008). Moreover, the use of new markers or antibodies, or their utilisation in a new tissue requires thorough validation, which takes time and effort (Miettinen 1990; Walker 2008). Other difficulties with this technique are the false-positive results it generates and the problem of reproducibility (Pillai et al. 1993), which are principally related to the lack of standardised controls or reference standards (Torlakovic et al. 2015). This lack of standards as well as the differences in the immunostaining protocols, the number of cases analysed and the cut-off used, among others, may explain the discrepancies observed in some studies mentioned in this review (Matkowski et al. 2009; Shabo et al. 2008).

The problem of proper reproducibility is one we have become particularly aware of in writing this review. Apart from using protocols from different commercial companies, different markers are sometimes used to identify the same cell population, while an antibody specifically designed to bind a particular epitope might also bind to others (Baker 2015). In addition, quantifying reproducibility also poses a problem, since no proper method of standardization exists (Jaraj et al. 2009; Sompuram et al. 2015), although some scientists have proposed reliable methods (Jaraj et al. 2009; Sompuram et al. 2015) and the use of automated analysis in recent decades

has improved this issue (Walker 2006). This makes IHC evaluation a subjective technique with problems of its application in research and clinical evaluation (Jaraj et al. 2009).

On the other hand, we consider that the differences in the results between studies for the same marker can also be caused by the patients themselves, the use of either tissue microarrays (TMAs) or whole biopsies, manual or computerized evaluation, or by the choice of cut-off value of markers related to OS or DFS, among other factors. These aspects make the search for solutions and improvements of IHC evaluation a priority.

Conclusions

The immune system, particularly TILs, TAMs and DCs, determine the capacity of the tissue to fight tumour growth. Even so, the specific niche stimulated by the malignant cells can turn this system against the organism, promoting tumoral establishment, invasion into nearby tissues and metastasis. This review summarizes the principal results of cancer research into the immune response, from the point of view of IHC. Although further research is needed, there is evidence that a diverse range of immune cells are active first in the invasion of the sentinel or axillary LNs, and then in the metastatic spread to distant organs. Our wish is to open the door to a new line of research, focusing on the LN as the turning point of BC progression, and as a decisive organ of great relevance to the study of biomarkers of prognosis. Future research should focus on establishing the differences in the immune response in each BC molecular subtype and on exploring the role of immune cells in preparing the niche for metastatic spread.

Acknowledgements

This work was funded by projects PI11/0488 and PI13/02501 of the Institute of Health Carlos III, which is the main public research body that funds, manages and carries out biomedical research in Spain, and co-funded with European Union ERDF funds (European Regional Development Fund). It was also supported by the Project AIDPATH FP7-PEOPLE Project ID: 612471. The biopsies were selected from the Banc de Tumors of the HTVC, member of the Xarxa de Bancs de Tumors de Catalunya (XBTC), sponsored by the Oncology Master Plan for Catalonia (Pla Director d'Oncologia de Catalunya).

Conflict of interest

The authors declare that they have no conflict of interest.

References

Ammar A, Mohammed RA, Salmi M, Pepper M, Paish EC, Ellis IO, Martin SG (2011) Lymphatic expression of CLEVER-1 in breast cancer and its relationship with lymph node metastasis. Anal Cell Pathol (Amst) 34:67-78 doi:10.3233/ACP-2011-0002

Aras S, Zaidi MR (2017) TAMeless traitors: macrophages in cancer progression and metastasis. Br J Cancer 117:1583-1591 doi:10.1038/bjc.2017.356

Asano K, Nabeyama A, Miyake Y et al. (2011) CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. Immunity 34:85-95 doi:10.1016/j.immuni.2010.12.011

Baker M (2015) Reproducibility crisis: Blame it on the antibodies. Nature 521:274-276 doi:10.1038/521274a

Baptista MZ, Sarian LO, Derchain SF, Pinto GA, Vassallo J (2016) Prognostic significance of PD-L1 and PD-L2 in breast cancer. Hum Pathol 47:78-84 doi:10.1016/j.humpath.2015.09.006

Bembenek A, Li J, Loddenkemper C, Kemmner W, Stein H, Wernecke KD, Schlag PM (2008) Presence of mature DC-Lamp+ dendritic cells in sentinel and non-sentinel lymph nodes of breast cancer patients. Eur J Surg Oncol 34:514-518 doi:10.1016/j.ejso.2007.05.013

Blenman KRM, He TF, Frankel PH et al. (2018) Sentinel lymph node B cells can predict disease-free survival in breast cancer patients. NPJ Breast Cancer 4:28 doi:10.1038/s41523-018-0081-7

Bogels M, Braster R, Nijland PG et al. (2012) Carcinoma origin dictates differential skewing of monocyte function. OncoImmunology 1:798-809 doi:10.4161/onci.20427

Buldakov M, Zavyalova M, Krakhmal N et al. (2017) CD68+, but not stabilin-1+ tumor associated macrophages in gaps of ductal tumor structures negatively correlate with the lymphatic metastasis in human breast cancer. Immunobiology 222:31-38 doi:10.1016/j.imbio.2015.09.011

Burugu S, Asleh-Aburaya K, Nielsen TO (2017) Immune infiltrates in the breast cancer microenvironment: detection, characterization and clinical implication. Breast Cancer 24:3-15 doi:10.1007/s12282-016-0698-z

Cimino-Mathews A, Ye X, Meeker A, Argani P, Emens LA (2013) Metastatic triple-negative breast cancers at first relapse have fewer tumor-infiltrating lymphocytes than their matched primary breast tumors: a pilot study. Hum Pathol 44:2055-2063 doi:10.1016/j.humpath.2013.03.010

Coffelt SB, Kersten K, Doornebal CW et al. (2015) IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. Nature 522:345-348 doi:10.1038/nature14282

Chang AY, Bhattacharya N, Mu J et al. (2013) Spatial organization of dendritic cells within tumor draining lymph nodes impacts clinical outcome in breast cancer patients. J Transl Med 11:242 doi:10.1186/1479-5876-11-242

Chen DS, Mellman I (2017) Elements of cancer immunity and the cancer-immune set point. Nature 541:321-330 doi:10.1038/nature21349

Chen Z, Chen X, Zhou E et al. (2014) Intratumoral CD8(+) cytotoxic lymphocyte is a favorable prognostic marker in node-negative breast cancer. PLoS One 9:e95475 doi:10.1371/journal.pone.0095475

de la Cruz-Merino L, Barco-Sanchez A, Henao Carrasco F et al. (2013) New insights into the role of the immune microenvironment in breast carcinoma. Clin Dev Immunol 2013:785317 doi:10.1155/2013/785317

de Melo Gagliato D, Cortes J, Curigliano G, Loi S, Denkert C, Perez-Garcia J, Holgado E (2017) Tumor-infiltrating lymphocytes in breast cancer and implications for clinical practice. Biochim Biophys Acta Rev Cancer 1868:527-537 doi:10.1016/j.bbcan.2017.10.003

Denkert C, Loibl S, Noske A et al. (2010) Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 28:105-113 doi:10.1200/JCO.2009.23.7370

Ferlay J, Soerjomataram I, Dikshit R et al. (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136:E359-386 doi:10.1002/ijc.29210

Fridman WH, Galon J, Pages F, Tartour E, Sautes-Fridman C, Kroemer G (2011) Prognostic and predictive impact of intra- and peritumoral immune infiltrates. Cancer Res 71:5601-5605 doi:10.1158/0008-5472.CAN-11-1316

Gadalla R, Hassan H, Ibrahim SA et al. (2019) Tumor microenvironmental plasmacytoid dendritic cells contribute to breast cancer lymph node metastasis via CXCR4/SDF-1 axis. Breast Cancer Res Treat doi:10.1007/s10549-019-05129-8

Gajewski TF, Meng Y, Harlin H (2006) Immune suppression in the tumor microenvironment. J Immunother 29:233-240 doi:10.1097/01.cji.0000199193.29048.56

Gardner A, Ruffell B (2016) Dendritic cells and cancer immunity. Trends Immunol 37:855-865 doi:10.1016/j.it.2016.09.006

Gingras I, Azim HA, Jr., Ignatiadis M, Sotiriou C (2015) Immunology and breast cancer: toward a new way of understanding breast cancer and developing novel therapeutic strategies. Clin Adv Hematol Oncol 13:372-382

Gokmen-Polar Y, Thorat MA, Sojitra P, Saxena R, Badve S (2013) FOXP3 expression and nodal metastasis of breast cancer. Cell Oncol (Dordr) 36:405-409 doi:10.1007/s13402-013-0147-3

Goto W, Kashiwagi S, Asano Y et al. (2018) Predictive value of improvement in the immune tumour microenvironment in patients with breast cancer treated with neoadjuvant chemotherapy. ESMO Open 3:e000305 doi:10.1136/esmoopen-2017-000305

Gu-Trantien C, Loi S, Garaud S et al. (2013) CD4(+) follicular helper T cell infiltration predicts breast cancer survival. J Clin Invest 123:2873-2892 doi:10.1172/JCI67428

Gupta R, Babb JS, Singh B, Chiriboga L, Liebes L, Adams S, Demaria S (2011) The numbers of FoxP3+ lymphocytes in sentinel lymph nodes of breast cancer patients correlate with primary tumor size but not nodal status. Cancer Invest 29:419-425 doi:10.3109/07357907.2011.585193

Hanahan D, Coussens LM (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21:309-322 doi:10.1016/j.ccr.2012.02.022

Hansen M, Andersen MH (2017) The role of dendritic cells in cancer. Semin Immunopathol 39:307-316 doi:10.1007/s00281-016-0592-y

Heiskala M, Leidenius M, Joensuu K, Heikkila P (2018) High expression of CCL2 in tumor cells and abundant infiltration with CD14 positive macrophages predict early relapse in breast cancer. Virchows Arch doi:10.1007/s00428-018-2461-7

Hou Y, Nitta H, Wei L et al. (2018) PD-L1 expression and CD8-positive T cells are associated with favorable survival in HER2-positive invasive breast cancer. Breast J 24:911-919 doi:10.1111/tbj.13112

Jaraj SJ, Camparo P, Boyle H, Germain F, Nilsson B, Petersson F, Egevad L (2009) Intra- and interobserver reproducibility of interpretation of immunohistochemical stains of prostate cancer. Virchows Arch 455:375-381 doi:10.1007/s00428-009-0833-8

Jubb AM, Soilleux EJ, Turley H et al. (2010) Expression of vascular notch ligand delta-like 4 and inflammatory markers in breast cancer. Am J Pathol 176:2019-2028 doi:10.2353/ajpath.2010.090908

Kakavand H, Vilain RE, Wilmott JS et al. (2015) Tumor PD-L1 expression, immune cell correlates and PD-1+ lymphocytes in sentinel lymph node melanoma metastases. Mod Pathol 28:1535-1544 doi:10.1038/modpathol.2015.110

Kalyuzhny AE (2009) The dark side of the immunohistochemical moon: industry. J Histochem Cytochem 57:1099-1101 doi:10.1369/jhc.2009.954867

Karahalli O, Acar T, Atahan MK, Acar N, Haciyanli M, Kamer KE (2017) Clinical and pathological factors affecting the sentinel lymph node metastasis in patients with breast cancer. Indian J Surg 79:418-422 doi:10.1007/s12262-016-1500-3

Kerjaschki D (2005) The crucial role of macrophages in lymphangiogenesis. J Clin Invest 115:2316-2319 doi:10.1172/JCI26354

Kim JM, Chen DS (2016) Immune escape to PD-L1/PD-1 blockade: seven steps to success (or failure). Ann Oncol 27:1492-1504 doi:10.1093/annonc/mdw217

Kim R, Emi M, Tanabe K, Arihiro K (2006) Immunobiology of the sentinel lymph node and its potential role for antitumour immunity. Lancet Oncol 7:1006-1016 doi:10.1016/S1470-2045(06)70975-5

Kohrt HE, Nouri N, Nowels K, Johnson D, Holmes S, Lee PP (2005) Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer. PLoS Med 2:e284 doi:10.1371/journal.pmed.0020284

Komohara Y, Fujiwara Y, Ohnishi K, Takeya M (2016) Tumor-associated macrophages: Potential therapeutic targets for anti-cancer therapy. Adv Drug Deliv Rev 99:180-185 doi:10.1016/j.addr.2015.11.009

La Rocca G, Anzalone R, Corrao S et al. (2008) CD1a down-regulation in primary invasive ductal breast carcinoma may predict regional lymph node invasion and patient outcome. Histopathology 52:203-212 doi:10.1111/j.1365-2559.2007.02919.x

Li M, Li A, Zhou S, Xu Y, Xiao Y, Bi R, Yang W (2018) Heterogeneity of PD-L1 expression in primary tumors and paired lymph node metastases of triple negative breast cancer. BMC Cancer 18:4 doi:10.1186/s12885-017-3916-y

Liu Y, Cao X (2016) Characteristics and significance of the pre-metastatic niche. Cancer Cell 30:668-681 doi:10.1016/j.ccell.2016.09.011

Loi S (2013) Tumor-infiltrating lymphocytes, breast cancer subtypes and therapeutic efficacy. OncoImmunology 2:e24720 doi:10.4161/onci.24720

Loi S, Sirtaine N, Piette F et al. (2013) Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol 31:860-867 doi:10.1200/JCO.2011.41.0902

Lorusso G, Ruegg C (2012) New insights into the mechanisms of organ-specific breast cancer metastasis. Semin Cancer Biol 22:226-233 doi:10.1016/j.semcancer.2012.03.007

Maguire A, Brogi E (2016) Sentinel lymph nodes for breast carcinoma: A paradigm shift. Arch Pathol Lab Med 140:791-798 doi:10.5858/arpa.2015-0140-RA

Malter W, Hellmich M, Badian M, Kirn V, Mallmann P, Kramer S (2018) Factors predictive of sentinel lymph node involvement in primary breast cancer. Anticancer Res 38:3657-3662 doi:10.21873/anticanres.12642

Mansfield AS, Heikkila PS, Vaara AT, von Smitten KA, Vakkila JM, Leidenius MH (2009) Simultaneous Foxp3 and IDO expression is associated with sentinel lymph node metastases in breast cancer. BMC Cancer 9:231 doi:10.1186/1471-2407-9-231

Mansfield AS, Heikkila P, von Smitten K, Vakkila J, Leidenius M (2011) Metastasis to sentinel lymph nodes in breast cancer is associated with maturation arrest of dendritic cells and poor co-localization of dendritic cells and CD8+ T cells. Virchows Arch 459:391-398 doi:10.1007/s00428-011-1145-3

Mansfield AS, Heikkila P, von Smitten K, Vakkila J, Leidenius M (2012) The presence of sinusoidal CD163(+) macrophages in lymph nodes is associated with favorable nodal status in patients with breast cancer. Virchows Arch 461:639-646 doi:10.1007/s00428-012-1338-4

Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P (2017) Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol 14:399-416 doi:10.1038/nrclinonc.2016.217

Matkowski R, Gisterek I, Halon A et al. (2009) The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer. Anticancer Res 29:2445-2451

Miettinen M (1990) Immunohistochemistry of solid tumors. Brief review of selected problems. APMIS 98:191-199

Mittal D, Gubin MM, Schreiber RD, Smyth MJ (2014) New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. Curr Opin Immunol 27:16-25 doi:10.1016/j.coi.2014.01.004

Miyan M, Schmidt-Mende J, Kiessling R, Poschke I, de Boniface J (2016) Differential tumor infiltration by T-cells characterizes intrinsic molecular subtypes in breast cancer. J Transl Med 14:227 doi:10.1186/s12967-016-0983-9

Muenst S, Schaerli AR, Gao F et al. (2014) Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. Breast Cancer Res Treat 146:15-24 doi:10.1007/s10549-014-2988-5

Nakamura R, Sakakibara M, Nagashima T et al. (2009) Accumulation of regulatory T cells in sentinel lymph nodes is a prognostic predictor in patients with node-negative breast cancer. Eur J Cancer 45:2123-2131 doi:10.1016/j.ejca.2009.03.024

Nathanson SD, Shah R, Rosso K (2015) Sentinel lymph node metastases in cancer: causes, detection and their role in disease progression. Semin Cell Dev Biol 38:106-116 doi:10.1016/j.semcdb.2014.10.002

Nielsen SR, Schmid MC (2017) Macrophages as key drivers of cancer progression and metastasis mediators. Inflamm 2017:9624760 doi:10.1155/2017/9624760

Nienhuis HH, Gaykema SB, Timmer-Bosscha H et al. (2015) Targeting breast cancer through its microenvironment: current status of preclinical and clinical research in finding relevant targets. Pharmacol Ther 147:63-79 doi:10.1016/j.pharmthera.2014.11.004

Ogiya R, Niikura N, Kumaki N et al. (2016) Comparison of tumor-infiltrating lymphocytes between primary and metastatic tumors in breast cancer patients. Cancer Sci 107:1730-1735 doi:10.1111/cas.13101

Padera TP, Meijer EF, Munn LL (2016) The lymphatic system in disease processes and cancer progression. Annu Rev Biomed Eng 18:125-158 doi:10.1146/annurev-bioeng-112315-031200

Peng Y, Butt YM, Chen B, Zhang X, Tang P (2017) Update on immunohistochemical analysis in breast lesions. Arch Pathol Lab Med 141:1033-1051 doi:10.5858/arpa.2016-0482-RA

Pillai R, Kannan S, Chandran GJ (1993) The immunohistochemistry of solid tumours: potential problems for new laboratories. Natl Med J India 6:71-75

Pinder SE (2010) Ductal carcinoma in situ (DCIS): pathological features, differential diagnosis, prognostic factors and specimen evaluation. Mod Pathol 23 Suppl 2:S8-13 doi:10.1038/modpathol.2010.40

Poindexter NJ, Sahin A, Hunt KK, Grimm EA (2004) Analysis of dendritic cells in tumor-free and tumor-containing sentinel lymph nodes from patients with breast cancer. Breast Cancer Res 6:R408-415 doi:10.1186/bcr808

Pruneri G, Vingiani A, Denkert C (2018) Tumor infiltrating lymphocytes in early breast cancer. Breast 37:207-214 doi:10.1016/j.breast.2017.03.010

Qin T, Zeng YD, Qin G et al. (2015) High PD-L1 expression was associated with poor prognosis in 870 Chinese patients with breast cancer. Oncotarget 6:33972-33981 doi:10.18632/oncotarget.5583

Ran S, Volk L, Hall K, Flister MJ (2010) Lymphangiogenesis and lymphatic metastasis in breast cancer. Pathophysiology 17:229-251 doi:10.1016/j.pathophys.2009.11.003

Raschioni C, Bottai G, Sagona A et al. (2018) CXCR4/CXCL12 Signaling and protumor macrophages in primary tumors and sentinel lymph nodes are involved in luminal B breast cancer progression. Dis Markers 2018:5018671 doi:10.1155/2018/5018671

Scully OJ, Bay BH, Yip G, Yu Y (2012) Breast cancer metastasis. Cancer Genomics Proteomics 9:311-320

Schoppmann SF, Birner P, Stockl J et al. (2002) Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. Am J Pathol 161:947-956 doi:10.1016/S0002-9440(10)64255-1

SEER Cancer Stat Facts: Female Breast Cancer. (2018). https://seer.cancer.gov/statfacts/html/breast.html. Accessed 8 November 2018 2018

Shabo I, Stal O, Olsson H, Dore S, Svanvik J (2008) Breast cancer expression of CD163, a macrophage scavenger receptor, is related to early distant recurrence and reduced patient survival. Int J Cancer 123:780-786 doi:10.1002/ijc.23527

Shiota T, Miyasato Y, Ohnishi K et al. (2016) The clinical significance of CD169-positive lymph node macrophage in patients with breast cancer. PLoS One 11:e0166680 doi:10.1371/journal.pone.0166680

Shou J, Zhang Z, Lai Y, Chen Z, Huang J (2016) Worse outcome in breast cancer with higher tumor-infiltrating FOXP3+ Tregs : a systematic review and meta-analysis. BMC Cancer 16:687 doi:10.1186/s12885-016-2732-0

Slodkowska J, Rojo MG (2011) Digital pathology in personalized cancer therapy. Folia Histochem Cytobiol 49:570-578

Smith HA, Kang Y (2013) The metastasis-promoting roles of tumor-associated immune cells. J Mol Med (Berl) 91:411-429 doi:10.1007/s00109-013-1021-5

Sobottka B, Pestalozzi B, Fink D, Moch H, Varga Z (2016) Similar lymphocytic infiltration pattern in primary breast cancer and their corresponding distant metastases. OncoImmunology 5:e1153208 doi:10.1080/2162402X.2016.1153208

Solinas G, Germano G, Mantovani A, Allavena P (2009) Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol 86:1065-1073 doi:10.1189/jlb.0609385

Sompuram SR, Vani K, Tracey B, Kamstock DA, Bogen SA (2015) Standardizing immunohistochemistry: A new reference control for detecting staining problems. J Histochem Cytochem 63:681-690 doi:10.1369/0022155415588109

Song JL, Chen C, Yuan JP, Sun SR (2016) Progress in the clinical detection of heterogeneity in breast cancer. Cancer Med 5:3475-3488 doi:10.1002/cam4.943

Soysal SD, Tzankov A, Muenst SE (2015) Role of the tumor microenvironment in breast cancer. Pathobiology 82:142-152 doi:10.1159/000430499

Spranger S, Spaapen RM, Zha Y, Williams J, Meng Y, Ha TT, Gajewski TF (2013) Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. Sci Transl Med 5:200ra116 doi:10.1126/scitranslmed.3006504

Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG (2014) Lymphangiogenesis and lymphatic vessel remodelling in cancer. Nat Rev Cancer 14:159-172 doi:10.1038/nrc3677

Tatara T, Mukohara T, Shimono Y et al. (2018) Expression of programmed death-1 in sentinel lymph nodes of breast cancer. J Surg Oncol 117:1131-1136 doi:10.1002/jso.24937

Torlakovic EE, Nielsen S, Vyberg M, Taylor CR (2015) Getting controls under control: the time is now for immunohistochemistry. J Clin Pathol 68:879-882 doi:10.1136/jclinpath-2014-202705

Treilleux I, Blay JY, Bendriss-Vermare N et al. (2004) Dendritic cell infiltration and prognosis of early stage breast cancer. Clin Cancer Res 10:7466-7474 doi:10.1158/1078-0432.CCR-04-0684

Van Bockstal M, Floris G, Galant C, Lambein K, Libbrecht L (2018) A plea for appraisal and appreciation of immunohistochemistry in the assessment of prognostic and predictive markers in invasive breast cancer. Breast 37:52-55 doi:10.1016/j.breast.2017.10.012

Waisman A, Lukas D, Clausen BE, Yogev N (2017) Dendritic cells as gatekeepers of tolerance. Semin Immunopathol 39:153-163 doi:10.1007/s00281-016-0583-z

Walker RA (2006) Quantification of immunohistochemistry--issues concerning methods, utility and semiquantitative assessment. I Histopathology 49:406-410 doi:10.1111/j.1365-2559.2006.02514.x

Walker RA (2008) Immunohistochemical markers as predictive tools for breast cancer. J Clin Pathol 61:689-696 doi:10.1136/jcp.2006.041830

WHO (2018) Cancer - Key Facts. http://www.who.int/en/news-room/fact-sheets/detail/cancer. Accessed 7 November 2018 2018

Yang J, Li X, Liu X, Liu Y (2015) The role of tumor-associated macrophages in breast carcinoma invasion and metastasis. Int J Clin Exp Pathol 8:6656-6664

Zhang C, Gao L, Cai Y et al. (2016) Inhibition of tumor growth and metastasis by photoimmunotherapy targeting tumor-associated macrophage in a sorafenib-resistant tumor model. Biomaterials 84:1-12 doi:10.1016/j.biomaterials.2016.01.027

Zhang S, Zhang D, Yi S et al. (2017) The relationship of lymphatic vessel density, lymphovascular invasion, and lymph node metastasis in breast cancer: a systematic review and meta-analysis. Oncotarget 8:2863-2873 doi:10.18632/oncotarget.13752

Zhao YC, Ni XJ, Wang MH, Zha XM, Zhao Y, Wang S (2012) Tumor-derived VEGF-C, but not VEGF-D, promotes sentinel lymph node lymphangiogenesis prior to metastasis in breast cancer patients. Med Oncol 29:2594-2600 doi:10.1007/s12032-012-0205-0

Zitvogel L, Apetoh L, Ghiringhelli F, Andre F, Tesniere A, Kroemer G (2008) The anticancer immune response: indispensable for therapeutic success? J Clin Invest 118:1991-2001 doi:10.1172/JCI35180

Table 1. Studies showing the role of lymphocytes and PD-L1 on the immune response to the primary tumour and the lymphatic node.

Authors	Adjuvancy/Neoadjuvancy	Diagnosis	Biopsies studied	Number of cases	Subtype	IHC	Main results
Matkowski et al. 2009	Adjuvancy	Invasive ductal breast carcinoma	Primary tumour	88 cases	Heterogeneous	CD4, CD8	Correlation between $CD8^+$ and $CD4^+$ lymphocytes with unfavourable prognosis, including ALN invasion
La Rocca et al. 2008	Non specified	Invasive ductal breast carcinoma	Primary tumour and regional lymph nodes	35 cases	Heterogeneous	CD1a, CD4, CD8, CD20	CD1a+ cells in the primary region correlate with those in the regional LN. Moreover, $CD4^+$ and $CD8^+$ cells are more highly expressed in lymphatic nodes without detectable metastasis
Miyan et al. 2016	Adjuvancy	Primary unilateral invasive breast cancer without clinical signs of regional or distant metastasis.	Whole tissue section of primary tumour and SLN	177 cases	Luminal A, B, B Her2, Her2, Basal-like	CD3, ζ-chain, FoxP3, CD8	High densities of $CD8^+$, $FoxP3^+$, $\zeta\text{-chain}^+$, $CD3^+$ lymphocytes are associated with more aggressive subtypes
Nakamura et al. 2009	Adjuvancy	Ductal breast carcinoma in situ and invasive ductal breast carcinoma	Positive and negative SLN	30 cases (training set) - 129 samples (validation set)	Heterogeneous	Foxp3, CD4, CD8	$Foxp3^+$ Treg lymphocytes correlate with pathological undetectable micrometastasis in the SLN
Mansfield et al. 2009	Non specified	Ductal, lobular and other carcinoma	Positive and negative SLN	47 cases	Heterogeneous	Foxp3, IDO, CD3, CD4	Positive SLN have a higher proportion of Foxp3 than negative SLN
Gökmen-Polar et al. 2013	Adjuvancy	Infiltrating ductal carcinoma and infiltrating lobular carcinoma	Positive and negative SLN	70 cases	Heterogeneous	Foxp3	No differences in Treg between positive and negative SLN
Gupta et al. 2011	Non specified	Invasive breast carcinoma: ductal, lobular and mixed	Positive and negative SLN	121 cases	Heterogeneous	Foxp3	In invasive ductal carcinoma, the number of Foxp3 Treg in the SLN correlates with the size of the primary tumour, but not with SLN metastases
Baptista et al. 2016	Adjuvancy	Non defined	Primary tumour	192 cases	Heterogeneous	PD-L1, PD-L2	PD-L1 is associated with higher overall survival and positive LN, but not with disease free survival.
Hou et al.2018	Adjuvancy	Primary HER2-positive breast carcinoma	Primary tumour	216 cases	HER2	Multiplex: PD-L1, PD1, CD8, CD163, CD3	PD-L1 and $CD8^+$ cells are good predictors of HER2 breast cancer prognosis. PD-L1 correlates with overall survival and the absence of LN metastasis
Tatara et al. 2017	Non specified	Non defined	Positive and negative SLN	65 cases	Luminal A-like, luminal B-like, HER2 and TNBC	PD-1	Positive SLN express more PD-1+ lymphocytes than downstream negative SLN
Li et al. 2018	Adjuvancy	Invasive ductal carcinoma with at least one tumour-positive axillary lymph node	Primary tumour and axillary lymph node-positive	101 cases	TNBC	PD-L1	PD-L1 expression is higher in the positive LN than in the corresponding primary tumour. PD-L1 in the LN is linked to a lower prognosis
Muenst et al. 2014	Non specified	Invasive ductal, invasive lobular, mucinous, and other breast carcinoma	Primary tumour	650 cases	Luminal A, B, B Her positive, HER2 and Basal-like	PD-L1	PD-L1 is associated with poor prognosis
Qin et al. 2015	Neoadjuvancy and Adjuvancy	Invasive breast carcinoma	Primary tumour	870 cases	Heterogeneous	PD-L1	PD-L1 is associated with poor prognosis, larger tumour size, grade, positive LN status, and ER and PR status

IHC Immunohistochemistry, *ALN* Axillary lymph node, *SLN* Sentinel lymph node, *LN* Lymph node, *HER-2* human epidermal growth factor receptor 2, *IDO* Indoleamine 2,3-dioxygenase, *PD-L1* programmed death ligand 1, *PD-L2* programmed death ligand 2, *PD1* Programmed cell death protein 1, *TNBC* Triple-negative breast cancer, *ER* Oestrogen receptor, *PR* Progesterone receptor

Table 2. Studies showing the role of macrophages on the immune response to the primary tumour and the lymphatic node.

Authors	Adjuvancy/Neoadjuvancy	Diagnosis	Biopsies studied	Number of cases	Subtype	IHC	Main results
Jubb et al. 2010	Adjuvancy	Breast adenocarcinoma	Primary tumour	296 cases	Heterogeneous	CD68, CD14, neutrophil elastase, DC-SIGN, CD31, CA9, CD123 and DII4	DII4 is a significant prognostic factor. CD68 and CD31 expression in the primary tumour are associated with LN invasion
Yang et al. 2015	Non specified	Invasive breast carcinoma	Normal and malignant breast tissue and LN	100 cases	Heterogeneous	CD68	$CD68^+$ macrophage infiltration correlates with worse survival and stimulates LN invasion
Shiota et al. 2016	Adjuvancy	In situ or invasive ductal carcinoma	Primary tumour and negative regional LN	167 cases	Heterogeneous	CD68, CD8 and CD169	M1 macrophages ($CD169^+$) and the proportion of M1/$CD68^+$ macrophages correlates with a better prognosis and no LN metastasis
Mansfield et al. 2012	Non specified	Ductal, lobular or other breast carcinoma	Positive and negative SLN	47 cases	Heterogeneous	CD163, ANK-1, IDO, CD3, CD8, Foxp3 and CD123	Increased $CD163^+$ macrophages in the SLN are linked to a higher risk of metastatic invasion of the SLN. In positive SLN, CD163 correlates with decreased infiltration of $CD8^+$ cells
Raschioni et al. 2018	Non specified	Invasive ductal luminal B breast carcinoma	Primary tumour and SLN	40 cases in the discovery cohort and 150 cases in the validation cohort	Luminal B	Vimentin, CD163, CXCR4, CXCL12	M2 macrophages ($CD163^+$) and the regulation of CXCR4 and CXCL12 in the primary tumour and the SLN are partially responsible for the aggressiveness of luminal B BC cells
Buldakov et al. 2017	Adjuvancy	Nonspecific invasive breast carcinoma T1-4N0-3M0	Primary tumour: soft fibrous stroma, coarse fibrous stroma, maximum stromal-and-parenchymal relationship, parenchymal elements and gaps of ductal tumour structures	37 cases	Heterogeneous	CD68, stabilin-1	High concentrations of $CD68^+$ in the gaps of ductal tumour structures is protects against metastases in the regional LN

IHC Immunohistochemistry, *DC-sign* Dendritic cell-specific Intercellular adhesion molecule-3-Grabbing Non-integrin (CD209), *DII4* Delta-like ligand 4, *LN* Lymph node, *SLN* Sentinel lymph node, *CXCR4* C-X-C chemokine receptor type 4, *CXCL12* C-X-C motif ligand 12, *ANK-1* Ankyrin-1, *IDO* Indoleamine 2,3-dioxygenase,

Table 3. Studies showing the role of dendritic cells on the immune response to the primary tumour and the lymphatic node.

Authors	Adjuvancy/Neoadjuvancy	Diagnosis	Biopsies studied	Number of cases	Subtype	IHC	Main results
Mansfield et al. 2011	Non specified	Ductal, lobular and other breast carcinoma	Positive and negative SLN	47 cases	Heterogeneous	CD1a, CD8, CD123 and CD208	In positive SLN, DC maturation is arrested
Treilleux et al. 2004	Adjuvancy	Invasive non-metastatic breast carcinoma	Primary tumour	1996 series: 152 cases. 1997 series: 103 cases	Heterogeneous	CD3, CD68, CD1a, CD208/DC-LAMP, CD207, hCCL19 and hCCL21	pDCs in breast cancer correlate with poor prognosis
Gadalla et al. 2019	Adjuvancy	Breast carcinoma	Primary tumour, ALN and blood samples	35 cases	Heterogeneous	CD303 and SDF-1	pDC stimulate cell migration and are found in higher proportions in patients with LN^+
Poindexter et al. 2004	Neoadjuvancy and adjuvancy	Breast carcinoma	Positive and negative SLN	50 cases (6 received chemotherapy before the biopsy)	-	CD3, HLA class II, CD83, CD1a, IL-10, IL-12	Negative SLN present a higher number of mature DC ($CD83^+$) compared to positive SLN
Ammar et al. 2011	Non specified	Primary invasive breast carcinoma	Primary tumour, tonsil and LN	148 cases	-	Stabilin-1, CD209, CD83 and CD68	Immature DC in the primary tumour are related to a higher risk of recurrence
Bembenek et al. 2008	Adjuvancy	Breast carcinoma	SLN and non-SLN (ALN)	79 cases	-	CD3, DC-LAMP, CD1a	Mature DC (DC-Lamp) in the SLN are related to decreased or negative metastasis in the SLN
La Rocca et al. 2007	Non specified	Invasive ductal breast carcinoma	Primary tumour and regional lymph nodes	35 cases	Heterogeneous	CD1a, CD4, CD8, CD20	$CD1a^+$ cells in the primary region correlate with those in the regional LN. Moreover, $CD4^+$ and $CD8^+$ cells are more highly expressed in lymphatic nodes without detectable metastasis
Chang et al. 2013	Adjuvancy	Breast carcinoma	Positive and negative SLN	59 cases	Luminal A, B, HER2, Basal, Unknown	Pan-cytokeratin AE1/AE3, CD1a, CD83, CD3,	In negative SLN, DC aggregate into large clusters of mature DCs, while in positive SLN, DCs are unclustered and have fewer mature DCs. Moreover, high clustering is related to DFS.

IHC Immunohistochemistry, *SLN* Sentinel lymph node, *DC-LAPM* Dendritic cell lysosomal associated membrane glycoprotein, *hCCL19* Human chemokine (C-C motif) ligand 19, *hCCL21* Human chemokine (C-C motif) ligand 21, *DC* Dendritic cells, *pDC* Plasmacytoid dendritic cells, *SDF-1* Stromal cell-derived factor 1, *ALN* Axillary lymph node, *LN* Lymph node, *DFS* Disease-free survival

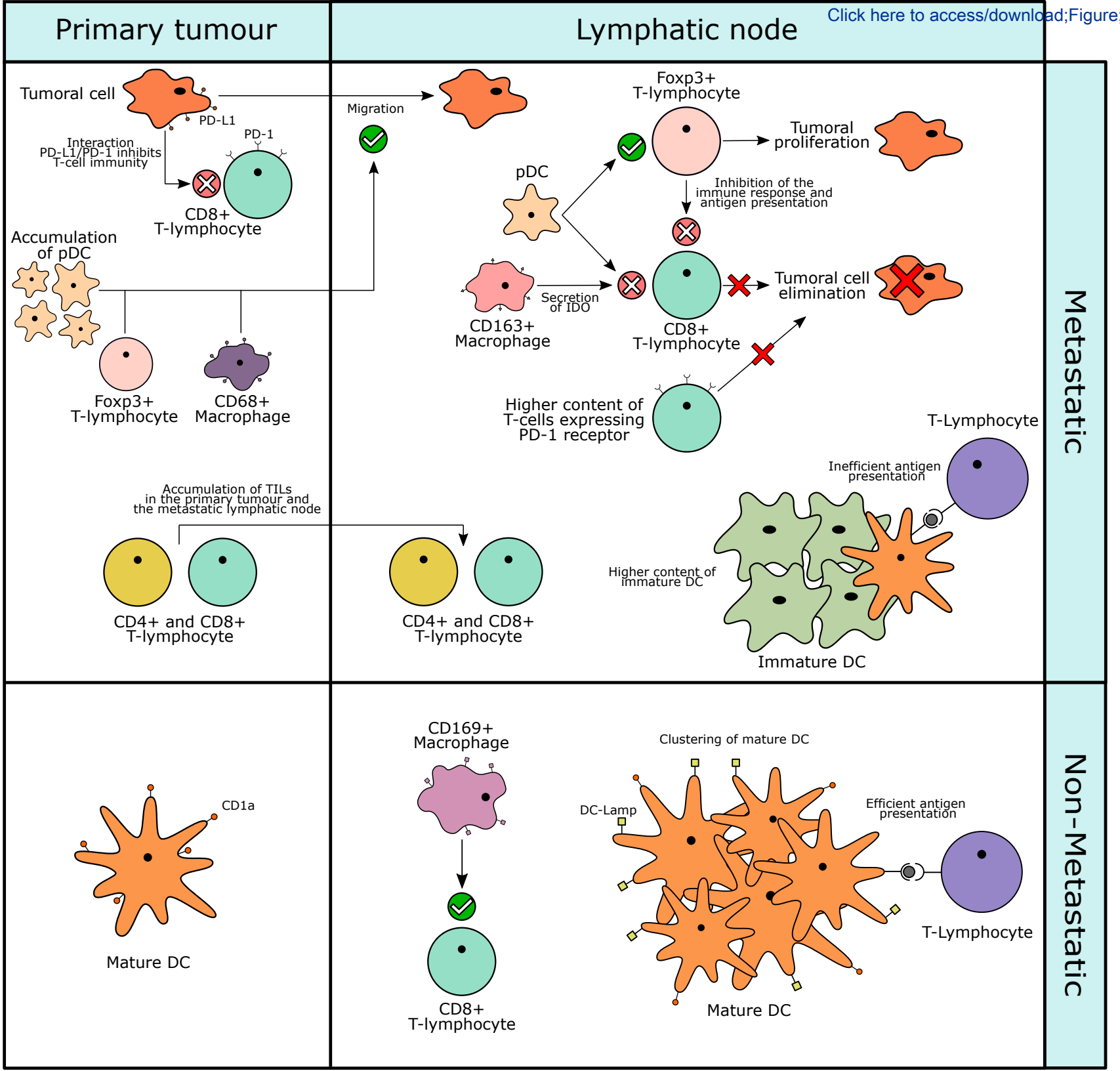
Table 4. Studies showing the role of immune cells and the immune response in the primary tumour and the lymphatic nodes on distant metastasis.

Authors	Adjuvancy/Neoadjuvancy	Diagnosis	Biopsies studied	Number of cases	Subtype	IHC	Main results
Cimino-Mathews et al. 2013	Neoadjuvant and adjuvant	Invasive ductal carcinoma and invasive lobular carcinoma	Primary tumour and metastasis collected after death (autopsy)	15 cases	Heterogeneous	CD3, CD4, CD8, CD20. FoxP3	Metastatic tumours have a lower concentration of TILs than the primary tumour, suggesting an increase in the tumoral immunosuppression during metastatic spread
Ogiya et al. 2016	Neoadjuvant (after collection of sample) and adjuvant	Early breast carcinoma and subsequent distant recurrence	Primary tumour and distant metastasis	25 cases	TNBC and $HER2^+$	CD4, CD8, PD-L1, PD-L1, HLA class 1	Metastasis show a lower percentage of TILs, $CD8^+$ and $CD4^+$ T cells compared to primary tumours
Sobottka et al. 2016	Neoadjuvant and adjuvant	Invasive-ductal or invasive-lobular breast carcinoma	Primary tumour and distant metastasis	87 cases	Heterogeneous	CD4, CD8, CD20,	TILs are found at a lower quantity in the metastatic site compared to the primary tumour, but follow a similar pattern of immune markers than the primary site
Coffelt et al. 2015	Not treated	Invasive lobular breast carcinoma. Orthotopic transplantation of tumour pieces into female recipient mice.	Primary tumour, lymph node and distant metastasis	Variable	-	Ly6B, Ly6G, CD34	γδ T cells activate neutrophil accumulation and differentiation into a phenotype that suppresses $CD8^+$ lymphocytes, promoting metastasis
Blenman et al. 2018	Non specified, but always after collection of sample	Non defined	SLN, tumour-invaded nodes compared to tumour-free lymph nodes, whole tissue section	76 cases/21 cases (validation cohort)	Heterogeneous/TNBC (validation cohort)	Multiplexed IHC: CD3, CD20, CD1a	High number of T cells and B cells predict longer DFS/ Lower number of B cells in the SLN were associated with poor prognosis
Chen et al. 2014	Non-treated	LN negative breast cancer	Primary tumour	396 cases	ER-, ER+, $HER2^-$, HER2+, TNBC	CD8	$CD8^+$ lymphocytes in the intratumoral region show protective effects against cancer
Heiskala et al. 2018	Non specified	Ductal and lobular breast cancers	Primary tumours and metastasis	137 cases	Heterogeneous	CD68, CD14, CD163, CD206, CD80 and CCL2.	$CD14^+$ macrophages are associated with rapid tumour metastasis
Shabo et al. 2008	Adjuvant	Stage II breast cancer	Primary tumour	133 cases	Heterogeneous	CD163	$CD163^+$ macrophages in the primary tumour are linked to a higher occurrence of distant metastases

IHC Immunohistochemistry, *TILs* Tumour infiltrating lymphocytes, *TNBC* Triple-negative breast cancer, *HER-2* Human epidermal growth factor receptor 2, *PD-L1* Programmed death ligand 1, *Ly6B* Lymphocyte antigen 6b precursor, *Ly6G* Lymphocyte antigen 6 complex locus G6D *SLN* Sentinel lymph node, *DFS* Disease-free survival, *ER* Oestrogen receptor, *PR* progesterone receptor, *CCL2* C-C Motif chemokine ligand 2

Fig. 1 Involvement of the immune microenvironment in the lymphatic node metastasis. A higher accumulation of tumour infiltrating lymphocytes (TIL), mainly $CD4^+$ and $CD8^+$, and plasmacytoid DC (pDC) is linked to a metastatic lymphatic node. At the same time, pDC, $Foxp3^+$ T lymphocytes and $CD68^+$ macrophages present in the primary tumour seem to be involved in the migration of tumoral cells from the primary tumour to the regional lymphatic nodes (LN). The tumour expresses Programmed Death-Ligand 1 (PD-L1), which interacts with Programmed Death 1 (PD-1) receptors in T lymphocytes, inhibiting the immune response. In the metastatic LN, pDC are found at higher concentrations and are correlated with an increased number of $Foxp3^+$ lymphocytes, and a lower activation of $CD8^+$ T lymphocytes. The lower activation of $CD8^+$ T lymphocytes is also affected by the secretion of indoleamine-pyrrole 2,3-diogenase (IDO) from $CD163^+$ M2 macrophages, which are stimulated by the primary tumour. Metastatic LN also have a higher concentration of immature DC, which suggests an inefficient antigen presentation and so, a deficient immune response. The $CD1a^+$ DC and those expressing the mature markers DC-Lamp, as well as the $CD169^+$ M1 macrophages are found in greater numbers in the primary tumour and LN of patients with non-metastatic LN, than in metastatic patients, which suggest a more efficient immune response against the tumour.

Figure



Hospital Verge de la Cinta

Carlos López

Molecular Biology and Research Section, Hospital de Tortosa Verge de la Cinta, IISPV,

URV, Tortosa, Spain

Tel: +34 977 519104 Email: clpclp3@gmail.com

Tortosa, 28 June 2019

Dear Reviewer,

Thank you for your clear revisions and valuable comments, which have enabled us to improve our manuscript.

We have modified the 3 small corrections.

1. Page 3, line 14: "other LNs"
2. Page 3, line 33: considered "as" a highly
3. Page 6, line 6: biomarker "for"

Yours faithfully,

Carlos López.