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REVIEW ARTICLE

Indicators of a pro-tumor immune response are evident at early stages of breast cancer

C. Zhang[1] · R. A. Kurt[2]

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Abstract

With advances in checkpoint inhibitor and CAR T-cell therapies, among other advances in immunotherapy, this is an exciting time to be a tumor immunologist. We are witnessing the transition of decades of work at the bench leading to substantial success in the clinic. While work continues developing new and improving existing immunotherapies, there remains a great deal of basic tumor immunology still to learn, information that can only lead to greater success in the clinic. One area in need of more attention is understanding the immune response at early stages of breast cancer. While there is no question that early diagnosis and treatment save lives, a greater understanding about the immune response during early stages of breast cancer may reveal information that could assist in monitoring individuals at risk of breast cancer, and could have implications for patients diagnosed at early stages of disease, and may provide important information about the origins of an immune-suppressive environment. Here, we review studies that have looked at the very early immune response to breast cancer focusing on patients with DCIS, before invasion in spontaneous transgenic murine mammary carcinoma models, and before transplantable or orthotopic murine mammary carcinoma models become palpable. The findings revealed that indicators of a pro-tumor immune response are already present at early stages of disease.

Keywords Ductal carcinoma in situ · Tumor associated macrophage · Early immune response · Pro-tumor environment

Introduction

Addressing the tumor-promoting and immune-suppressive environments is a challenge that must be overcome for treating patients with breast cancer. The importance of having a thorough understanding of tumor-associated macrophages (TAM), for example, is underscored by clinical data that reveal TAM correlate with poor prognosis and negatively impact efficacy of checkpoint-inhibitor therapy in patients with cancer [1–8]. However, although it has been over 40 years since altered metabolism and effector function of TAM were reported [9], exactly when and how pro-tumor TAM arise has yet to be well defined. The current paradigm is that tumor progression over time creates an environment that leads to the generation of pro-tumor TAM which then further potentiates tumor progression. This model, however, is largely based on analysis of cells isolated from advanced tumors, or the periphery of humans or mice at advanced stages of disease. While it is challenging to capture a sufficient number of white blood cells from early sites of breast cancer for characterization, several studies have indicated that there is not a clear anti-tumor phenotype even at early time points and that an immune-suppressive environment may be present earlier than generally appreciated. For example, Carron et al. [10] used a p53−/− mammary carcinoma model and found that there was an increase in pro- and anti-tumor macrophages before the tumor was even palpable, and that the macrophages present at this early stage of disease contributed to tumor progression. Zhang et al. [11] found macrophages with some M1 and M2 characteristics at early sites of three different murine mammary carcinomas. In patients, macrophages are also present at early sites of breast cancer, yet we still do not know what these cells are doing at this site even though McKee et al. [12] reported almost 20 years ago that the presence of macrophages was among the characteristics for high-grade ductal carcinoma in situ (DCIS). It is likely, however, that the TAM present

✉ R. A. Kurt
kurtr@lafayette.edu

[1] Department of Molecular and Cellular Biology, University of California Berkeley, Berkeley, CA 94720, USA

[2] Department Biology, Rockwell Integrated Science Center, Lafayette College, Easton, PA 18042, USA

at early stages of disease are not affected by a hypoxic environment or chronic inflammation; factors often ascribed to the generation of pro-tumor TAM. It is for some of these reasons that we became interested in studying the very early immune response to breast cancer, and we became acutely aware of the paucity of information available. This is unfortunate because information about the immune response at early stages of the disease may assist in monitoring individuals at risk of breast cancer, and could have implications for patients diagnosed at early stages of disease, and may provide information about the origins of an immune-suppressive environment. In this review, we highlight studies that have looked at the early immune response to breast cancer focusing on patients with DCIS, before invasion (hyperplasia, adenoma, or early carcinoma) in transgenic murine models and before transplantable or orthotopic murine mammary carcinoma models become palpable, often within 7 days of tumor delivery.

Immune infiltration is common before patients progress to invasive breast cancer

In patients, DCIS can be used to investigate the early immune response to breast cancer. In an early immunophenotyping study, Lee et al. [13] investigated 41 DCIS samples and found two different patterns of immune infiltrates. They found clusters of B ($CD20^+$) and T ($CD3^+CD8^+$) cells around involved ducts which were associated with increased vascularity, poor differentiation, and HER2 expression, and they found diffuse infiltrates of T cells and macrophages ($CD68^+$) in the stroma although these were less predominant than the B and T cell clusters. Interestingly, the investigators also looked at specimens from patients with invasive breast cancer and found the main pattern of inflammation was the diffuse T-cell and macrophage infiltrates, suggesting that these cells persist as the tumors progress. Yet, no information about cell subtype or effector function was assessed in that study. McKee et al. [12] found macrophages were more common in high-grade than non-high-grade DCIS, and Sharma et al. [14] looked a little deeper at macrophages in DCIS. They found high-grade DCIS was associated with a colony stimulating factor-1 (CSF-1, M-CSF, a well-known chemotactic and survival factor for macrophages [15]) gene signature, and of the 285 DCIS samples examined by immunohistochemistry (IHC), 19% contained the macrophage markers CD64, CSFR and CD163 [14]. If CD163 functions to identify immune-suppressive or M2 TAM, then these data indicate that M2 TAM are already present in a subset of those with DCIS. Notably, a similar incidence was found in specimens from patients with an invasive disease, suggesting that $CD163^+$ macrophages persist as the tumors progress. Campbell et al. [16] went further with subtype analysis using IHC and multispectral imaging to investigate white blood cells in high-grade and non-high-grade DCIS. They found high-grade DCIS had more $FoxP3^+$, $CD68^+$, $HLADR^+$, $CD4^+$, and $CD20^+$ cells than non-high-grade DCIS. Although none of these cells were associated with patient outcome, TIL ($CD4^+$, $CD8^+$, $CD20^+$) were associated with high-risk features, such as hormone receptor negativity and HER2 expression, $CD68^+$ cells correlated with high-grade and hormone-receptor negativity, $CD115^+$ macrophages correlated with high Ki67 status and HER2 expression, and while $CD68^+$ cells bearing an M2 TAM marker ($CD206^+$) were present, they did not correlate with any clinical parameters. Finally, the presence of Tregs and M2 TAM are not the only indicators of a pro-tumor phenotype at early sites of breast cancer. Thompson et al. [17] reported that 81% of DCIS specimens contained $PD\text{-}L1^+$ TIL, and triple negative specimens contained TIL with higher levels of PD-L1.

Finally, we would be remiss if we did not point out that there is evidence of an immune infiltrate earlier than DCIS. Degnim et al. [18] analyzed samples from normal breast tissue and biopsies from patients with benign breast disease using IHC and found a higher density of T cells ($CD4^+$, $CD8^+$), B cells ($CD20^+$), macrophages ($CD68^+$) and dendritic cells ($CD11c^+$) in benign lesions than in normal breast tissue. Although the strongest association was with the macrophages and dendritic cells, the only cells associated with risk of disease progression were with B cells, and a fewer of these cells here were associated with increased risk of disease progression [18]. Along this same line, Hussein and Hassan [19] found more white blood cells in benign lesions than normal tissue. They examined 53 mastectomy samples from patients with breast cancer and found the number of T cells ($CD3^+$), B cells ($CD20^+$) and macrophages ($CD68^+$) increased from normal tissue to benign lesions, benign lesions to DCIS, and DCIS to invasive disease [19].

Transgenic mouse models

The role of macrophages in tissue development and support of mammary stem cells may help explain the presence of macrophages at premalignant or early sites of breast cancer. For instance, Gyorki et al. [20] reported that macrophages were required for mammary stem cell function and their progeny. They found that osteopetrotic (op/op) mice, which are deficient in CSF-1 and, therefore, deficient in macrophages or treatment with clodronate liposomes resulted in a decrease in mammary stem cells. These data support the contention that stem cell function in the mammary gland is impaired by the absence of macrophages in the mammary gland, and these data may also help explain how macrophages help with mammopoiesis and development of

the mammary gland by accumulating around the terminal end buds and assisting with ductal growth [21, 22].

To bring some consistency to this review, we focus on data from PyMT mice analyzed prior to week 10 which is often before many mice display invasive disease [23]. With respect to immune infiltration in this model, H&E staining has demonstrated mainly macrophages and neutrophils at the adenoma stage, with more macrophages than neutrophils at the early carcinoma stage [23]. By crossing op/op mice with PyMT mice, Lin et al. [24] found that the presence or absence of macrophages ($F480^+$) at early sites (before week 10) did not affect tumor incidence or growth. However, macrophages correlated with progression from early to late carcinoma, invasion, and metastasis. Wang et al. [25] used a slightly different PyMT system and reached a similar conclusion. They decreased CSF by targeting the steroid receptor coactivator-1 (SRC-1) which also results in a decrease in macrophages (defined as $CD11b^+$ rather than $F480^+$ in this study). At 4–8 weeks of age, there was a decrease in macrophages at the tumor site, with no significant change in tumor initiation or growth, as with the PyMT op/op mice intravasation and metastasis were affected by the absence of macrophages [25]. It is worth pointing out that while the results were not statistically significant, there was a slight trend toward earlier tumorigenesis in that study [25]. Strachan et al. [26] found a CSFR-targeting reagent (BLZ945) also decreased macrophages at 9–11 week tumor sites, and this also resulted in no delay in tumor growth in the PyMT mice. Finally, DeNardo et al. [27] looked at B cells ($B220^+$), T cells ($CD4^+$, $CD8^+$) and macrophages ($CD68^+$) in PyMT mice at the early tumor sites by IHC and found each of these cell types was present at the hyperplasia stage, and were elevated at the early carcinoma stage with more B cells than T cells, but removal of these cells did not influence tumor latency or growth [27]. Thus, in the PyMT model, macrophages appear to play a role in invasion and metastasis, but B cells, T cells and macrophages do not appear to be critical for tumor incidence or growth.

Kirma et al. [28] used a MMTV CSF transgenic model and found the presence of macrophages ($CSF\text{-}1R^+$ cells) were higher in and around preneoplastic lesions and mammary tumors. Although there was no information about what the macrophages were doing at these sites, the long latency in this model (12 months) suggests that macrophages alone may not be enough for tumor progression. Some of the data supporting the contention that macrophages at early sites exhibit a pro-tumor phenotype comes from an inducible transgenic model (MMTV-iFGR1) which has been used to study preneoplastic progression in the mammary gland [29]. This model makes use of inducible fibroblast growth factor receptor-1 expression in the mammary epithelium and upon signaling, there is development of lateral buds off the terminal end buds in the mammary gland within 3 days, hyperplasia at 4 weeks, and invasive lesions appearing at 4–6 weeks. Following early stages of breast cancer progression, Schwertfeger et al. [29] found macrophages ($F4/80^+$) were recruited before bud formation (8–24 h), and removal of the macrophages resulted in a decrease in lateral budding formation, as well as a decrease in proliferation (Ki67) of the epithelial cells supporting the contention that macrophages are important for mammary epithelial proliferation at early stages. The authors also looked at the presence of other white blood cells and found no B cells, T cells or neutrophils. Subsequently, Bohrer and Schwertfeger [30] showed in this same model that decreased TGF-β1 expression, decreased SMAD3 activity, and increased expression of CXCR2-binding chemokines may be related to the early pro-tumor phenotype of these macrophages. Finally, Campbell et al. [31] used a murine model dependent on expression of rat prolactin ligand in the mammary tissue (NRL-PRL mice) in which the mice generate spontaneous estrogen receptor-positive mammary tumors in nulliparous females. While they did not look at early tumor sites, they did find some T cells ($CD8^+$) and macrophages ($F4/80^+$) in age-matched pre-neoplastic sites, and looking at the whole tissue, they found pro- (*Ifng*) as well as anti-inflammatory (*Tgfb1, Arg1*, *Nos2*) factors at these early sites [31].

Transplantable/orthotopic mouse models

The **4T1** tumor is commonly used as a model for stage IV disease as it is aggressive and spontaneously metastasizes to the lungs, liver and brain, and some investigators have looked at the immune response at early stages of disease using this model. Balogh et al. [32] found DC in 8-day **4T1** tumor sites. Of the $CD45^+$ cells, 18% were $CD11c^+$, $CD8^+$, and $CD103^+$, and of these DC, 19% were MHC Class II^+, 11% were $CD40^+$, and 6% were $CD86^+$. Luo et al. [33], using a **4T1** matrigel model, found macrophages ($CD68^+$) by IHC at day 6 tumor sites, but the cells were not quantified and no further analysis of the cells was conducted. Our lab [11] used a gelfoam model to study early **4T1** tumor sites (days 1 and 3 post-tumor delivery) and found approximately 40% of the infiltrating cells were macrophages ($F4/80^+$) and 20% were neutrophils ($Ly6G^+$). Further analysis of these cells revealed production of TNF-α and TGF-β, the cells were phagocytic, and by day 3, the macrophages exhibited a higher dependence on oxidative phosphorylation and the neutrophils showed a decrease in production of reactive oxygen species (ROS) relative to day 1 cells. Using a particular novel model, Steenbrugge et al. [34] looked at early tumor sites (1 week) using **4T1** transfected with luciferase and delivered into the duct of the mammary fat pad in an attempt to model DCIS. While they did not find many

white blood cells (less than 5% of the tumor area), they did find $CD163^+$ macrophages and neutrophils ($Ly6G^+$), with many more macrophages than neutrophils present. No information about effector function was assessed, but co-cultures with **4T1** and the RAW264.7 cell lines revealed a decrease in IL-12 and an increase in TGF-β production after 96 h, suggesting that the **4T1** tumor can exert a rapid impact on this macrophage cell line [34]. Finally, Bunt et al. [35] reported a small increase (23% of tumor bearing mice versus 18% of naïve mice) in $CD11b^+Gr1^+$ cells (a phenotype sometimes used for myeloid derived suppressor cells) in the blood of 4T1 tumor-bearing mice 8–10 days after tumor delivery, suggesting that systemic effects may also appear relatively early in this model.

Matory et al. [36] looked at **EMT6** as well as **410** and **DA3** tumors by IHC and reported macrophages ($F4/80^+$) were present at days 1 and 5, and $CD4^+$ and $CD8^+$ T cells were present at day 5. The authors did not quantify the cells or assess effector function. Stewart and Beethman [37] reported macrophages (phagocytic$^+$ cells) made up about 27% of day 7 EMT6 tumor digests and that these cells were also cytotoxic for the EMT6 tumor. Akporiaye et al. [38] looked at the immune infiltrate in day 7 EMT6 tumors and found macrophages ($Mac1^+$ and by morphology) were the most numerous white blood cell, followed by neutrophils (by morphology), then lymphocytes ($Thy1.2^+$ and by morphology). The only effector function assessed was phagocytic activity of the macrophages which were phagocytic at day 7, and they did not lose this activity until day 21. Our lab [11] used the gelfoam model to study early EMT6 tumor sites (days 1 and 3) and found approximately 40% of the infiltrating cells were macrophages ($F4/80^+$) and 20% were neutrophils ($Ly6G^+$). Further analysis of the macrophages revealed a significant increase in TNF-α and TGF-β production between day 1 and day 3 post-tumor delivery, and by day 3, the macrophages exhibited a greater dependence on oxidative phosphorylation and the macrophages and neutrophils showed a decrease in ROS production relative to day 1 cells. Finally, although they did not conduct in vivo or ex vivo analysis, Stevenson et al. [39] found that after 4 h of co-culturing activated macrophages (peritoneal exudate cells) with EMT6 tumor cells, the tumor cells stopped proliferating. However, the results were transient, and after 24–48 h, the tumor cells started proliferating again [39].

With the **168** breast cancer model, approximately 20–40% of the cells at early tumor sites (days 1–3) are macrophages ($F4/80^+$) and neutrophils ($Ly6G^+$) [11]. In addition, these cells produce TNF-α and TGF-β and are phagocytic. Similar to the 4T1 and EMT6 tumors, by day 3, the macrophages exhibit higher dependence on oxidative phosphorylation, and the macrophages and neutrophils showed a decrease in production of reactive oxygen species relative to day 1 cells [11].

Using the **410.4** breast cancer model, Robinson et al. [40] found macrophages ($F4/80^+$), neutrophils ($Gr1^+$) and T cells ($CD8^+$) present at 1-week tumor sites though these cells were not quantified and no further analysis was conducted. However, the authors did find decreased tumor development if they targeted macrophages with the chemokine-receptor antagonist Met-CCL5, suggesting that macrophages at the early tumor sites have a role in tumor development in this model. An early immune infiltrate is also evident with the **TSA** breast cancer model. Musiani et al. [41] used the TSA model ± cytokine transfection and conducted early histological analysis (days 1 through 7). At the non-transfected control sites, neutrophils ($Gr1^+$ and by morphology) were first seen around days 10–13 which is when the tumors are first palpable in this model.

Carron et al. [10] used a p53−/− mammary epithelial cell transplantable tumor model. In this model, there are hyperplastic lesions at week 8, nodules at week 16, and palpable tumors at week 24. By week 8, they found a significant number of $F4/80^+/CD204^+$ and $F4/80^+/CD206^+$ macrophages, and co-culturing the tumor cells with the RAW264.7 macrophage line or bone marrow-derived macrophages for 2 h resulted in increased expression of pro-(*Tnfa*, *Il6*) as well as anti-(*Arg1*, *Tgfb*, *Vegfa*) inflammatory factors. Moreover, macrophage depletion delayed progression of pre-invasive lesions and eventual tumor formation, suggesting an important role for early macrophages in this model. In another novel model, Lu et al. [42] used immortalized human mammary epithelial cells transformed with HRasv12 in nude mice and found depletion of macrophages in the mammary fat pad decreased tumor initiation, and if human or mouse macrophages were co-injected with the tumor cells, then there was an earlier tumor onset and a higher tumor incidence, suggesting a role for macrophages in early stages of disease in this model. Additionally, co-culturing macrophages with tumor cells resulted in proliferation and elevated expression of IL-6 and IL-8 in the tumor cells, implying a contact dependent mechanism [42].

Concluding remarks

This review aims to bring attention to a crucial stage of disease which is difficult to study, but has potential implications of monitoring individuals at risk of breast cancer, those diagnosed at early stages of disease, and the origins of an immune-suppressive environment. Collectively, studies investigating early sites of breast cancer in patients provide ample evidence of an early immune infiltrate (Table 1, Fig. 1). B cells, T cells and macrophages are present in benign lesions with more of these cells appearing as the tumors progress to DCIS and then to invasive disease. While it is still not completely clear what these cells are doing at

Table 1 Data from patients with early-stage breast cancer

Stage	Type of analysis	Immune cells found	Pro-tumor indicators	Anti-tumor indicators	References
Benign breast disease	Compared normal breast tissue to benign lesions	CD4 and $CD8^+$ T cells, B cells, macrophages and DC	More of these cells in benign lesions than in normal tissue	Fewer B cells correlated with risk of disease progression	[18]
DCIS	53 Mastectomy samples	B cells, T cells, macrophages	Increasing number of these cells as the tumors progress; infiltrating ductal carcinoma > DCIS > benign lesions > normal tissue		[19]
DCIS	IHC of 66 DCIS samples	Macrophages	High grade DCIS had more macrophages than non-high grade DCIS		[12]
DCIS	IHC of 41 DCIS samples	B cells, $CD8^+$ T cells, and macrophages	B and T cell clusters associated with increased vascularity, poor differentiation and her2 expression		[13]
DCIS	IHC of 285 high grade DCIS	Macrophages	High grade DCIS associated with CSF-1 gene signature 19% of specimens had $CD64^+$, $CSF1R^+$, $CD163^+$ macrophages		[14]
DCIS	IHC of 27 DCIS samples	B cells, T cells	81% of DCIS samples contained $PD\text{-}L1^+$ TIL with higher levels of PD-L1 expression in TNBC		[17]
DCIS	Compared 52 cases of high grade and 65 cases of non-high grade DCIS by IHC and multispectral imaging	B cells, T cells, and macrophages; none correlated with patient outcome	High grade DCIS had more $FoxP3^+$, $CD68^+$, $HLADR^+$, $CD4^+$, and $CD20^+$ cells than non-high grade DCIS B cells, T cells and macrophages correlated with some high risk features ($CD206^+$ cells did not)		[16]

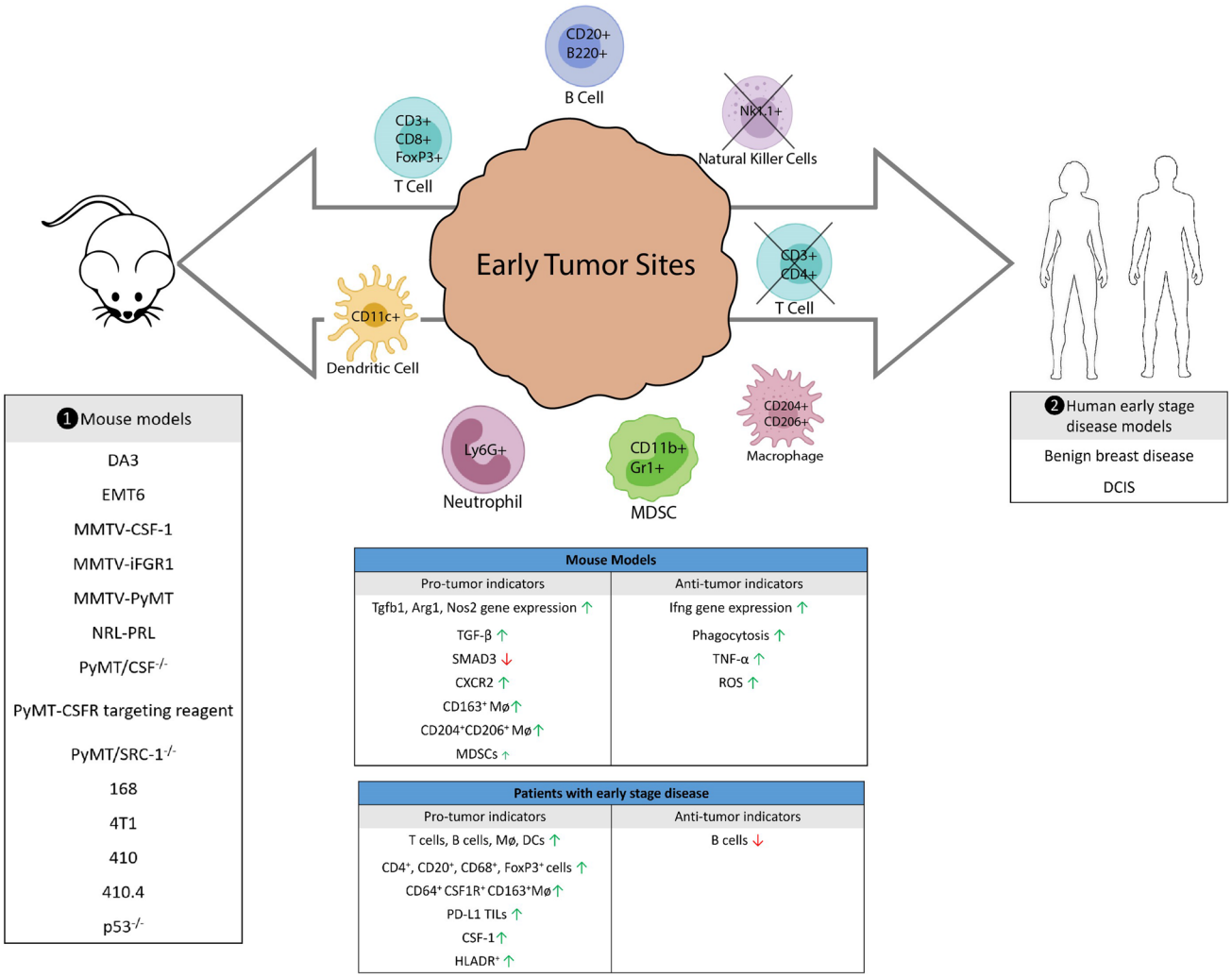

Fig. 1 Immune microenvironment associated with early sites of breast cancer

the early tumor sites, there are correlative data that support the contention that these cells contribute to early stages of tumor progression. The presence of cells expressing markers commonly associated with an immune-suppressive or pro-tumor phenotype, such as $FOXP3^+$ cells, PD-$L1^+$ TIL, $CD206^+$, and $CD163^+$ macrophages, also underscores the need for more studies to determine how these cells are recruited or generated, and what their functions are at the early tumor sites in patients with breast cancer.

Mouse models reveal evidence of macrophages and neutrophils with some studies also finding T and B cells (Table 2, Fig. 1). Except for the neutrophils, these data are consistent with what is found in patients with early-stage disease. Similar to studies in humans, there is no definitive conclusion that can be drawn about the role of these cells, if any, at the early tumor sites in mice. For instance, macrophages have been shown to have a role in cancer initiation and development in some mouse models (410.4, $P53^{-/-}$ epithelial model), but not others (PyMT). There remains a paucity of information about what factors influence the early presence or generation of white blood cells with pro- or anti-tumor phenotypes at the early tumor sites; although as described in this review, there are some particularly nice models that have potential to deliver answers. The spontaneous transgenic models are valuable for studying early sites even though they pose a challenge due to differences in the rates at which individual mice reach a particular stage of disease (i.e., early carcinoma is seen in a relatively wide time frame; 8–12 weeks of age in PyMT mice). The **4T1** DCIS model [32], the p53−/− epithelial cell model [10], and inducible transgenic model [27] provide alternative systems to get around some of the timing challenges with the spontaneous models, and although not an ideal physiologically system, the gelfoam model [11, 38] allows capture of a sufficient number of cells from early tumor sites for functional analysis.

Table 2 Data from early stages of disease in murine mammary carcinoma models

Model	Found	Pro-tumor indicators	Anti-tumor indicators
NRL-PRL	Macrophages and $CD8^+$ T cells at preneoplastic sites [31]	*Tgfb1*, *Arg1*, *Nos2* expression at pre-neoplastic site [31]	*Ifng* expression at pre-neoplastic site [31]
MMTV-PyMT	Macrophage and neutrophils [23, 26], B cells [27], $CD4^+$ and $CD8^+$ T cells [27], but did not influence tumor latency or growth [27]		
PyMT crossed with CSF-$1^{-/-}$ mice, PyMT crossed with SRC-$1^{-/-}$ mice, PyMT mice treated with a CSFR targeting reagent	Macrophages, but did not impact tumor incidence or growth [24–26]		
MMTV-CSF-1	Macrophages present around pre-neoplastic lesions and mammary tumors [28]		
MMTV-iFGR1	Macrophages, but no B cells, T cells or neutrophils present at pre-neoplastic sites [29]	Macrophages important at pre-neoplastic site for epithelial cell proliferation [29], pro-tumor phenotype of macrophages at early site correlated with decreased TGF-β expression, decreased SMAD3 activity, and increased expression of CXCR2 binding chemokines [30]	
4T1	Dendritic cells [32], macrophages [11, 33, 34], and neutrophils [11, 34]	Macrophages and neutrophils make TGF-β [11], $CD163^+$ macrophages present [34], slight increase in MDSC in blood of early tumor bearing mice [35]	Macrophages and neutrophils phagocytic, and make TNF-α and ROS [11][a]
410, DA3	Macrophages, $CD4^+$ and $CD8^+$ T cells [36]		
EMT6	Macrophages [11, 36–38], T cells [36, 38], and neutrophils [11, 38]	Macrophages and neutrophils make TGF-β [11]	Macrophages phagocytic [11, 37, 38], cytotoxic [56], and make TNF-α and ROS [11] Neutrophils phagocytic and make TNF-α and ROS [11]
168	Macrophages and neutrophils [11]	Macrophages and neutrophils make TGF-β [11]	Macrophages and neutrophils phagocytic, and make TNF-α and ROS [11]
410.4	Macrophages, neutrophils, and $CD8^+$ T cells [40]	Macrophages contribute to tumor development [40]	
TSA	Neutrophils [41]		
P53$^{-/-}$ epithelial model	Macrophages [10]	$CD204^+$, $CD206^+$ macrophages present, macrophages contribute to tumor formation [10]	
Immortalized human mammary cells in nude mice		Macrophages enhance tumor development [42]	

[a] TNF-α and ROS are included in the anti-tumor indicator category although they could also be included as pro-tumor indicators [43–46]

Overall, we believe a more thorough understanding about the immune events taking place at the very early stages of breast cancer can provide information that may eventually lead to greater success in the clinic. Data from patients and animal models reveal indications that a pro-tumor phenotype are present earlier than generally appreciated, yet there is not a clear understanding of what the macrophages, T cells or B cells are doing at these early tumor sites. Although it is too early to tell whether targeting the early immune infiltrate will prove to be beneficial for patients diagnosed with early-stage breast cancer, we anticipate that more attention to this area of tumor immunology holds promise for moving the field forward.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by the authors.

Informed consent For this retrospective review formal consent is not required.

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