# IMMUNOTHERAPY AS AN EVOLVING APPROACH FOR THE TREATMENT OF BREAST CANCER

EDITED BY: Mai F. Tolba, Adriana Albini, Basel K. Al-Ramadi, Emile Rugamika Chimusa, Sara M. Tolaney and Cesar Augusto Santa-Maria

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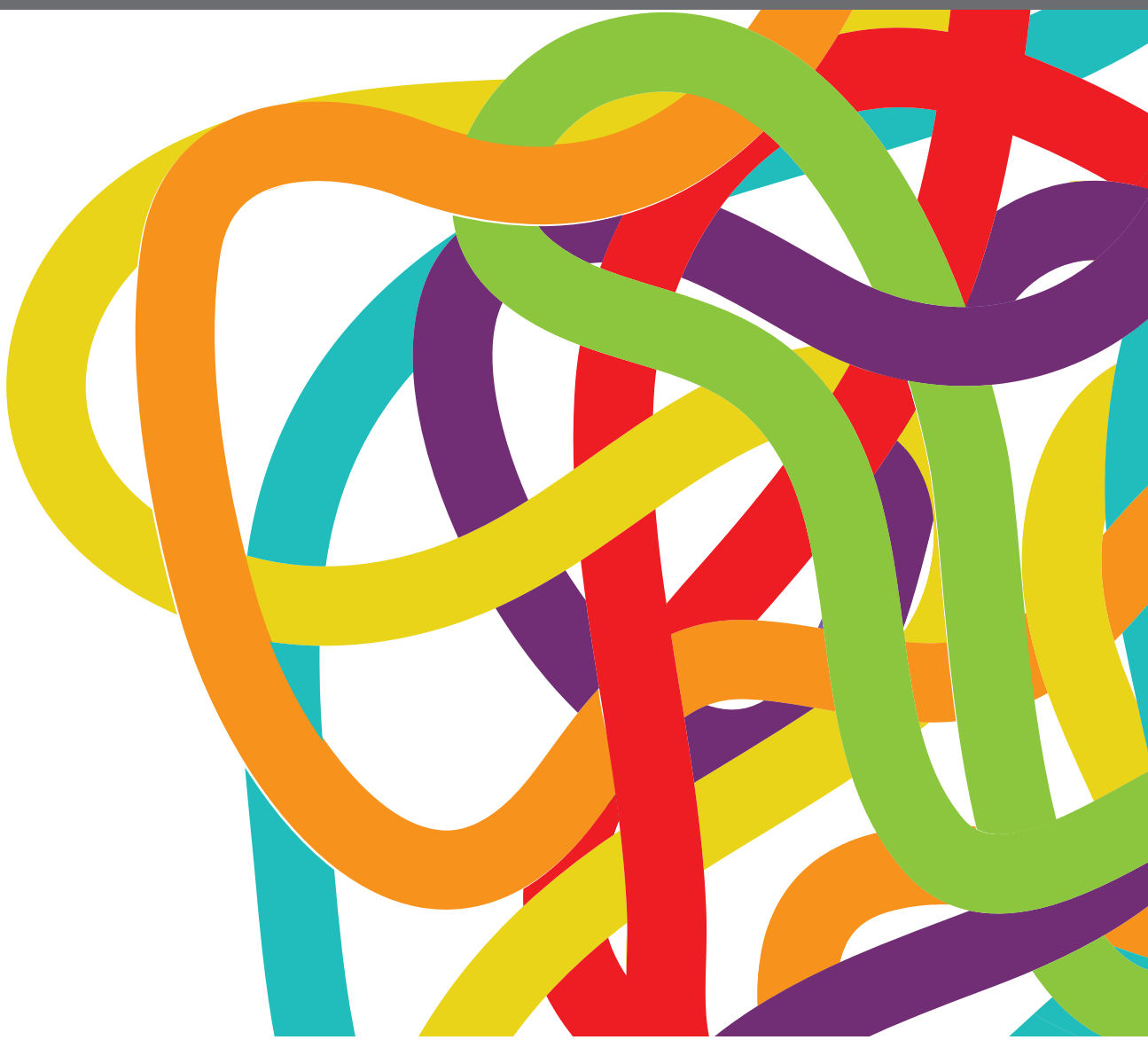

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# IMMUNOTHERAPY AS AN EVOLVING APPROACH FOR THE TREATMENT OF BREAST CANCER

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EDITORIAL
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# Editorial: Immunotherapy as an Evolving Approach for the Treatment of Breast Cancer

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## Editorial on the Research Topic

## Immunotherapy as an Evolving Approach for the Treatment of Breast Cancer

Novel therapies have improved outcomes of breast cancer (BC) patients, but still many progress to the metastatic disease, which remains very difficult to cure. Hormonal and targeted therapies including monoclonal antibodies against HER2 have become routine treatment in BC. In recent years oncology has made great advances by tackling the immune system as a new pillar for cancer therapy. Initial work exploring immunotherapy focused on triple-negative breast cancer (TNBC) since it was known to have higher rates of PD-L1 expression, higher prevalence of tumor-infiltrating lymphocytes (TILs), and higher mutational burden (1). Clinical trials for antibodies targeting PD-1/PD-L1 in metastatic TNBC have demonstrated promising therapeutic outcomes (1, 2). Despite a very modest response rate to checkpoint inhibition as monotherapy in TNBC, patients who achieved response were found to have prolonged overall survival (1). Therefore, the main challenge is to develop strategies to boost the tumor response to immunotherapy in order to increase the percentage of patients benefiting from therapy. The beginning of 2019 witnessed the first FDA accelerated approval of immunotherapy for the treatment of patients with metastatic PD-L1+ TNBC (3, 4). The first approved combination comprises atezolizumab (anti-PD-L1 monoclonal antibody) together with nab-paclitaxel chemotherapy. This combination represented the first step of introducing immunotherapy to the standard treatment protocol of breast cancer and revolutionized the landscape of treatment for metastatic TNBC. We have since seen approval for pembrolizumab in combination with chemotherapy for first line PD-L1+ metastatic TNBC based on the KEYNOTE-355 study (5), and even more recently we now have approval for pembrolizumab with chemotherapy for the treatment of early stage TNBC, based on results from the KEYNOTE-522 trial (6). While there is a benefit in adding checkpoint inhibitors to chemotherapy in TNBC, not all patients respond to immunotherapy. This has underscored the need for novel strategies to expand the benefits of checkpoint inhibitors for broader populations of patients including patients

with advanced hormone receptor-positive (HR+) BC as well as HER-2 positive tumors that are refractory to the standard therapy (2). Early data generated from immunotherapy studies with those other BC subtypes showed clues of improved therapeutic outcomes potentially within certain subsets of patients (2). There are therefore several registration studies for early-stage HR+ disease along with early and advanced HER2+ disease. Moreover, the development of biomarker predictors of benefit and resistance to immunotherapy remains one of the top research priorities for optimizing the application of cancer immunotherapy in the different patient cohorts.

The articles published under this Research Topic fall into two sections. Section I includes articles presenting basic research outcomes or literature reviews highlighting molecular targets and pathways to be tackled to enhance the tumor response to immune checkpoint inhibitors, while section II comprises articles providing rationale for newly established BC immunotherapy clinical trials and/or preliminary outcomes.

## SECTION I STUDIES

The articles under this section provide an overview of novel approaches to be adopted in order to enhance the tumor response to immunotherapy. In contrast to normal cells, cancer cells display rapidly adaptive responses to the conditions of oxygen and nutrient insufficiency in a cell survival tactic known as "Metabolic Reprogramming" (7, 8). These changes of tumor cellular bioenergetics include the switch to aerobic glycolysis, a phenomenon known as Warburg effect, are essential for tumor development, invasion, metastasis and resistance to therapies (8). TNBC is known to be a highly glycolytic tumor, providing fuel for growth-promoting biosynthetic pathways and exhibits elevated glucose uptake and a glycolytic gene-expression signature (9, 10). This cancer subtype generates an immunosuppressive tumor microenvironment which is hostile for T-cells and contributes to TNBC immune evasion (11). Thus metabolic reprograming is an attractive approach to reshape the tumor immune environment and bypass immune evasion (Naik and Decock). Moreover, several studies offered an extensive overview for novel molecular targets beyond PD-1/PD-L1 such as ITM2A, VEGFR, STING, TLRs and others (Zhang et al.; Tabana et al.). Other studies discussed the significance of cutting the crosstalk between the tumor cells and other components within the tumor microenvironment (TME) including immune cells, extracellular matrix components and others (Salemme et al.; Deligne and Midwood). Finally, two comprehensive literature reviews underscored the promise of cellular immunotherapies as well as a series of immunotherapy combinations under development for TNBC (Fuentes-Antrás et al.; Thomas et al.).

Naik and Decock discussed how tumor metabolism shapes the local immune environment, with particular emphasis on the aerobic glycolysis-coupled lactate metabolism in TNBC. In addition to the well-established role of metabolic reprograming in accelerating tumor cell proliferation, invasion, metastasis and angiogenesis, the review highlighted the immunosuppressive effects of a lactate-rich microenvironment through modulation of tumor-infiltrating T-cells, natural killer (NK) cells, dendritic cells, Tregs and myeloid-derived suppressor cells as well as tumor-associated macrophages. These data support the rationale for targeting intra-tumoral metabolic landscape to augment the anti-tumor response to immunotherapy and improve the outcomes in highly glycolytic tumors such as TNBC.

Zhang et al. contributed an original research article in which they investigated the tumor suppressor role of the integral membrane protein 2A (ITM2A) in BC and how it is correlated to PD-L1 expression. This study showed that the differentially expressed genes (DEGs) screened based on RNA-sequencing data of MCF-7 cells overexpressing ITM2A were associated with immune response. ITM2A was shown to induce PD-L1 expression in BC cells and boost TILs numbers in the tumor microenvironment. The authors concluded that the overexpression of ITM2A reduced the aggressiveness of BC cells and had a favorable effect on outcomes in BC patients.

Tabana et al. reviewed novel immunological targets beyond PD-1/PD-L1 axis that can be exploited to tune up the tumor immune microenvironment and enhance the outcomes of immunotherapies. Those included engaging stimulator of interferon (IFN) genes (STING), toll like receptors (TLRs), vascular endothelial growth factor receptor (VEGFR) signaling, cytokines along with cyclooxygenase-II (COXII)/prostaglandin E2 (PEG2) axis. Tackling CSF-1/CSF-1R axis as well as adenosine signaling also showed promising outcomes. The modulation of tryptophane and arginine catabolism using inhibitors for indoleamine-2,3-dioxygenase (IDO1) and tryptophan-2,3-dioxygenase (TDO), and arginase 1 was also covered.

Salemme et al. depicted the crosstalk between the tumor cells and the immune TME in BC. In particular the authors presented an updated view of the pro- and anti-tumor activities of the main immune cell populations present in breast TME, with emphasis on the role of cytokine-signaling, cell–cell contact- and microvesicle-based mechanisms. Additionally, this review highlighted the current clinical trials assessing the efficacy of investigational strategies proposed to revert immunosuppression such as chimeric antigen receptor (CAR)-T and CAR-NK cells, cancer vaccination, immunogenic cell death-inducing chemotherapy, DNA methyl transferase and histone deacetylase inhibitors, cytokines or their inhibitors and other immunotherapies in BC.

Deligne and Midwood discussed the controversial role of macrophages and extracellular matrix in BC. Extracellular matrix (ECM) molecules such as tenascin-C, fibronectin and collagen are commonly upregulated within the tumor stroma. Such molecules were reported to exert a complex influence over the behavior of tumor-associated macrophages (TAM). They can either restrict or enhance TAMs intra-tumoral infiltration and drive their polarization towards or away from a pro-tumoral phenotype. On the other hand, TAMs can modulate the production of matrix molecules within the tumor to augment tumor growth and metastasis. The authors suggested that targeting specific immunomodulatory domains of the ECM to reinstate an efficient anti-tumor immune response, and effectively control tumor growth and spread, is emerging as a promising approach offering a new angle in the management of BC.

Fuentes-Antrás et al. outlined a clinically oriented overview of preclinical and clinical data regarding the use of cellular immunotherapies in BC. Cellular therapies aim to harness the immune system as a tool against antigenic heterogeneity and the broad repertoire of immune escape mechanisms occurring in advanced BC. This approach encompasses multiple strategies including the adoptive transfer of TILs, dendritic cells, NK cells, and engineered immune components such as CAR constructs and engineered T cell receptors.

Thomas et al. demonstrated multiple promising future combinations of immune-checkpoint inhibitors in TNBC. The article focused on assessing combinatorial approaches utilizing immune checkpoint inhibitors to enhance both innate and adaptive immune responses, or to establish a more immune favorable environment for cancer vaccines. This article also highlighted the limitations for predictive biomarkers of immunotherapy response. The authors concluded that combination of predictive biomarkers such as PD-L1 expression, intra-tumoral TILs, and stromal TILs density together with tumor mutational burden (TMB), TCR diversity and immune gene signatures will more likely yield improved performance versus each of these biomarkers alone which warrants further investigation.

## SECTION II STUDIES

This section includes articles providing rationale for newly established breast cancer immunotherapy clinical trials and/or preliminary outcomes. Among those is the SOLTI-1805 TOT-HER3 trial that focuses on patients with HR+/HER2- BC as well as the PELICAN-IPC trial which focused on HER2-inflammatory BC. This is in addition to the BrEAsT study which investigated immunotherapy/HDACI combination in both TNBC and HER2+ metastatic BC.

Pascual et al. presented the ongoing early phase 1 trial "SOLTI-1805 TOT-HER3". In this window-of-opportunity study the human epidermal growth factor receptor 3 (HER3)-directed antibody-drug conjugate (ADC) patritumab deruxtecan is given to patients with early-stage hormone receptor-positive (HR+)/human epidermal growth factor receptor 2-negative (HER2-) breast cancer. The primary endpoint is the CelTIL score, a novel tumor microenvironment (TME) biomarker based on the percentage of tumor cellularity and stromal TILs.

Bertucci et al. contributed the rationale and design of the PELICAN-IPC 2015-016/Oncodistinct-003 study (NCT03515798) which is an open-label, randomized, non-comparative, phase II study. PELICAN-IPC is assessing the efficacy, and safety of pembrolizumab in combination with chemotherapy in the neoadjuvant setting in HER2-negative inflammatory breast cancer (IBC). This type of breast cancer is extremely aggressive and is known for very low long-term survival. The mainstay for IBC management was through deploying neoadjuvant chemotherapy protocol. Adding panitumumab (anti-EGFR mAb) to the routine chemotherapy backbone has shown promising outcomes in the HR-/HER2- IBC (12). The PELICAN-IPC trial is the first one to investigate the efficacy of immune checkpoint inhibitors specifically in patients with IBC which is a hard-to-treat form of BC. It is noteworthy that the PELICAN-IPC 2015-016 trial is ongoing, and the estimated study completion date is by 2022.

Gatti-Mays et al. presented the supporting preclinical data and the design of the BrEAsT phase 1b clinical trial (NCT04296942). The study is enrolling patients with advanced/metastatic TNBC or HR-/HER2+ to receive a tetratherapy combination: BN-Brachyury (a poxvirus vaccine encoding a tumor-associated antigen), bintrafusp alfa (a bifunctional protein composed of the extracellular domain of the TGF receptor fused to a human IgG1 anti-PD-L1), entinostat (a histone deacetylase inhibitor), and the HER2-directed ADC ado-trastuzumab emtansine. The study is designed to assess the safety and efficacy of the combination.

Kim et al. contributed a case series of 5 patients with metaplastic BC treated with anti-PD-1-based therapy at a single center. Metaplastic breast cancer (MBC) is known to be a rare and chemo-refractory subtype of BC with poor prognosis and limited treatment options. It is noteworthy that 3 out of the 5 cases demonstrated a response to therapy, albeit limited in duration. One of the responding cases exhibited low-level hormone receptor expression and pleomorphic lobular features, whereas the other cases were TNBC. Responses were observed in tumors with intermediate PD-L1 expression (CPS 1-10). The extensive characterization of MBC was not feasible due to the small sample size in this series. However, in this series the authors also demonstrated a method of interrogating for unique immunologic and/or genomic features of individual tumor cases, relative to a parent cohort.

Schreiber et al. provided a retrospective analysis for the clinical outcomes for patients with metastatic BC treated with immunotherapy agents in Phase I clinical trials. A total of 43 patients with different BC subtypes were identified to be treated with an immunotherapy agent as single agent (72.1%) or combined with chemotherapy (27.9%). All patients had received an average of 2 prior lines of chemotherapy in the metastatic setting. The analysis showed that patients who had a progression-free survival (PFS) of >6 months were more likely to have been treated with a combination of immunotherapy plus chemotherapy compared to patients with a PFS < 6 months (77.8% v. 14.7%), demonstrating the added benefit of using chemotherapy in combination with immunotherapy in metastatic BC irrespective of BC subtype.

In summary, immunotherapy continues to represent an attractive option for patients with TNBC, with emerging strategies being explored in the different subtypes of BC. The emerging data elucidated additional angles for the complex interplay between the different components of the TME along with the ECM and how that contributes to the tumor immune escape. This largely contributes to developing promising strategies that simultaneously target multiple key pathways in order to enhance the therapeutic outcomes for immunotherapies. Yet, further research is still necessary to determine the mechanisms of resistance, identify predictive biomarkers, and to develop optimal combination regimens. These efforts are ongoing in order to provide the most effective, least toxic regimens to the patients that are most likely to benefit.

## AUTHOR CONTRIBUTIONS

MFT drafted the editorial. SMT, AA, CS, and BA-R contributed comments on the manuscripts they edited. All editors revised and approved the final copy of the editorial before submission.

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# Adoptive Cell Therapy in Breast Cancer: A Current Perspective of Next-Generation Medicine

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Immunotherapy has become a cornerstone in the treatment of cancer and changed the way clinicians and researchers approach tumor vulnerabilities. Durable responses are commonly observed with immune checkpoint inhibitors in highly immunogenic tumors, while the infusion of T cells genetically engineered to express chimeric antigen receptors (CARs) has shown impressive efficacy in certain types of blood cancer. Nevertheless, harnessing our own immunity has not proved successful for most breast cancer patients. In the era of genomic medicine, cellular immunotherapies may provide a more personalized and dynamic tool against tumors displaying heterogeneous mutational landscapes and antigenic pools. This approach encompasses multiple strategies including the adoptive transfer of tumor-infiltrating lymphocytes, dendritic cells, natural killer cells, and engineered immune components such as CAR constructs and engineered T cell receptors. Although far from permeating the clinical setting, technical advances have been overwhelming in recent years, with continuous improvement in traditional challenges such as toxicity, adoptive cell persistence, and intratumoral trafficking. Also, there is an avid search for neoantigens that can be targeted by these strategies, either alone or in combination. In this work, we aim to provide a clinically-oriented overview of preclinical and clinical data regarding the use of cellular immunotherapies in breast cancer.

**Keywords: adoptive cell therapy, breast cancer, TIL, TCR, CAR, dendritic cell, natural killer cell, tumor antigen**

## INTRODUCTION

Breast cancer (BC) is a leading cause of death worldwide and remains mostly incurable in advanced stages (1). Tumor initiation and progression is continuously controlled by innate and adaptive immune cells, which falter as cancer cells undergo mesenchymal dedifferentiation and/or evolve different mechanisms of tumor escape (2). In general, BC is not regarded as an inflamed tumor, triple negative BC (TNBC) and $HER2^{+}$ tumors being more immunogenic than the most common luminal A-like subtype (3). Immunotherapeutic strategies against BC have traditionally been based on "passive immunotherapy" such as the HER2 blocking antibody trastuzumab. Encouraged by the success of

immune checkpoint inhibitors (ICIs) in melanoma and lung cancer, numerous trials have tested the use of this "active immunotherapy" in BC with overall disappointing results (4). In the metastatic setting, the most significant achievement was observed in the IMpassion130 phase III trial, which demonstrated an increase in progression-free survival in TNBC patients receiving atezolizumab plus nab-paclitaxel compared to nab-paclitaxel alone (7.2 vs 5.5 months) (5). This humble benefit did not lead to a better overall survival and was not recapitulated when using paclitaxel as concomitant chemotherapy nor consistently associated to any predictive biomarker other than PD-L1 (6). Findings seem to be more clinically meaningful in the neoadjuvant setting, in which an increased pathological complete response rate has been reported in patients receiving atezolizumab (58 vs 41% for total population, 69 vs 49% in PD-L1 positive tumors) (7). This body of evidence underscores the need of a better understanding of the tumor-immune interaction, escape mechanisms, and the role of the microenvironment when a high tumor burden exists. Globally, the use of ICIs in BC would at best provide a nonspecific approach, guided by poorly understood biomarkers, to harnessing a debilitated immune system against a cold tumor. Instead, the development of omic-scale repositories and high-throughput technologies enable us to decode the genomic traits of each unique tumor and calls for the design of more specific and flexible immunotherapies, capable of targeting oncogenic addictions and overcoming temporal and spatial mutational heterogeneities. Thus, the aim of our work is to bridge the complex body of evidence on the different types of adoptive cell therapy (ACT) and the clinicians who everyday care for BC patients.

# T CELL THERAPY

## Tumor-Infiltrating Lymphocytes (TIL) Therapy

The adoptive transfer of lymphocytes to treat BC has been attempted in numerous occasions. Allogeneic stem cell transplants in addition to high-dose chemotherapy achieved successful long-term outcomes but arouse significant safety concerns, whereas ACT with autologous circulating lymphocytes conditioned *in vitro* was better tolerated but showed less efficacy (8–11). Tumor-infiltrating lymphocytes (TILs) include a subset of naturally occurring T cells capable of targeting neoantigens encoded by genes harboring nonsynonymus somatic mutations (12, 13). BC, particularly $HER2^+$ and luminal-like tumors, have been traditionally considered as poorly immunogenic, with low numbers of TILs and a limited burden of neoantigens (3, 14, 15). However, a robust correlation exists between increased stromal TILs and a better prognosis in TNBC (16–19).

Adoptive transfer of autologous TILs was first described as a treatment modality by Rosenberg and colleagues in 1987 (**Figure 1A**) (20). Substantial objective responses have been observed in patients with tumors with high mutation rates such as melanoma, lung or bladder cancer (20–22). However, with few exceptions, the infusion of unselected heterogenous TILs appears mostly ineffective in epithelial malignancies (23–27). In order to boost tumor recognition and killing efficacy, TIL therapy has been refined by selecting TILs reactive for tumor antigens (TAs) identified by whole-exome sequencing and RNA sequencing. Zacharakis et al. recently described the case of a 49 year-old woman with $ER^+/HER2^-$ metastatic BC refractory to multiple lines of chemotherapy, who exhibited a complete durable regression after ACT with TA-specific TILs in conjunction with IL-2 and an anti-PD1 agent (28). In this particular case, the genomic analysis of a right breast subcutaneous lesion revealed the presence of 62 nonsynonymous somatic mutations, of which the mutant versions of 4 proteins rendered the highest TIL reactivity. Further, a relevant impact of the concomitant anti-PD1 therapy was unlikely since no expression of PD-L1 was detected in tumor biopsies. A similar approach was used for a pulmonary metastasis of a TNBC patient, where an immunogenic mutation was found among 72 nonsynonymous mutations (29). However, outcome data from this tailored TIL therapy was not reported. Four clinical initiatives have been registered to date in ClinicalTrials.gov and are briefly displayed in **Table 1**. Notably, only two of them incorporate preconditioning with non-myeloablative chemotherapy regimens, and one of them will address the role of an anti-PD1 agent as concurrent medication. In sum, the transfer of selected autologous TILs primed against multiple MHC-restricted TAs may provide a safe and personalized option for patients with advanced BC.

## Engineered T Cell Receptor (TCR) and Chimeric Antigen Receptor (CAR) Therapy

Gene transfer-based strategies have been developed to overcome the main challenges of TIL therapy, including the low yield of TIL expansion, the low affinity of human TCRs for TAs, and the immune tolerance elicited by the downregulation of MHC molecules and TAs (30). Both TCR and chimeric antigen receptor (CAR) gene transfer endow polyclonal T cells with reactivities that are not naturally present against TAs of choice and thus provide an adaptable and highly subtle tool for personalized medicine (**Figures 1A, B**) (31).

The majority of engineered αβTCRs recognize epitopes presented by MHC molecules, thereby narrowing down the group of potential targets to those which are MHC-restricted, and exhibit an increased specificity recognition and affinity for tumor cells (**Figure 1A**) (32, 33). Mounting clinical evidence on several tumor types along with preclinical data on BC underscores the rationale for TCR use in BC patients (34–37). Of note, in both hormone-dependent and independent BC cell lines and in xenograft mice, Li et al. reported a notable enhancement of anti-tumor cytotoxicity by $CD8^+$ T cells transduced with an MHC-A2-restricted placenta-specific 1 (PLAC1)-TCR molecule (38). However, to the best of our knowledge, evidence on humans is still lacking, with many ongoing clinical trials testing intravenous infusions of TCR-engineered T cells against TAs such as HER2, NYESO-1, and MAGE-A3 (**Table 1**). Interestingly, some of them will assess the value of adding anti-PD1 therapy to enhance immune reconstitution after lymphodepleting chemotherapy and cytotoxicity.

In order to bypass the limitations of MHC restriction of conventional αβTCRs, intensive research has focused on the development of CARs and, more recently, on the γδT cell

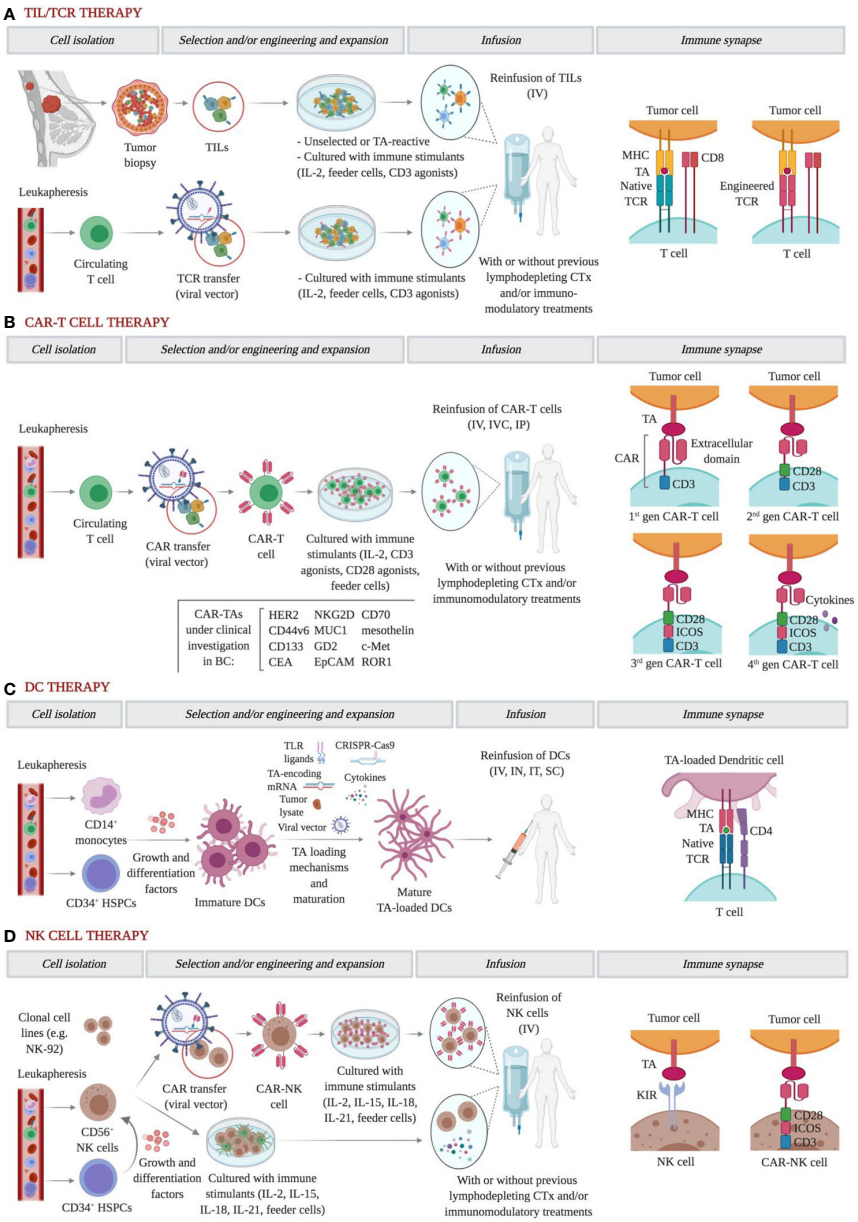


**FIGURE 1** | Graphical representation of the main approaches of adoptive cell therapy in breast cancer. **(A)** In general, TILs are enzymatically isolated, activated with high-dose IL-2, and eventually expanded for therapeutic use. More recently, they can also be screened for a high avidity for TAs. TCR transfer, usually using viral vectors on circulating T cells, endows T cells with TCRs with high affinity for TAs. Further, to help condition the body for the T cell transplant, patients often receive a non-myeloablative lymphodepleting chemotherapy regimen before IV infusion, which facilitates the access to growth-promoting cytokines and removes suppressor cells. The role of concomitant immunomodulatory therapies is yet to be elucidated. In both approaches, recognition of cognate TAs is MHC-restricted. **(B)** CAR engineering of circulating T cells has been progressively refined. First-generation CARs include only a CD3ζ chain as intracellular signaling domain; second-generation CARs add a single co-stimulatory domain, such as CD28, 4-1BB (CD137), CD27, or OX40; third-generation CARs add two or three co-stimulatory domains; fourth-generation CARs, also known as TRUCKs (T cells redirected for antigen-unrestricted cytokine-initiated killing) are further armored with potent antitumor cytokines and co-stimulatory ligands. CARs target a wide range of surface TAs in an MHC-independent manner, and multiple trials are currently testing the feasibility and efficacy of different administration routes. **(C)** DCs can be generated from PBMNC and HSPCs and become mature after being pulsed using a growing set of TA loading mechanisms. In trials, DCs are infused IV but also as IT or IN vaccines. **(D)** NK cells for ACT can be obtained from clonal cell lines, primary NK cells, or HSPCs. Whether they undergo CAR engineering or remain unmodified, NK cells ligate cognate TAs in an MHC-independent manner. After co-culture with immune stimulants and feeder cells, NK cells are infused IV with or without prior lymphodepleting chemotherapy and/or immunomodulatory treatments. TILs, tumor-infiltrating lymphocyte; IL, interleukin; TA, tumor antigen; TCR, T-cell receptor; MHC, major histocompatibility complex; CAR, chimeric antigen receptor; DC, dendritic cell; PBMC, peripheral blood mononuclear cell; HSPC, hemopoietic pluripotent stem cell; NK, natural killer. IV, intravenous; IVC, intraventricular; IP, intraperitoneal; IN, intranodal; IT, intratumoral; SC, subcutaneous. Figure created with *BioRender.com*.

**TABLE 1 |** Clinical trials of ACT in breast cancer.

| Antigen | Coadjuvants | Phase | Stage | Phenotype | Route | Precondition | NCT | Status |
|---|---|---|---|---|---|---|---|---|
| **TIL therapy** | | | | | | | | |
| Unselected TAs | None | I | IV | TN | IV | Yes | NCT04111510 | Recruiting |
| Unselected TAs | None | I | IV | Mixed | IV | No | NCT01462903 | Unknown |
| Unselected TAs | Anti-PD1 | II | IV | Mixed | IV | Yes | NCT01174121 | Recruiting |
| Unselected TAs | Trastuzumab | I | IV | HER2+ | IV | No | NCT00301730 | Completed |
| **TCR therapy** | | | | | | | | |
| Neoepitopes | None | II | IV | Mixed | IV | Yes | NCT04102436 | Recruiting |
| Neoepitopes | Anti-PD1 | I | IV | HR+ | IV | No | NCT03970382 | Recruiting |
| Neoepitopes | Anti-PD1 | II | IV | Mixed | IV | Yes | NCT03412877 | Recruiting |
| NYESO-1 | None | I | IV | Mixed. HLA-A0201+, NY-ESO-1+ | IV | Yes | NCT03159585 | Completed |
| NYESO-1 | None | I | IV | Mixed. HLA-A0201+, NY-ESO-1+ | IV | Yes | NCT02457650 | Unknown |
| MAGE-A3 | None | I/II | IV | HLA-DP0401/02+, MAGE-A3+ | IV | Yes | NCT02111850 | Active, not recruiting |
| NYESO-1 | None | II | IV | HLA-A2+, NYESO-1+ | IV | Yes | NCT01967823 | Completed |
| **CAR-T cell therapy** | | | | | | | | |
| HER2 | None | I | IV | HER2+ | IV | Yes | NCT04511871 | Recruiting |
| HER2, GD2, CD44v6 | None | I/II | III, IV | GD2, CD44v6, HER2+ | IV | No | NCT04430595 | Recruiting |
| CD44v6 | None | I/II | NR | CD44v6 | IV | No | NCT04427449 | Recruiting |
| CEA | None | I/II | IV | CEA+ | IV | No | NCT04348643 | Recruiting |
| NKG2D | None | I | IV | TN | IV | No | NCT04107142 | Not yet recruiting |
| MUC1 | None | I | IV | TN. MUC1+ | IV | Yes | NCT04025216 | Recruiting |
| MUC1 | None | I | IV | Mixed | IV | No | NCT04020575 | Recruiting |
| HER2 | CAdVEC oncolytic virus | I | Unresectable, IV | HER2+ | IV | No | NCT03740256 | Not yet recruiting |
| HER2 | None | I | IV (brain, leptomeningeal) | HER2+ | IVC | No | NCT03696030 | Recruiting |
| CEA | None | I | IV (carcinomatosis, malignant ascites) | CEA + | IP | No | NCT03682744 | Active, not recruiting |
| GD2 | None | I | IV | Mixed | IV | Yes | NCT03635632 | Recruiting |
| EpCAM | None | I | Unresectable, IV | EpCAM+ | IV | No | NCT02915445 | Recruiting |
| CEA | Low dose IL-2 | I | IV (liver) | CEA+ | Hepatic artery | No | NCT02850536 | Active, not recruiting |
| CD70 | None | I/II | Unresectable, IV | CD70+ | IV | Yes | NCT02830724 | Recruiting |
| Mesothelin | None | I | IV | HER2-. Mesothelin+ | IV | Yes | NCT02792114 | Recruiting |
| ROR1 | None | I | IV | TN. ROR1+ | IV | Yes | NCT02706392 | Recruiting |
| CD133 | None | I/II | IV | CD133+ | IV | No | NCT02541370 | Completed |
| CEA | Low dose IL-2 | I | IV (liver) | CEA+ | IV | No. | NCT02416466 | Completed |
| Mesothelin | Anti-PD1 | I/II | IV (pleural) | Mesothelin+ | Pleural | Yes | NCT02414269 | Recruiting |
| cMet | None | I | IV | TN. cMet+ | IT | No | NCT01837602 | Completed |
| **DC therapy** | | | | | | | | |
| HER2/HER3 | Anti-PD1, IFNa2b | II | IV | TN, HER2+ | SC | No | NCT04348747 | Not yet recruiting |
| Neoepitopes | None | 1 | II, III | TN | NR | No | NCT04105582 | Recruiting |
| NR | None | I | IV | Mixed | IT | No | NCT03638765 | Not yet recruiting |
| HER2 | None | II | I-III, IV in CR | HER2+ | IN | No | NCT03630809 | Recruiting |
| NR | None | I/II | IIA, III, IV | Mixed | NR | No | NCT03450044 | Completed |
| HER2 | None | I | II, III | HER2+ | IN | No | NCT03387553 | Recruiting |
| GFBP2, HER2, IGF1R | None | II | I-III | HER2+ | IN | No | NCT03384914 | Recruiting |
| NR | CIK, anti-PD1 | I/II | IV | Mixed | IV | No | NCT02886897 | Unknown |
| NR | CIK | II | IV | Mixed | NR | No | NCT02491697 | Active, not recruiting |
| TBVA | None | I | IV | Mixed | SC | No | NCT02479230 | Completed |
| MUC-1 | None | I | IV | Mixed | NR | No | NCT02140996 | Unknown |
| HER2 | None | I | III (N2) | HER2+ | IN | No | NCT02063724 | Active, not recruiting |
| HER2 | None | I/II | DCIS | HER2+ | IT, IN | No | NCT02061332 | Completed |
| HER2 | None | I | I-III | HER2+ | IN | No | NCT02061423 | Active, not recruiting |

*(Continued)*

**TABLE 1** | Continued

| Antigen | Coadjuvants | Phase | Stage | Phenotype | Route | Precondition | NCT | Status |
|---|---|---|---|---|---|---|---|---|
| Cyclin B1/WT-1/ CEF | None | I/II | II-III | TN, ER+ | IN, SC | No | NCT02018458 | Completed |
| HER2 | None | I | IV | HER2+ | SC | No | NCT01730118 | Completed |
| HER2 | None | II | II-III | TN, ER+ | NR | No | NCT01431196 | Completed |
| WT1 | None | I/II | III (N2), IV | TN | SC | No | NCT01291420 | Unknown |
| p53 | None | I/II | IV | p53+ | SC | No | NCT01042535 | Completed |
| Survivin, hTERT, p53 | None | I | IV | Mixed | SC | No | NCT00978913 | Completed |
| OFP/iLRP | None | I/II | IV | Mixed | SC | No | NCT00879489 | Unknown |
| NR | None | II | II-III | TN, ER+ | IT, IN | No | NCT00499083 | Completed |
| HER2 | None | I | IV | HER2+ | SC | No | NCT00197522 | Completed |
| HER2 | None | I | Local relapse, IV | HER2 | NR | No | NCT00162929 | Completed |
| HER2 | None | I | DCIS | HER2+ | IN | No | NCT00107211 | Completed |
| p53 | None | I/II | III | p53+ | SC | No | NCT00082641 | Completed |
| CEA | None | I | IV | Mixed | IV | No | NCT00004604 | Completed |
| **NK cell therapy** | | | | | | | | |
| HER2 | None | I/II | IV | HER2+ | IV | Yes | NCT04319757 | Recruiting |
| NR | Anti-PD1/PD-L1 | I | IV | Mixed | IV | Yes | NCT03841110 | Recruiting |
| NR | None | I/II | All | All | IV | No | NCT03634501 | Recruiting |
| NR | Trastuzumab | I | IV | HER2+ | IV | No | NCT03319459 | Active, not recruiting |
| MUC1 | None | I/II | IV | TN, MUC1+ | IV | No | NCT02839954 | Unknown |
| HER2 | Trastuzumab | I/II | IV | HER2+ | IV | No | NCT02030561 | Unknown |
| NR | None | II | IV | Mixed | IV | Yes | NCT01105650 | Completed |

*TA, tumor antigen; PD1, programmed death receptor 1; PD-L1, programmed death receptor ligand 1; CIK, cytokine-induced killer cell; TIL, tumor infiltrating lymphocyte; TCR, T-cell receptor; DC, dendritic cell; NK, natural killer; TN, triple negative; HLA, human leukocyte antigen; HR, hormone receptor; IV, intravenous; IVC, intraventricular; IP, intraperitoneal; IN, intranodal; IT, intratumoral; SC, subcutaneous.*

compartment. γδT cells exhibit potent anti-tumor responses by bridging innate and adaptive immunities, since they incorporate both γδTCRs and killer cell immunoglobulin-like receptors (KIRs) (39, 40). Also, γδT cell ligand recognition requires the expression of accessory costimulatory molecules, which may prevent harmful self-reactivity. Infiltration by γδT cells has been associated with improved outcomes in a small cohort of TNBC patients (41). Consistently, ACT of γδT cells together with trastuzumab improved control of tumor growth as compared to trastuzumab alone in a mouse model of $HER2^+$ BC (42). However, the function of the γδT cells may be extremely pleiotropic. In this regard, Peng et al. described a BC-infiltrating γδT cell subset with strong immunosuppressive effects on T cells and DCs regulated *via* the Toll-like receptor 8, thus suggesting that its depletion or reversal could enhance anti-tumor responses (43). ACT with unmodified or engineered γδT cells emerges as an appealing prospect for BC immunotherapy, but further functional characterization and data on clinical interventions are still required (44).

On the other hand, CAR-T cells are T cells engineered to express an artificial receptor with a modular design consisting of an extracellular ligand-binding domain, usually a single-chain antibody, a hinge, a transmembrane domain, and a cytoplasmic signaling domain, with increasing complexity and functionality across the four generations of CAR constructs (**Figure 1B**) (45–47). Compared to TILs, CAR-T cells are not as affected by the hurdles of isolation, expansion, and persistence limitation of natural tumor-specific T cells. Moreover, CAR recognition occurs in an MHC-independent manner, which helps overcome MHC downregulation as a mechanism of tumor escape, and can also recognize carbohydrate and glycolipid antigens (46). Yet, cognate antigens are consequently restricted to surface molecules. Numerous preclinical studies *in vitro* and *in vivo* have evaluated the use of CAR-T cells armed to specifically target TAs in BC, with HER2-CAR constructs attracting the most attention and achieving robust tumor regressions (48–60). To our knowledge, only one phase I trial has been published testing a HER2-CAR in BC patients. In the study by Lum et al., 23 metastatic BC patients independent of their HER status received 8 infusions of anti-CD3/HER2 bispecific antibody-armed T cells. In the evaluable patients at 14.5 weeks, 13 patients experienced clinical benefit, including 2 objective responses (61). Notwithstanding, serious adverse events have been reported following the use of HER2-CARs. The first evidence on the clinical use of HER2-CAR-T cells was a case report of a patient with metastatic $HER2^+$ colon cancer in whom the administration of a 3$^{rd}$ generation HER2-CAR was followed by multiple cardiac arrests, respiratory distress, and multiorgan damage (62). This harm was attributed to an inflammatory cytokine release elicited by the immune-mediated recognition of HER2 in normal epithelial tissues, which is referred to as "on-target, off-tumor" toxicity.

Besides HER2, the single injection of accessible lesions with CAR-T cells targeting c-Met, a cell-surface protein tyrosine kinase aberrantly expressed in BC, in a group of 6 patients with metastatic BC comprised by two $ER^+$ tumors and 4 TN tumors, did not render measurable responses but elicited extensive tumor necrosis and loss of c-Met immunoreactivity at the injection site, and also translated into detectable levels of c-Met-CAR-T cell mRNA in peripheral blood (63). Similarly, Specht et al. recently communicated preliminary safety results of a phase I trial targeting ROR-1, a

tyrosine kinase protein expressed in TNBC and associated with a worse prognosis (64, 65). Interestingly, patients received a 2nd generation ROR1-CAR engineered with a truncated EGFR molecule to permit the elimination of infused cells in case of toxicity (64). Only 6 patients had been enrolled with no adverse events observed, but further update is expected to support this innovative approach.

A considerable number of trials are testing CAR constructs against multiple TAs in BC (**Table 1**). We expect that these studies also convey relevant information about on-target, off-tumor effects, and the benefits of the different administration routes, preconditioning or concomitant immunomodulatory therapies. In addition, it seems clear that a thorough genomic-scale understanding of molecular vulnerabilities and antigenic shifts will be of paramount importance in the design of CAR-based strategies.

## DENDRITIC CELL (DC) THERAPY

Dendritic cells (DCs) are particularly well-suited for BC immunotherapy due to their ability to sensitize $CD8^+$ T cells and also $CD4^+$ T cells capable of generating memory T cells and contribute with additional cytotoxicity against tumors (66). DCs have been found infiltrating BC specimens in nearly half of the patients with either early or advanced disease, but are mostly relegated to the periphery, functionally compromised, and show a poor correlation with outcome (67–70).

Autologous DCs may be fused with tumor cells or pulsed with tumor lysates or TAs to activate T cells against tumors (**Figure 1C**) (71–74). Across these strategies, DCs may be either exposed to one particular neoantigen or to the entire repertoire of TAs, including those yet to be identified. In contrast to what was observed in TIL and TCR therapies, DCs can be obtained in large numbers from bone marrow precursors and monocyte-derived DCs from peripheral blood (75). The pioneering study by Brossart et al. evaluated the vaccination with autologous DCs pulsed with HER2 or MUC1-derived peptides in 7 BC patients. Although the clinical outcomes were disappointing, peptide-specific T cell responses could be detected even at 9 months after initiation of vaccinations, and T cell responses against epitopes not used for vaccination were identified as a result of cross priming (76). More encouraging objective responses were achieved by Avigan et al. in a phase I trial testing the vaccination with DCs fused with autologous tumor cells in 16 patients with metastatic BC (77). These included 2 patients attaining a partial response and 6 patients attaining a stable disease, although the anti-tumor effects were not maintained over time. In the neo/adjuvant setting, vaccination with autologous HER2-pulsed DCs achieved a modest rate of pathological complete responses in $HER2^+$ BC patients, which yet correlated poorly with immune surrogates in peripheral blood (78). This study, however, demonstrated that intralesional and intranodal routes of administration may not substantially differ in terms of anti-tumor efficacy, thus facilitating vaccination when tumor locations are challenging. Likewise, the trial conducted by Qi et al. in stage II-IIIA $ER^-/PR^-$ BC patients reported a 3-year relapse-free survival of 71% versus 31%, with and without vaccination, respectively (79). Other promising approaches consist of adding cytokine adjuvants, such as IL-2, or targeting both the innate and adaptive immune systems by complementing DCs with cytokine-induced killer cells, although the response to these strategies has so far been humble or confused by the effect of concurrent chemotherapies (80–82). More than 20 trials are registered to date testing DC vaccinations in BC patients of all major pathological and most of them are designed to pulse DCs with TAs of choice (**Table 1**). Although ACT with DCs has not yet materialized in a relevant clinical benefit, we believe that the role of DCs as stimulators of T-cell response and long-term memory, and their safety and ease of manufacture, may justify further development alone or in combination with other T cell therapies.

## NATURAL KILLER (NK) CELL THERAPY

Different from the previous approaches, NK cells represent an attractive asset for cancer immunotherapy due to their innate ability to eliminate cancer cells in an MHC-independent and non-TA-restricted manner. The "loss of self" mediated by the downregulation of MHC molecules as a mechanism of tumor escape hinders the recognition of cancer cells by $CD8^+$ T cells but unleashes the activity of NK cells, which are regulated by the interplay of activating and inhibitory receptors such as KIRs and natural killer group 2D (NKG2D) (83, 84).

Activated NK cells can be manufactured in large numbers *ex vivo* from primary NK cells, hemopoietic stem cells, and clonal cell lines, of which the NK-92 is approved by the US FDA for use in clinical trials (**Figure 1D**) (85–87). So far, adoptive transfer of autologous NK cells has been tested in a wide range of solid malignancies with poor clinical efficacy, which has been explained by the immunosuppressive state of the host and because the inhibitory receptors on autologous NK cells matched molecules exhibited on the tumor cell surface (87–89). Anecdotally, a report by Tian et al. described a partial response in a patient with progressing metastatic $HER2^+$ BC who underwent treatment with trastuzumab-treated NK cells, which was consistent with an increased activation and expansion of NK cells mediated by trastuzumab *in vitro* (90). Allogeneic NK cells, however, have not proved to do much better in BC patients, with only one phase II trial published describing 4 patients with stabilized disease from a total of 6 patients evaluated at 4–6 weeks from infusion and after pre-conditioning with lymphodepleting chemotherapy and total body irradiation (91).

In order to enhance their cytotoxic properties, NK cells are also being modified with the addition of CARs against specific TAs. Compared to CAR-T cells, CAR-NK cells are theoretically less potent due to their lack of clonal expansion, relatively short lifespan, and less cytotoxic cytokines (87). Although CAR-T cells may mediate more incisive and long-term responses, the use of CAR-NK cells would minimize the risk of cytokine release syndrome and tumor-lysis syndrome, thereby increasing

overall treatment safety (92). Importantly, CAR-NK therapy is expected to be much less expensive, considering that NK cells can be derived from multiple sources. Encouraging results have been reported in a phase I/IIa trial using cord blood-derived CAR-NK cells targeting CD19 in patients with relapsed or refractory non-Hodgkin's lymphoma and chronic lymphocytic leukemia, with up to 64% of patients achieving a complete response (93). In BC, tissue factor (TF) was recently described by Hu as a novel and common yet selective molecule on TNBC, whose targeting by TF-CAR NK cells resulted in an increased cytotoxicity against TNBC cell lines and was effective and safe for the treatment of TNBC in an orthotopic mouse model (94). Chen et al. recapitulated these findings when investigating the effect of EGFR-CAR NK cells in TBNC cell lines and in mice pre-inoculated with brain metastases (95). To the best of our knowledge, there is not published data on human trials on BC to date, although several initiatives can be found registered in the Clinical Trials.gov repository including multiple trials evaluating the intravenous infusion of *ex vivo* expanded, autologous NK cells and also the administration of NK cells incorporating HER2- and MUC1-CAR constructs (**Table 1**).

## CONCLUDING REMARKS

ACT offers a growing toolkit to overcome antigenic heterogeneity and the broad repertoire of immune escape mechanisms occurring in advanced BC. To fully capitalize these set of highly personalized treatments, we must address both approach-specific and cross-cutting challenges. ACT with autologous TILs may benefit from the standardization of TIL assessment in routine biopsies and the effective expansion of those TILs with the highest anti-tumor reactivity. Gene transfer-based TCR therapies increase antigen specificity but still fail to target those not presented by the MHC, whereas CAR engineering may provide additional versatility but entails elevated costs and significant on-target, off-tumor toxicity. Additionally, although DC and NK cell therapies may have not achieved relevant tumor responses, their better safety profile and reduced costs make them suitable companions for multimodal strategies. The successful transition of the different ACTs to the clinic poses a number of common considerations. The discovery of TAs that can guide ACT against BC is critically linked to its success and relies on comprehensive strategies integrating genomic sequencing, *in silico* prediction, and immunogenicity evaluation. Methodological refinement is also required to improve our ability to isolate immune components and modify them *ex vivo* and *in vivo*, and to enhance cell persistence and intratumor trafficking. Finally, clinical trials testing ACTs will progressively need to be more adaptable, explore the reliability of predictive biomarkers, and generate quality data from small sample sizes. Both puzzling and fascinating, this is the path ahead to materialize ACT and transform the therapeutic landscape of BC patients.

## AUTHOR CONTRIBUTIONS

JF-A, KG-H, and AO contributed to the conception and scope of the study. JF-A and AO wrote the first draft. JF-A and KG-H composed the figures/tables. All authors contributed to the article and approved the submitted version.

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# PELICAN-IPC 2015-016/Oncodistinct-003: A Prospective, Multicenter, Open-Label, Randomized, Non-Comparative, Phase II Study of Pembrolizumab in Combination With Neo Adjuvant EC-Paclitaxel Regimen in HER2-Negative Inflammatory Breast Cancer

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Inflammatory breast cancer (IBC) is a highly aggressive entity with a poor outcome and relative resistance to treatment. Despite progresses achieved during the last decades, the survival remains significantly lower than non-IBC. Recent clinical trials assessing PD-1/PD-L1 inhibitors showed promising results in non-IBC. Pembrolizumab, an anti-PD-1 monoclonal antibody, revolutionized the treatment of different cancers. Several recent studies suggested a potential interest of targeting the immune system in IBC by revealing a more frequent PD-L1 expression and an enriched immune microenvironment when compared with non-IBC. Here, we describe the rationale and design of PELICAN-IPC 2015-016/Oncodistinct-003 trial, an open-label, randomized, non-comparative, phase II study assessing efficacy, and safety of pembrolizumab in combination with anthracycline-containing neoadjuvant chemotherapy in HER2-negative IBC. The trial is ongoing. The

primary endpoint is the pCR rate (ypT0/Tis, ypN0) in overall population and the co-primary endpoint is safety profile during a run-in phase. Key secondary objectives include tolerability, invasive disease-free, event-free and overall survivals, as well as collection of tumor and blood samples for translational research.

**Clinical Trial Registration:** https://clinicaltrials.gov/ (NCT03515798).

**Keywords: immune checkpoint inhibitor, inflammatory breast cancer, neoadjuvant therapy, PDL1, pembrolizumab**

## INTRODUCTION

Inflammatory breast cancer (IBC) is an uncommon (less than 5% of all BC) and very aggressive form of locally advanced BC. IBC has a clinical definition, which includes a rapidly (less than 6 months) enlarging, erythematous (which has to occupy at least one-third of the breast) and edematous breast (known as "peau d'orange"), which often presents without any underlying breast mass (1–3). Women with IBC are typically diagnosed at a younger age than patients with non-IBC (4, 5). IBCs are more frequently ductal than non-IBCs, with more frequent high grade, axillary lymph node involvement and metastases (more than 30%) at diagnosis (6, 7).

Biologically also, IBCs differs from non-IBCs, with more frequent hormone receptor (HR)-negativity and HER2-positivity (˜40% *versus* 15% in non-IBC) (8, 9), and a more angiogenic phenotype (10). IBCs display higher vascularity and an increased microvessel density (11, 12), and frequently include the presence of dermal lymphovascular emboli (13, 14). During the last two decades, IBC clinical tumor samples have been profiled using high-throughput molecular profiling technologies, mainly based on transcriptome analysis, in order to better delineate the molecular biology of disease (15). In 2013, the World IBC Consortium identified a robust 79-gene expression signature discriminating IBCs *versus* non-IBCs samples independently form the molecular subtypes (16). This signature notably suggested that alterations in TGF-β and immune response pathways are involved in the biology of IBC. Therefore, a particular tumor immune microenvironment is likely to participate into the unique biological patterns associated with IBC. Such importance of the tumor stroma has then been underlined by other research groups (17).

Significant therapeutic progresses were achieved during the past 50 years using a multidisciplinary approach, including neoadjuvant chemotherapy (NACT), followed by surgery and radiation therapy, and adjuvant anti-HER2 treatment and/or endocrine therapy – when indicated. However, the survival of IBC patients, when matched stage for stage, remains inferior to that of non-IBC patients. Research efforts are ongoing for many years to improve the treatment of disease. Due to the scarcity of the disease, its rapid progression and its unfavorable outcome, IBC-specific clinical trials have been rare. When they are not excluded, IBC patients are included in non-specific studies, being considered as locally advanced BC. Here, we present the rationale and the design of PELICAN-IPC 2015-016/Oncodistinct-003 trial, an open-label, multicentric, randomized, non-comparative, phase II study evaluating efficacy and safety of pembrolizumab in combination with neoadjuvant chemotherapy in HER2-negative IBC.

### Neoadjuvant Chemotherapy in IBC

Historically, the long-term survival was dramatically low (<5%) when patients were treated with loco-regional treatment only, suggesting the strong metastatic potential of IBC. Incorporation of multi-agent NACT in the therapeutic strategy significantly improved the prognosis, and achievement of pathological complete response after chemotherapy was identified as a favorable prognostic factor.

Advances in IBC have been made paralleling locally advanced non-IBC such as multi-agent NACT including anthracycline-based regimen with addition of taxanes, and more recently with incorporation of anti-HER2 targeted therapies (trastuzumab, pertuzumab, neratinib, trastuzumab-emtansine) in HER2 amplified disease (18–22). Three IBC-specific trials evaluated addition of bevacizumab in HER2-positive (23) and HER2-negative (24) IBC in the neoadjuvant and adjuvant setting, and panitumumab (an anti-EGFR monoclonal antibody) in HER2-negative IBC in the neoadjuvant setting (25). Results were promising with bevacizumab in HER2-positive and with panitumumab in triple-negative (TN) IBC, but these drugs are not recommended in routine. To our knowledge there is no ongoing IBC-specific study further evaluating these agents. Importantly, these trials showed the feasibility of IBC-dedicated clinical trials, with more than 50 patients enrolled per year in the two French multicentric trials (23, 24). However, and despite the benefit of NACT, the results are insufficient, with a 5-year survival remaining between 30% and 50%. Thus, it remains crucial to improve the results by optimizing neoadjuvant systemic regimen.

### Immune Microenvironment in IBC

Escape from immune destruction is an important way set up by cancers to promote cell transformation and favor tumor growth, which has been known for decades in various tumor models. In BC, this process was more recently enlightened. Thus, various features associated with immune response have a significant predictive impact on therapeutic efficacy and survival. In BC and also IBC, tumor infiltrating lymphocytes (TIL) (26–28) and immune gene expression signatures have shown a prognostic impact, in particular for ER-negative and/or high proliferating tumors (29–32). Interestingly, studies on small BC series have also indicated that some NACT regimens, such as anthracyclines-taxanes combinations, could favor the attraction

of lymphocytes to the tumor bed (33, 34). The Programmed cell death 1 (PD-1) receptor-ligand interaction is a major inhibitor pathway hijacked by tumors to suppress immune control (35–41). Under physiological conditions, when PD-1, which is expressed on the cell surface of activated T-cells, is engaged by its ligands, Programmed death-ligand 1 and 2 (PD-L1 and/or PD-L2), it mitigates lymphocyte activation and promotes T-regulatory cell development and function, allowing to terminate the immune response. PD-L1 and PD-L2 are either constitutively expressed or induced in various tissues, including different neoplastic diseases. PD-L2 regulates T-cell activation in lymphoid tissues, whereas PD-L1 serves to limit unneeded T-cell function in peripheral organs and tissues. Several studies have examined PD-L1 expression in BC at ARN and protein levels, using different scoring systems: various expression rates have been reported ranging from less than 2% to 55%, with discordant prognostic impact (42–49). Our group retrospectively analyzed *PD-L1* mRNA expression in 45 BC cell lines and 5,454 clinical BC. Compared to normal tissue, we found PD-L1 expression as increased in 20% of clinical samples, and in almost 40% of basal-like subtypes. Expression of PD-L1 was associated with biological evidences of major cytotoxic immune response, such as TCR-related gene expression, indicative of a high T-cell infiltration. PD-L1 overexpression was not associated with survival in the overall population, but with better metastasis-free survival (MFS) and overall survival (OS) in basal-like tumors, independently from the clinico-pathological features. The pCR rate after NACT was higher in case of increased PD-L1 expression (50% *versus* 21%) (48).

Few studies have been specifically dedicated to IBC. In the World IBC Consortium series including 87 informative IBC samples (50), we identified and validated a robust 107-gene signature associated with pCR and strongly enriched for genes involved in both adaptative and innate immunity. In a cohort of 306 BC samples (51), including 112 IBC samples, PD-L1 was overexpressed in 38% of IBC samples compared to normal breast tissue. Such overexpression correlated with aggressive molecular subtypes (TNBC or basal-like and HER2-positive subtypes) and with a higher pCR rate to NACT as well as biological signs of antitumor T-cell cytotoxic response. There was no correlation with MFS and specific OS. Microenvironment of "PD-L1-high" IBC samples was in favor of a strong local cytotoxic immune response, with higher expression of T-cell-specific and CD8+ T-cell-specific gene signatures, and higher expression of T-cell receptor-related genes. In addition, these tumors displayed features of T-cell activation. However, some T-cells infiltrating the tumor had a phenotype of exhausted T-cells. Similar observations were reported at the protein level (52). In a recent study including 143 patients with IBC and 142 control subtype-matched patients with non-IBC, PD-L1 IHC expression on immune cells (SP142 antibody) was more frequent in IBC (42.9%) than in non-IBC (23.7%), and correlated with higher pCR rate and stromal TIL infiltration (53). This later was associated with improved overall survival in a multivariate model. Finally, recent next-generation sequencing studies have shown that IBC samples display higher tumor mutational burden (TMB) than non-IBC samples, independently from the molecular subtypes and tumor stage (54, 55). Such increased TMB in IBC might lead to increased tumor antigen-based attraction of cytotoxic T-cells and better sensitivity to immune checkpoint inhibitors.

## Pembrolizumab and Other Anti-PD1/PD-L1 Agents in BC

Pembrolizumab, a humanized immunoglobulin (IgG4) monoclonal antibody (mAb), binds PD-1 with a high specificity, blocks the interaction with PD-L1 and PD-L2, and reactivates inhibited T-cells, which is expected to increase the antitumor immune response. This drug and other immune checkpoint inhibitors (ICI) targeting the PD-1/PD-L1 axis showed evidences of antitumor activity in several cancers, with a favorable toxicity profile compared to conventional chemotherapy. They are already registered in various indications, especially in the management of non-small cell lung cancer, melanoma, renal carcinoma, and classical Hodgkin lymphoma.

When administered as single agent in advanced BC, pembrolizumab, and other ICI such as atezolizumab or avelumab generated moderate but detectable antitumor activity, with objective response rate ranging between 3 to 18% (56). Of note, efficacy was higher in patients with TNBC, minimal pre-treatment exposure, and PD-L1- and/or TILs-positive tumors. Yet, in the KEYNOTE119 randomized phase III study involving pre-treated advanced TNBC, pembrolizumab was not better than chemotherapy at physician's choice (57).

There is also a solid rationale to combine anti-PD1/PD-L1 agents with chemotherapy in BC, which may have significant immunomodulatory effects, and may in turn increase the antitumor activity of PD-1 pathway inhibition (58, 59). Indeed, even though cytotoxic drugs have historically been considered as immunosuppressive, they can also have pro-immune properties (60–67) by i) depleting immuno-suppressive cells, including regulatory T-cells and myeloid-derived suppressor cells, which stimulate a quiescent anti-tumor immune response, ii) inducing an immunogenic cell death, iii) improving presentation of tumor antigens by upregulating their expression or that of the major histocompatibility complex (MHC) class I molecules, iv) up-regulating co-stimulatory molecules (B7-1) or down-regulating co-inhibitory molecules (PD-L1 or B7-H4) expressed on tumor or immune cells, thus boosting the activity of T-cell effectors, and vi) enhancing tumor cells sensitivity to T-cell–mediated lysis through fas-, perforin-, and granzyme B–dependent mechanisms.

Recent results from clinical studies in TNBC have confirmed the potential for combination of chemotherapy and ICI. First, in the IMpassion130 phase III randomized study, first-line atezolizumab plus nab-paclitaxel improved progression-free survival over nab-paclitaxel alone in advanced TNBC. Benefit was restricted to patients with PD-L1-positive tumors, in which a strong numerical advantage in OS was suggested, leading to approval by both FDA and EMA (68). Very recently, first results of the KEYNOTE-355 phase III randomized trial (69), comparing several chemotherapy regimens (nab-paclitaxel, paclitaxel or

carboplatine plus gemcitabine) plus placebo *versus* chemotherapy plus pembrolizumab in the same setting, have confirmed IMPassion130 results in terms of progression-free survival for patients with PD-L1 combined Positive Score (CPS) 10. However, statistical significance was not achieved in PD-L1 CPS 1 patients and OS data are still immature. In addition, IMpassion 131 failed to demonstrate any advantage for atezolizumab in combination with paclitaxel over paclitaxel alone (70). IMpassion 132, which evaluates atezolizumab with capecitabine or carboplatin-gemcitabine is still ongoing (71). Second, a significant improvement in pCR rate was recently reported when pembrolizumab was added to NACT (carboplatin/paclitaxel followed by AC) in non-metastatic TNBC, while preliminary analysis suggested a possible and promising advantage in event-free survival (72, 73). A similar improvement in pCR was recently demonstrated with atezolizumab when combined with anthracyclines/taxanes but carboplatin-free NACT in IMpassion 031 trial (74). Yet, results from other studies evaluating anti-PD-L1 antibodies such as durvalumab, another anti-PD-L1 antibody in combination with anthracyclines/taxanes (75), or atezolizumab in combination with anthracyclines-free regimen (NeoTripp trial: NCT002620280) NACT in TNBC failed to significantly improve pCR rates (76). Thus, the role of ICI in NACT of early BC remains to be defined. Of note, in both advanced and early settings, no new signal of toxicity was detected, and tolerance was similar to what expected with ICI in other tumor types.

# METHODS OF PELICAN-IPC 2015-016/ ONCODISTINCT-003 STUDY

The currently insufficient results of NACT in IBC, the relatively peculiar immune microenvironment of IBC when compared to non-IBC, and efficacy of pembrolizumab in BC led us to launch the PELICAN trial.

## Study Design and Participants

PELICAN-IPC 2015-016/Oncodistinct-003 is a prospective, multicenter, open-label, randomized, non-comparative, phase II study evaluating pembrolizumab in combination with NACT in HER2-negative IBC. The trial was registered in ClinicalTrials.gov database (NCT03515798). The study, promoted by Institut Paoli-Calmettes (Marseille, France) is being conducted in up to 21 centers (13 in France, 8 in Belgium), 10 of them being activated on January 2020. Patients are eligible to the study, if they have a previously untreated, histologically-confirmed diagnosis of HER2-negative IBC as defined according to 8$^{th}$ American Joint Committee on Cancer (AJCC) classification: breast erythema, edema and/or *peau d'orange*, occupying at least 1/3 of the breast, with or without underlying palpable mass, duration of history of no more than 6 months. The main other inclusion and exclusion criteria are listed in **Table 1**. In PELICAN trial, all HER2-negative IBC patients are eligible, resulting in a mixed population of triple-

**TABLE 1** | Eligibility criteria of the PELICAN-IPC 2015-016/Oncodistinct-003 trial.

| |
|---|
| Inclusion criteria |
| - HER2 negative tumors by immunohistochemistry (IHC 0 or 1+) or fluorescent/chromogenic *in situ* hybridization (FISH- or CISH-) |
| - Hormone receptors status known |
| - Previously untreated, histologically confirmed diagnosis of breast cancer and confirmed inflammatory breast cancer |
| - No metastases |
| - No organ dysfunction, especially adequate cardiac, kidney, liver and hematologic function |
| - At least 18 years |
| - Performance status (ECOG) 0 or 1. ECOG 2 may be considered if good rationale provided and discussed |
| - A female participant if she is not pregnant, not breastfeeding, if she is a woman of childbearing potential (WOCBP), who agrees to the follow contraceptive guidance during the treatment period and for at least 12 months after the last dose of cyclophosphamide and 4 months after the last dose of pembrolizumab, whichever come last. Abstinence is acceptable |
| - A male participant must agree to use a contraception during the treatment period and for at least 6 months after the last dose of study treatment and refrain from donating sperm during this period |
| Exclusion criteria |
| - Bilateral breast cancer |
| - Prior allogeneic stem cell or solid organ transplantation |
| - WOCBP who has a positive serum pregnancy test within 72 h prior to randomization |
| - Current participation in or recent participation in a study of an investigational agent or use of an investigational device within 4 weeks prior to the first dose of study treatment |
| - Active CNS disease or carcinomatous meningitis |
| - Diagnosis of immunodeficiency or is receiving systemic steroid therapy (in dosing exceeding 10 mg daily of prednisone equivalent) or any other form of immunosuppressive therapy within 7 days prior to the first dose of study drug, |
| - Known history of active bacillus tuberculosis |
| - Severe hypersensitivity (grade 3) to pembrolizumab and/or any of its excipients |
| - Known additional malignancy that is progressing or has required active treatment within the past 3 years. Note: participants with basal cell carcinoma of the skin, squamous cell carcinoma of the skin, or carcinoma in situ (e.g., breast carcinoma, cervical cancer *in situ*) that have undergone potentially curative therapy are not excluded. |
| - Active autoimmune disease that has required systemic treatment in the past 2 years |
| - Active infection requiring systemic therapy or history of Human Immunodeficiency Virus, Hepatitis B or C |
| - Delivery of a live vaccine within 30 days prior to the first dose of study drug |
| - Known psychiatric or substance abuse disorders that would interfere with cooperation with the requirements of the trial |
| - Prior therapy with an anti-PD-1, anti-PD-L1, or anti PD L2 agent or CTLA-4 |

negative and hormone receptor-positive/HER2-negative tumors. While we acknowledge that this is a significant limitation of the study, this is justified by the rarity of the disease and the anticipated difficulties of recruitment if restricted to a single IBC subtype. In addition, previous studies in the field, which provided the basis to our statistical hypothesis, were performed in a similar setting. Moreover, the design includes stratification on hormone receptor status and will allow a specific analysis in triple negative subtype, the most likely to benefit according to recent results (see Statistics section).

## Study Procedures and Treatment

Patients are to be randomly assigned within 28 days from initiation of screening with a 2/1 ratio between NACT without (arm A) or with (arm B) pembrolizumab. The randomization procedure is assessed with block and is stratified by centers and hormone receptor status (positive HR is defined as tumor cell staining by immunohistochemistry ≥10% for ER and/or PR). To increase the randomness of the assignments, the permuted-block randomization schedule is generated within varying block sizes. A minimum and maximum number of patients of each phenotype (TN/non-TN) are respected in order to keep the adequate power.

In the experimental arm, pembrolizumab (intravenous administration at a dose of 200 mg every 3 weeks starting on cycle 2) is combined with conventional anthracycline/taxane-based NACT. Since anthracyclines have been shown to strongly induce immunogenic cell death, IFN gamma production and dendritic and T-cell tumor infiltration in mouse models (60, 61, 64, 66, 67), pembrolizumab is started on cycle 2, assuming that it should maximize potential sequential synergism. In addition, differing pembrolizumab initiation on cycle 2 should help evaluating the safety of the combination, by identifying patients with specific chemotherapy-induced toxicities. In the initial version of the protocol, the anthracyclines part of NACT was different according to the HR status: non-TN IBC (HR-positive and HER2-negative) patients were to receive 3-weekly 5-fluorouracil 500 mg/m², epirubicin 100 mg/m², cyclophosphamide 500 mg/m² (FEC100) from C1 to C4, whereas TN IBC (HR-negative and HER2-negative) were to receive epirubicin 90 mg/m² and cyclophosphamide 600 mg/m² every 2 weeks with G-CSF support, i.e., a dose dense (DD-EC) regimen from C1 to C4. A subsequent amendment homogenized the anthracycline-based schedule and all patients are now to receive DD-EC, whatever the HR status. Following anthracyclines sequence, all patients receive weekly paclitaxel for 12 injections (from C5 to C8). Details of design are shown in **Figure 1**.

Mastectomy with axillary lymph node dissection is to be performed within 4–6 weeks after the last chemotherapy administration. Pathological analysis is assessed by the local pathologist of each center and by a centralized reviewer. Radiotherapy starts 3 to 6 weeks after the surgery. Dose and frequency are left to investigator's discretion and according to the site's standard practice. Adjuvant endocrine therapy (if HR-positive disease) and/or capecitabine (if residual disease in TN IBC and no DPD enzyme deficit identified by plasma uracil dosage) is to be given after radiotherapy completion according to the site's standard practice. After local treatment is completed, patients are followed every 4 months (+/- 28 days) for 2 years, then every 6 months for 3 years, and every year until the end of the study.

## Outcomes

The primary endpoint is a central evaluation of pCR rate (as defined as ypT0/Tis, ypN0 following 8th AJCC classification) of the resected breast specimen and all sampled ipsilateral lymph nodes following NACT with/without pembrolizumab. A co-primary endpoint of safety is also included to determine if combining pembrolizumab and DD-EC exposes to significant toxicity. Thus, a run-in safety phase is to be conducted in order to stop the trial in case of unacceptable toxicity, as defined by the incidence of dose limiting toxicities (DLTs).

The secondary endpoints include the safety profile and tolerability of the combination, pCR rate by local assessment, invasive disease-free, event-free, and overall survivals (iDFS, EFS, and OS) in each arm. Since identification of predictive

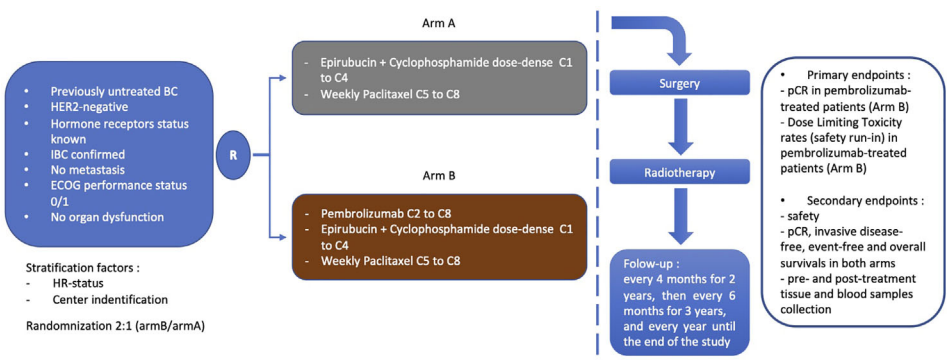


**FIGURE 1** | Design of PELICAN-IPC 2015-016/Oncodistinct-003 trial. BC, breast cancer; IBC, inflammatory breast cancer; HR, hormone receptor; pCR, pathological complete response.

biomarkers for pembrolizumab efficacy is of critical importance, pharmacodynamics measurements and search for biological/immunological correlates are planned on pre- and post-treatment tissue and blood samples regularly collected before, during, and after treatment.

### Statistics

The PELICAN trial is designed and powered to demonstrate that experimental group (arm B) achieves a pCR rate higher than a predefined undesirable rate of 20%. To reject the null hypothesis of a truly inefficient regimen (H0: p≤20%) at 5% error risk, following a Simon's 2-stage optimal procedure, a total of 54 patients in arm B is necessary to obtain a power of 90% assuming a true pCR rate of 40%. Furthermore, to reach a power of 80% to reject the null hypothesis at 5% error risk in HR-negative (arm B), there must be at least 32 HR-negative patients and no more than 25 HR-positive patients to be recruited in arm B. A total of 27 patients are planned to be enrolled in the control arm (arm A), leading to a total of 81 patients planned. Predefined pCR of 20% was set-up according to several studies demonstrating a pCR rate between 20% and 28% in HER2-negative IBC receiving anthracyclines-taxanes NACT. The duration of enrolment is planned to 24 months. The randomized phase II design was selected to provide a control arm not directly compared to the experimental arm but allowing verifying the expected pCR rate with a conventional NACT in the selected population.

According to a 2-stage optimal design, an interim analysis is planned when 19 patients will be evaluable for the primary endpoint (pCR) in the experimental arm. At this interim evaluation, the study will stop for futility if no more than 4 patients have a documented pCR. If the patient accrual continues, the true pCR rate following NACT plus pembrolizumab will be estimated at the end of study with 90% confidence interval in all patients who received at least one dose of pembrolizumab. The overall hypothesis of a truly ineffective experimental arm will be accepted if the lower bound of the above estimated 90% bilateral confidence interval (CI) is inferior to 20%, or equivalently if no more than 15 patients out of a total of 54 evaluable patients have a documented pCR. In addition, the true pCR rate in patients enrolled in the HR-negative (TN IBC) strata will also be estimated. In this subgroup analysis, the true pCR rate will be estimated with 90% bilateral CI using an exact method for binomial proportions in one-stage clinical trials. The hypothesis of a truly ineffective experimental arm in this subgroup of interest will be accepted if the lower bound of the above estimated 90% exact bilateral CI is inferior to 20% and rejected otherwise.

According to standard practice in phase I studies, run-in phase conducted will enroll a maximum of 6 patients who completed 21 days after the first administration in two consecutive sub-cohorts (3+3). If at least 2 out of the 3 patients enrolled in the first sub-cohort report a DLT episode, the accrual of patients will be stopped, and the combination will be declared too toxic to warrant further investigation. If 1 or less than 1 patient (≤1 patient) reported a DLT in the first sub-cohort, 3 additional patients will be enrolled in the run-in phase. At the end, the combination will be declared sufficiently safe if less than 2 patients report a DLT out of the 6 evaluable patients enrolled in the run-in phase.

The incidence of reported adverse events during the treatment period will be summarized according to the treatment arm, by primary system organ class, CTCAE v5.0 severity grade, type of adverse event, and relationship to the study drug. Locally assessed pCR rate will be estimated with 90% exact confidence intervals. Time-to-event outcomes will be censored at the time of last follow-up visit. IDFS, EFS and OS will be estimated using the Kaplan-Meier method. Pointwise estimations for 3-year and 5-year IDFS, EFS, and OS will be provided with corresponding 90% asymptotic confidence interval.

## CONCLUSION

To our knowledge, the PELICAN-IPC 2015-016/Oncodistinct-003 study (NCT03515798) is the first one to investigate the efficacy of ICI in patients with IBC, a rare but difficult-to-treat form of BC. Pembrolizumab is combined to chemotherapy in the neoadjuvant setting. Even if the recent most promising results remain modest in BC compared with more immunogenic cancers such as lung cancer or melanoma, they are significant notably in metastatic TNBC when combined to chemotherapy. Furthermore, IBC display few molecular characteristics that may suggest higher efficiency than in non-IBC: more frequent TN subtype, more frequent PD-L1-positivity and higher TMB independently from the molecular subtypes. Enrolment began in July 2018 and the estimated study completion date is 2022.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Protection des Personnes IIe de France VII (hôpital de Bicêtre, Le Kremlin Bicêtre, FRANCE). The participants or legal guardian provided written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AG was involved in the conceptualization of the manuscript. AB wrote the first draft of the manuscript. FB and AG revised the manuscript. AB was in charge of the tables and figure. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Lactate Metabolism and Immune Modulation in Breast Cancer: A Focused Review on Triple Negative Breast Tumors

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Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer associated with poor prognosis, early recurrence, and the lack of durable chemotherapy responses and specific targeted treatments. The recent FDA approval for immune checkpoint inhibition in combination with nab-paclitaxel for the treatment of metastatic TNBC created opportunity to advocate for immunotherapy in TNBC patients. However, improving the current low response rates is vital. Most cancers, including TNBC tumors, display metabolic plasticity and undergo reprogramming into highly glycolytic tumors through the Warburg effect. Consequently, accumulation of the metabolic byproduct lactate and extracellular acidification is often observed in several solid tumors, thereby exacerbating tumor cell proliferation, metastasis, and angiogenesis. In this review, we focus on the role of lactate acidosis in the microenvironment of glycolytic breast tumors as a major driver for immune evasion with a special emphasis on TNBCs. In particular, we will discuss the role of lactate regulators such as glucose transporters, lactate dehydrogenases, and lactate transporters in modulating immune functionality and checkpoint expression in numerous immune cell types. This review aims to spark discussion on interventions targeting lactate acidosis in combination with immunotherapy to provide an effective means of improving response to immune checkpoint inhibitors in TNBC, in addition to highlighting challenges that may arise from TNBC tumor heterogeneity.

**Keywords: triple negative breast cancer, lactate acidosis, immunotherapy, tumor metabolism, Warburg effect, metabolic reprogramming, anti-tumor immunity, immunosuppression**

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## INTRODUCTION

Inter- and intra-tumor heterogeneity of breast tumors are a major causal factor for prognostic and drug response disparities. Among the breast cancer molecular subtypes, triple negative breast cancer (TNBC), accounting for 15–20% of all breast cancers, is defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (Her-2) expression (1). TNBCs are particularly characterized by poor prognosis, early recurrence, and

increased risk of metastasis, cumulatively accounting for 25% of all breast cancer-related deaths (2). In addition, the lack of hormone receptor expression renders TNBC tumors refractory to the targeted therapeutics currently being implemented for the treatment of hormone receptor positive breast cancer subtypes, essentially limiting treatment options to chemotherapy. Although TNBC tumors initially respond well to chemotherapy, they develop resistance and display early recurrence rates (3). In addition, the molecular heterogeneity within TNBCs has led to its classification into several intrinsic subtypes, further adding to the predicament of developing personalized approaches to treat TNBCs (4, 5).

Immunotherapy has revolutionized the treatment of several cancer types, particularly melanoma, lymphoma, renal cell cancer, and non-small cell lung cancer (6). This treatment modality involves activating the host immune system to recognize and eliminate tumor cells. Numerous types of cancer immunotherapy are being trialed and implemented, as reviewed in detail elsewhere (7). Immune checkpoint blockade (ICB) has progressed most prominently as an effective immunotherapy by targeting inhibitory T cell regulatory molecules such as programmed cell death-1 (PD-1), its ligands programmed cell death ligand 1/2 (PD-L1/L2), and cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), thereby re-invigorating the anti-tumor immune response (8). In 2019, the US Food and Drug Administration (FDA) approved the use of Atezolizumab, a blocking antibody targeting PD-L1, in combination with nab-paclitaxel chemotherapy for first-line treatment of unresectable, PD-L1 positive, locally advanced, or metastatic TNBC. Although this is the only immunotherapy currently available to TNBCs, there are several clinical trials evaluating the efficacy of ICB in TNBCs as monotherapy or in combination with other treatment modalities (9).

Some key factors that influence the response to immunotherapy in solid tumors include the extent of tumor immune infiltration and the expression of immune checkpoint molecules. Within the breast cancer subtypes, TNBCs are considered to be the most immunogenic (10), in part due to higher levels of tumor-infiltrating lymphocytes (TILs), and higher tumor mutational burden and neoantigen load. Concordantly, TNBCs are enriched in the expression of immune checkpoint molecules, either on tumor cells or on infiltrating immune cells (11, 12). These properties provide rationale for the responsiveness of TNBCs to ICB compared to other breast cancer subtypes. Nonetheless, considering the heterogeneity of this subtype, only a small proportion of TNBCs indicate an immunomodulatory phenotype amenable to targeting with immunotherapy. Ali et al. reported that only 20% of TNBCs expressing core basal markers exhibit PD-L1 expression. Moreover, single-agent ICB response rates in unselected metastatic TNBC patient cohorts still remain low with limited durability (13).

Thus, improving the efficacy of immunotherapy in TNBCs requires a better understanding of factors that influence tumor immune infiltration and immune evasion. In this regard, tumor metabolism is known to play a critical role in shaping the tumor and immune microenvironment. Within the scope of this review, we will discuss the molecular factors driving the glycolytic nature of TNBCs, and explore their role in lactate-mediated modulation of the anti-tumor immune response. Finally, we will assess the clinical benefit of combining targeting of lactate metabolism with immune checkpoint blockade to improve the efficacy of immunotherapy in TNBCs.

## METABOLIC PLASTICITY IN TNBC

Under normal conditions, oxidative phosphorylation (OXPHOS) is the preferred mode of energy generation in somatic cells, including normal mammary epithelial cells. Particularly during lactation, glucose uptake is significantly increased in the mammary cells, the major proportion of which is metabolized to lactose in the Golgi apparatus (14). Under circumstances of oxygen deprivation, cells may switch from aerobic OXPHOS to glycolytic metabolism to reduce the generation of reactive oxygen species (ROS) and hence alleviate hypoxic stress (15). Likewise, rapidly dividing tumor cells rewire cellular metabolism to meet the high bioenergetic and anabolic demands of growing tumors in a nutrient-deprived microenvironment. This tumor characteristic or cancer hallmark is known as the 'Warburg effect', whereby tumors shift their metabolic preference from OXPHOS to aerobic glycolysis, even under oxygen-rich conditions (16). The shift to aerobic metabolism is thought to result from both intrinsic and extrinsic cues (17). Intrinsically, oncogenic mutations, aberrant expression of microRNAs and transcription factors, and cumulative mitochondrial defects in tumor cells instigate metabolic reprogramming (18–20). Extrinsic cues that promote metabolic reprogramming include reduced oxygen and nutrient availability, decreased extracellular pH, and microenvironment interactions with immune and stromal cells and the extracellular matrix (ECM). TNBC tumors often exhibit several of these features, rendering them more sensitive to metabolic reprogramming. TNBC cells show increased rates of glycolysis, as inferred from increased glucose uptake, overexpression of glycolytic enzymes, and increased oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), in comparison to other breast cancer subtypes (21–23). Furthermore, TNBC cell lines display more glycolytic dependence compared to luminal breast cancer cell lines whereby treatment with the glycolysis inhibitor 2-deoxyglucose (2-DG) markedly reduced cell proliferation in TNBC cells (24). To gain insight into how the glycolytic nature of TNBCs may affect anti-tumor immunity and how this can be exploited for therapeutic purposes, it is important to identify the key molecules involved in the metabolic adaptation. In this context, we will explore any alterations in molecular determinants of glucose uptake, lactate to pyruvate interconversion, and lactate transport.

### Aberrant Expression of Glucose Transporters

The Warburg effect observed in tumors depends on the availability of glucose as a substrate. Glucose uptake into the

cell is mediated by GLUT transporters, a family of transmembrane proteins, of which GLUT1 is the most widely expressed isoform in cancers, particularly in basal-like TNBC (25). In TNBC, GLUT1 overexpression correlates with higher histological tumor grade (26). Interestingly, silencing of GLUT1 in TNBC models reduces both cell proliferation and invasive potential, thus highlighting the role of GLUT1 and indirectly glucose scavenging in supporting the aggressive tumor behavior of TNBC (27). The expression of glucose transporters is regulated by c-Myc, a basic region helix–loop–helix leucine zipper (bHLHZip) transcription factor serving as a hub in regulating a broad range of cancer-related signaling pathways (28). Oncogenic mutations in c-Myc leading to overexpression are often observed in TNBC tumors whereby c-Myc functions antagonistically with MondoA, a nutrient-sensing transcription factor allowing cells, to adapt to changes in glycolytic flux (18, 29). Mechanistically, c-Myc upregulation in TNBCs directly suppresses the MondoA-dependent induction of thioredoxin-interacting protein (TXNIP), an inhibitor of glucose uptake and glycolysis, through competitive binding of the TXNIP promoter region (29). As TXNIP regulates the mRNA expression and protein stability of GLUT1, its suppression by c-Myc eventually results in enhanced glucose metabolism (30). In concordance, a $Myc^{high}/TXNIP^{low}$ signature correlates with poor clinical outcome in TNBC but not in non-TNBC subtypes (31). Moreover, this correlation was more prominent in the presence of p53 mutations which are frequently found in TNBC tumors, suggesting an indirect association between tumor mutation status and metabolism (19). Of interest, a familial genetics study reported a homozygous point mutation in the *TXNIP* gene that completely suppressed its expression, leading to lactate acidosis in the affected individuals (32). The presence of mutant TXNIP variants in breast cancer is yet unknown. Expression of GLUT1 can also be regulated through hypoxia response elements by hypoxia-inducible factor (HIF)-1a whose expression is correlated with *BRCA1* and basal phenotypes in breast cancer such as those observed in TNBC (33, 34). Another mechanism that supports GLUT1 stabilization, specifically in basal-like TNBC cells, involves the suppression of GLUT1 endocytosis and Akt-mediated degradation by the GTPase-activating protein USP6NL (35). Thus, TNBC tumors are intrinsically primed for enhanced glucose uptake to support their glycolytic phenotype. Although several long non-coding RNA, such as ANRIL and HOTAIR, have been shown to regulate GLUT expression in various tumor types, no reports are available yet for breast cancer (36).

## Upregulation of Lactate Dehydrogenases

Lactate dehydrogenases (LDHs) are key enzymes in glycolysis, regulating the interconversion of pyruvate to lactate. There are five L-lactate dehydrogenase isoforms that are composed of different combinations of LDH-M (M for muscle) and LDH-H (H for heart) subunits: LDH-1 (H4), LDH-2 (H3M1), LDH-3 (H2M2), LDH-4 (H1M3), and LDH-5 (M4) (37). The LDH-M and LDH-H subunits are encoded by the *LDHA* and *LDHB* genes and are alternatively denoted as LDHA and LDHB, hence, LDH-5 (M4) and LDH-1 (H4) are often referred to as LDHA and LDHB respectively. The LDH isoforms are associated with different tissue specificity with LDH-1/LDHB predominantly being expressed in the heart, LDH-5/LDHA in striated muscle, LDH-2 in the reticuloendothelial system, LDH-3 in the lungs, and LDH-4 in the kidneys. Additionally, there is a sixth isoform, LDHC or LDHX, that is composed of four LDHC subunits and is exclusively expressed in testis tissue (38). LDHA and LDHC preferentially catalyze pyruvate to L-lactate conversion, while LDHB has a higher affinity for lactate, thus collectively determining the rate of glycolysis.

In addition to their widespread expression in normal tissues, LDHA and LDHB are often overexpressed in tumor tissues, including TNBC. Furthermore, elevated circulating total LDH levels have been found to predict clinical outcome and treatment response to chemotherapy in advanced TNBC patients (39). LDHA expression is significantly upregulated in TNBC tumors compared to non-TNBC tumors and is associated with shorter overall- and disease-free survival (40). Increased tumoral and serum LDHA levels have also been correlated with brain metastasis and poor survival in patients with TNBC (41). In line with this finding, knocking down LDHA expression in the syngeneic 4TI TNBC mouse model decreased tumor-derived lactate levels, tumor growth rate and metastases (42). LDHB is also upregulated in TNBC (24) and PAM50 basal-like subtypes (43). The function of LDHB in breast cancer or more specifically TNBC remains ambiguous. The role of LDHB in promoting lysosomal acidification required for autophagy-associated vesicle maturation and protease activation has been reported as a mechanism by which LDHB can promote tumor cell proliferation and survival in some cancer types (44). High LDHB expression in basal-like breast cancer has been associated with better pathological complete response rates to neoadjuvant chemotherapy (43). LDHB has been reported to complement the role of LDHA in colon adenocarcinoma and melanoma models with metabolic pressure (45). More specifically, knockout of both LDHA and LDHB was required to suppress glycolysis under hypoxic conditions and hence, curb tumor growth, but under normoxic conditions the tumor cell metabolism shifted to OXPHOS as an energy source. Although the substrate preference of LDHA and LDHB differs, these observations indicate that substrate affinity and the extent of metabolic adaptation in tumors may vary depending on both tumor-specific intrinsic and extrinsic cues. The LDHC isoenzyme is an immunogenic germline-specific antigen that is re-expressed in a wide variety of cancer types (46, 47). Particularly, high levels of circulating LDHC in serum and tumor-derived exosomes are negatively correlated with breast cancer prognosis (48). Expression of LDHC has been reported to play a role in propagating TNBC tumor cell invasion and migration (49). To date, LDHC has been implicated in glycolysis and energy metabolism of sperm only (50).

From the current literature, LDHA appears to be a key enzyme in TNBC-associated lactate acidosis. Studies in different cancer types have reported that LDHA overexpression stems from mechanisms involving transcriptional, post-

transcriptional, and post-translational regulation (37). For instance, HIF-1a, c-Myc and the forkhead box M1 (FOXM1) transcription factor have been shown to bind to the LDHA promoter region to regulate its transcription (51). However, it remains to be understood if these regulatory mechanisms are ubiquitous across different cancers or alternative modes of regulation exist in TNBC. Moreover, the metabolic role of LDHB and LDHC in TNBC require thorough investigation.

## Dysregulation of Lactate Transport and Metabolic Symbiosis

The concentration of lactate in solid tumors has been reported to be chronically high (up to 50 mM) in comparison to physiological levels in the blood (up to 2 mM) (15). Quantification of lactate concentration in freshly excised tumors from a small cohort of 30 breast cancer patients using double quantum filtered magnetic resonance spectroscopy indicated that a higher tumor grade was associated with increased lactate concentration (52). A trend of increased mean lactate concentration (8.4 mM) was also reported in a small group of six TNBC tumors compared to nine non-TNBC tumors (7.2 mM) in the same study, however this observation needs confirmation in a larger population. Lactate was initially thought to be a mere waste metabolite from aerobic glycolysis. However, we now know that lactate has an active tumorigenic role as a biosynthetic precursor, signaling molecule and regulator of extracellular acidosis, and has therefore been referred to as an "oncometabolite" (53). Considering the excessive rate of glycolysis in tumors, the intracellular concentration of lactate can accumulate rapidly, serving as a rate-limiting step within the glycolysis pathway and impairing enzymatic function and cell proliferation. To avoid excessive pools of intracellular lactate, lactate is transported across the plasma membrane by the monocarboxylate family of transporters (MCTs) that are encoded by solute carrier 16 (*SLC16*) genes. Among these transporters, MCT1 (SLC16A1) and MCT4 (SLC16A3) have been extensively characterized in multiple tumors (54). MCTs are passive symporters transporting lactate anions in conjunction with protons, implying their function in equilibrating the lactate concentration and pH gradient across the intra- and extra-cellular compartments. Generally, MCT1 is involved in lactate import or export depending on the cell type and context while MCT4 primarily functions in lactate efflux from glycolytic cells into the microenvironment.

According to the Warburg effect, tumor cells undergo a metabolic switch to aerobic glycolysis whereby glycolytic tumor cells expressing MCT4 export lactate and oxidative tumor cells or stromal cells with high MCT1 expression import lactate to use as an energy source through OXPHOS. In contrast, the reverse Warburg effect offers a state of metabolic symbiosis with reciprocal interactions between tumor and stromal cells, whereby glycolytic stromal cells provide lactate as a fuel to oxidative tumor cells (55). The existence of the reverse Warburg effect in TNBC tumors is under debate with some studies advocating for the traditional Warburg effect or a mixed model while others provide experimental evidence for the presence of the reverse Warburg phenotype (**Figure 1**). In support of the former, an immunohistological study by Choi et al. classified 740 breast cancer cases into different metabolic subgroups based on the expression of metabolic markers such as GLUT1 and MCT4 (56). Tumors were either considered to be of the Warburg type (glycolytic tumor cells and non-glycolytic stromal cells), reverse Warburg type (non-glycolytic tumor cells and glycolytic stromal cells), mixed type (glycolytic tumor cells and stromal cells), or null type (non-glycolytic tumor cells and stromal cells). Based on this classification, the majority of TNBC tumors displayed a Warburg or mixed metabolic phenotype, both characterized by high MCT4 expression, while luminal-type breast tumors mainly belong to the reverse Warburg or null metabolic phenotype, consistent with their metabolically inactive and less aggressive clinical presentation. In accordance, MCT4 expression strongly correlates with worse survival in TNBC as compared to luminal-type breast cancer (23, 56). TNBC tissue microarrays indicated that basal-like TNBC tumors in particular expressed glycolysis markers such as GLUT1 and MCT4, whereas non-basal-like TNBCs were represented by a glutaminolysis or mitochondrial metabolism phenotype (57). Furthermore, MCT4 ablation in the TNBC cell line MDA-MB-468 reduced cell viability and lactate secretion, enhanced OXPHOS, sensitized cells to mitochondrial respiration inhibitors, and impeded orthotopic tumor growth (58).

In support of the reverse Warburg phenotype, Witkiewicz et al. identified that MCT4 expression in stromal cells, but not tumor cells, was associated with poor survival in TNBC (59). In addition, loss of stromal caveolin-1, an indicator of hypoxia, has been associated with selective MCT4 stromal and MCT1 tumor expression and poor clinical outcome in TNBC (60). Combining positive stromal MCT4 with negative stromal Caveolin-1 expression improved stratification of TNBC cases with a high risk of recurrence and metastasis. Moreover, MCT1 expression in tumor cells showed a strong positive correlation with LDHB expression in TNBC tumors, corroborating the presence of the reverse Warburg effect (24). More specifically, basal-like TNBC tumors demonstrate increased MCT1 expression that is associated with a high proliferative index and histological grade (61). Of note, silencing of MCT1 in basal-like TNBC models disrupted lactate export and tumor growth *in vivo* (62), suggesting that MCT1 can adapt for bidirectional lactate transport in tumors.

In addition to the classical Warburg, reverse Warburg and mixed metabolic phenotype models, few studies have suggested the existence of a hybrid metabolic state in TNBC tumors and metastatic lesions (**Figure 1**) whereby tumor cells exhibit both high glycolytic and OXPHOS activity, allowing these tumors to switch between metabolic phenotypes for their bioenergetic demands in response to microenvironmental cues (63, 64). Targeting both glycolysis and OXPHOS in metastatic TNBC cells was required to eliminate this metabolic plasticity and hence, reduce their proliferation and survival.

Mechanistically, elevated MCT1 in TNBCs has been attributed to low levels of its regulatory miRNA miR-342-3p (65). In addition, the stability and localization of MCT1 and

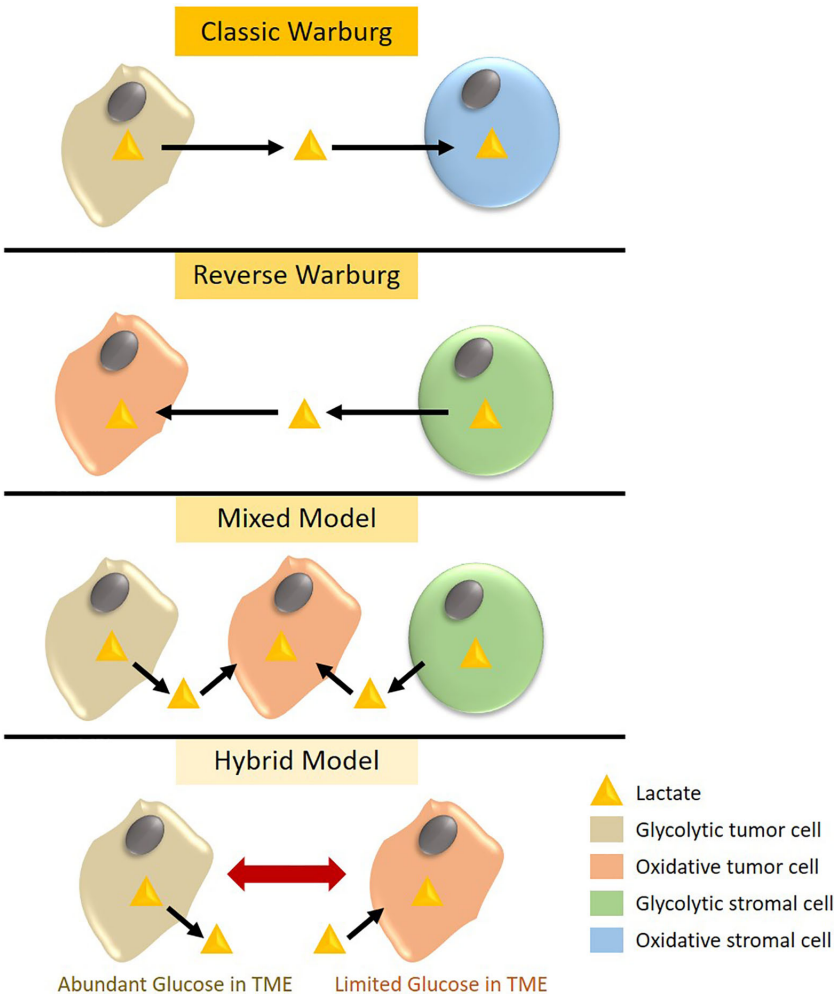


**FIGURE 1** | Metabolic phenotypes observed in triple negative breast cancer (TNBC). According to the classic Warburg theory, glycolytic TNBC cells expressing high levels of the lactate transporter MCT4 export lactate, which is taken up by MCT1-expressing stromal cells to generate energy through oxidative phosphorylation (OXPHOS). Alternatively, MCT4 expressing glycolytic stromal cells can export lactate that is used by oxidative tumor cells in a phenomenon called the reverse Warburg effect. The mixed model represents metabolic symbiosis in heterogeneous tumors whereby glycolytic tumor and stromal cells generate lactate to feed oxidative tumor cells. Lastly, the hybrid model depicts metabolic plasticity in TNBC tumor cells that can switch between a glycolytic and oxidative phenotype based on extrinsic cues and glucose availability in the tumor microenvironment (TME).

MCT4 are regulated by the chaperone glycoprotein CD147 that is upregulated in TNBCs compared to other breast cancer subtypes. CD147 expression is directly correlated with high tumor grade, basal markers, shorter progression-free and overall survival, and poor response to chemotherapy in TNBC (66, 67). Furthermore, the lactate sensing G-protein-coupled receptor 81 (GPR81), also known as hydrocarboxylic acid receptor 1 (HCAR1) has been implicated in an autocrine feedback loop that regulates MCT1 and/or MCT4 expression and their chaperone CD147 (68–70). GPR81 is highly expressed in many tumor types including breast cancer, in particular hormone receptor positive breast cancers where it is associated with improved overall survival and lower risk of distant metastasis (69, 71, 72). Silencing of GPR81 in hormone receptor positive breast tumor cells reduced the expression of specifically MCT1 but not of MCT2 or MCT4, resulting in decreased lactate uptake, extracellular acidification, and inhibition of tumor cell proliferation and survival (69). Hence, GPR81 may support the OXPHOS phenotype in these breast tumors by sensing and regulating influx of extracellular lactate. However, the role of GPR81 in TNBC-associated lactate signaling has not yet been reported and mandates future investigation. It is plausible that the high levels of lactate in the TNBC micromilieu constitutively activate GPR81, resulting in a negative feedback loop yielding reduced levels of GPR81 in glycolytic TNBC tumors. Alternatively, additional previously unidentified lactate-sensing GPCRs may play a role in TNBCs. Expression of GPR81 in tumor cells can be regulated through an autocrine feedback loop of lactate by the induction of Signal transducer and activator of transcription 3 (STAT3) that directly

binds to the GPR81 promoter to induce its expression (73). Interestingly, lactate-induced expression of GPR81 has been shown to trigger the tumor expression of the immune checkpoint ligand PD-L1, indicating an additional dimension of lactate-mediated immune dysregulation in the tumor milieu to dampen anti-tumor immunity (74), as will be discussed in the following section.

## LACTATE-RICH ENVIRONMENT MEDIATES IMMUNOSUPPRESSION

Normal mammary gland architecture comprises of diverse cell types, including immune cells, which are essential at various stages of mammary organogenesis (75, 76). During malignant transformation, the mammary gland undergoes considerable reorganization of the tissue architecture as well as changes in cellular composition and cellular properties (77). Likewise, the tumor microenvironment of breast tumors is comprised of numerous cell types, including tumor cells, cancer-associated fibroblasts, various cell types forming vascular networks and immune cells. The composition and functionality of this complex landscape is ultimately shaped by a network of interacting extracellular cues such as lactic acid, subsequently influencing the anti-tumor immune response (**Table 1**) (93). Here, we will specifically discuss lactate-mediated changes in anti-tumor immunity in TNBC, focusing on pro-inflammatory immune cell subsets such as T lymphocytes, natural killer cells, dendritic cells, as well as immune suppressive myeloid-derived suppressor cells, T regulatory cells, and tumor-associated macrophages.

### T Lymphocytes

The number of tumor infiltrating lymphocytes (TILs) has consistently been identified as a prognostic and predictive biomarker in early stage TNBC (94). However, as tumors progressively grow larger, metabolic competition ensues and impairs the activity of various immune cell subpopulations (95). Cytotoxic CD8+ lymphocytes (CTLs) profoundly rely on glycolysis for proliferation and activation of their effector function (96). Thus, high rates of glycolysis in TNBCs offer a competitive advantage for tumor cells by restricting cytotoxic T cell metabolism and functionality. In addition, there is a feedforward mechanism whereby the lactate-rich environment

**TABLE 1** | Impact of lactate acidosis on immune cells in the tumor microenvironment.

| Immune cell | Effect of lactate acidosis | References |
|---|---|---|
| **T lymphocytes** | - Diminished lactate export<br>- Decreased glycolysis, proliferation, and cytotoxicity<br>- Inhibited expression of IFN-γ and IL-2 cytokines<br>- Enhanced mitochondrial dysfunction and ROS production<br>- Increased apoptosis<br>- Polarization to iTregs | (78–80) |
| **NK cells** | - Decreased tumor infiltration, proliferation, and cytotoxicity<br>- Inhibited expression of activation receptors NKG2D and NKp46<br>- Dampened expression of IFN-γ, perforin, and granzyme<br>- Enhanced mitochondrial dysfunction and ROS production<br>- Impaired proliferation and differentiation of NKT cells | (81–83) |
| **DCs** | - Lactate sensed by GPR81 and imported by MCTs<br>- Decreased glycolysis<br>- Hindered maturation, activation, and antigen presentation<br>- Impaired priming of T cells<br>- Inhibited expression of IFN-α, IL-6, and IL-12 cytokines<br>- Upregulated expression of IL-10<br>- Increased production of kynurenine that induces Tregs | (72, 84, 85) |
| **MDSCs** | - Increased proliferation and immunosuppressive activity<br>- Induced development by tumor-derived G-CSF and GM-CSF | (81, 86) |
| **Tregs** | - Metabolic adaptation to suppress glycolysis and increase OXPHOS<br>- Increased survival and proliferation | (87, 88) |
| **Monocytes** | - Diminished lactate export<br>- Decreased glycolysis<br>- Inhibited expression of IFN-γ and TNF-α cytokines<br>- Upregulated expression of IL-17 and IL-23 cytokines | (89, 90) |
| **TAMs** | - Lactate sensed by GPR132 and imported by MCTs<br>- Polarization from M1 to anti-inflammatory/pro-tumorigenic M2<br>- Increased OXPHOS<br>- Upregulated expression of pro-tumorigenic ARG1, VEGF, and CCL5<br>- Enhanced secretion of immunosuppressive cytokines that subdue TIL cytotoxicity and promote Treg induction | (91, 92) |

*ARG1, arginase 1; CCL5, CC chemokine ligand 5; DCs, dendritic cells; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; GPR, G-protein receptor; IFN-γ, interferon gamma; IL, interleukin; MCT, monocarboxylate transporters; MDSC, myeloid-derived suppressor cells; NK, natural killer cells; NKG2D, natural killer group 2 member D; NKT, natural killer T cells; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TAMs, tumor-associated macrophages; TIL, tumor infiltrating lymphocyte; TNF- α, tumor necrosis factor alpha; Treg, T regulatory cell; VEGF, vascular endothelial growth factor.*

of glycolytic tumors interferes with lactate export in cytotoxic T cells, which depends on an active lactate gradient, therefore resulting in increased intracellular lactate levels that inhibit metabolism, proliferation, and production of interferon (IFN)-γ (78). In concordance, Lim et al. observed that epidermal growth factor receptor (EGFR) signaling in TNBC cells and murine models promoted aerobic glycolysis and lactate efflux, subsequently dampening the activation of CTLs and the production of IFN-γ and interleukin (IL)-2 (79). Similar observations have also been reported for highly glycolytic melanomas, wherein $LDHA^{high}$ tumors dampen IFN-γ-producing CD8+ T cells due to lactate acidosis (80). Conversely, reducing LDHA-mediated lactic acid production has been found to enhance T cell-mediated tumor killing, improve IFN-γ-producing T cell infiltration, and reduce melanoma tumor size (97–99). Furthermore, tumor-derived lactate enhances mitochondrial dysfunction and excess ROS production in naïve T cells, leading to apoptosis, by a mechanism involving the inhibition of focal adhesion kinase (FAK) family-interacting protein of 200 kDa (FIP200), a suppressor of the pro-apoptotic Bcl-2 family of proteins (100).

Molecular mechanisms driving this phenomenon involve the ability of lactic acid to inhibit IFN-γ transcription by preventing the upregulation of nuclear factor of activated T cells (NFAT), which is required for T cell and natural killer (NK) cell activation (80). Additionally, suppressed IFN-γ production has been linked to diminished mitogen-activated protein kinase (MAPK)/p38 and c-Jun N-terminal kinase (JNK) activity stemming from impaired T-cell receptor (TCR) activation under conditions of lactate acidosis in tumors (97). Finally, lactate acidosis, resulting from increased release of protons during lactate transport, has been shown to directly affect CTL cytolytic activity, cytokine secretion, and TCR activation, by lowering the pH in the tumoral niche (101).

## Natural Killer Cells

NK cells are innate effector lymphoid cells with anti-tumor cytolytic activity that is orchestrated by the secretion of pro-inflammatory cytokines and cytotoxic granules. In TNBC, NK cell infiltration has been associated with improved survival (102, 103). The inhibitory effect of lactate on NK cell cytotoxic activity has been reported for numerous cancers and involves downregulation of the expression of IFN-γ, perforin, granzyme, and the activating receptor NKp46 (81, 104). In line with this observation, glycolytic melanomas with high LDHA expression and lactate secretion show reduced NK cell activity and infiltration (80). In breast cancer specifically, tumor-infiltrating NK cells display decreased expression of the NKG2D activating receptor as compared to their counterparts in normal tissue (82). Inhibition of the lactate transporter MCT1 in the syngeneic 4T1 TNBC mouse model reduced lactate efflux and tumor growth, accompanied by an increased frequency of NKG2D/perforin/CD107a-expressing NK cells with improved cytotoxicity. Lactate-rich colorectal cancer liver metastasis exhibits a scarcity of NK cells with mitochondrial dysfunction and excessive ROS production leading to apoptosis, which could be recapitulated by treating healthy liver resident NK cells with lactic acid *in vitro* (105).

Invariant NKT cells, with properties of both NK and T cells, can also elicit an anti-tumor immune response by rapidly producing pro-inflammatory and immunomodulatory cytokines and cytotoxic perforin/granzyme B granules. Activation of NKT cells entails glucose uptake *via* the GLUT1 transporter and a glycolytic switch in metabolism, which is dependent on mTOR complex (mTORC) signaling (106). Exposure to high lactate levels inhibits NKT survival and proliferation. Mechanistically, acidosis induced by tumor-derived lactic acid inhibits the mTOR pathway and nuclear translocation of promyelocytic leukemia zinc-finger (PLZF), a regulator of NKT expansion and functional differentiation, resulting in impaired production of IFN-γ and IL-4 (83). The role, functional status, and prognostic value of NKT cells in TNBC remain to be investigated.

## Dendritic Cells

Dendritic cells (DCs) are a specialized class of antigen presenting cells involved in antigen processing and cross-presentation to CD8+ T cells. DC-mediated tumor rejection has been attributed to their ability to sense tumor-derived nucleic acids and activation of the type-I IFN system. Similar to CTLs, DCs rely on a metabolic switch from OXPHOS to glycolysis for activation, thus potentially ensuing metabolic competition within the tumor microenvironment (107). Lactic acid was shown to impair DC maturation, activation, cross-presentation, type-I IFN response, and antigen degradation (84, 108). In a syngeneic 4T1 TNBC mouse model, MCT-mediated lactate uptake by plasmacytoid DCs (pDCs), natural type I interferon–producing cells with antigen-presenting potential, inhibited their glycolysis capacity and thus IFN-α production while inducing the production of tryptophan-derived kynurenine and subsequent proliferation of T regulatory cells (Tregs) (85). In addition, GPR81 expressed on pDCs senses extracellular lactate and mobilizes intracellular calcium, which further has an inhibitory effect on DC activation and IFN-α expression. Lactate-dependent acidosis also inhibits DC differentiation through the induction of IL-10 production with concomitant loss of IL-12 (109). Similarly, lactate-mediated activation of GPR81 in DCs was found to abrogate antigen presentation, secretion of pro-inflammatory cytokines IL-6 and IL-12 and T cell function, and was associated with increased tumor growth in murine breast cancer models (72). In line with these findings, one study reported a high frequency of tumor-derived DCs with suppressed IFN-α production in aggressive, highly proliferative TNBC tumors, enabling the sustenance and expansion of Tregs and priming of anti-inflammatory IL-10-secreting CD4+ T cells (110).

Thus, the lactate-induced tolerogenic phenotype of tumor-infiltrating DCs indirectly impacts the priming of T lymphocytes and promotes an immunosuppressive cytokine profile and Treg expansion, collectively reinforcing tumor immune escape.

## Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSC) are immunosuppressive immune cells that restrict T cell function, proliferation, and TCR signaling, and promote differentiation of Tregs (111). In TNBC tumors, glycolytic gene expression profiles (including *LDHA*) correlate with MDSC gene signatures and both associate with reduced survival (86). Increased glycolysis and hence lactate production was found to induce MDSC development and immunosuppression in murine TNBC models through the activation of the LDHA/AMP-activated protein kinase (AMPK)-Unc-51 Like Autophagy Activating Kinase 1 (ULK1)/autophagy axis, thereby promoting the expression of granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) (86). Conversely, glycolytic restriction enhanced T cell immunity, reduced tumor growth and metastasis, and prolonged survival in the TNBC murine model (86). Depletion of LDHA to lower lactate production also decreased the frequency and immunosuppressive activity of MDSCs in a highly glycolytic murine pancreatic tumor model (81). This effect was directly attributed to lactate-mediated induction of MDSC proliferation, as observed by *in vitro* experiments supplementing lactate to human peripheral blood mononuclear cell co-cultures. Interestingly, as MDSCs rely on glycolysis for proliferation and their immunosuppressive activity by evading ROS-mediated apoptosis and enhancing mTOR pathway activation (112, 113), it remains to be understood how MDSCs thrive with metabolic competition in glycolytic tumor environments.

## T Regulatory Cells

Immunosuppressive Tregs undergo metabolic adaptation in low-glucose, lactate-rich tumor microenvironments. Specifically, an upregulation of the Treg-specific transcription factor forkhead box P3 (FOXP3) mediates induction of OXPHOS, alongside suppression of c-Myc expression and glycolysis (87). This metabolic reprogramming in Tregs, which are particularly enriched in TNBC tumors (114), makes them less dependent on glycolysis and enables the cells to efficiently turnover lactate into pyruvate. In addition, an increased nicotinamide adenine dinucleotide (NAD):NADH ratio in Tregs compensates for the lack of glycolytic activity and hence, renders them resistant to the inhibitory anti-glycolytic effects of lactate observed in T cells, and can polarize conventional T cells into induced Tregs (iTregs) that thrive on the metabolic symbiosis with glycolytic tumor cells (88).

## Tumor-Associated Macrophages

Tumor-associated macrophages (TAMs) are abundant in tumors, wherein extracellular stimuli guide their polarization between the pro-inflammatory "M1" subtype and anti-inflammatory "M2" subtype (115). In comparison to the glycolytic metabolism in M1 macrophages, M2 TAMs rely on OXPHOS to meet their bioenergetic demands—a trait that may additionally support the metabolic symbiosis between highly glycolytic TNBC tumor cells and M2 TAMs (116). Indeed, several studies have reported that M2 TAMs in TNBC tumor stroma positively associate with higher grade, larger tumor size, and poor survival whereas an inverse correlation has been observed in luminal A breast tumors that primarily depend on OXPHOS (117, 118). Moreover, co-culturing monocytes (precursors of macrophages and DCs) with the TNBC cell line MDA-MB-231 induced an M2 macrophage phenotype (119). Taken together, it can be envisaged that the lactate-rich landscape in TNBC tumors drives re-education of TAMs to an M2 phenotype. Indeed, tumor-derived lactate can induce TAM polarization to the M2 immunosuppressive phenotype by binding to the lactate-sensitive receptor GPR132 (91, 120). In turn, the M2 TAMs promote breast tumor cell migration and invasion *in vitro* and metastasis *in vivo*, thus supporting a positive feedback loop between tumor cells and pro-tumorigenic M2 TAMs. Concordantly, high GPR132 expression in breast cancer tumors correlated with the expression of M2 macrophage markers and low metastasis-free and relapse-free survival. Moreover, abrogating the lactate/GPR132 axis impedes M2 polarization and breast cancer metastasis in mice. In addition to lactate sensing by GPR132, lactate uptake by MCTs in TAMs also mediates M2 polarization (91). Lactate-induced TAM polarization and its pro-tumorigenic effects in breast cancer has been attributed to TAM-specific extracellular signal-regulated kinase (ERK)/STAT3 activation, stimulated expression of vascular endothelial growth factor (VEGF) and arginase-1 (ARG1), and stabilization of HIF-1a (91, 121, 122). Another mode of lactate-associated paracrine signaling between TAMs and breast tumor cells was reported by Lin et al., who showed that tumor cell-derived lactate induced the Notch pathway in TAMs to generate CC chemokine ligand 5 (CCL5) which then binds to its receptor CCR5 on breast tumor cells to promote aerobic glycolysis, migration, and epithelial-to-mesenchymal transition (EMT) (123). Besides driving tumorigenesis, M2 macrophages also secrete immunosuppressive cytokines that subdue the cytotoxicity of TILs and promote the differentiation of Tregs (92).

Furthermore, elevated levels of extracellular lactate prevent the expulsion of lactate generated in macrophage precursor monocytes, prompting a negative feedback mechanism for glycolysis and tumor necrosis factor (TNF) release (89). In toll-like receptor (TLR)-activated monocytes, lactic acid was also observed to induce the IL-23/IL-17 pathway, thus polarizing the immune response towards a pro-tumorigenic Th17 profile while suppressing the anti-tumor Th1 response (90, 124). Consistent with this observation, Th17 cytokines are upregulated in TNBCs compared to other breast cancer subtypes, especially in "immune-cold" tumors that are devoid of TILs (125). Thus, lactate imposes adverse effects on not only macrophage function and polarization, but also on its precursor monocytes.

# TARGETING LACTATE-MEDIATED IMMUNE EVASION IN TNBC: POTENTIAL STRATEGIES AND CHALLENGES

Metabolic reprogramming, lactate accumulation, and metabolic competition promotes immunosuppression in the tumor

microenvironment and is thus capable of modulating the efficacy of immunotherapy. In line with this, elevated tumor glycolysis has been reported as a negative prognostic indicator in immunotherapy. For instance, melanoma tumors that are refractory to adoptive T cell therapy (ACT) display high rates of glycolysis and reduced TILs (98). Likewise, elevated baseline serum LDH levels are associated with limited clinical benefit from ICB treatment in several tumor types including TNBC (126–128). Nevertheless, ICB therapy has shown promising results in certain glycolytic cancers such as TNBC. The success of ICB in these tumors might be in part the result of an anti-metabolic effect on both tumor and immune cells. PD-L1 expression on tumor cells has been found to support tumor glycolysis *via* the activation of mTOR/Akt pathway (95). Hence, anti-PD-L1 ICB may not only release T cell inhibition but also impair tumor glycolysis, lactate production, and metabolic competition between immune and tumor cells. In addition, ICB therapy may induce a shift in the metabolic needs of cytotoxic immune cells. While activation of T cell effector function relies on glycolysis, ligation or inhibition of PD-1 on T cells inhibits glycolysis and instead switches to fatty acid oxidation, which is crucial for maintaining T cell memory function and long-term anti-tumor activity (129). This phenomenon further allows T memory cells to thrive by reducing their dependence on glucose and hence avoiding metabolic competition within the tumor microenvironment. In addition to harnessing the anti-metabolic potential of ICBs, there is also evidence supporting exploiting tumor acidity to improve treatment response to ICB. In a preclinical study, Johnston et al. show that activation of the checkpoint molecule V-domain immunoglobulin suppressor of T cell activation (VISTA) was more prominent under acidic conditions such as those found in highly glycolytic tumors with lactate acidosis (130). Blocking VISTA with a monoclonal antibody could reverse the immunosuppressive activity, particularly in combination with anti-PD-1, leading to enhanced T cell infiltration, dampened expression of checkpoint receptors on T cells (PD-1, LAG-2, and TIM-3), and subsequent increased anti-tumor activity in MC38 colorectal carcinoma-bearing mice. Further investigation in mice and cynomolgus macaque models showed that acidic pH-selective anti-VISTA antibodies preferentially accumulated in tumor tissue, suggesting minimal risk of off-target effects even though VISTA is expressed by leukocytes. Therefore, combining immunotherapy with strategies to either mitigate tumor glycolysis and lactate levels or specifically

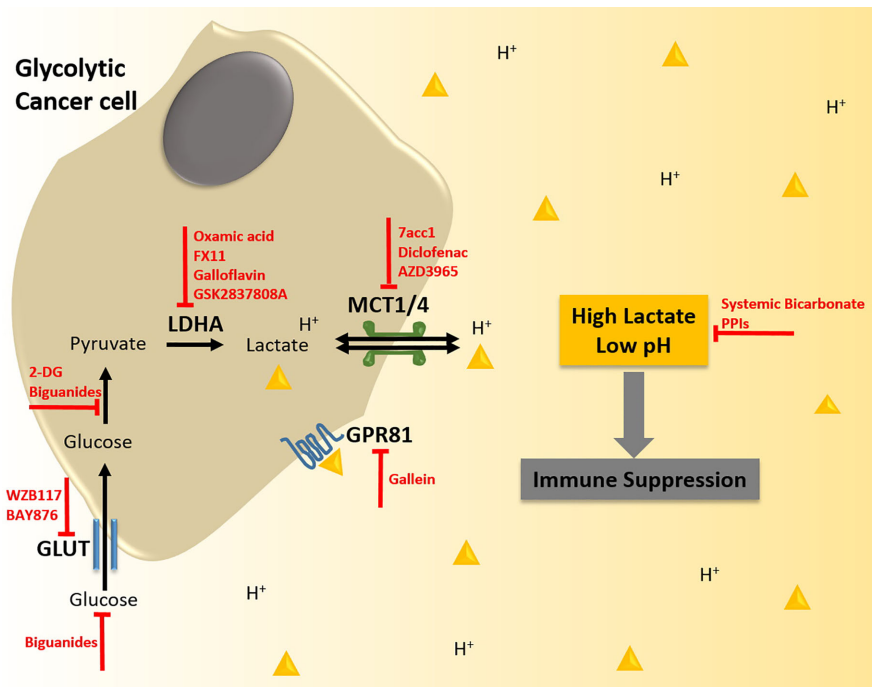


**FIGURE 2** | Strategies to target lactate biogenesis and acidosis to enhance immunotherapy response in triple negative breast cancer (TNBC). TNBC tumor cells display enhanced rates of glycolysis. This metabolic phenotype is supported by the increased expression of glucose transporters (GLUTs) that import glucose into the cell, and of lactate dehydrogenase A (LDHA) that converts the glycolytic intermediate pyruvate into lactate. The augmented production of lactate in TNBC tumors is also associated with higher expression of monocarboxylate transporters (MCTs), which shuttle lactate coupled to protons ($H^+$) out of the tumor cell resulting in excessive levels of lactic acid in the tumor microenvironment (TME) and reduced pH. Lactate acidosis in the TME creates an immunosuppressive milieu, which can antagonize the efficacy of immunotherapy. Thus, anti-metabolic strategies could alleviate lactic acid-induced immunosuppression and potentiate immunotherapy such as Adoptive T cell therapy (ACT), Chimeric Antigen Receptor T cell (CAR-T) therapy, and Immune Checkpoint Blockade (ICB), thereby synergistically inhibiting tumorigenesis. Potential strategies to abrogate lactate biogenesis and acidosis include specific targeting of GLUTs, LDHA, MCTs, and the lactate-receptor GPR81 with small molecule inhibitors, inhibition of glucose-pyruvate conversion, systemically lowering the availability of glucose by treatment with biguanides, and buffering the intra-tumoral pH with bicarbonate therapy or proton pump inhibitors (PPIs).

render immune cells resistant to the hostile tumor microenvironment may prove advantageous in improving therapeutic response in TNBCs (**Figure 2**). Here, we speculate on the potential of anti-metabolic strategies to enhance the efficacy of immunotherapy and their associated challenges.

## Targeting Molecular Mediators of the Warburg Phenotype

Inhibiting tumor glucose uptake, glycolysis and lactate transport have been proposed to reduce both tumor growth and immunosuppression, thus rendering these strategies compelling candidates for combination therapy. Specific and potent inhibitors of the GLUT transporters have been identified and investigated for their anti-tumor activity in pre-clinical studies (131). For instance, BAY-876 and WZB117 GLUT-1 inhibitors have shown anti-proliferative effects in breast tumor cells (132). In particular, a subset of TNBC tumors expressing the retinoblastoma (Rb) tumor suppressor with high glycolytic activity and low OXPHOS are sensitive to GLUT1 inhibition with BAY-876 (133). Since GLUTs are ubiquitously expressed, the impact of their inhibition at peripheral organs still needs to be well documented. Treatment with 2-DG, a non-metabolizable glucose analog and inhibitor of hexokinase, restricts tumor glycolysis and growth. Furthermore, combining 2-DG with mitochondria-targeting agents synergistically eradicates metabolic plasticity and enhances tumor regression in a TNBC xenograft model (134). Inhibition of glycolysis by 2-DG also dampens tumor cell production of G-CSF and GM-CSF in TNBC models, thus restricting MDSC development (86). Similarly, Dichloroacetate (DCA), an agent that shifts metabolic flux from glycolysis to OXPHOS, has shown efficacy in restricting tumor growth, specifically in tumor types with dysfunctional mitochondrial function such as TNBC (135). However, glycolysis inhibition beyond tumor cells could adversely affect T cell activation and trigger the induction of immunosuppressive Tregs and M2 TAMs (136). Notably, it has been argued that inhibiting glycolysis could drive T cells to a memory phenotype, a silver lining for long-term anti-tumor response (137, 138). Another approach to reduce glucose uptake in tumor cells and improve ICB response involves limiting glucose availability using anti-hyperglycemic biguanide drugs such as metformin and phenformin and glucose-limiting dietary interventions (139, 140). In murine B16 and MC38 melanoma models, combining anti-PD-1 treatment with metformin significantly reduced tumor growth by metabolic remodeling, reduced tumor hypoxia and improved T cell infiltration and function, as compared to either treatment alone (141). This combination treatment is currently under investigation in human clinical trials for advanced melanoma and non-small cell lung cancer (NSCLC) (NCT04114136) (142). Further, metformin can induce PD-L1 glycosylation and degradation thereby enhancing CTL activity and improving the efficacy of immunotherapy (143). While these studies appear promising, it should be noted that the effects of systemic interventions are pleiotropic and require careful investigation for off-target effects in combination with immunotherapy.

Targeting lactate dehydrogenases, in particular LDHA, offers another lucrative approach to alter the balance in tumor metabolic needs and to shape the composition and orientation of the immune microenvironment. Treatment with LDHA inhibitors such as oxamic acid, FX11, galloflavin, and 1-(phenylseleno)-4-(trifluoromethyl) benzene (PSTMB) demonstrate anti-proliferative effects in TNBC and other cancer models (144–146). Furthermore, treatment with oxamic acid enhanced CTL IFN-γ production, promoted DC differentiation, improved TNF secretion in monocytes, and abrogated M2 macrophage polarization in *in vitro* co-culture models (78, 89, 109, 120). Similarly, LDHA knockdown enhanced T cell infiltration and reduced the number of TAMs, leading to improved survival in the murine 4T1 TNBC model (147). LDHA depletion was also found to decrease MDSC development, improve NK cell cytotoxicity and hence, enhance anti-tumor immune response in multiple murine tumor models (81, 86). Interestingly, LDHA and PD-L1 are both negatively regulated by miR-34a and correlate with poor prognosis in TNBC, providing rationale for combining ICB therapy with LDHA inhibition (148). In accordance, lactate-mediated upregulation of PD-L1 has been observed in lung cancer and melanoma (74, 149). LDHA abrogation in the murine B16F10 melanoma model improved response to anti-PD-1 treatment, accompanied by an increase in tumor infiltration of CD8+ T cells and NK cells, increase in production of IFN-γ and granzyme B, and decrease in Treg infiltration (149). Moreover, combining the LDHA inhibitor GSK2837808A with ACT in a syngeneic murine melanoma model profoundly improved the anti-tumor response and survival compared to either LDHA inhibition or ACT alone (98). However, LDHA inhibitors have not yet successfully transitioned into clinical trials due to limited membrane permeability and on-/off-target toxicity (150). Moreover, the impact of LDHA inhibition on the viability and cytotoxicity of TILs needs to be explored extensively considering their need of glycolysis for activation.

As lactate transporters also play a key role in metabolic adaptation, their inhibition may provide another way to induce a metabolically favorable TME for immune cells. Indeed, MCT1/4 inhibition improved CD8+ T cell functionality *in vitro*, and the MCT4 inhibitor 7acc1 enhanced NK cell cytotoxicity and attenuated tumor growth in the murine 4T1 TNBC model (147). Although the MCT1/2 inhibitor AR-C155858 did not show any effect on tumor growth in the murine 4T1 TNBC model (151), its analogue AZD3965 is currently being assessed in a phase I clinical trial in solid tumors, diffuse large B cell lymphoma, and Burkitt's lymphoma (NCT01791595). Interestingly, the non-steroidal anti-inflammatory drug (NSAID) diclofenac was found to be a potent inhibitor of MCT1/4 and to reduce intra-tumoral lactate levels, concomitant with inhibition of tumor growth and Treg infiltration in a glioma model (152). A more recent study explored the molecular mechanisms of diclofenac-mediated tumor inhibition using various co-culture and murine tumor models (153). Of note, the authors found that diclofenac alone or in combination with the MCT1/2 inhibitor AZD3965 didn't negatively impact T cell viability and effector functions despite reducing the glycolytic

activity of the cells due to their metabolic adaptability and shift to OXPHOS. Treatment of 4T1 cells with diclofenac reduced the expression of MCT1 and LDHA, while increasing major histocompatibility complex (MHC)-I and MHC-II surface expression. Furthermore, diclofenac increased tumor infiltration of activated T cells and IFN-γ+ NK cells and delayed tumor growth in the 4T1 TNBC mouse model. Combining diclofenac treatment with single anti-PD-1 or dual checkpoint blockade (anti-PD-1 plus anti-CTLA-4) inhibited tumor growth and increased treatment response in two murine models, 4T1 TNBC and B16F10 melanoma. Although encouraging, the efficacy and safety profile of this combination treatment remains to be confirmed. Recent pre-clinical reports have also hypothesized that pharmacological blockade of GPR81 may prove advantageous in improving response to immunotherapy by enhancing DC antigen presentation and dampening PD-L1 expression in lactate-rich environments (72). Blocking GPR81-mediated lactate signaling by gallein decreased the frequency of intra-tumoral Tregs and delayed tumor growth in the murine 4T1 model (85). Of importance, *Gpr81*-null mice did not exhibit any detrimental phenotypes, indicating that off-target effects of targeting GPR81 may be minimal. Nevertheless, high affinity GPR81 inhibitors are yet to be identified.

Collectively, the promising findings of the aforementioned studies suggest that inhibition of glycolysis through LDH and/or MCT inhibition may improve treatment response to ICB. However, it is paramount to minimize the risk of off-target effects since it has become evident that immune cells and tumor cells exploit common metabolic mechanisms and display an overlap in expression of the major players in lactate biogenesis and export. Moreover, ubiquitous expression of candidate targets such as LDHA and MCT1/4 in normal tissues necessitates extensive risk assessment of small-molecule inhibitors before considering combination with immunotherapy.

## Targeting of Metabolic Lactate Acidosis

One important aspect of lactic acid-mediated immunosuppression within solid tumors is the detrimental effect of the accompanying acidosis. Thus, repurposing drugs that modulate systemic metabolism may represent an opportunity to improve the response to immunotherapy. Oral bicarbonate therapy has been extensively used to treat metabolic acidosis associated with chronic kidney disease. Pre-clinical evidence for its utility in cancer therapy was provided by a study that demonstrated its ability to buffer intra-tumoral pH and inhibit tumor growth, concomitant with increased CD8+ T cell infiltration in murine melanoma and pancreatic tumor models (154). In addition, oral bicarbonate improved NK cell infiltration and IFN-γ production in a murine lymphoma model, resulting in delayed tumor growth (104). Moreover, combining bicarbonate therapy with anti-PD-1 or anti-CTLA-4 checkpoint blockade or ACT improved tumor regression in comparison to either treatment alone in murine cancer models. The efficiency of bicarbonate therapy to improve cancer immunotherapy response in humans remains to be confirmed.

Likewise, multiple proton pump inhibitors (PPIs), commonly used as antacids, are being clinically investigated for their ability to modulate intra-tumoral pH in solid tumors (155). PPIs can be administered as prodrugs that are activated in low pH microenvironments to subsequently interact with and inhibit the activity of H+/K+-ATPase, thus making them well-tolerated and safe even at high doses. Treatment with the PPI Esomeprazole showed an increase in tumor pH and improved effector function of TILs in B16 melanoma xenografts without increasing activation of T cell subsets isolated from peripheral organs (101). Combining PPI treatment with ACT enhanced the anti-tumor effect and overall survival. Surprisingly, several clinical studies assessing the efficacy of PPIs in combination with anti-PD-1/PD-L1 therapy have shown either no effect or an adverse effect on ICB response in melanoma and NSCLC patients (156, 157). It is important to note here that individual PPIs have different acid neutralizing abilities, and therefore reduce the diversity of the gut microbiome at varying degrees, which in turn is known to affect the response to ICB. It may be prudent to assess gut microbiome diversity and consider history of antibiotics use prior to treatment of cancer patients with acidosis-reducing agents and ICB.

## Targeting Immunometabolism

In addition to reducing the hostility of the TME, an alternative strategy involves engineering autologous T cells to optimize metabolic adaptation and confer more resistance to glucose-limiting, lactate-rich conditions (158, 159). For example, overexpression of phosphoenolpyruvate carboxykinase 1 (PCK1), a regulator of gluconeogenesis, could increase the production of phosphoenolpyruvate (PEP) that is required for sustained T cell effector function (160). Mechanistically, PEP suppresses sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) activity in glucose-deprived T cells and improves Ca2+ flux and NFAT signaling required for T cell cytotoxicity. Adoptive transfer of PCK1-overexpressing T cells into the B16 murine melanoma model demonstrated improved production of CD4+ T cell derived IFN-γ and increased expression of the M1 macrophage CD86 marker on TAMs, collectively suppressing tumor growth and improving survival. Similarly, overexpression of PPAR-gamma coactivator 1α (PGC1α) restores mitochondrial dysfunction and biogenesis in tumor-infiltrating T cells supporting enhanced anti-tumor efficacy in B16 melanoma mice (161). Engineering chimeric antigen receptor (CAR) T cells to include the 4-1BB/CD137 signaling domain promotes the development of CD8+ memory T cells with an OXPHOS phenotype that may be beneficial to withstand metabolic competition within glycolytic TNBC tumors, as opposed to inclusion of the CD28 domain that induces a glycolytic phenotype in T cells (162). Thus, metabolic preconditioning of immune cells by ACT can enhance their persistence and effector function within the TME.

# CONCLUSION

Anti-cancer therapy has proven most effective in combinatorial settings, as tumors can quickly adapt to extrinsic cues. As such, improving long-term response rates to immunotherapy requires both direct and indirect modulation of anti-tumor immunity

through a better understanding of the tumor-immune cell interface. One of the many unanswered questions pertains to the feasibility of targeting tumor cell metabolism without negatively affecting immune cell metabolism in order to enhance immunotherapy response. Ideally, metabolic interventions should aim to target unique vulnerabilities of tumor cells, without dampening anti-tumor immune function and eliciting undesirable effects on peripheral organs—the paradigm of "cellular selectivity based on demand" (136). Although direct intra-tumoral injection of anti-metabolic agents and immunotherapy in solid tumors such as breast tumors is lucrative in principle (163), the effect of this mode of delivery on normal mammary function requires investigation. This is particularly relevant in the case of lactating breast cancer patients since mammary epithelial cells considerably rely on glucose uptake for lactose biosynthesis. In this regard, gaining insight into the potential role of tumor-specific antigens, such as LDHC, in cancer metabolism could aid the development of specific inhibitors to circumvent the risk of adversely impacting normal cells. Alternatively, small molecules inhibiting lactate biogenesis or export could be delivered specifically to tumor cells using polymeric nanocarriers that are responsive to tumor-specific cues such as pH-sensitive nanoparticles that could facilitate drug delivery to lactate-rich tumor microenvironments (164).

The timing and sequence of the combinatorial approach also requires optimization. For instance, targeting glycolysis or lactate transport in tumor cells prior to ACT may reduce unfavorable effects on immune function. As anti-metabolic therapies are developed for cancer treatment, their efficacy and effects on anti-tumor immune response requires close monitoring. Moreover, the impact of metabolic heterogeneity as observed between TNBC subtypes in addition to intra-tumoral heterogeneity, on the response to immunotherapy and combinatorial approaches requires in-depth investigation (165). Lastly, well-designed interventional studies examining intra-tumoral or systemic biomarkers to enable stratification of TNBC patients that may benefit from combining immunotherapy with anti-metabolic strategies are essential. Such biomarkers could include genetic mutations or variants that have been associated with metabolic reprogramming in TNBC (166) such as mutant p53 (19), *BRCA1* mutations (167, 168), c-Myc amplification (169), and Rb expression (133). Notably, investigation of congenital lactic acidosis has identified distinct genetic variants that result in defective mitochondrial function and drive the pathogenesis of the disease (170) and hence, assessment of the effect of these genetic alterations on tumor metabolic phenotype would be of interest in the search for prognostic biomarkers.

Although this review focuses on TNBC, it is important to note that endocrine-resistant luminal breast cancer and trastuzumab-resistant HER2+ breast cancer also exhibit metabolic reprogramming, whereby the glycolysis rate and associated lactate acidosis are increased (17, 171–173). Hence, in analogy with the observed metabolic changes in TNBC, it is likely that the glycolytic TME in treatment-resistant luminal and Her2+ tumors could disrupt immune surveillance and negatively affect the response to immunotherapy. However, in comparison to TNBC tumors, HER2+ and in particular luminal or hormone receptor positive tumors display a significantly lower infiltration of TILs (174), potentially explaining the inferior efficacy of immunotherapy in these tumors. Moreover, in contrast to TNBC, the presence of a sparse TIL infiltration in hormone receptor positive tumors has been associated with worse clinical outcome (175, 176). These observations highlight the importance of considering the immune landscape characteristics of each breast cancer subtype as well as the auxiliary role of lactate acidosis in modulating anti-tumor immunity in order to predict immunotherapy response. Immunotherapy strategies such as ACT and CAR-T therapy have shown great potential to improve the immune permissiveness of luminal breast tumors (177), and could likely be used in combination with anti-metabolic strategies in endocrine-resistant tumors.

To conclude, identification and development of the next generation of immune-based therapeutic approaches that can improve the intra-tumoral metabolic landscape and hence augment the anti-tumor response is gaining interest and necessitates extensive research in this direction.

## AUTHOR CONTRIBUTIONS

AN conceived and drafted the manuscript and designed the figures and table. JD conceived and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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# ITM2A as a Tumor Suppressor and Its Correlation With PD-L1 in Breast Cancer

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**Background:** High expression of integral membrane protein 2A (ITM2A) was reported to be associated with favorable prognosis in several solid tumors including breast cancer. This study aimed to investigate the role of ITM2A in breast cancer, especially in respect to tumor microenvironment.

**Methods:** ITM2A expression was evaluated based on qRT-PCR results on breast cancer specimens, as well as TCGA and GEO datasets. The influence of ITM2A expression on breast cancer cell proliferation and tumor growth were evaluated by CCK-8 assay, clonogenic assay, and murine xenograft models. Transwell assay was performed to observe the changes of invasion and migration capacity in breast cancer cells. To determine the biological functions of ITM2A, differentially expressed genes (DEGs) were screened based on RNA-sequencing data of MCF-7 cells overexpressed ITM2A. Then, functional annotation on DEGs was given by Gene Ontology and KEGG analysis. The stimulation on programmed cell death ligand 1 (PD-L1) expression when ITM2A overexpressed was determined by flow cytometry. Meanwhile, the correlation on expression levels between PD-L1 and ITM2A was tested *via* qRT-PCR on 24 breast cancer tissues, as well as public database.

**Results:** We demonstrated that ITM2A was frequently downregulated in breast cancer. Patients with high expression levels of ITM2A had longer overall survival and relapse free survival. Overexpression of ITM2A inhibited proliferation and impaired cells capacity of invasion and migration *in vitro* and *in vivo*. The DEGs in breast cancer cells overexpressed ITM2A were found to be associated with immunity responses. Moreover, ITM2A was found to facilitate breast cancer cells to express PD-L1. The correlation between PD-L1 and ITM2A was verified with both qRT-PCR assay and public database. Additionally, it was found that breast cancer had higher ITM2A expression frequently had more tumor-infiltrating lymphocytes (TILs).

**Conclusion:** In summary, we found that high expression of ITM2A reduced the aggressivity of breast cancer cells and had a favorable effect on outcomes of patients

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with breast cancer. Moreover, ITM2A induced PD-L1 expression in breast cancer cells was accompanied with higher TILs numbers in tumor microenvironment.

**Keywords: ITM2A, breast cancer, prognosis, PD-L1, immune infiltration**

## INTRODUCTION

Integral membrane protein 2A (ITM2A) belongs to the Type II Integral Membrane protein (ITM2) family, along with ITM2B and ITM2C (1). This family belongs to the BRICHOS superfamily. ITM2A is preferentially expressed in T lineage cells among hematopoietic cells (2). Now we concentrate on the biological functions that ITM2A performs in breast cancer.

Breast cancer is frequently diagnosed among females globally. In the United States, it is estimated that 281,550 new cases of invasive breast cancer will be diagnosed in females in 2021 (3). Simultaneously an estimated 43,600 breast cancer deaths will occur. The treatments targeting breast cancer have been continually developed and advanced for more than 100 years. Those treatments include mastectomy, conserving surgery, endocrine therapy, and common anti-tumor regimens—chemotherapy and radiation therapy. Currently, the 5-year survival of patients with breast cancer is over 90% (4). Nevertheless, the survival of patients with triple-negative breast cancer (TNBC) is quite poor, resulting from lack of robust treatment strategies (5). The remarkably heterogeneous TNBC tumor microenvironment has added disadvantage to treatments. Whenever the clinical stage, chemotherapy is the primary established treatment option for patients with TNBC (5). In addition, metastatic breast cancer frequently has poor clinical outcomes with a 5-year survival rate at 26% (4). It is urgent to explore robust regiments to improve outcomes of patients with TNBC or metastatic breast cancer.

As a novel therapeutic approach that releases the brake on effector T cells activation, immune checkpoint blockade (ICB) therapy has achieved success in several solid malignancies. The most deeply investigated ICBs, including anti-cytotoxic T lymphocyte associated antigen 4 (CTLA-4), anti-programmed death 1 (PD-1), and anti-programmed death ligand 1 (PD-L1), are developed to bypass the immune checkpoint, with the aim of rescuing and enhancing the functions of antitumor T effector cells (6). Pembrolizumab is a representative reagent of anti-PD-1 monoclonal antibody. There was a phase 2 trial that demonstrated that women with high risk, stage II/III, ERBB2-negative breast cancer had improvement in pathological complete response (pCR) rate when pembrolizumab was added to standard neoadjuvant chemotherapy over patients who received chemotherapy alone (7). Pembrolizumab also has showed durable antitumor activity as first-line therapy for patients with PD-L1-positive metastatic TNBC (mTNBC) (8), as well as demonstrated durable antitumor activity in a subset of patients with previously treated mTNBC (9). Currently, a phase 3 clinical trial showed that among patients with stage II/III TNBC, patients who received pembrolizumab plus neoadjuvant chemotherapy had a significantly higher rate of pCR than those who received placebo plus neoadjuvant chemotherapy (10). Normally, the response rate in patients with other solid tumors when receiving anti-PD-1/PD-L1 antibodies hangs on multifactorial parameters, including PD-L1 expression, tumor mutational load/microsatellites status, and intensity of intratumoural CD8+ cytotoxic T cells (11–13). In breast cancer, PD-L1 expressed on the surface of tumor cells as well as infiltrating lymphocytes (14). Meanwhile, PD-L1 expression associated with tumor-infiltrating lymphocytes (TILs) was found to be a positive prognostic feature in breast cancer (14, 15).

In this study, we explored if ITM2A could influence the proliferation and aggressivity of breast cancer cells. Meanwhile, RNA-sequencing (RNA-seq) of breast cancer cells that overexpressed ITM2A was conducted. We found ITM2A was associated with immunity responses. More importantly, ITM2A was found to induce PD-L1 expression as well as be associated with TILs.

**Abbreviations:** DMFS, Distant Metastasis Free Survival; RFS, Relapse Free Survival; OS, Overall Survival; DSS, Disease Specific Survival.

## METHODS

### Public Database Analysis

For comparing ITM2A expression between breast cancer samples and normal samples, gene expression profiles (GSE29413 containing 12 normal tissues and 54 breast cancer samples; GSE61304 containing 4 normal tissues and 58 breast cancer tissues) were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). In addition, gene expression date of n = 110 breast cancer tissues and paired normal tissues were selected from TCGA (https://portal.gdc.cancer.gov/). Tumor Immune Estimation Resource (TIMER; https://cistrome.shinyapps.io/timer/) was utilized to exam the correlation between ITM2A expression and TILs (16).

### Specimens Collection and Processed

Human tumor samples were obtained from patients diagnosed with invasive breast cancer who had not received neoadjuvant chemotherapy. A total of 24 breast cancer tissues and paired adjacent tissues were obtained during surgery at the Department of Thyroid and Breast Surgery, Tongji Hospital, Tongji Medical College of HUST. Specimens were immersed in cold RNA later solution overnight and then stored at -80°C. Each informed consent was signed by patients and approval of experiment on human specimens was received from the Ethics Committee of Tongji Hospital.

### Survival Analysis

For survival analysis, the online tools—Kaplan-Meier Plotter (http://kmplot.com/) (17) and PrognoScan (http://www.

prognoscan.org/) (18)—were used to detected the overall survival (OS), progression free survival (PFS), and distant metastasis free survival (DMFS) of patients grouped by the median of ITM2A mRNA.

## Cell Culture

MCF-7 and MDA-MB-231 cells were obtained from the Chinese Academy of Science cell bank (Wuhan, China). The cell lines were authenticated by using short tandem repeat (STR) DNA profiling (ABI 3730XL Genetic Analyzer, Life Technologies, Waltham, MA, USA). MCF-7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen, USA) at 37°C with 5% $CO_2$. MDA-MB-231 cells were cultured in L-15 (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, USA) at 37°C. These cells passaged less than 30 times during our experiment.

## Transfection

The MCF-7 and MDA-MB-231 cells were grown in 6-well plate to arrive at 50%–60% or 70%–80% confluence respectively. Then cells were transfected with 2.0 ug empty vector or ITM2A plasmid using X-tremeGENE HP DNA Transfection Reagent (Roche, USA) according to the manufacturer's instructions. After transfection for 48 h, cells were harvested or passaged for subsequent experiments.

## Quantitative Real-Time PCR

Total RNA was extracted from tissues or cell pellets using Trizol reagent (Invitrogen, USA) and reversely transcribed using PrimeScript RT Reagent Kit (Takara Bio Inc.). The expression levels of interested genes were measured in triplicate using SYBR Green qPCR Mix (Toyobo, China). Primer sequences were as follows. ITM2A Forward: 5′-ATCCTGCAAATTCCCTTCGTG-3′, Reverse: 5′- CAGGTAAGCAGTCATTCCCTTT-3′; PD-L1 Forward: 5′- CT GTCACGGTTCCCAAGGAC -3′, Reverse: 5′-GGTCTTCCTC TCCATGCACAA-3′; GAPDH Forward: 5′- CTCACCGGATG CACC AATGTT -3′ Reverse: 5′-CGCGTTGCTCACAAT GTTCAT -3′. Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method, and GAPDH was used as an internal control (19).

## Immunoblotting Analysis

For immunoblotting, cells were lysed in iced RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, USA). After centrifuging in high speed, the lysates were purified and then were separated in 5–12% SDS-PAGE gels. The lysates were transferred to PVDF membranes and blocked for 1 hour with 5% bevor serum albumin (BSA). The primary antibodies we used were as follows: anti-ITM2A (Proteintech, Wuhan, China); anti-α-Tubulin (Proteintech, Wuhan, China). Membranes were incubated with a species-matched HRP-conjugated secondary antibody for 1 h. α-Tubulin was used as loading controls to quantify the results.

## Clonogenic Assay

For clonogenic assay, cell lines transduced with empty vector or ITM2A were seeded in 6-well plates (1,000 cells per well), typically for 14 days. Cells were fixed with 4% formaldehyde for 15 min followed by crystal violet staining for 15 min.

## Cell Counting Kit-8 (CCK-8) Assay

Cell viability was determined using CCK-8 (DOJINDO, Japan) assay. Briefly speaking, cells transfected with indicated plasmid were plated in 96-well plates and cultured for 24–72 hours. Then the medium in each well was replaced with 100 ul fresh culture medium supplemented with 10 ul CCK-8. After incubation for 2 hours at room temperature in the dark, the absorbance at 450 nm wave length was read by microplate reader (Bio-Rad, USA). Cell viability was evaluated based on the absorbance value relative to wells plating no cells.

## Transwell Migration and Invasion Assay

Migration and invasion assay were performed according to the manufacturer's instructions (Corning, USA). Briefly, about $5 \times 10^4$ MDA-MB-231 cells transfected with empty vector or ITM2A plasmid were plated into upper chamber coated with 200 ul L-15 with 10% FBS. In the lower chamber, 500 ul medium with 40% FBS was used as a chemotactic agent. Nineteen hours later, the insert was removed. Cells in the microporous membrane were washed with PBS and then stained with crystal violet at room temperature for 10–15 min. As invasion assay, the upper chamber was coated with Matrigel besides medium and 10% FBS, and the incubation time was 36 hours. Cells in the microporous membrane were counted in five random fields per chamber.

## Flow Cytometric Analysis

The FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) was used to detect apoptosis rate of cells. The collected fresh cell pellets were washed using cold PBS and then suspended by 1 × binding buffer. Next, 5 ul of Annexin V and 5 ul of PI was added into 300 ul of 1 × binding buffer contained around $1 \times 10^5$ cells. Cells were gently mixed and then incubated for 15 min at room temperature in the dark. Then cells were analyzed on the FACS Calibur System (Beckman Coulter). The PE Mouse Anti-Human PD-L1 (Cat 329706; BioLegend, Inc.), APC Mouse Anti-Human PD-L2 (Cat 345507; BioLegend, Inc.), and PerCP/Cyanine5.5 Mouse Anti-Human B7-H3 (Cat 351009; BioLegend, Inc.) were used to detect PD-L1, PD-L2, and B7-H3 in MCF-7 and MDA-MB-213 cells. First, fresh cell pellets were collected and washed with PBS, then were suspended in 300 ul of PBS containing 1‰ BSA and incubated with corresponding antibody for 30 min on ice in the dark. Finally, staining cells were washed and then analyzed on FACS Calibur System (Beckman Coulter). FlowJo (ver. 10.0) was used for data acquisition and analysis.

## Mouse Xenograft Studies

Female BALB/c null mice between the ages of 4–6 weeks were purchased from the Beijing HFK Bio-Technology Co., Ltd. The mice were bred in a specific-pathogen free facility. Prepared MCF-7 cells were inoculated subcutaneously under axilla ($1 \times 10^6$ cells per mouse). Once the tumors were tangible, its volume was calculated using the formula $0.5 \times (\text{minor tumor axis})^2 \times (\text{major tumor axis})$ once in 3 days. Before the nude mice were

sacrificed, magnetic resonance imaging examination (plain scan and enhanced) was conducted to check the growth of tumors in the body. All animal procedures were performed in accordance with the approved Guide for the Care and Treatment of Laboratory Animals of Tongji Hospital and approved by the Ethics Committees of Tongji Hospital.

## RNA Sequencing on MCF-7 Cells and DEGs Screening

MCF-7 cells were transfected with 2.0 ug empty vector or ITM2A plasmid as above described. Three days after transfection, the MCF-7 cells were collected using trypsin (Cat 0458-250G; Lifescience). Then total RNA was derived from cells pellets using Trizol (Invitrogen, USA) according to the manufacturer's protocol. A total amount of 2 ug RNA per samples was sent to BerryGenomics (Beijing, China) for next generation sequencing. Briefly, mRNA was purified from total RNA and cDNA was synthesized. Then the 3' ends of DNA fragments were adenylated and adaptor with hairpin loop structure were ligated. After PCR, the libraries were sequenced on an Illumina NovaSeq platform to generate 150 bp paired-end reads, according to the manufacturer's instructions. The DEGs between ITM2A overexpressed cells and nature control cells were identified using the DEGseq R package according to below criterions: the adjusted $p < 0.05$ and $|\log2\text{-fold-change}| > 2$.

## Functional Annotation and Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed on RNA-Seq profiles of 1,053 breast cancer stratified by ITM2A mRNA expression levels using GSEA software as previously described (20). For DEGs derived from ITM2A overexpressed cells, the R packages "clusterProfiler" and "enrichplot" were used to conduct Gene Ontology (GO) function and Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (21). It was considered statistically significant when the adjusted $p < 0.05$.

## Statistical Analysis

Statistics analysis in this experiment was conducted by SPSS 22.0 and GraphPad 8.0. Comparisons between two groups were determined by two tailed Student's t-test or chi-square test. The data were expressed as mean ± standard deviation from at least three independent experiments. The correlation of gene expression was accessed by Pearson's correlation coefficient. *P*-value < 0.05 was taken as statistically significant and "ns" represented *P*-value ≥ 0.05; "*" represented *P*-value < 0.05, and "**" represented *P*-value < 0.01.

# RESULT

## ITM2A Was Decreased in Breast Cancer and Positively Associated With Favorable Survival

First, we analyzed ITM2A expression in collected specimens and found that ITM2A was decreased in breast cancer compared to normal tissues (**Figure 1A**). The down regulation of ITM2A in breast cancer tissues was further verified in GEO and TCGA datasets (**Figures 1B, C**). This result was square with the research performed by Cefan Zhou et al. (22). Additionally, ITM2A was observed to be decreased in acute myeloid leukemia (23), cervical cancer (24), and ovarian cancer (25). Based on the TIMER database, there were more than 10 types of cancer with lower expression of ITM2A (**Supplementary Figure 1**). We then evaluated the prognostic value of ITM2A mRNA in patients with breast cancer. It was proved that patients with increased ITM2A had longer overall survival (OS) (HR = 0.57 [0.46–0.71], Log-rank $P = 2.4\times10^{-7}$) (**Figure 1D**), disease free survival (DFS) (HR = 0.67 [0.60–0.75], Log-rank $P = 1.3\times10^{-12}$) (**Figure 1E**), and distant metastasis free survival (DMFS) (HR = 0.64 [0.53–0.78], Log-rank $P = 8.1\times10^{-6}$) (**Figure 1F**). PrognoScan is a database for met-analysis of the prognostic value of genes (26). In PrognoScan, high expression of ITM2A in BC was found to be associated with longer relapse free survival (RFS), disease specific survival (DSS), as well as OS and DMFS (**Table 1**). Collectively, we found that ITM2A was positively associated with favorable survival.

## ITM2A Inhibited Migration and Promoted Apoptosis of Breast Cancer Cells

To get an in-depth understanding of the role of ITM2A in the tumorigenicity of breast cancer, plasmid encoding ITM2A (OE-ITM2A) or empty vector was transfected into two frequently used breast cancer cell lines: MCF-7 and MDA-MB-231 cells. Overexpression of ITM2A by OE-ITM2A transfection was validated by qRT-PCR (**Figure 2A**) and immunoblotting (**Figure 2B**). Meanwhile, ITM2A overexpressed cells had a reduced capacity to immigrate and invade (**Figures 2C, E**). Moreover, we observed a higher apoptosis rate in OE-ITM2A transfected cells than in empty vector transfected cells using FASC (**Figures 2D, F**). Our findings demonstrated that ITM2A could inhibit the migration and invasion of breast cancer cells. At the same time ITM2A had the ability to promote apoptosis in breast cancer cells.

## Overexpression of ITM2A Decreased the Proliferation of Breast Cancer In Vitro and In Vivo

To validate the inhibitory proliferation in breast cancer cells with ITM2A overexpression in the long term, we then seeded cells transfected with plasmid into 6-well plates and counted the number of clones 14 days later. It was proved that both MCF-7 and MDA-MB-231 formed fewer clones when OE-ITM2A was transfected (**Figures 3A, B**). Meanwhile, cell viability assays showed that proliferation of breast cancer cells was significantly attenuated after OE-ITM2A transfection (**Figures 3C, D**) at 72 hours. To explore how the ITM2A expression influence the breast cancer growth *in vivo*, we then planted MCF-7 cells that transfected with OE-ITM2A or empty vector under axilla of female BALB/c null mice. Those mice did not receive any treatments and were sacrificed 20 days later. It was proved that ITM2A overexpressed tumors were notably smaller than ITM2A normally expressed tumor with respect to MRI test and tumor size (**Figures 3E–G**). These results,

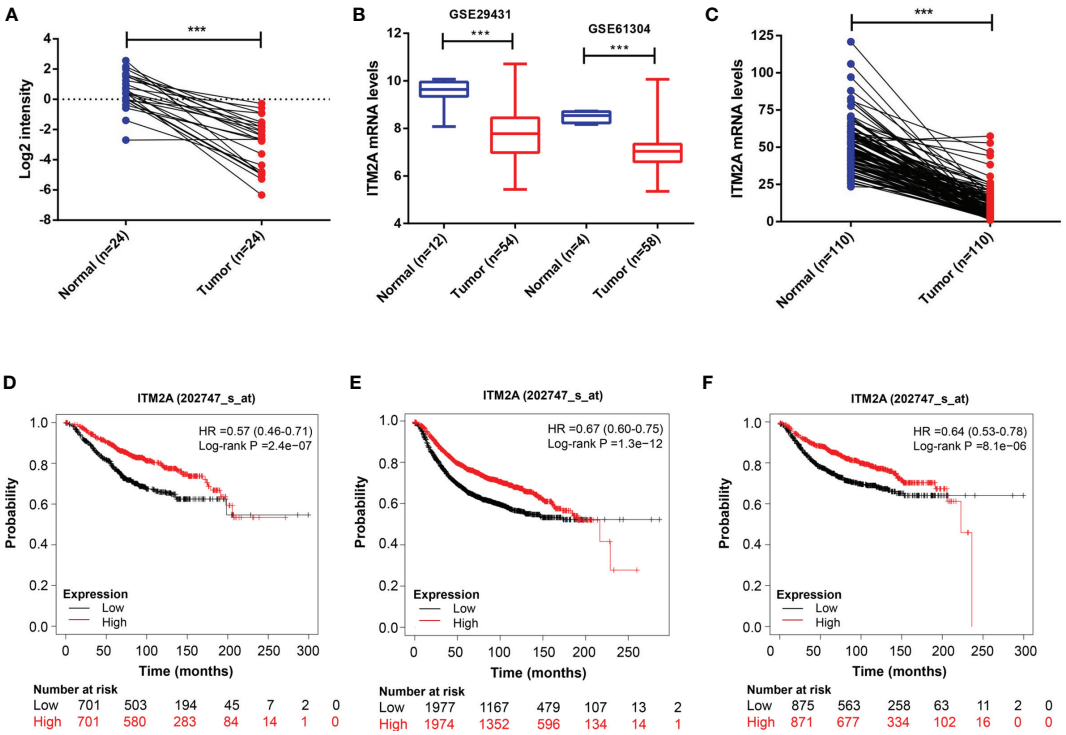


**FIGURE 1** | ITM2A was down-regulated in breast cancer and positively associated with favorable outcomes. **(A)** Comparison of ITM2A expression in breast cancer tissues with that of paired adjacent normal tissues. **(B)** Boxplot showing expression level of ITM2A in cancer tissues and normal tissues in the breast profile GSE29431 and GSE61304. **(C)** Comparison of ITM2A expression in cancer tissues with that of paired adjacent normal tissues in the breast TCGA dataset. **(D-F)** Kaplan-Meier survival curves depicting the OS **(D)**, RFS **(E)**, and DMFS **(F)** of patients with breast cancer stratified by ITM2A mRNA levels. ***p < 0.001. OS, overall survival; RFS, relapse free survival; DMFS, distant metastasis free survival.

in summary, demonstrated that ITM2A could impair the proliferation of breast cancer *in vitro* and *in vivo*.

## Overexpression of ITM2A Induced Immunity Relate Responses

In order to understand the biological role of ITM2A in breast cancer cells, we performed RNA sequencing (RNA-seq) on MCF-7 cells that transfected with OE-ITM2A plasmid or vectors. The differentially expressed genes (DEGs) were derived (**Supplementary Figure 2**) and then mapped to GO terms and KEGG pathways. The most DEGs that map to GO terms were chemokine and interleukin, such as CCL3, CXCL8, IL24, and TNF (**Figure 4A**), which indicated a pro-inflammatory effects (27, 28). Consistent to these genes, the top ranked GO terms

**TABLE 1** | Survival analysis of ITM2A mRNA in breast cancer patients (the PrognoScan database).

| Dataset | Endpoint | Probe ID | Number | COX P-value | HR [95% CI-low CI-up] |
|---|---|---|---|---|---|
| GSE19615 | DMFS | 202747_s_at | 115 | 0.0342 | 0.39 [0.16–0.93] |
| GSE19615 | DMFS | 202746_at | 115 | 0.0166 | 0.43 [0.22–0.86] |
| GSE11121 | DMFS | 202747_s_at | 200 | 0.0370 | 0.70 [0.51–0.98] |
| GSE11121 | DMFS | 202746_at | 200 | 0.0080 | 0.57 [0.37–0.86] |
| GSE2034 | DMFS | 202747_s_at | 286 | 0.0042 | 0.75 [0.62–0.91] |
| GSE2034 | DMFS | 202746_at | 286 | 0.0241 | 0.75 [0.58–0.96] |
| GSE1456 | RFS | 202747_s_at | 159 | 0.0050 | 0.66 [0.49–0.88] |
| GSE1456 | OS | 202746_at | 159 | 0.0005 | 0.40 [0.26–0.62] |
| GSE1456 | DSS | 202747_s_at | 159 | 0.0031 | 0.60 [0.43–0.84] |
| GSE1456 | OS | 202747_s_at | 159 | 0.0017 | 0.63 [0.47–0.84] |
| GSE1456 | RFS | 202746_at | 159 | 0.0001 | 0.43 [0.28–0.66] |
| GSE1456 | DSS | 202746_at | 159 | 0.0001 | 0.37 [0.22–0.62] |
| GSE3494 | DSS | 202746_at | 236 | 0.0361 | 0.68 [0.47–0.98] |
| GSE3494 | DSS | 202747_s_at | 236 | 0.0266 | 0.74 [0.57–0.97] |
| GSE49226 | DFS | 202747_s_at | 249 | 0.0433 | 0.80 [0.65–0.99] |

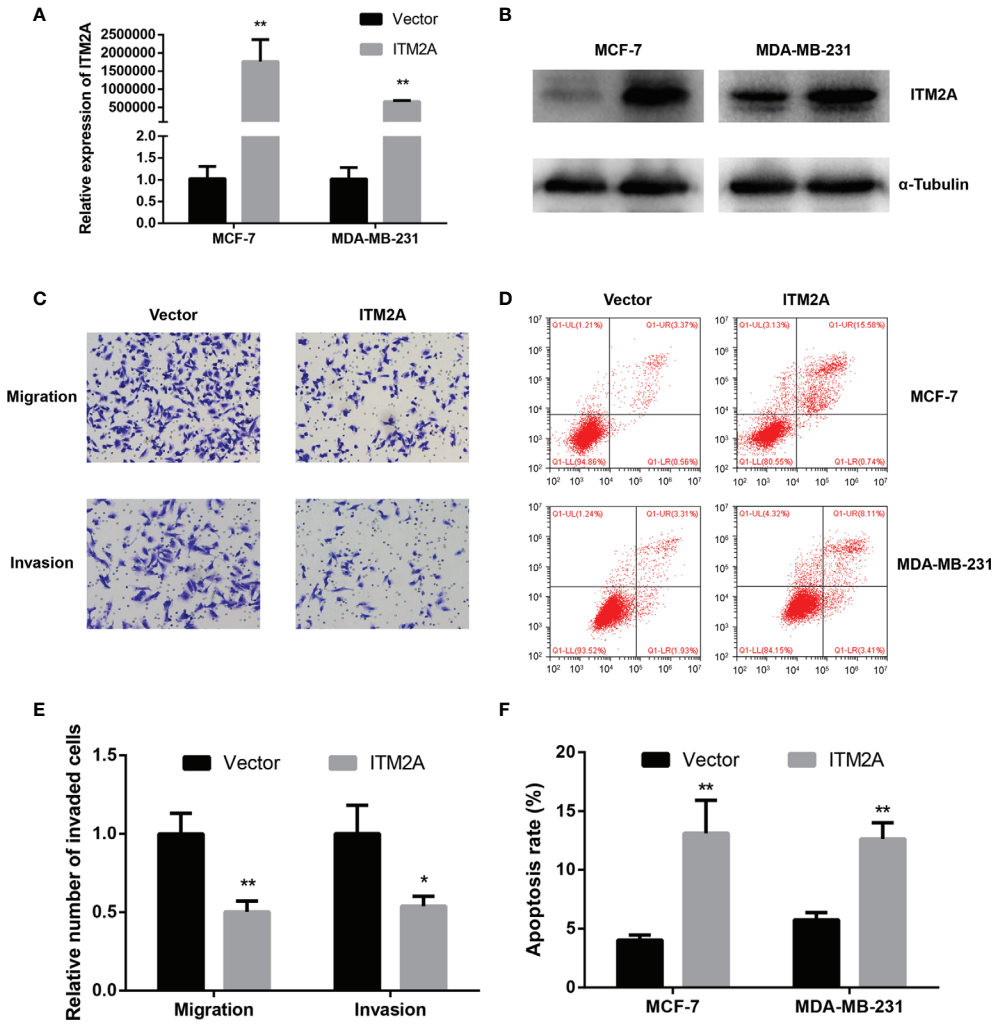


**FIGURE 2** | Overexpression of ITM2A inhibited migration and promoted apoptosis of breast cancer cells. MCF-7 and MAD-MB-231 cells were transfected with the indicated plasmid. qRT-PCR **(A)** and immunoblotting **(B)** were used to test ITM2A expression in mRNA and protein levels 48 hours after transfection. **(C, E)** Migration and invasion assay in MDA-MB-231 cells. **(D, F)** Apoptosis rate in MCF-7 and MDA-MB-231 cells 48 hours after transfection. *p < 0.05, **p < 0.01.

concentrated on cell chemotaxis (**Figure 4A**). When matched with KEGG pathway, the DEGs enriched in immunity related response (**Figure 4B**). The top three ranked pathways were cytokine-cytokine receptor interaction, NF-Kappa B signaling pathway, and viral protein interaction with cytokine and cytokine receptor. Additionally, GSEA analysis implied ITM2A was active in immunity related pathways (**Supplementary Figure 3**). To our knowledge the association between ITM2A and immunity response in breast cancer has never been reported. Thus, we focused on the role of ITM2A in breast cancer on context of immunity.

## ITM2A Increased PD-L1 Expression in Breast Cancer Cells

Anti-PD-L1 therapy has been approved in TNBC treatment and PD-L1 expression levels were related to clinical response to these therapies (8). In addition, some evidence demonstrated other signaling roles of the PD-L1 molecule, including pro-survival, reducing mTOR activity, and glycolytic metabolism (29). We then explored if the ITM2A expression is associated with PD-L1 expression. Analysis based on the TIMER database revealed a positive correlation between ITM2A and PD-L1 in lumina and basal subtypes (**Figure 5A**). In addition, this correlation was verified by qRT-PCR, which quantified the relative expression levels of ITM2A and PD-L1 of 24 breast cancer specimens (**Figure 5B**). Furthermore, ITM2A could significantly upregulate the PD-L1 expression in both MCF-7 and MDA-MB-231 cells (**Figures 5C, D**), along with PD-L2 (**Supplementary Figures 4A, B**) and B7-H3 (**Supplementary Figures 4C, D**). PD-L2 is the second ligand for PD-1 and was reported to be associated with PD-L1 expression in melanoma, lung, and kidney cancer (30). Those results demonstrated that ITM2A could upregulate PD-L1, PD-L2, and B7-H3 expression in breast cancer cells.

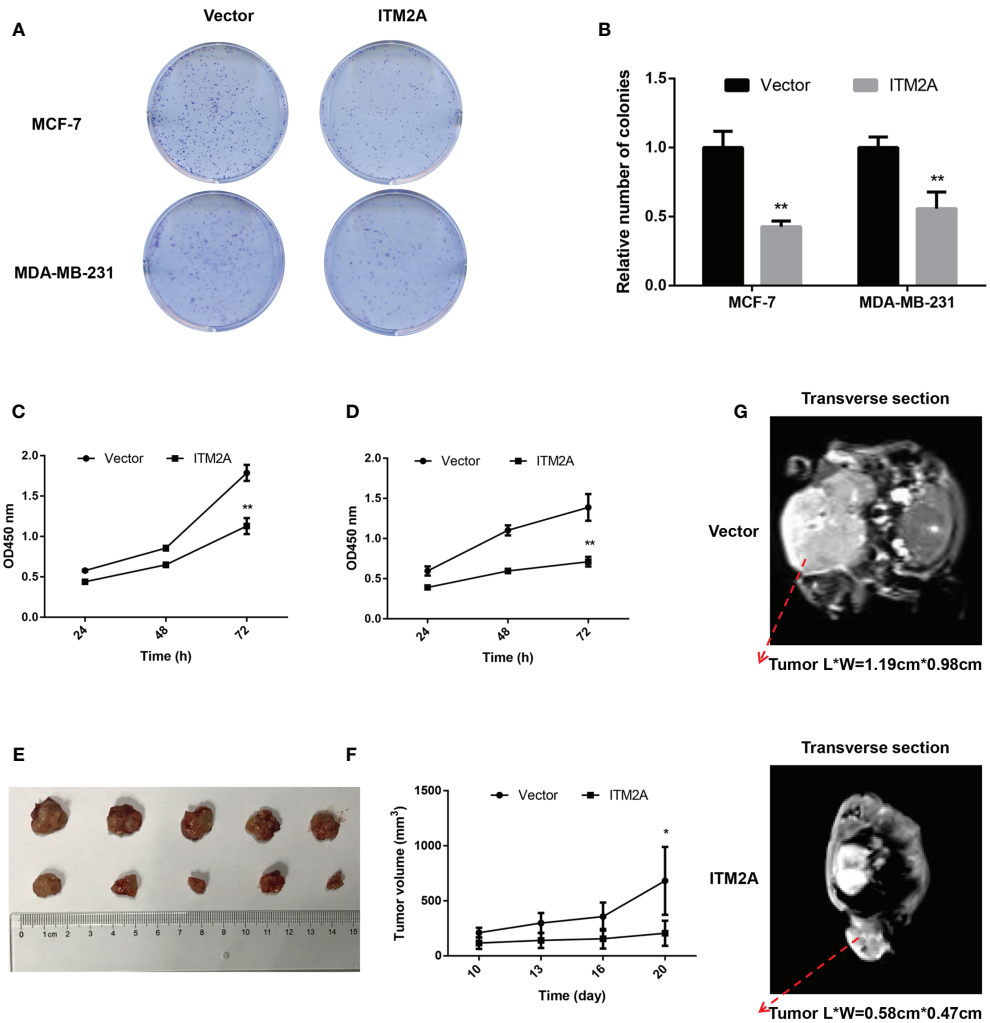


**FIGURE 3** | ITM2A overexpression decreased proliferation of breast cancer *in vitro* and vivo. **(A, B)** Clone formation was stained with crystal violet 14 days after seeded. **(C, D)** Growth curves of MCF-7 (left) and MDA-MB-231 cells (right) transfected with the indicated plasmid were measured by CCK-8 assay. **(E-G)** ITM2A expression inhibited the proliferation of breast cancer cells *in vivo*. **(E)** Photographs of dissected tumors from sacrificed mice. **(F)** Growth curves of indicated tumors in BALB/c null mice. **(G)** Representative images of enhanced MRI in transverse section. "L" represents the major tumor axis and "W" represents the minor tumor axis. *p < 0.05, **p < 0.01.

## ITM2A Expression Was Positively Correlated With TILs Quantity

The TILs have been considered as reliable biomarkers to predict breast cancer patients' response to chemotherapy, as well as ICB (31, 32). Above results indicated the correlation between ITM2A and immunity response, as well as PD-L1 expression. We then examined if the ITM2A could predict the TILs intensity. The correlation between ITM2A expression and six TILs types (B cells, CD8+ cells, CD4+ cells, macrophage, neutrophil, and dendritic cell) were analyzed based on TIMER database. A positive correlation ranged from intermediate to high between ITM2A and CD8+ T cells was frequently observed over all subtypes of breast cancer (**Figures 6A–D**). The similar results were found in another five common cancer types, including lung adenocarcinoma (**Supplementary Figure 5A**), lung squamous cell carcinoma (**Supplementary Figure 5B**), colon adenocarcinoma (**Supplementary Figure 5C**), head and neck cancer (**Supplementary Figure 5D**), and prostate adenocarcinoma (**Supplementary Figure 4E**).

## DISCUSSION

Before this study, there were a handful of studies that investigated the functions of ITM2A in tumor. A previous study found that ITM2A could inhibit ovarian cancer growth and induce G2/M cell cycle arrest, indicating that ITM2A was a novel tumor suppressor in ovarian cancer (25). Another study demonstrated that ITM2A inhibited breast cancer cells growth *via* enhancing autophagy induction through a mTOR-dependent manner (22). In this study, we explored the roles that ITM2A played in breast cancer.

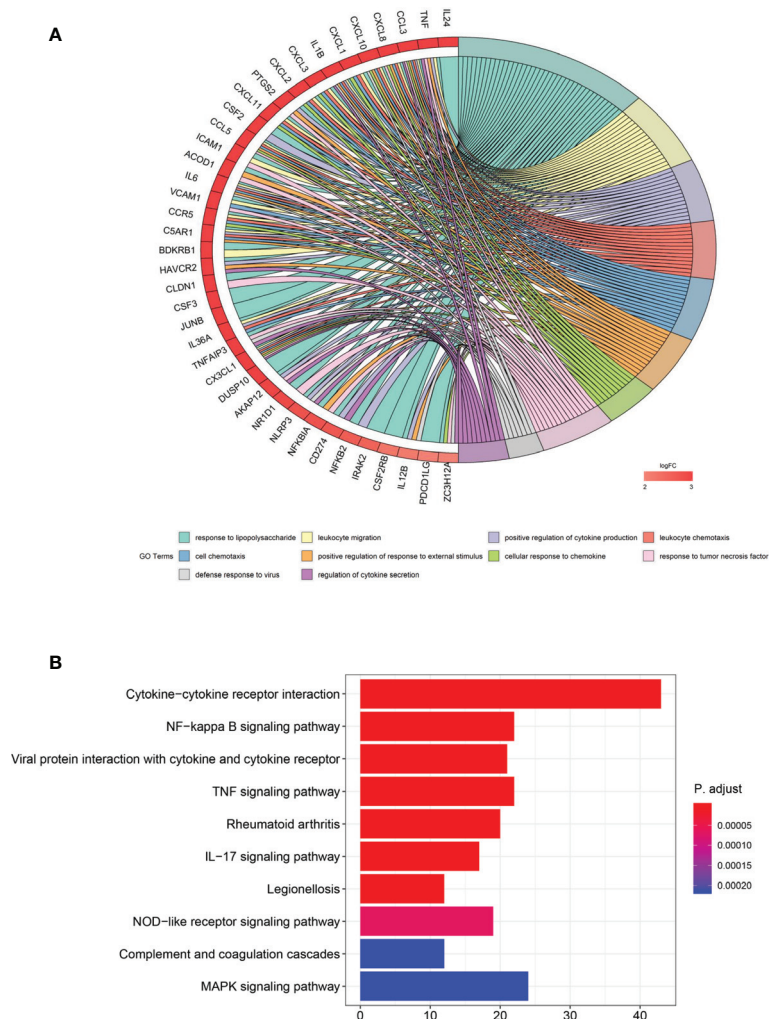


**FIGURE 4** | Overexpression of ITM2A induced immunity related response. **(A)** DEGs were derived based on RNA-seq data on MCF-7 cells that overexpressed ITM2A. Then the DEGs were mapped to GO terms and the top 10 ranked GO terms are showed. **(B)** The top-10 ranked KEGG pathways in which DEGs enriched. DEGs, differentially expressed genes; RNA-seq, RNA-sequencing; GO, gene ontology.

It was confirmed that ITM2A was decreased in breast cancer tissues. ITM2A inhibited breast cancer cells growth *in vitro* and *in vivo*, and reduced the aggressivity of breast cancer *via* impairing its immigratory and invading capacity. We also found that high expression of ITM2A was associated with a longer OS and RFS, etc. However, ITM2B or ITM2C expression has no correlation with OS and PFS of patients with breast cancer (data not shown). With studies that reported that loss of ITM2A was a poor OS factor of cervical cancer (33), hepatocellular carcinoma (34), and acute myeloid leukemia (23), together with the fact that ITM2A has been observed to significantly decrease in those carcinoma types, we implied that ITM2A might be a novel tumor suppressor in those carcinomas that included breast cancer. Consistent to this point, our study demonstrated that upregulation of ITM2A reduced the aggressivity of breast cancer cells. Also, we found that ITM2A was correlated with immune response. Additionally, it was observed that ITM2A not only positively correlated with intensity of TILs and PD-L1 expression but also stimulated expression of PD-L1, PD-L2, and B7-H3 in breast cancer cells.

It is contentious to consider how critical the ITM2A is for the development of T effector cells (T effs). Tzong-Shyuan Tai et al. demonstrated that ITM2A plays a minimal role in development of T cells. On the other hand, other studies reported that overexpression of ITM2A in murine thymocytes resulted in a partial downregulation of CD8 in the CD4+CD8+ double positive thymocytes (35). What's more, a study reported that ITM2A might be a susceptibility gene for graves' disease (GD) in the Xq21.1 locus, strengthening the role of ITM2A in the immune system. This provoked our concentration on the association between ITM2A and tumor immunity in breast cancer. On the one hand, existing evidence demonstrated an association between ITM2A and immunity although this association was unclear. On the other hand, ITM2A is probably closely related to TILs and shapes the tumor

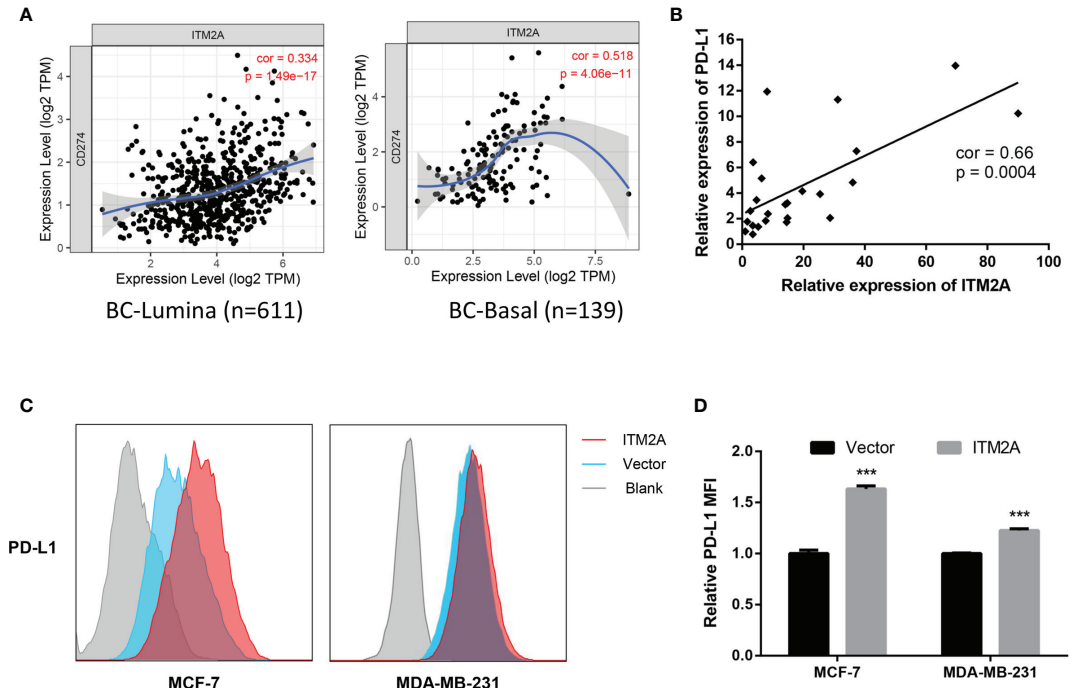


**FIGURE 5** | ITM2A increased PD-L1 expression in breast cancer cells. **(A)** Correlations between PD-L1 and ITM2A expression in lumina breast cancer (left) and basal breast cancer (right) based on TIMER database. **(B)** Correlation between PD-L1 and ITM2A expression in collected 24 breast cancer specimens. **(C, D)** MCF-7 and MDA-MB-231 cells were transfected with indicated plasmid. PD-L1 expression in these cells 48 hours after transfection was tested by flow cytometric analysis. ***p < 0.001.

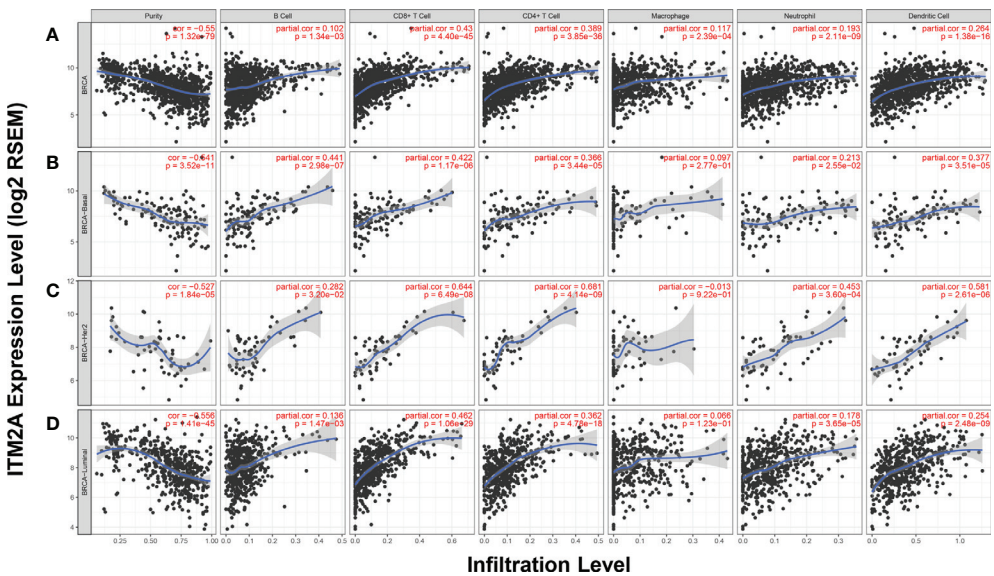


**FIGURE 6** | ITM2A expression was positively correlated with TILs quantity. Correlations between ITM2A expression and six TILs types in breast cancer were evaluated based on TIMER database. Analysis in all subtypes of BC **(A)**, basal **(B)**, HER-2 **(C)**, and luminal subtypes **(D)**.

microenvironment, which influences patients' outcomes and treatment strategy once it is proved to play roles in T cells development. There are abundant studies showing that TILs can provide prognostic value for patients with breast cancer. For example, a pooled analysis of 3,771 patients treated with neoadjuvant therapy evaluated the correlation between TILs and prognosis in different subtypes of breast cancer. It was proved that increased TILs were associated with longer DFS in

TNBC and HER2-positive breast cancer, longer OS in TNBC, and shorter OS in luminal B tumors (36). Our study demonstrated the positive correlation between ITM2A expression and TILs in breast cancer. Additionally, TILs and PD-L1 are helpful to choose patients who will receive more benefits from anti-PD-1/PD-L1 therapy. Patients with breast cancer that has over 1% cells stained with anti-PD-L1 antibody have higher rate of pRC than patients who expressed less than 1% anti-PD-L1 stained cells (8). Thus, we evaluated the relevance between ITM2A and TILs and PD-L1 expression based on TIMER. Importantly, we confirmed that ITM2A could stimulate PD-L1 expression in breast cancer cells. Collectively, high expression of ITM2A in breast cancer was accompanied with high intensity of TILs and abundant PD-L1 expression. This work provokes further study about predictive value of ITM2A in patients when they receive ICB treatments.

There are many limits in our study. We evaluated the correlations between ITM2A expression and TILs basing on a single database without verification. We did not explore the mechanism that ITM2A overexpression upregulates the PD-L1 expression. Those questions remain to be answered in further study.

## CONCLUSION

In summary, we found that ITM2A played a tumor suppressor role in breast cancer aggressivity, and had favorable effects on outcomes of patients with breast cancer. Meanwhile, ITM2A induced PD-L1 expression in breast cancer cells while accompanied with higher TILs numbers in the tumor microenvironment.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Tongji Hospital. The patients/participants provided their written informed consent to participate in this study. All animal procedures were performed in accordance with the approved Guide for the Care and Treatment of Laboratory Animals of Tongji Hospital and approved by the Ethics Committees of Tongji Hospital.

## AUTHOR CONTRIBUTIONS

WC designed the experiments and supervised the study. RZ and TX performed the experiments. TX and RZ collected, analyzed, and interpreted the data. YX, ZW, and XL participated in revising the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.581733/full#supplementary-material

**Supplementary Figure 1 |** ITM2A expression levels in different cancers. ITM2A expression levels in different human cancers from TCGA database were shown by TIMER database. **p < 0.01, ***p < 0.001. TCGA, the Cancer Genome Atlas; TIMER, the Tumor Immune Estimation Resource.

**Supplementary Figure 2 |** DEGs between MCF-7 cells that overexpressed ITM2A and expressed ITM2A normally. Volcano plot shows the 511 up-regulated genes (red) and 190 down-regulated genes (green) in ITM2A overexpression MCF-7 cells **(A)**. Heatmap of DEGs **(B)**.

**Supplementary Figure 3 |** GSEA analysis implied ITM2A was active in immunity related response. GSEA was performed on RNA-Seq profiles of 1,053 breast cancer stratified by ITM2A mRNA expression levels and the top-10 ranked KEGG pathways were showed. GSEA, Gene Set Enrichment Analysis.

**Supplementary Figure 4 |** ITM2A increased PD-L2 and B7-H3 expression in breast cancer cells. MCF-7 and MDA-MB-231 cells were transfected with indicated plasmid. PD-L2 **(A, B)** and B7-H3 **(C, D)** expression in these cells 48 hours after transfection were tested by flow cytometric analysis. ***p < 0.001.

**Supplementary Figure 5 |** ITM2A expression was positively correlated with TILs quantity across common cancers. TIMER database was used to assess the correlation between ITM2A expression and six types of TILs enrichment cross five common cancers. TIMER analysis in lung adenocarcinoma **(A)**, lung squamous cell carcinoma **(B)**, colon adenocarcinoma **(C)**, head and neck cancer **(D)**, and prostate adenocarcinoma **(E)**. TIMER, the Tumor Immune Estimation Resource; TILs, tumor infiltration lymphocytes.

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REVIEW
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# Macrophages and Extracellular Matrix in Breast Cancer: Partners in Crime or Protective Allies?

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Solid cancers such as breast tumors comprise a collection of tumor, stromal and immune cells, embedded within a network of tumor-specific extracellular matrix. This matrix is associated with tumor aggression, treatment failure, chemo- and radio-resistance, poor survival and metastasis. Recent data report an immunomodulatory role for the matrix in cancer, *via* the creation of niches that control the migration, localization, phenotype and function of tumor-infiltrating immune cells, ultimately contributing to escape of immune surveillance. Macrophages are crucial components of the immune infiltrate in tumors; they are associated with a poor prognosis in breast cancer and contribute to shaping the anti-tumor immune response. We and others have described how matrix molecules commonly upregulated within the tumor stroma, such as tenascin-C, fibronectin and collagen, exert a complex influence over macrophage behavior, for example restricting or enhancing their infiltration into the tumor, and driving their polarization towards or away from a pro-tumoral phenotype, and how in turn macrophages can modify matrix production in the tumor to favor tumor growth and metastasis. Targeting specific domains of matrix molecules to reinstate an efficient anti-tumor immune response, and effectively control tumor growth and spread, is emerging as a promising field offering a new angle for cancer therapy. Here, we review current knowledge on the interactions between tumor-associated macrophages and matrix molecules that occur within the tumor microenvironment of breast cancer, and discuss how these pathways can be targeted for new immunotherapies for hard to treat, desmoplastic tumors.

**Keywords: extracellular matrix, macrophages, breast cancer, tumor microenvironment, immunotherapy, immune infiltrate**

## INTRODUCTION

The extracellular matrix (ECM) is a complex network of secreted molecules that, in healthy conditions, serves to define tissue architecture and stiffness, and program cell behavior including supporting cell adhesion, survival and migration. The matrix comprises collagens, proteoglycans and glycoproteins (including fibronectin, laminin, osteopontin, tenascin-C), along with a variety of matrix-associated molecules such as glycosaminoglycans, enzymes such as proteases, cross-linkers and kinases, and soluble factors such as chemokines, growth factors and cytokines (1). The study of the stroma during neoplasia reveals a deeply reorganized composition compared to healthy tissues, at both the cellular and molecular levels. In particular, tumors comprise a highly heterogeneous and dysregulated ECM network, embedding tumor cells and cancer-associated fibroblasts (CAF), as well as newly developed blood vessels (2). In breast cancer, ECM accumulation and desmoplasia in general are associated with a poor prognosis (3), and increased matrix deposition can predict breast tumor formation (4). Well-studied changes in the breast TME include abnormal matrix molecule expression, permanent remodeling, destruction by proteolytic enzymes and concomitant repair (5). Moreover, changes in individual matrix constituents have also been associated with breast cancer aggressiveness and metastasis. For example, tenascin-C, a protein which is not detected in most healthy adult tissues is notably re-expressed during tumorigenesis (6), deposition of collagen types I, III and V is particularly affected, with progressive fibril linearization and thickening over time during breast carcinogenesis (7–9), and epithelial upregulation of the ubiquitous matrix glycoprotein fibronectin (10) as well as enrichment of specific splice variants containing the oncofetal extra domain A (EDA) or extra domain B (EDB) (11) are observed. At the functional level, the unique matrix composition of tumors can influence all aspects of carcinogenesis, from angiogenesis, EMT, metastasis to immune surveillance (12). However, the molecular mechanisms defining cell-matrix interactions in the TME are not yet completely understood, highlighting the need for a better understanding of the role of the ECM during cancer evolution.

Macrophages have a central role in cancer immune surveillance in general (13) and in breast cancers in particular, where they are associated with a poor prognosis (14) and with negative hormone receptor status and malignant phenotype (15). However the prognostic role of tumor-associated macrophages (TAM) is not as clear cut as reported for other immune subsets, such as Th1 or Treg cells (16), as TAM may be also associated with a positive outcome in some cancers like colorectal carcinoma (14). This discrepancy may be explained by the extraordinary phenotypic plasticity of these cells, which are easily modified depending on local stimuli from the tumor microenvironment (TME). TAMs with a "M1-like" phenotype are characterized by tumor-killing functions, inflammatory cytokines production such as TNFα, IL-1β, IL-6, and IL-8, nitric oxide (NO) and reactive oxygen species (ROS), as well as improved priming capacities towards T cells *via* upregulated MHC class I and II presentation and associated co-stimulatory molecules (17). On the other extremity of this spectrum, "M2-like" alternatively activated macrophages present tumor-facilitating characteristics, characterized by secretion of immunosuppressive effectors such as TGFβ and IL-10, promotion of tissue remodeling and expression of inhibitory checkpoint molecules such as PD-1 (18). However, the phenotypic spectrum of TAM is much more complex than initially described. In breast cancer, TAM can express in the same cells a combination of M1-like and M2-like signature genes that correlate along the same activation trajectory (19), and TAM subsets that exert pro-angiogenic capacities *via* the expression of pro-angiogenic factors and vascular promotion (20), or favor the formation of pre-metastatic niches in breast cancer (21), have been identified.

A close relationship between TAM and the tumor-specific ECM network has been known for almost 40 years (22), with more recent studies reporting an increasingly complex crosstalk between these two components of the TME, comprising multiple layers of molecular and cellular cues reciprocally influencing both TAM biology and ECM composition. In this review, we will describe how the tumor-specific ECM can modify TAM phenotype, function and migration in breast cancer and how in turn TAM can influence the ECM network to favor tumor growth and spread. We will explore how the understanding of these mechanisms can be exploited to offer novel therapeutic solutions for cancers in need of novel treatments, drug resistant or poorly immune infiltrated "cold" tumors.

## ECM Favoring Macrophage Infiltration in the Tumor

The prognostic impact of the immune infiltrate in tumors has historically been defined by cell density (23). The density of the TAM infiltrate varies between different cancer types, but these cells are particularly abundant in breast cancers, where they can represent up to 50% of the tumor mass (24). Correlative data suggest an association between the composition of the tumor-specific ECM and TAM infiltrate. For example, a higher deposition of hyaluronan, a glycosaminoglycan of the ECM, correlated with higher macrophage counts and poor outcome in a cohort of 278 people with breast cancer, regardless of their tumor subtype (25, 26). Moreover, ECM stiffness and activation of TGFβ signaling, classically associated with fibrosis, both positively correlated with the number of macrophages at the invasive front in 20 breast cancer patients (9). Similar associations have also been observed in murine models of breast tumorigenesis. For example, when Pten, a gene involved in tumor growth regulation, is inactivated in the stromal fibroblasts of mice mammary glands, in MMTV-ErbB2/neu

**Abbreviations:** CAF, cancer-associated fibroblasts; CCL, chemokine ligand; CCR, chemokine receptor; DAMP, damage associated molecular pattern; ECM, extracellular matrix; EDA, Extra Domain A; FBG, fibrinogen-like globe domain; GM-CSF, granulocyte-monocyte colony stimulating factor; IFNγ, interferon gamma; IL, interleukin; M-CSF, macrophage colony stimulating factor; MMP, matrix metalloproteinase; NF-KB, nuclear factor kappa b; TAM, tumor-associated macrophages; TGFβ, transforming growth factor beta; TLR, toll like receptor; TNBC, triple negative breast cancer; TNFα, tumor necrosis factor alpha; TME, tumor microenvironment.

mice, the spontaneous tumorigenesis observed in mice expressing wild type levels of stromal Pten was decreased compared to mice lacking stromal Pten, a phenomenon associated with both collagen I deposition and increased macrophage infiltration (27). Furthermore, in a MMTV-PyMT/colla1$^{tm1jae}$ model of spontaneous mammary tumorigenesis, increased collagen deposition within the tumor was associated with higher TAM numbers, an effect dependent on COX2 expression, and in which COX2 blockade limited TAM and collagen levels (28). Similarly, constitutive expression of CCL2 in the mammary epithelium, which leads to increased macrophage infiltration, was associated with increased stromal deposition of collagen, that could elevate the risk of cancer development (29). Also, the overexpression of CCL2 by breast stromal cells transplanted into mouse mammary glands leads to enhanced TAM infiltration, concomitant with increased collagen expression. Both of these effects were ablated by depletion of CD11b expressing cells (30). These data suggest that tumors can manipulate the CCL2/CCR2 pathway to facilitate the infiltration of tumor prone collagen-producing macrophages.

Together these studies demonstrate a positive correlation between TAM density, ECM remodeling and tumor progression, although it is difficult to distinguish cause and effect. The underlying reasons for altered TAM density are also not known; it is possible that this phenomenon may result directly from higher TAM infiltration, but could also arise from changes in monocyte infiltration and subsequent differentiation, and/or changes in monocyte and macrophage survival (**Figure 1**).

More information has come from colocalization studies, which imply direct matrix-TAM interactions within the TME in experimental breast cancer. For example, using an orthotopic mammary tumor model, in which grafted tumor cells were engineered to express high or low levels of tenascin-C, we observed not only more numerous TAM in tenascin-C high tumors, but that TAM were exclusively present inside "tracks" formed by tenascin-C deposition. Treatment of mice with function blocking anti-tenascin-C antibodies caused TAM to accumulate at the edge of the tumor, compared to higher numbers within the tumor stroma in untreated mice (**Figure 2**) (31). These data indicate the capacity of ECM molecules to promote TAM infiltration during tumorigenesis, and demonstrate a role for the tumor specific-matrix in controlling the spatial positioning of TAM once within the TME. Conversely, matrix molecules may also restrict TAM infiltration; for example, blockade of the EDA domain of fibronectin in a mouse colon cancer model reduced tumor growth and led to increased infiltration of macrophages in the tumor (32), with a direct interaction of Fn-EDA with macrophages demonstrated by immunofluorescence (33). These

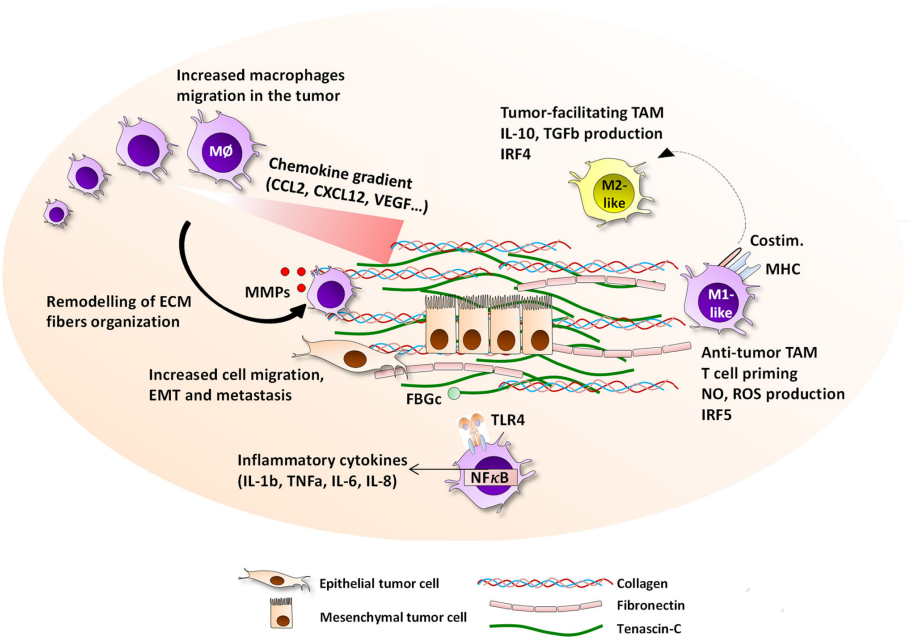


**FIGURE 1** | Interactions between the ECM and macrophages in the tumor microenvironment. The tumor-specific ECM network has a panel of possible interaction pathways with macrophages, all of which ultimately impact the evolution of cancer growth and prognosis. ECM molecules including tenascin-C, collagen, fibronectin, osteopontin, hyaluronan, versican, and thrombospondin, are highly upregulated in primary and metastatic breast cancer and embed epithelial tumor cells, and are produced by tumor cells, CAF, or immune cells. The ECM presence is associated with an increased migration of macrophages to the tumor site, with which they directly interact *via* the expression of integrins including αMβ2, α2β2, α2β1, αvβ5, or α9β1, or are guided by patterns of chemokine-matrix gradients. On site, macrophages are able to degrade the ECM fibers by secreting MMP2, 9, 13, and 14, and reorganize the collagen fibers. Together with their capacity to help cancer cell migration, intra- and extravasation, and initiating the EMT process, ECM help TAM contribute to accelerated metastasis. The ECM network is able to drive TAM towards either pro-angiogenic, anti-tumor M1-like or anti-tumor M2-like phenotype depending of the local contexture. Moreover, the EDA and FBG domains of fibronectin and tenascin-C respectively are TLR4 ligands that can trigger inflammatory responses in myeloid cells.

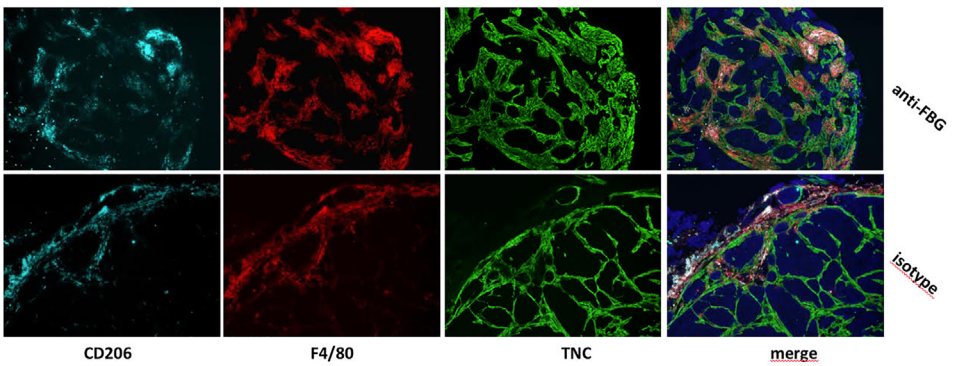


**FIGURE 2** | *In vivo* blockade of the FBG domain of tenascin-C diminishes TAM numbers and restrict their presence to tumor edge in a mammary tumor model. Mammary tumors from mice that were treated with a blocking anti-FBG antibody (upper panels) or a control isotype (lower panels) were collected 21 days after engraftment and stained with anti-CD206 (light blue), anti-F4/80 (red), and anti-TNC (green) antibodies.

data reveal not only the versatility of the effect of ECM molecules on TAM infiltration, but also indicate that this function may be limited to specific domains of these large multimodular molecules. However, little is known at the molecular level as to if, and if so how, these matrix molecules directly control TAM migration and positioning in the breast TME, or whether changes in the matrix indirectly affect immune cell infiltration.

Several cellular and molecular pathways have been brought forward from studies outside of breast cancer to explain how matrix molecules can interact with macrophages to promote their infiltration. TAM adhere directly to fibronectin and collagens in the ECM *via* integrins such as αMβ2 and α2β2 (34). These interactions have been shown to contribute to TAM motility and functions within breast (35), lung and colon (36), and prostate (37) cancers. However, binding to other matrix components, using other integrins, may also play a role. For example αvβ5, expression by TAM in glioblastoma serves as a receptor for the matrix glycoprotein osteopontin, whose interaction provides a chemotactic signal that is essential for macrophage recruitment (38). In addition to cell-matrix adhesion directly mediating TAM infiltration, the ECM also serves as a reservoir for soluble factors including chemokines. Chemokine-matrix interactions, in particular chemokine binding to matrix-resident glycosaminoglycans, are important in controlling not only local concentrations of these soluble factors, but also their oligomerization state, resistance to proteolysis, activity and signaling capabilities (39, 40). As such changes in the tumor-specific matrix may alter the capacity to bind and retain secreted molecules classically produced by tumor cells, thus impacting cell infiltration. For example, CCL2, a major player responsible for monocyte/macrophage infiltration into tumors, is known to rely on glycosaminoglycans within the extracellular matrix to effectively signal (41, 42). Moreover, other matrix-chemokine mediated mechanisms may also be at play in modifying TAM migration. In a mouse model of bladder cancer, versican, a large extracellular matrix proteoglycan, enhances lung metastasis in a manner dependent on the presence of CCL2 and of macrophages in the TME (43). One could argue that signals from the TME could induce parallel production of both versican and CCL2 by tumor cells, which signals could synergize in favor of metastasis colonization.

Together, these studies demonstrate that in breast cancer, there is a correlation between changes in expression levels of ECM molecules such as hyaluronan, collagen, fibronectin or tenascin-C with intratumoral TAM density, linked to disease outcome (**Table 1**). Moreover, data are emerging that the tumor-specific ECM creates sub-tumoral niches or tracks to control the distribution of TAM within the TME. Studying other cancer models has revealed a number of proposed mechanisms to explain how the ECM network may promote TAM infiltration, which includes providing an adhesive substrate for cells to attach to, and move along, and the patterning of TAM-attracting chemokines to provide migration permissive gradients. Their evaluation offers new clues for a better understanding of the mechanisms at stake in breast cancer.

## ECM as a Modulator of TAM Phenotype in the TME

As the complexities of macrophage biology continue to emerge, it is clear that moving beyond a simple consideration of intra-tumoral TAM density, to take into account the nature of the TAM infiltrate, is essential. If the tumor-specific ECM network is able to modulate the infiltration of myeloid cells in the tumor tissue, it can also directly impact their polarization and activation status, by driving them either towards tumor-facilitating or anti-tumor phenotype. *In vitro*, the impact of matrix on cultured macrophages and macrophage cell lines has been well documented, and most report that the ECM drives an M2-like phenotype. This was first noted in a historical paper from 1983, where Kaplan demonstrated the immunomodulatory role of collagen by cultivating human primary monocytes on collagen, which blunted their capacity to kill cancer cells (22). Similarly, overexpression of the anti-inflammatory transcription factor ATF3 (activating transcription factor 3) in RAW 264.7 macrophage cell line lead to an upregulation of tenascin-C, which was directly responsible for subsequent M2 differentiation and increased migration (54), whilst a hyaluronan

**TABLE 1** | TAM and ECM interactions described in breast cancer studies.

| Effect | Disease or model | ECM | Effect of ECM | Ref |
|---|---|---|---|---|
| **ECM favoring macrophage infiltration in the tumor** | | | | |
| Correlation TAM infiltration and ECM | Human breast cancers | COLL | TAM number, TGFβ signaling and ECM stiffness all positively correlate | (9) |
| | MMTV-ccl2 model | COLL | CCL2 epithelial overexpression leads to increased TAM and increased COLL | (29) |
| | Human breast cancers | HA | HA and TAM correlate with a poor outcome | (25) |
| | MMTV-ErbB2/neu model | COLL | Inactivation of Pten leads to COLL remodeling and TAM infiltration | (27) |
| | MMTV-PyMT/colla1$^{tm1jae}$ model | COLL | Increased COLL deposition leads to higher TAM number in the TME | (28) |
| Colocalization of TAM and ECM | NT193 TNC$^{+/-}$ cells orthotopic | TNC | TAM in the TME are trapped in TNC tracks | (31) |
| Linked TAM infiltration ECM | CCL2$^{+/-}$ cells orthotopic | COLL | CCL2-attracted TAM directly lead to stromal COLL deposition in tumor | (30) |
| **ECM as a modulator of TAM phenotype in the TME** | | | | |
| ECM driving M2 TAM | 4T1 cells orthotopic | COLL | IL-6 and COLL drive TAM towards wound healing phenotype | (44) |
| | NT193 TNC$^{+/-}$ cells orthotopic | TNC | TNC drives pro-tumoral M2-like TAM phenotype *via* TLR4-FBG interaction | (31) |
| ECM driving pro- angiogenic TAM | 4T1 cells orthotopic | FN | TAM drive metastasis by recruiting VEGFR+ myeloid cells and promoting FN expression | (21) |
| | Breast carcinoma cells, breast xenograft | HA | High molecular weight HA drives pro-angiogenic behavior in breast TAM | (45) |
| **Macrophages as shapers of the tumor ECM** | | | | |
| TAM reorganize collagen fibers | E0771 cells orthotopic | COLL | TAM reorganize COLL fibers to favor metastasis | (46) |
| TAM as ECM producers | Her2$^{+/-}$ and ccl5$^{+/-}$ cells orthotopic | COLL | CCL5 leads to the recruitment of TAM expressing COLL | (47) |
| | 4T1 cells orthotopic | OPN | MDSC drive metastasis and immune suppression by producing OPN | (48) |
| **The regulation of EMT and metastasis by ECM and TAM** | | | | |
| Cell migration promoting TAM | MMTV-PyMT model | COLL | TAM by COLL-rich tumor border support tumor cell intravasation | (49) |
| | 4T1 cells orthotopic | SPARC | TAM-derived SPARC favors metastasis *via* integrin-dependent tumor cell invasion | (50) |
| EMT promoting TAM | MMTV-PyMT model | VSC | Myeloid cells-derived versican drives EMT and favor lung metastasis | (51) |
| | SPARC$^{+/-}$ breast cancer cells | SPARC | Tumor cells derived SPARC induces EMT *via* the immunosuppressive functions of MDSC | (52) |
| **The need for CAF in the TAM-ECM relationship** | | | | |
| CAF-TAM-ECM crosstalk | MMTV-PyMT model | COLL | Fibroblast-derived FAP signaling cleaves collagen and increases TAM adhesion | (53) |
| | NT193 TNC$^{+/-}$ cells orthotopic | TNC | Tumor-derived TNC switches TAM phenotype, but not CAF-derived TNC | (31) |

*COLL, collagen; FN, fibronectin; HA, hyaluronan; OPN, osteopontin; SPARC, secreted protein acidic and rich in cystein; TNC, tenascin-C; VSC, versican.*

and collagen mix drove the upregulation of the hyaluronan receptor CD44 in THP-1 macrophage cell line, together with the upregulation of a group of M2-related genes like CD163, IL-10 and CCL22 (55).

These studies support the idea that in breast cancer, tumors can manipulate ECM production to highjack the immune infiltrate, and switch the phenotype of TAM from efficient tumor killing cells to tumor-facilitating cells, further helping the tumor to thrive. But driving macrophages towards M2-like polarization is not the only impact induced by the ECM; these external signals can also affect another major pro-tumoral function of macrophages in tumors - to enhance or accelerate the neoangiogenesis required for the growth of cancer (56). The culture of macrophages with conditioned media of breast carcinoma cells in the presence of high molecular weight hyaluronan lead to an increased production of angiogenic factors such as VEGF, and increased endothelial cell migration. In line with this, macrophages primed with high molecular weight hyaluronan increased the number of blood vessels in breast carcinoma xenograft models (26, 45). Similarly, the chemical inhibition of CYP4A (Cytochrome P450 4A) in TAM in mouse models of breast cancer skewed their phenotype away from M2-like, and decreased the recruitment of VEGFR1+ myeloid cells and the expression of fibronectin by fibroblasts, altogether contributing to the metastatic process (21).

The molecular mechanisms by which the tumor-specific ECM may orientate the activation and polarization of macrophages and the immune response in the TME includes a variety of secreted factors, in particular those controlled by toll-like receptors (TLR) and NF-κB dependent inflammatory signaling. Indeed, it has been known for a long time that not only infectious triggers can drive a TLR-dependent responses in myeloid cells expressing these receptors, but endogenous triggers, or DAMPs (damage associated molecular pattern), also activate these cells during "sterile" inflammation. DAMP-mediated TLR signaling is involved in the pathogenesis of inflammatory diseases such as auto-immune diseases, atherosclerosis and cancer, where it can initiate and maintain a deleterious chronic immune response (57). ECM domains that are highly expressed in cancer have been identified as TLR-engaging DAMPs, and are likely to be involved in the skewing of the TAM phenotype and functions. For example, the fibrinogen-like globe (FBG) domain of tenascin-C was identified more than 10 years ago as a ligand of TLR4, and is able to engage aberrant inflammatory responses in TLR4-expressing myeloid cells *via* the release of TNFα, IL-6, and IL-8 (58), that are distinct from LPS-dependent responses (59), prolonging inflammation in arthritis models. Recently, we used an orthotopic grafting murine model of breast cancer to demonstrate that the tumor-derived tenascin-C is able to switch the phenotype of TAM towards a M2-like, pro-tumoral polarization, in a FBG/TLR4 dependent fashion (31). The triggering of this pathway generated a deleterious inflammatory contexture that helped the tumor escape from immune surveillance and supported a pro-metastatic environment, demonstrating a role for the TLR4 engagement by tenascin-C for tumor growth and spread. Similarly, a pro-tumorigenic role for

versican-mediated TLR activation has been reported. When produced by lung lewis carcinoma tumor cells, versican acts as a ligand for TLR2 and TLR6 expressed by macrophages, generating a strong TNFα response from these cells, and ultimately acting as a help for metastasis (60), *via* mechanisms that could include TNFα-dependent stimulation of cancer cells proliferation, intravasation and extravasation.

However, little is still known about the impact of ECM-driven inflammation on macrophages phenotypes and functions in breast cancer, including the role of other TLR-binding ECM proteins and the importance of the local microenvironment. It is however noteworthy that matrix-mediated TLR ligation is not always tumor supportive. For example, fibronectin, in which the alternatively spliced EDA domain is also able to bind TLR4 (61), activates inflammatory responses in TLR4-expressing cells. This has led to the use of the EDA domain as an adjuvant for a protein vaccine derived from HPV (human papillomavirus) against HPV + cervical carcinoma, which was able to generate antigen-specific CD8 T cells and eradicate tumors (62) *via* the activation and maturation of myeloid cells, hence triggering anti-tumor responses. Another example of matrix being able to modulate the orientation of tumor immunity one way or another is thrombospondin-1. Thrombospondin-1 can indeed generate enhanced expression of TNFα in bone marrow derived macrophages upon triggering of its receptor CD36, *via* signaling mediated by TLR4 and NF-κB (63), and the addition of exogenous thrombospondin-1 to a murine macrophage cell line *in vitro* blocked IL-10 production induced upon ionizing radiations (64), suggesting a tumor-facilitating role. But on the other hand, thrombospondin-1 can also exert a protective activity against carcinogenesis *in vivo*, as its absence during skin carcinogenesis limits cancer growth *via* its anti-inflammatory properties, including decreasing the levels of IL-6 and IL-12 and limiting the local infiltration of neutrophils and macrophages (65), highlighting a dual and probably context-dependent role of this protein during carcinogenesis.

Matrix-mediated pro- and anti-tumoral effects may be accounted for by the fact that different stimuli have different effects, despite using the same receptor, or receptor family. However, for one TLR4 ligand, the story is more complex, exerting distinct effects depending on the disease model or even the cellular source. Whilst we showed that tumor-derived tenascin-C drives M2 macrophage polarization in experimental mammary tumors, contradictory findings to ours have been found in glioblastoma. The absence of expression of CD47 on tumor cells increased the expression of tenascin-C in the TME, which in turn triggered TLR4-dependant inflammation in macrophages, characterized by high levels of TNFα secretion and activation of STAT-3 dependent signaling, together with an increased phagocytosis of tumor cells, suggesting an anti-tumor role for this ECM molecule in this model (66). Data from mouse models of cardiac pathologies show that up-regulation of tenascin-C during disease was associated with the shifting of macrophage phenotype towards M1-like *via* the engagement of TLR4 (67). However in hepatocellular carcinoma, Nong et al. demonstrated that macrophage-derived TNFα induces the production of tenascin-C by cancer cells in an NF-kB dependent pathway, promoting cell migration and tumor aggressiveness (68). Indeed, when we deleted host tenascin-C we found that, in contrast to deletion of tumor-derived tenascin-C, this resulted in diminished M1-like macrophage behavior (31). These data suggest that whilst host-derived matrix can be used to trigger TLR-mediated anti-tumoral immune responses, tumor-derived matrix can trigger TLR-mediated tumor supportive phenotypes, which may explain why pre-clinical global TLR4 blockade has provided mixed results to date.

These data highlight how the cell source of the matrix, the local microenvironment and specific tissue pathology can influence the pro- or anti-tumoral role of the ECM on immunity. Moreover, it is however important to note that the *in vitro* impact of any single matrix molecule on macrophage phenotype may be more complex than a black and white dichotomy, as highlighted by Huleihel et al. This group exposed macrophages to ECM bioscaffolds (69) and observed that macrophages turned either M1-like or M2-like depending on the tissular origin of the ECM. Moreover, pre-activation of macrophages with IFNγ and LPS lead to a decrease of inflammatory responses in all ECM stimuli tested, altogether indicating that not only the ECM network composition can influence macrophage activation status, but that the inflammatory contexture may also orientate their polarization.

Together, these studies reveal the role of the immunomodulatory properties of the ECM to be a double edged sword in the shaping of the immune response, as ECM molecules can drive an M2 phenotype in TAM as well as triggering TLR and NF-κB dependent inflammatory responses based on the specific cues of the local cellular and tissular microenvironment. Understanding the mechanisms by which the ECM network shape the TAM phenotype may offer clues about events occurring during breast cancer. Although it is likely that the levels of ECM expression and the number of macrophages may modulate these responses, the cellular source of ECM molecules, which alternatively spliced domains can be differentially expressed by different cell types, is also an important factor to take in account for the capacity of ECM to modulate macrophages, and will be discussed below.

## Macrophages as Shapers of the Tumor ECM

Whilst the tumor-associated matrix is able to modify macrophage infiltration, organization and phenotype, in turn, macrophages are also capable of directly modulating the organization and composition of the ECM network. This phenomenon has been well described in particular *via* the capacity of macrophages to secrete matrix metalloproteinases (MMP), enzymes that are able to degrade ECM proteins and that are key determinants for facilitating cell migration (70). In breast cancer, MMP-2, 9, 13 and 14 are involved in a broad spectrum of actions including the remodeling of the ECM, cell migration and metastasis, as well as neo-angiogenesis (71). Several studies further suggest that macrophages have a crucial role in the organization of the ECM. In CSF-KO mice, where numbers of circulating and mammary glands resident macrophages are greatly reduced due to their dependency on CSF, mammary

tissue levels of collagen were unaffected, however collagen fibrillogenesis into long fibers was impaired (72). Moreover, the depletion of TAM dramatically altered collagen fibrillar microstructure in the tumor, changes that were associated with an increase in the number of lung metastases in an orthotopic engraftment breast tumor model (46). This could be explained by the involvement of macrophage-derived MMPs, contributing to the degradation of the matrix and as a consequence help for cancer cell intravasation and extravasation. These data suggest a role for TAM in the organization, or re-organization, the matrix, but other studies show that TAM may also play a role in *de novo* synthesis of the tumor specific ECM.

Macrophages can directly secrete ECM components. For example, this was demonstrated by an RNA expression study in primary human cells, showing that most myeloid cells are able to secrete tenascin-C upon activation (73). Similarly, macrophages are also able to produce different types of collagen upon a TLR4-dependent activation (59), further demonstrating the importance of TLR triggers is this crosstalk. Moreover, the presence of IL-6 and collagen in a triple negative mammary tumor model drove TAM towards a "wound healing" phenotype characterized by the production of effectors of the inflammatory phase of wound healing IL-1β, IL-6 and osteopontin in that in this context facilitated the trans-endothelial migration of tumor cells (44). Whilst a link between macrophage residence in the TME and matrix synthesis in the breast remains to be further explored, data are available from other tumor sites. For example, using an orthotopic model of colorectal carcinoma, Afik et al. showed that the ECM composition in tumors is markedly different in TAM high or deficient tumors as TAM activate matrix remodeling programs upon their differentiation from monocytes (74). In particular, TAM were able to express unique ECM and ECM-associated genes in the TME, including collagen type I VI and XIV, collagen synthesis and assembly as well as matrix cross linkers gene sets, leading to an impaired deposition, cross-linking and linearization of the matrix in presence of TAM, and altogether shaping the tumor invasiveness (74). The idea has been broached that TAM have a unique impact on ECM remodeling compared to other macrophage subsets. This was investigated in a study showing that TAM from ovarian carcinoma are remarkably similar at the transcriptomic and protein expression levels to resident peritoneal macrophages, sharing features such as phagocytic and antigen presentation capabilities levels (75). However, TAM had a non-overlapping gene expression signature not shared with monocyte-derived macrophages and resident peritoneal macrophages, mainly composed of matrix-remodeling genes and collagen fiber organization. These data indicate that these macrophage subpopulations acquire particular capacities to manipulate the ECM in tumors (75). How these cells acquire these novel capabilities remains unclear but this re-programming may be induced by tumors cells in order to aid tumor escape from immunosurveillance.

In a model of mammary tumor engraftment by tumor cells with a conditional Her2 downmodulation inducible by doxycycline which leads to tumor regression, the following tumoral recurrence is associated with an increased tumor production of CCL5, a chemokine involved in many aspects of tumor progression in breast cancer (76). The authors link this secretion with the recruitment of CCR5 expressing macrophages that express genes of collagen and collagen-deposition factors such as procollagen C-endopeptidase enhancer 1 and asporin (47), suggesting that the CCL5/CCR5 pathway can be manipulated by tumors to provoke macrophage to directly deposit collagen into the tumors, favoring their recurrence.

The functional implications of TAM-derived ECM networks are also emerging, with data showing that immune cell-mediated matrix synthesis directly contribute to tumor growth. For example, in mouse models of mammary tumors cell lines orthotopic engraftment, the use of osteopontin wt or ko mice showed that myeloid derived suppressor cells (MDSC) exerted a more immunosuppressive impact at the metastatic site in presence than in absence of osteopontin (48). In non-small cell lung cancer, the concomitant tissue detection and quantification of macrophages and osteopontin revealed that osteopontin produced by TAM is also associated with progression and poor survival (77) and in colorectal cancer co-culture models, the expression by cancer cells of osteopontin receptor CD44 drives its production by TAM, which in turn favors tumor cells tumorogenicity (78). On the contrary, Szulzewsky et al. showed that glioblastoma-associated monocytes are the main producers of osteopontin in the TME and that it exerts an anti-tumor effect, as opposed to tumor-derived osteopontin which has little impact (79). This suggests that TAM-derived osteopontin can be used as a communication tool by macrophages to interact with cancer cells and impact the TME. This idea of an intermediate signal used by macrophages to shape the ECM in breast cancer can also be applied to TGFβ, which not only can be produced by tumor-facilitating M2-like TAM and is a key regulator of carcinogenesis in tumors such as breast cancer (80), but is also a key signal involved in fibrosis and ECM production (81). Moreover, TGFβ in conjunction with tenascin-C are associated with an epithelial to mesenchymal transition (EMT) of breast cancer cells (82), and stimulation of macrophages by TGFβ force them to produce type VI collagen (83). Finally, although not in models of tumorigenesis, but during experimental dermal remodeling, CCR2 expressing macrophages directly degraded collagen and fibrins, and the addition of GM-CSF selectively enhanced their collagen endocytosis capacities, likely *via* the proliferation-inducing capacities of this cytokine (84). These data suggest an involvement of the CCL2/CCR2 axis in matrix remodeling and tissue repair mechanisms, in favor of a positive reinforcement loop between the ECM and macrophages; it is for example known that collagen degradation products can play a chemotactic role towards macrophages, which may contribute to their recruitment to the tumor site (85).

These data demonstrate how macrophages can modify the composition and the organization of the ECM network in tumors by producing specific ECM or ECM associated components or by reshaping collagen fibers, showing that TAM-ECM crosstalk occurs in both directions.

## The Regulation of EMT and Metastasis by ECM and TAM

One of the best studied roles of the ECM network in breast cancer is its contribution to driving metastatic transition (86). The role of TAM in this process have also been extensively studied *via* their capacity to secrete matrix metalloproteinase MMPs that are critical for the digestion of ECM prelaminar to the migration of cancer cells outside the primary tumor site (87), and this has been reviewed elsewhere (88, 89). However, other mechanisms of interplay between TAM and ECM have also been reported in breast cancer models, such as in driving epithelial to mesenchymal transition (EMT). For example, versican, a matrix proteoglycan, is produced by monocytes in pre-metastatic lesions where it aids breast cancer cell transformation in MMTV-PyMT mice (51), whilst expression of the ECM glycoprotein SPARC (secreted protein acidic and rich in cysteine) by breast tumor cells induces EMT dependently on the presence of MDSC (52) in breast cancer cell engraftment, and expression of SPARC by macrophages induces cell migration and metastasis in triple-negative breast cancer cells engraftment (50). Moreover, Wyckoff et al. elegantly used intravital microscopy in a MMTV-PyMT model to show that the cancer cell intravasation observed by live imaging in mammary tumors was helped by perivascular macrophages that accumulated by collagen-rich tumor border in association with the EGF and CSF-1 pathways (49). Together, these data provide clues for a better understanding of one of the tumor-facilitating roles of the ECM-TAM collaboration, which is the promotion of early events crucial for metastasis in epithelial cancers including EMT and cancer cell migration.

## The Need for CAF in the TAM-ECM Relationship

The presence of a specific ECM network within tumors is the result of the contribution of many cellular players including not only tumor cells and immune cells, but also CAF. CAF are critical for breast cancer evolution and important producers of extracellular matrix in tumors (90, 91). The interactions between CAF and TAM are becoming increasingly well characterized, for example co-culture of monocytes with pancreatic tumor cells with CAF drives monocytic differentiation into cells with an M2-like macrophage phenotype (92). Moreover, CAF and TAM crosstalk can synergize to increase the invasiveness of the tumor by increasing cell mobility, as well as favoring neoangiogenesis programs (93). Indeed, a role for the ECM in this intercellular interplay is also emerging; endogenous fibroblasts and TAM may also matrix molecules as a means to communicate with one another in the breast TME. For example, fibroblast synthesis of FAP (fibroblast activation protein), a membrane-bound serine protease that can cleave collagen fibers and thus increase TAM adhesion in an MMTV-PyMT breast cancer model (53). In our work, using a syngeneic orthotopic grafting model of breast cancer, the absence of tenascin-C production by tumor cells was compensated by CAF-derived tenascin-C, meaning that total tumor levels of this matrix protein were not altered when grafting tumors with high or low tenascin-C expression levels. However, tenascin-C derived from each cellular source had a dramatically different impact on the TAM phenotype; only tumor-derived tenascin-C could induce a pro-tumor TAM phenotype, as opposed to CAF-derived tenascin-C (31). These data together pinpoint CAF as contributors of the TAM-ECM crosstalk in the breast TME and potential target to block these pro-tumor processes.

# CONCLUSIONS AND PERSPECTIVES

A growing number of studies report ever increasingly complex interactions between the ECM and the tumor myeloid compartment. These extend beyond the degradation of ECM by TAM-derived MMPs and integrin-mediated TAM-matrix cell adhesion. As discussed here, tumor-specific ECM and TAM crosstalk has multiple faces; the ECM can shape macrophage infiltration, positioning and phenotype, driving cells towards anti- to pro-tumoral, inflammatory, pro-angiogenic or pro-metastatic depending on the local tissue contexture, whilst macrophages can systematically modify the organization of ECM fibers, as well as the composition of the ECM network (**Figure 1**, **Table 1**). The coordination of the ECM–TAM crosstalk ultimately impacts the orientation and strength of the innate and adaptive anti-tumor immune response, and thus modulates the rate of the primary tumor growth and metastasis. The importance of these interactions for cancer evolution therefore represents a pool of promising biomarkers and myriad of potential therapeutic targets for new anti-cancer immunotherapies in breast cancer.

Moreover, the interaction between the ECM and TAMs is not static, but constantly changing. As the tumor develops, grows, and spreads, changes in the tumor microenvironment brought by cooperative anabolism and catabolism of CAFs, proliferating tumor cells and infiltrating immune cells contribute to dynamically modify the biochemical and mechanical signals mediated by the ECM that shapes cell behavior. At present, we have a limited understanding of the complexities of this evolving TAM-ECM relationship, despite the recent increase in the number of interactions being discovered and functionally characterized, and with it the perspective of novel biomarkers (94). Furthermore, more detailed investigation into pathways that are universal amongst tumors, compared to tumor-, or tissue-, specific changes will be of importance. This approach may indeed shape our understanding of how the crosstalk between ECM and TAM may have a different impact on cancer evolution depending on disease stage, tumor type and contexture as well as articulation with co-treatments, and become critical for the success of new immunotherapies.

The field of immunotherapies in general, and in cancer in particular, is indeed evolving in giant strides, as represented by the success of T-cell targeting immune checkpoints inhibitors anti-PD1, anti-PD-L1 and anti-CTLA4, that revolutionized treatment of cancer patients (95). But immune checkpoints inhibitors face limitations, due to induced resistance, lack of target expression, or lack of immune infiltrate in "cold" tumors (96, 97). In breast cancer,

in particular, we see highly heterogeneous pathology (98), with a complex organization of the therapeutic landscape. Triple negative breast cancer (TNBC) that are defined by their lack of expression of progesterone receptor, estrogen receptors and Her2-neu particularly suffer from an absence of viable long-term therapeutic options, and although anti-PD-L1 immune checkpoints inhibitors have recently given promising results after a phase III clinical trial (99). To this end, and because of the importance of macrophages in this cancer, several macrophage-targeting options have been extensively explored, mainly including blocking the CCL2/CCR2 or the CSF1/CSFR1 pathways (100, 101), but they have faced mitigated success. Targeting specific domains of the tumor-specific ECM in order to reorient the immune response is in this context an emerging option which efficacy has been explored in different situations. In our model of mammary tumor expressing or not tenascin-C, the blockade of the TLR4-binding domain of tenascin-C, FBG, with a neutralizing antibody (102) lead to a reversion of the TAM phenotype towards a tumor-killing type, and was associated with decreased tumor growth and smaller lung metastases (31). Moreover, the therapeutic combination of this FBG-targeting therapy with an anti-PD-L1 antibody further diminished the tumor growth. Similar options were investigated against the EDA alternatively spliced domain of fibronectin, by developing a fusion protein with an anti-EDA sequence and calreticulin, an alarmin serving as a pro-phagocytosis signal towards myeloid cells. In a colon cancer model, its therapeutic use helped slow down tumor growth and this treatment worked synergistically with an anti-PDL1 antibody (32), confirming the interest for combining this type of matrix-targeting therapy modulating the macrophage innate response with T-cell targeting with immune checkpoints inhibitors to unlock different levels of tumor-associated immune suppression. Arribillaga et al. used the capacity of the EDA domain of fibronectin to activate myeloid cells *via* binding TLR4 (61) to develop an immunogenic antigen delivery tool by coupling EDA with proteins, activating dendritic cells, which were then able to effectively prime specific T cells (103), highlighting again the dual role of ECM domains of the activation of innate immune cells. Other options have been explored to use the immunomodulatory properties of fibronectin, including against the alternatively spliced EDB domain, which neutralizing sc-Fv fragment L19 was coupled with IL-2 to trigger immunogenicity (104). Thrombospondin-1 could also be used as a target, and its neutralization blocked the osteoclast differentiation from monocytes formation occurring during myeloma bone (105). Another way by which therapies investigate the blocking or enhancement of the ECM-macrophages interaction consists in targeting membrane-bound molecules serving as receptors for ECM or ECM-associated proteins. It is the case for CD47, a ligand of thrombospondin-1, which can be expressed by cancer cells and act as a "don't eat me" signal towards macrophages, hence a good target for cancer (106). Anti-CD47 neutralization has shown promising anti-tumor properties by promoting the phagocytosis of cancer cells by TAM, and evidenced potential to synergize with other tumor-targeting therapies (107). Finally, the blockade of the interaction between ECM and TAM can be performed *via* integrin blockade. $\alpha4\beta1$ in particular, is an integrin expressed by monocytes and necessary to their homing into the tumor as well as a receptor for the EDA domain of fibronectin (108) and its blockade on TAM limited the macrophage-induced neoangiogenesis (36), helping control the tumor growth. Finally, it is noteworthy that CSFR1 inhibitors were able to block the migration of macrophages induced by fibronectin (109). The exploration of how macrophages and the matrix work together to shape the TME provides a large panel of pathways that leads the tumor growth and spread, and the understanding of these signaling is still at its beginning. The majority of these studies report the tumor-facilitating roles of the TAM and ECM crosstalk, and these pathways constitute powerful and relatable predictive biomarkers for cancer evolution and metastasis. But they also provide invaluable indications for new pathways to target in novel therapy for cancers that are lacking options, like the TNBC. Preclinical studies suggest that targeting immunomodulatory domains of the ECM can be an efficient and safe way to reinstate an efficient innate immune response, and is working well as combination therapies, at a time where multi-hit therapy paves its way in the cancer therapeutic landscape.

## AUTHOR CONTRIBUTIONS

CD and KM wrote the review. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Immune Checkpoint Inhibitors in Triple Negative Breast Cancer Treatment: Promising Future Prospects

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Immunotherapy has emerged as the fifth pillar of cancer treatment alongside surgery, radiotherapy, chemotherapy, and targeted therapy. Immune checkpoint inhibitors are the current superheroes of immunotherapy, unleashing a patient's own immune cells to kill tumors and revolutionizing cancer treatment in a variety of cancers. Although breast cancer was historically believed to be immunologically silent, treatment with immune checkpoint inhibitors has been shown to induce modest responses in metastatic breast cancer. Given the inherent heterogeneity of breast tumors, this raised the question whether certain breast tumors might benefit more from immune-based interventions and which cancer cell-intrinsic and/or microenvironmental factors define the likelihood of inducing a potent and durable anti-tumor immune response. In this review, we will focus on triple negative breast cancer as immunogenic breast cancer subtype, and specifically discuss the relevance of tumor mutational burden, the plethora and diversity of tumor infiltrating immune cells in addition to the immunoscore, the presence of immune checkpoint expression, and the microbiome in defining immune checkpoint blockade response. We will highlight the current immune checkpoint inhibitor treatment options, either as monotherapy or in combination with standard-of-care treatment modalities such as chemotherapy and targeted therapy. In addition, we will look into the potential of immunotherapy-based combination strategies using immune checkpoint inhibitors to enhance both innate and adaptive immune responses, or to establish a more immune favorable environment for cancer vaccines. Finally, the review will address the need for unambiguous predictive biomarkers as one of the main challenges of immune checkpoint blockade. To conclude, the potential of immune checkpoint blockade for triple negative breast cancer treatment could be enhanced by exploration of aforementioned factors and treatment strategies thereby providing promising future prospects.

**Keywords: triple negative breast cancer, immune checkpoint blockade, predictive biomarkers, tumor mutational burden, tumor infiltrating lymphocytes, combination therapy, programmed death-1 (PD-1), programmed death ligand-1 (PD-L1)**

## INTRODUCTION

Breast cancer constitutes a major health problem worldwide, accounting for 30% of all female cancer cases and 15% of female cancer-related deaths (1). Clinically, breast tumors are categorized into hormone receptor positive (HR+) tumors expressing the estrogen (ER) and/or progesterone (PR) receptors, Human Epidermal Receptor 2 (Her2)-enriched tumors with overexpression of Her2 in the absence of HR expression, and triple negative tumors lacking expression of all three receptors. Standard treatment of these clinical subtypes consists of surgery, radiotherapy, chemotherapy, hormonal therapy, anti-Her2 targeted therapy or a combination thereof. In recent years, with -omics based profiling becoming more accessible and affordable, molecular profiling of tumors has started to enter clinical routine such as the multigene OncotypeDX, Mammaprint and ProSigna tests (2–4). Each of these assays uses distinct gene signatures to predict the risk of recurrence of early stage, hormone receptor positive (and negative) breast cancer. In addition, the OncotypeDX test helps to predict the likely benefit of adjuvant chemotherapy in early stage HR+ cancer. The more recent Prosigna test not only provides a 10-year risk of recurrence score but also classifies breast tumors into distinct prognostic molecular subtypes based on the Prediction Analysis of Microarray 50 (PAM50) gene signature. This signature forms the basis of the PAM50 classifier that has provided major insights into the molecular heterogeneity of breast tumors (5, 6). More specifically, the classifier categorizes breast tumors into four distinct molecular subtypes with different response to treatment and clinical outcome: luminal A (LA), luminal B (LB), Her2-enriched (Her2+), and basal-like (BL). Furthermore, stratification of breast tumors based on the presence of tumor infiltrating lymphocytes (TILs) and differential expression of immune-related genes revealed further heterogeneity with prognostic significance (6–9). Using a gene signature composed of immune-regulatory genes, chemokine ligands and genes involved in T helper 1 (Th1) signaling and effector immune functions, approximately 30% of basal-like and Her2-enriched breast tumors can be classified as tumors with an immune favorable phenotype as compared to 5–10% of luminal type tumors (9). In this review, we will look into cancer cell-intrinsic and/or microenvironmental factors that have a likely effect on shaping the tumor immune phenotype, and will discuss the emerging potential of immune checkpoint inhibitors (ICIs) in triple negative breast cancer treatment in particular.

## POTENTIAL OF IMMUNOTHERAPY IN (TRIPLE NEGATIVE) BREAST CANCER: PARAMETERS TO BE CONSIDERED

Cancer immunotherapy is considered the new pillar of cancer treatment, shifting the focus from the tumor to the tumor microenvironment and was awarded the Nobel Prize for physiology or medicine in 2018. Numerous immunotherapy approaches have proven effective in generating durable clinical responses, with the greatest success stories to date coming from treatment with immune checkpoint inhibitors (10–13). It is well known that tumors adopt various mechanisms to evade detection and eradication by the immune system, including the activation of inhibitory pathways governed by immune checkpoints. Treatment with ICIs releases the immune system from these inhibitory signals and reinvigorates the anti-tumor immune response as demonstrated by numerous studies and clinical trials using monoclonal antibodies against cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), programmed death-1 (PD-1), and programmed death ligand-1 (PD-L1) (14–18). In breast cancer, especially triple negative breast cancer, treatment with ICIs has been found to improve clinical outcome (18). Overall, immune checkpoint inhibition is well tolerated and is associated with a relatively mild toxicity profile. However, immune-related adverse events may develop and need to be closely monitored, including the development of colitis, thyroid dysfunction, hypophysitis, skin rash, pneumonitis, and inflammatory arthritis (19).

The success of immunotherapy largely depends on the immunogenic nature of the tumor, exemplified by the higher response rates in malignant melanoma and non-small cell lung carcinoma (20, 21). Traditionally, breast cancer has been considered an immune silent cancer type that is less likely to benefit from immunotherapy. Increasing evidence, however, indicates that breast cancer constitutes a varied spectrum of tumors with different degrees of immunogenicity whereby triple negative breast cancer is believed to be a more immunogenic subtype (7–9, 22, 23). Moreover, multiple factors derived from tumor cells or from within the tumor micro- or macro-environment dictate the immune contexture of a tumor and hence responsiveness to immunotherapy, including the tumor mutational burden (TMB) and neoantigen load, diversity of the immune infiltrate and the microbiome.

### Tumor Mutational Burden and Neoantigen Load

The tumor mutational burden is defined as the total number of somatic nonsynonymous mutations in the coding region of genes that may result in the generation of abnormal proteins or neoantigens (24, 25). A high TMB and number of predicted neoantigens has been associated with a better response to immune checkpoint inhibitor therapy in various cancer types (26–30). In breast cancer, most tumors harbor a low TMB (1mut/Mb) and only 5% of all tumors are characterized by a high tumor mutational burden (≥ 10 mut/Mb) of which most are metastatic (31, 32). More specifically, TNBCs have a higher TMB compared to Her2-enriched and HR+ tumors (33, 34). Analysis of the TCGA and METABRIC breast cancer datasets demonstrate an improved overall survival (OS) for patients with tumors featuring a high TMB and favorable immune-infiltrate disposition (FID), irrespective of the type of treatment. Luminal A tumors with a high TMB/FID phenotype were associated with the best survival rates, whereas TNBCs with a high TMB and poor immune-infiltrate disposition were

associated with the worst prognosis (25). Conversely, immune-rich TNBC tumors with lower mutation and neoantigen counts have been associated with better prognosis, likely due to a reduced clonal heterogeneity as a result of immunosurveillance (35).

Furthermore, tumors with somatic or germline *BRCA1/2* mutations are believed to be more immunogenic due to the dysregulation of homologous recombination-based DNA repair, leading to increased genomic instability and higher mutational burden (36). However, *BRCA1/2* mutation-associated breast tumors display a great variability in immunogenicity with approximately 50% of tumors displaying an absent or mild tumor lymphocyte infiltrate and moderate neoantigen load, suggesting that only a subset of *BRCA1/2* breast tumors may benefit from immune-based therapy (37). In line with this, at best 1 out of 5 patients with triple negative breast cancer, the most common form of *BRCA1* mutation-associated breast cancer, has been shown to benefit from single agent PD-1 blockade (38–40). Interestingly, genomic analysis of 115 *BRCA1/2* breast tumors revealed an inverse association between homologous recombination deficiency (HRD) and immunogenicity despite a higher mutational burden and neoantigen load (41). Moreover, hormone receptor status further stratified *BRCA1/2* breast tumors with low-HRD TNBC tumors being more immunogenic than high-HRD HR+ tumors (41). This unexpected inverse correlation of high TMB, resulting from homologous recombination deficiency, and immunogenicity is supported by a pan-cancer analysis that demonstrated that large somatic copy number alterations are associated with reduced immunogenicity, possibly due to disruption of genes involved in the regulation of immune cell recruitment (42). In accordance, PTEN, another important regulator of DNA damage repair and hence mutational burden, is frequently impaired in tumors and loss of PTEN has been associated with poor response to PD-1 blockade (43, 44). For instance, patients with metastatic TNBC (mTNBC) who carry *PTEN* mutations had a significant lower response rate to PD-1/PD-L1 inhibitors (45). Moreover, in the absence of PTEN-mediated inhibition of the PI3K-Akt pathway, the use of an Akt inhibitor combined with chemotherapy and PD-L1 blockade significantly improved the overall response rate of metastatic TNBC patients compared to combination treatments of chemotherapy with PD-L1 blockade or Akt inhibition (46). Together, these findings suggest that in a proportion of breast tumors ICI response is not dictated by TMB per se but rather by specific genomic events that disrupt a functional immune response.

## Diversity of Immune Infiltrate

In addition to cancer cell-intrinsic features, the tumor microenvironment plays a prominent role in determining anti-tumor immunity and response to immunotherapy. Understanding the complexity of the interplay between tumor cells and components of the immune system offers a unique opportunity to explore combination treatments that can help to reshape the tumor microenvironment into an immune favorable phenotype. Immunohistochemical analyses of tumor immune infiltrates has resulted in the classification of tumors into distinct immune phenotypes: "hot", "cold-immune desert", and "cold-excluded" tumors (47–49). Immunological "hot" tumors often have a high TMB and number of neoantigens, and have a high likelihood of provoking an anti-tumor immune response. They are also called "inflamed tumors" as they are characterized by a considerable infiltration of T cells although these are not fully functional. Overall, hot tumors are associated with a better response to ICIs through the activation of the present immune infiltrate (50) and examples include melanoma, non-small cell lung cancer, head and neck cancer, kidney, liver, and bladder cancer. Immunological "cold" tumors either exhibit a lack or paucity of a T cell infiltrate, the so-called "immune desert" tumors, or feature a phenotype whereby T cells have been excluded from the tumor core and aggregate at the tumor boundaries, the so-called "immune excluded" tumors. Tumors with an "immune excluded" phenotype reflect the ability to induce a T cell- mediated immune response, however, the response is impaired by the inability to penetrate the tumor tissue. The presence of immunosuppressive immune cell subsets within the tumor or tumor microenvironment can alter both the infiltration and functional status of the T cell infiltrate and hence reduce the potential benefit from ICI therapy (48). Many studies are looking into ways to turn "cold" tumors into "hot" tumors to achieve higher responsiveness to immune checkpoint blockade. Here, we will discuss some of the factors to be considered in addition to the density and localization of the immune infiltrate such as the cellular composition and functional orientation of the immune cell infiltrate and of tertiary lymphoid structures (TLS), the expression of immune checkpoints, and the enrichment of prognostic immune gene signatures.

Tumor infiltrating lymphocytes or TILs represent the major infiltrating immune cell subpopulation defining a favorable immune microenvironment in tumors. The density of TILs is indicative of the magnitude of anti-tumor immunity and is emerging as a prognostic and predictive biomarker for immunotherapy response in a wide range of cancers (51–53). The seminal work by Galon et al. introduced the immunoscore concept in colorectal cancer, an immunohistochemically-based scoring system of CD8+ TILs in the center and invasive margin of a tumor with independent prognostic connotation (47). Subsequent work consolidated the prognostic value of the immunoscore in colorectal cancer and multiple other cancers (53–56). A recent study on the predictive value of the immunoscore in colorectal cancer patients suggests that patients with a low immunoscore do not benefit from a longer treatment with oxaliplatin-based chemotherapy as opposed to patients with intermediate or high immunoscore values (55). This observation seems counterintuitive as one could argue that patients with low immunoscore and higher risk of recurrence would more likely benefit from longer treatment. However, it is important to consider the interactions of the chemotherapeutic agents with the immune response. Oxaliplatin is known to elicit bona fide immunogenic cell death and 5-fluorouracil decreases the number of myeloid derived suppressor cells (MDSCs) while enhancing the cytotoxic T cell function, however, these effects depend on the presence of an active tumor immune microenvironment. Therefore, tumors with a low immunoscore and weak cytotoxic T cell activity may not experience additional benefit from increasing the treatment duration. More studies are needed to

validate these findings as the follow-up time of the current study was rather short with 4.3 years. In breast cancer, the immunoscore has not yet been established as a prognostic and/or predictive biomarker, however, a plethora of studies supports the importance of the tumor immune microenvironment in defining breast cancer clinical outcome. Numerous studies have demonstrated an association of breast tumor infiltration by cytotoxic T lymphocytes with better survival (57–60). In particular, TNBC and Her2-enriched tumors feature high TIL counts, which are associated with better clinical outcome, and suggest greater immunogenicity and likely benefit from immune-based interventions (61, 62). Higher densities of TILs have also been associated with greater response rates to chemotherapy (62–65).

In line with the immunoscore concept, spatial distribution of lymphocytes beyond intratumoral lymphocytes could provide added value for predicting survival and treatment response in breast cancer. High densities of stromal T lymphocytes have been associated with improved breast cancer specific survival of patients with TNBC and Her2-enriched tumors (66). Moreover, one study expanded the immunoscore concept by quantifying the density of immunosuppressive FoxP3 T regulatory cells (Treg) in addition to CD3+ and CD8+ T cells (67). Interestingly, they were able to develop a prognostic scoring system that could distinguish molecular breast cancer subtypes. Joint analysis of immunosuppressive CD163+ tumor associated macrophages (TAMs) with cytotoxic CD8+ T lymphocytes resulted in a novel immune infiltrate scoring model with favorable prognosis, as defined by high CD8+ and low CD163+ cell counts in the tumor center and low CD8+ and high CD163+ in the invasive tumor margin (68, 69). These findings highlight the importance of capturing a complete picture of the tumor immune microenvironment, accounting for both cytotoxic T cells and immunosuppressive immune cell populations. This notion is further supported by the ongoing discussion on the prognostic value of tertiary lymphoid structures within the tumor or tumor microenvironment. Several studies in a range of cancer types have reported a favorable outcome for patients with a high number of TLS, irrespective or in addition to a high TIL count (70, 71). In TNBC, high TIL counts in combination with moderate to high TLS counts have been associated with improved disease free survival (DFS) (70). On the other hand, a number of studies have reported conflicting data that do not support a favorable prognostic value for TLS (69). Notably, TLS can exert a dual effect on anti-tumor immunity, serving as an *in situ* niche of cytotoxic T cells as well as of immunosuppressive cells such as T regulatory cells and hence high TLS counts can be associated with better or worse prognosis (72). High TLS counts have been associated with better DFS in patients with Her2-enriched tumors whereas no prognostic value was observed in Her2-negative breast cancer patients. Therefore, it is clear that the current definition of the tumor immune microenvironment needs to be revisited in order to account for TLS cellular composition and functional orientation.

This brings us to the pivotal role of the activation status of the tumor immune infiltrate which is partly controlled by the expression of immune checkpoints. The presence of infiltrating T lymphocytes has been associated with elevated expression of PD-L1 (73–75), corroborating the therapeutic potential of immune checkpoint blockade in tumors with a high T cell immune infiltrate density. In accordance, high TIL scores in patients with TNBC and Her2-enriched tumors predict a better response to PD-1 inhibitors, counteracting the increased PD-L1 expression (51, 76, 77). In a study involving more than 3,000 breast cancer patients, the relevance of TILs for chemotherapy response and prognosis in patients of different breast cancer subtypes was assessed (78). Increased TIL counts were associated with a survival benefit and better response to neoadjuvant chemotherapy in Her2-enriched breast cancer and TNBC. In contrast, a different role for TILs was observed in luminal breast cancer where an increase in TILs was associated with adverse prognostic effects. Furthermore, combined analysis of TIL density and PD-L1 tumor expression indicated that the DFS of TNBC patients with low-TIL tumors (< 30% stromal) was significantly worse compared to patients with high-TIL tumors, with the most unfavorable DFS and OS for patients with low-TIL and high PD-L1 (> 50%) (75). Furthermore, the presence of specifically tissue resident memory T cells in the TIL infiltrate of TNBC tumors has been associated with better response rates and overall survival in patients who received chemotherapy or PD-1 inhibition (51, 79, 80). Interestingly, characterization of TILs after treatment with PD-1/PD-L1 inhibitors revealed an increase in expression of various immune checkpoints including PD-1, CTLA-4, T cell immunoglobulin and mucin domain-containing protein 3 (Tim3) and Lymphocyte-activation gene 3 (Lag3) in CD4+ T cell subsets suggesting the presence of a compensatory inhibitory mechanism mediated by CD4+ T regulatory cells (81). These findings underscore the need to identify, quantify, and phenotype all components of the immune microenvironment including immunosuppressive regulators. Great efforts are expended to develop strategies to deplete immunosuppressive cells from the tumor microenvironment, to impede their infiltration and to impair their functionality, or to induce cytotoxic T cell expansion, survival and function by modulating cytokine levels (82–84). Importantly, any of these strategies could be combined with immune checkpoint inhibitors. In this context, it is important to note that PD-1 and CTLA-4 are not only expressed on activated T cells, but also on T regulatory cells. Hence, treatment with anti-PD-1 and/or anti-CTLA4 antibodies may result in the additional release of Treg-mediated suppression of T cell activation, strengthening the anti-tumor immunity (85–88). Of note, additional factors besides immune checkpoint expression probably affect ICI response and clinical outcome as for instance, only a small proportion of metastatic PD-L1 positive TNBC patients (8–20%) respond to PD1/PD-L1 therapy (76).

In an attempt to comprehensively capture the immune contexture of a tumor, numerous immune gene signatures have been developed. The first prognostic immune signature describing the functional orientation of the tumor immune microenvironment was established in colorectal cancer and was composed of genes involved in Th1 and cytotoxic T cell function, including interferon- γ (IFN-γ), granulysin (GNLY), perforin (PRF1), and granzymes (GZMs) (47). This signature

was subsequently validated in other cancer types including breast cancer (89, 90). In addition, Hendrickx et al. demonstrated that the Immunologic Constant of Rejection (ICR) 20-gene signature can differentiate immune favorable and immune unfavorable breast cancer subtypes, and recently refined and validated its prognostic value in a pan-cancer study (9, 91) Furthermore, they showed that MAPK pathway regulation could modulate the intratumoral response in breast cancer (9). A meta-analysis of approximately 18,000 human tumors identified complex associations between 22 distinct leukocyte subsets and cancer survival (92). Using the CIBERSORT algorithm for relative immune cell abundance, the authors demonstrated that tumor-associated neutrophil and plasma cell signatures are significant but opposite predictors of survival in breast cancer. Further, a T cell- inflamed gene expression profile exhibited predictive value to identify pan-cancer patients that will more likely benefit from PD-1 inhibition (93). Paradoxically, subsets of breast cancer patients with high expression of immune-associated signatures have been identified to experience poor outcome (94), suggesting the presence of additional complexity beyond the current information provided by bulk tumor immune signatures. Moreover, some studies have demonstrated differences in spatial distribution of immune gene signatures (95). For instance, integration of CD8+ T cell localization and matched stromal and epithelial tumor gene expression signatures revealed distinct, spatial, tumor immune microenvironment-subtypes of treatment-naive TNBC tumors, each characterized by a specific metagene signature (96).

## Gut and Breast Microbiome

The gut microbiome is a recognized master modulator of the development and maintenance of a healthy immune system (97, 98). Perturbation of the normal microbiota—dysbiosis—is often observed in disease and changes the interactions between the gut microbiota, intestinal epithelium, and host immune system (99). Many studies have shown that gut microbiota shape the immune system and the host metabolism. In addition to regulating local, intestinal immune responses, changes in gut microbiota can have systemic effects on the innate and adaptive immunity. While the encounter of microbial molecules by Toll-like receptors provoke a local immune response in the gut, the escape of microbial factors from the gut can modulate immune function, causing systemic infection or inflammation which favors the development of immune-mediated and metabolic diseases (100). Thus, understanding how the gut microbiota impact anti-tumor immunity could provide insight into how it might influence tumor development, progression and treatment response. In breast cancer, a collection of microbial genes known as the estrobolome has been shown to affect estrogen metabolism, resulting in higher circulating levels of estrogen and hence an increased risk of hormone-dependent breast cancer (101). Furthermore, the gut microbiome has been found to be involved in the regulation of tumor progression and the response to anticancer therapies (102–106). For instance, gut microbiome dysbiosis has been shown to promote cancer cell dissemination in a HR+ breast cancer mouse model through increased fibrosis and collagen deposition (107). Several studies have identified distinct microbial signatures in breast cancer patients, however, further studies are needed to define their diagnostic and therapeutic implications (108–110). Furthermore, few studies demonstrated that the composition of the gut microbiota could influence the response to immunotherapy, including immune checkpoint. For instance, comparing the gut and oral microbiome of melanoma patients treated with anti-PD-1 immunotherapy revealed significant differences in the diversity and composition of gut microbiota in patients that responded to treatment versus non-responders (103, 105). Furthermore, exposure to broad-spectrum combination antibiotics (fluoroquinolones, ß-lactam$^{+/-}$ or macrolides) during anti-PD1/PD-L1 treatment has been shown to significantly decrease progression-free survival (PFS) and OS of patients with advanced non-small cell lung, renal cell carcinoma, and urothelial carcinoma, suggesting that the overall diversity of the microbiota and the presence of specific clades determines the responsiveness to immunotherapy (104).

Historically, breast tumor tissue has been considered a sterile environment, however, recent studies suggest the existence of a local, breast microbiome. Indeed, the composition of breast tissue with abundance of fatty tissue, extensive vasculature, and lymphatic drainage makes it a favorable environment for the growth of bacteria (111). Comparison of microbial signatures across multiple cancer types revealed cancer type specific microbial signatures that differ between the respective tumors and adjacent normal tissues whereby breast cancer was associated with a particularly rich and diverse microbiome. Furthermore, the breast microbiome has been shown to differ from normal to benign to malignant tissues, as well as between breast cancer subtypes, and in relation to response to immunotherapy (112, 113).

# IMMUNE CHECKPOINT INHIBITION IN TRIPLE NEGATIVE BREAST CANCER

ICI therapy has become the most successful immune-based intervention to generate durable responses in a variety of tumors. Monoclonal antibodies against PD-1/PD-L1 and CTLA-4 have emerged as powerful tools to release the inhibitory regulation of T cell activation (114, 115). To date, multiple blocking monoclonal antibodies have been approved by the US Food and Drug Administration (FDA) including the anti CTLA-4 antibody ipilimumab, anti-PD1 antibodies pembrolizumab, nivolumab and cemiplimab and anti-PD-L1 antibodies atezolizumab, avelumab and durvalumab (116, 117). Treatment response to immune checkpoint inhibitors varies greatly with only a small proportion of patients experiencing better survival rates (118, 119). Hence, there is a growing need for predictive biomarkers of ICI response. Furthermore, few preclinical studies are investigating the benefit of targeting multiple immune checkpoints including PD-1, CTLA-4, Tim3, and Lag3 (120). Currently, the majority of breast cancer studies focus on inhibition of the PD1/PD-L1 pathway. A single-arm pilot study investigating the combination of PD1/PD-L1 blockade with CTLA-4 inhibition reported an objective

response rate (ORR) of 43% in patients with metastatic TNBC, whereas no responses were observed in patients with HR+ breast cancer (121). We will focus our discussion on anti-PD1/PD-L1 mono- and combination therapy in TNBC (**Figure 1**) given that it is the most immunogenic breast cancer subtype and hence will more likely benefit from treatment with ICIs.

## PD1/PD-L1 Antibody Monotherapy

PD1/PD-L1 monotherapy has demonstrated promising durable responses in patients with advanced, metastatic TNBC (**Table 1**). The safety profile and clinical activity of the anti-PD1 inhibitor pembrolizumab was first studied in heavily pretreated patients with advanced, PD-L1 positive triple negative breast cancer, head and neck cancer, urothelial cancer or gastric cancer in the KEYNOTE-012 (NCT01848834) clinical trial. Interim analysis revealed an overall response rate of 18.5% in mTNBC patients with the median duration of response ranging from 15.0 to 47.3 weeks (38). In a subsequent phase II clinical trial, KEYNOTE-086 (NCT02447003), PD-L1 positive mTNBC patients who received no prior systemic treatment for metastatic disease showed the highest ORR of 21.4% with a median duration of response of 10.4 months at data cut-off, and PFS and OS of 2.1 and 18.0 months, respectively (80). In comparison, heavily pretreated, PD-L1 positive mTNBC patients experienced an ORR of 5.7% with median PFS and OS of 2.0 and 9.0 months, respectively (122). Both studies demonstrated a manageable safety profile and durable clinical activity of single agent pembrolizumab treatment in PD-L1 positive mTNBC, in particular in the first-line setting. Next, the randomized phase 3 KEYNOTE-119 trial (NCT02555657) investigated the efficacy of pembrolizumab monotherapy versus chemotherapy (capecitabine, gemcitabine, eribulin, vinorelbine) in pretreated, PD-L1 positive mTNBC. Initial results revealed no significant improvement in PFS (HR = 1.35) nor in OS (HR = 0.86) for patients receiving pembrolizumab, although there was a trend for better survival with higher PD-L1 score (123). At the date of data cut-off (11$^{th}$ April 2019), the median follow-up time was 9.9 months for the pembrolizumab cohort and 10.9 months for the chemotherapy cohort, hence, differential survival outcomes may become more apparent as the study matures. However, these findings may also suggest that pembrolizumab monotherapy is more effective as first line treatment in mTNBC.

In addition to blocking PD-1, antibodies have been developed that target PD-L1, thereby disrupting PD-L1/CD80 binding in addition to PD-L1/PD1 and resulting in an augmented anti-tumor immune response by both T cells and antigen presenting

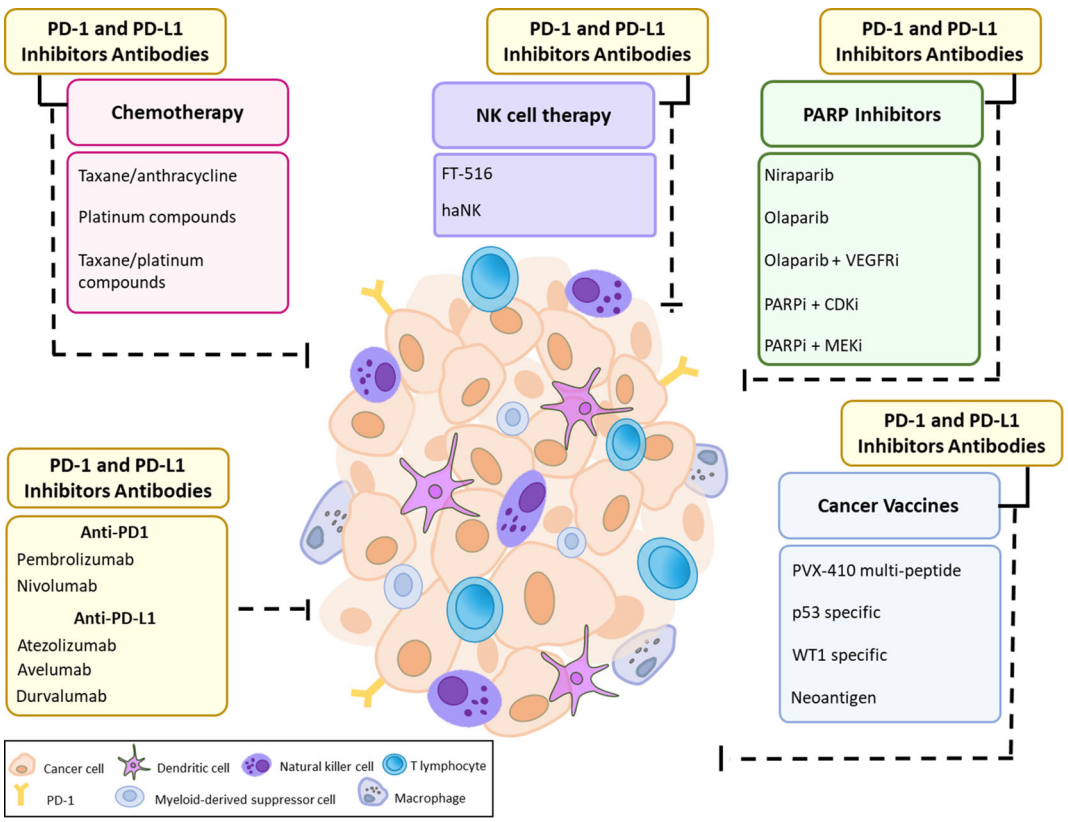


**FIGURE 1** | Current Approaches for PD-1 and PD-L1 immune checkpoint inhibition in TNBC. The efficacy of PD-1 and PD-L1 therapy may be hampered due to cancer cell-intrinsic interactions and/or microenvironmental factors along with the expression of immune checkpoint molecules such as PD-L1 that define a potent and durable anti-tumor immune response. Immune checkpoint blockade could be used as monotherapy or in combination with different therapeutic approaches, including chemotherapy, PARP inhibitors with or without VEGFR/CDK/MEK inhibitors, cancer vaccines, and NK cell therapy.

**TABLE 1** | PD1/PD-L1 antibody monotherapy in metastatic TNBC.

| NCT Number | Other IDs | Intervention | Trial status/interim results | Ref |
|---|---|---|---|---|
| **NCT01848834** | KEYNOTE-012/MK-3475-012, 2012-005771-14, 142453, 3475-012 | pembrolizumab | completed<br>ORR 18.5% | (38) |
| **NCT02447003** | KEYNOTE-086/MK-3475-012, 3475-086, 2015-000294-13, 152987 | pembrolizumab | completed<br>ORR 5.7%<br>PFS 2.0 mths, OS 9.0 mths<br>first line setting:<br>ORR 21.4%<br>PFS 2.1 mths, OS 18.0 mths | (80, 122) |
| **NCT02555657** | KEYNOTE-119/MK-3475-119, 3475-119, 2015-001020-27, 153082 | pembrolizumab vs chemotherapy | active<br>no difference in PFS and OS | (123) |
| **NCT01375842** | PCD4989g, 2011-001422-23, GO27831 | atezolizumab | completed<br>ORR 6% (12 vs 0%*)<br>OS (10.1 vs 6.0 mths*)<br>first line setting:<br>ORR 24%<br>OS 17.6 mths | (124) |
| **NCT01772004** | JAVELIN/EMR 100070-001, 2013-002834-19 | avelumab | completed<br>ORR 5.2% (22.2 vs 2.6%**) | (40) |
| **NCT02926196** | A-Brave, 2016-000189-45 | avelumab | recruiting | |

*ORR, overall response rate; OS, overall survival; PFS progression free survival.*
**PD-L1 cutoff 1%; **PD-L1 cutoff 10%.*

cells (125). In breast cancer, studies have investigated the safety profiles and efficacy of two anti PD-L1 antibodies, atezolizumab and avelumab. The clinical activity of single agent atezolizumab treatment was evaluated in a multi-cohort phase I study (NCT01375842) involving patients with locally advanced or metastatic solid malignancies or hematologic malignancies. In mTNBC, the ORR in first line atezolizumab treatment reached 24% with a median OS of 17.6 months compared to 6% in pretreated patients (124). PD-L1 expression in at least 1% of tumor infiltrating immune cells was associated with higher ORR (12 versus 0%) and better OS (10.1 versus 6.0 months). Further, higher levels of PD-L1 positivity (> 10%) were associated with better ORR and OS, albeit not significantly. The phase 1b JAVELIN trial (NCT01772004) on avelumab reported an ORR of 3.0% in metastatic breast cancer, and an ORR of 5.2% in mTNBC (40). In line with previous reports, higher response rates were observed in PD-L1 positive versus negative patients (16.7 vs 1.6%) using a PD-L1 cutoff of 10%, in particular in TNBC patients (22.2 vs 2.6%). To conclude, although the response rates of single agent ICIs in mTNBC may be modest, the durable responses of a subset of PD-L1 positive patients suggest that combination treatment of immune checkpoint blockade with other treatment modalities may provide a favorable outcome.

## PD1/PD-L1 Antibody-Chemotherapy Combination Treatment

Chemotherapy has been shown to increase tumor cell antigen release, induce the expression of MHC Class I molecules, neoantigens and PD-L1, and promote dendritic cell activation thus potentially augmenting the released immune response following or during ICI treatment (126–128). In line with this rationale, combination regimens of PD1/PD-L1 inhibitors with chemotherapy have shown promising results in metastatic, locally advanced and early stage TNBC (**Table 2**).

The majority of studies on PD1 inhibition in TNBC has investigated the safety profile and clinical activity of pembrolizumab. Interim analysis of the phase 3 KEYNOTE-355 (NCT02819518) study reveals a significant improvement of PFS (5.6 vs 9.7 months) in strong PD-L1 positive, untreated mTNBC patients who received pembrolizumab in addition to chemotherapy (nab-paclitaxel, paclitaxel, gemcitabine/carboplatin) (129). Results from the phase 2 BR-076 (NCT02755272) clinical trial on pembrolizumab in combination with gemcitabine/carboplatin in mTNBC are pending. The KEYNOTE-150/ENHANCE 1 (NCT02513472) trial of pembrolizumab plus the microtubule inhibitor eribulin mesylate demonstrated an ORR of 25.6% with a median PFS of 4.1 months (130). The phase 2 TONIC trial (NCT02499367) evaluated the efficacy of PD1 blockade with nivolumab in pre-treated mTNBC (cyclophosphamide, cisplatin, doxorubicin). Of note, nivolumab therapy preceded by doxorubicin resulted in an ORR of 35 compared to 23% for cisplatin and 17% for patients without preceding chemotherapy, suggesting that pretreatment with chemotherapy can induce an inflamed tumor microenvironment (117). In comparison with metastatic TNBC, significant more studies have been conducted in locally advanced or early stage TNBC. In the phase 2 I-SPY 2 (NCT01042379) study the addition of pembrolizumab to taxane- and anthracycline-based neoadjuvant chemotherapy doubled the estimated pathological complete response (pCR) rates of early stage patients with Her2-negative breast cancer including triple negative breast cancer (131). These promising results provided the rationale for the phase 1 KEYNOTE-173 (NCT02622074) trial to investigate the toxicity and anti-tumor activity of adding pembrolizumab to six commonly used neoadjuvant chemotherapy regimens in untreated, locally advanced TNBC. The toxicity profile of the combination treatments were similar to what has been observed for the individual treatments, suggesting a manageable safety profile.

**TABLE 2 |** PD1/PD-L1 antibody chemotherapy combination treatment in TNBC.

| NCT Number | Other IDs | Intervention | Disease setting | Trial status/interim results | Ref |
|---|---|---|---|---|---|
| **NCT02819518** | KEYNOTE-355/MK-3475-355, 3475-355, 2016-001432-35, 163422 | pembrolizumab + nab-paclitaxel or paclitaxel or gemcitabine/carboplatin | metastatic | active<br>first line setting:<br>PFS 9.7 mths | (129) |
| **NCT02755272** | BR-076 | pembrolizumab + gemcitabine/ carboplatin | metastatic | recruiting | |
| **NCT02513472** | KEYNOTE-150, ENHANCE 1, E7389-M001-218 | pembrolizumab + eribulin mesylate | metastatic | active<br>ORR 25.6%<br>PFS 4.1 mths | (130) |
| **NCT02499367** | TONIC, N15TON | cyclophosphamide, cisplatin or doxorubicin followed by nivolumab | metastatic | active<br>ORR 35% (doxorubicin)<br>first line setting:<br>ORR 17% | (117) |
| **NCT01042379** | I-SPY 2, 097517 | neoadjuvant pembrolizumab + paclitaxel, followed by AC | locally advanced | recruiting | (131) |
| **NCT02622074** | KEYNOTE-173/MK-3475-173, 3475-173, 2015-002405-11 | neoadjuvant pembrolizumab + chemotherapy combination (nab-paclitaxel, paclitaxel, doxorubicin, cyclophosphamide, carboplatin) | locally advanced | completed<br>first line setting:<br>pCR 60% | (132) |
| **NCT03036488** | KEYNOTE-522/MK-3475-522, 3475-522, 2016-004740-11, 173567 | neoadjuvant pembrolizumab + paclitaxel-carboplatin followed by adjuvant pembrolizumab | locally advanced | active<br>first line setting:<br>pCR 64.8% | (133) |
| **NCT01633970** | GP28328, 2012-001422-10 | atezolizumab + nab-paclitaxel | locally advanced, metastatic | active<br>ORR 39.4%<br>PFS 5.5 mths | (134) |
| **NCT02425891** | IMpassion130, WO29522, 2014-005490-37 | atezolizumab + nab-paclitaxel | metastatic | active<br>first line setting:<br>ORR 53%<br>OS 25 mths | (135) |
| **NCT03125902** | IMpassion131, MO39196, 2016-004024-29 | atezolizumab + paclitaxel | locally advanced, metastatic | active<br>first line setting | |
| **NCT03371017** | Impassion132, MO039193, 2016-005119-42 | atezolizumab + gemcitabine/carboplatin or capecitabine | locally advanced, metastatic | recruiting | |
| **NCT02685059** | GeparNuevo, GBG89 | neoadjuvant durvalumab + nab-paclitaxel + EC | early stage | unknown<br>pCR 53% | (136) |
| **NCT02620280** | NeoTRIPaPDL1, FM-14-B02, 2014-005017-23 | neoadjuvant atezolizumab + nab-paclitaxel + carboplatin, followed by AC or EC or FEC | early high risk, locally advanced | active | (137) |
| **NCT03197935** | Impassion031, WO39392, 2016-004734-22 | neoadjuvant atezolizumab + nab-paclitaxel, followed by AC | early stage | active<br>pCR 57.6% | (138) |
| **NCT03281954** | NSABP B-59/GBG 96-GeparDouze, 2017-002771-25, MO39875 | neoadjuvant atezolizumab + paclitaxel + carboplatin, followed by adjuvant atezolizumab + AC or EC | early stage | recruiting | |
| **NCT03498716** | Impassion030, WO39391, 2016-003695-47, BIG 16-05, AFT-27, ALEXANDRA | atezolizumab + paclitaxel, followed by atezolizumab + AC or EC | locally advanced | recruiting | |

*AC, doxorubin + cyclophosphamide; EC, epirubicin + cyclophosphamide; FEC, fluorouracil + epirubicin + cyclophosphamide; ORR, overall response rate; OS, overall survival; pCR, pathological complete response; PFS, progression free survival.*

Furthermore, combination treatment showed promising clinical activity with pCR rates of 60% across all treatment cohorts (132). In accordance with other studies, higher pre-treatment PD-L1 expression was associated with better outcome. Similarly, interim analysis of the phase 3 KEYNOTE-522 trial (NCT03036488) demonstrated that addition of pembrolizumab to paclitaxel-carboplatin chemotherapy in the neoadjuvant setting, followed by adjuvant pembrolizumab increased the pCR rates from 51.2 to 64.8% in untreated, locally advanced TNBC patients (133). Of note, the trial design does not allow the comparison of adjuvant pembrolizumab versus placebo treatment following neoadjuvant chemotherapy alone.

In addition to PD1 blockade, several clinical trials aim to study the safety and efficacy of PD-L1 inhibition in combination with chemotherapy, in particular in metastatic TNBC patients. The phase 1b clinical study NCT01633970 reported an ORR of 39.4% with a median PFS of 5.5 months for locally advanced or metastatic TNBC patients treated with atezolizumab plus nab-paclitaxel (134). PD-L1 positive mTNBC patients showed a non-significant higher ORR (41.4 vs 33.3%), PFS (6.9 vs 5.4 months) and OS (21.9 vs 11.4 months), irrespective of treatment history. Furthermore, although not statistically significant, patients who received the treatment regimen in first line setting experienced a higher ORR (53.8 vs 30.0%), longer PFS (8.6 vs 5.1 months) and OS (24.2 vs 12.4 months), providing evidence for a more favorable outcome compared to atezolizumab monotherapy where an ORR of 24% and median PFS of 1.6 months was observed (124, 134). The phase 3 randomized IMpassion130 trial

(NCT02425891) supports these findings, demonstrating a clinically meaningful improvement in OS of 7 months (25.0 vs 18.0 months) for PD-L1 positive mTNBC patients who received first line atezolizumab plus nab-paclitaxel treatment (135). Interim results show that addition of pembrolizumab increased the ORR from 33 to 53% (128). In 2019, the FDA and European Medicines Agency (EMA) granted accelerated approval for the use of atezolizumab plus nab-paclitaxel as first line treatment of PD-L1-positive, unresectable, locally advanced or metastatic TNBC. The subsequent phase 3 IMpassion131 trial (NCT03125902) will evaluate the safety and efficacy of atezolizumab plus paclitaxel as a first-line therapy in patients with either locally advanced or metastatic TNBC. The IMpassion132 trial (NCT03371017) will investigate whether atezolizumab plus chemotherapy (gemcitabine/carboplatin, capecitabine) may benefit pretreated, inoperable locally advanced or metastatic TNBC patients who were not eligible for the IMpassion130 trial. So far, limited information is available on the effect of PD-L1 blockade in combination with chemotherapy for early stage TNBC. Results from the randomized phase 3 GeparNuevo study (NCT02685059) suggest that combining durvalumab with taxane-anthracycline based neoadjuvant chemotherapy provides clinical benefit in early TNBC with an increase in pCR from 44 to 53% (136). As of July 2020, no interim results are available for the phase 3 NeoTRIPaPDL1 (NCT02620280) clinical trial that aims to evaluate the anti-tumor activity of neoadjuvant atezolizumab plus carboplatin and nab-paclitaxel, followed by adjuvant chemotherapy in early stage high risk or locally advanced TNBC. Preliminary results were presented at the San Antonio Breast Cancer Symposium 2019 and revealed slightly higher pCR rates with pembrolizumab addition (137). The phase 3 NSABP B-59 (NCT03281954) trial of neoadjuvant chemotherapy (paclitaxel plus carboplatin) with atezolizumab, followed by adjuvant atezolizumab and chemotherapy is currently in the recruiting stage. A recent study released interim results from the Impassion031 (NCT03197935) trial on the combination treatment of neoadjuvant atezolizumab with sequential nab-paclitaxel and anthracycline-based chemotherapy in early stage TNBC. Patients who received atezolizumab plus chemotherapy showed a pathologic complete response rate of 57.6 versus 41.1% in patients who received chemotherapy plus placebo (138). In PD-L1 positive patients, the pathologic complete response reached 69% for patients who received atezolizumab plus chemotherapy and 49% for patients treated with chemotherapy plus placebo. Of note, there are two ongoing studies in locally advanced TNBC that evaluate the effect of chemotherapy with PD-L1 blockade in adjuvant setting. The Impassion30 (NCT03498716) trial will study the efficacy of atezolizumab in combination with adjuvant chemotherapy, while the A-Brave (NCT02926196) study focuses on avelumab.

## PD1/PD-L1 Antibody-Targeted Therapy Combination Treatment

Triple negative tumors feature a higher tumor mutational burden and extensive genomic instability with defects in the DNA damage response (139). As such, combination therapy strategies targeting distinct oncogenic pathways in conjunction with immunotherapy could offer a promising approach for TNBC treatment. The current clinical trials exploring such combination therapies are summarized in **Table 3**. For instance, Poly (ADP-Ribose) Polymerase inhibitors (PARPi) that target the homologous recombination repair pathway and induce synthetic lethality in *BRCA1/2* mutation carriers have been approved for the treatment of TNBC patients with germline mutations in *BRCA1/2* (143). The use of PARPi in combination with immune checkpoint blockade in this subset of TNBC patients has the potential to trigger a stronger anti-tumor immune response as a result of the activation of infiltrating T cells following the release of tumor antigens by PARPi-induced cell death. Furthermore, PARPi have been shown to upregulate PD-L1 expression in cell line and animal models providing further rationale for combining treatment with PD1/PD-L1 inhibitors (144). The KEYNOTE-162/TOPACIO (NCT02657889) study reported an ORR of 29% in mTNBC patients treated with a combination of pembrolizumab and the PARPi niraparib. The presence of *BRCA* mutations was associated with a higher ORR of 67% (140). Of note, the ORR

**TABLE 3** | PD1/PD-L1 antibody-targeted therapy combinations in locally advanced or metastatic TNBC.

| NCT Number | Other IDs | Intervention | Trial status/interim results | Ref |
|---|---|---|---|---|
| **NCT02657889** | TOPACIO/KEYNOTE-162 | pembrolizumab + niraparib | active<br>ORR 29% (67%*) | (140) |
| **NCT03167619** | DORA, 3000-PN162-01-001 | durvalumab + olaparib | recruiting | |
| **NCT03801369** | STUDY00018504, NCI-2019-00388, STUDY00018504 | durvalumab + olaparib | recruiting | |
| **NCT02849496** | NCI-2016-01130, 1608018258, 10020, UM1CA186644/86/88/89/91, UM1CA186709 | atezolizumab + olaparib | recruiting | |
| **NCT02484404** | 150145, 15-C-0145 | durvalumab + olaparib + VEGFRi | recruiting | |
| **NCT02734004** | MEDIOLA, D081KC00001, 2015-004005016 | durvalumab + olaparib +/- VEGFRi | active | (141) |
| **NCT02322814** | COLET, WO29479, 2014-002230-32 | atezolizumab + taxanes + MEKi | active<br>ORR 29–34% | (142) |
| **NCT03106415** | MC1632, NCI-2017-00496, P30CA015083 | pembrolizumab + MEKi | recruiting | |
| **NCT03971409** | 187519, NCI-2019-01531, TBCRC 047, BRE16-279, | avelumab + MEKi | recruiting | |

*MEKi, MEK inhibitors; ORR, overall response; VEGFRi, VEGFR inhibitors; *BRCA mutant.*

was higher than what has been reported for anti-PD1 monotherapy in similar patient populations (122, 124). Additionally, several clinical trials have been designed to evaluate the combination of PD-L1 inhibition with PARPi in mTNBC, including two phase 2 studies combining durvalumab with the PARPi olaparib (DORA/NCT03167619 and NCT03801369), and a phase 2 study on atezolizumab plus olaparib (NCT02849496). Furthermore, triplet combination treatments of PD-L1 inhibition with PARPi and VEGF inhibitors are currently on the way. For instance, the doublet or triplet combination of durvalumab with olaparib and the VEGFR inhibitor cediranib is the focus of a phase 1/2 study (NCT02484404) in advanced or recurrent solid cancer. Preliminary results show that the recommended dose was tolerable and yielded a 67% clinical benefit rate in nine women with pretreated recurrent solid tumors of which 1 TNBC (141). Results from the MEDIOLA (NCT02734004) clinical trial are pending. This open basket study aims to compare the safety and efficacy of durvalumab in combination with the PARPi olaparib or in combination with olaparib plus the VEGF inhibitor bevacizumab in patients with advanced solid tumors including *BRCA1/2*-deficient breast cancer. Furthermore, it would be of interest to study the clinical benefit of combining PARPi, PD1/PD-L1 blockade and cyclin dependent kinase (CDK) inhibitors. Cyclin dependent kinases are well known master regulators of cell cycle progression and DNA repair pathways, and CDK inhibitors have been shown to sensitize breast cancer cells to PARPi which may further augment the treatment response to immune checkpoint blockade (145). Furthermore, CDK4/6 inhibitors have been found to promote anti-tumor immunity through the stimulation of effector T cell activity, inhibition of proliferation of immunosuppressive regulatory T cells, induction of fibroblast-derived pro-inflammatory cytokines and increased cell surface antigen presentation (146, 147). Another strategy to combine immune checkpoint blockade with targeted therapy involves the inhibition of the MAPK pathway, which is often dysregulated in TNBC and is associated with increased cell proliferation and resistance to apoptosis (148). The phase 2 COLET (NCT02322814) study evaluated the added benefit of combining the MEK1/2 inhibitor cobimetinib with atezolizumab and paclitaxel/nab-paclitaxel as first line treatment in locally advanced or metastatic TNBC. Interim analysis reveals an ORR of 34% in combination with paclitaxel and 29% with nab-paclitaxel (142). In addition, clinical trials using the MEK inhibitor binimetinib in combination with pembrolizumab (NCT03106415) or avelumab (InCITe/NCT03971409) in locally advanced or metastatic TNBC are currently ongoing.

## PD1/PD-L1 Antibody-Vaccine Combination Treatment

The use of peptide vaccines for the treatment of metastatic cancer patients has been challenged by low response rates, however, using a multi-peptide vaccine approach the response rates have increased to 9.9% in different cancer types (149, 150). Moreover, combining cancer vaccines with immune checkpoint inhibitors may enhance the anti-tumor immune response elicited by the vaccine. The current clinical trials using PD/PD-L1 antibody-vaccine combination treatments are summarized in **Table 4**. Few ongoing trials are investigating the efficacy of combining cancer vaccines with pembrolizumab, using either the multi-peptide vaccine PVX-410 (NCT03362060), or specific vaccine targeting p53 (NCT02432963) or WT1 (NCT03761914) in advanced TNBC. Additionally, there are few clinical trials exploring the efficacy of combining durvalumab with the multi-peptide vaccine PVX-410 (NCT02826434) or with a neoantigen vaccine (NCT03199040, NCT03606967), and of atezolizumab with a neoantigen vaccine (NCT03289962).

## PD1/PD-L1 Antibody-Natural Killer Cell Combination Treatment

Natural killer (NK) cells form the first line natural defense against abnormal cells and infection with a wide range of pathogens. However, tumor cells have found ways to escape NK cell-mediated immunosurveillance such as the shedding of stress-inducible ligands MHC class I polypeptide–related sequence A (MICA) and MICB, which are exclusively expressed in stressed or transformed cells (151, 152). This results in downregulation of the activating Natural killer group 2 member D (NKG2D) receptor and reduced susceptibility to NK cytotoxicity due to reduced cell surface density of the ligands. NK-based immunotherapy studies are investigating the use of vast numbers of *ex vivo* expanded autologous NK cells, strategies to boost NK cell activity or target inhibitory NK receptors, and the development of genetically engineered NK cells to overcome the immunosuppressive environment (153–155). NK-based immunotherapy in combination with PD-1/PD-L1 immune checkpoint blockade is relatively less studied, with only two clinical trials in TNBC as shown in **Table 5**. The combination of avelumab with iPSC-derived NK cells (FT-516) expressing a high-affinity, non-cleavable variant of the NK activating receptor

**TABLE 4** | PD1/PD-L1 antibody-vaccine combination treatment in locally advanced or metastatic TNBC.

| NCT Number | Other IDs | Intervention | Trial status |
|---|---|---|---|
| **NCT03362060** | 17-328 | pembrolizumab + PVX-410 | recruiting |
| **NCT02432963** | 15002, NCI-2015-00653 | pembrolizumab + p53-specific vaccine | active |
| **NCT03761914** | SLS17-201/MK3475-770 | pembrolizumab + WT1-specific vaccine | recruiting |
| **NCT02826434** | 16-132 | durvalumab + PVX-410 | active |
| **NCT03199040** | 201710109, 1R01CA240983-01 | durvalumab + neoantigen DNA vaccine | recruiting |
| **NCT03606967** | NCI-2018-01581, 10146, UM1CA186704 | durvalumab + Nab-paclitaxel+ neoantigen vaccine | unknown |
| **NCT03289962** | GO39733, 2017-001475-23 | atezolizumab + neoantigen vaccine | recruiting |

*PVX-410, multi-peptide vaccine (XBP1 $US_{184-192}$; XBP1 $SP_{367-375}$; $CD138_{260-268}$; and $CS1_{239-247}$).*

**TABLE 5 |** PD1/PD-L1 antibody-NK cell combination treatment in advanced or metastatic TNBC.

| NCT Number | Other IDs | Intervention | Trial status | Ref |
|---|---|---|---|---|
| **NCT04551885** | FT516-102 | Avelumab + FT-516 | Recruiting | |
| **NCT03387085** | QUILT-3.067 | Avelumab + haNK + IL-15 + vaccine + chemoradiation | Active<br>ORR 67%<br>PFS (13.7 mths) | (156) |

*FT-516, iPSC-derived NK cells with hnCD16; IL-15, interleukin 15; NK, natural killer cell; ORR, overall response rate; PFS, progression free survival.*

CD16 (hnCD16) is currently under investigation in multiple advanced solid cancers, including TNBC (NCT04551885). Furthermore, the ongoing landmark trial QUILT-3.067 (NCT03387085) evaluates the safety and efficacy of NK cell combination immunotherapy in patients with refractory, metastatic or unresectable TNBC tumors. The study is unique in design as it combines the use of immune checkpoint inhibition (avelumab) with high-affinity NK (haNK) cell therapy, IL-15 cytokine administration, cancer vaccines and metronomic chemoradiation to stimulate both the innate and adaptive immune system. Interim results of nine patients demonstrate an overall response rate of 67% with a disease control response rate of 78% and complete response rate of 22% (156). Notably, the duration of the treatment responses with a median PFS of 13.7 months is very promising in comparison to the historical PFS of 3 months.

## PREDICTIVE BIOMARKERS IN IMMUNE CHECKPOINT INHIBITION

Immune checkpoint blockade has entered clinical practice as first- or second-line treatment for a number of cancers, however, it remains a challenge to select patients that will benefit the most. PD-L1 expression is widely used as predictive biomarker due to its association with better response rates to PD1/PD-L1 blockade for patients with mTNBC. As described above, stronger PD-L1 positivity has been associated with better overall response rates, progression-free, and overall survival in metastatic TNBC patients treated with ICI monotherapy or in some cases with a chemotherapy combination (40, 124, 129, 134). Routine clinical testing of PD-L1 expression is currently conducted using five distinct FDA-approved companion diagnostic immunohistochemistry tests (157). Nevertheless, the use of different antibody clones (22C3 for pembrolizumab, 28-8 for nivolumab, SP263 for durvalumab, SP142 for atezolizumab, and 73-10 for avelumab), biomarker staining platforms, scoring systems and cut-off values for PD-L1 positivity makes it very difficult to consolidate the predictive value of PD-L1 expression across tumor types and across studies. Moreover, some assays define PD-L1 positivity solely based on tumor cell surface expression while others quantify cytoplasmic plus cell surface PD-L1 expression of tumors and immune cells. The prospective multi-institutional Blueprint study compared the performance of all five PD-L1 antibody clones in non-small cell lung cancer specimens (158). They reported good concordance among three antibodies (22C3, 28-8, and SP263), while the fourth antibody clone (73-10) demonstrated superior sensitivity and the fifth clone (SP142) underperformed with lower sensitivity. Similarly, high concordance has been reported between clones 22C3, 28-8, and SP142 in primary and metastatic urothelial carcinomas with the lowest sensitivity again being associated with SP142 (159). PD-L1 scoring of head and neck squamous cell carcinoma, urothelial carcinoma and breast cancer revealed a higher inter-observer variability for clone SP142 as compared to clones SP263 and 22C3 (160). In TNBC, few studies compared the performance of the FDA-approved assays and corroborated the previous findings in which SP142 detected significant less PD-L1 positivity compared to SP263 and 22C3 (161–163). A recent study involved 19 pathologists from 14 different institutions to evaluate the sensitivity and reproducibility of SP142 and SP263 staining in advanced TNBC (164). This study reported PD-L1 positivity in 58% of cases using SP142 and in 78% with SP263, with decreased observer agreement of 41% at eight observers for SP142 and 46% at 10 observers for SP263. Despite the lower performance of SP142, the SP142-based Ventana test currently remains the companion diagnostic test for the first FDA-approved immunotherapy regimen of atezolizumab plus nab-paclitaxel treatment of patients with metastatic, locally advanced or unresectable tumors, based on the results from the Impassion130 trial (128, 135). Of note, soluble PD-L1 (sPD-L1) has been detected in the peripheral blood of patients with advanced non-small cell lung cancer, multiple myeloma, diffuse large B-cell lymphoma, and renal cell carcinoma whereby high levels are associated with poor prognosis (165–168). High pre-treatment sPD-L1 levels were associated with worse outcome in melanoma patients treated with ipilimumab or pembrolizumab, which could possibly reflect a larger tumor burden and/or an exhausted immune response that cannot be reinvigorated by immune checkpoint blockade (168). In contrast, an increase in post-treatment sPD-L1 was associated with partial response. These findings highlight the need for less ambiguous, more reproducible predictive biomarkers for immune checkpoint inhibition.

Two emerging predictive biomarkers are the number of tumor infiltrating lymphocytes and the tumor mutational burden. Increased number of TILs have been associated with better overall survival in TNBC patients treated with ICI monotherapy or in combination with chemotherapy (117, 136). The relative importance of intratumoral TILs (iTILs) versus stromal TILs (sTILs) has not clearly been defined yet and might differ between tumor types. In breast cancer, both iTILs and sTILs have been correlated with clinical outcome and chemotherapy response (59, 60, 63, 78, 169). Moreover, in metastatic TNBC sTILs have been correlated with treatment response to pembrolizumab, atezolizumab, and nivolumab (117, 124). Thus, the International Immuno-Oncology Biomarker Working Group published guidelines for the assessment of stromal and intra-tumoral TILs in a wide range of solid tumor

types (170). However, robust scoring of sTILs is hindered by differences in relative iTIL and sTIL distribution, inaccurate delineation of tumor boundaries, small areas of intratumoral stroma, presence of necrosis and extracellular mucin (171). Furthermore, tumor mutational burden has been correlated with higher objective response rates to anti-PD1 or anti-PD-L1 monotherapy across 27 solid tumor types (29). Interestingly, in breast cancer lower response rates were observed than expected based on TMB suggesting that TMB might not be a good predictive biomarker in these tumors. We believe that a combination of predictive biomarkers such as PD-L1 expression, iTIL and sTIL density together with TMB, TCR diversity and immune gene signatures will more likely yield improved performance over each of these biomarkers alone, therefore warranting further investigation.

## CONCLUSION

To conclude, the results of immune checkpoint blockade clinical trials in TNBC are promising, in particular in metastatic setting. The FDA-approval of atezolizumab plus nab-paclitaxel for metastatic TNBC marks the first licensed immunotherapy regimen for breast cancer. Combining immune checkpoint inhibition with chemotherapy, PARP inhibitors, cancer vaccines or NK cell therapy holds great potential to increase the clinical benefit in TNBC. Nevertheless, we highlight here that the selection of patients with the highest likelihood of benefit from these treatments requires reliable predictive biomarkers as well as a better understanding of cancer cell-intrinsic and/or microenvironmental factors that define a potent and durable anti-tumor immune response.

## AUTHOR CONTRIBUTIONS

RT drafted the manuscript. GA-K designed the figure and tables and critically revised the manuscript. JD conceived and critically revised the manuscript and tables. All authors contributed to the article and approved the submitted version.

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# Tackling Immune Targets for Breast Cancer: Beyond PD-1/PD-L1 Axis

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The burden of breast cancer is imposing a huge global problem. Drug discovery research and novel approaches to treat breast cancer have been carried out extensively over the last decades. Although immune checkpoint inhibitors are showing promising preclinical and clinical results in treating breast cancer, they are facing multiple limitations. From an immunological perspective, a recent report highlighted breast cancer as an "inflamed tumor" with an immunosuppressive microenvironment. Consequently, researchers have been focusing on identifying novel immunological targets that can *tune up* the tumor immune microenvironment. In this context, several novel non-classical immune targets have been targeted to determine their ability to uncouple immunoregulatory pathways at play in the tumor microenvironment. This article will highlight strategies designed to increase the immunogenicity of the breast tumor microenvironment. It also addresses the latest studies on targets which can enhance immune responses to breast cancer and discusses examples of preclinical and clinical trial landscapes that utilize these targets.

**Keywords: breast cancer, immunotherapy, therapeutic agents, immune targets, PD-1, PD-L1**

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## INTRODUCTION

The Global Cancer Statistics (GLOBOCAN 2018) report of 2018 flags breast cancer as the second most diagnosed cancer, with a prevalence of ∼11.6% of all cancer cases (1). Breast cancer is the first diagnosed cancer and the leading cause of death among women, with over 450,000 mortalities annually (2). Based on the status of the tumor receptors, three types of breast cancers have been reported: estrogen/progesterone receptor-positive (ER+), human epidermal growth factor receptor 2-positive (HER2+), and triple-negative (TNBC) breast cancer (3). ER+ breast cancer is the most diagnosed breast cancer, with an incidence rate of ∼80% (4, 5). Recently, the reactivation of the immune system has emerged as a strategy for cancer treatment other than traditional methods (6). Due to the immunological quiescent nature of breast tumors, immunotherapy has not been considered as a strategy for breast cancer treatment. However, this strategy has been reconsidered following the identification of tumor immune infiltrates. Since tumor-infiltrating lymphocytes (TILs: CD8+ cytotoxic T cells and helper CD4+ cells, regulatory T cells, B cells, NK cells), tumor-associated macrophages and myeloid-derived suppressor cells (MDSCs) are observed in some breast tumors (7, 8). Hence, the alteration and manipulation of the immune responses are now the focus of breast cancer therapeutic strategies (9). The discovery of inhibitory immune checkpoints has revolutionized cancer treatment (10). Understanding their role in promoting immunosuppression in the tumor microenvironment (TME) has resulted in the use of checkpoint inhibitors (generally monoclonal antibodies), which can reactivate immune cells (11, 12). Checkpoint inhibitors that target PD-1 or CTLA-4 have been used for treating metastatic breast cancer (13).

However, the response rates were lower than other types of cancers; the overall response rate to anti-PD-1 (Pembrolizumab) was only 18.5% when used as monotherapy for patients with advanced triple-negative breast cancer (TNBC) (14). However, the KEYNOTE 355 study was initiated in 2016 to compare the effectiveness of using pembrolizumab in combination with chemotherapy with placebo plus chemotherapy for treating patients with unresectable locally advanced or metastatic PD-L1-positive TNBC (ClinicalTrials.gov Identifier: NCT02819518). Reports from this study indicated that pembrolizumab combined with several chemotherapy agents showed a statistically significant and clinically meaningful improvement in progression-free survival with 9.7 months vs. only 5.6 months with using chemotherapy alone in these patients. Pembrolizumab combined with chemotherapy showed adverse event rates 68% while 67% with chemotherapy. This combination was generally well-tolerated, with no safety concerns (15, 16). Based on the results of this trial, the FDA approved the use of pembrolizumab (anti-PD1) in combination with chemotherapy for the treatment of unresectable locally advanced or metastatic PD-L1-positive TNBC, in November 2020.

Nevertheless, identifying novel targets and developing new therapeutic agents are needed for breast cancer treatment. Other therapeutic targets that can modulate immune responses against breast tumors are currently under investigation. Co-stimulatory receptors are promising targets, which can improve anti-tumor immunity in breast cancer (13). Purinergic ectoenzymes attenuate the immune response by increasing the level of extracellular adenosine, which has immunosuppressive properties (17, 18). Inhibiting purinergic ectoenzymes will increase the anti-tumor immune responses (19). Similarly, targeting the immunosuppressive enzyme arginase 1 (ARG1), could also improve anti-tumor immune responses (20, 21). Studies have shown that various cytokines, chemokines, growth factors, and their receptors such as vascular endothelial growth factor (VEGF), VEGF Receptor (22), CXC receptor 1(CXCR1), CCL2 receptor (CCR2) (23), colony-stimulating factor-1 (CSF-1) (24) and toll-like receptors (TLRs) (25) are essential for breast tumor proliferation and metastasis. Furthermore, studies on targeting tryptophan catabolism enzymes, such as indoleamine-2,3-dioxygenase (IDO1/IDO2), and tryptophan-2,3-dioxygenase (TDO/TDO2), which are expressed by many immune cells and solid tumors, including breast cancer are underway (26). Moreover, the development of agents, which can modulate the COX2/PGE2 (27) and STING (28) signaling pathways, are ongoing.

The effects of blocking different immune checkpoints in breast cancer have been recently reviewed by Swoboda A, and Nanda R (29). Furthermore, the effectiveness of combining PD1/PD-L1 blockade with chemotherapy, targeted therapies and radiotherapy for the treatment of metastatic breast cancer has been reviewed by Page et al. (30). In this review, we will discuss the pathways that modulate immune responses to breast cancer (**Figure 1**). We will also discuss novel therapies and clinical trials designed to target these pathways (**Table 1**).

## STIMULATORY CHECKPOINTS

A major characteristic of tumors is the paucity of, or ability to downregulate the expression of co-stimulatory molecules and upregulate co-inhibitory receptor expression (31, 32). The ligation of co-stimulatory molecules expressed by antigen-presenting cells (APCs) with their receptors on T cells provides the second signal necessary for T cell activation and differentiation. Hence, the use of co-stimulatory molecule agonist antibodies, is a strategy which may enhance T cell function in the TME (31, 32) (**Figure 2A**). Targeting co-stimulatory molecules that belong to the tumor necrosis factor receptor (TNFR) family such as OX40, ICOS, GITR, CD40L, and 4-1BB with agonist antibodies have been found to improve T cell function, with favorable outcomes in some cancer patients [reviewed in Moran et al. (33)].

OX40 (i.e., CD134) is expressed by TILs in various types of cancers, including breast cancer (34), while its receptor OX40L, is upregulated on monocytes, neutrophils, macrophages and dendritic cells. Studies have shown that OX40–OX40L signaling reduces immunosuppression mediated by regulatory T cells (Tregs) and enhances the expansion and proliferation of T cells (34). A study to assess the safety and tolerability of the OX40 agonist (PF-04518600) alone, or in combination with the 4-1BB agonist, PF-05082566, in patients with metastatic carcinoma, including TNBC was concluded in December 2020 (ClinicalTrials.gov Identifier: NCT02315066), (35). However, a clinical study that had planned to test the agonistic anti-OX40 antibody, MEDI6469, in combination with immune checkpoint inhibitors in patients diagnosed with advanced solid tumors, was terminated (32, 35, 36). Another phase I/II study, which investigated the use of MEDI6469 in combination with radiation for the treatment of metastatic breast cancer has been completed (ClinicalTrials.gov Identifier: NCT01862900). An additional phase I study has been initiated to investigate the effectiveness of using a CD40 agonist, ABBV-927 plus OX40 agonist ABBV-368 in combination or without the PD1 inhibitor, budigalimab in patients with advanced solid tumors, including TNBC (ClinicalTrials.gov Identifier: NCT03893955). Observations from a recent study indicated that OX40 agonists enhanced the production of IL-2 by conventional TILs, which increases the proliferation of both tumor-infiltrating Tregs and conventional T cells. Hence, in contrast to what has been postulated by previous studies, Tregs retain their immunosuppressive abilities in response to OX40 agonist treatment. However, results from this study also indicate that Tregs acquire a Th1 phenotype (IFN-g and granzyme B production) in response to OX40 agonist treatment (37). These observations imply that OX40 agonist treatment may be more suitable for combination therapies for cancer treatment. The importance of investigating the sequence of administering monoclonal antibodies in combination treatments that include anti-PD1 and OX40 agonists has been highlighted by Messenheimer et al. (38). They showed that using a preclinical model of oncogene-driven mammary cancer that concurrent administration of anti-PD1 antibody and an OX40 agonist compromised tumor regression. In contrast, sequential administration of the OX40

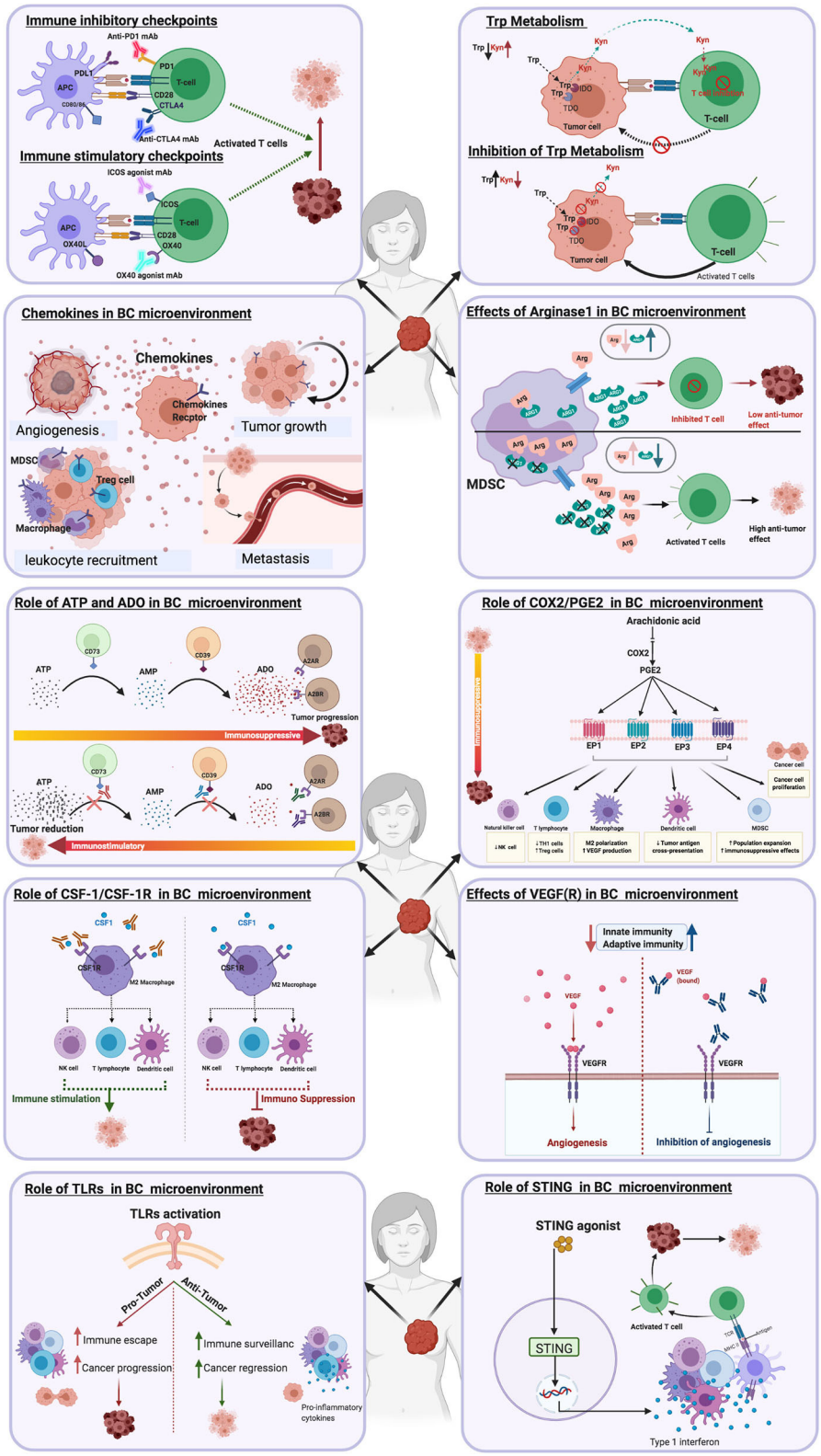


**FIGURE 1 |** Immune targets in breast cancer immunotherapy.

**TABLE 1 |** Examples of clinical trials of Immune targets in breast cancer immunotherapy.

| Target | Drugs | Company | With combination | Phase | Clinicaltrials.gov identifier (selected trials) |
|---|---|---|---|---|---|
| Anti-(PD-1) | Pembrolizumab | Merck | +Nab-paclitaxel/Paclitaxel/ Gemcitabine/Carboplatin | III | NCT02819518 |
| Anti-(PD-L1) | Atezolizumab | Genentech/Roche | – | I | NCT01375842 |
| | | | + Nab-paclitaxel | III | NCT02425891 |
| | Avelumab | Merck | – | III | NCT02926196 |
| Anti-(CTLA-4) | tremelimumab | AstraZeneca | +Exemestane/ durvalumab | II | NCT02997995 |
| | | | + Durvalumab | I/II | NCT01975831 NCT02536794 |
| | Ipilimumab | Bristol-Myers Squibb (BMS) | + Nivolumab/ cobimetinib | I/ II | NCT01928394 |
| | | | + Enoblituzumab | I | NCT02381314 |
| Anti-(LAG-3) | IMP321/Eftilagimod alpha | Immutep | +Paclitaxel | II | NCT02614833 |
| Anti-(TIM-3) | MBG453 | Novartis | + Spartalizumab | I/II | NCT02608268 |
| OX40 agonists | | | | | |
| | GSK3174998 | GlaxoSmithKline | Alone/ with Pembrolizumab | I | NCT02528357 |
| | MEDI-0562 | MedImmune | – | I | NCT02318394 |
| | MEDI-6383 (OX40L-Fc) | MedImmune | Alone/with MEDI-4736 | I | NCT02221960 |
| | PF-04518600 | Pfizer | Alone/with PF-05082566 | I | NCT02315066 |
| | MEDI-6469 | MedImmune | +Radiation | I | NCT01862900 |
| | BMS-986178 | Bristol-Myers Squibb | Alone/ with nivolumab ± ipilimumab | I/II | NCT02737475 |
| | ABBV-368 | Idera Pharmaceuticals | +ABBV-927 ± budigalimab | I | NCT03893955 |
| GITR agonist | | | | | |
| | INCAGN01876 | Incyte | Nivolumab and/ or ipilimumab | I/II | NCT03126110 |
| | INCAGN01876 | Incyte | Pembrolizumab and/ or epacadostat | I/II | NCT03277352 |
| | TRX518 | Leap Therapeutics | + Cyclophosphamide +/or Avelumab | I/II | NCT03861403 |
| 4-1BB agonist | PF-05082566 (Utolimumab) | Pfizer | + Trastuzumab – Emtansine | I | NCT03364348 |
| | PRS-343 | Pieris Pharmaceuticals, Inc. (PIRS) | +Atezolizumab | Ib | NCT03650348 |
| CD40 agonist | CDX-1140 | Celldex Therapeutics | Alone or with Pembrolizumab | I | NCT03329950 |
| ICOS agonist | JTX-2011 | Jounce Therapeutics | Nivolumab/Ipilimumab/ Pembrolizumab | I/II | NCT02904226 |
| IDO1 inhibitor | | | | | |
| | Indoximod | NewLink Genetics | - | I | NCT00739609 |
| | | | +Docetaxel/paclitaxel | II | NCT01792050 |
| | Epacadostat | Incyte *Corporation* | + INCMGA00012 and Epacadostat | I/II | NCT03328026 |
| | | | +/or Itacitinib with INCB050465 | I | NCT02559492 |
| Targeting Arginase-1 | Arginase-1 peptide vaccine | IO Biotech ApS. | | I | NCT03689192 |
| CXCR4 antagonist | balixafortide | Polyphor | +Eribulin | III | NCT03786094 |
| CCR5 antagonist | Leronlimab | CytoDyn, Inc. | - | - | NCT04313075 |
| CD73 antagonists | ±Oleclumab (*MEDI9447*) | IMFINZI® | + Carboplatin + Paclitaxel +Durvalumab | I/II | NCT03616886 |
| | CPI-006 | Corvus Pharmaceuticals | Alone/ with Ciforadenant +Pembrolizumab | I | NCT03454451 |
| A2AR antagonist | CPI-444 Ciforadenant | Corvus Pharmaceuticals | Alone/ with Combination+ Atezolizumab | I | NCT02655822 |

*(Continued)*

**TABLE 1** | Continued

| Target | Drugs | Company | With combination | Phase | Clinicaltrials.gov identifier (selected trials) |
|---|---|---|---|---|---|
| PGEP4R blocker | AAT-007 | Applied Therapeutics | - | II | NCT02538432 |
| CSF1R blocker | LY3022855 | Imclone Llc | Alone/ with Durvalumab or Tremelimumab | I | NCT02718911 |
| | Pexidartinib (PLX-3397) | *Daiichi Sankyo* | + Eribulin | I/II | NCT01596751 |
| | Emactuzumab (RG7155) | Roche | +Atezolizumab | | NCT02323191 |
| | | | +RG7876 | I | NCT02760797 |
| VEGFR blocker | Ramucirumab | Eli Lilly and Company | +Docetaxel | III | NCT00703326 |
| | Lucitanib | Clovis Oncology, Inc. | – | II | NCT02202746 |
| TLR7 agonist | 852A | Pfizer | – | II | NCT00319748 |
| | Imiquimod | NYU Langone Health | – | II | NCT00899574 |
| STING agonist | ADU-S100 (MIW815) | Novartis Pharmaceuticals | +Spartalizumab | I | NCT03172936 |
| | E7766 | Eisai Inc. | - | I | NCT04144140 |

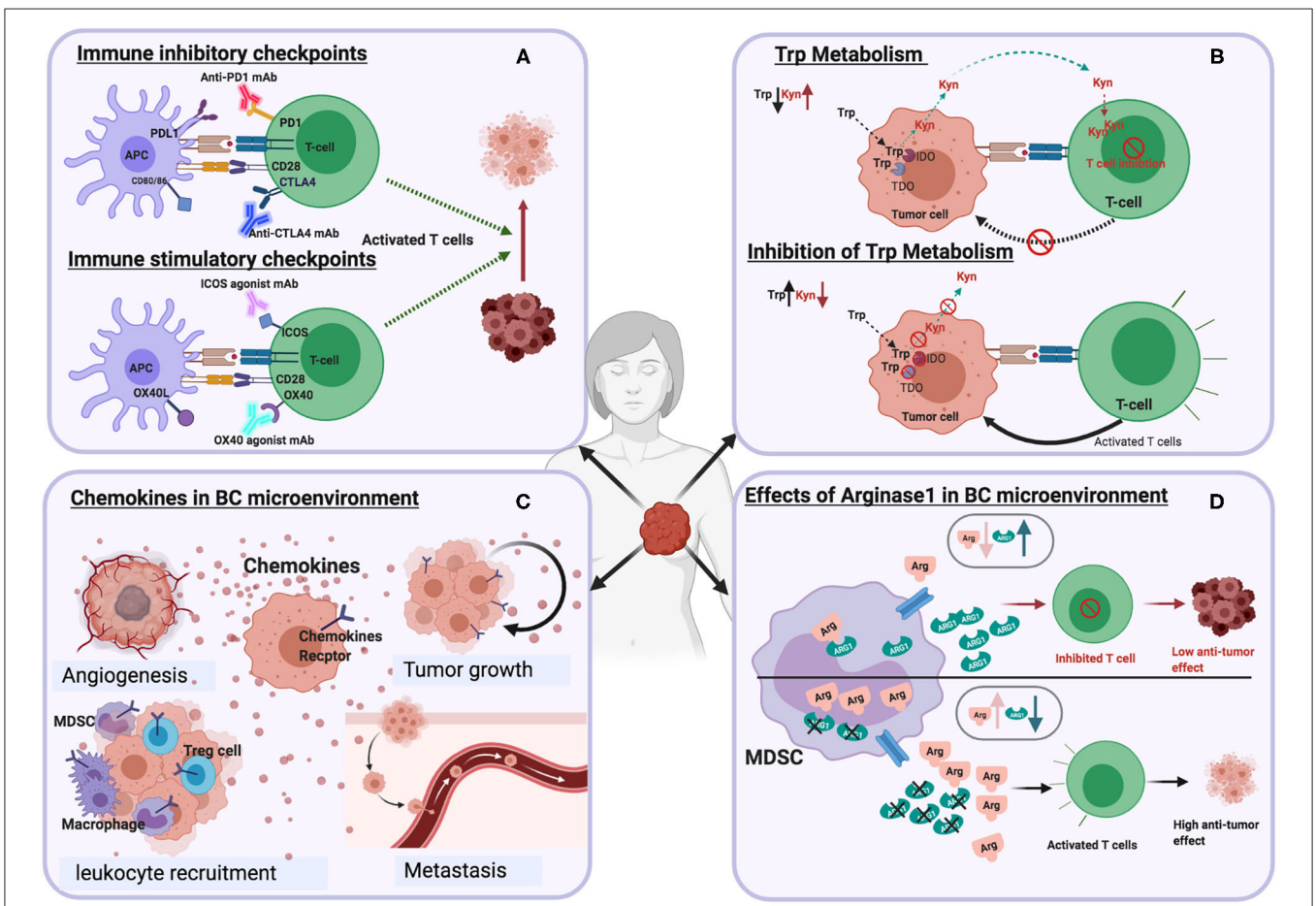


**FIGURE 2** | Schematic illustrations depicting the effects of different immune targets on breast cancer **(A)** Immune checkpoints **(B)** Tryptophan metabolism **(C)** Chemokines **(D)** Arginase enzyme.

agonist and anti-PD1 facilitated tumor elimination, which was dependent on CD4+ and CD8+ T cell responses (38). These results indicate that sequential, rather than simultaneous administration of OX40 agonists and anti–PD-1 can revert PD-1 resistance and improve responses to combination therapy. Consequently, one of the approaches in a Bristol-Myers Squibb

(BMS) clinical study (39) involves exploring the effectiveness of sequentially administering an OX40 agonist, BMS-986178, anti-PD1 (Nivolumab), an allogeneic autophagosome-enriched vaccine, DPV-001 and cyclophosphamide in TNBC patients (ClinicalTrials.gov Identifier: NCT02737475).

Another co-stimulatory molecule, the inducible co-stimulator (ICOS), is mainly expressed by activated CD4+ and CD8+ T cells and constitutively by Tregs. ICOS binds to its ligand, ICOS-L (B7RP1), expressed by APCs, epithelial cells, endothelial cells and tumor cells (40). ICOS-mediated co-stimulation does not induce IL-2 production, hence it is regarded as less potent relative to co-stimulation elicited by CD28 (41, 42). However, various clinical studies have shown that high expression of ICOS by T cells in patients treated with PD-1 and CTLA-4 checkpoint inhibitors correlates with positive treatment responses (43, 44). Hence, current immunotherapy strategies include the administration of ICOS or ICOS-L agonists with CTLA-4 checkpoint inhibitors (43, 45). A Phase 1/2 first in-human clinical trial has been set up to evaluate JTX-2011, an agonist monoclonal antibody that binds to ICOS, alone or in combination with checkpoint inhibitors for the treatment of advanced solid tumors, including TNBC breast cancer (46). A recently completed phase 1 clinical trial, which involved the use of another ICOS agonist, GSK3359609, in combination with anti-PD-1 shows promising anti-tumor activity in anti-PD-1/L1 naive patients with head and neck squamous cell carcinoma (HNSCC) (32, 36). Furthermore, the findings from this study indicate that GSK3359609 is also suitable for monotherapy of HNSCC in patients with anti-PD-1/L1 - experienced HNSCC (GSK Press Release September 28, 2019).

The glucocorticoid-induced TNFR related protein (GITR) is preferentially expressed on NK cells and T cells, particularly Tregs. GITR interaction with its ligand, GITRL, on dendritic cells, boosts effector T cell differentiation and IL-2 production (11, 13). Importantly, GITR has been detected on lymphocytes and carcinoma cells from a subset of breast cancer tumor specimens (47). Furthermore, observations from a study by Krausz et al. indicated that Tregs from tumor-positive lymph nodes from advanced breast cancer patients express increased levels of GITR, compared to tumor-negative lymph nodes (48). The potential for GITR-mediated co-stimulation to promote high effector CD8+ T cell to Treg ratios, is now harnessed as an immunotherapy strategy (49, 50). In fact, the first in-human phase 1 trial of GITR agonism with the anti-GITR antibody TRX518, has been initiated and a report indicates reduction in circulating and intratumoral Tregs at similar levels (51). However, a combination of GITR agonism with PD-1 blockade has been postponed due to sub-optimal clinical responses induced by TRX518 (51, 52). A clinical trial using another anti-GITR agonistic mAb, INCAGN01876, in combination with pembrolizumab and epacadostat for the treatment of advanced or metastatic malignancies is underway (ClinicalTrials.gov Identifier: NCT03277352).

CD40 is upregulated on the surface of activated APCs and its interaction with its ligand (CD40-L), expressed on activated B cells and T cells, leads to the initiation and progression of cellular and humoral adaptive immunity (53, 54). CD40 is also expressed in breast and lung carcinomas and carcinomas of the urinary bladder, nasopharynx, and colon, in contrast to normal non-proliferating tissues, which are CD40-negative (55, 56). Observations from a study approximately two decades ago by Tong et al., indicated that the interaction of soluble recombinant CD40L with CD40+ human breast cancer cell lines directly inhibits breast cancer cell growth. By examining primary tumor biopsies, they also found that infiltrating ductal, lobular carcinomas and carcinomas expressed CD40 while benign epithelial tissues of these biopsies exhibited weaker expression of CD40 (57). Interestingly, tumor infiltrating lymphocytes from most of the breast cancers examined expressed very low levels of CD40L (57). Other studies have suggested that CD40 may induce apoptosis in breast carcinoma cells by upregulating Fas expression induced by CD40 ligation (58).

A clinical study of CDX-1140, a CD40 agonist, for use as a monotherapy or in combination with the anti-PD-1 mAB, pembrolizumab, has been initiated in patients with advanced malignancies, including breast cancer (ClinicalTrials.gov Identifier: NCT03329950). Furthermore, results from a recent orthotopic breast cancer study suggest that combination treatment using anti-PD-1 and a CD40 agonist promote tumor immunogenicity (59).

4-1BB (CD137) is another member of the TNFR family of co-stimulatory molecules. It is expressed on many hematopoietic cells, including T cells and NK cells. Its ligand, 4-1BBL (CD137L), is predominantly expressed on APCs. 4-1BB:4-1BBL ligation potentiates CTL responses, induces antibody-dependent cell-mediated cytotoxicity in NK cells and modulates the activity of CD4+ T cells, B cells, DCs, monocytes and macrophages (60). For instance, CD8+ TILs from TNBC tumors were successfully propagated with a 4-1BB agonistic antibody (urelumab) (61). Based on these properties, harnessing the 4-1BB signaling pathway through the use of agonistic monoclonal antibodies can serve as a cancer immunotherapy strategy.

Significant breast tumor reduction in xenograft models has been achieved by targeting 4-1BB, combined with trastuzumab (anti-HER2) and rituximab (anti-CD20) treatment (32, 62, 63). In 2017, a clinical trial to investigate the optimal dosage and side effects of the 4-1BB agonist, utomilumab with trastuzumab emtansine or trastuzumab in patients with metastatic HER2-positive breast cancer was initiated (ClinicalTrials.gov Identifier: NCT03364348). However, a dependency of 4-1BB agonists on the Fcγ receptor–mediated hyperclustering and liver toxicity in patients, have been reported (64). Consequently, strategies that will restrict 4-1BB agonism to the TME, thereby minimizing off-target toxicities, have been proposed. A recent study has adopted a protein engineering approach to develop proteins that simultaneously target 4-1BB and tumor stroma or tumor antigens (65).

## AMINO ACID CATABOLISM

Amino acid metabolism is an immune regulatory mechanism (52). The breakdown of amino acids, particularly tryptophan and arginine by immunoregulatory myeloid cells, is one mechanism whereby T cell proliferation and activation are suppressed

(29). Furthermore, these catabolic pathways are harnessed by solid tumors to induce the development of immunosuppressive tumor microenvironments and poor anti-tumor T cell responses. Hence, the use of inhibitors of arginase-1 and indolamine-2, 3- dioxygenase-1 enzymes, which catabolise L-arginine and tryptophan, respectively, are now exploited as new cancer immunotherapy strategies.

## Catabolism of Tryptophan

Tryptophan is the rarest essential amino acid found in food. It is a precursor to the synthesis of niacin (vitamin B3), neurotransmitter serotonin, and the hormone melatonin. Tryptophan metabolism is associated with immune regulation and tumor progression (66). Tryptophan catabolism occurs through the kynurenine pathway with the aid of two enzymes, indoleamine-2,3-dioxygenase (IDO1) and tryptophan-2,3-dioxygenase (TDO), which catalyze the first rate-limiting step by facilitating the oxidative breakdown of the tryptophan indole group. The generation of kynurenine (Kyn) and the concomitant release of kynurenine metabolites by myeloid cells, suppresses T cell and NK cell activity. The activities of IDO and TDO have been investigated due to their link with various diseases, including diabetes, mental disorders, inflammatory, and cancer (67, 68) (**Figure 2B**).

### Indoleamine-Pyrrole 2,3-Dioxygenase (IDO1)

The upregulation and sustained expression of IDO by tumor cells is a well-characterized immunosuppressive strategy, orchestrated in conjunction with MDSCs and Tregs (69). IDO1 and TDO, through their catalytic activity, function as tryptophan *sinks*, leading to the suppression of T cell proliferation, apoptosis and Tregs differentiation. Indeed, T cell activation and function are highly dependent on the levels of tryptophan in their microenvironment, as the zeta chain of TCR complex is downregulated upon tryptophan withdrawal. IDO1 also suppresses anti-tumor responses through the generation of L-kynurenine, an endogenous agonist of the arylhydrocarbon receptor (AhR). AhR activation promotes the differentiation of Tregs and the concomitant upregulation of IDO1 by DCs (70). Furthermore, long-term expression of IDO1 by DCs is facilitated when IDO functions as a signal-transducing molecule (70).

The expression of IDO has been observed in breast carcinomas, particularly among triple negative (TNBC) basal-like breast cancers (71, 72). In a study by Dill et al., the authors assessed 281 primary and metastatic breast cancers and identified a correlation between IDO1 and PD-L1 expression, particularly in high-grade TNBC (73). Their observations imply that IDO1 expression contributes to the resistance of breast cancer to anti-PD-1/PD-L1 treatment.

A positive correlation between the high expression of PD-1 by T cells and high levels of kynurenine in the plasma and the TME of breast cancer patients has also been reported (74). IFN-γ produced by CD8+ T cells induces the production of IDO and kynurenine by CD45 negative tumor cells. Kynurenine promotes the translocation of AhR from cytosol to the nucleus of *in vitro*-treated and tumor-infiltrating CD8+ T cells and subsequently upregulates PD-1 (60).

IDO1 also induces cancer progression in a non-immune manner by regulating angiogenesis (59). The expression of IDO and levels of CD105+ micro vessel density by breast cancer specimens were found to be associated with metastasis and poor prognosis (75). Furthermore, MCF-7 cells which produce high levels of IDO significantly induced the proliferation of human umbilical vein endothelial (HUVEC) cells (75). Thus, the pharmacological modulation of IDO1 and other enzymes that target amino acids have been included in cancer therapy strategies (20). Preclinical and clinical studies to test the efficacy of IDO inhibitors for cancer treatment are discussed extensively in a recent review (76).

A number of studies in which IDO1 is targeted alone or in combination with immune checkpoint inhibitors have been proposed. In 2017, a phase II clinical study investigated the effect of the combined use of chemotherapy and the IDO1 inhibitor, 1-Methyl-D-tryptophan (Indoximod) in metastatic breast cancer patients (ClinicalTrials.gov Identifier: NCT01792050). Results from the phase I study indicated no drug-drug interactions and partial responses in breast cancer and patients with other metastatic tumors (77). Four of the breast cancer patients achieved a reduction in tumor burden; a patient that had hitherto only received only adjuvant endocrine therapy achieved the best response (77). Results from another phase I study on the use of a small molecule inhibitor of IDO1 (Navoximod) alone, or in combination with a PD-L1 inhibitor (Atezolizumab) to treat TNBC and other solid tumors indicated tolerability, partial responses and complete responses in some patients (78). However, there were no clear benefits associated with the use of atezolizumab with navoximod (78). Results from another phase I/II study of another IDO inhibitor, Epacadostat, used in combination with anti-PD-1 (pembrolizumab) for the treatment of TNBC and ovarian cancer indicated tolerability, safety and anti-tumor activity (79). However, in another study, there was no difference in progression-free or overall survival in patients with unresectable stage III or IV melanoma administered with Epacadostat in combination with anti-PD1 (pembrolizumab), compared to placebo plus pembrolizumab (80). Hence, the usefulness of IDO1 inhibition as a strategy to enhance anti-PD-1 therapy activity in cancer yet to be clarified.

Other approaches which utilized nanodelivery systems designed to use Indoximod in conjunction with a-PD-L1 or the induction of immunogenic cell death using doxorubicin for breast cancer treatment, have also been investigated (81). Taken together, the outcomes of these studies suggest that IDO1 inhibitors can be used as standard-of-care treatment for breast cancer and other solid tumors, alone or in combination with other cancer therapeutic strategies.

### Tryptophan-2,3-Dioxygenase (TDO)

Unlike IDO1, which is induced in immune cells such as DCs, TDO is constitutively expressed in the liver, where it regulates tryptophan homoeostasis in the blood (82–84). Similar to IDO1, TDO suppresses T cell activation by tryptophan depletion and is also overexpressed in the microenvironment of various tumors, including breast cancer (26). Preclinical studies have demonstrated that TDO expression by breast cancer

cells is associated with increased cancer cell migration and invasion (66, 85). In a study by Greene et al., the authors demonstrated that triple-negative breast cancer (TNBC) cells use TDO to suppress CD8+ T-cell viability (86). Furthermore, in an earlier preclinical study, D'Amato et al., showed that NF-kB-dependent upregulation of TDO and AhR is linked to anchorage-independent cell survival and anoikis resistance of TNBC cells (85). These observations imply that the overexpression of TDO by tumors such as TNBC is associated with disease metastatic.

Results from preclinical studies investigating the impact of TDO inhibition using knockout mice or compounds have shown that deletion of the TDO gene (*TDO2*) in mice results in tryptophan accumulation in the blood and neurologic changes, which may be associated with serotonin production (84) Consequently, the utilization of TDO inhibitors may have safety implications with respect to liver and CNS complications. Dose-dependent reduction of the 4T1 breast or CT26 colon tumor growth was achieved by dual inhibition of IDO and TDO using a lead compound, CB548, in a mouse preclinical model (87). Also, the administration of CMG017, another dual inhibitor of IDO and TDO, to tumor-bearing mice resulted in reduced kynurenine concentration, differential expression of immune-related genes and the infiltration of effector CD8+ T cells in the TME (87). Furthermore, co-administration of CMG017 with checkpoint inhibitors (a-PDL1 and a-CTLA-4) to tumor-challenged mice resulted in tumor regression and the establishment of memory CD8+ T cell responses (87).

In 2017, a phase I study was initiated to investigate the safety, pharmacokinetics, pharmacodynamics and efficacy of HTI-1090, a small molecule dual inhibitor of IDO1 and TDO, in patients with advanced solid tumors (ClinicalTrials.gov Identifier: NCT03208959). Although this study was completed in 2019, the outcomes are yet to be disclosed. The utilization of other TDO and IDO1 inhibitors such as 680C9, LM101 are still under preclinical investigation.

## Catabolism of Arginine

### Arginase

L-arginine is a non-essential amino acid that plays a vital role in cellular activity such as metabolic programming and maintenance of T cell fitness (88, 89). The administration of L-arginine to breast tumor-bearing BALB/c mice suppressed tumor growth significantly and prolonged the survival time of treated mice. L-arginine supplementation also enhanced the levels of IL-10, TNF-α, IFN-γ; macrophage and T cell numbers and suppressed the activity of MDSCs. The activity of arginase enzymes (ARG1 and ARG2), which catalyze L-arginine into ornithine and urea, is increased in the TME of multiple cancers including breast cancer. Arginase enzymes facilitate localized immune suppression mediated by cancer-associated fibroblasts (ARG2), MDSCs, DCs, tumor-associated macrophages (TAMs) and tumor-infiltrating macrophages (ARG1) (90, 91). These cells in turn, produce ARG1 in response to a milieu of tumor cues, such as HIF-1α, M-CSF, GM-CSF, IL-4, IL-13 and IL-6 (89). Another key enzyme associated with L-arginine metabolism, nitric oxide synthase (NOS), produces nitric oxide (NO) from L-arginine and oxygen. In low L-arginine conditions, characteristic of tumor sites, NOS can induce the production of superoxide anion, which can combine with NO to generate various reactive nitrogen species that can also hamper T cell activity at tumor sites (89).

The reduction of extracellular arginine by ARG1 leads to suppression of T cell function (**Figure 2D**) by the activation of GCN2 kinase, which blocks the expression of several cell cycle genes such as cdk4, cyclin D3, and CD3 (21). High levels of ARG1 have been identified in the serum of preoperative breast cancer patients compared to healthy controls (92). In addition, elevated ARG1 is expressed by MDSCs from patients diagnosed with early-stage breast cancer, which is reduced upon surgical tumor resection (2).

A number of preclinical strategies that target ARG1 have been implemented with promising results. The cell viability and arginase activity of a TNBC cell line with high levels of arginase (MDA-MB-468), were decreased in response to L-lysine induced arginase inhibition, in comparison to a cell line with less arginase levels (MDA-MB-231) (93). The treatment of tumor bearing mice (CT26, 4T1, B16, and LLC) with CB-1158, a small molecule inhibitor of ARG1, elicited increased cytotoxic T cell infiltration and decreased myeloid cell numbers (71). This correlated with increased activation markers, cytokine production and expression of interferon genes. Furthermore, CB-1158 efficacy was enhanced when combined with checkpoint blockade, chemotherapy and adoptive cell therapy (94).

Treatment with the arginase inhibitor (INCB001158) alone inhibited plasma arginase activity with concomitant increase in the plasma arginine in a colorectal carcinoma patient cohort. INCB001158 used in combination with a-PD-1 (pembrolizumab) for the treatment of advanced/metastatic solid tumors. INCB001158-pembrolizumab combination treatment elicited increased frequencies of intratumoural CD8+ T cells and a 7% partial response (ClinicalTrials.gov Identifier: NCT02903914). A clinical study has been initiated to evaluate the safety, toxicity and immune correlates of administering an Arginase-1 peptide vaccine (ARG1-18,19,20) to patients with breast cancer and other solid tumors (ClinicalTrials.gov Identifier: NCT03689192).

# CHEMOKINES AND CHEMOKINE RECEPTORS

Chemokines and their receptors play a pivotal role in various biological and pathological processes, including chronic inflammation, tissue development, hematopoiesis, and immune modulation (95). Many studies revealed chemokines' role as essential mediators of immunity, angiogenesis (96), metastasis (97), drug resistance (98), breast cancer occurrence and progression (**Figure 2C**) (23, 99, 100). Chemokines have been classified into four main groups, CXC, CC, XC, and $CX_3C$. The CXC family consists of 17 subfamily members (CXCL1-CXCL16), while CC family is the largest subgroup (CCL1-CCL28). The XC family has two subgroups (XCL1 and XCL2), while there is only one $CX_3C$ chemokine ($CX_3CL1$) (95, 101).

Tumor cell migration and the ensuing invasion into specific organs occur in response to receptor-ligand interactions, the rearrangement of the actin cytoskeleton and multiple environmental cues which favor trafficking. Mueller et al., in investigating the role of chemokine receptors in promoting breast cancer metastasis almost two decades ago, found that breast cancer cells express CXCR4 and CCR7 (90). Consequently, targeting chemokines and their receptors has been evaluated in preclinical and clinical cancer immunotherapy studies. The detailed roles of chemokines in cancer biology have been reviewed elsewhere (23, 95, 102). We will highlight a few examples of the roles of chemokine–chemokine receptor interactions in the breast cancer microenvironment.

## CXCR Family

CXCL8 (IL-8) is a chemokine whose physiological effects are mediated by two receptors, namely CXCR1 and CXCR2 (103). CXCR2 (IL-8 receptor) is expressed on MDSCs, neutrophils, lymphocytes, and breast cancer cells. CXCR2 and CXCL8 regulate breast cancer progression in the TME by modulating several related pathological processes, including promoting breast cancer growth, angiogenesis, invasion, metastasis, and reducing cancer cell sensitivity to chemotherapy (99, 104, 105). CXCR2 modulates the trafficking of neutrophils from the bone marrow to breast cancer sites, leading to increased tumor growth (106). CXCR2 also induces the migration of MDSCs, thus, promoting local immunosuppression (107). Studies show that cancer patients with high levels of CXCR2 have low overall survival and poor prognosis (108). The CXCL8-CXCR2 axis can also stimulate the transcription of VEGF and activate its receptor,VEGFR2, in endothelial cells by the NF-κB pathway (109). Like CXCR2, CXCR1 is expressed significantly in breast cancer stem cells, which increases the growth of breast cancer when stimulated by inflammation or tissue damage (110). Consequently, targeting the CXCL8-CXCR1/CXCR2 axis has been adopted as a breast cancer therapy strategy (111). The utilization of reparixin, a small molecular weight antagonist of CXCR1/2 as a breast cancer therapeutic agent has been investigated in preclinical and clinical studies (99, 112). Results from a phase Ib trial on the co-administration of reparixin and paclitaxel to patients with HER-2- negative metastatic breast cancer yielded a 30% response rate (88). In another study on the treatment of women with HER-2- negative operative breast cancer with reparixin only, the frequency of cancer stem cells, indicated by aldehyde dehydrogenase, CD44+/CD24- expression, was reduced (113).

Several studies have assessed the impact of CXCR4 in breast cancer cell survival, proliferation, angiogenesis, migration, and metastasis (114, 115). CXCR4 induces breast cancer metastases by binding to its ligand stromal cell-derived factor-1α (SDF-1), which is overexpressed in the bone marrow, liver, lung, and breast tumors sites (100, 116). CXCR4 promotes cancer cell proliferation by activating several signaling pathways, including Src/ERK1-2, PI3K/AKT, STAT3, and NF-κB. The cross-link between CXCR4 and other pathways such as Notch, Wnt, and SHH is also associated with increased breast cancer growth (117). Injecting immunocompromised mice subcutaneously with a CXCR4-low-expressing breast cancer cell line (MCF-7), resulted in reduced tumor growth compared to mice inoculated with the MDA-MB-231 cell line, which expresses high levels of CXCR4 (118). Also, results from a human study in which surgically resected ductal carcinomas were evaluated, indicate that high CXCR4 expression correlates with extensive nodal metastasis (119). Preclinical studies of CXCR4 inhibitors have demonstrated its ability to attenuate the proliferation and metastasis of breast tumors; AMD3100 is a CXCR4 antagonist that decreases lung metastases in breast cancer (120). However, Lefort et al., have shown that AMD3100 and TN14003, another CXCR4 inhibitor, impair only the growth and metastasis of HER2 breast cancers, but not TNBC (121).

In contrast to the preclinical outcomes, the efficacy of CXCR4 blockade in clinical trials has not shown clear success with respect to dosage and the manifestation of undesirable side effects. In a clinical study by Pernas et al., the safety, tolerability, pharmacokinetics, and preliminary phase 1 dose-escalation activity of the CXCR4 antagonist, balixafortide, in combination with eribulin (antineoplastic) chemotherapy, was assessed in patients with relapsed metastatic breast cancer who had hitherto received chemotherapy (96). Partial responses were observed and serious side effects occurred in 30 and 38% of the study patients, respectively. Furthermore, two patients died from septic shock and pneumonia, respectively (96). Based on the observations of the Phase 1 trial, a phase 3 study has been set up to investigate the safety, efficacy and tolerability of intravenous balixafortide administered with eribulin compared to eribulin monotherapy for the treatment of HER2 negative, locally recurrent or metastatic breast cancer patients (ClinicalTrials.gov Identifier: NCT03786094).

## The CCR Family

CCL2 is overexpressed in tumor cells, including breast, ovarian, and lung cancer. CCL2 stimulates the migration of macrophages that express the chemokine CCL2 receptor (CCR2), into the TME. It also induces cancer proliferation and invasion (122). CCL2 can induce the migration of various breast cancer cell lines, including T47D, MCF-7, and ZR-75-1 (123). Studies using breast tumor xenografts show that blocking CCL2-CCR2 axis suppresses the recruitment process of inflammatory monocytes, increases tumor growth, and promotes metastasis and invasion (124). These studies suggest that CCL2-CCR2 signaling promotes breast cancer progression, and targeting this pathway might be adopted as a breast cancer therapy strategy.

CCL5/CCR5 pathway also plays a critical role in promoting breast cancer progression. CCL5 ligand is overexpressed in breast cancer cells, mesenchymal stem cells (MSCs), and infiltrating leukocytes. Results from a clinical study indicate that levels of CCL5 in breast cancer patients are higher than that of healthy controls (125). CCL5 can maintain the immunosuppressive activity of human MDSCs (126). The CCL5 receptor (CCR5) is also upregulated on breast cancer cells (127). A study conducted on breast cancer patients showed that 50% of breast tumors express CCR5, with >95% TNBC tumors being CCR5+ (128). The blockade of CCR5 suppresses breast cancer proliferation, migration, colony formation, and metastasis (129). Therefore,

targeting CCR5 could be promising strategy for metastatic breast cancer. Met-CCL5, a competitive CCR5 inhibitor, reduces breast cancer proliferation and infiltrating macrophages in animal preclinical models (130). Treatment with maraviroc, CCR5 antagonist, significantly suppresses bone metastasis in a xenograft rat model implanted with breast cancer cells (MDA-MB-231) (131). Leronlimab (PRO 140) is another CCR5 antagonist under investigation in breast cancer clinical trials (129, 132, 133).

## PURINERGIC SIGNALING

Purinergic signaling plays a prominent role in inflammation and cancer. It modulates cell growth, migration, and cell death (134). In this pathway, two potent molecules (ATP and Adenosine) involved in the immune response are released into the TME (**Figure 3A**) (135). Intracellular ATP levels are sustained at millimolar concentrations under physiological conditions, while extracellular levels are regulated in nanomolar concentrations. However, in the TME, ATP concentrations arise due to release from necrotic or apoptotic cells (136). Adenosine concentrations in solid tumors are also higher than that of healthy tissues (137, 138). It is well-reported that ATP and Adenosine have opposite effects. ATP is immunostimulatory as it enhances the activation of dendritic cells (DC), macrophages, IL-1β secretion, and cytotoxicity of CD8+ T cells. Hence, ATP activity can mediate the suppression of proliferating cancer cells. Adenosine, on the other hand, has immunosuppressive properties. It inhibits immune effector cells, DC maturation, cytokine production and stabilizes immunosuppressive Tregs (139). Purinergic cell surfaces ectoenzymes (P2Xs, P2Ys, CD73, CD39, and CD38), mediate the biological activities of ATP and Adenosine, and adenosine receptors (A1R, A2AR, A2BR, A3R), are overexpressed by breast cancer cells and tumor-infiltrating immune cells (19). Several therapeutic agents are developed to target these receptors to enhance anti-tumor immune responses against breast cancer.

### The P2 Family

The pyrogenic receptors P2Xs (ion channel receptors) and P2Ys (G protein-coupled receptors) are overexpressed on several immune cells within the TME (140). Among the pyrogenic receptors, P2X7 receptor (P2X7R) has been studied extensively

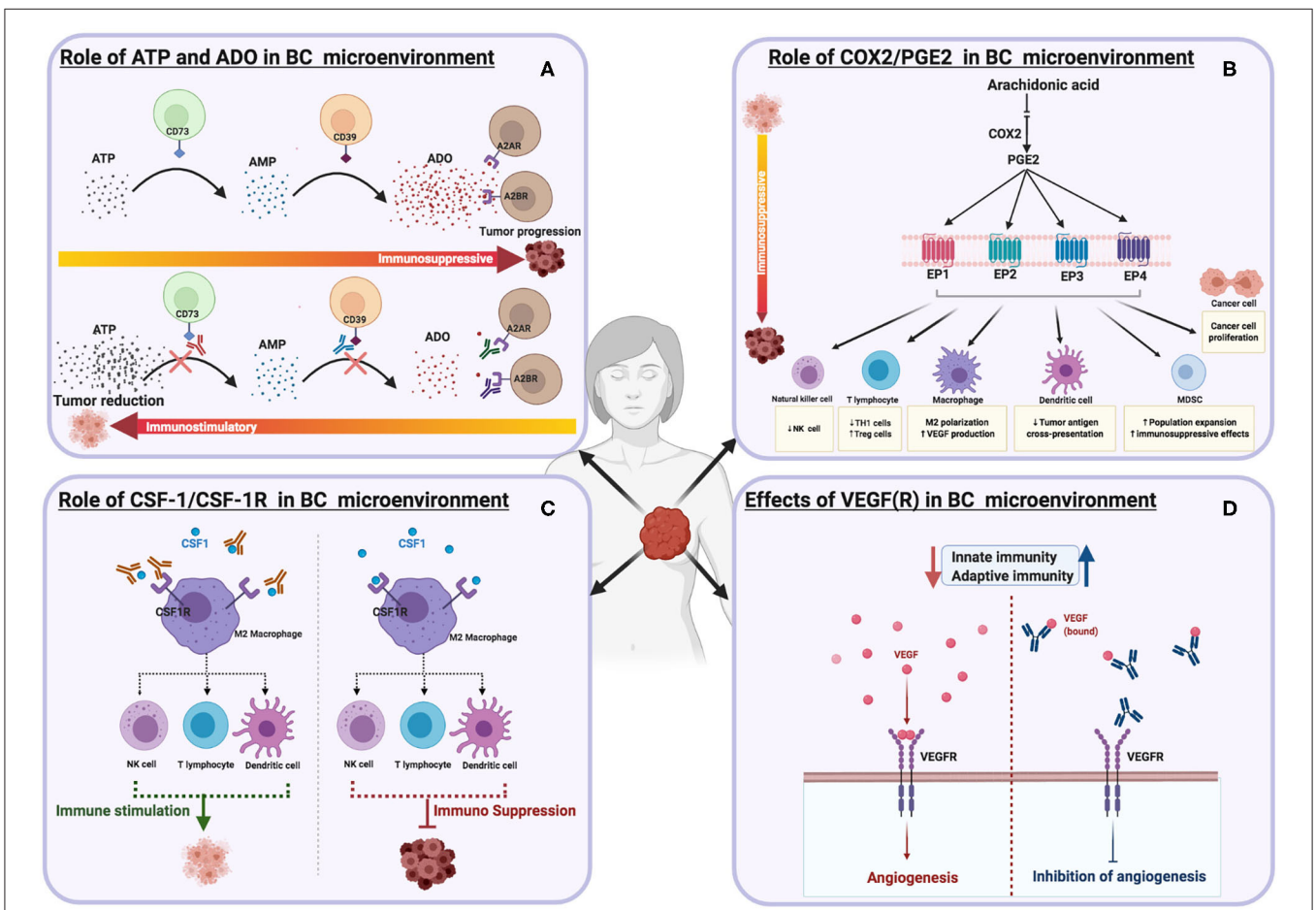


**FIGURE 3 |** Schematic illustrations depicting the effects of different immune targets on breast cancer **(A)** ATP and Adenosine signaling **(B)** COX2/PGE2 pathways **(C)** CSF-1/CSF-1R **(D)** VEGF(R).

due to its contrasting effects (134). In some studies the role of P2X7 in inducing antitumor immune responses by activating NK cells, CD4+, and CD8+ effector T cells, and promoting Treg apoptosis, has been shown (141, 142). Two P2X7 receptor agonists ATPγS and BzATP, reduce tumor growth and metastasis (143, 144). Other pieces of evidence propose the P2X7 receptor as promoters of tumor progression, mediated by inducing tumor growth, metastasis, and survival (145). P2X7R is upregulated in various tumors, including malignant breast cancers, and its expression is higher in tumors compared to the healthy tissue. This indicates that P2X7 can be used as an effective early cancer biomarker (40, 146). Many inhibitors that target P2X7R have been developed, such as Anthraquinone Emodin, which can potently suppress invasive breast cancer cells *in vitro* (147). AZ10606120 is another P2X7R antagonist reported to be a potent inhibitor of tumor growth (91).

### CD39 and CD73

ATP and ADP are converted into AMP by the catalytic activity of CD39, while AMP is irreversibly converted to adenosine by CD37 (148). CD39 and CD73 are expressed significantly by breast cancer and various immune cells, including T cells, NK cells, B cells, MDSC, macrophages, and neutrophils (17). The high expression of CD39 and CD73 results in increasing adenosine levels in the TME, which in turn stimulates the adenosine A2A and A2B receptors. The adenosine A2A and A2B receptors promote tumor progression by triggering angiogenesis, tumor cell survival, and metastasis (149–151). They also increase the immunosuppressive efficacy of Tregs, macrophages, MDSCs and development of effector T cells. Breast cancer patients with positive clinical outcomes exhibited low expression of CD39 and CD73 compared to patients with poorer clinical outcomes, which indicates that CD39 and CD73 can serve as biomarkers of patients' progress (152–154). Blocking CD73 and CD39 promoted anti-tumor responses; anti-CD73 mAbs, enhances the cytotoxicity of CD8+ T cells and inhibits the activity of Tregs and MDSCs (155). Small molecules against CD73 such as LaSOM 63 and APCP, inhibit tumor progression and increase the efficacy of effector T cells (150, 156). Preclinical studies indicate that anti-CD73 mAbs can hinder metastasis in human breast cancer (157). Three CD73 antagonists (MEDI9447, BMS-986179, CPI-006), which target TNBC are currently under clinical investigation (158). Similarly, preclinical studies of anti-CD39 monoclonal antibodies, BY40 and BA54G, have demonstrated anti-tumor efficacy (159). Therapeutic agents that target CD39 are still in the developmental stage (160).

### Adenosine A2A Receptor (A2AR) and Adenosine A2B Receptor (A2BR)

Extracellular adenosine stimulates the immunosuppressive pathway through engagement with specific G-protein-coupled adenosine receptors such as (A2a and A2b) (160). A2aR (high affinity receptor) is upregulated on a variety of immune cell subsets, including monocytes, macrophages, DCs, T cells, and natural killer T (NKT) cells. Adenosine signaling pathway through the A2aR suppresses T cell proliferation by increasing the expression of anti-inflammatory cytokines (IL-10, TGF-β) and reducing the expression of pro-inflammatory cytokines (IFN-γ, IL-2) (161). It also triggers increased expression of immune checkpoints such as LAG-3, PD-1, and CTLA-4 (162, 163). A2aR is overexpressed in many cancer cells, including breast cancer cells. Activation of A2aR leads to an increase in the proliferation of MCF-7 breast cancer cells (164). A2aR promotes proliferation and metastasis by stimulating various signaling pathways, including PLC/PKC, ERK-MAPK, PI3K/AKT/mTOR (165). CPI-444, an A2AR antagonist, is used as monotherapy or combined with anti-PD-L1 (Atezolizumab) to treat TNBC (166). A2bR, on the other hand, is a low-affinity receptor which needs more Adenosine to be activated. A2bR is overexpressed by macrophages, DCs, and multiple tumors such as breast tumors (167, 168). Its upregulation is associated with poor survival and worse prognosis in human TNBC (169). *In vitro* activation of A2bR, increases the growth and migration of breast cancer (MDA-MB-231) cells (170). Results from an *in vivo* study indicate that blocking A2bR reduces the metastasis of TNBC and enhances the activities of chemotherapy and immune checkpoint inhibitors (169). Several studies indicate that stimulating A2bR promotes tumor growth and metastasis through the activation of the ERK1/2 and angiogenesis pathways; blocking this receptor reverses these effects (19, 171, 172). A selective A2bR blocker (ATL801) promotes the inhibition of bladder and breast cancer growth when injected intratumorally (173).

## TARGETING THE COX2/PGE2 PATHWAYS

Increased levels of COX2 enzymes have been reported in nearly half of breast cancer patients (174), with other studies reporting a range of 17 to 84% (175, 176). The silencing of COX-2 expressed by the human breast cancer cell line, MDA-MB-231, inhibits cell migration *in vitro* and metastasis *in vivo* (177). PGE2, an enzymatic product of COX2, functions by signaling through one of the four G-protein coupled receptors (EP1, EP2, EP3, and EP4) (**Figure 3B**). The COX2/PGE2 axis promotes breast cancer progression by increasing cancer migration, metastasis, and angiogenesis (178–180). In addition, PGE2 regulates different immune cells- it suppresses the proliferation of CD4+ T cells by reducing intracellular calcium release and suppressing the activity of the p59 protein tyrosine kinase (181, 182). PGE2 decreases the production of effector cytokines, such as IL-2 and IFN-γ, and it can also inhibit NK cell function and B cell proliferation (183–185). PGE2 elevates cAMP by the stimulation of its receptors, EP2 and EP4 (186). COX2/PGE2 and its receptors are potential target(s) for breast cancer therapy. Preclinical studies indicate that celecoxib, a selective COX-2 inhibitor, reduces breast cancer metastasis (176, 187). The daily intake of COX-2 inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of breast cancer occurrence significantly (188). The PGEP4 receptor blocker (AAT-007) is currently in phase 2 for the treatment of patients with solid tumors, including breast cancer (179). A newer version of the PGEP4 receptor antagonist called (AAT-008) has shown significant bioavailability and pharmacological profiles in preclinical investigations (189). The PGE2 EP1 antagonist (ONO-8711) suppresses breast cancer progression in rats (190). Using different breast cancer cell lines *in vitro*, the

PGEP3 receptor antagonist (L798,106), demonstrated potency in reducing breast cancer proliferation and migrations (191).

## CSF-1/CSF-1R

Activated macrophages are divided, for simplicity, into anti-tumor (M1) macrophages and pro-tumor (M2) macrophages. M1 macrophages are activated by GM-CSF, IFN-γ, LPS, and other cytokines. M1 macrophages, referred to as "fight" macrophages, play a significant role in producing pro-inflammatory cytokines and inducing anti-tumor immune responses (192, 193). The growth factor, GM-CSF, regulates the differentiation of DCs and macrophages (194, 195). Results from *in vivo* studies indicate that GM-CSF suppresses breast cancer growth and metastasis (196). In contrast, M2 macrophages induce tumor proliferation, therapy resistance, tumor invasion, angiogenesis, and metastasis. M2 macrophages are polarized by colony-stimulating factor 1 (CSF1), IL-13, IL-10, IL-4, TGF-β, and prostaglandin E2 (197, 198). The upregulation of CSF-1 signaling correlates with increased breast cancer progression (**Figure 3C**) (199). CSF1R is expressed by both M1/M2 TAMs, MDSCs, neutrophils, and DCs (200). CSF1/CSF1R signaling increases angiogenesis, cancer growth, metastases, invasion, CD8+ T cell suppression, tumor macrophage recruitment, and resistance to therapy (201, 202). CSF1 can also stimulate VEGF production (196). Blocking CSF1 in breast cancer-bearing mice reversed these effects and increased mouse survival rate (203). There are currently many therapeutic agents that target CSF1 and its receptor CSF1R, in preclinical or clinical development stages. For example, LY3022855, a CSF1R blocker used as a single agent or in combination with Durvalumab (anti-PDL1 mAb) or Tremelimumab (anti-CTLA4 mAb) for patients with a solid tumors, including breast cancers (24). Pexidartinib is another inhibitor of CSF1R that is used in combination with a microtubule inhibitor (Eribulin) for breast cancer patients (24). Anti- CSF1R (Emactuzumab) combined with Atezolizumab (anti-PDL1 mAb) are used to treat TNBC (24, 204).

## VASCULAR ENDOTHELIAL GROWTH FACTOR A (VEGF-A)

VEGF binding to its receptors promotes the progression, proliferation, and metastasis of breast cancer (**Figure 3D**) (22, 205, 206). Among the five identified VEGF subfamilies (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E), VEGFA, also called VEGF, is the dominant and most researched isoform (207). VEGF isoforms bind with varying affinities to VEGFR1, VEGFR2, and VEGFR3, which mediates VEGF downstream signaling (208). VEGFA is overexpressed in several types of cancer, including breast cancer (209), and plays a vital role in angiogenesis (210). VEGF halts the differentiation and activation of DCs and promotes the exhaustion of CD8+ T cells by increasing the expression of inhibitory receptors, such as PD-1, TIM-3, LAG-3, and CTLA-4 (211). High VEGF plasma levels in breast cancer patients is associated with a significant reduction of DCs in the peripheral blood of cancer patients. The appearance of immature DCs in the blood correlates with the duration and disease stage; surgical removal of tumors showed a partial reversal of the noted effects (212). On the other hand, inhibiting VEGF increases tumor-infiltrating effector T-cells and reduces the recruitment of Tregs and MDSCs to the TME (213). Blocking VEGF stops the growth of tumor blood vessels in murine models and promotes cancer cell death and tumor-shrinkage (214). Therefore, targeting VEGF and its receptor VEGFR are key therapeutic targets for breast cancer treatment. Many angiogenesis inhibitors have been approved by the FDA, however, only a few have been tested in breast cancer patients such as bevacizumab, which binds to VEGFA and blocks its efficacy (215). Bevacizumab was the first FDA approved antiangiogenic agent (216, 217). In 2008, it was approved to be used in combination with chemotherapy to treat metastatic HER2-negative breast cancer (218). However, it showed several adverse side effects and poor overall survival, which led the FDA to revoke its approval in 2011 (219, 220). An example of a VEGFR inhibitor is DC101, a monoclonal antibody which binds to VEGFR2, and exhibits potential antiangiogenic efficacy against breast tumors in xenograft models. In another *in vivo* study, DC101 enhanced tumor-specific CD8+ T cells and accelerated tumor regression. Combining DC101 with neu-specific vaccination also suppressed tumor progression and increased the activity of CD8+ T cells (221). Ramucirumab, a VEGFR2 blocker, has shown preclinical and clinical promise in targeting breast cancer angiogenesis, growth, and metastasis (222). Axitinib is a small molecule that binds selectively to VEGFR-1,−2, and−3, and blocks their activities (223); murine studies indicate its potency in inhibiting breast cancer growth (224). However, clinical studies have only demonstrated its activity in combination with chemotherapy (paclitaxel). Sorafenib is another small molecule VEGFR blocker; reports indicate encouraging clinical trial results from the treatment of breast cancer patients. However, the utilization of sunitinib, a VEGFR inhibitor, has not shown any clinical benefit in breast cancer patients (225).

Overall, the preclinical results obtained from the use of anti-VEGF agents showed a significant decrease in tumor angiogenesis. However, the outcome of clinical trials exhibited an average response (22, 226).

## TOLL-LIKE RECEPTOR (TLR)

TLRs are expressed by both cancer and immune cells (227, 228). Among the thirteen TLRs (TLR1-13) that have been characterized, ten (TLR1-10) were identified in humans, six of which are expressed on the cell surface TLR (1, 2, 4, 5, 6, and 10) and four on endosomal membranes (229).

Several TLRs are upregulated in human breast tumors. TLR4 is the most expressed among the TLR family, on breast cancer cells (MDA-MB-231 cells). Deletion of the TLR4 gene resulted in an increase in cell death and suppression of IL-6 and IL-8 expression (230). The overexpression of TLR9 in human breast cancer enhances tumor cell invasion, which is mechanistically linked to the induction of MMP13 and COX-2 secretion (231). Various studies have reported positive correlations between

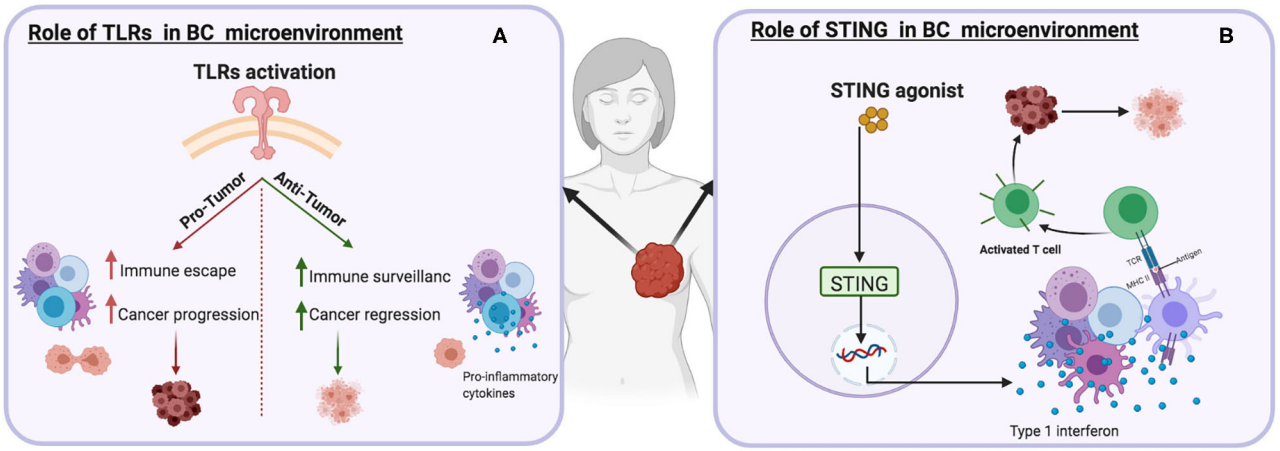


**FIGURE 4 |** Schematic illustrations depicting the effects of different immune targets on breast cancer **(A)** Toll-like receptor(s) **(B)** Stimulator of interferon genes protein.

TLR expression and the activation of the immune system. TLR stimulates DCs and macrophages and promotes the secretion of pro-inflammatory cytokines and the facilitation of anti-tumor immune responses (232, 233). The role of TLRs as pro-tumor agents has also been investigated (234). The function of TLRs in cancer can be described as a "double-edged sword" (**Figure 4**). On the one hand, agonists that bind to TLR(s) on tumor cells can promote cancer progression by promoting immune escape and cancer cell proliferation and survival. The engagement of TLR4 expressed by human breast cancer cells results in increased production immunosuppressive factors such as NO, VEGF, and MMPs, thereby promoting the tumor invasion (230, 235, 236).

On the other hand, activating TLR5 in the breast cancer mouse model resulted in anti-proliferative efficacy through the promotion of necrosis, increased neutrophil infiltration and down-regulation of cyclin B1, cyclin D1, and cyclin E2 (237). TLR3 expressed by human and mouse breast cancer cells promotes apoptosis by inducing type I IFN signaling (238). Preclinical studies have demonstrated that TLR agonists, combined with other therapeutic agents, can potentially reduce and suppress tumor progression (239, 240). The different roles of TLRs are linked to the proximal signaling pathways stimulated in cancer cells and immune cells. For example, even though TLR5 is overexpressed in both gastric and breast cancers, it has opposite effects as it suppresses the proliferation of breast cancer and induces the growth of gastric cancer cells (237, 241). Many TLR agonists have been investigated for clinical use. The TLR5 agonist, flagellin, suppresses breast cancer by induction of caspase-1 activation-dependent pyroptosis. It also enhances the expression of granzyme B, TNF-α, and IFN-γ in CD8+ T cells (242, 243). The TLR3 ligand, poly-AU, increases the survival rate in patients with TLR3-positive breast cancer (244). Imiquimod is a well-tolerated TLR7 agonist that can promote the rejection of immune-mediated skin metastasis in breast cancer patients (245) 852A is another TLR7 agonist used for the treatment of metastatic breast cancer patients (240).

## STIMULATOR OF INTERFERON GENES PROTEIN (STING)

Various studies have suggested that STING (stimulator of interferon (IFN) genes) expression is not only confined to innate and adaptive immune cells (246–248), but is also expressed in various tumors, including breast cancer (249). STING stimulators have shown great potential for activating immune cells, enhancing anti-tumor immunity by inducing a variety of pro-inflammatory cytokines and chemokines (246, 247, 250), priming and activation of T cells (251), enhancement of antigen presentation, promotion of cancer cell death, inducing the recognition and apoptosis of cancer cells by T cells (249, 252, 253). A previous study has revealed the role of STING in promoting death in 4T1 breast cancer cells by increasing the caspase-3 pathway cascade (249). Similarly, the overexpression of STING in two breast cancer cell lines, T47D or MCF-7 has been shown to increase caspase 3 and/or 7 activity (252). The deletion of STING expressed by melanoma cell lines results in the suppression of cytokines (IFN-γ) and chemokines (CCL5 and CXCL10) production (254). Furthermore, STING knockout mice exhibit reduced NK cell responses by mediating the downregulation of perforin, granzyme B, and IFN-γ (253, 254). Numerous STING stimulators are now under clinical investigation for the treatment of various types of cancers. The utilization of ADU-S100 (MIW815), a STING agonist, is currently being tested in combination with anti-PD-1 (spartalizumab) for the treatment of patients with solid tumors, including PD-1-naïve TNBC (255).

## CONCLUSIONS

Following several years of preclinical and clinical research, our understanding of how the immune system responds to cancer has increased. The limited success of immune checkpoints, like CTLA-4 or PD-1, in clinical trials for breast cancer patients, has prompted research to find alternative

targets. Many new emerging data reported novel pathways that stimulate immune responses against breast tumors. These newly discovered pathways are likely to be the future targets of breast cancer immunotherapy.

## AUTHOR CONTRIBUTIONS

YT and IO drafted the manuscript. KB, AS, and SE edited and modified the manuscript. All authors contributed to the article and approved the submitted version.

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# Improving the Odds in Advanced Breast Cancer With Combination Immunotherapy: Stepwise Addition of Vaccine, Immune Checkpoint Inhibitor, Chemotherapy, and HDAC Inhibitor in Advanced Stage Breast Cancer

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Breast tumors commonly harbor low mutational burden, low PD-L1 expression, defective antigen processing/presentation, and an immunosuppressive tumor microenvironment (TME). In a malignancy mostly refractory to checkpoint blockade, there is an unmet clinical need for novel combination approaches that increase tumor immune infiltration and tumor control. Preclinical data have guided the development of this clinical trial combining 1) BN-Brachyury (a poxvirus vaccine platform encoding the tumor associated antigen brachyury), 2) bintrafusp alfa (a bifunctional protein composed of the extracellular domain of the TGF-βRII receptor (TGFβ "trap") fused to a human IgG1 anti-PD-L1), 3), entinostat (a class I histone deacetylase inhibitor), and 4) T-DM1 (ado-trastuzumab emtansine, a standard of care antibody-drug conjugate targeting HER2). We hypothesize that this tetratherapy will induce a robust immune response against HER2$^+$ breast cancer with improved response rates through 1) expanding tumor antigen-specific effector T cells, natural killer cells, and immunostimulatory dendritic cells, 2) improving antigen presentation, and 3) decreasing inhibitory cytokines, regulatory T cells, and myeloid-derived suppressor cells. In an orthotopic HER2$^+$ murine breast cancer model, tetratherapy induced high levels of antigen-specific T cell responses, tumor CD8$^+$ T cell/Treg ratio, and augmented the presence of IFNγ- or TNFα-producing CD8$^+$ T cells and IFNγ/TNFα bifunctional CD8$^+$ T cells with increased cytokine production. Similar effects

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were observed in tumor $CD4^+$ effector T cells. Based on this data, a phase 1b clinical trial evaluating the stepwise addition of BN-Brachyury, bintrafusp alfa, T-DM1 and entinostat in advanced breast cancer was designed. Arm 1 (TNBC) receives BN-Brachyury + bintrafusp alfa. Arm 2 ($HER2^+$) receives T-DM1 + BN-Brachyury + bintrafusp alfa. After safety is established in Arm 2, Arm 3 ($HER2^+$) will receive T-DM1 + BN-Brachyury + bintrafusp alfa + entinostat. Reimaging will occur every 2 cycles (1 cycle = 21 days). Arms 2 and 3 undergo research biopsies at baseline and after 2 cycles to evaluate changes within the TME. Peripheral immune responses will be evaluated. Co-primary objectives are response rate and safety. All arms employ a safety assessment in the initial six patients and a 2-stage Simon design for clinical efficacy (Arm 1 if ≥ three responses of eight then expand to 13 patients; Arms 2 and 3 if ≥ four responses of 14 then expand to 19 patients per arm). Secondary objectives include progression-free survival and changes in tumor infiltrating lymphocytes. Exploratory analyses include changes in peripheral immune cells and cytokines. To our knowledge, the combination of a vaccine, an anti-PD-L1 antibody, entinostat, and T-DM1 has not been previously evaluated in the preclinical or clinical setting. This trial (NCT04296942) is open at the National Cancer Institute (Bethesda, MD).

**Keywords: metastatic breast cancer, bintrafusp alfa, entinostat, BN-Brachyury, TGF-β**

## INTRODUCTION

Breast cancer has historically been considered immunologically cold with most tumors having a relatively low mutational burden [mean 1.63 mutations per megabase (1)] and low programmed death-ligand 1 (PD-L1) expression (2, 3). In addition, it is hypothesized that the intrinsic resistance to immunotherapy observed in breast cancer may be due to low neoantigen levels, defective antigen presentation, and the presence of transforming growth factor beta (TGF-β) and other immunosuppressive signals in the tumor microenvironment (TME), collectively promoting exclusion of effector T cells and natural killer (NK) cells from the tumor (4–7). Together, these defects create an immunosuppressive TME that impedes the generation of an effective anti-tumor immune response as is reflected by the modest results observed with immune checkpoint blockade (ICB) monotherapy in breast cancer (3, 8, 9).

The FDA recently granted the first approved indication for ICB use in breast cancer, with the use of the anti-PD-L1 antibody atezolizumab in combination with nab-paclitaxel for patients with PD-$L1^+$ triple-negative breast cancer (TNBC) (10). In this new era, multimodal therapies may help shift the TME to a more immunopermissive environment, thus allowing proper engagement of the immune system to successfully eradicate the tumor.

Here, we present preclinical data and the resulting innovative clinical trial design for a phase 1b clinical trial that will evaluate safety and efficacy of combination immunotherapy through the stepwise addition of Bavarian Nordic (BN)-Brachyury, bintrafusp alfa, T-DM1, and entinostat in advanced breast cancer.

## RATIONALE FOR TETRATHERAPY COMBINATION IN ADVANCED BREAST CANCER

### BN-Brachyury

BN-Brachyury vaccine is a recombinant poxvirus vaccine against the transcription factor brachyury, a tumor-associated antigen that plays an important role in the epithelial-to-mesenchymal transition in breast cancer (11–13). BN-Brachyury is comprised of two replication incompetent recombinant viral vaccines, i.e., modified vaccinia Ankara (MVA-BN-Brachyury; prime) and fowlpox (FPV-Brachyury; boost). Clinical data with various brachyury vaccines have consistently demonstrated generation of a brachyury-specific T cell response in breast cancer patients (14, 15). A recently completed phase 1 study of BN-Brachyury prime-boost vaccines showed the vaccine was well tolerated and generated brachyury-specific immune responses in patients with advanced solid tumors (16).

**Abbreviations:** ACP, American College of Pathology; ADCC, Antibody-dependent cellular cytotoxicity; AE, Adverse event; BrEAsT, BN-Brachyury, Entinostat, Ado-trastuzumab Emtansine and M7824 in Advanced Stage Breast Cancer; cfDNA, Cell free DNA; CR, Complete response; CTCAE, Common Terminology Criteria for Adverse Events; DLT, Dose limiting toxicity; EBAT, entinostat, Bintrafusp alfa, Ad-Twist vaccine, and T-DM1; ER, Estrogen receptor; FISH, Fluorescence in situ hybridization; FPV, Fowlpox virus; HBV, hepatitis B virus; HDACi, Histone deacetylase inhibitor; HCV, hepatitis C virus; HIV, human immunodeficiency virus; ICB, Immune checkpoint blockade; IHC, Immunohistochemistry; i.p., Intraperitoneally; ITT, Intention to treat; i.v., Intravenously; MDSC, Myeloid-derived suppressor cell; MVA, Modified vaccinia Ankara; NK, Natural killer; NSCLC, Non-small cell lung cancer; ORR, Objective response rate; PD-1, Programmed cell death protein 1; PD-L1, Programmed death ligand 1; PFS, Progression-free survival; PR, Partial response; PR, Progesterone receptor; s.c., Subcutaneously; TGF-β, Transforming growth factor beta; TIL, Tumor infiltrating lymphocyte; TME, Tumor microenvironment; TNBC, Triple-negative breast cancer; Tregs, Regulatory T cells.

## Bintrafusp Alfa

Bintrafusp alfa (*previously designated M7824*) is a novel bifunctional fusion protein composed of a monoclonal antibody against human PD-L1 fused to the soluble extracellular domain of human TGF-β receptor II (TGF-βRII), which functions as a TGF-β "trap" (17–19). Preclinically, bintrafusp alfa has synergized with vaccines (20). Phase 1 studies have demonstrated an acceptable safety profile and have suggested some signs of clinical benefit (21, 22). A small pilot study of bintrafusp alfa in TNBC demonstrated a response rate of 9.1%, which is comparable to other ICB in unselected TNBC (22). Multiple phase II studies are ongoing with bintrafusp alfa in breast cancer (NCT03579472, NCT03524170) [clinicaltrials.gov].

## Entinostat

Entinostat is a class 1 histone deacetylase inhibitor (HDACi) that has been shown to increase sensitivity of breast cancer cells to antigen-specific $CD8^+$ T cell mediated lysis *in vitro* (23). This immunogenic modulation was observed against a broad range of tumor-associated antigens (TAAs) including brachyury and was associated with increased expression of antigen processing machinery and tumor immune recognition. HDACi have been shown to restore expression of MHC class I proteins and antigen processing and presentation molecules (23, 24). Entinostat exposure also increased the sensitivity of breast cancer cells (MDA-MB-231) to NK cell-mediated attack through direct lysis and *via* anti-PD-L1-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) (25). These effects were associated with increased expression of NK ligands on tumor cells and augmented NK activation and cytolytic function upon entinostat exposure *in vitro*. Furthermore, entinostat increased the expression of human PD-L1 in PC-3 (prostate) carcinoma xenografts *in vivo* (25). Others have also shown upregulation of PD-L1 on tumors with entinostat treatment (26), which has been attributed to the epigenetic modulation observed with HDACi (24). In addition, the combination of entinostat with nivolumab and ipilimumab in patients with advanced HER2 negative breast cancers demonstrated altered myeloid-derived suppressor cell (MDSC) signaling pathway and increased immune infiltration (27). Preliminary results of the ENCORE-601 trial involving entinostat with pembrolizumab in checkpoint refractory non-small cell lung cancer (NSCLC) demonstrated clinical efficacy in multiple patients regardless of prior checkpoint treatment or PD-L1 status (28). However, despite strong preclinical evidence regarding the combination of entinostat with ICBs, trials results have been inconsistent. In ENCORE-602 (29) and ENCORE-603 (30), entinostat was combined with an anti-PD-L1 antibody in patients with advanced TNBC (ENCORE-602) or advanced epithelial ovarian cancer (ENCORE-603). The combination did not improve clinical efficacy in these populations and a higher number of grade 3 and 4 treatment related adverse events (AEs) were seen in the combination arms, with the most frequent toxicities seen being neutropenia and fatigue (29, 30). However, with dose modifications, most patients were able to continue on therapy. In addition, there were no increases in immune-related adverse events in the combination arms over the anti-PD-L1 monotherapy arm.

## T-DM1

Ado-trastuzumab emtansine (T-DM1 or Kadcycla®) is an antibody-drug conjugate used in the second- and third-line treatment of metastatic $HER2^+$ breast cancer ($HER2^+$ BC). T-DM1 activates ADCC, dendritic cell maturation, and increases tumor infiltrating lymphocytes (TILs), PD-L1 expression, and immunomodulatory cytokines (31, 32). The KATE2 trial evaluated T-DM1 +/- atezolizumab. There was no significant improvement in the primary trial endpoint of progression-free survival (PFS) in the intention to treat (ITT) population. Exploratory analysis of overall survival data in the ITT population is still maturing but an interim report at ESMO 2019 showed that the median survival had not been met in either treatment arm. In addition, survival analysis stratified by PD-L1 expression demonstrated a trend towards longer survival in PD-L1+ patients (94.3% in PD-L1+ vs 87.9% in PD-L1-) but this was not significant (HR 0.55, 95% CI 0.22 to 1.38) (33, 34).

# PRECLINICAL DATA SUPPORTING TETRATHERAPY IN ADVANCED BREAST CANCER

Previously, Christmas et al. (35) demonstrated synergistic anti-tumor activity with the triple combination of entinostat, an anti-programmed cell death protein 1 (PD-1) antibody and a HER2-targeted antibody in HER2/neu transgenic breast cancer models, resulting in significant reduction in tumor size and improved survival. This was associated with reprogramming of granulocytic MDSCs in the TME to become less suppressive, increased $CD8^+$ effector T cells, and reduced regulatory T cells (Tregs) compared to single agent HER2-targeted therapy. Based upon this preliminary data along with the known immune effects and anti-tumor activity of BN-Brachyury (14, 16, 36), entinostat (23, 25, 35, 37), bintrafusp alfa (38–40), and T-DM1 (31, 32), we developed a preclinical hypothesis that the 4-agent combination with entinostat (E), bintrafusp alfa (B), Ad-Twist vaccine (A), and T-DM1 (T) (EBAT) would provide superior anti-tumor activity and immune effects in a breast cancer model (**Figure 1**) compared to the triplet (BAT), doublet (BA) or single agents (E, T, or B). To our knowledge, the combination of a vaccine, an anti-PD-L1 antibody, entinostat, and T-DM1 has not been previously evaluated in the preclinical or clinical setting.

To test this hypothesis, we examined the antitumor effects and immune correlates of the 4-agent combination therapy in the HER-2 expressing TuBO murine breast cancer model. Of note, since TuBO cells do not express brachyury, Twist was used as a model antigen. Twist is well-expressed in TuBO tumor cells and shares functional features with brachyury in multiple aspects of tumor progression. Similar to brachyury, Twist is a transcription factor that has also been implicated in the control of tumor plasticity and as a driver of metastatic progression (11, 13, 41, 42). Overexpression of either brachyury or Twist has been associated with poor prognosis for multiple carcinomas, including breast cancer (11, 43, 44). In preclinical studies, a vaccine targeting Twist demonstrated significant anti-tumor and anti-metastatic effects as a monotherapy (45).

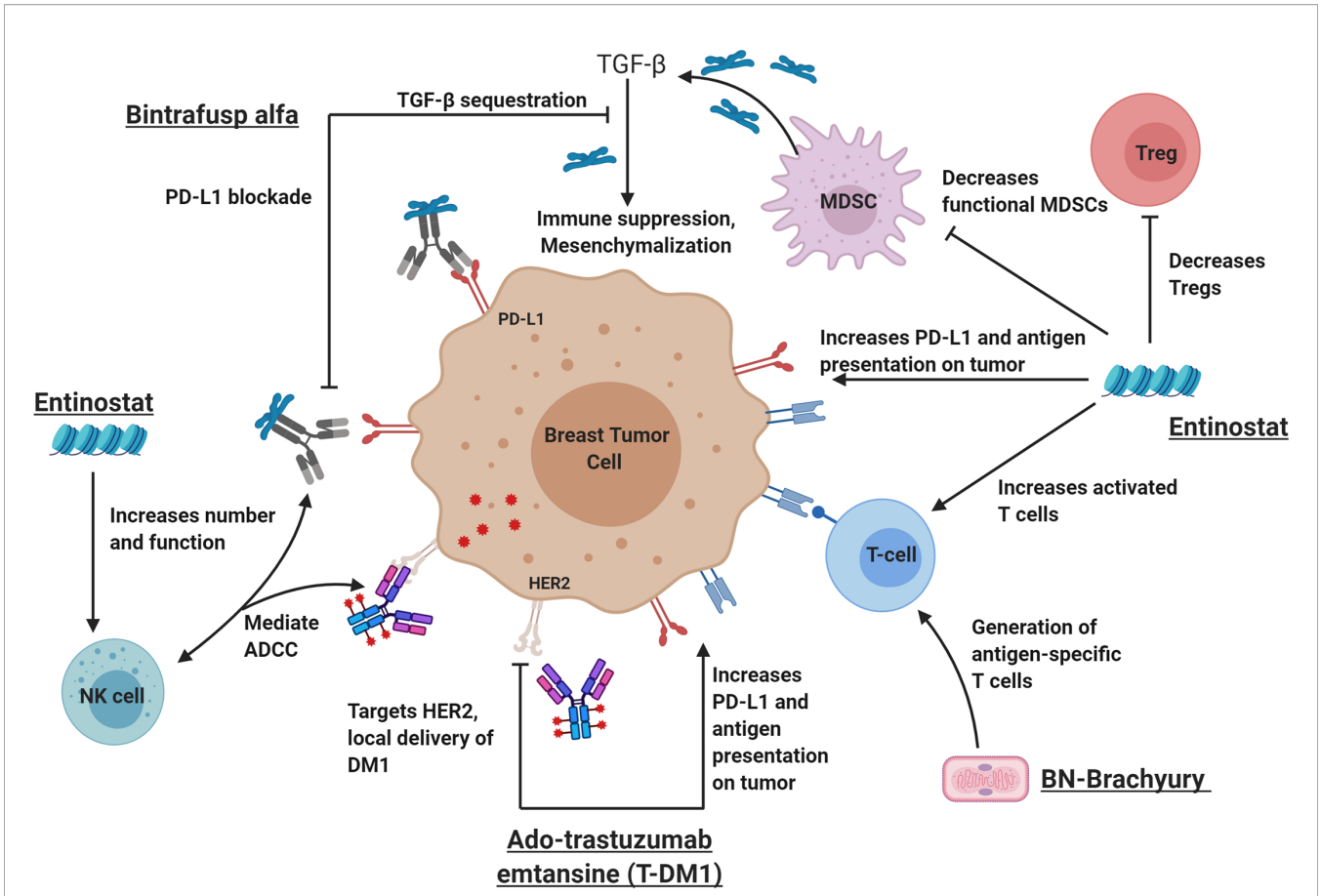


**FIGURE 1** | The rational combination of immunotherapy agents can augment key components of a successful anti-tumor immune response. From left to right: Bintrafusp alfa inhibits PD-L1 and sequesters TGF-β in the tumor microenvironment leading to activation of immune effector cells (including natural killer cells) and decreased tumor plasticity. Ado-trastuzumab emtansine (aka T-DM1l, Kadcyla) is an antibody drug conjugate that targets the HER2 receptor and allows the delivery of the anti-microtubule chemotherapy called DM1. Like other HER2-targeting antibodies, T-DM1 also mediates ADCC, increases antigen presentation on tumor cells and increases PD-L1 expression on the tumor and immune cells. BN-Brachyury vaccines generate brachyury-specific T cell responses in breast cancer. Brachyury is a transcription factor involved in tumor plasticity, metastasis, chemoresistance, and poor clinical outcomes in breast cancers. Entinostat is an HDACi that increases antigen presentation, increases activated T cells and decreases Tregs. In addition, entinostat is also known to decrease HER2 resistance through various mechanisms. It is hypothesized that this tetratherapy will induce a robust immune response against breast cancer with improved tumor control. ADCC, antibody-dependent cell-mediated cytotoxicity; HDACi, histone deacetylase inhibitor; MDSCs, myeloid-derived suppressor cells; NK cell, natural killer cell; PD-L1, programmed death-ligand 1; TGF-β, transforming growth factor beta. This figure was created by MG-M using BioRender.com.

TuBo murine breast tumor cells were orthotopically implanted subcutaneously (s.c.) into the mammary fat pad of female Balb/c mice. Tumor volume was measured twice weekly. When tumors reached 200mm$^3$, mice were randomized based on tumor size and treatment initiated as per the schematic (**Figure 2A**). Mice received entinostat (E) or control (C) chow. The Ad-Twist vaccine (A) was administered s.c. at a non-tumor site. Bintrafusp alfa (B) and T-DM1 (T) were administered intraperitoneally (i.p.) and intravenously (i.v.), respectively. Animals were monitored by the veterinarian staff for signs of toxicity, including weight loss, signs of pain, lethargy, or any abnormal behavior. No toxicity was reported in this study. Animals were sacrificed on day 34 and immune populations in the tumor were examined (**Figures 2D–K** and **Supplemental Figure S1**). As shown in **Figure 2**, combination therapy with entinostat, bintrafusp alfa, Ad-Twist vaccine, and T-DM1 (EBAT) induced the highest level of anti-tumor activity of all treatment groups (**Figures 2B, C**, and **Supplemental Figure S1**), with 100% of tumors regressing at day 32 (**Figure 2B** and **Supplemental Figure S1a**). While not statistically significant, there was a trend towards EBAT therapy enhancing CD8$^+$ T cell tumor infiltration (**Figure 2D**), but no change in the infiltration of Tregs (**Supplemental Figure S1b**). However, the ratio of CD8$^+$ T cells to CD4$^+$ Tregs (**Figure 2E**) was increased, suggesting a more immunopermissive TME. Furthermore, functional analyses revealed EBAT increased the functional activity of T cells. There was a significant increase in splenic Twist-specific CD8$^+$ T cell responses upon EBAT treatment compared to monotherapy, dual or triple agent regimens (**Figure 2F**). Analysis of tumor CD8$^+$ T cell responses (**Figures 2G–K**) upon T-cell receptor stimulation demonstrated that EBAT treatment induced the highest proportion of CD8$^+$ T cells producing IFNγ (**Figure 2G**) and TNFα (**Figure 2I**), as well as multifunctional IFNγ/TNFα double

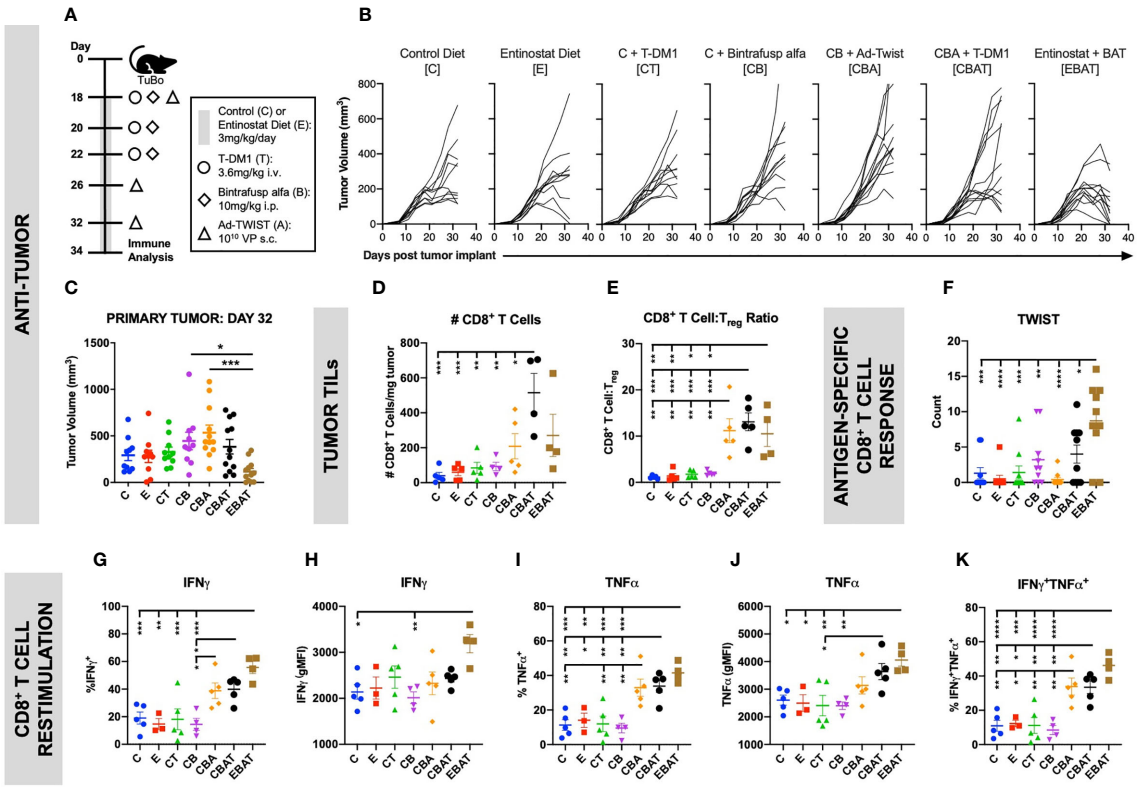


**FIGURE 2** | Combination therapy resulted in improved anti-tumor immune response in an orthotopic HER2$^+$ murine breast cancer model. TuBo breast tumor cells ($4\times10^5$) were orthotopically implanted into the mammary fat pad of female Balb/c mice on day 0 and treated with either control (C) or entinostat (E) diet, T-DM1 (T), bintrafusp alfa (B), and/or Ad-Twist vaccine (A) according to the schedule and doses in **(A)**. **(B, C)** Graphs show primary tumor growth curves of individual mice in each treatment group over the entire study duration **(B)**, and at day 32 **(C)**. For **(B, C)**, data from 1 experiment, n=11–12 mice/group. **(D–K)** Two days after the last vaccination, tumor, and spleen immune cells were isolated. Graphs show the number of CD8$^+$ T cells **(D)**, or the ratio of CD8$^+$ T cells to $T_{reg}$ **(E)** in the tumor. **(F)** Splenocytes were incubated with a Twist peptide pool overnight and IFNγ production was determined by ELISPOT. **(G–K)** Tumor CD8$^+$ T cells were stimulated with αCD3 and αCD28 for 4 h and cytokine production was analyzed by flow cytometry. Graphs show frequency of total IFNγ$^+$ **(G)**, IFNγ production on a per cell basis **(H)**, frequency of total TNFα$^+$ **(I)**, TNFα production on a per cell basis **(J)**, and frequency of IFNγ/TNFα-double producing **(K)** CD44$^{hi}$ CD8$^+$ T cells. For **(D–K)**, data from 1 experiment, n=3-5 mice/group. All graphs show mean ± SEM. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons. Statistical significance was set at p <0.05. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

positive T cells (**Figure 2K**). Furthermore, EBAT induced a significant increase over control treatment in IFNγ (**Figure 2H**) and TNFα (**Figure 2J**) expression on a per cell basis, which was not observed with monotherapy, dual or triple agent regimens. Similar results were observed in effector CD4$^+$ T cells (**Supplemental Figure S1c-g**). Collectively these results suggest that EBAT tetratherapy induces superior immune-mediated anti-tumor activity relative to monotherapy, dual or triple agent regimens in a preclinical model of HER2$^+$ breast cancer.

## CLINICAL TRIAL DESIGN

### Patient Eligibility

The BrEAsT (BN-Brachyury, Entinostat, Ado-trastuzumab emtansine, and Bintrafusp alfa) study enrolls patients (female and male) with histologically or cytologically confirmed metastatic TNBC or ER$^-$/PR$^-$/HER2$^+$ breast cancer. In this study, hormone receptor negative is defined by immunohistochemistry (IHC) as estrogen receptor (ER) < 10%, progesterone receptor (PR) < 10%, in order to include patients with low ER$^+$ tumors, where the clinical benefit of endocrine therapy is unclear (46). This relaxed definition is supported by the new 2020 American College of Pathology (ACP) guidelines (46) and by molecular analysis showing similar ER gene signature scores in tumors with ER < 1% (negative) and ER 1 to 9% (47). Furthermore, gene expression signatures in tumors with ER 1 to 9% demonstrated that 48% had gene expression signatures that were basal-like, with only 8% of these tumors being identified as luminal B (47). For Arm 1 (TNBC), HER2 negative or unamplified breast cancer is defined as IHC 0 or 1+ or IHC 2+ with FISH average HER2 copy number < 4.0 signals per cell or HER2/CEP17 < 2.0 with average HER2 copy number < 4.0 signals per cell. For Arms 2 and 3 (HER2$^+$), HER2 positivity is defined as HER2 amplified by IHC 3+ or FISH average with HER2 copy number ≥ 6 signals per cell or HER2/CEP17 ≥ 2.0.

Patients must be ≥ 18 years old with an Eastern Cooperative Oncology Group performance status of ≤ 1, adequate organ function including cardiac function (Ejection Fraction ≥ 50%) and bone marrow function. All patients must have measurable disease per RECISTv1.1 and HER2$^+$ patients must have biopsiable disease. At least 1 prior treatment in the metastatic setting is required. Patients with known PD-L1 positive TNBC (positive according to the SP142 assay) must have received prior treatment with atezolizumab + nab-paclitaxel. TNBC patients with ER 1%–9% must have received treatment with at least two lines of endocrine treatment (tamoxifen, aromatase inhibitor, or fulvestrant), with one line including a CDK4/6 inhibitor plus endocrine therapy for metastatic cancer and be considered endocrine therapy resistant. HER2$^+$ patients must have received prior treatment for metastatic disease with a taxane, trastuzumab, and pertuzumab (THP).

There is a required treatment washout period of 3 weeks for chemotherapy, 4 weeks for radiation, anti-PD-1/-L1 therapy, and other investigational agents. Asymptomatic or brain metastases treated > 6 weeks are allowed. Well controlled human immunodeficiency virus (HIV), hepatitis B virus (HBV) or treated hepatitis C virus (HCV) is allowed. Exclusion criteria include symptomatic brain metastases or clinically significant bleeding (≤ 3 months from study entry). Patients with active autoimmune conditions requiring immunosuppression are excluded. Concurrent use of immunosuppressive drugs including therapeutic prednisone is not allowed. Due to the potential for cardiac dysfunction and myocarditis, patients with a history of myocarditis are excluded.

Baseline screening assessments include a full history and physical exam, assessment of functional status, basic laboratory evaluations (complete blood count, comprehensive metabolic panel, coagulation studies, thyroid function), viral studies (HBV, HCV and HIV), cardiac evaluation with an electrocardiogram and a 2D echocardiogram and imaging studies (CT CAP and bone scan) to confirm measurable disease.

## Study Design

This is an open label, phase 1b trial with three arms to evaluate BN-Brachyury, entinostat, bintrafusp alfa +/- T-DM1 in advanced breast cancer (**Figure 3**). Arm 1 will evaluate BN-Brachyury and bintrafusp alfa in TNBC. Arms 2 and 3 will evaluate BN-Brachyury, bintrafusp alfa, T-DM1 +/- entinostat in ER$^-$/PR$^-$/HER2$^+$ breast cancer. If a patient is removed from treatment, that patient will not be allowed to enroll on another study arm. Up to 51 patients will be treated on this study with an accrual ceiling set at 55 patients.

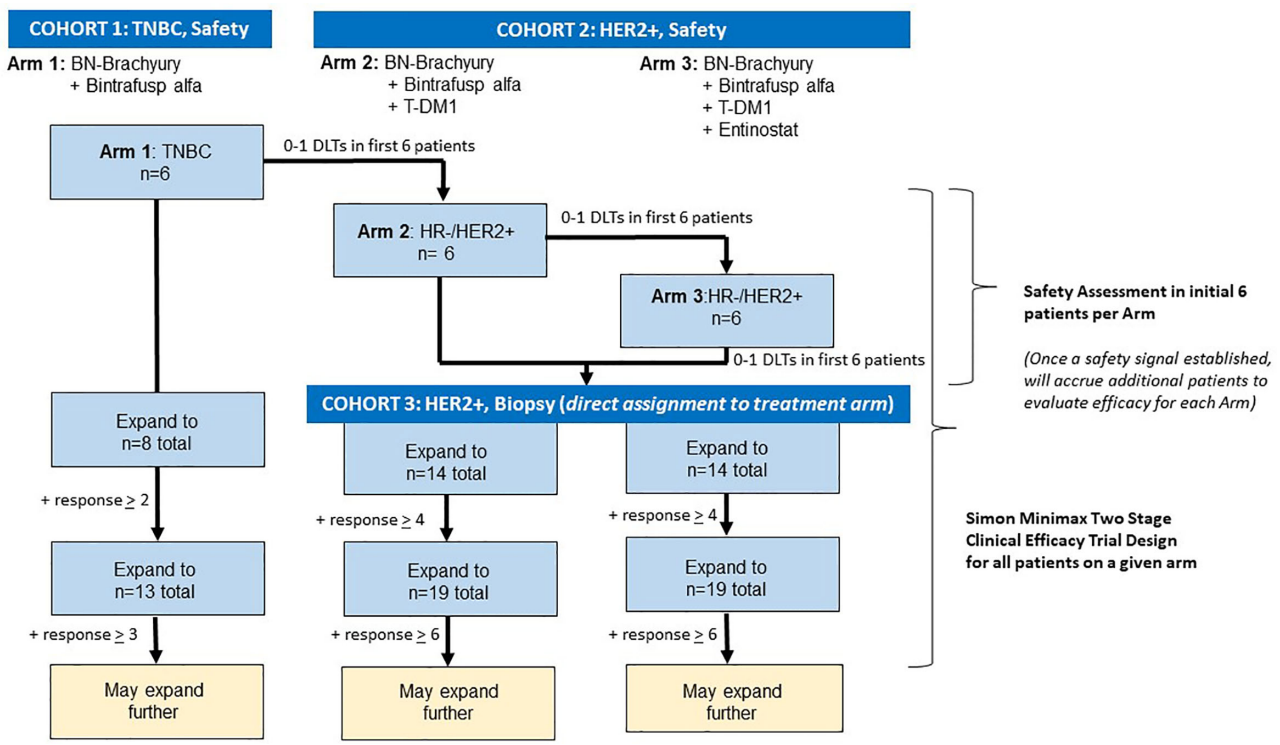


**FIGURE 3** | Trial schema. The BrEAsT study contains three separate, single arm phase 1b trials that evaluate the safety and efficacy of the sequential addition of immunotherapy agents with the goal of rapidly escalating to the 4-drug combination to lead to the best anti-tumor control and immune infiltration seen in the preclinical studies. Each arm starts with a safety assessment of the drug combinations with DLT assessments in the first six patients on each arm. Following the demonstration of safety, additional patients will be enrolled using a Simon 2-stage clinical efficacy design. DLT, dose limiting toxicity.

## Drug Administration

Treatment cycles are 21 days in length. Patients in Arm 1 will receive BN-Brachyury in a prime-boost fashion. The priming doses of MVA-BN-Brachyury consist of one injection in each extremity (four injections per priming dose) with each injection consisting of $2 \times 10^8$ infectious units (IU) per 0.5 ml administered s.c. on day 1 of cycles 1 and 2. The boosting dose of FPV-Brachyury consists of one injection s.c. with 1 x $10^9$ IU/0.5 ml every 3 weeks until cycle 9, then every 12 weeks. Bintrafusp alfa will be administered IV every 3 weeks using a 2,400 mg flat dose. T-DM1 is given at the standard dose of 3.6 mg/kg IV every 3 weeks. Patients will self-administer entinostat 5 mg by mouth every week while on trial (**Table 1**). Dose reductions for bintrafusp alfa (1,800 mg IV q3 weeks), entinostat (3 mg po q7 days, 2 mg po q7 days) and T-DM1 (per FDA package insert) are permitted. A prophylactic dose of a 5-HT3 antagonist or dopamine blocker will be given prior to T-DM1 and patients in Arm 3 will be prescribed an oral anti-emetic to self-administer as needed due to the known gastrointestinal side effects of entinostat.

During cycle 1, patients will return to the clinic on day 8 for a safety visit, which includes a physical exam, basic labs and repeat electrocardiogram. Starting with cycle 2, patients will be clinically assessed every 3 weeks and undergo evaluation with physical exams, functional status assessment and basic laboratory evaluations (CBC, CMP). Patients who receive T-DM1 will undergo a 2D echocardiogram every 84 days as recommended by current guidelines.

## Dose-Limiting Toxicity

Dose-limiting toxicities (DLTs) are based on the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE), version 5. A DLT is defined as an adverse event or abnormal laboratory value assessed as suspected to be trial treatment related (possible, probable or definite) and unrelated to disease or disease progression that occurs within the 30 days of the first treatment. Any grade ≥3 non-hematologic, non-hepatic adverse event will be considered a DLT with the following exceptions: nausea and/or vomiting or electrolyte imbalances that persist for 48 h despite supportive care. DLTs will also include grade ≥ 3 fatigue lasting ≥ 7 days, hematologic abnormalities (grade ≥ 4 neutropenia lasting ≥ 7 days, grade ≥ 3 febrile neutropenia, grade ≥ 4 thrombocytopenia), grade ≥ 3 bleeding events, and liver abnormalities (grade ≥ 4 elevation in AST or ALT, grade ≥ 4 elevation in bilirubin).

## Endpoints

### Safety Assessments

Up until the time the first three patients complete at least one cycle of treatment on each arm, accrual will proceed slowly (in Arm 1, no more than one patient per 6 days; in Arms 2 and 3, no more than one patient per 28 days) to more closely monitor safety, and after three patients receive at least 1 cycle of investigational agents, a safety review will be conducted before additional patients are enrolled. If there is 1 or more DLT in the first three patients on a given arm, accrual of the next three patients on the associated arm will proceed slowly with no more than one patient per 21 days (1 cycle) to continue to closely monitor safety. On the basis of the monitoring criteria, if two of the first two patients or if three patients experience DLT within the first six patients, then the trial will halt accrual and the treatment regimen may be modified. The review will include a per-patient listing of all reported AEs to date, including actions required for dosing, to more fully review the nature, frequency, severity, and timing of the events. This information combined with fewer DLTs may also result in modification of the treatment regimen. Throughout enrollment of all arms, DLTs within the 30 days of first treatment will be summarized with pre-specified criteria based on sequential boundaries to pause enrollment to more fully review safety if excessive numbers of DLTs are observed.

### Objective Responses

Patients will undergo restaging with CT chest, abdomen and pelvis +/- bone scan every 6 weeks (2 cycles). The co-primary endpoints of this study are safety and efficacy, with efficacy being

**TABLE 1** | Trial agents and dosing schedules*.

| Agent | Manufacturer | Dose | Arms |
|---|---|---|---|
| BN-MVA-Brachyury | Bavarian Nordic | **DL1:** 4 injections SC per dose q3 weeks x 2 doses (1 injection = $2x10^8$ Inf.U/0.5 ml) | 1, 2, 3 |
| FPV-Brachyury | Bavarian Nordic | **DL1:** 1 injection SC per dose q3 weeks x 4 doses then q12 weeks (1 injection = $1x10^9$ Inf.U) | 1, 2, 3 |
| Bintrafusp alfa | EMD Serono | **DL1:** 2400mg IV q3 weeks<br>**DL-1:**1,800 mg IV q3 weeks | 1, 2, 3 |
| T-DM1 | Standard of Care | **DL1:** 3.6mg/kg IV q3 weeks<br>**DL-1:** 3.0 mg/kg IV q3 weeks<br>**DL-2:** 2.4 mg/kg IV q3 weeks | 2, 3 |
| Entinostat | Syndax | **DL1:** 5 mg po q 7 days<br>**DL-1:** 3 mg po q7 days<br>**DL-2:** 2 mg po q7 days | 3 |

*1 Cycle = 21 days; q = every.*

**The stepwise addition of agents with continuous safety assessment will assist in determining any potential additive toxicities of the agent combination. Dose modifications for agents will be performed for toxicity based upon known treatment-related adverse events of each respective agent. One or more agents may be decreased or held at any time based upon high grade toxicity possibly/probably/definitely attributed to a specific trial drug or drugs. DL1, dose level 1; DL-1, dose level minus 1; DL-2, dose level minus 2.*

defined as an objective response in a measurable lesion as defined by RECIST version 1.1. Secondary endpoints of this study are PFS in all treatment arms and change in TILs in Arms 2 and 3.

### Correlative Studies

Peripheral blood samples for exploratory analysis will be collected on day 1 of cycles 1, 2, 3 and 6. Peripheral blood mononuclear cells will be evaluated for changes in immune cell subsets. A flow-based assay to interrogate over 123 peripheral immune cell subsets (48, 49) will be employed to detect any changes in different phenotypes of the following: $CD8^+$ T cells, $CD4^+$ T cells, Tregs, dendritic cells, B cells, NK cells, NKT cells, MDSCs, and total monocytes. In addition, the generation of brachyury-specific T cells will be analyzed using a flow-based assay (14–16) that simultaneously detects antigen-specific $CD8^+$ and $CD4^+$ cells, and multifunctional subsets of each; the assay is also non-MHC restricted. In patients who receive entinostat, histone acetylation will be evaluated as a surrogate marker for entinostat pharmacodynamics. Plasma and serum will be evaluated for pharmacokinetics, brachyury-specific antibodies, soluble factors including sCD27 and sCD40, TGF-β levels, and cell free DNA (cfDNA). Patients with $HER2^+$ breast cancer will undergo a tumor biopsy at baseline and after 2 cycles. Biopsy samples will be evaluated for changes in the immune microenvironment, HER2 expression, PD-L1 expression, tumor mutational burden, as well as epigenetic changes induced by the agent combination.

Materials and methods pertaining to preclinical studies are described in **Supplementary Materials**.

## Statistical Considerations and Plans

### Arm 1

In similar subjects with TNBC (unselected for PD-L1 expression) who received a checkpoint inhibitor, the objective response rate (ORR) was approximately 8%–10% (50–52). Recently presented data on the use of bintrafusp alfa in TNBC demonstrated a clinical response rate of 9.1% (22). The goal is to first determine if using BN-Brachyury plus bintrafusp alfa in a small pilot arm is safe and if this combination could improve this ORR by a modest amount in advanced breast cancer. Since a HER2 targeting agent is not included in this initial pilot arm, the patient population for BN-Brachyury plus bintrafusp alfa is limited to TNBC patients. Accrual of the first three patients on trial in this arm will be slow (no more than one patient per 6 days) in order to allow for monitoring of toxicity. If there are 0–1 patients with DLTs among the six patients enrolled on this arm, accrual to this arm will continue by using a Simon optimal 2-stage phase II trial design (53) to rule out an unacceptably low partial response (PR) + complete response (CR) rate of 10% ($p0 = 0.10$) in favor of an improved response rate of 35% ($p1 = 0.35$). With 1-sided alpha=0.10 (probability of accepting a poor treatment=0.10) and beta=0.20 (probability of rejecting a good treatment=0.20), the first stage will enroll eight evaluable subjects, and if 0 to 1 of the 8 have a clinical response, then no further subjects will be accrued. If 2 or more of the first 8 subjects have a response, then accrual would continue until a total of 13 evaluable subjects have been enrolled. If there are two subjects with a response out of 13 subjects, this would be an uninterestingly low response rate and the arm would not further expand. If there were 3 or more of 13 (23.1%) who experienced a response, this would be sufficiently interesting to warrant further study in later trials. If the response rate is 10% (unacceptable level), the probability of early termination after the first stage is 81.3%.

### Arms 2 and 3

Following determination of safety in Arm 1 (0–1 with DLTs among the first six patients enrolled), patients who are $HER2^+$ will undergo an initial safety evaluation and then will be assigned in an alternating fashion to the second and third arms. Of note, due to the small number of patients and the initial safety assessment in six patients per arm, randomization is not feasible since these six initial patients will also be used in the efficacy assessment. Provided that there are 0–1 patients with DLTs among the six patients enrolled on Arm 2, this arm will be temporarily closed, and patients will be enrolled on Arm 3 for a safety evaluation. Provided that there are 0–1 patients with DLTs among the six patients enrolled on Arm 3, patients with $HER2^+$ breast cancer will be directly allocated in an alternating fashion to Arms 2 and 3. In Arms 2 and 3, accrual of the first three patients in each arm will proceed slowly with no more than one patient enrolled every 28 days in order to allow for safety monitoring. If there is one or more DLTs in the first three patients on any arm, accrual of the next three patients on the associated arm will proceed slowly with no more than one patient per 21 days (one cycle) to closely monitor safety. If there are two or more patients with a DLT among the first six treated on either arm, the trial will halt accrual pending an amendment to detail how the study will proceed from that point forward.

Real world data from similar patients with metastatic $HER2^+$ breast cancer who received second-line T-DM1 after first-line treatment with THP demonstrated a response rate of 18% and a duration of treatment of 4 to 5 months (54, 55). The primary objective is to determine if either arm could improve upon the 18% response rate by a modest amount. Each arm will be conducted using a Simon minimax 2-stage phase II trial design (53) to rule out an unacceptably low PR+CR rate of 18% ($p0 = 0.18$) in favor of an improved response rate of 40% ($p1 = 0.40$). With 1-sided alpha=0.10 (probability of accepting a poor treatment=0.10) and beta=0.20 (probability of rejecting a good treatment=0.20), the first stage will enroll 14 evaluable subjects in each arm, and if 0 to 3 of the 14 have a clinical response, then no further subjects will be accrued. If 4 or more of the first 14 subjects have a response in an arm, then accrual would continue until a total of 19 evaluable subjects have been enrolled in that arm. If there were 6 or more of 19 (31.6%) who experience a response, this would be sufficiently interesting to warrant further study of that combination in later trials. If accrual ends to one arm because of insufficient activity, the other arm will remain open to enroll patients directly. If the response rate is 18% (unacceptable level), the probability of termination after the first stage is 76.5% in each arm. There will be no adjustment for the multiplicity of the three arms.

## DISCUSSION

We hypothesize that combining these four agents will lead to a robust immune response against $HR^-/HER2^+$ breast cancer with improved response rates when compared to historical controls. The immune effects of the standard of care therapy T-DM1 may be enhanced through combination with entinostat, bintrafusp alfa, and the BN-Brachyury vaccine. In a tumor that generally does not respond to checkpoint monotherapy, this combination of agents may help to augment the three key components of a successful anti-tumor immune response (3). Furthermore, the use of novel combination approaches is in keeping with the Cancer Moonshot Task Force's mandate, which called for the use of innovative strategies to rapidly translate new agents from bench to bedside. Rational combination of immune therapies based on preclinical data is a plausible strategy to achieve this aim and is especially warranted in treating patients who have exhausted most, if not all, therapeutic options. Enhancing immunity *via* several complementary mechanisms is a promising means to produce objective responses in an ever-increasing portion of patients who may benefit from immunotherapy.

While one of the primary objectives is response rate, we acknowledge that due to small numbers, this study is not powered to fully examine clinical efficacy even if the primary endpoint of response rate is met. If the co-primary objective of response rate is met, the trial agents would likely be evaluated further in a larger phase 2 clinical trial where clinical efficacy could be better assessed with ample power.

Since the preclinical data demonstrated the best anti-tumor activity with the 4-agent combination, this study was designed to allow for rapid escalation to the 4-agent regimen. However, since these agents have not been used in combination, we are required to demonstrate safety of the agent combinations while assessing the potential clinical impact of the respective agents. A Simon optimal design was used in Arm 1 (TNBC; bintrafusp alfa + BN-Brachyury) in order to minimize the sample size needed since preclinical data did not suggest significant improvement in tumor control with this doublet. A Simon minimax design was used in Arms 2 (HER2; bintrafusp alfa + BN-Brachyury + T-DM1) and 3 (HER2; bintrafusp alfa + BN-Brachyury + T-DM1 + entinostat) due to the need to generate informative data on clinical efficacy while limiting the number of patients who would be exposed to these agents in the event there is toxicity or even decreased efficacy of the active agents. Furthermore, due to the time it took to develop this novel trial and for the trial to progress through all of the scientific and regulatory assessments, statistics were based on the data available at the conception of the trial concept in 2017 and 2018. While more recently released response rates from the KATE2 study demonstrated ORR of around 40%–45% in both the T-DM1 arm and the T-DM1+atezolizumab arm, only half of the patients in the KATE2 trial had received prior pertuzumab (33). The specified thresholds in the BrEAsT trial to proceed to a phase 2 trial are not significantly different from the response rates documented in KATE2 and since prior treatment with pertuzumab is required, we would expect the ORR to be slightly lower than the T-DM1 arm from the KATE2 trial as has been documented (54, 55). If one or more arms of the trial were to advance to a phase 2 clinical trial, a more rigorous threshold for clinical efficacy would be employed as is the case with transition of most early clinical trials to larger phase 2 clinical trials.

Preclinical data presented here support this combination of agents and show that tetratherapy increases the functionality of $CD4^+$ and $CD8^+$ T cells in the TME, which is associated with augmented anti-tumor efficacy relative to the triplet, doublet or singlets. This trial design in which the safety and efficacy of various combinations of immunotherapy agents are able to be evaluated relatively quickly is just one in a series of quick efficacy seeking trials (QuEST) that are being conducted at the National Cancer Institute, National Institutes of Health (Bethesda, MD) (56). The BrEAsT trial is now open and accruing patients at the Center for Cancer Research at the National Cancer Institute, National Institutes of Health (NCT04296942).

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the National Institutes of Health (NIH) Intramural Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

The study hypothesis was designed primarily by MG-M, SRG, LMC, SS, SL, JS, and JLG. Preclinical data was performed by SG, YO, KK, KH, CP, RD, and CJ. All authors contributed to the design and drafting of the clinical trial protocol described in this manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.581801/full#supplementary-material

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REVIEW
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# The Crosstalk Between Tumor Cells and the Immune Microenvironment in Breast Cancer: Implications for Immunotherapy

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Breast cancer progression is a complex process controlled by genetic and epigenetic factors that coordinate the crosstalk between tumor cells and the components of tumor microenvironment (TME). Among those, the immune cells play a dual role during cancer onset and progression, as they can protect from tumor progression by killing immunogenic neoplastic cells, but in the meanwhile can also shape tumor immunogenicity, contributing to tumor escape. The complex interplay between cancer and the immune TME influences the outcome of immunotherapy and of many other anti-cancer therapies. Herein, we present an updated view of the pro- and anti-tumor activities of the main immune cell populations present in breast TME, such as T and NK cells, myeloid cells, innate lymphoid cells, mast cells and eosinophils, and of the underlying cytokine-, cell–cell contact- and microvesicle-based mechanisms. Moreover, current and novel therapeutic options that can revert the immunosuppressive activity of breast TME will be discussed. To this end, clinical trials assessing the efficacy of CAR-T and CAR-NK cells, cancer vaccination, immunogenic cell death-inducing chemotherapy, DNA methyl transferase and histone deacetylase inhibitors, cytokines or their inhibitors and other immunotherapies in breast cancer patients will be reviewed. The knowledge of the complex interplay that elapses between tumor and immune cells, and of the experimental therapies targeting it, would help to develop new combination treatments able to overcome tumor immune evasion mechanisms and optimize clinical benefit of current immunotherapies.

**Keywords: breast cancer, cancer immunotherapy, tumor microenvironment (TME), immune checkpoint inhibitors (ICI), immunosuppression**

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## INTRODUCTION

Breast cancer (BC) still represents the most frequent cancer in women and the second cause of cancer deaths worldwide (1). Treatment options have improved the outcome of BC patients, but still many patients progress to metastatic disease, which remains very difficult to cure. The failure of specific therapies may be ascribed to the fact that most anti-cancer drugs currently used mainly target cancer cells. Indeed, emerging evidence suggests that BC is not only composed of neoplastic cells but also of the tumor microenvironment (TME) consisting of different cell types, including endothelial cells, several stromal cell types, and immune cells. The cells composing the TME undergo a complex interplay with cancer cells through either cell–cell contacts or the production of extracellular matrix complexes and soluble factors that shape the microenvironment (2). The continuous and dynamic interaction between cancer cells and the TME can either promote or hinder cancer progression. In particular, tumor infiltrating immune cells protect from tumor progression by eliminating immunogenic neoplastic cells, but in the meanwhile they can contribute to tumor resistance to therapies, shaping tumor immunogenicity and selecting resistant tumor clones able to escape the immune response (3). Although BC was previously considered as a poor immunogenic cancer that does not respond to immunotherapies due to a low mutational burden (4), the notion of the role exerted by the immune system in BC progression has led to the application of this type of treatments also in this tumor. The introduction of immunotherapies improved the outcome of many BC patients, however, data from the clinics have underlined that it is strongly influenced by the composition of the immune TME. Indeed, immune cells have been implied in the development of resistance mechanisms to immunotherapy in BC, which hampers the establishment of durable responses, leading to disease progression (5).

Therefore, a deeper knowledge of BC TME and of the role that the different tumor infiltrating immune cell populations exert on cancer progression and response to therapies would allow the development of more effective treatments for BC. Furthermore, the identification of TME-related characteristics associated with a good or poor response to therapies would facilitate patient stratification and therapeutic decisions. In this light, in this paper we summarize the role exerted by the main immune cell populations present in the TME in BC progression, their influence on immunotherapies, and we discuss novel therapeutic strategies able to counteract the tumor-promoting activities of BC TME.

## MAJOR PLAYERS IN BC IMMUNE MICROENVIRONMENT

During the evolutionary history of a tumor, a complex and dynamic communication between tumor cells and the cells in the TME is established, shaping several tumor hallmarks such as sustained proliferative signaling, avoidance of immune destruction, induction of angiogenesis, and activation of invasion and metastasis (6). Importantly, different types of immune cells play specific roles, establishing a strong crosstalk network with cancer cells (**Figure 1**). In this sense, tumor immunoediting by innate and adaptive immune cell populations that together constitute the so-called Breast Cancer Immune Microenvironment (BCIM) is an important determinant of tumor progression. Immunoediting is a dynamic process that occurs in three steps, notably Elimination, Equilibrium, and Escape. The Elimination is the first step, also called immunosurveillance, in which transformed cells are destroyed by a competent immune system able to activate a strong immune response against cancer. During the Equilibrium phase, tumor cells that survived the Elimination phase and immune cells reciprocally shape each other. A balance is established between the tumor and the immune system with a selection pressure on tumor cells, which are genetically unstable and rapidly mutating. Tumor cell variants that have acquired resistance to elimination then enter the Escape phase, the final step of the process, when the tumor grows and becomes clinically apparent. The Escape phase is characterized by the progressive establishment of an immunosuppressive TME (7).

Based on the activity of the innate and adaptive immune cell populations involved in the immunoediting process, we can identify two major subclasses of immune cells: the immunosuppressive and the immunostimulating cells. Several lines of evidence have demonstrated that the presence of these cells within the BCIM significantly impacts on BC progression and treatment response. In particular, infiltration of tumors by immunostimulating immune cells such as some macrophages, lymphocytes, natural killer (NK) cells, innate lymphoid cells (ILCs), dendritic cells (DCs) and eosinophils is crucial for tumor control (8). The anti-cancer immune response generated by these cells is, however, inhibited by the action of immunosuppressive

**Abbreviations:** ADCC, Antibody-Dependent Cellular Cytotoxicity; APCs, Antigen-Presenting Cells; ARG1, Arginase 1; BC, Breast Cancer; BCIM, Breast Cancer Immune Microenvironment; bFGF, basic Fibroblast Growth Factor; CAR, Chimeric Antigen Receptor; CCL, CC-chemokine ligand; COX-2, Cyclooxygenase-2; CRTH2, Chemoattractant-homologous Receptor expressed on Th2 cells; CSCs, Cancer Stem Cells; CSF-IR, Colony-Stimulating Factor-1 Receptor; CSFs, Colony-stimulating factors; CTLA-4, Cytotoxic T Lymphocyte Antigen 4; CXCL, C-X-C-chemokine Ligand; DCs, Dendritic Cells; DFS, Disease Free Survival; ECP, Eosinophil Cationic Protein; EDN, Eosinophil-Derived Neurotoxin; EMT, Epithelial–Mesenchymal Transition; EPX, Eosinophil Peroxide; Evs, Extracellular Vesicles; G–, Granulocyte; GM–, Granulocyte-macrophage; HMGB1, High-Mobility Group Box 1 protein; ICD, Immunogenic Cell Death; ICI, Immune Checkpoint Inhibitor; Ics, Immune Checkpoints; IFN, Interferon; IL, Interleukin; ILCs, Innate Lymphoid Cells; IMCs, Immature Myeloid Cells; LAG-3, Lymphocyte activation gene-3; M, macrophage; MBP, Major Basic Protein; MCs, Mast cells; MDSCs, Myeloid-Derived Suppressor Cells; M-DSCs, Monocytic MDSCs; MHC, Major Histocompatibility Complex; MMPs, Matrix metalloproteinase; MUC, Mucin; NGF, Nerve Growth Factor; NK, Natural Killer; NO, Nitric Oxide; OS, Overall Survival; PD-1, Programmed Death 1; PDGF, Platelet-Derived Growth Factor; PG, Prostaglandin; PGD2, Prostaglandin D2; PlGF, Placental Growth Factor; PMN-MDSCs, Polymorphonuclear MDSCs; PNT, Peroxynitrite; RNS, Reactive Nitrogen Species; ROS, Reactive Oxygen Species; SCF, Stem Cell Factor; TAAs, Tumor Associated Antigens; TAMs, Tumor Associated Macrophages; TCR, T Cell Receptor; TGFβ, Transforming Growth Factor-β; TIGIT, T cell immunoglobulin and ITIM domain; TILs, Tumor Infiltrating Lymphocytes; TIM-3, T-cell Immunoglobulin and Mucin domain-containing molecule 3; TME, Tumor Microenvironment; TNBC, TripleNegative Breast Cancer; Tregs, Regulatory T Cells, VEGF, Vascular Endothelial Growth Factor.

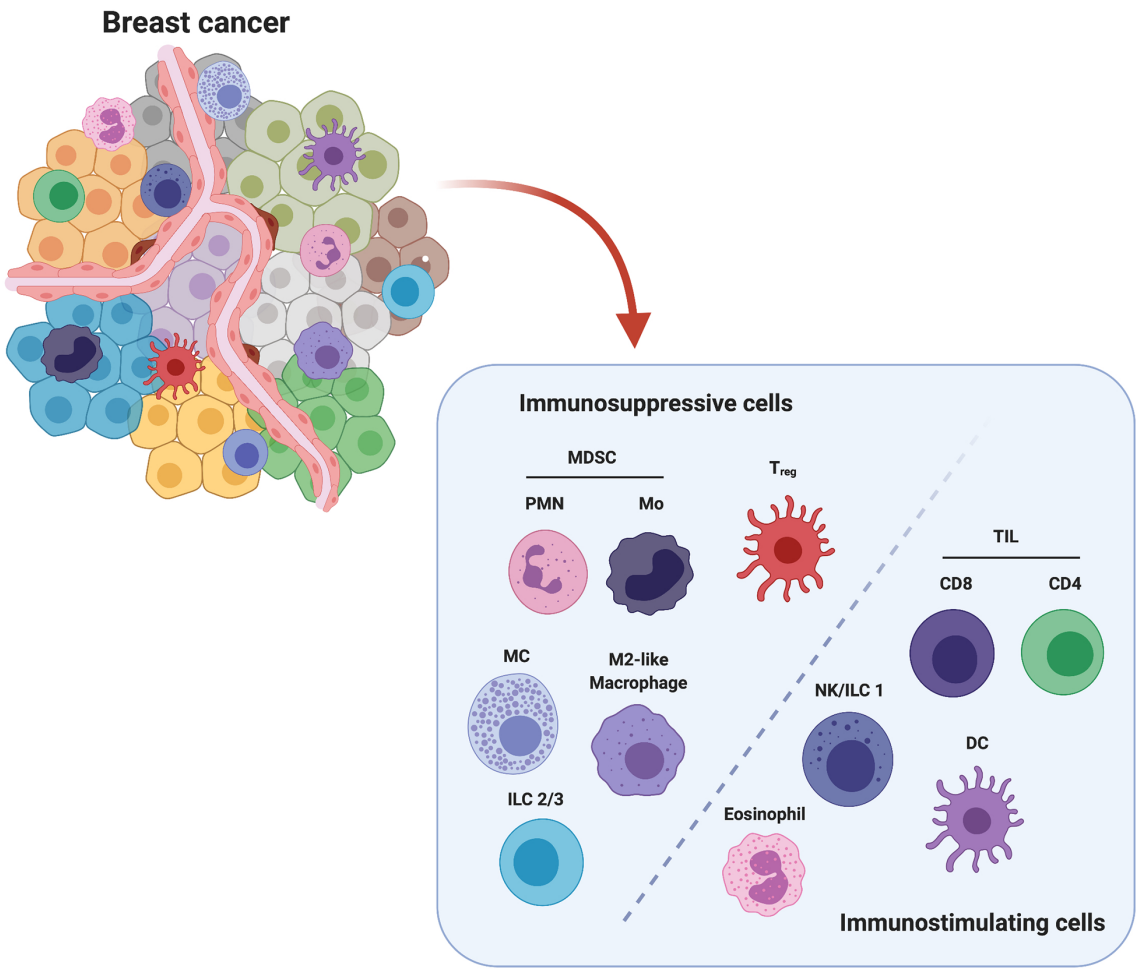


**FIGURE 1** | Major players in immune breast TME. Among all cell populations present in breast TME, polymorphonuclear (PMN) and monocytic (Mo) Myeloid-Derived Suppressor Cells (MDSCs), Mast Cells (MCs), Innate Lymphoid Cells Type 2 and 3 (ILC2/3), M2-like Tumor Associated Macrophages (TAMs) and FoxP3$^+$ regulatory T cells (Treg) are considered to exert an immunosuppressive action, while Tumor Infiltrating CD4$^+$ and CD8$^+$ Lymphocytes (TILs), Natural killer (NK) cells/Innate Lymphoid Cells Type 1 (ILC1), Dendritic cells (DCs) and Eosinophils are associated with an anti-tumor activity. Created with BioRender.com.

cells, such as myeloid-derived suppressor cells (MDSCs), mast cells (MCs), regulatory T cells (Tregs), and type 2-polarized tumor-associated macrophages (M2-like TAMs), which are intrinsically associated with the developing TME (9).

Here, we briefly describe the major immune subpopulations present in BCIM, with a particular attention to their impact on BC patient's prognosis and to their influence on the response to current immunotherapies. In addition, we review the state of the art of the therapeutic strategies aiming at reverting immunosuppression in order to potentiate anti-cancer immune responses.

## Immunosuppressive Cells

### Myeloid-Derived Suppressor Cells

MDSCs are a heterogeneous population of progenitors and precursors of myeloid cells. The molecular mechanisms behind their generation and their true origins are still debated, and different theories proposed. Upon an increased demand for myeloid cells, immature myeloid cells (IMCs) can undergo a process known as emergency myelopoiesis, expanding in the bone marrow and migrating into the periphery. Or else, IMCs may also expand and become functionally active MDSCs extramedullary (in organs such as spleen) (10). Conversely, in pathologic conditions such as cancer, several cytokines, chemokines and factors, such as for example granulocytic-colony stimulating factor (G-CSF) (11), C-X-C-chemokine ligand (CXCL)2, CC-chemokine ligand (CCL)2, CCL5 (12) CXCL5, and CXCL12 (13) (see below *Cytokine and Soluble Factors-Mediated Mechanisms*) secreted by the tumor cause the block of their differentiation as well as their mobilization from the bone marrow and accumulation into the primary and secondary neoplastic lesions (10). Based on the different cell surface antigen expressions, two subsets of MDSCs have been identified: polymorphonuclear or granulocytic MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). In mice, the PMN-MDSCs and M-MDSCs are identified by a CD11b$^+$Ly6G$^+$Ly6C$^{low}$ and a CD11b$^+$Ly6G$^-$Ly6C$^{high}$ phenotype, respectively, whereas, in humans, PMN-MDSCs are CD11b$^+$CD14$^-$CD15$^+$CD33$^+$ cells,

and M-MDSCs are $CD11b^{+}CD14^{+}$ $CD15^{-}CD33^{+}HLA^{-}DR^{-/low}$ cells. Other hypotheses suggest that M-MDSCs and PMN-MDSCs may represent reprogrammed or activated monocytes and granulocytes (10). Nowadays, it is widely accepted that these IMCs, through the secretion of several soluble factors as well as the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (see below), are able to induce severe anergy of effector immune cells, to recruit Tregs and to promote the M2-like TAM polarization, thus generating a strong immunosuppressive TME. In particular, MDSCs are able to recruit Tregs at the tumor site throughout the expression on their membrane of the immune stimulatory receptor CD40. The same receptor is exploited by MDSCs to directly inhibit T-cell proliferation by its binding with the ligand CD40L expressed on T-cell plasma membrane (14, 15). Recently, MDSCs have also been associated with the formation of the pre-metastatic niche, to the stimulation of angiogenesis and the maintenance of cancer stem cells (CSCs), a small population of cells responsible for tumor initiation and metastases (16–18). Several studies have shown that MDSCs are associated with poor prognosis in BC patients. Notably, Kumar et al. reported that MDSCs are more enriched in triple-negative BC (TNBC) patient samples compared to non-TNBC (19), and high levels of circulating MDSCs significantly correlate to liver and bone metastases and higher levels of circulating tumor cells (20). In summary, many lines of evidence suggest that MDSCs play a detrimental role in BC progression.

## Mast Cells

MCs are innate immune cells characterized by their cargo of inflammatory mediators stored in cytoplasmic granules, which are released upon encountering the appropriate stimuli, such as IgE, that play a central role in allergic diseases (21). MC degranulation is known to have beneficial roles in response against pathogens, such as helminths, bacteria, and viruses.

They are distributed in diverse tissues throughout the body and, like other immune cells, originate into the bone marrow from the hematopoietic stem cell progenitor which can become a committed MC progenitor that through the bloodstream migrates to peripheral tissues to complete maturation (22). Their differentiation, growth, and survival are strongly regulated by tissue microenvironmental factors, of which stem cell factor (SCF), the ligand of the c-Kit receptor, and interleukin (IL)-3 are the best-characterized (23).

Interestingly, other endogenous factors such as IL-4, IL-6, IL-9, IL-10, IL-33, nerve growth factor (NGF), and transforming growth factor $\beta$ (TGF-$\beta$) contribute to MC maturation and function (22). Inside the tumor, MCs are able to suppress the anti-tumor immune response by inducing an adenosine-mediated immunosuppressive crosstalk with MDSCs and Tregs and by limiting the adaptive immunity through IL-13 secretion (24, 25). However, the influence of MCs in BC prognosis is still much debated. MCs, through the secretion of the great variety of bioactive components contained inside the cytoplasmic granules, may exert both pro- and anti-tumor effects. In particular, *in vitro* and *in vivo* studies indicate that MCs exhibit a pro-tumor activity through the promotion of lymphatic and blood vessel formation, tumor growth, and metastasis (26). On the other hand, Samoszuk et al. demonstrated that depletion of MCs with imatinib enhanced tumor growth in a murine model of BC, supporting MC anti-tumoral role (27). Another study associates MCs with a greater survival and favorable prognosis (28). Consistently, Rajput et al. reported that in a cohort of 4.444 invasive BC patients with a long term follow-up, stromal MCs correlate with a good prognosis (29).

## M2-Like Tumor Associated Macrophages

Macrophages are terminally differentiated myeloid cells which are responsible for the elimination of infectious agents and the regulation of adaptive immunity. For many years, macrophage biological origin was attributed to bone marrow-derived progenitors and blood monocyte intermediates that differentiate into mature cells once seeded into organs (30). However, several genetic tracing data revealed that multiple macrophage populations develop from embryonic progenitors and are able to self-renew by local proliferation of mature, differentiated cells. Each tissue microenvironment has been demonstrated to influence macrophage morphological and functional characteristics (31). Based on their functional role, macrophages have been classified in two different subtypes: anti-tumoral M1-like and pro-tumoral M2-like polarized TAMs (32). In mice, both M1- and M2-like TAMs are characterized by the expression of markers such as CD11b, F4/80 and colony-stimulating factor-1 receptor (CSF-1R) and low levels of expression of the myeloid differentiation marker Gr1, whereas major histocompatibility complex (MHC) class II glycoproteins and CD206 are used to distinguish between M1- and M2-like TAMs, respectively. In humans, macrophages are identified by the expression of CD68, CD312, CD115, and other markers. However, it is important to note that TAM phenotypes are much more complex and categorizing them into binary states is not completely correct (33). Several data indicate that the pro-tumoral M2-like TAMs within the BCIM play pivotal roles in promoting tumorigenesis and metastasis formation *via* both non-immune and immune related mechanisms. The non-immune role of TAMs consists in the release of numerous angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF), that stimulate angiogenesis within the tumor, as well as in the secretion of many signaling molecules, including EGF, matix metalloproteinases (MMPs), CCL2, CCL18, and macrophage (M)-CSF that consequently activate tumor cell epithelial–mesenchymal transition (EMT), invasion, and metastasis (34, 35). The pro-tumoral M2-like TAM infiltration contributes to establish an immunosuppressive microenvironment. For example, it has been reported that M2-like TAMs, through the secretion of TGF-$\beta$, as well as IL-10, suppress $CD8^{+}$ T cell functions by direct transcriptional repression of genes encoding functional mediators, such as perforins, granzymes, and cytotoxins (34, 36). Moreover, in virtue of their high expression levels of enzymes such as arginase 1 (ARG1) and indoleamine 2,3-dioxygenase 1, M2 TAMs deplete the TME of the amino acids arginine and tryptophan, which are essential for T and NK cell proliferation and survival (35) (see below). Several studies demonstrated that

M2-TAMs are a poor prognostic factor in BC (37–39). In particular, M2-TAMs promote tumor growth by facilitating immunosuppression, angiogenesis, and inflammation, and can also promote tumor recurrence after conventional therapies (30, 39). Consistently, CSF1-expressing TAMs are associated with more aggressive tumors, in a cohort of 47 BC patients (33). Moreover, signatures of M2-like TAM infiltration correlate with a poor prognosis in luminal and triple negative subgroups of BC (40).

### FoxP3$^+$ Regulatory T Cells

Tregs are a distinct specialized subpopulation of T cells that act to suppress immune response. Tregs represent half of the $CD4^+CD25^+$ T cell population. In addition, a small number of $CD8^+FoxP3^+$ Tregs have also been identified in a large cohort of BC patients (41). Physiologically, Tregs are involved in the regulation of T and B lymphocyte activation as well as in the homeostasis of cytotoxic lymphocytes (9, 42). The normal thymus produces FoxP3-expressing $CD25^+CD4^+$ Tregs. In addition to these naturally occurring Tregs, some naive $CD25^-CD4^+$ T cells may also differentiate to Tregs in the periphery (43). Tregs are also involved in a broad spectrum of pathologies such as autoimmunity, allograft rejection, and hypersensitivity. Their role in immunosuppression is indisputable since they can disrupt the host immune response through a multitude of mechanisms involving cell–cell contacts and the production of immunosuppressive cytokines and metabolites, thus sustaining tumor progression and aggressiveness. Tregs appear to have a major role in disrupting the immune control of cancers and are therefore associated with worse patient outcome (44).

Higher numbers of Tregs in the peripheral blood of BC patients compared with healthy controls have been reported, and their ability to infiltrate tumors increases with tumor stage and correlates with poor prognosis in invasive BCs (41, 45). Tregs are recruited in the TME by several chemokines and cytokines produced by tumor cells, cancer associated fibroblasts or immunosuppressive cells. CXCL12 is one of the main factors that induce Treg recruitment. Interestingly, the expression of CXCL12 and its receptor CXCR4 is increased by hypoxia, which could further promote Treg infiltration in breast tumors, especially in the basal-like subtype (46). Related to the different BC subtypes, it has been described that Treg infiltration signature is associated with poor prognosis in luminal, triple negative and $HER2^+$ BC. Interestingly, Peng et al. also reported that, in a cohort of 122 patients with primary invasive ductal BC, patients with a low $FoxP3^+/CD8^+$ ratio showed a higher disease free survival (DFS) than patients with an higher $FoxP3^+/CD8^+$ ratio (47). Moreover, the depletion of Tregs in advanced primary tumors induces a strong $CD4^+$ T cell and interferon (IFN)$\gamma$-dependent anti-tumor response (45). In particular, the interferon (IFN) $\gamma$ derived from the $CD4^+$ cells, but not from the $CD8^+$ and NK cells, is responsible for the tumoricidal effects after Treg depletion in PyMT breast carcinomas (48).

## Anti-Tumor Immune Cells

### Tumor Infiltrating T cells

TILs include all the cells with a lymphocytic nature infiltrating the tumor tissues. Of particular interest are cytotoxic ($CD8^+$) and helper ($CD4^+$) T-lymphocytes (49) that constitute an essential part of the adaptive immunity. $CD8^+$ T-lymphocytes are the major effector cells involved in tumor elimination by recognizing tumor-associated- and neo-antigens presented by MHC class I (47). $CD4^+$ T cells can support and help the $CD8^+$ T population during the anti-tumor response *via* the secretion of a wide range of effector cytokines. In general, TIL abundance in tumors is fundamental for the establishment of an important immune response against cancer. Indeed, a huge literature is consistent with a positive correlation between TILs and good prognosis of BC patients. For example, an increased number of TILs positively correlates with increased DFS and overall survival (OS) in both TNBC and HER2-positive BC patients treated with neoadjuvant chemotherapy. Surprisingly, this correlation is completely lost in luminal A tumors (50). Further studies are needed to elucidate the underlying mechanisms, which might be related to the effects of the endocrine therapy on the immune system in Luminal A patients.

### Natural Killer Cells

NK cells derive from a common lymphoid progenitor into the bone marrow and then spread to primary and secondary lymphoid tissues, as well as within non-lymphoid tissues including the lungs, liver, and the peripheral blood (51, 52). Phenotypically, they are identified as $CD3^-NK1.1^+$ in mice, while in humans two main subsets exist: cytotoxic $CD56^{dim}CD16^+$ cells and cytokine-producing $CD56^{bright}CD16^-$ cells (51). In both mice and humans, NK cells can be divided in four subsets, corresponding to different maturation stages, based on the expression of CD27 and CD11b surface markers. Immature NK cells do not express the two markers. During maturation, they acquire CD27 expression and then CD11b, while fully mature NK present in peripheral blood are nearly all $CD11b^+$ $CD27^-$. These different phenotypes correspond to different cell functions, with $CD27^+$ cells showing the best ability to secrete cytokines, and $CD11b^+$ $CD27^-$ displaying high cytolytic function (53, 54). NK cells play an important role in cancer immunosurveillance, eliminating a variety of transformed cells through the release of cytolytic granules containing perforins and granzymes. Differently from T-lymphocytes, NK cells participate in the innate immunity and can recognize and kill altered cells without prior sensitization. Moreover, NK cells recognize and eliminate cells that do not express MHC class I, a mechanism that many cancer cells, and BC CSCs in particular, exploit to escape from T cell-mediated cytotoxicity (55, 56). For these reasons, NK cells are the most effective immune cell subpopulation to control and eventually eliminate abnormal cells. However, in BC and several other solid cancer types, tumor infiltrating NK cells display a $CD56^{bright}CD16^-$ phenotype and secrete invasion-associated enzymes such as MMP9 and, similarly to decidual NK cells, exert pro-angiogenic functions through the secretion of VEGF and angiogenin (57, 58). VEGF induces tumor vessel growth and exerts immunosuppressive functions, promoting the proliferation of immunosuppressive cells, limiting T-cell recruitment and enhancing T-cell exhaustion (59). This shift in NK cell function may be induced by several factors present in the breast TME, as previously described for lung cancer, where TGF-$\beta$, adenosine,

and prostaglandins downregulate NK activating receptors and induce the production of VEGF and placental growth factor (PIGF) that promote cancer progression (55, 60, 61). Interestingly, the balance between pro- and anti-tumor activity exerted by NK cells differs in the different BC subtypes. Indeed, a strong presence of NK cells that in turn is associated with a good prognosis has been found in $ER^+$ and $HER2^+$ BC patients, while NK cell infiltration correlates with poor prognosis in TNBC patients (40).

## Innate Lymphoid Cells

ILCs are immune cells deriving from the common lymphoid progenitor and belong to the innate counterparts of T cells. In effect, ILCs have been proposed as the evolutionary precursors of T cells that do not express antigen-specific receptors (62). They are tissue resident cells extremely rare in the peripheral blood (63, 64), able to detect changes in the local microenvironment through receptors for cytokines that are released during tissue damage, and to trigger the adaptive immunity (65). Based on their hallmarks, such as their cytokine signature and phenotype, ILCs are divided into three major groups: ILC1s, ILC2s, and ILC3s, even if two additional immune cell types, NK cells and lymphoid tissue inducer cells, are also included in the ILC family (66).

In response to IL-12, IL-15, and IL-18, ILC1s secrete IFN$\gamma$ that is extremely important to induce macrophages and DCs to eliminate bacteria and to present antigens. ILC2s secrete type-2 cytokines such as IL-5, IL-9, IL-13, and amphiregulin, which on one hand are involved in the expulsion of helminths and in helping to repair the damaged tissues, while on the other hand are able to enhance Treg functions and thus immunosuppression (67). ILC3s, instead, produce IL-22 and IL-17 that are able to stimulate the secretion of antimicrobial peptides and mucus by epithelial and goblet cells, respectively (68, 69).

Like NK cells, ILC1s are dependent on IL-15 and exhibit potent cytotoxic activities against tumor cells, limiting tumor growth in mammary preclinical model (70, 71). In BC, Irshad et al. identified an interesting mechanism through which ILC3s, together with stromal cells, are able to promote lymphatic metastasis by modulating the local chemokine milieu. In particular, in a preclinical mouse model of TNBC, CCL21-dependent ILC3 recruitment into the primary tumor stimulates CXCL13 production by the stromal cells, which in turn promotes the production of the cancer cell motile factor RANKL that induces cell migration (72). Moreover, in BC an enrichment of ILC2s in tumors compared to healthy tissue was observed, and IL-33 administration in 4T1 BC cell model accelerates tumor growth and the development of lung and liver metastases, which is associated with increased intratumoral infiltration of ILCs, MDSCs and Tregs (73, 74). However, the real contribution of ILCs in cancer disease is still a matter of debate. Whether the enrichment of ILCs into the tumor site results from newly recruited cells or from local *in situ* proliferation is another open question.

## Dendritic Cells

DCs are specialized antigen-presenting cells able to orchestrate an efficient anti-tumor immunity as well as to participate in the immune tolerance. Mouse and human conventional DCs derive from common DC precursors in the bone marrow. There are two main subsets of DCs, monocytic DCs (mDCs) that are generally $CD11c^+$, and plasmacytoid DCs (pDCs) (75, 76). DCs induce an efficient T lymphocyte activation and anti-tumor immune response stimulation through the process of antigen presentation on MHC class I and II molecules to T-lymphocytes, as well as by producing immunomodulatory signals through cell–cell contacts and soluble factors (77).

DCs have been found in many cancer types, including BC, where they are poorly activated and often dysfunctional, since the TME promotes their production of IL-10 and TGF-$\beta$, which contribute to the expansion of Tregs (77). Moreover, an increase of DCs has been observed in the peripheral blood of BC patients, with higher levels in HER2-positive BC patients compared to HER2 negative ones, suggesting differences between the different BC subtypes (78). However, the prognostic role of DCs in patients remains unclear, likely due to their heterogeneous composition that comprises cells at different maturation stages. In a recent study about metastatic BC, Holsbø and Olsen analyzed gene expression profiles in patient blood samples and examined genes and gene sets associated with risk of BC metastasis. Among the top genes, pDC-related genes and processes were identified (79). This was in line with another study, in which pDC infiltration in primary localized BC correlates with an adverse outcome, suggesting their contribution in tumor progression (80). On the other hand, Bailur and colleagues' results suggest a positive association between circulating pDCs and BC survival (76, 80). Similarly, the presence of $CD83^+$ mature intratumor DCs strongly associated with better patient survival in node-positive tumors (81), and $CD11c^+$ mDCs positively correlated with T cell infiltration and OS in TNBC patients (82). Moreover, different subsets of DCs can have different correlations with therapeutic response in BC patients. Indeed, a significant increase of DCs in the blood was noted in BC patients whose tumors showed a good pathological response following neoadjuvant capecitabine and docetaxel preceded by adriamycin and cyclophosphamide regimens. However the presence of a decreased amount of intratumoral $CD1a^+$ DCs did not show any significant correlation with response to therapy, in both primary breast tumors and metastatic axillary lymph nodes (83, 84).

## Eosinophils

Eosinophils are innate immune cells involved in the protective immune response of the host against helminthes (85), viral (86) and microbial pathogens (87). Human eosinophils derive from $CD34^+CD117^+$ pluripotent hematopoietic stem cells in the bone marrow, where they complete their maturation and subsequently enter into the bloodstream (88).

Phenotypically, eosinophils are characterized as $CD11b^+Gr\text{-}1^{lo}F4/80^+$ cells. These markers are also found on macrophages, but eosinophils can be distinguished due to their high granularity, lack of expression of MHC-II and expression of the sialic acid-binding lectin Siglec-F (89).

Eosinophils are recruited from the blood into the sites of inflammation where, upon activation, they can release an array of inflammatory mediators such as for example cationic proteins

(major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxide (EPX), and eosinophil-derived neurotoxin (EDN)) that are unique to eosinophils and are important in the defense against parasitic infections (90). Noteworthy, IL-5 together with IL-3 and GM-CSF, is crucial for supporting the maturation of human eosinophils in the bone marrow (91) and mediates their survival by NF-κB-induced Bcl-xL, which inhibits apoptosis.

Evidence indicates the presence of eosinophils in the TME of several human hematological and solid tumors, including BC, even if the mechanisms responsible of the eosinophil infiltration into the tumors are not completely known (92–94). However, some data show that the high-mobility group box 1 protein (HMGB1), IL-1α, and IL-33 potentially trigger eosinophil recruitment (95). Moreover, macrophages and MCs can recruit eosinophil *via* the production of VEGFs (96, 97) and/or the release of histamine and prostaglandin $D_2$ (PGD2) through the activation of the chemoattractant-homologous receptor expressed on Th2 cells (CRTH2) (98) and H4 receptor (99), respectively.

Into the TME, eosinophils influence other leukocytes, such as T cells, NK cells, DCs and macrophages. In particular, they are able to recruit and activate T cells through CXCL9, CXCL10, and CCL5, to attract NK cells by IL-6, IL-12, and CXCL10 production, and to induce M1 polarization (100). Therefore, the presence of eosinophils into the tumor or in bloodstream is a favorable prognostic factor for most cancers, although evidence for a pro-tumorigenic role for eosinophils is reported (101). In BC eosinophils appear to be anti-tumorigenic, enhancing the patients' ability to respond against disease (102). In particular, Ownby et al. reported that BC patients with eosinophil counts of less than 55/$mm^3$ had significantly higher risk of recurrent disease than patients who had normal or high levels of eosinophils (102). Moreover, a study on a cohort of 930 BC patients reported a benefit for relative eosinophil count (REC)-high *vs* REC-low in BC-specific survival and in time to treatment failure (93).

## MECHANISMS OF IMMUNOSUPPRESSION IN BREAST TME

During tumor progression, several immunosuppressive mechanisms appear, with a huge advantage in terms of growth, aggressiveness and resistance to treatments for cancer cells. As reported above, the BCIM contains specific immune sub-populations that, through complex and dynamic mechanisms, are able to inhibit the host anti-tumor immune response, by affecting the activity of the main immunostimulating populations. It is important to note that, to generate a tumor immunosuppressive microenvironment, the presence of the immunosuppressive cells inside the tumor lesion is absolutely indispensable. Several anti-inflammatory mechanisms used by BC cells to mobilize and recruit the immunosuppressive mediators have been identified. Here we summarize the main communication strategies that the tumor cells apply to recruit these pro-tumor immune cells as well as the mechanisms through which these cells inhibit the activity of the anti-tumor immune cells, distinguishing between cytokine/soluble factors, cell–cell contact and exosome-mediated mechanisms (**Figure 2**).

### Cytokine and Soluble Factors-Mediated Mechanisms

Colony-stimulating factors (CSFs) are essential for the proliferation, activity and differentiation of the myeloid-cell lineage. G-, GM- and M-CSF are the main components of this family. Interestingly, BC cells can upregulate the expression of these CSFs through a variety of mechanisms, promoting the mobilization and infiltration of specific MDSC populations into the tumors (12, 103, 104). In particular, the mTOR pathway drives G-CSF expression in *in vivo* preclinical models of BC, where, notably, the CSC compartment exhibits an elevated production of G-CSF, thus identifying a positive correlation between CSCs and immunosuppressive TME (11). In addition, it has been demonstrated that tumors actively reprogram metabolic pathways to evade effective anti-tumor immunity. Interestingly, a high glycolytic rate is associated with an increased secretion of both G-CSF and GM-CSF in TNBC cells (105).

Equally, also the chemokines play an important and fundamental role in the regulation of the TME. In particular, the secretion of CXCL2 and CCL22 by ΔNp63-carrying BC cells has been reported to be associated with MDSC infiltration. Importantly, CCL2 and CCL5 have been identified to be important chemokines implicated in monocyte and/or M-MDSC migration to tumors (12). Instead, CXCL5 and CXCL12 (SDF-1) play an important role in PMN-MDSC recruitment into the primary tumor in a BC mouse model with the deletion of *Tgfbr2* (13).

Once recruited inside the tumor, the MDSCs explicate a strong immunosuppressive activity both directly, through the continuous production of reactive oxygen species (ROS), nitric oxide (NO) and several cytokines and, indirectly, by attracting additional immunosuppressive populations. In particular, it is widely reported that M-MDSCs are able to produce mainly $O_2^-$, $H_2O_2$, and peroxynitrite (PNT), while PMN-MDSCs mainly release NO and arginase, which deplete L-arginine from the TME, inhibiting T cell function. These MDSC-derived ROS, NRS, and PNT are able to modify the T cell receptor (TCR) and the CD8 molecules, inducing the block of T-cell immune activity (106). Interestingly, MDSCs can directly block the entry of $CD8^+$ T cells into tumors, by producing high levels of PNT, as well as are able to inhibit T-cell proliferation, strongly impairing the anti-tumor immune response (12, 107). MDSCs as well as the BC cells themselves can also produce immunosuppressive cytokines, such as IL-10, IL-6 and TGF-β, inducing inflammation that may facilitate immune suppression (108, 109). Moreover, to amplify the immunosuppression mechanisms repertoire, MDSCs are able to attract Tregs in a CCR5-dependent manner by secreting CCL4 and CCL5 (12). In addition, to further increase the complexity of the immunosuppressive network, Tregs have been identified as an important source of IL-10 in the TME. High IL-10 production levels amplify the immunosuppressive

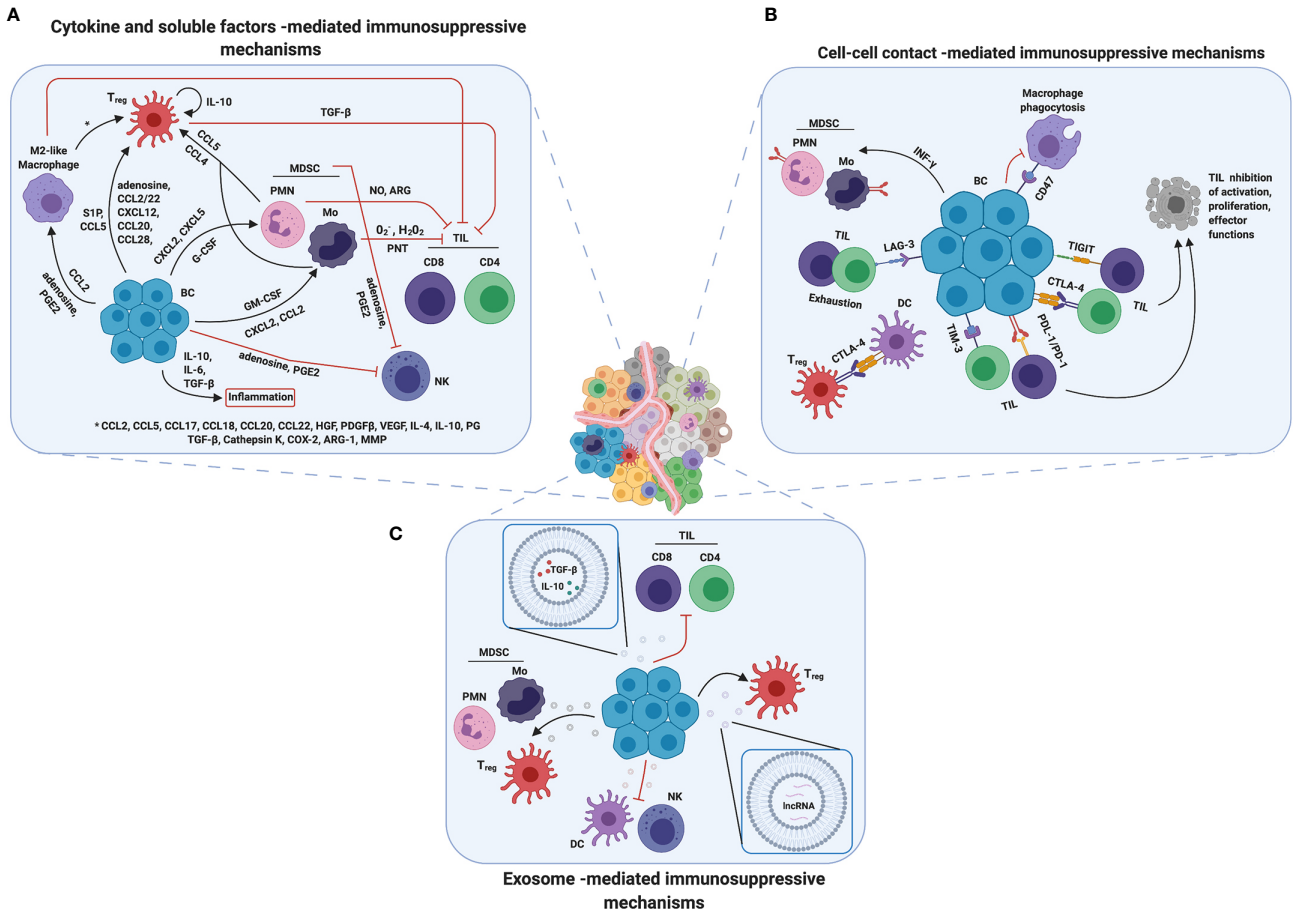


**FIGURE 2** | Mechanisms of immunosuppression in breast TME. Breast cancer cells developed several mechanisms to promote immunosuppression. **(A)** Cytokines and soluble factors are the main players in cell communication and signaling and they are able to mediate immune cell recruitment, mobilization and/or tumor infiltration. Moreover, they promote inflammation and contribute in changing TME composition, making it more immune suppressive. **(B)** Another strategy adopted by breast cancer cells is to overexpress on their surface immune checkpoint receptors such as PD-L1 or CTLA4, inducing cell–cell contact mediated death or anergy in T cells and suppressing immune response against tumor. **(C)** Finally, tumor derived exosomes could induce a reprograming in both immune suppressive and immune cells promoting tumor progression and survival by a wild range of molecules through different mechanisms. Created with BioRender.com.

mechanisms sustaining the expression of FoxP3, TGF-$\beta$R, and TGF-$\beta$. TGF-$\beta$ plays a complex role in BC progression, since it acts as a tumor-suppressor in normal and premalignant cells and as a tumor promoter during the more advanced phases of tumor development, with several epigenetic modification of its signaling partners and target genes controlling this dual role (110). Indeed, while under physiological conditions TGF-β inhibits mammary ductal growth and epithelial stem cell self-renewal, when released in the TME it induces EMT and the secretion of matrix components that stimulate invasion and metastatic spreading, and, together with VEGF, recruits endothelial cells and promotes their proliferation, favoring angiogenesis (111). Moreover, TGF-$\beta$ participates in the downregulation of IL-2 expression, which is a requirement for T cell proliferation (44). Concomitantly, TGF-$\beta$ favors the Treg infiltration in tumor tissues, which could also be directly induced by cancer cells through the expression of several chemokines, such as S1P, CXCL12, CCL20, CCL5, CCL28, and CCL2/22 (44).

As reported above, also TAMs, mainly as pro-tumoral M2, are abundant in the BCIM. TAMs originate primarily from bone marrow-derived blood monocytes/M-MDSC recruited in the TME and induced to rapidly differentiate into macrophages (12). Moreover, one of the main mechanisms found in different types of cancer, including BC, is the secretion of CCL2 through which the cancer cells are able to attract and increase the TAM abundance into the TME (112, 113). The presence of TAMs has been associated with the secretion of an array of chemokines, cytokines, and enzymes able to induce immunosuppression and to downregulate the activation of immune cells involved in the anti-tumor response. Notably, chemokines such as CCL2, CCL5, CCL17, CCL18, CCL20 and CCL22, cytokines such as hepatocyte growth factor (HGF), PDGF-B, VEGF, IL-4, IL-10, prostaglandin (PG) and TGF-β and enzymes, such as Cathepsin K, cyclooxygenase-2 (COX-2), ARG1 and MMPs secreted by TAMs can directly inhibit both $CD8^+$ and $CD4^+$ T cell effector function as well as recruit Tregs

into the tumor lesion (114). In particular, PGE2, the major product of COX-2, plays a pivotal role in BC progression, though the binding to seven transmembrane G-protein-coupled receptors expressed on several immune cell subsets (115). Inhibition of its production by unselective COX inhibitors such as aspirin or other non-steroidal anti-inflammatory drugs has been associated with a reduced risk of developing BC (116), which constitutively expresses high amounts of COX-2 (117). PGE2 is secreted by both cancer cells and immune cells present in the TME, where it promotes the differentiation of MDSCs, from bone marrow progenitors, and DCs and their recruitment and activation, the M2 polarization of macrophages and their expression of programmed death ligand (PD-L)1. In addition, it suppresses NK anti-metastatic activity by reducing the expression of their activating receptors, stimulates the induction of Th2 cells and Tregs while inhibiting Th1 polarization, overall inducing immunosuppression (115).

Besides chemokines, cytokines, and eicosanoids, an immunosuppressive role is also played by metabolites produced by cancer cells, such as adenosine. Adenosine is a purine nucleoside present at low levels in healthy tissues, but released in high amounts in inflamed tissues and in the TME, where it acts as a danger signal. Adenosine is produced by the ectoenzyme CD73 from AMP, generated by CD39 starting from ATP. These two ectoenzymes are expressed at high levels on MDSCs and tumor cells from different cancers, and correlate with poor response to therapy in TNBC patients (118). Adenosine binds four different G-protein-coupled receptors that have been found to be expressed on multiple immune subsets. It exerts several immunosuppressive activities, such as the inhibition of activation and proliferation of $CD4^+$ T and NK cells, induction of Tregs, skewing of DCs to tolerogenic or regulatory subsets and of macrophages to the M2 phenotype (61, 118) (**Figure 2A**).

## Cell–Cell Contact-Mediated Immunosuppressive Mechanisms

An additional strategy by which BC cells are able to evade immune destruction is mediated by cell-cell contact. Plasma membrane receptors such as the Programmed Death 1 (PD-1) and the Cytotoxic T lymphocyte antigen 4 (CTLA-4) are responsible for the T cell anti-tumor suppression activity, leading to tumor escape from the immune surveillance (119). In normal conditions, PD-1 is expressed on T and B lymphocytes, providing peripheral tolerance and protection against autoimmunity, while its ligand PD-L1 is mainly expressed on the surface of antigen-presenting cells. In pathological conditions, such as cancer, the cells can acquire the capability to overexpress PD-L1 and PD-L2. Although the mechanism is not completely understood, the PD-1/PD-L1/PD-L2 axis is able to induce anergy and/or apoptosis of $PD\text{-}1^+$ T cells, attenuating the anti-tumor immune response and promoting Treg immunosuppressive activity (120, 121). Interestingly, a higher PD-L1 expression has been observed in $HER2^+$ BC and TNBC subtypes rather than in the Luminal subtypes (122, 123).

In addition, CTLA-4, which belongs to the immunoglobulin superfamily, is expressed mainly on activated T cells, playing the role of T cells activity inhibitor. In fact, CTLA4 is homologous to CD28, a T-cell co-stimulatory protein, able to bind CD80 and CD86 on antigen-presenting cells. Thanks to its role in inhibiting the immune response against the tumor, CTLA-4 correlates with a poor prognosis in BC patients. Interestingly, besides on T cells, CTLA-4 is often expressed on BC cells (124, 125). Although its exact role in BC cells is still unknown, it might contribute to the regulation of PD-L1 expression and cell proliferation, as observed in lung cancer (126). Moreover, BC cells not only express these receptors on their surface, but they can also induce PD-1 expression in other immune cell populations, enhancing their immunosuppressive function. In particular, it has been described that tumor cells can modulate PD-L1 expression on MDSCs through the release of cytokines such as IFN$\gamma$. In fact, IFN$\gamma$-activated pSTAT1 is able to activate IRF1 protein, leading to its binding on a specific sequence in the *cd274* promoter, enhancing PD-L1 transcription. In fact, IFN$\gamma$ is highly expressed in cells of the tumor tissues and its neutralization significantly decreased $PD\text{-}L1^+$ MDSCs in the TME *in vivo* (127).

Furthermore, previous works demonstrated that also Tregs, accumulated in BC microenvironment, express high levels of CTLA-4 and PD-1, participating in T cell inhibition (128).

Interestingly, in addition to PD-L1 and CTLA-4, BC cells often upregulate other immune checkpoint (IC) markers as a mechanism of resistance to current inhibitors (129). For instance, T-cell Immunoglobulin and Mucin domain-containing molecule 3 (TIM-3) correlates with the presence of other IC markers such as lymphocyte activation gene (LAG)-3 and PD-L1 (129). TIM-3 is an IC receptor that is emerging as a target for cancer immunotherapy. It is expressed on both tumor and immune cells, and contributes to immune tolerance (130). LAG-3 is a cellular receptor expressed by activated T lymphocytes and is associated with T cell exhaustion (131), and it is commonly upregulated with PD-1 (132). Additionally, the T cell immunoglobulin and ITIM domain (TIGIT) co-inhibitory receptor (131), is highly expressed on $CD8^+$ and $CD4^+$ TILs in TNBC, while its ligands are present on antigen presenting cells and cancer cells (133). These three ICs, due to their properties, have been proposed as prognostic markers in BC, together with CD47 (131, 132, 134, 135). The CD47 receptor is expressed on the surface of several types of cancer cells and functions as an anti-engulfment signal that protects cells from phagocytosis by macrophages (136, 137). In particular, it is highly expressed on TNBC, and it has been associated with EMT and poor prognosis (135) (**Figure 2B**).

## Exosomes and Microvesicles as Important Players in Sustaining Tumor Progression

Due to their lipid double layer, extracellular vesicles (EVs) are able to carry stably active biological molecules and have a crucial role in cellular communication and trafficking in both physiological and pathological conditions. Exosomes are a subclass of EVs involved in intercellular communication that are released by all cell types, including cancer cells. Cancer

exosomes have been demonstrated to mediate the main steps of tumor progression, in particular through the modulation of immune response, TME reprogramming and metastasis formation (138). It has been reported that BC cells often release exosomes containing TGF-$\beta$ and IL-10, leading to T cells suppression (139–141). In particular, it has been shown that tumor-derived EVs are predominantly taken up by MDSCs, inducing MDSC immunosuppressive functions (142).

Moreover, it has been shown that tumor-derived exosomes could carry PD-L1 on their membrane surface. Besides inhibiting effector T cell recruitment and activation, exosome PD-L1 confers resistance to ICI therapy. Their ability to competitively bind to PD-L1 antibodies may contribute to the still largely unknown mechanisms of resistance of exosomal PD-L1 (143).

Numerous studies have underlined the role of exosomes in processes involved in tumor progression and survival, modulating immune cells such as DCs, T cells, macrophages, and NK cells and exerting a pro-inflammatory effect (144). For example, BC-derived exosomes can induce a pro-inflammatory response in macrophages localized at distant sites through the activation of NF-κB, which in turn stimulates production of inflammatory cytokines (145). In particular, palmitoylated proteins on the cancer exosome surface are able to bind to TLR2 enhancing NF-κB activation. In turn, activated macrophages prepare pre-metastatic niches that favor colonization by tumor cells (145). Furthermore, despite the molecular mechanism is not fully understood, it has been shown that tumor-derived EVs are able to increase the expansion of $CD4^+CD25^+FoxP3^+$ Treg cells, inducing their suppressor activity and at the same time blocking the proliferation of activated $CD8^+$ T cells (141, 146).

Interestingly, it has been demonstrated that BC-derived exosomes can contain and transmit also non-coding RNA, such as lncRNA SNHG16, which is able to induce CD73 in γδ1 Treg cells, enhancing their immunosuppressive effect *via* adenosine generation (147). Further studies have underlined the presence in EVs of miRNAs able to contribute to tumor progression. For instance, BC-secreted exosomal miR-105 could induce a metabolic program in cancer associated fibroblasts by activating the MYC signaling, adapting them to a different metabolic environment (148, 149). Another example is miR-503 that can enhance polarization of the microglia from a tumor-suppressive M1 to a tumor-promoting M2 phenotype, thus contributing to brain metastasis in BC (150). Interestingly, hypoxic conditions favor the release of immunosuppressive exosomes by BC cells. In fact, hypoxia increases the EV content of two immunosuppressive factors, TGF-β1 and miR-23a, which inhibit NK cell function by directly targeting the expression of CD107a and decreasing the cell surface expression of the activating receptor NKG2D (151) (**Figure 2C**).

# IMPORTANCE OF THE TME IN RESPONSE AND RESISTANCE TO IMMUNOTHERAPY

## Immunotherapy in BC

Immunotherapy has entered the clinical practice for BC patients as early as 1998, with the FDA-approval of the humanized HER2 monoclonal antibody trastuzumab, followed by other HER2 targeting antibodies (152). These drugs improved overall survival of patients affected by early or advanced $HER2^+$ BC. However, tumors often display intrinsic or acquired resistance mechanisms, and most patients eventually experience disease progression (153).

Besides these passive immunotherapies, active immunotherapy for BC has been extensively studied. Although encouraging results came from preclinical analysis, most of the clinical trials with vaccines targeting tumor associated antigens (TAA) such as HER2 or mucin (MUC)1 failed to significantly improve patients' outcome (154). Currently, new vaccines based on tumor-specific neo-antigens and shared oncoantigens that play a key role in the biology of CSCs are giving promising results that will hopefully pave the way for their clinical translation (155–159). Recently, immunotherapy options for BC treatments have expanded, with the introduction of the ICI atezolizumab (a PD-L1 antibody) in combination with chemotherapy for the treatment of patients with $PD\text{-}L1^+$ unresectable locally advanced or metastatic TNBC (152). However, the Phase III double-blind IMpassion130 trial (ClinicalTrials.gov NCT02425891) demonstrated a clinically meaningful but not statistically significant difference in OS between patients treated with nab-paclitaxel plus atezolizumab or placebo, and a complete response rate of only 10.3% in PD-L1+ patients subjected to the combinatory treatment (160, 161).

Altogether, the results coming from the different BC immunotherapy regimens applied so far either in the clinical practice or in clinical trials suggest that multiple tumor cell intrinsic and extrinsic mechanisms of resistance need to be targeted to increase their efficacy. In particular, it is becoming increasingly evident that the immunosuppressive activity of the TME greatly affects tumor response to immunotherapy (5).

## The Role of TME in the Response to HER2-Targeted Therapies

The composition of the TME is key in determining the sensitivity of $HER2^+$ BCs to HER2-targeted therapies (153). Indeed, several studies have shown that the presence of TILs and the expression of immune-associated gene signatures in pre-treatment biopsies are associated with longer DFS in $HER2^+$ BC patients treated with anti-HER2-based therapy in the neoadjuvant or adjuvant settings (50). This is mainly due to the ability of immune cells to enhance trastuzumab anti-cancer activity. In fact, NK-dependent antibody-dependent cellular cytotoxicity (ADCC) plays a central role in trastuzumab-mediated cancer cell killing (162). Moreover, trastuzumab-induced HER2 internalization leads to HER2 presentation in MHC class I molecules, which can activate anti-tumor $CD8^+$ T cells (163). Therefore, although the main mechanisms responsible for primary or acquired resistance to trastuzumab and to the other HER2-targeted therapies are cancer cell-intrinsic (153), the presence of M2 macrophages and other immunosuppressive cells in the TME significantly impairs the efficacy of HER2-targeting antibodies (164). Interestingly, trastuzumab treatment can increase the immune evasive properties of BC cells through the induced secretion of TGF-$\beta$, IL-6 and other immunosuppressive cytokines that, in turn, recruit immunosuppressive cells (165, 166). Indeed, the

presence of a high number of TILs in patients with residual disease after neoadjuvant therapy was associated with worse DFS (167), probably due to an increase in Treg cells (168), indicating that immune-mediated resistance mechanisms need to be inhibited in BC patients to guarantee a good response to HER2-targeted therapy.

### TME Mediates Resistance to Immune Checkpoint Inhibitors

The poor response of most BCs to single-agent ICI therapy reflects intrinsic or acquired resistance (169). The mechanisms responsible for acquired resistance to ICIs in BC are currently unclear. However, lack of TILs and the presence of high numbers of MDSCs and other immunosuppressive cells correlate with low response (170). Of note, the TME composition in primary cancer differs from that in metastases, and clinical and preclinical data have demonstrated that primary BC are more responsive to ICIs than their corresponding lung or liver metastases, demonstrating that TME is important in determining response to immunotherapy (171). Indeed, many cells within the TME can impair the response to ICIs by inhibiting effector T cells (172), and depletion of intra-tumor MDSCs or Treg cells improved responsiveness to PD-1/PD-L1 blockade in preclinical models of BC (173, 174). Therefore, association of ICIs with therapies that revert the immunosuppressive activity of TME may improve their efficacy.

## STRATEGIES TO REVERT IMMUNE SUPPRESSION AND IMPROVE CANCER IMMUNOTHERAPY

The growing understanding of the mechanisms that cause resistance to immunotherapy will pave the way to the development of combination strategies that associate immunotherapy with drugs able to revert TME immunosuppression. Indeed, several studies have demonstrated that therapies that either recruit T or NK cells or reduce immunosuppressive factors in the TME can sensitize poorly immunogenic tumors to immunotherapy (175) (**Figure 3**).

The first strategy to improve the effectiveness of immunotherapy, and in particular of ICIs, is to recruit and activate effector cells such as anti-tumor T lymphocytes, since ICIs are not able to unleash antitumor responses if fully primed T cells are not present at the tumor site (176). This effect may be obtained with adoptive cell transfer therapy, and in particular with the administration of chimeric antigen receptor (CAR) T cells. Several clinical trials with CAR T cells specific for different tumor antigens such as HER2 (NCT03696030), EpCAM (NCT02915445), MUC1 (NCT04020575 and NCT02587689) and mesothelin (NCT02792114), alone or in combination regimens, are currently ongoing in BC patients. Till now, CAR T efficacy in solid tumors has demonstrated limited, mostly due to the presence of physical barriers that limit their infiltration in the tumor and to the immunosuppression exerted by the TME, but their combination with ICIs and other immunotherapies is expected to ameliorate cancer patient outcomes (177). However, it must be taken into account that, due to the difficulties in finding BC specific antigens, CAR T cells have been generated that targets TAAs, and therefore they can induce cytokine release syndrome and other severe reactions (178), as observed in a patient who died of pulmonary distress 5 days after receiving HER2-targeting CAR T cells (179), rising safety concerns.

Anti-cancer vaccination is a promising alternative to induce T cell recruitment in the tumor. Recently, cancer vaccines have been repositioned as a way to activate an immune response whose brakes are then removed by ICIs (154). A phase I clinical trial is testing the association of the personalized cancer vaccine RO7198457—an mRNA-based vaccine targeting an unspecified amount of tumor-associated antigens expressed in the patient's tumor—with atezolizumab in patients with TNBC and other solid tumors (NCT03289962). Moreover, several clinical trials are currently recruiting patients affected by TNBC or other advanced BCs that will be treated with vaccination in association with the anti-PD-1 pembrolizumab or the anti-PD-L1 durvalumab (NCT04024800; NCT03362060; NCT03632941; NCT03789097; NCT04634747; NCT04418219; NCT03199040; NCT03606967; NCT02643303)). In the next years, the results coming from these trials will clarify the effectiveness of combined therapies based on ICIs and vaccination for BC treatment. Interestingly, cancer vaccines targeting CSCs can also synergize with HER2-targeted immunotherapy, as we have recently demonstrated in a preclinical model (180). Thus, multiple combination strategies might be developed in the next years to further improve BC treatment.

Another strategy to induce T cell activation and increase the efficacy of immunotherapy in BC patients is its association with cytotoxic chemotherapies that can induce immunogenic cell death (ICD), with the subsequent release of tumor antigens that prime T cells. Not all cytotoxic agents lead to ICD, but doxorubicin, mitoxantrone, paclitaxel and oxaliplatin do (181, 182). Moreover, these immunomodulating drugs improve immunotherapy by downregulating PD-L2 and upregulating MHC class I expression on tumor cells, increasing their immunogenicity (183). Several clinical trials testing the combination of ICIs with immunogenic chemotherapy have been performed (some examples are NCT02555657; NCT02622074; NCT03139851; NCT02425891), and the results from the IMpassion130 trial (NCT02425891) have led to the FDA approval of atezolizumab in association with nab-paclitaxel for first line, metastatic, PD-L1$^+$ TNBC (160, 161).

Very recent data have shown that ICIs can act not only on T cells but also on NK cells, which express several ICs that inhibit their cytotoxic function, such as PD-1, TIM3, TIGIT, LAG-3 and CD96 (184). Several clinical trials are ongoing investigating the effects of ICIs on NK cells in different solid cancers, as reviewed in (185). Besides the classical ICs, NK cells express specific inhibitory receptors such as KIRs and NKG2A, and several inhibitory receptor blocking antibodies are currently undergoing clinical evaluation in solid cancers. Monalizumab, a mAb targeting NKG2A, is currently being tested in combination with trastuzumab in metastatic HER2$^+$ breast cancer (NCT04307329). However, the study of these novel drugs in BC patients is still

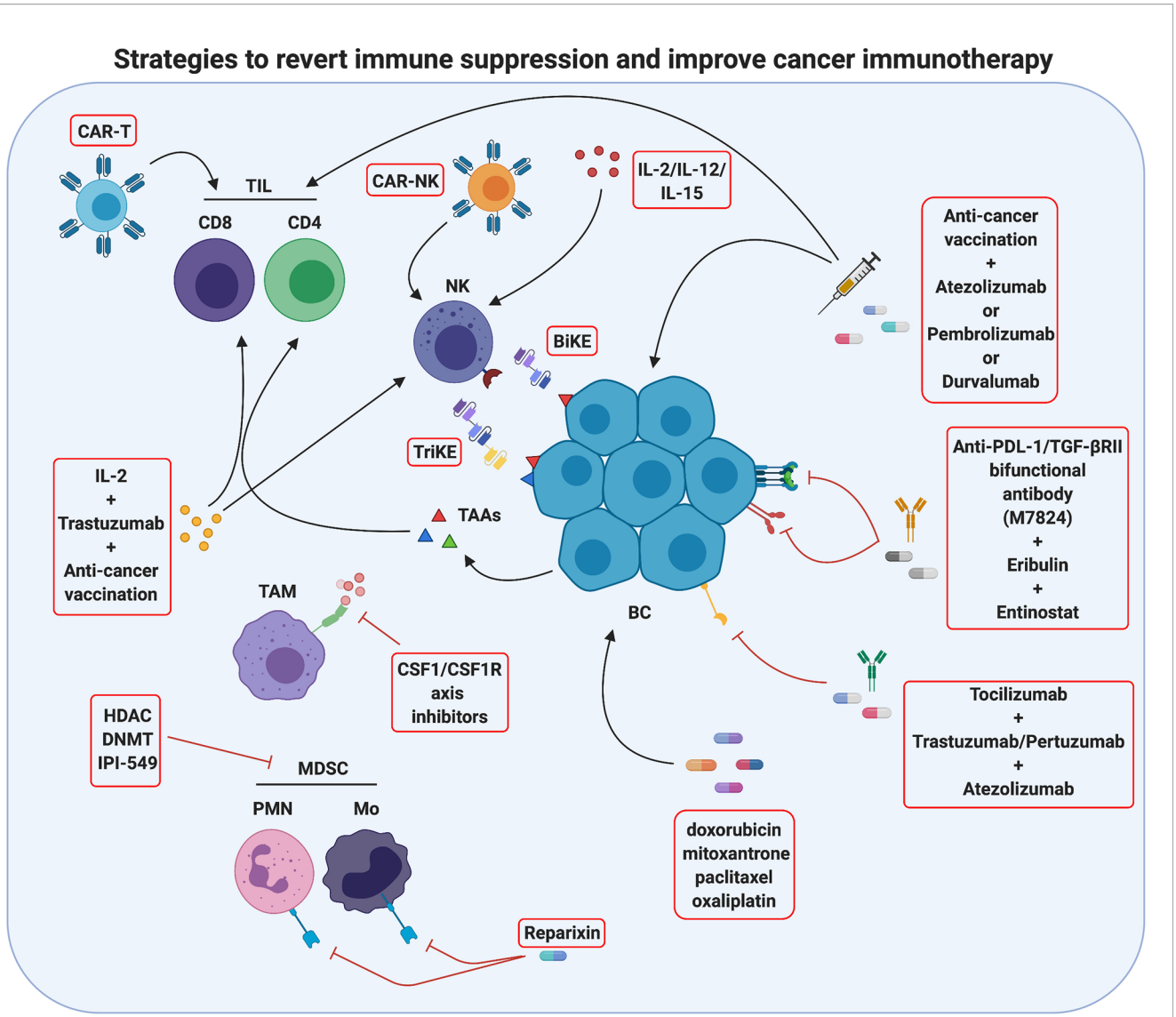


**FIGURE 3** | Strategies to revert immune suppression and improve cancer immunotherapy. Actually, several methods used in breast cancer treatment take advantage of immunomodulation mechanisms and are promising tools for tumor immunotherapy. One strategy is to improve immune cell activity against the tumor by innovative therapies such as CAR-T/CAR-NK administration, which employs patient's T/NK cells engineered with chimeric receptors targeting antigens characteristics of cancer cells, or as anti-cancer vaccination, which stimulates the activation of the patient's tumor-specific T cells. An additional method ongoing in clinical studies is the use of monoclonal antibodies against immune checkpoints or immune checkpoints inhibitors (ICIs) to block T-cell suppression, and the use of BiKE and TriKE reagents to induce antitumor NK cell activation. Moreover, an antitumor immune response may be activated by chemotherapy drugs inducing immunogenic cell death. A second strategy to improve tumor immunotherapy is to revert the immunosuppressive activity of TME often displayed in several breast cancers. In this sense, cytokine pharmacological modulation or inhibition of specific immunosuppressive pathways can be performed. The effects of single or combined therapies are actually studied. Created with BioRender.com.

limited. Nevertheless, since NK cells represent an attractive tool for cancer immunotherapy thanks to their ability to kill cancer cells in an MHC-independent manner, other NK-based immunotherapies have been developed (186). Besides stimulation with cytokines (such as IL-12, IL-15 or IL-2, discussed below), the anti-tumor effects of endogenous NK cells can be stimulated by administration of bispecific and trispecific killer cell engagers (BiKE and TriKE, respectively), constituted by antibodies targeting CD16 or NKG2D and one or two tumor antigens (61). TriKEs can also be engineered to sustain NK cell proliferation *in vivo*, through the insertion of a modified IL-15 cross-linker (51). BiKe and TriKE specific for HER2 or EpCam were developed for BC, and, during the revision of this paper, GT Biopharma announced the initiation of clinical development of TriKE therapy for the treatment of $HER2^{+}$ breast and gastrointestinal cancers, using a tri-specific scFv recombinant fusion protein conjugate composed of anti-CD16 and anti-HER2 antibodies, and a modified form of IL-15 (61).

Recently, adoptive NK cell therapy strategies have been explored in preclinical and clinical studies. Although adoptive transfer of autologous NK cells expanded *ex vivo* induced only very limited antitumor effect in patients with solid cancers, partially due to the immunosuppressed state of patients' NK cells, a phase I clinical trial in patients with treatment-refractory $HER2^+$ solid cancers treated with trastuzumab, bevacizumab, and autologous *in vitro* expanded NK cells reported preliminary antitumor activity, supporting the assessment of this approach in phase II trials (187). Alloreactive human pluripotent stem cell- or PBMC-derived NK cells have been widely investigated. However, in BC patients a phase II trial with allogeneic NK cell administration after lymphodepleting chemotherapy and total body irradiation gave poor results (188).

The difficulties in obtaining large amounts of NK cells led to the development of NK cell lines, among which EBV-transfected NK-92 is the only approved by the FDA for use in clinical trials (189). A clinical trial associating the infusion of NK-92 cells to the IL-15 super-agonist N-803 that promotes enhanced NK cell function, several chemotherapeutic drugs and vaccines targeting CEA, Ras and MUC-1, is currently recruiting TNBC patients who have progressed on standard of care therapy (NCT03387085). In order to improve their efficacy, NK cells expressing tumor-targeting CARs were generated. Autologous, allogeneic and NK cell lines can all be engineered to express CARs. Most CAR-NKs developed so far were tested in hematological malignancies, and some clinical trials are currently evaluating the safety and efficacy of PD-L1 or HER2-targeting CAR-NK therapy in solid tumors, although, to the best of our knowledge, there is not published data on human trials on BC up to now (186). Of note, the identification of CD142 (also known as tissue factor) as an antigen highly expressed in TNBC cells and CSCs, led to development of CAR-NKs specific for this aggressive type of BC, which led to positive results in preclinical studies (190), and similar results were obtained with EGFR-CAR NK cells (191), opening the way for a clinical development. Although CAR-NK therapy is still under evaluation, it displays potential advantages over CAR-T cell therapy. Indeed, NK cells release mainly IFN$\gamma$ and GM-CSF, which are relatively safer than the cytokines released by activated CAR-T cells (IL-6 and TNF-$\alpha$) that can cause cytokine release syndrome. Finally, CAR-NK cells can kill target cells in both CAR-dependent and CAR-independent manners, increasing their efficacy (51).

Another strategy to improve tumor immunotherapy is to revert the immunosuppressive activity of TME that characterizes most BCs. To this end, both drugs that deplete immunosuppressive cells and inhibitors of inflammatory cytokines have been tested. No selective MDSC inhibitors are currently known; however, many existing drugs reduce systemic and intratumor MDSCs, potentiating immunotherapy over time (15). DNA methyl transferase (DNMT) and histone deacetylase (HDAC) inhibitors, besides increasing tumor cell intrinsic immunogenicity through the upregulation of MHC class I and the antigen processing machinery (192), exert this effect (193). The HDAC inhibitor romidepsin is being evaluated in association with nivolumab and cisplatin in TNBC (NCT02393794), while the DNMT inhibitor decitabine in combination with pembrolizumab, followed by standard neoadjuvant chemotherapy, is under evaluation for locally advanced $HER2^-$ BC (NCT02957968). Recently, a key role of PI3K$\delta$ and PI3K$\gamma$ isoforms in promoting integrin4-dependent MDSC recruitment in the TME and in stimulating the immunosuppressive polarization of MDSCs and TAMs has been shown (194). Therefore, the PI3K$\delta$ and PI3K$\gamma$ inhibitor IPI-549 is under evaluation in combination with atezolizumab and nab-paclitaxel in TNBC patients (NCT03961698).

Since in BC the dominant TAM phenotype is that of tumor promoting M2, which is associated with poor prognosis (195), macrophage depletion or re-education to anti-tumor M1 is an attractive approach for TME modulation (196). The most widely used strategy to date has been TAM depletion from the TME through inhibition of CSF-1/CSF-1R axis. CSF-1/CSF-1R inhibitors have been administered either as a monotherapy (NCT02265536) or in association with chemotherapy (NCT01596751 and NCT02435680). However, the available results from other cancer types showed only modest efficacy (196). This could be partially due to the ability of chemotherapy to recruit $Tie^+$ macrophages in the TME, which in turn promote cancer cell dissemination (197). Therefore, a phase I clinical trial that evaluates the efficacy of the Tie2 kinase inhibitor rebastinib in combination with paclitaxel and the microtubule inhibitor eribulin mesylate in patients with advanced BC is currently ongoing (NCT02824575).

Aberrant overexpression of many proinflammatory cytokines has been reported in breast tumors, with a different profile during cancer progression (108). The modulation of cytokines present in the TME can be pharmacologically performed in order to either increase cytokines that promote anti-tumor immune responses or inhibit those that favor tumor progression (198). Among the anti-tumoral cytokines, IL-2 is one of the most studied, since it potentiates the activation of both cytotoxic T and NK cells, and can therefore enhance ADCC (198). IL-2 (aldesleukin or its pegylated more stable form bempegaldesleukin) administration has therefore been associated with trastuzumab, cancer vaccines or ICIs in several clinical trials in BC patients. However, the few results available so far indicate only a modest benefit (NCT00784524; NCT00003199; NCT03435640). This could be ascribed to the induction of compensatory immunosuppressive mechanisms, such as increased expression of IC molecules, secretion of inhibitory cytokines such as IL-10 and TGF-$\beta$, triggering of Treg cells and MDSCs, and activation of intracellular suppressors of cytokine signaling proteins that terminate the antitumor response (198). Therefore, many strategies that inhibit immunosuppressive cytokines have been developed and tested in a multitude of clinical trials in BC patients. TGF-$\beta$, IL-6 and IL-8 are among the most promising cytokines to be targeted, since their overexpression has been associated with advanced disease, higher risk of recurrence, stemness, therapeutic resistance as well as immune suppression (199–202). Several TGF-$\beta$ targeting agents are under analysis in BC patients. An anti-PD-L1/TGF-$\beta$RII bifunctional antibody (M7824) is currently undergoing clinical evaluation either as a single agent in stage II–III $HER2^+$ BC (NCT03620201) or in combination with radiation (NCT03524170), with eribulin (NCT03579472) or with a

brachyury-targeting virus-based vaccine plus trastuzumab emtansine or the class I HDAC inhibitor entinostat in TNBC patients (NCT04296942). Similarly, the selective TGF-βR1 inhibitor galunisertib is under evaluation in combination with paclitaxel in TNBC patients (NCT02672475). For what concerns IL-6, the neutralizing IL-6 receptor antibody tocilizumab—FDA-approved for the treatment of cytokine release syndrome in CAR T-treated patients—is emerging as a potential new therapeutic in BC. Two clinical trials are recruiting patients to test its administration in combination with either trastuzumab and pertuzumab in metastatic $HER2^+$ BC or with atezolizumab and nab-paclitaxel in advanced TNBC patients (NCT03135171 and NCT03424005). In preclinical models of TNBC, IL-8 inhibition was shown to revert the mesenchymal phenotype, decrease MDSCs in the TME and enhance tumor cell killing by T and NK cells (202), providing the rational for combining IL-8 inhibitors with immunotherapy or chemotherapy. Reparixin, a small molecule inhibitor of the IL-8 receptors CXCR1 and CXCR2, has been tested in clinical trials (NCT01861054; NCT01861054) in $HER2^-$ BC patients, reporting a 30% response rate in 27 patients and a decrease in the aldehyde dehydrogenase CSC marker in about 25% of patients (203). Besides cytokines, molecules involved in the production of the immunosuppressive metabolite adenosine represent promising targets for BC therapy. In this light, clinical trials are currently ongoing in TNBC and other solid tumors combining immunotherapy with pembrolizumab or atezolizumab and inhibitors of CD73 or adenosine receptors (CPI-006 and CPI-444, respectively; NCT03454451 and NCT02655822), although the results have not yet been published (204).

## CONCLUSIONS

The introduction of immunotherapy has revolutionized the treatment of several cancer types, shifting the focus from cytotoxic therapies toward treatments that boost anti-tumor immune responses. However, only a small percentage of patients affected by BC currently benefit from immunotherapy. Indeed, the clinical efficacy of immunotherapy is limited to a subset of patients, and secondary resistance often develops in responding patients, further constraining the possibility of immunotherapy of substantially improving the outcome of BC patients.

A plethora of mechanisms contribute to the low efficacy displayed by immunotherapy in general, and of ICIs in particular, when administered as a single agent in the majority of BC patients, and it is now well known that the TME plays a pivotal role in the resistance mechanisms. Indeed, tumor progression is strictly intertwined with modifications of its TME that promote cancer cell proliferation while inhibiting the effector functions of anti-tumor immune responses, generating an immunosuppressive microenvironment that finally results in tumor outgrowth and metastatic dissemination. This immunosuppressive milieu generated by the crosstalk between cancer cells and immune and stromal cells present in the TME significantly dampens the protective anti-tumor immune responses activated by immunotherapies, thus resulting in treatment failure. The awareness of the existence of these mechanisms has shed light on the need to develop combination therapies that support the effect of ICIs and other immunotherapies by either expanding the activation and recruitment of effector cells, such as T lymphocytes and NK cells, or by inhibiting immunosuppressive cells and soluble factors. Of note, recent evidence from the literature and the clinics is expanding the focus of immunotherapy from its traditional T cell-centric view to a broader vision. Indeed, others and we have previously suggested that the humoral response plays a key role in immunotherapy-induced anti-cancer responses (157, 205, 206). This is particularly important considering that CSCs from BC and many solid cancers downregulate antigen-processing and presentation, thus escaping T cell responses (155). For the same reason, NK cells are emerging as new potential allies in cancer immunotherapy. Hundreds of clinical trials are currently testing different combinations of drugs, sometimes obtaining encouraging results. However, we must be conscious that BC and its TME represent a very heterogeneous and dynamic system that changes over time as the result of a complex crosstalk between neoplastic cells, immune cells and cancer therapies. This implies that a deeper understanding of the role played by the innate and adaptive immune response in individual BCs, and the characterization of the TME features that mostly influence the efficacy of immunotherapy, are needed to develop more effective treatments able to simultaneously activate anti-tumor immune responses and hinder the mechanisms leading to tumor immune escape. To this end, the identification of new predictive biomarkers of response to ICIs and combined therapies, which could help to stratify patients and guide the therapeutic decision, is urgently needed. Many efforts to define an immune signature distinctive of BC patients that positively respond to immunotherapy have been made, but clear-cut data are still missing (122, 207). The identification of personalized biomarker profiles, although representing a demanding challenge, may represent in the next years an important tool that could improve the development of optimal personalized combination therapies able to significantly improve BC prognosis.

## AUTHOR CONTRIBUTIONS

LC, VS, and GC searched for current literature on the topic and wrote the manuscript. FC, PD, and LC reviewed the manuscript and finalized it for publication. All authors contributed to the article and approved the submitted version.

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# Clinical Outcomes for Patients With Metastatic Breast Cancer Treated With Immunotherapy Agents in Phase I Clinical Trials

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**Background:** Immuno-oncology (IO) agents have demonstrated efficacy across many tumor types and have led to change in standard of care. In breast cancer, atezolizumab and pembrolizumab were recently FDA-approved in combination with chemotherapy specifically for patients with PD-L1-positive metastatic triple-negative breast cancer (TNBC). However, the single agent PD-1/PD-L1 inhibitors demonstrate only modest single agent efficacy in breast cancer. The purpose of this study was to investigate the efficacy of novel IO agents in patients with metastatic breast cancer (MBC), beyond TNBC, treated in phase I clinical trials at the University of Colorado.

**Methods:** We performed a retrospective analysis using a database of patients with MBC who received treatment with IO agents in phase I/Ib clinical trials at the University of Colorado Hospital from January 1, 2012 to July 1, 2018. Patient demographics, treatments and clinical outcomes were obtained.

**Results:** We identified 43 patients treated with an IO agent either as a single agent or in combination. The average age was 53 years; 55.8% had hormone receptor-positive/HER2-negative breast cancer, 39.5% TNBC and 4.7% HER2-positive. Patients received an average of 2 prior lines of chemotherapy (range 0-7) in the metastatic setting. Most patients (72.1%) received IO alone and 27.9% received IO plus chemotherapy. Median progression-free survival (PFS) was 2.3 months and median overall survival (OS) was 12.1 months. Patients remaining on study ≥ 6 months (20.9%) were more likely to be treated with chemotherapy plus IO compared to patients with a PFS < 6 months (77.8% v. 14.7%). No differences in number of metastatic sites, prior lines of chemotherapy, breast cancer subtype, absolute lymphocyte count, or LDH were identified between patients with a PFS ≥ 6 months vs. < 6 months.

**Conclusions:** Our phase I experience demonstrates benefit from IO therapy that was not limited to patients with TNBC and confirms improved efficacy from IO agents in combination with chemotherapy. A subset of patients with MBC treated in phase I clinical trials with an IO agent derived prolonged clinical benefit. Predictors of response to immunotherapy in breast cancer remain uncharacterized and further research is needed to identify these factors.

**Keywords: immunotherapy, metastatic breast cancer, PD-L1 inhibitors, phase I clinical trials, PD-1 inhibitors**

## INTRODUCTION

Breast cancer is the most common cancer in women and patients with metastatic breast cancer (MBC) have a 5-year overall survival of only 27% (1). While prognosis depends on biologic subtype, there remains a critical unmet need for novel therapeutic options to improve survival for patients with MBC.

The development of immuno-oncology (IO) therapeutics has changed the way we treat many cancers, most dramatically with inhibitors of programmed cell death-1 (PD-1), its ligand (PD-L1) and cytotoxic T-lymphocyte-associated antigen (CTLA-4) (2–4). The first approval in metastatic breast cancer came in 2019 with the approval of atezolizumab in combination with nab-paclitaxel for patients with PD-L1-positive (tumor-infiltrating immune cells ≥ 1%) metastatic triple-negative breast cancer (TNBC) (5). This was followed in 2020 by the approval of pembrolizumab in combination with chemotherapy including paclitaxel, nab-paclitaxel or gemcitabine plus carboplatin in patients with PD-L1-positive (combined positive score ≥10) metastatic TNBC (5, 6).

TNBC and human epidermal growth factor receptor 2 (HER2)-positive breast cancers are perceived as being more immunogenic compared to luminal breast cancers based on a higher mutational burden, higher tumor-infiltrating lymphocyte (TIL) rates and higher PD-L1 expression (7–10). Higher TIL expression is associated with increased pathologic complete response (pCR) rates in patients treated with neoadjuvant chemotherapy and improved prognosis in HER2-positive and TNBC (9, 11). PD-L1 is expressed in 20-50% of breast cancers and varies depending on the specific antibody clone and evaluation on tumor cells or immune cells in the tumor microenvironment (10). Expression is higher in TNBC and HER2-positive breast cancer compared to hormone receptor (HR)-positive/HER2-negative tumors (10, 12). In patients with TNBC and HER2-positive breast cancer treated with neoadjuvant chemotherapy, PD-L1 expression correlates with a higher pCR rates and improved clinical outcomes (13–15).

Despite the increased immunogenicity of TNBC, response rates to IO monotherapy with pembrolizumab range from 23% for PD-L1-positive patients treated in the first-line setting to approximately 5% for patients previously treated with chemotherapy regardless of PD-L1 status (16, 17). While there is a subset of patients with TNBC who are exceptional responders to immunotherapy and experience long-term disease control, the efficacy of IO monotherapy generally is no better than palliative chemotherapy and combinations of IO plus chemotherapy are more active (5, 6, 18).

The activity of IO agents in luminal breast cancers is more limited with response rates ranging from 11% to 30% for pembrolizumab in patients with advanced PD-L1 positive, HR-positive HER2-negative breast cancer and 3% with avelumab in a similar patient population (19, 20). In the neoadjuvant setting, the addition of pembrolizumab to an anthracycline and taxane-containing chemotherapy backbone resulted in an increased pCR rate in patients with HR-positive/HER2-negative breast cancer in the I-SPY2 clinical trial (21). There are numerous ongoing clinical trials evaluating IO agents in combination with chemotherapy, radiotherapy, other immune checkpoint inhibitors or cancer vaccines (22).

Over the last decade, there has been a rapid increase in the development of diverse IO agents targeting numerous pathways extending beyond CTLA-4 and PD-1/PD-L1. The recent approval of atezolizumab and pembrolizumab in combination with chemotherapy for patients with PD-L1-positive TNBC allows for an IO option for a subset of patients with metastatic breast cancer. Outside of this limited indication, the opportunity for many patients to receive treatment with an IO agent has been in the setting of a clinical trial. Given the great enthusiasm for IO agents in general for the treatment of cancer and the promise of durable responses for some patients, we observed high enrollment of patients with metastatic breast cancer in phase I clinical trials evaluating IO agents at our site.

The purpose of this study was to evaluate clinical outcomes for patients with metastatic breast cancer who were treated in phase I clinical trials containing at least one IO agent at the University of Colorado Cancer Center. We included patients with all breast cancer subtypes who were treated with many different types of IO agents ranging from PD-1/PD-L1-inhibitors to cancer vaccines. While each phase I trial enrolled a small number of patients with metastatic breast cancer, we sought to combine these patients into one dataset to explore outcomes for IO agents in a phase I breast cancer population.

## MATERIALS AND METHODS

We performed a retrospective analysis using a database of patients from the electronic medical record system (EMRS) with MBC who received treatment with IO agents in phase I/Ib clinical trials at the University of Colorado Hospital from

January 1, 2012 – July 1, 2018. All data was stored in a secure online database and the study was performed in accordance with local IRB guidelines. Phase I trials included all protocols that studied single agent or multi-agent investigational drugs that had phase I or phase Ib in the title. For patients in phase Ib/II trials, only patients enrolled in the phase Ib portion of the study were included for analysis. Patients were included in the study if they received an agent considered to directly target or modulate immune cells or immune cell signaling (an IO agent).

Patient characteristics including age, sex, presence of metastatic disease at diagnosis, number of sites of metastases, lines of prior systemic therapy, HR and HER2 receptor status, Eastern Cooperative Oncology Group Performance Status (ECOG PS), radiation within 30 days of IO and mean lab values were collected *via* chart review using the EMRS. HR and HER2 receptor status was based on local pathology report also found in the EMRS. Other data collected included: time of treatment discontinuation, disease progression and death. We did not collect PD-L1 status as this was not uniformly performed for all patients with MBC during the time period of this study at our institution.

Investigational treatments were administered at the University of Colorado Hospital as part of a clinical trial that received institutional review board (IRB) approval. All patients provided written informed consent prior to enrollment in these phase I clinical trials.

## Endpoints and Statistical Methodology

Cohort characteristics were summarized using counts with percentages for categorical variables and with the mean with quartiles for continuous variables. The association between cohort characteristics and progression-free survival (PFS) was evaluated with the Wilcoxon rank-sum test for continuous variables and the Fisher Exact test for categorical variables due to low cell counts. The Wilcoxon rank-sum test was chosen to account for the non-normal distribution of the continuous variables.

PFS was defined as the time from study enrollment to the date of discontinuation for progressive disease, initiation of a new anti-cancer therapy, or death. Overall survival (OS) was defined as the time from study enrollment to the date of death. Patients lost to follow-up were censored at the last follow-up date. For patients who remained on study, the date of analysis (May 1$^{st}$, 2019) was used to censor the patient outcomes. The median number of months for OS and PFS were calculated using the Kaplan-Meier method with *p*-values determined by log-rank test. *p*-values were reported based on a null hypothesis of no difference against a two-sided alternative. Analyses were performed using SAS 9.4 (SAS Institute; Cary, NC).

# RESULTS

## Baseline Patient Characteristics

A total of 43 patients with MBC were treated with a wide range of IO agents in phase I/Ib clinical trials at the University of Colorado Hospital during the period of our study. The average age was 53 years (range 33-71) and all patients were female (**Table 1**). ECOG PS was 0 in 53.5% of patients and 1 in 46.5% of patients. Most patients had 3 or more sites of metastasis (51.6%). On average, patients received two prior lines of chemotherapy (range 0-7) in the metastatic setting. Most patients had HR-positive/HER2-negative breast cancer (55.8%), followed by TNBC (39.5%) and HER2-positive disease (4.7%). In the phase I/Ib clinical trials, 72.1% of patients received single or combination immunotherapy and 27.9% received an IO agent plus chemotherapy.

Patients with HR-positive/HER2-negative breast cancer all received hormonal therapy prior to enrollment in the phase I/Ib clinical trials (**Supplemental Table 1a**). The most common prior therapy administered in the HR-positive/HER2-negative group, included capecitabine (45.8%) and everolimus (45.8%). Around one-fifth (20.8%) of patients with HR-positive/HER2-negative cancers received a cyclin-dependent kinase (CDK) 4/6 inhibitor prior to enrollment in phase I/Ib clinical trials. A little less than half (44.4%) of HR-positive/HER2-negative patients received a CDK 4/6 inhibitor and one patient (5.6%) received alpelisib following progression on phase I/Ib clinical trials (**Supplemental Table 1b**). In TNBC, the most common prior therapy administered in any setting was carboplatin with gemcitabine (64.7%) and doxorubicin, cyclophosphamide, paclitaxel/docetaxel (47.1%) (**Supplemental Table 1c**). Around one-quarter (23.5%) of patients with TNBC received sacituzumab govitecan prior to enrollment in phase I/Ib trials. The most common therapy received post-progression in TNBC was eribulin (28.6%) (**Supplemental Table 1d**). One patient (7.1%) with TNBC received sacituzumab govitecan following progression. Prior therapies received in HER2-positive cancers are listed in **Supplemental Table 1e**.

## Phase I Clinical Trials Including IO Agents

In the phase I studies included in this analysis, patients were treated with PD-L1/PD-1 inhibitors without chemotherapy (N=12, 27.9%), IO agents other than PD-L1/PD-1 inhibitors without chemotherapy (N=19, 44.2%) or any IO agent plus chemotherapy (N= 12, 27.9%) (**Figure 1**). Patients treated with PD-L1/PD-1 inhibitors without chemotherapy also received other agents targeting vascular endothelial growth factor (VEGF), indoleamine 2,3-dioxygenase 1 (IDO), OX40, CD38, and T-cell immunoreceptor with Ig and ITIM domains (TIGIT). Trials containing IO agents other than PD-L1/PD-1 inhibitors without chemotherapy included agents targeting IL-10 inhibitor, toll-like receptor 9 (TLR9) agonist and cancer vaccines. Trials with an IO agent in combination with chemotherapy included agents targeting PD-L1, cancer vaccines, nab-paclitaxel, cyclophosphamide and FOLFOX chemotherapy.

## Clinical Outcomes

The median PFS and OS for all patients with MBC enrolled in phase I clinical trials including any IO agent was 2.3 months (95% CI, 2.07-2.60) and 12.1 months (95% CI, 8.35-14.27), respectively (**Figure 2**). Patients who received an IO agent plus chemotherapy had an improved PFS (5.9 months [95% CI, 2.60-10.45] vs. 2.1 months [95% CI, 1.55-2.30], p<0.001) and OS (18.4 months [95% CI, 11.54-28.60] vs. 9.5 months [95% CI, 5.39-13.84] p=0.015) compared to those who received an IO agent without chemotherapy (**Table 2**, **Figure 3**). In subgroup analysis for patients with HR-positive/HER2-negative breast cancer,

**TABLE 1** | Baseline Patient Characteristics.

| | Total Patients | PFS <6 months | PFS ≥ 6 months | p-value |
|---|---|---|---|---|
| Total Number Patients (N) | 43 | 34 | 9 | – |
| Age (years) | | | | |
| Mean | 52.58 | 52.71 | 52.11 | 0.9167± |
| Range | (33-71) | (33-71) | (39-62) | |
| Sex | | | | |
| Male | 0 (0%) | 0 (0%) | 0 (0%) | – |
| Female | 43 (100%) | 34 (100%) | 9 (100%) | |
| Metastatic disease at diagnosis | 3 (7.14%) | 3 (9.09%) | 0 (0%) | 1.0000* |
| Number of Metastatic Locations | | | | |
| 1 | 9 (20.93%) | 6 (17.65%) | 3 (33.33%) | 0.4284* |
| 2 | 12 (27.91%) | 9 (26.47%) | 3 (33.33%) | |
| 3+ | 22 (51.16%) | 19 (55.88%) | 3 (33.33%) | |
| Lines of chemotherapy in metastatic setting | | | | |
| Mean | 2.14 | 2.09 | 2.33 | 0.8179± |
| Range | (0-7) | (0-5) | (0-7) | |
| Receptor status | | | | 1.0000* |
| HR+/HER2- | 24 (55.81%) | 19 (55.88%) | 5 (55.56%) | |
| HER2+ | 2 (4.65%) | 2 (5.88%) | 0 (0%) | |
| TNBC | 17 (39.53%) | 13 (38.24%) | 4 (44.44%) | |
| Treatment | | | | |
| PD-L1/PD-1 | 12 (27.91%) | 11 (32.35%) | 1 (11.11%) | 0.0015* |
| IO + Chemotherapy | 12 (27.91%) | 5 (14.71%) | 7 (77.78%) | |
| Other IO, No Chemo | 19 (44.19%) | 18 (52.94%) | 1 (11.11%) | |
| ECOG PS | | | | |
| 0 | 23 (53.49%) | 18 (52.94%) | 5 (55.56%) | – |
| 1 | 20 (46.51%) | 16 (47.06%) | 4 (44.44%) | |
| Radiation within 30 days of IO | 3 (6.98%) | 1 (2.94%) | 2 (22.22%) | 0.1060* |
| Lymphocyte count (k/uL) | | | | |
| Mean (SD) | 1.23 (0.68) | 1.13 (0.47) | 1.59 (1.14) | 0.2948± |
| Alkaline Phosphatase (U/L) | | | | |
| Mean (SD) | 99.72 (56.82) | 105.5 (62.42) | 77.89 (14.18) | 0.1389± |
| LDH (U/L) | | | | |
| Mean (SD) | 449.71 (787.83) | 500.22 (863.14) | 217.4 (99.02) | 0.6313± |

*Fisher Exact Test.*
*±Wilcoxon rank-sun Test.*
*ECOG PS, Eastern Cooperative Oncology Group Performance Status; SD, Standard Deviation.*

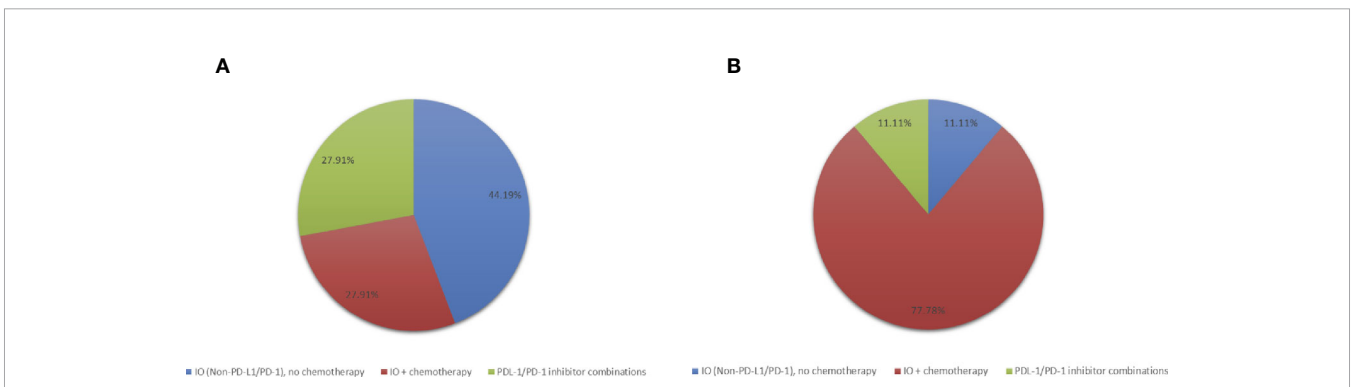


**FIGURE 1** | Pie-charts showing **(A)** Treatment distribution in all patients (N=43) **(B)** Treatment distribution in patients with PFS ≥ 6 months (N=9).

median PFS was prolonged in patients treated with IO plus chemotherapy compared to IO alone (5.6 months [95% CI, 2.6-8.1] vs. 2.2 months [95% CI, 2.0-2.4], p = 0.0096) (**Figure 3**). There was also a trend towards improved OS in these patients (17.2 months [95% CI, 11.5-31.0] vs. 11.0 months [95% CI, 5.4-14.8], p= 0.276). Similar findings were observed in patients with TNBC with improved median PFS (10.5 months [95% CI, 2.5-NE (Not Estimable)] vs. 1.8 months [95% CI, 0.6-2.5], p=0.008) and OS (24.2 months [95% CI, 6.6-NE] vs. 6.0 months [95% CI, 0.7-14.4], p=0.0193) in patients treated with IO plus chemotherapy versus IO alone (**Figure 3**).

We identified 9 patients (20.9%) with PFS ≥ 6 months which we considered to be consistent with clinical benefit (**Table 1**). Of these, 5 had HR-positive/HER2-negative breast cancer and 4

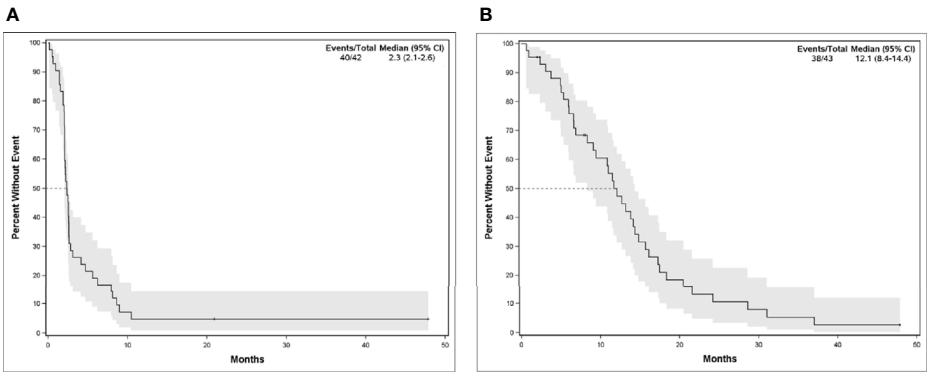


**FIGURE 2** | **(A)** Progression-Free Survival and **(B)** Overall Survival for Patients with Metastatic Breast Cancer in Phase I/Ib Clinical Trials Treated with IO Agents.

**TABLE 2** | Progression-Free Survival (PFS) and Overall Survival (OS) for Patients Who Received IO Plus Chemotherapy Compared to Patients Who Received IO Only.

| | IO + Chemotherapy | IO Only | p-value[2] |
|---|---|---|---|
| Median PFS (months)[1] | 5.88 (95% CI, 2.60-10.45) | 2.07 (95% CI, 1.55-2.30) | <0.001 |
| Median OS (months) | 18.38 (95% CI, 11.54-28.60) | 9.47 (95% CI, 5.39-13.84) | 0.015 |

*CI, confidence interval*
[1]*One observation dropped due to unknown reason going off-study in calculation of PFS.*
[2]*p-values generated using log-rank test.*

patients had TNBC. Patients with PFS ≥ 6 months, were treated with IO plus chemotherapy (N=7, 77.8%) and IO alone (N=2, 22.2%) (**Table 1**, **Figure 1**). Patients with PFS ≥ 6 months were more likely to receive an IO agent plus chemotherapy compared to those with PFS < 6 months (77.8% vs. 14.7%). No significant differences in prior lines of therapy, lymphocyte count, alkaline phosphatase, or lactate dehydrogenase (LDH) were identified between patients with PFS < 6 months and ≥ 6 months.

Five patients (11.6%) had PFS ≥ 9 months (range 9 months to >36 months) which we considered to be consistent with durable response. All but one of these patients were treated with IO plus chemotherapy and four of the five patients had TNBC. The best response observed in our study was in a 59-year-old woman with TNBC metastatic to her chest wall, lymph nodes and lungs who was treated with anti-PD-L1 and chemotherapy in the second-line setting. Chemotherapy was discontinued after 4 cycles due to neuropathy and she continued on single agent anti-PD-L1 for another 11 cycles before developing immune-mediated pneumonitis requiring discontinuation of immunotherapy. She had a complete clinical response to therapy and remains with no evidence of disease 3.5 years later.

## DISCUSSION

Our study looked at clinical outcomes in patients with previously-treated metastatic breast cancer treated in phase I clinical trials that included an IO agent. We included patients with all breast cancer subtypes treated with many different IO agents targeting PD-1/PD-L1, but also other immune checkpoints and cancer vaccines. Patients were previously treated with an average of 2 prior lines of chemotherapy in the metastatic setting, approximately 20% of patients with HR-positive/HER2-negative disease previously received CDK 4/6 inhibitors and 23.5% of patients with TNBC received prior sacituzumab govitecan. Our study demonstrates that regardless of breast cancer subtype or specific IO target, patients with metastatic breast cancer (TNBC or endocrine-resistant HR-positive/HER2-negative) treated with combinations of IO plus chemotherapy had prolonged PFS and OS compared to patients treated with IO agents alone. Our study found limited efficacy for IO agents administered without chemotherapy, including novel immune checkpoint inhibitor combinations, in patients with previously-treated metastatic breast cancer.

In our study, the median PFS for all patients with previously-treated metastatic breast cancer who received an IO agent in a phase I clinical trial was a modest 2.3 months. This is consistent with other reports of outcomes for similar patients treated in phase I trials (23). However, a unique finding of our study looking specifically at patients receiving IO agents was the observation of durable responses (PFS ≥ 9 months) in 11.6% of patients including one patient who experienced a durable remission lasting many years after stopping therapy for toxicity. Additionally, 20.9% of patients had PFS ≥ 6 months consistent with clinical benefit. Durable responses to immunotherapy observed in our study are consistent with what has been observed in other larger trials of IO agents in breast cancer and other solid tumors where durable remissions can occur even in patients with widespread metastatic disease (24–26).

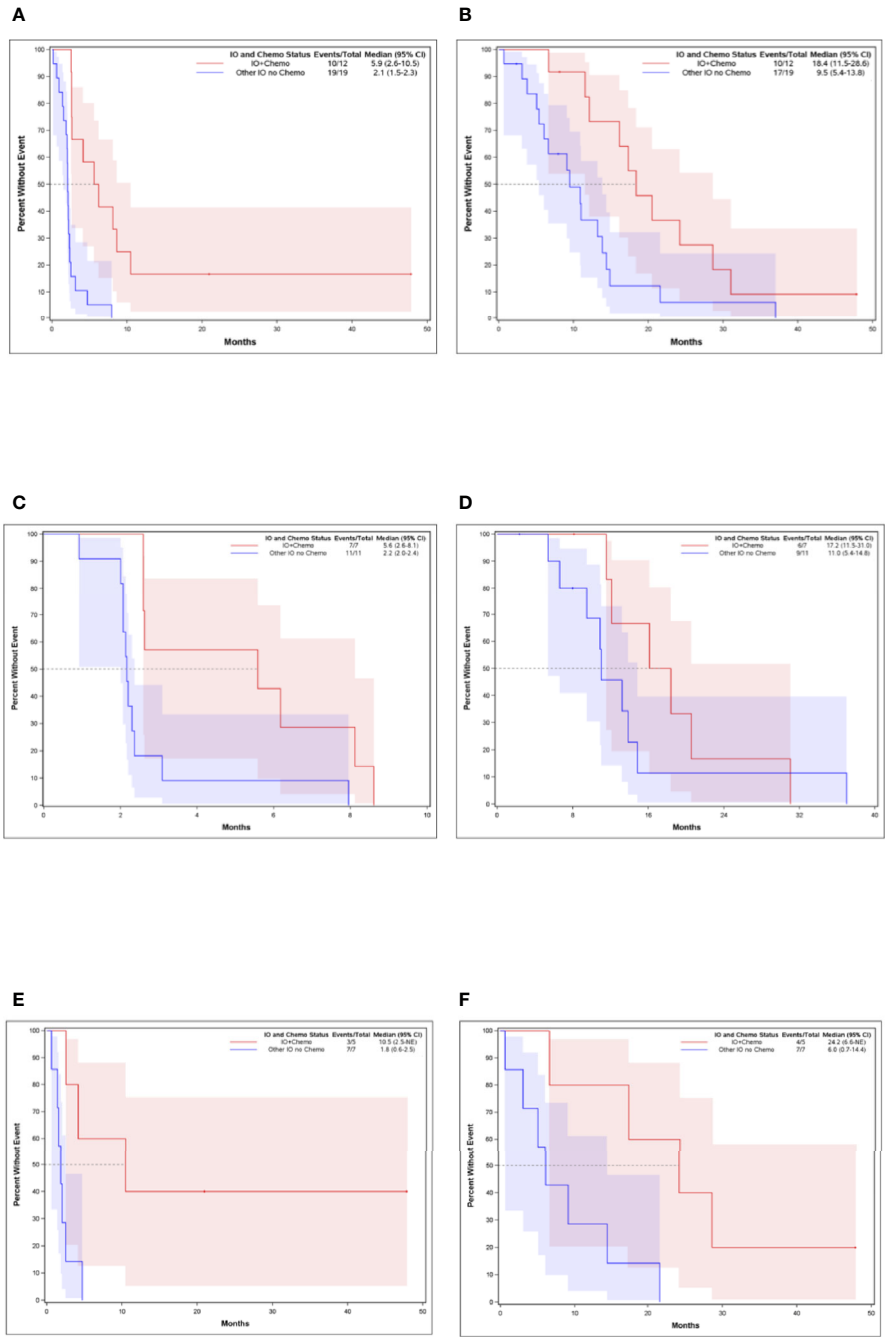


**FIGURE 3** | Kaplan-Meier Curves for Patients Who Received IO and Chemotherapy Compared to Patients Who Received IO Without Chemotherapy **(A)** Progression-Free Survival for all patients **(B)** Overall Survival for all patients **(C)** Progression-Free Survival for HR-positive, HER-2 negative breast cancer **(D)** Overall Survival for HR-positive, HER-2 negative breast cancer **(E)** Progression-Free Survival for TNBC **(F)** Overall Survival for TNBC.

The majority of patients in our study who experienced durable long-term responses (PFS > 9 months) were patients with metastatic TNBC treated with IO plus chemotherapy consistent with the now proven benefit of FDA-approved regimens in PD-L1-positive metastatic TNBC. Notably, the efficacy of the combination of atezolizumab and nab-paclitaxel in patients with PD-L1-positive TNBC was confirmed in the phase III first-line Impassion130 trial resulting in FDA-approval following the observation of preliminary efficacy in a Phase Ib trial including previously-treated patients (5, 26). Despite our

patients being somewhat heavily pretreated, a subset of patients with TNBC still derived long-term benefit from IO + chemotherapy when treated in a phase I clinical trial setting.

There were patients in our study with metastatic HR-positive/HER2-negative breast cancer, resistant to endocrine therapy, that derived benefit from IO plus chemotherapy including one patient with a durable response (PFS > 9 months) and 5 patients with PFS > 6 months. The efficacy of single agent PD-1/PD-L1-inhibitors in HR-positive HER2-negative breast cancer is modest with response rates lower than in TNBC (16, 17, 19, 20, 27, 28). Our results support the many ongoing clinical trials of IO agents in combination with chemotherapy in patients with endocrine-resistant HR-positive/HER2-negative breast cancer (22).

Clinical benefit in our study was greater in patients treated with IO plus chemotherapy and this finding was observed in patients with both endocrine-resistant HR-positive/HER2- breast cancer and TNBC which is also consistent with other studies in breast cancer demonstrating modest response rates with IO agents alone (16, 17, 19, 20, 26, 29, 30). Patients in our study with HR-positive/HER2-negative breast cancers, had a median PFS of 5.6 vs. 2.2 months (p=0.0096) in those treated with IO plus chemotherapy compared to IO monotherapy. OS was also improved however this result was not statistically significant.

There are few studies which have examined IO agents in combination with chemotherapy in a metastatic HR-positive/HER2-negative population. Interestingly, a recently released study examining survival of HR-positive/HER2-negative MBC patients treated with eribulin with or without pembrolizumab did not find improvement in OS or PFS in the IO plus chemotherapy group, which differs from our findings (31). In the I-SPY2 trial, the combination of pembrolizumab with chemotherapy led to a more than doubling of the pCR rate in patients with early stage HR-positive/HER2-negative cancers who had a MammaPrint that was not in the low risk range (21). The limited efficacy of IO monotherapy in HR-positive/HER2-negative breast cancer has been hypothesized to be potentially related to lower PD-L1 expression, tumor-infiltrating lymphocytes (TILs) and tumor mutation burden (TMB) in this disease subset (9, 31–33). Current data suggest that the addition of chemotherapy to IO agents may have multiple favorable effects including stimulation of the immune system by release of tumor neoantigens and recruitment of antigen-presenting cells (22, 34). Moreover, IO plus chemotherapy combination may delay the development of resistance to treatment (35).

Our study relays the importance of phase I clinical trials, often thought as a last resort for patients with advanced malignancy. Enrollment in phase I clinical trials remains a viable option for select patients with previously-treated metastatic breast cancer (23). Of the patients examined, 11.6% had durable response and one patient with metastatic TNBC remains disease free after 3.5 years. It is estimated that only 3-5% of United States adult cancer patients are enrolled in clinical trials (36). However, when comparing breast cancer (BC) patients to the general population, BC patients appear to obtain clinical benefit from phase I therapies with similar toxicity (36). As a result of phase I trials, atezolizumab with nab-paclitaxel is FDA approved for metastatic TNBC as is tucatinib in the treatment of HER2-positive MBC (5, 26, 37, 38). Phase I trials are important for discovering promising therapies and should continue to be utilized.

While our study showed benefit of IO plus chemotherapy in a metastatic TNBC and HR-positive population, there were several limitations. Limitations to our study included the retrospective nature of the analysis and our inability to include PD-L1-expression as a variable. We included patients treated with a diverse range of IO agents making our population heterogeneous and patients were all treated at a single academic center. There were overall very modest patient numbers and very few patients with HER2-positive breast cancer were treated in these studies. Another limitation to our study was that only a fifth of patients received prior therapy with CDK 4/6 inhibitors. As CDK 4/6 inhibitors are standard first line therapies, our study may not be able to be extrapolated to patients who did receive this therapy prior to IO.

The success of the combination of IO plus chemotherapy in TNBC highlights the potential for activity of new therapies in early phase clinical trials in carefully selected patients. Our study demonstrates that the benefit derived from novel IO agents is not limited to a TNBC population. Despite these benefits, larger, multi-center trials are needed in order to better understand the use of IO agents in all breast cancer subtypes.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Colorado Multiple Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AS, JD: manuscript writing and submission. AS, JD, JK, JW: material preparation and data collection. AN, DG: statistical analysis and figure development. JD, JK, VB, PK: study review and monitoring. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.640690/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

HYPOTHESIS AND THEORY
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# SOLTI-1805 TOT-HER3 Study Concept: A Window-of-Opportunity Trial of Patritumab Deruxtecan, a HER3 Directed Antibody Drug Conjugate, in Patients With Early Breast Cancer

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**Background:** Preclinical data support a key role for the human epidermal growth factor receptor 3 (HER3) pathway in hormone receptor (HR)–positive breast cancer. Recently, new HER3 directed antibody drug conjugates have shown activity in breast cancer. Given the need to better understand the molecular biology, tumor microenvironment, and mechanisms of drug resistance in breast cancer, we designed this window-of-opportunity study with the HER3 directed antibody drug conjugate patritumab deruxtecan (HER3-DXd; U3-1402).

**Trial Design:** Based on these data, a prospective, multicenter, single-arm, window-of-opportunity study was designed to evaluate the biological effect of patritumab deruxtecan in the treatment of naïve patients with HR-positive/HER2-negative early breast cancer whose primary tumors are ≥1 cm by ultrasound evaluation. Patients will be enrolled in four cohorts

according to the mRNA-based ERBB3 expression by central assessment. The primary endpoint is a CelTIL score after one single dose. A translational research plan is also included to provide biological information and to evaluate secondary and exploratory objectives of the study.

**Trial Registration Number:** EudraCT 2019-004964-23; NCT number: NCT04610528.

**Keywords: Breast Cancer, ERBB3, HER3, U3-1402, patritumab deruxtecan, HER3-DXd, CelTIL Score**

## INTRODUCTION

HER3, encoded by the *ERBB3* gene, is broadly expressed in various types of human cancer. HER3 has been associated with poor patient outcomes (1) and therapeutic agent resistance, including resistance to anti-EGFR, anti-HER2 inhibitors (2), and endocrine therapy (3, 4). HER3 belongs to the type I transmembrane tyrosine kinase family of receptors and activates intracellular signaling pathways, mainly the PI3K/AKT and MAPK/ERK pathways, upon dimerization with other HER family members (2, 5). These observations have resulted in the development of investigational HER3 directed agents in HER3-expressing breast cancer and other solid tumors.

Patritumab deruxtecan (HER3-DXd; U3-1402), a potential first-in-class HER3 directed antibody drug conjugate (ADC), is currently under development to act on these previously mentioned targets (6). In addition to its antitumor efficacy by binding HER3 ligand and the release of the cytotoxic payload in the tumor cells (7), patritumab deruxtecan enhanced the infiltration of innate and adaptive immune cells in preclinical models (8). These preclinical data have shown that patritumab deruxtecan can elicit potent antitumor immunity even in the setting of tumors insensitive to PD-1 and PD-L1 immune checkpoint inhibitors and that its efficacy is more pronounced in the presence of PD-1 inhibition, suggesting that patritumab deruxtecan sensitizes insensitive tumors to PD-1 blockade and has synergistic effects (8).

In the clinical setting, an early report of a clinical trial suggested that patritumab deruxtecan could be safely administered and it demonstrated promising antitumor efficacy (the overall response and the disease control rate were 42.9 and 90.5%, respectively) in heavily pretreated HER3-expressing metastatic breast cancer (9); these results are in accordance with more recent preliminary data from heavily pretreated EGFR-mutated non-small cell lung carcinoma patients, in whom the overall response rate was 25%, and the disease control rate was 70% (10).

Although no validated HER3 assay has been established to date, recent studies support the role of HER3 immunohistochemistry (IHC) as a potential biomarker (11–13). However, there are important limitations with IHC-based assays, such as different sensitivities of the antibodies used, their low dynamic range, their subjectivity in scoring, and their difficulty in establishing suitable cut-offs. Therefore, clinical implementation of a robust genomic assay would represent an important advancement. To overcome these limitations, we plan to test the prospective use of an mRNA-based *ERBB3* expression assay using the nCounter platform (Nanostring Technologies, Seattle, USA) developed by our group (14).

The role of the host immune system in breast cancer is becoming an important topic to study for several reasons. First, the immune response has a fundamental role in the efficacy of drug therapy. In all breast cancer subtypes, baseline high TIL grade is associated with a significantly higher pCR rate after neoadjuvant chemotherapy (15). Second, the recent success of therapeutic agents capable of activating immune responses to cancer, such as anti-PD1/PDL1 or anti-CTLA4 inhibitors, allows innovative treatment strategies (16). Third, high tumor-infiltrating lymphocytes (TILs) counts and immune-related gene expression signatures in the primary tumor are consistently associated with better survival in triple-negative breast cancer and HER2-positive breast cancer (15, 17–19). On the other hand, the prognostic value of assessing TILs in HR-positive/HER2-negative breast cancer remains unclear according to a few studies (15, 20).

The TOT-HER3 (a window-of-opportunity study of patritumab deruxtecan, a HER3 directed ADC in operable breast cancer according to *ERBB3* expression) trial is designed to assess whether a single dose of patritumab deruxtecan can increase immune infiltration and the lysis of tumor cells during short-term preoperative treatment in hormone receptor (HR)-positive/HER2-negative primary breast cancer. Short-term preoperative studies are a validated strategy for evaluating the impact of targeted therapies using the decrease in tumor cellularity and the increase in immune infiltration as a surrogate endpoint of treatment benefit (21, 22). The primary endpoint of TOT-HER3 is changes in the CelTIL score, a novel combined biomarker based on stromal TILs and tumor cellularity. Access to tumor tissue before and after the investigational treatment enables comprehensive analysis of biomarker changes, thus providing critical insights into the optimal patient population, biomarker predictive value, and potential mechanisms of primary resistance (23, 24).

## METHODS

### Study Design and Treatment

This is a prospective, multicenter, single-arm, window-of-opportunity study evaluating the biological effect of patritumab deruxtecan in treatment naïve patients with early breast cancer, whose primary tumors are $\geq$1 cm by ultrasound evaluation (**Figure 1**). The study will include up to 80 patients with HR-positive/HER2-negative tumors.

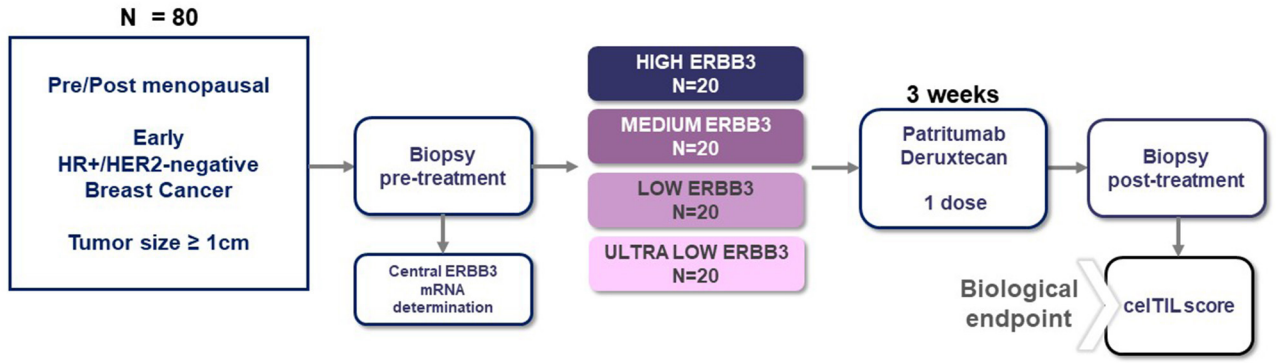


**FIGURE 1 |** TOT-HER3 trial design.

**TABLE 1 |** Main/key eligibility criteria.

| Inclusion Criteria | Exclusion criteria |
|---|---|
| 1. Written informed consent form.<br>2. Premenopausal or post-menopausal women and men, age ≥ 18 years.<br>3. ECOG Performance Status 0–1.<br>4. Histologically confirmed non-metastatic primary invasive adenocarcinoma of the breast untreated and recently diagnosed, with all the following characteristics:<br>- At least one lesion that can be measured in at least 1 dimension with ≥ 1 cm in the largest diameter measured by ultrasound.<br>- Absence of distant metastasis (M0) as determined by institutional practice.<br>- in the case of a multifocal or multicentric tumor, the largest lesion must be ≥1 cm and designated the "target" lesion for all subsequent tumor evaluations and biopsies.<br>5. Patient must have biopsiable disease.<br>6. Estrogen (ER)-positive and/or Progesterone (PgR)-positive and HER2-negative tumor by the most recent American Society of Clinical Oncology—College of American Pathologists (ASCO-CAP) guidelines: ER and PgR defined as IHC nuclear staining >1% and HER2 negative locally assessed.<br>7. Ki67% ≥ 10% locally assessed.<br>8. Available pretreatment FFPE core needle biopsy evaluable for PAM50 and *ERBB3* mRNA expression.<br>9. Baseline LVEF ≥ 50%<br>10. Adequate organ functions<br>11. Absence of any psychological, familial, sociological, or geographical condition potentially hampering compliance with the study protocol and follow-up schedule; those conditions should be discussed with the patient before registration in the trial. | 1. Inoperable locally advanced or inflammatory (i.e., inoperable stage III) breast cancer.<br>2. Bilateral invasive breast cancer.<br>3. Patients in whom a primary tumor excisional biopsy was performed.<br>4. Any prior treatment for primary actual invasive breast cancer.<br>5. Prior treatment with a HER3 antibody, topoisomerase I inhibitor, with an ADC that consists of an exatecan derivative that is a topoisomerase I inhibitor (e.g., DS-8201) and with a govitecan derivative (e.g., IMMU-132).<br>6. Medical history of symptomatic congestive heart failure or serious cardiac arrhythmia requiring treatment; myocardial infarction within 6 months prior to enrolment or unstable angina.<br>7. QT interval corrected using Fridericia's formula to >450 ms in males and > 470 ms in females.<br>8. Any factor that increases the risk of corrected QT interval prolongation or risk of arrhythmic events, such as congenital long QT syndrome, family history of long QT syndrome, or unexplained sudden death under 40 years of age in first-degree relatives.<br>9. Medical history of clinically significant lung diseases or who are suspected to have these diseases by imaging at the screening period.<br>10. Clinically significant corneal disease.<br>11. Known hypersensitivity to either the drug substance components or inactive ingredients in the drug product or history of severe hypersensitivity reactions to other monoclonal antibodies.<br>12. Clinically severe pulmonary compromise resulting from intercurrent pulmonary illnesses including, but not limited to, any underlying pulmonary disorder and any autoimmune, connective tissue, or inflammatory disorders with potential pulmonary involvement or prior pneumonectomy. |

Adult female patients (≥18 years old) with pre/post-menopausal status will be eligible if they have not been previously treated and have histologically confirmed stage I–IIIA invasive breast cancer, with primary tumors equal to or larger than 1 cm in diameter (as measured by ultrasound), clinical nodal status of 0–2, HR-positive and HER2-negative according to ASCO/CAP guidelines, and Ki67% ≥ 10% determined locally. Patients should also have an Eastern Cooperative Oncology Group (ECOG) performance status of 0–1 and adequate hematological counts, hepatic and renal function, and left ventricular ejection fraction ≥ 50%. Patients will be excluded if they have received prior anticancer therapy. Detailed inclusion and exclusion criteria can be found in **Table 1**.

All patients will undergo pretreatment tumor tissue acquisition. Central determination of *ERBB3* mRNA expression will be performed in FFPE core biopsies, and patients will be enrolled in four cohorts, according to the expression of *ERBB3* based in quartiles and defined by the pre-specified cutoffs, to ensure a broad representation of HR-positive/HER2-negative tumors with different *ERBB3* expression. The number of slots available per cohort will be limited to 20 patients each.

After confirmation of all the eligibility criteria, patients will be enrolled, and a single dose of patritumab deruxtecan will be

administered by intravenous infusion at a dose of 6.4 mg/kg. A second optional biopsy will be performed in the same lesion 3–7 days after patritumab deruxtecan's administration. A third biopsy post-treatment of the same lesion will be mandatory 21 (±3) days after the administration of patritumab deruxtecan, independently of the subsequent treatment. Thereafter, patients will be considered either for definitive surgery or primary medical treatment (e.g., neoadjuvant chemotherapy) at the discretion of the treating physician.

## Primary Endpoint—The CelTIL Score

To answer the primary objective of the trial, we will evaluate CelTIL score differences between baseline and post-treatment samples in all patients regardless of their *ERBB3* mRNA expression. The CelTIL score is based on the percentage (%) of tumor cellularity and the % of stromal TILs. Histopathologic analysis of the proportion of TILs will be done in whole sections of tumor tissue stained with hematoxylin and eosin (H&E). TILs will be quantified according to the 2014 guidelines developed by the International TILs Working Group (25). Percentages of TILs and tumor cellularity at baseline and D21 will be scored in slides of core biopsies from patients enrolled in the trial blinded from clinic–pathologic and outcome data.

The CelTIL score was developed based on day 15 tumor samples from the PAMELA trial (22). The neoadjuvant PAMELA trial treated 151 HER2+ breast cancer patients with trastuzumab-lapatinib (and endocrine therapy if HR-positive) (26). Tumor cellularity and the TILs score measured at day 15 following anti-HER2 therapy was associated with pathologic complete response (pCR). A combined score, CelTIL, considering both variables was derived: CelTIL score = $-0.8 \times$ tumor cellularity (in %) + $1.3 \times$ TILs (in %). The CelTIL score was validated in the PAMELA (26) and LPT109096 (27) phase II neoadjuvant trials as an early readout of the probability of a pCR. High CelTIL scores identify tumors that have high immune infiltration and reduced tumor cellularity (22).

In a third study, the CelTIL score was performed in tumor samples of 196 patients with early-stage HER2-positive disease treated with standard trastuzumab-based chemotherapy from the NeoALTTO phase III trial (28). This study randomized 455 women with HER2-positive early breast cancer to lapatinib (Arm A), trastuzumab (Arm B), or trastuzumab and lapatinib (Arm C) for 6 weeks, followed by an assigned anti-HER2 treatment combined with paclitaxel weekly. The CelTIL score was independently associated with event free survival, overall survival, and pCR (29). Early and absolute changes in the CelTIL score following neoadjuvant therapy were associated with tumor shrinkage at surgery in other three neoadjuvant trials (30). Taken together, these results demonstrated that high TILs and low tumor cellularity following one cycle of treatment provided

**TABLE 2 |** Primary and secondary objectives and endpoints.

| **Primary objective** | **Primary endpoint** |
|---|---|
| To evaluate if one dose of U3-1402 increases the value of the CelTIL score between baseline and post-treatment samples in all included patients with early breast cancer. | Mean change in the CelTIL score per central assessment in paired samples after one dose of U3-1402 at C1D21 (±3).<br>CelTIL score = $-0.8 \times$ tumor cellularity (in %) + $1.3 \times$ TILs (in %). The minimum and maximum unscaled CelTIL scores will be −80 and 130. This unscaled CelTIL score will then be scaled to reflect a range from 0 to 100 points. |
| **Secondary objectives** | **Secondary endpoints** |
| To identify a significant increase in the CelTIL score after one dose of U3-1402 between baseline and post-treatment samples within each of the four *ERBB3* cohorts. | Mean change in the CelTIL score at C1D21 of treatment in paired samples in ultralow, low, medium, and high *ERBB3* cohorts. |
| To determine the association of the levels of baseline *ERBB3* expression with changes in the CelTIL score after one dose of U3-1402 in all patients and within each ERBB3 cohort. | Correlation between *ERBB3* mRNA baseline levels and changes in the CelTIL score at C1D21 in paired samples in all patients and in ultralow, low, medium, and high ERBB3 cohorts. |
| To determine the association of HER3 IHC expression with changes in the CelTIL score after a single dose of U3-1402 in all patients and within each *ERBB3* cohort. | Correlation between HER3 IHC levels per central assessment and changes in the CelTIL score at C1D21 in paired samples in all patients and in ultralow, low, medium, and high *ERBB3* cohorts. |
| To evaluate the changes in CelTIL across the four PAM50 intrinsic subtypes. | CelTIL score at the C1D21 score according to intrinsic subtype: Luminal A, Luminal B, HER2-enriched, and Basal-like subtypes. |
| To evaluate the antiproliferative activity of one dose of U3-1402 between baseline and post-treatment samples. | Complete Cell Cycle Arrest (CCCA) determined per central assessment by IHC Ki67 < 2.7% at C1D21. |
| | Differences in differential expression [mean suppression = 100–[geometric mean (post-treatment/pre-treatment 100)]] of proliferative genes (*BIRC5, CCNB1, CDC20, CDCA1, CEP55, KNTC2, MKI67, PTTG1, RRM2, TYMS,* and *UBE2C*). |
| To evaluate the association of *ERBB3* mRNA expression with HER3 IHC expression. | Correlation coefficients between both biomarkers. |
| To evaluate the changes of HER3 expression. | HER3 IHC at baseline, at D3-D7 (optional), C1D21. |
| To describe the safety and tolerability of U3-1402. | Type, incidence, severity (as graded by the NCI CTCAE v. 5.0), seriousness, and attribution to the study medications of AEs and any laboratory abnormalities. |

independent and additional predictive information in patients with primary breast cancer following neoadjuvant treatment, also suggesting that CelTIL could be a surrogate for treatment efficacy in the neoadjuvant setting.

Secondary endpoints, summarized in **Table 2**, include mean change in the CelTIL score in ultralow, low, medium, and high ERBB3 cohorts, correlation between *ERBB3* mRNA and HER3 IHC baseline levels and changes in the CelTIL score, the CelTIL score according to PAM50 intrinsic subtype, antiproliferative activity, and safety. Exploratory and translational research endpoints include the assessment of predictive and prognostic biomarkers.

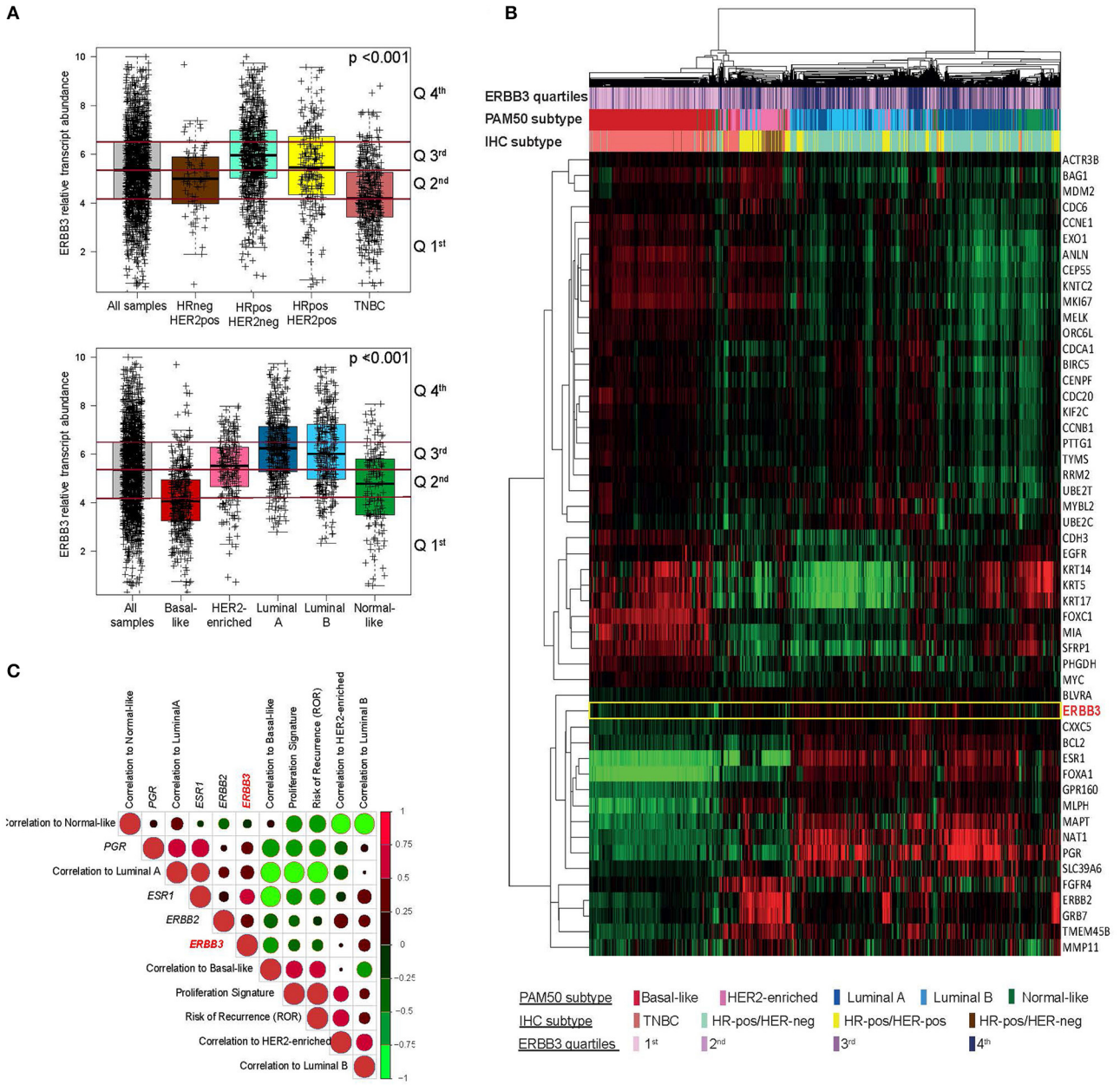


**FIGURE 2 |** Measurement of ERBB3 expression in breast cancer using the nCounter platform. **(A)** Box plots of *ERBB3* gene expression in breast tumors as classified by hormone receptor and *HER2* expression and intrinsic subtype. **(B)** Unsupervised hierarchical clustering using the 50 PAM50 genes and *ERBB3* (rows) and 1,580 tumor samples (columns). Each colored square on the heatmap represents the relative median signature score for each sample with the highest expression being red, the lowest expression being green, and the average expression being black. **(C)** Pearson correlation between *ERBB3*, single genes, and PAM50 gene expression signatures evaluated in breast cancer samples.

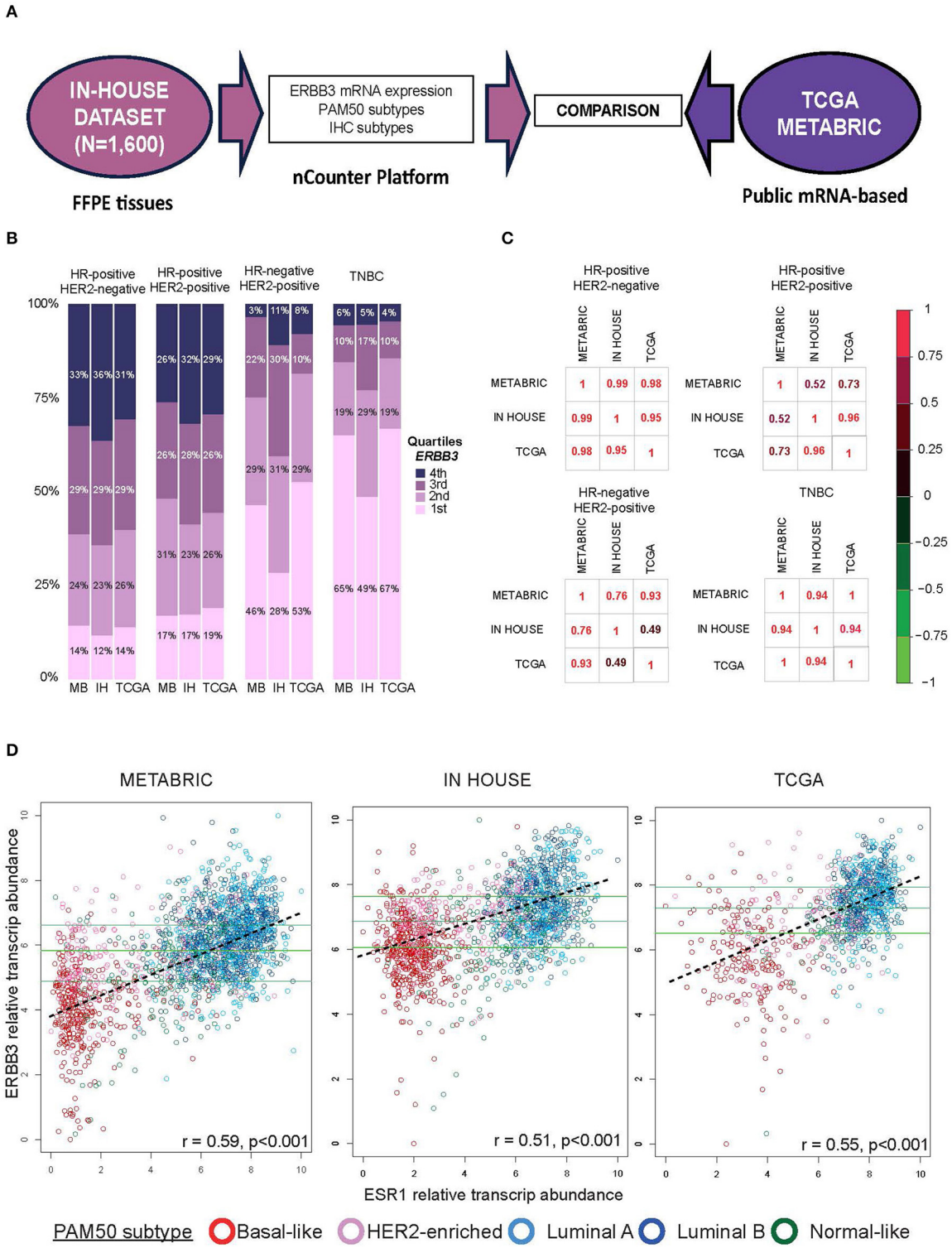


**FIGURE 3 |** Comparing *ERBB3* expression across datasets **(A)** Evaluation of *ERBB3* cutoff in breast cancer samples from patients with early breast cancer included in IN-HOUSE, METABRIC, and TCGA. **(B)** Proportion of samples in each immunohistochemistry subtype based on the ERBB3 cohort. Each bar is colored according to the ERBB3 distribution in each cohort. **(C)** Correlation coefficients of proportions of tumor samples within each quartile based on the IHC subtype between the three datasets. **(D)** Scatter plots of *ERBB3* vs. *ESR1* expression for samples from METABRIC, IN-HOUSE, and TCGA cohorts, colored by subtype. The three horizontal lines indicate the cutoffs of each cohort. Discontinued line in each figure represents the regression line. Pearson correlation coefficient (r) with significance (*p*-value) is presented in each figure.

## Measuring *ERBB3* mRNA

Each patient will be assigned to one of the four cohorts according to their *ERBB3* mRNA expression in the baseline sample determined by the nCounter Platform. The cutoffs to be used in this trial were determined as follows.

To date, we have analyzed *ERBB3* mRNA using the nCounter platform in 1,600 tumor samples using formalin-fixed paraffin-embedded tumor samples with IHC data. Among these samples with IHC data, 65% were HR-positive and 18% were HER2-positive. The IHC subtype distribution is as follows: (1) 51.9% HR-positive/HER2-negative, (2) 29.9% triple-negative breast cancer (TNBC), (3) 13.5% HR-positive/HER2-positive, and (4) 4.7% HR-negative/HER2-positive.

In this nCounter dataset, the range of *ERBB3* mRNA expression has an 18.6-fold difference in gene expression (i.e., from the lowest to the highest *ERBB3* value), and the interquartile range is 1.5 (in log base 2), which is equal to a difference in expression of 2.9-fold.

Large expression variability across and within each IHC-based and PAM50 subtype was observed. *ERBB3* expression was statistically significantly higher in HR-positive tumors ($P < 0.001$; **Figure 2A**). *ERBB3* expression varied statistically significantly according to the intrinsic subtype ($P < 0.001$; **Figure 2A**), with the Luminal A subtypes showing the highest median expression, followed by the Luminal B, HER2-enriched, and Basal-like.

Using quartiles, the proportion of *ERBB3*-high tumors within each IHC subtype ranged from 4% in TNBC to 36% in HR+/HER2-negative when percentile 75th in the combined matrix was used as the cutoff to define *ERBB3*-high (**Figure 2A**).

Next, we explored the association of *ERBB3* expression with PAM50 breast cancer-related genes in the combined matrix (**Figure 2B**). As expected, *ERBB3* high correlated [correlation coefficients [r] $> 0.50$] with a group of five genes, including *ESR1* and *FOXA1*, which are significantly enriched in luminal and hormone response biology. Concordant with this single-gene analysis, moderate correlation ($r = 0.53$) was found between *ERBB3* and PAM50 Luminal A signature and negative correlation ($r = -0.25$) between *ERBB3* and PAM50 Basal-like, proliferation, and risk of recurrence signatures (**Figure 2C**).

## Evaluating *ERBB3* Expression in Independent Datasets

In order to examine the consistency of the cutoff points, results from the in-house nCounter dataset were compared to two independent cohorts (i.e., METABRIC and TCGA datasets). METABRIC includes 1,992 breast cancer samples analyzed by the Illumina HT 12 IDATS platform, and TCGA includes 1,101 breast cancer samples analyzed by HiSeq Illumina sequencers (**Figure 3A**).

Using quartiles, **Figure 3B** shows the proportion of tumors within each quartile based on their IHC subtype between our in-house dataset, METABRIC, and TCGA. **Figure 3C** shows the correlation coefficients among the three datasets in the different IHC-group tumors. In HR-positive/HER2-negative, the correlation coefficients of the proportions between the three datasets were remarkably similar. In the other subtypes, the correlation coefficients among the datasets were between 0.49 and 0.99. A relationship between *ERBB3* and *ESR1* expression was seen to be moderately correlated across the three datasets (**Figure 3D**); the correlation coefficients among the datasets were between 0.51 and 0.59.

## Statistical Analysis

The study would require a sample size of 72 (number of pairs samples) to achieve a power of 80% using a level of significance of 5% (two sided), for detecting a mean difference between pairs of 13 CelTIL score. It is assumed that the standard deviation of the differences is 38.6, which is the standard deviation observed in 403 patients with CelTIL data across the four SOLTI trials (30). Assuming a 10% drop-out or lack of available tissue, 80 patients will be recruited.

No formal statistical comparison will be carried out between cohorts. Statistical analyses will be performed to estimate the proportions or means (or medians) for all variables including confidence interval calculations.

# CONCLUSION

We propose the TOT-HER3 study, the first window of opportunity trial to evaluate the biological effect of patritumab deruxtecan in patients with HR-positive/HER2-negative early breast cancer. High *ERBB3* mRNA gene expression is observed across all subtypes of breast cancer, although it predominates in HR-positive/HER2-negative disease suggesting a role for HER3 directed therapies in this disease. We will analyze *ERBB3* expression using a clinically applicable assay in FFPE primary tumors.

This information can provide insight for improving the design of future clinical trials in the HR-positive/HER2-negative breast cancer through the selection of patients who will mostly benefit from this drug. The use of a quantitative method such as *ERBB3* mRNA expression, which offers the opportunity to identify different cutoffs, might potentially improve treatment personalization. In addition, the results of TOT-HER3 could help identify patients most likely to benefit from HER3 directed ADCs across cancer types.

# DATA AVAILABILITY STATEMENT

Data from Breast tumor samples with available RNASeqv2 data at the TCGA portal was downloaded. Metabric expression data are available at the European Genome-Phenome Archive (https://ega-archive.org/), which is hosted by the European Bioinformatics Institute, under accession number EGAS00000000083. The rest of the data are available upon reasonable request.

## AUTHOR CONTRIBUTIONS

All authors participated in the design and/or interpretation of the reported results and participated in the acquisition and/or analysis of data. In addition, all authors participated in drafting and/or revising the manuscript and provided administrative, technical, or supervisory support.

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# A Case Series of Metastatic Metaplastic Breast Carcinoma Treated With Anti-PD-1 Therapy

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Metaplastic breast cancer is a rare and often chemo-refractory subtype of breast cancer with poor prognosis and limited treatment options. Recent studies have reported overexpression of programmed death ligand 1 (PD-L1) in metaplastic breast cancers, and there are several reports of anti-PD-1/L1 being potentially active in this disease. In this case series, we present 5 patients with metastatic metaplastic breast cancer treated with anti-PD-1-based therapy at a single center, with 3 of 5 cases demonstrating a response to therapy, and one of the responding cases being a metaplastic lobular carcinoma with low-level hormone receptor expression. Cases were evaluated for PD-L1 expression, tumor infiltrating lymphocytes (TILs), DNA mutations, RNA sequencing, and T-cell receptor sequencing. Duration of the response in these cases was limited, in contrast to the more durable responses noted in other recently published reports.

**Keywords: metaplastic breast cancer, TNBC, immunotherapy, PD-L1, PI3K**

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## INTRODUCTION

Metaplastic breast cancer (MBC) is a rare and aggressive subtype of breast cancer, comprising approximately 1% of all breast cancers, and is defined histologically as tumors that have epithelial differentiation into squamous and/or mesenchymal components, with multiple components often co-existing in the same tumor (1, 2). The current WHO classification of breast tumors further divides metaplastic carcinoma into additional subtypes: low grade adenosquamous, fibromatosis-like metaplastic, squamous cell, spindle cell, metaplastic with mesenchymal differentiation (including chondroid, osseous, or other types), mixed metaplastic, and myoepithelial carcinomas (3). There is limited understanding of the prognostic implications of various subtypes, and therefore are all clinically treated as a single entity (4). MBCs tend to present with a larger size, less frequent axillary nodal involvement, and have a higher rate of developing distant metastasis compared to other breast cancers (5, 6). They are frequently negative for estrogen receptor (ER), progesterone receptor (PR), and Human epidermal growth factor 2 (HER2) overexpression, with 85-89% of cases noted to be triple negative in recent analyses (6–9). However, compared to other triple negative breast cancers (TNBC), MBCs tend to have worse outcomes across all clinical stages, with 3-year overall survival for stage IV disease of 15% vs 22% for TNBC, and 64% for all other breast cancer types in one recent analysis of the National Cancer Database (10). MBCs also have poor response

rates to cytotoxic chemotherapy compared to other types of breast cancer (5, 11, 12). As a result, there has been interest in evaluating novel strategies, including targeted therapies and immunotherapy (12, 13). The potential utility of immunotherapy for this disease has been highlighted by recent reports of metastatic MBC with durable responses to immune checkpoint blockade (14–16). Here, we present a case series of 5 patients with metastatic MBC treated with anti-PD-1 therapy.

# MATERIALS AND METHODS

## Patients

4 of the 5 patients were treated on a phase 1b trial evaluating the safety of paclitaxel or capecitabine in combination with the anti-PD-1 antibody, pembrolizumab. Inclusion criteria for this trial included ER/PR <1% by IHC, HER2 negative (IHC 0-1 or IHC2 with ISH HER2/CEP17 <2), measurable disease by RECISTv1.1, ECOG 0-1, and investigator-determined indication for paclitaxel or capecitabine in the 1st or 2nd line setting (17). One additional patient was treated with compassionate use nivolumab with bicalutamide and was not part of the trial. Because bicalutamide was discontinued shortly after commencing therapy, this case is still described in the series. Baseline biopsies prior to receiving anti-PD-1 therapy were available for all patients, as were post-treatment biopsies for Cases 1 and 3. All biopsies were reviewed by a pathologist to confirm the diagnosis of MBC (**Figures 1A**, **2A**, **3A**, **4A**, **5A**). All biopsies were also evaluated for PD-L1 expression in both tumor cells and immune cells with the Ventana PD-L1 SP263 assay and were reviewed by a pathologist for scoring (**Figures 1B**, **2B**, **3B**, **4B**, **5B**). A combined positive score (CPS), defined as the total number of PD-L1 staining cells (tumor cells, lymphocytes, and macrophages) divided by the total of viable tumor cells, multiplied by 100, is reported, with a CPS ≥ 1 considered positive per manufacturer insert, though recent trials in breast cancer have identified a higher cut-off of CPS ≥10 for clinical activity (18, 19). TILs were also scored by a pathologist per the International TILs Working Group guidelines for evaluating TILs in breast cancer (20).

## Biomarker Assessment

When tissue was available, additional exploratory biomarker immune profiling was conducted. Cases 2, 4, and 5 were evaluated with a multiplexed immunofluorescence (mIF) panel as part of the clinical trial of pembrolizumab + chemotherapy in which they were enrolled (17). These cases were compared to the non-metaplastic TNBC cases from the same clinical trial, also evaluated with mIF.

5μm Formalin Fixed Paraffin Embedded (FFPE) slides were stained and microwave treated in citrate buffer pH 6.0 to present cross-reactivity between antibodies. Tissue slides were incubated with DAPI as counterstain and coverslipped with VectaShield mounting media (Vector Labs). Whole slides were scanned and digitized at 10x magnification (PerkinElmer Vectra 3.0) for gross visualization of the tumor, with regions of interest scanned at 20x ($0.36mm^2$) for quantification. The maximum possible number of non-overlapping regions of interest, as determined as areas with viable tumor and visible immune cells, were obtained for each slide. InForm software (PerkinElmer, package 2.4) was used according to manufacture instructions to segment and phenotype cells, with cells identified as cytokeratin-positive tumor cells, CD3-positive CD8-negative FoxP3-negative T-cells (Helper T-cells), CD3-positive CD8-positive T-cells (Cytotoxic T-cells), CD3-positive FoxP3-positive T-cells (Regulatory T-cells), and CD163-positive cells (Macrophages). PD-L1 quantitative immunofluorescence was also measured for each cell, which recent studies have found to be comparable to clinical PD-L1 scores (21, 22).

## Genomic Assessment

Cases were evaluated for targetable DNA mutations with a solid tumor mutation panel, although the commercial panels used varied as they were ordered at the discretion of the treating physician. All panels were processed similarly, with FFPE tissue sections examined by a pathologist and genomic DNA extracted from areas of viable tumor. Mutations were screened for by massively-parallel sequencing-by-synthesis.

RNA sequencing was performed on Cases 1, 2, 4, and 5 as part of exploratory analyses of the clinical trial. FFPE tissue sections were deparaffinized followed by RNA extraction and purification using the Qiagen AllPrep DNA/RNA FFPE kit. 85ng of input RNA was used to prepare sequencing libraries using the Illumina TruSeq RNA Exome kit. Sequencing of the RNA Exome libraries was performed on the Illumina HiSeq 4000 instrument at 2 x 76 read paired end configuration. Gene expression counts were quantified using salmon-v.0.11.2 (23). Differential gene expression analysis was performed using the R software package edgeR (24). Previously identified genes of interest in MBC were evaluated, including *AKT1, CCND3, CCNE1, CDK2NB, CDKN2A, CREB1, CREBBP, EGFR, KDM6A, KMT2D-MLL2, MKI67 (Ki-67), MTOR, MYC, Nanog, NF2, CD274 (PD-L1), PI3K, PIK3RI, PTEN*, and *TP53* (8, 9, 25, 26).

Peripheral blood T-cell receptor (TCR) sequencing was performed in cases 1, 2, 4, and 5, and on n=21 non-metaplastic metastatic TNBC patients from the phase Ib trial. Peripheral blood mononuclear cells (PMBCs) were collected at baseline and at regular intervals during treatment, and T-cell DNA was extracted and submitted for deep sequencing using the immunoSEQ Assay (Adaptive Biotechnologies). T-cell richness was estimated by the nonparametric model iChao1 function, and clonality index was calculated as the square root of the Simpson's diversity index.

## Statistical Methods

For the purpose of hypothesis generation, immune and genomic profiles were constructed for individual patients using the above biomarkers data. For each biomarker outcome, raw scores were converted into modified z-scores, based upon underlying median and median absolute deviations of the outcomes across a cohort of TNBC patients treated on the aforementioned phase Ib chemo-immunotherapy clinical trial. Because of the limited sample size, this analysis was conducted primarily for hypothesis generation and to identify possible outlier features of the case tumors, which could potentially assist with characterizing the unique clinical response profiles of each case in the series.

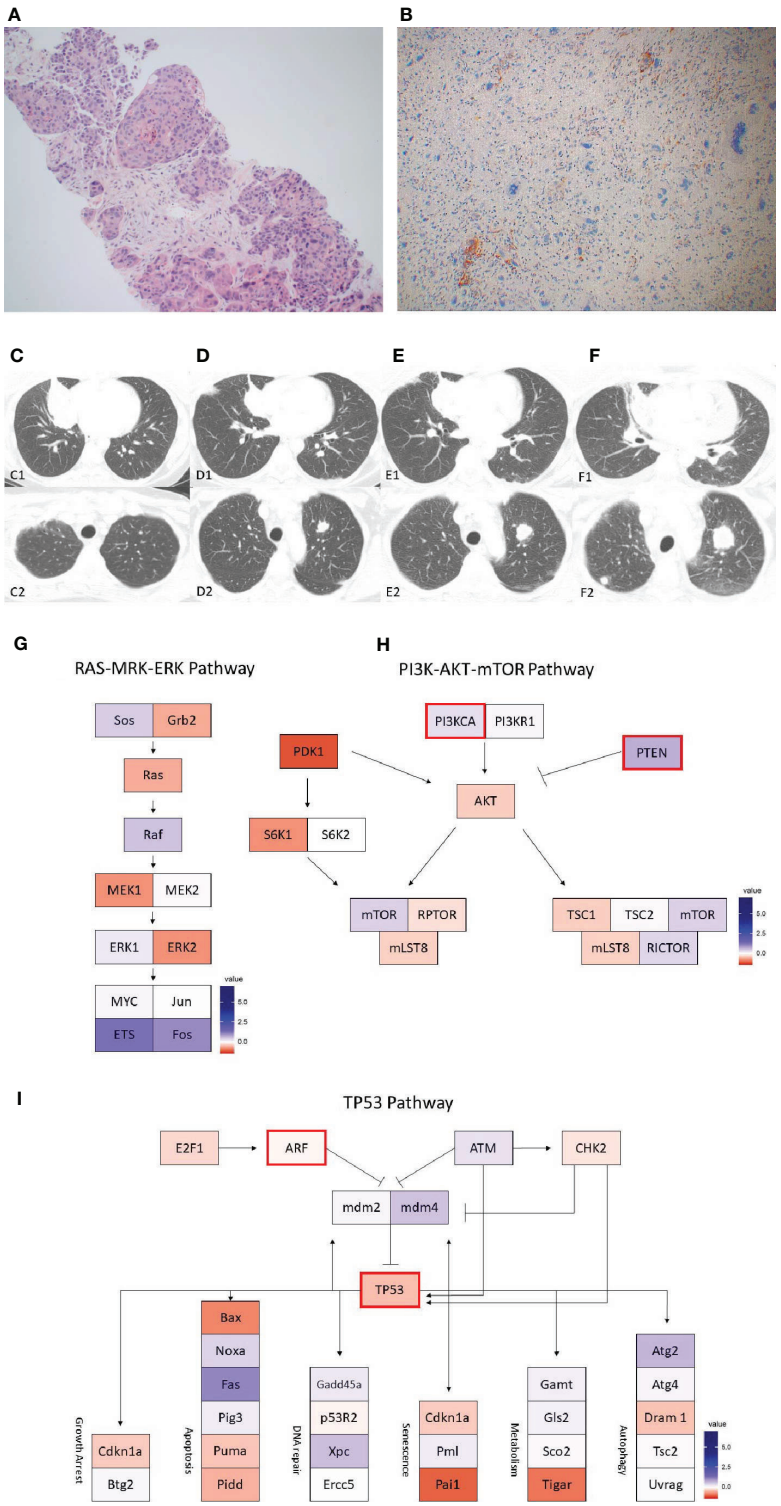


**FIGURE 1** | Case 1 **(A)** H&E image, showing metaplastic carcinoma with chondroid differentiation **(B)** PD-L1 by the Ventana PD-L1 SP263 assay **(C–F)**. Radiographic changes in Case 1 from **(C)** week 0, **(D)** 12 weeks, **(E)** 16 weeks, and **(F)** 24 weeks. Images C1 to F1 showing regression of the dominant right lung mass, then regrowth. Images C2 to F2 showing growth of an initially non-target left lung nodule. Images **(G–I)** show RNA expression heatmaps with modified z-scores of expression *vs.* non-metaplastic TNBC cases in pathways of interest for metaplastic breast cancer **(G)** RAS-MEK-ERK, **(H)** PI3K-AKT-mTOR **(I)** TP53. Genes with DNA mutations are outlined in red.

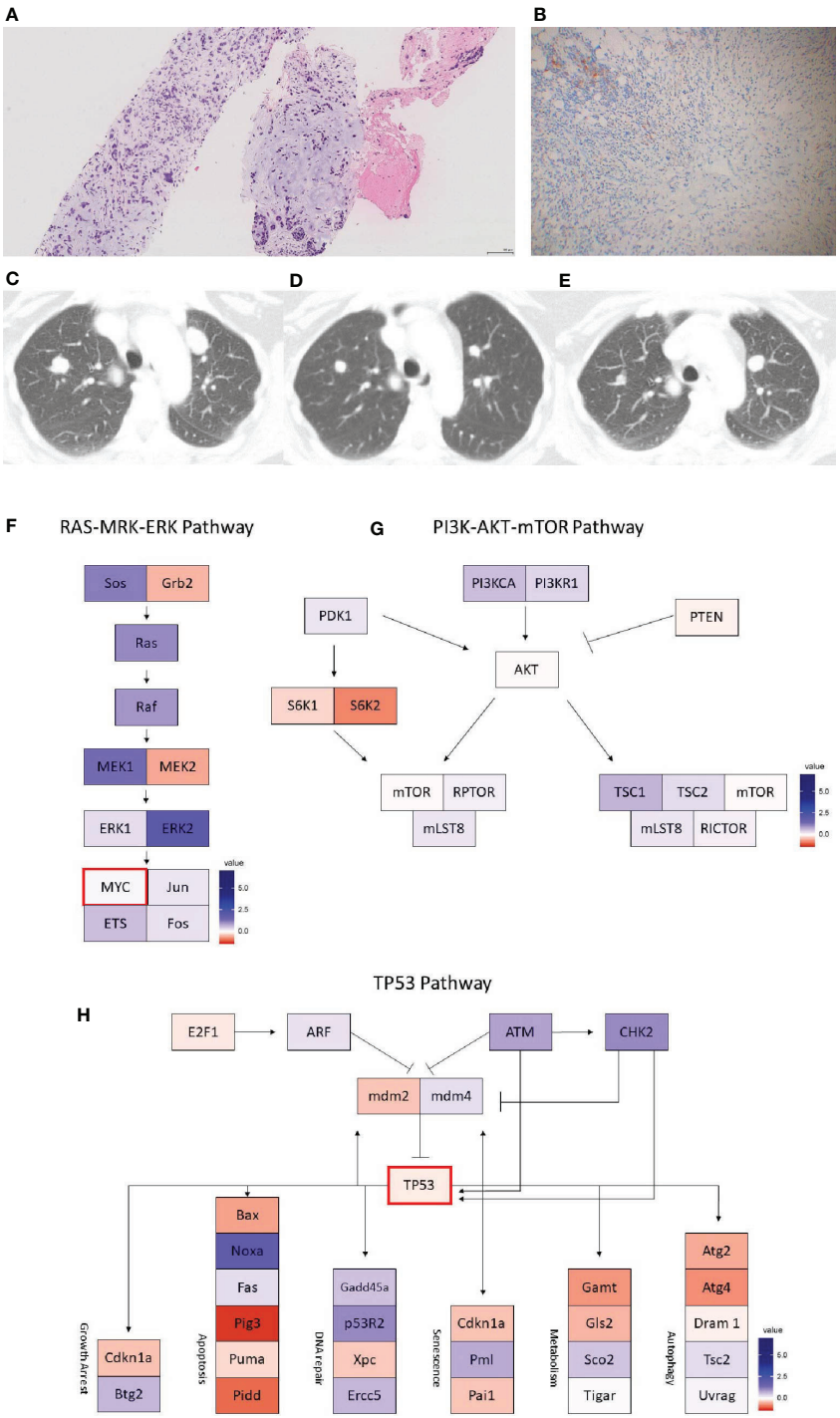


**FIGURE 2** | Case 2 **(A)** H&E image, showing metaplastic squamous carcinoma **(B)** PD-L1 by the Ventana PD-L1 SP263 assay **(C–E)**. Radiographic changes from **(C)** week 0, **(D)** 12 weeks, and **(E)** 24 weeks. A mixed, but overall partial response by RECIST criteria is noted initially **(D)** followed by progression **(E)**. Images **(F–H)** show RNA expression heatmaps with modified z-scores of expression *vs.* non-metaplastic TNBC cases in pathways of interest for metaplastic breast cancer **(F)** RAS-MEK-ERK, **(G)** PI3K-AKT-mTOR **(H)** TP53. Genes with DNA mutations are outlined in red.

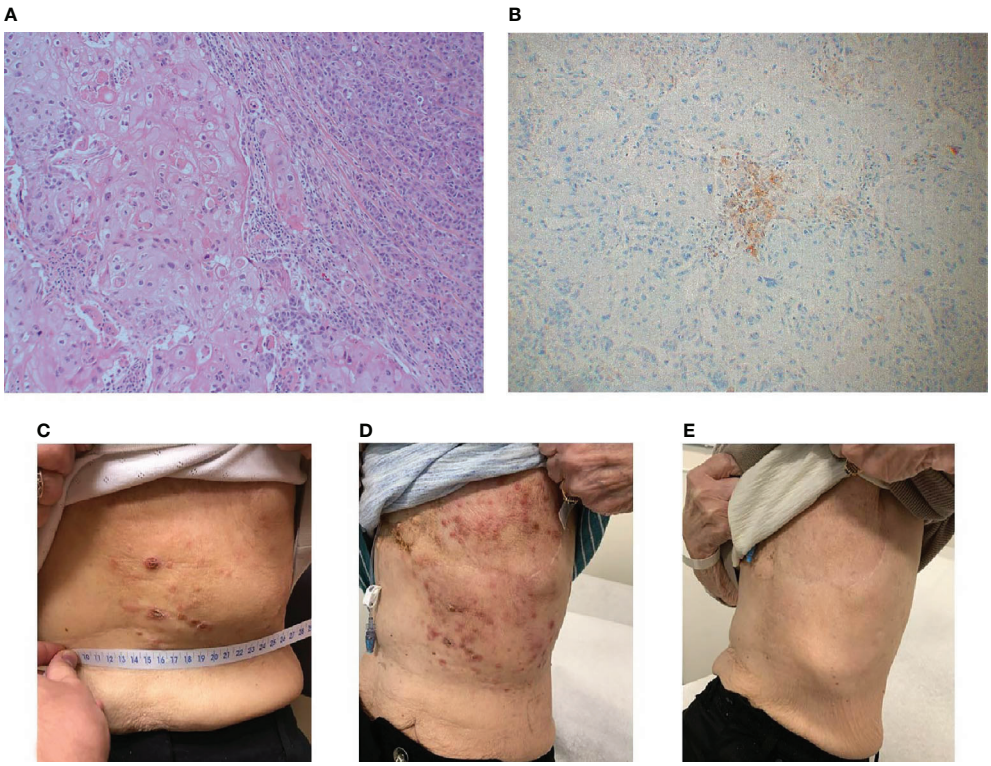


**FIGURE 3** | Case 3 **(A)** H&E image, showing mixed metaplastic squamous carcinoma and pleomorphic invasive lobular carcinoma **(B)** PD-L1 by the Ventana PD-L1 SP263 assay **(C–E)**. Lesions at baseline **(C)** initially appeared worsened at 4 weeks **(D)**, then demonstrated a complete clinical response by week 14 **(E)**.

# RESULTS

## Case 1

The patient is a 63-year-old woman found to have a right breast mass on screening mammography, with biopsy showing a grade 3 invasive ductal carcinoma, ER-, PR-, HER2- (2+ IHC, ISH 3.04, ratio1.27). MRI additionally noted a small enhancing mass of the left breast, biopsy showing a concurrent grade 1 invasive ductal carcinoma with associated low-grade DCIS, ER >95%, PR 30%, HER2- (1+ IHC). She was treated with neoadjuvant therapy on the I-SPY trial with paclitaxel + ganetespib followed by doxorubicin + cyclophosphamide with a brief clinical response, followed by re-growth. She underwent bilateral mastectomy and sentinel lymph node biopsy, pathology consistent with metaplastic carcinoma, 3.1 cm x 2.8 cm with lymphovascular invasion, negative for perineural invasion, 2/2 intramammary lymph nodes involved with no extracapsular extension, 0/7 axillary nodes positive, 1/2 sentinel nodes with micro-metastatic carcinoma, no extracapsular extension, 0/11 additional axillary lymph nodes, and an RCB score of 3.835, class RCB-III (corresponding with suboptimal response and prognosis) (27). No residual carcinoma was detected on the left, 0/2 sentinel lymph nodes involved. She received adjuvant radiation therapy. However, follow up imaging noted an 8.3 cm right middle lobe perihilar mass with complete occlusion of the bronchus intermedius. Biopsy was obtained by bronchoscopy, with pathology showing a poorly differentiated malignant neoplasm consistent with metaplastic breast cancer, ER-, PR-, HER2-. She received palliative bronchoscopic debulking.

She was enrolled in a phase Ib trial, receiving pembrolizumab (200 mg IV every 3 weeks) with capecitabine (2000mg twice daily by mouth on days 1-7, every 2 weeks) (17). Per trial protocol, CT imaging of the chest, abdomen, and pelvis were obtained at baseline and every 12 weeks thereafter to assess for response by RECIST v1.1. Imaging at 12 weeks showed an overall partial response, though with mixed findings showing significant shrinkage of her dominant tumor, but enlargement of a left lung lesion (**Figures 1C–F**). The left lung lesion was biopsied and was consistent with metaplastic breast cancer. She received palliative radiation to her right lung mass. On follow up at 23 weeks, had developed new scalp lesions, which were biopsied and consistent with metaplastic breast cancer. She subsequently enrolled in hospice.

PD-L1 expression and TILs were evaluated by a pathologist on pre- treatment and post- treatment biopsies. PD-L1 expression on tumor cells was 0% on both pre- treatment and post- treatment biopsies, but 10% and 40% respectively on immune cells. CPS measured 5 on the pre-treatment biopsy and 1.5 on post-treatment biopsies, both above the threshold for positivity of ≥ 1, but below the ≥10% threshold. TILs were 20% in the pre-treatment biopsy but decreased to 1% in the post-treatment biopsy.

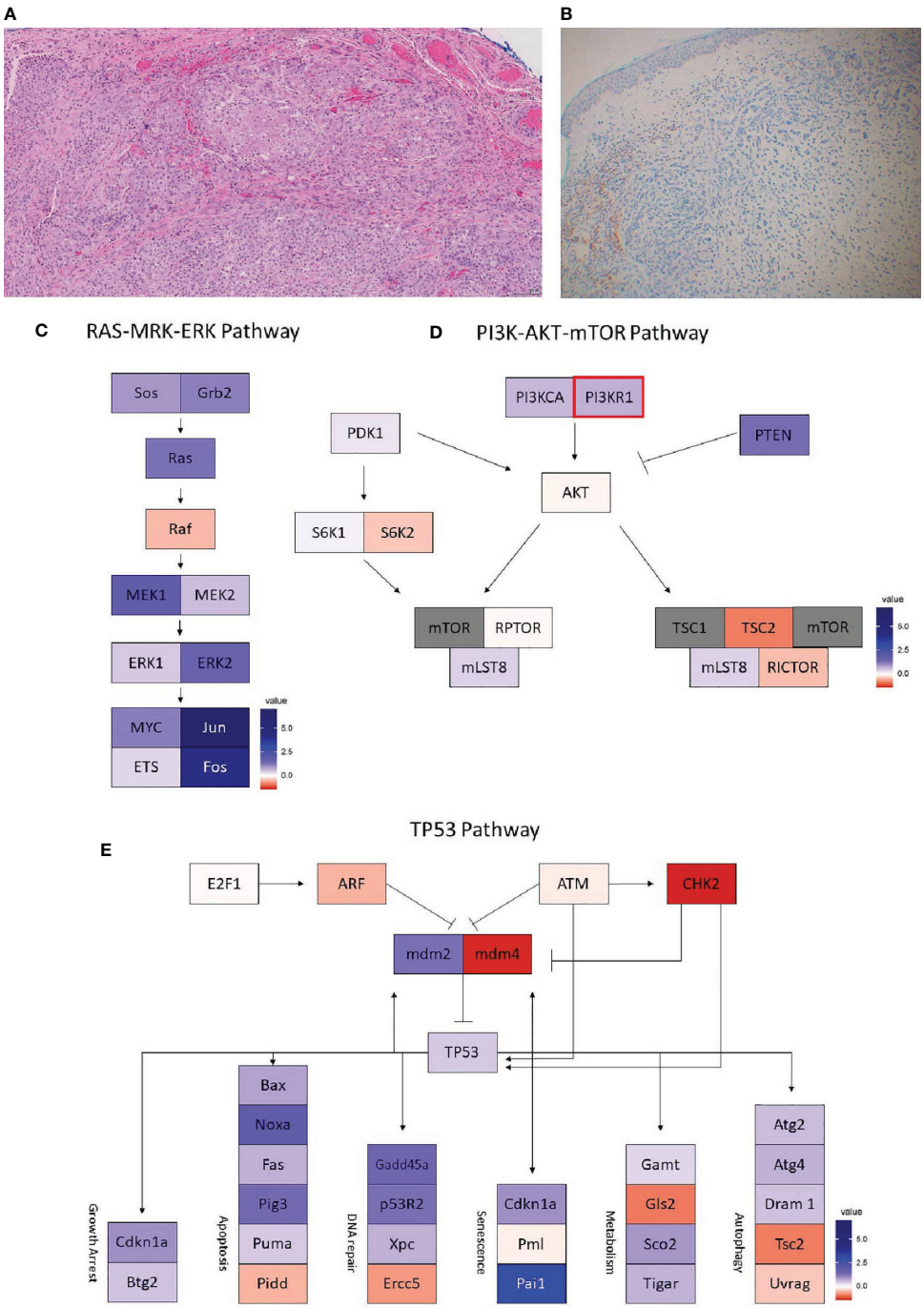


**FIGURE 4** | Case 4 **(A)** H&E image, showing metaplastic squamous carcinoma **(B)** PD-L1 by the Ventana PD-L1 SP263 assay. Images **(C–E)** show RNA expression heatmaps with modified z-scores of expression vs. non-metaplastic TNBC cases in pathways of interest for metaplastic breast cancer **(C)** RAS-MEK-ERK, **(D)** PI3K-AKT-mTOR **(E)** TP53. Genes with DNA mutations are outlined in red.

DNA mutations noted included *PIK3CA, TP53, PTEN, CDKN2A*. In a comparison of RNA expression, there were no marked differences in expression within the *TP53* or the *RAS/MRK/ERK* pathways, but *PDK1* appeared less expressed within the *PI3K* pathway compared to other cases (**Figures 1G–I**).

## Case 2

The patient is a 58-year-old woman who presented with a gradually enlarging right breast, biopsy revealing a grade 3 invasive ductal carcinoma, ER-, PR-, HER2- (IHC 0, FISH ratio 1.23). Right axillary lymph node biopsy was positive for

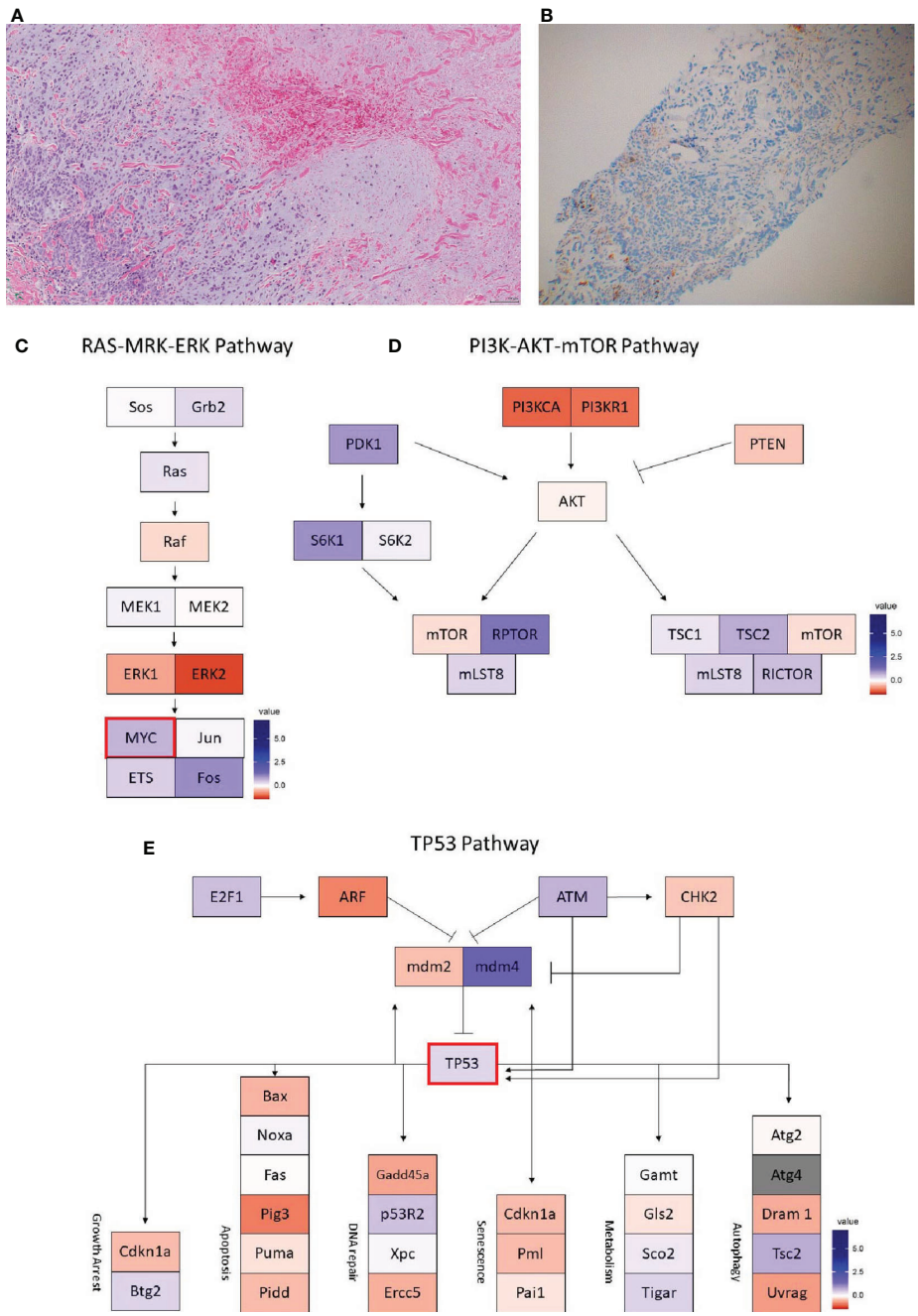


**FIGURE 5** | Case 5 **(A)** H&E image, showing metaplastic squamous carcinoma **(B)** PD-L1 by the Ventana PD-L1 SP263 assay. Images **(C–E)** show RNA expression heatmaps with modified z-scores of expression vs. non-metaplastic TNBC cases in pathways of interest for metaplastic breast cancer **(C)** RAS-MEK-ERK, **(D)** PI3K-AKT-mTOR **(E)** TP53. Genes with DNA mutations are outlined in red.

metastatic breast carcinoma. She received neoadjuvant dose-dense doxorubicin + cyclophosphamide, followed by paclitaxel, with decrease in the right breast mass but increase in an axillary dominant node on follow up ultrasound. She underwent lumpectomy and axillary lymph node dissection, with pathology showing a grade 3 invasive ductal carcinoma, 4.0 cm, with an additional 8 mm focus, 3/19 lymph nodes positive with the largest at 2.4 cm, negative for lymphovascular invasion. She received adjuvant radiation to the right breast. She later presented for follow up and reported increasing mid-back pain, with MRI of the T- and L-spine without evidence of metastasis to the spine, but found enhancing pulmonary lesions. CT chest noted bilateral lung lesions, with core biopsy showing an ER-, PR-, HER2- breast cancer with metaplastic features with focal chondroid differentiation.

She enrolled in the aforementioned phase Ib trial of capecitabine + pembrolizumab. Follow up CT scans at 12 weeks showed a partial response, with an overall shrinking of

multiple lung nodules, while also noting growth of other smaller nodules (**Figures 2C–E**). However, follow up scans at 24 weeks showed clear progression of disease and she was taken off the trial. She remains on 6th line therapy with sacituzimab as of March 2021, with addition lines including eribulin, gemcitabine, cisplatin, and paclitaxel.

On pre-treatment biopsy, PD-L1 expression was noted on 0% of tumor cells and 10% of immune cells, with a CPS of 5, above the threshold for positivity of ≥ 1, but below the ≥ 10 threshold. PD-L1 scoring by mIF was relatively low. TILs were scored as 15%. Immune cell counts were lower for CD8+ Cytotoxic T-cells, CD163+ Macrophages, and FOXP3+ Regulatory T-cells compared to non-metaplastic cases, but CD3+ Helper T-cells were higher than in non-metaplastics (**Table 1**, **Figure 6**). DNA mutations of interest included *TP53, MYC*, and *DICER1*. No significant patterns of increased or decreased expression was found in RNA analysis of the *TP53* or *PI3K* pathways. Higher expression was seen within the *RAS/MEK/ERK* pathway (**Figures 2F–H**).

## Case 3

The patient is an 82-year-old woman with a prior history of right sided stage IIB breast cancer in 2001, treated with mastectomy and axillary lymph node dissection, ER+, PR+, HER2-. She received adjuvant chemotherapy with cyclophosphamide, epirubicin, and 5-FU for 6 cycles, and additionally received radiation, 5 years of tamoxifen and 7 years of aromatase inhibitors (letrozole and exemestane). She had normal surveillance mammographies until November 2015 where she was found to have calcifications and possible distortion in the left upper outer breast. Biopsy found grade II pleomorphic invasive lobular carcinoma, ER 2%, PR-, HER2- (IHC 2+, ISH 1.8, ratio 1.06). She had a left breast mastectomy with sentinel lymph node biopsy, with a 5 mm residual invasive lobular carcinoma, with additional foci ranging from 1-3 mm, grade II, with negative margins, and extensive lymphovascular invasion, 2/2 sentinel nodes positive. She received adjuvant cyclophosphamide, methotrexate and 5-FU.

She developed a local chest wall recurrence, biopsy showing a metaplastic breast carcinoma with a component of pleomorphic lobular carcinoma associated with squamous differentiation, ER 20%, PR-, HER2- (IHC 1+ ISH 2.3, ratio 1.1), with androgen receptor staining positive in 30% of tumor cells. She received radiation, and then was started on fulvestrant + palbociclib, but had disease progression. She then started on exemestane + everolimus, but again had progressing skin lesions. She was then started on 3rd line compassionate use nivolumab with off-label bicalutamide as the patient had wanted to avoid further chemotherapy, and had not previously responded to ER-directed therapy. Bicalutamide was held after 2 weeks of treatment, with concerns for fluid retention and swelling. At 1 month follow up she had worsening skin lesions, but nivolumab was continued with the possibility of a flare reaction causing the exam findings rather than disease progression. 2 months into treatment skin lesions appeared to be crusting over, and at 4 months appeared to have a complete response (**Figures 3C–E**). She continued on therapy for an additional 4 months when new skin lesions were noted on her back and trunk and a biopsy confirmed disease recurrence.

PD-L1 expression on tumor cells was 2% of pre-treatment and 0% of post-treatment tumor cells were positive for PD-L1, compared to 50% of both pre-treatment and post-treatment immune cells. CPS was above the threshold for positivity of ≥ 1, and a higher threshold of ≥ 10 in the pre-treatment sample with a CPS of 10, though only above the ≥ 1 threshold in the post-treatment sample with a CPS of 3. TILs were scored as 30% in the pre- treatment and 15% in the post-treatment samples. DNA mutations included *PIK3CA, TP53, AKT1, CDH1, KMT2D*. Further genomic and immunoprofiling was unavailable for this case, as this patient was not a part of the clinical trial.

## Case 4

The patient is a 60-year-old woman who presented with a painful large left breast mass. Biopsy of the left breast showed grade 3 invasive ductal carcinoma with focal spindle cell features, also noted on left axillary biopsy, ER-, PR-, HER2- (IHC 0, ISH 1.55, ratio 0.86). She received 4 cycles of neoadjuvant dose dense doxorubicin + cyclophosphamide with minimal response, followed by 4 cycles of carboplatin + weekly paclitaxel with some response. She underwent a left modified radical mastectomy, with pathology showing a 4.4 cm grade 3 IDC with metaplastic features, and extensive lymphovascular invasion, clear surgical margins, and 4/7 axillary lymph nodes involved with extranodal extension. Prior to receiving adjuvant radiation, a subcutaneous nodule was found inferior to her mastectomy incision, with excisional biopsy showing 3 foci of recurrent/residual IDC with sarcomatoid features, with one focus extending beyond the excisional margin. She received adjuvant radiation, and a subsequent PET scan and brain MRI were without evidence of residual disease. She then presented with left arm swelling, CT chest, abdomen, pelvis found enlarged lymph nodes in the neck and chest, multiple pulmonary nodules, small hypodensities in the liver measuring less than 5 mm, and sclerotic-appearing lesions in the manubrium. A brain MRI and

**TABLE 1** | Immune cell counts in Case 2 by mIF.

| Patient | Median raw cell count per ROI (CD3+) | Z-score *vs.* Non-metaplastic (CD3+) | Median raw cell count per ROI (CD8+) | Z-score *vs.* Non-metaplastic (CD8+) | Median raw cell count per ROI (CD163+) | Z-score *vs.* Non-metaplastic (CD163+) | Median raw cell count per ROI (FOXP3+) | Z-score vs. Non-metaplastic (FOXP3+) |
|---|---|---|---|---|---|---|---|---|
| **Case 2** | 10.7 | 0.85 | 14.5 | -0.51 | 16.9 | -0.67 | 5.1 | -0.82 |

*ROI, region of interest; CD3+, CD3-positive CD8-negative FoxP3-negative T-cells (Helper T-cells); CD8+, CD3-positive CD8-positive T-cells (Cytotoxic T-cells); CD163+, CD163-positive cells (Macrophages); FOXP3+; CD3-positive FoxP3-positive T-cells (Regulatory T-cells).*

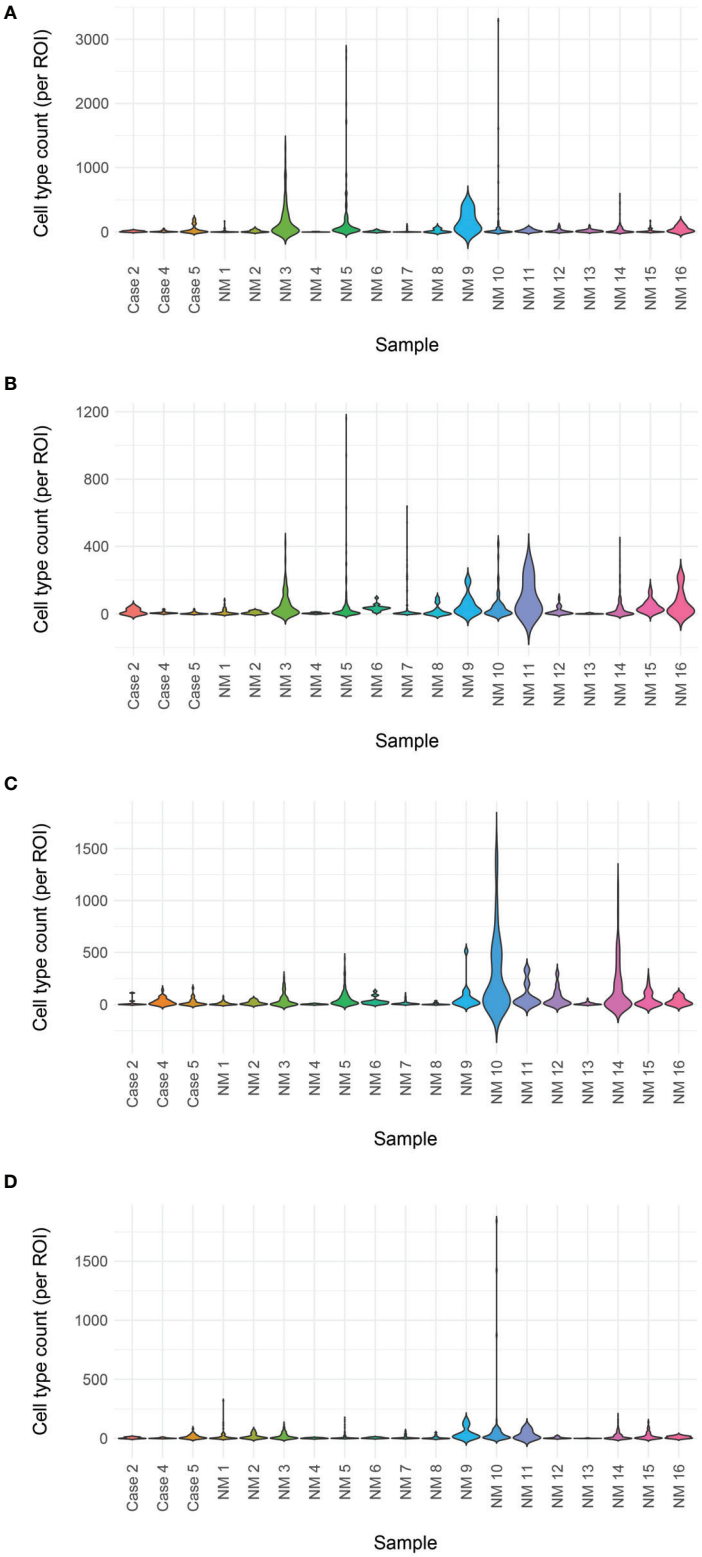


**FIGURE 6** | Immune cell counts by mIF. Total immune cell counts for metaplastic Cases 2, 4, and 5 plotted with non-metaplastic (cases identified as 'NM') TNBC from the same clinical trial in a violin plot. No clear difference is noted between the metaplastic cases and non-metaplastic cases **(A)** Helper T-cells **(B)** Cytotoxic T-cells **(C)** Macrophages **(D)** Regulatory T-cells.

bone scan showed no evidence of metastases. An ultrasound-guided FNA of a neck nodule on the right showed extensive necrosis and degenerated atypical cells, consistent with a necrotic carcinoma.

She was enrolled in the same phase Ib trial of capecitabine + pembrolizumab. Follow up imaging at 12 weeks noted a mixed response with growth of some nodes and regression of others, but she did have a new bony metastasis at T11 and was taken off of the trial.

On pre-treatment biopsy, PD-L1 expression was noted on 0% of tumor cells and 2% of immune cells, with a CPS of 0.5, under the threshold for positivity of ≥ 1. PD-L1 scoring by mIF was lower than the median of cases evaluated. TILs were scored as 2%. Immune cell counts by mIF noted higher CD163+ Macrophages than in non-metaplastic cases, and lower FOXP3+ Regulatory T-cells, which were 4$^{th}$ lowest among the 19 evaluable cases. CD3+ Helper T-cells and CD8+ Cytotoxic T-cell counts were similar to non-metaplastic cases (**Table 2**, **Figure 6**). DNA mutations of interest included *PIK3R1, CHEK2, NF1*, and *NCOR1*. RNA expression in the *TP53* pathway found decreased *MDM4* and *CHK2*, but otherwise was without a clear pattern of increased or decreased expression. The *PI3K* pathway noted increased *PTEN*, but otherwise was again without a clear pattern through the rest of the pathway. Strong expression was seen in the *RAS/MRK/ERK* pathway, particularly of *JUN* and *FOS* (**Figures 4C–E**).

## Case 5

This is a 62-year-old woman who had a small left breast lump that rapidly grew into a fungating mass. Skin punch and core needle biopsies showed metaplastic carcinoma with extensive necrosis and dermal direct extension, ER-, PR-, HER2- (IHC 0, ISH 3.05, ratio 0.72). Staging CT scan revealed a large left breast mass measuring 13.6 cm with a large left axillary node measuring 7.4 cm, numerous bilateral pulmonary metastasis, a suspected metastatic pancreatic neck mass measuring 1.8 cm, and a soft tissue lesion surrounding the right 10$^{th}$ rib, without other definite bone metastases, but bone scan noted multiple bone metastases.

She received paclitaxel (80mg/m2 IV weekly on days 1, 8, 15 of each 3-week cycle) with pembrolizumab (200 mg IV every 3 weeks). Following initiation, she had a mild infusion reaction to paclitaxel, but was maintained on therapy with dexamethasone pretreatment. The patient felt her breast mass shrank initially, but on follow up appointment prior to cycle 3, her mass appeared larger and repeat CT of the chest, abdomen, and pelvis showed progressive disease at multiple foci with a new pathologic fracture of the L-spine. She received palliative radiation to her spine and was taken off the trial and started on a DAE regimen (doxorubicin 30mg/m2 IV q3wk, bevacizumab 15mg/kg q3wk, and everolimus 5mg PO daily). She developed disease progression and subsequently enrolled in hospice.

A pre-treatment biopsy was available for review and PD-L1 expression was noted on 0% of tumor cells and 10% of immune cells, with a combined positive score of 2, above the threshold for positivity of ≥ 1, but below the threshold of >10. PD-L1 scoring by mIF noted relatively low expression. TILs were scored as 5%. Immune cells by mIF noted higher FOXP3+ Regulatory T-cells than the median of non-metaplastic cases, as well as compared to the other metaplastic cases, but overall populations were low for all cases. CD3+ Helper T-cell counts were near median values, with both CD8+ Cytotoxic T-cell and CD163+ Macrophages lower than non-metaplastic cases (**Table 3**, **Figure 6**). DNA mutations of interest included *TP53* and *MYC*. No significant patterns were noted in RNA expression in the *TP53* and *RAS/MEK/ERK* pathway, though ERK2 was significantly lower in the *RAS/MEK/ERK pathway*. In the *PI3K* pathway, *PIK3CA* and *PIK3R1* were relatively lower, but no pattern of reduced expression was noted in the rest of the pathway (**Figures 5C–E**).

## Comparative Biomarker Assessment of Metaplastic *versus* Non-Metaplastic TNBCs

The small sample size in this series prohibited extensive characterization of MBC. However, because data in MBC are

**TABLE 2** | Immune cell counts in Case 3 by mIF.

| Patient | Median raw cell count per ROI (CD3+) | Z-score *vs.* Non-metaplastic (CD3+) | Median raw cell count per ROI (CD8+) | Z-score *vs.* Non-metaplastic (CD8+) | Median raw cell count per ROI (CD163+) | Z-score *vs.* Non-metaplastic (CD163+) | Median raw cell count per ROI (FOXP3+) | Z-score *vs.* Non-metaplastic (FOXP3+) |
|---|---|---|---|---|---|---|---|---|
| **Case 4** | 5.5 | 0.04 | 5.5 | -0.33 | 18.5 | 0.92 | 1.5 | -0.67 |

*ROI, region of interest; CD3+, CD3-positive CD8-negative FoxP3-negative T-cells (Helper T-cells); CD8+, CD3-positive CD8-positive T-cells (Cytotoxic T-cells;, CD163+, CD163-positive cells (Macrophages); FOXP3+, CD3-positive FoxP3-positive T-cells (Regulatory T-cells).*

**TABLE 3** | Immune cell counts in Case 5 by mIF.

| Patient | Median raw cell count per ROI (CD3+) | Z-score *vs.* Non-metaplastic (CD3+) | Median raw cell count per ROI (CD8+) | Z-score *vs.* Non-metaplastic (CD8+) | Median raw cell count per ROI (CD163+) | Z-score *vs.* Non-metaplastic (CD163+) | Median raw cell count per ROI (FOXP3+) | Z-score *vs.* Non-metaplastic (FOXP3+) |
|---|---|---|---|---|---|---|---|---|
| **Case 5** | 6.5 | 0.22 | 2 | -0.65 | 4.5 | -0.47 | 5.5 | 0.52 |

*ROI, region of interest; CD3+, CD3-positive CD8-negative FoxP3-negative T-cells (Helper T-cells); CD8+, CD3-positive CD8-positive T-cells (Cytotoxic T-cells); CD163+, CD163-positive cells (Macrophages); FOXP3+, CD3-positive FoxP3-positive T-cells (Regulatory T-cells).*

limited due to the rarity of this disease, it was of interest to conduct an informal, hypothesis-generating descriptive comparison of immunoprofiles using MBC versus non-MBC specimens from the aforementioned phase Ib trial.

## PD-L1

PD-L1 expression by mIF was generally lower in the metaplastic cases vs the non-metaplastic TNBCs, with all 3 cases evaluated below the median in PD-L1 expression (**Figure 7**). However, clinical PD-L1 scoring by CPS >1 showed that 4 of 5 metaplastic cases were positive by this definition with only Case 4 below this threshold. When using a higher cutoff of CPS ≥10 for positivity as in other recent trials of pembrolizumab in triple negative breast cancer, only Case 3 met the threshold (18).

## Immune Cells

Given the heterogeneity of MBC, comparisons were made between each metaplastic case and n=14 evaluable non-metaplastic TNBC cases to evaluate for outlier factors to differentiate metaplastic and non-metaplastic TNBC, rather than against all other cases including the 2 other metaplastic cases (n=16) in an attempt to identify the unique differences in each metaplastic case against TNBC, rather than a cohort that would include other metaplastic cases. Evaluation of immune cells by mIF demonstrated overall lower median raw cell counts across regions of interest in the metaplastic cases compared to the median of non-metaplastic TNBC cases. No obvious outliers were noted in comparison to non-metaplastic TNBC. Cases 2, 4, and 5 had positive z-scores in comparing CD3+ Helper T-cells to non-metaplastic TNBC, but all scores were <1 (**Table 4**). To additionally evaluate heterogeneity in MBC, the variance in immune cell counts between regions of interest was evaluated. A median absolute deviation was calculated and overall, less variance was seen in metaplastic cases compared to non-metaplastic cases (**Table 5**).

## RNA and TCR Sequencing

Comparison of RNA sequencing did not demonstrate significant differences between metaplastic and non-metaplastic cases in multiple genes of interest, but did note multiple outlier genes, with an arbitrary cutoff of a modified z-score >3 in 2 or more metaplastic cases selected to identify possible outliers: *SOX8, CIC, COL9A3, ZFAND1, UBE2W, C2orf40, ENY2, RBM39, TGS1, DPY19L4, CLEC18A, ACAN, SLC25A32, VIRMA, IGF2, NOTUM, WWP2, NPIPB11, UPK1B, GABPB1, NR4A1, SLC25A42, FBXO25*. RNA expression in pathways of interest in MBC are further presented in **Figures 1**, **2**, **4**, **5** and **8**.

TCR sequencing did not find significant changes in T-cell diversity by richness or clonality at baseline or during treatment between metaplastics and non-metaplastics. Evaluating the clonotype structure, metaplastics as a group vs non-metaplastics did not have significant differences in the amounts of higher frequency or lower frequency clones (**Figure 9**). However, Case 2 and Case 4 had a greater proportion of high-prevalence clones compared to other cases at baseline with Case 2 being a responder and Case 4 being a non-responder.

# DISCUSSION

Our case series provides additional evidence of clinical activity of chemo-immunotherapy for MBC, a rare subtype of breast cancer for which limited outcomes data are available. In this series, we describe clinical responses in 2/4 cases treated with chemotherapy plus pembrolizumab. Of interest, we also report

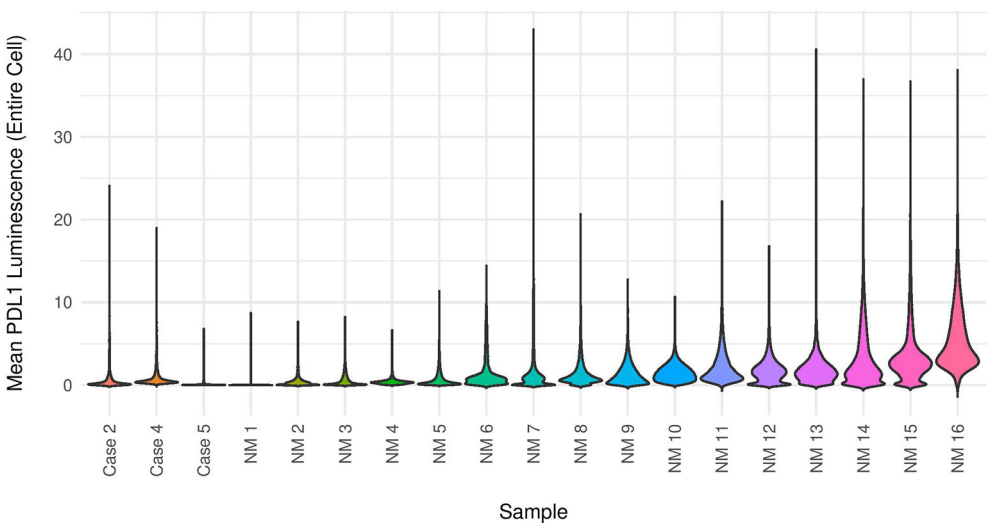


**FIGURE 7** | PD-L1 mIF violin plot. Mean PD-L1 quantitative immunofluorescence in baseline biopsies for metaplastic Cases 2, 4, and 5 as well as non-metaplastic (cases identified as 'NM') TNBC from the same clinical trial in a violin plot. A trend towards lower mean PD-L1 expression is noted in the metaplastic cases.

**TABLE 4** | Comparison of mIF cell counts.

| Patient | Median raw cell count per ROI (CD3+) | Z-score *vs.* Non-metaplastic (CD3+) | Median raw cell count per ROI (CD8+) | Z-score *vs.* Non-metaplastic (CD8+) | Median raw cell count per ROI (CD163+) | Z-score *vs.* Non-metaplastic (CD163+) | Median raw cell count per ROI (FOXP3+) | Z-score *vs.* Non-metaplastic (FOXP3+) |
|---|---|---|---|---|---|---|---|---|
| **Case 2** | 10 | 0.85 | 3.5 | -0.51 | 2.5 | -0.67 | 1 | -0.82 |
| **Case 4** | 5.5 | 0.04 | 5.5 | -0.33 | 18.5 | 0.92 | 1.5 | -0.67 |
| **Case 5** | 6.5 | 0.22 | 2 | -0.65 | 4.5 | -0.47 | 5.5 | 0.52 |
| **Non-metaplastic (n=16)** | 5.25 | n/a | 9 | n/a | 9.25 | n/a | 3.75 | n/a |

*ROI, region of interest; CD3+, CD3-positive CD8-negative FoxP3-negative T-cells (Helper T-cells); CD8+, CD3-positive CD8-positive T-cells (Cytotoxic T-cells); CD163+, CD163-positive cells (Macrophages); FOXP3+, CD3-positive FoxP3-positive T-cells (Regulatory T-cells).*

*Raw cell counts for immune cells as a median across regions of interest, quantified by a multiplexed immunofluorescence panel and calculated modified z-scores comparing metaplastic cases to n=16 non-metaplastic TNBC. Stromal and intra-tumor immune cells were not differentiated due to low numbers of immune cells within areas of tumor.*

**TABLE 5** | Variance in immune cells by mIF reported as median absolute deviation.

| Cell-type | Non-metaplastic (n=14) | Metaplastic (Cases 2, 4, 5) |
|---|---|---|
| CD3+ | 3.75 | 1 |
| CD8+ | 7.25 | 1.5 |
| CD163+ | 6.75 | 2 |
| FOXP3+ | 2.25 | 0.5 |

*CD3+, CD3-positive CD8-negative FoxP3-negative T-cells (Helper T-cells); CD8+, CD3-positive CD8-positive T-cells (Cytotoxic T-cells); CD163+; CD163-positive cells (Macrophages), FOXP3+; CD3-positive FoxP3-positive T-cells (Regulatory T-cells).*

a fifth MBC case of a complete clinical response to nivolumab and bicalutamide. These data are supportive of previously published reports of clinical response in MBC. Adams reported a case of metastatic MBC with a large chest wall lesion that dramatically responded to nab-paclitaxel + pembrolizumab, with an ongoing response at 6 months (14), whereas Al Sayed et al. reported a case of chemo-refractory metastatic MBC treated with durvalumab + paclitaxel with a complete clinical response reported without recurrence at 2 years (15). In comparison, clinical response rates to chemo-immunotherapy among non-MBC TNBCs were 8/24 in the parent phase Ib clinical trial. Otherwise, a recent report of an MBC cohort within the DART trial (NCT02834013) of dual anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab) therapy reported responses in 3 of 17 patients (18%), with ongoing responses at 23, 25, and 27 months (16).

## Duration of Response and Mixed Responses

One notable observation from our series is that clinical responses were less durable than previously reported in published case reports, with progression free survival (PFS) of 5.3, 5.7 and 8.0 months for Cases 1, 2 and 3 respectively. Of note, the non-metaplastic TNBC responders (n=8) in the same trial as Cases 1 and 2 had an average PFS of 6.9 months, arguing that duration of response to chemo-immunotherapy in metaplastic breast cancer may not appreciably differ from non-metaplastic TNBC. However, notably, Case 3 which had the longest PFS was an ER+ tumor, treated with bicalutamide in addition to anti-PD-1 therapy, and not a TNBC, limiting direct comparisons.

A classic histologic trademark of MBC is intralesional heterogeneity, with the potential for having multiple regions of the tumor exhibiting distinct histologic features. In a recent analysis, it has also been suggested that intralesional histologic heterogeneity may reflect underlying genomic heterogeneity (28, 29). We evaluated for heterogeneity of radiographic response in our case series, and observed that Cases 1 and 2 had partial responses by RECIST v1.1, but had a mixed picture, with target lesions both shrinking and enlarging on initial follow up imaging. Case 4 also noted regression of some target lesions, but overall had disease progression by RECIST v1.1. Mixed responses, defined as the presence of simultaneously regressing and progressing target lesions, have been previously reported in studies with immunotherapy, with one study of stage IV melanoma treated with immune checkpoint blockade reporting 22% of patients with a mixed response. However, the majority of these cases do eventually become clear responders or progressors, and the phenomenon of a mixed response may be an artifact of the kinetics of immunotherapy, rather than being a separate outcome (30). In comparison, of 15 evaluable non-metaplastic TNBC, just 2 cases had similar mixed responses to chemo-immunotherapy (17). The limited sample size in this series prohibits drawing conclusions, however as additional MBC patients receive chemo-immunotherapy across the globe, it would be of interest to further evaluate the hypothesis that MBC could experience heterogeneous clinical responses. Because of the aggressive nature of this disease, and limited standard-of-care systemic options, it may be of value to consider locoregional therapy such as radiotherapy, to address progressive lesions in the setting of otherwise-responding disease. Notably, a recent study has shown promising activity and safety of radiotherapy + pembrolizumab in metastatic TNBC, with an objective response rate of 17.6% in a phase II trial of n=17 patients, although it is uncertain whether any of these were MBC. Another recent study in metastatic hormone receptor+/HER2- breast cancer did not show any responses with this combination in a heavily pre-treated group of n=8 patients (31, 32).

## PD-L1 Status and Response

Increased PD-L1 expression has been reported in multiple studies of MBC, with one study of 75 MBCs reporting PD-L1

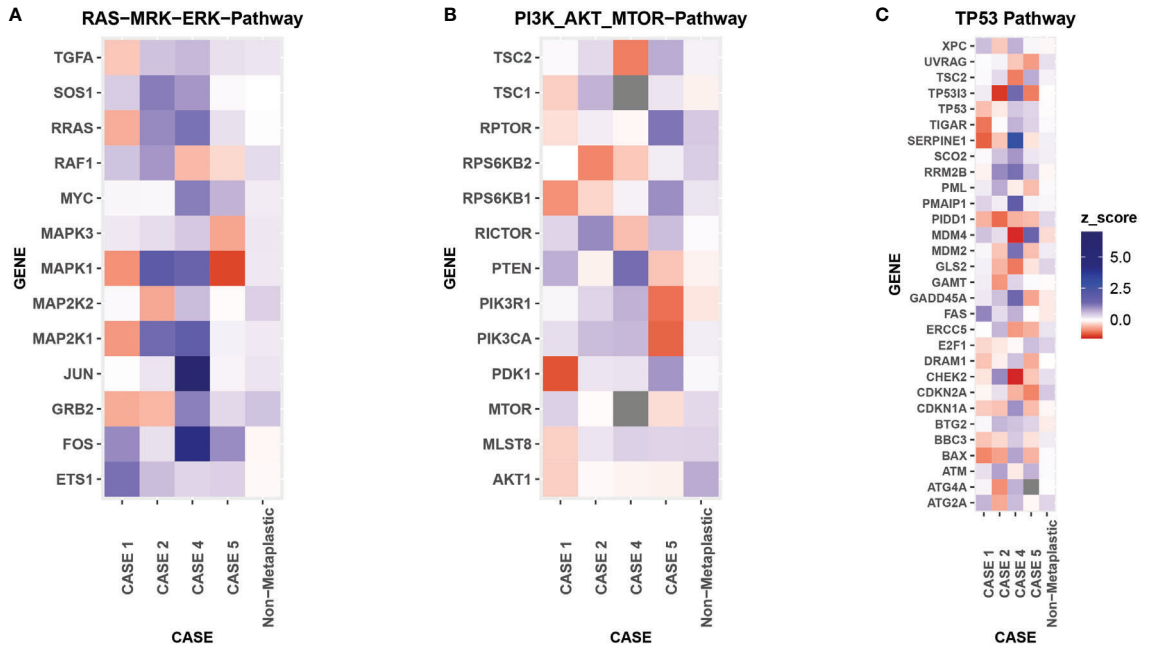


**FIGURE 8** | RNA Heat Map. RNA expression heatmaps with modified z-scores of expression vs non-metaplastics represented for each patient for 3 molecular pathways of interest in metaplastic breast cancer, **(A)** RAS-MRK-ERK pathway, **(B)** PI3K-AKT-mTOR pathway, **(C)** TP53 pathway.

overexpression in 46% of cases, with overexpression defined as 2+ staining in >5% of tumor cells, compared to just 9% in TNBC and 6% in HER2+ or ER/PR+ tumors (8). However, other studies have shown conflicting reports on rates of PD-L1 overexpression, potentially due in part to differences in how PD-L1 expression is measured and defined, with one study reporting 0% (0/18) expression (≥1% on tumor cells, SP142), and another reporting 50% (7/14) expression (>1% on immune cells and >+ by IHC, SP263) (25, 33). PD-L1 overexpression in MBCs may be related to epithelial to mesenchymal transition (EMT), which is thought to be related to the pathogenesis of MBC. MBC has been found to express markers of EMT including *ZEB1*, a repressor of E-cadherin and Yes-associated protein (34, 35). EMT may also explain the high rates of metastatic disease in MBC and has also been found to upregulate PD-L1 expression in breast cancer (36). Mutations of the *PI3K* pathway could also contribute to the overexpression of PD-L1 in MBCs (36, 37).

In this series, 4 of 5 cases exhibited modest PD-L1 expression, considered positive using the CPS overexpression by the CPS≥1 cutoff, but with only one case being positive by the ≥10 cutoff. In the phase III first-line KEYNOTE-355 trial, pembrolizumab was shown to improve outcomes in the CPS ≥10 group, but not the CPS≥1 group (18). In an exploratory analysis, this finding was also confirmed in the second/third-line trial of pembrolizumab versus chemotherapy, where an improvement in overall survival was noted in CPS ≥20, but not in CPS≥1 or CPS≥10 (19).In our series, 2 of the 3 MBC responders had a CPS of 1-10, with the 3$^{rd}$ with a CPS of 10. These data raise the hypothesis that responses could be achieved in MBC even with modest PD-L1 expression levels. Because of the unmet need and absence of effective systemic options for MBC, further clinical investigation is warranted to determine whether the addition of anti-PD-1/L1 to chemotherapy would be effective for MBC cases with CPS 1-10.

## Genomic Profiling and PI3K Inhibition

Within previously identified genes of interest, 4 of 5 cases in our cohort had mutations of TP53, and 3 of 5 patients had mutations in the *PI3K* pathway, with Case 1 and Case 3 with *PIK3CA* mutations and Case 4 with a *PIK3R1* mutation (**Table 6**). This is particularly of interest in the context of immunotherapy as activating mutations of the *PI3K* pathway and loss of its antagonist *PTEN* have been found to have multiple effects on the tumor microenvironment. Loss of *PTEN* has been associated with increased expression of immunosuppressive cytokines, decreased tumor infiltration by T-cells, decreased T-cell mediated cell death, and increased PD-L1 expression (37, 38). Activating mutations of the *PI3K* pathway have been associated with resistance to PD-1/PD-L1 inhibition, by decreased expression of *IFN-γ* and granzyme B, and decreased CD8+ T-cell infiltration (39). Use of *PI3K* inhibitors has been found to result in decreased PD-L1 expression, increased CD8+ T-cells, and inhibition of regulatory T-cells, restoring the anti-tumor immune response (37, 40). Murine mammary models have suggested improved response to anti-PD-L1 therapy when used in combination with *PI3K* inhibitors (38, 41). Given the potential synergy of PI3K inhibition and immune therapy, a combination approach may warrant further investigation in this group of patients with high incidence of PI3K pathway alterations.

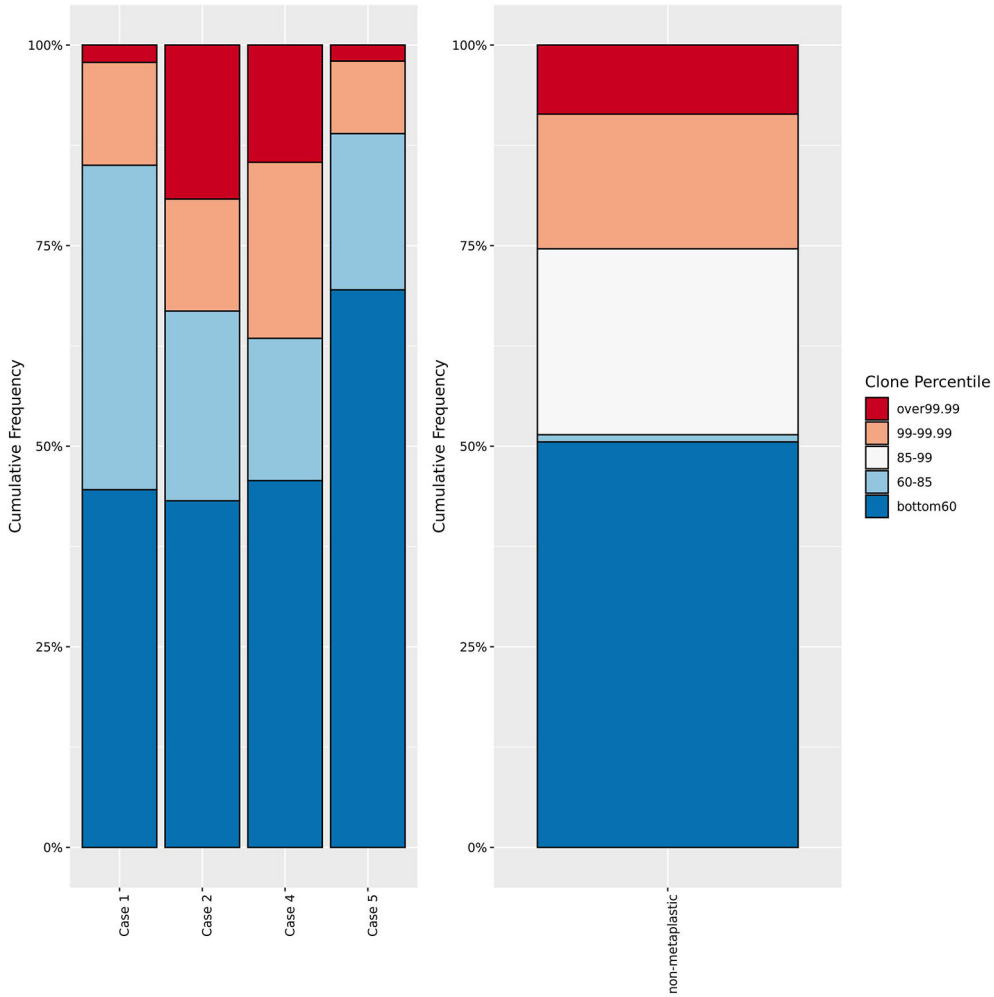


**FIGURE 9** | T-cell receptor sequencing clone frequency. Comparison of T-cell receptor sequencing clone frequency for metaplastic cases versus non-metaplastic TNBC prior to treatment. No significant difference in the percentage of low, low-middle, high-middle, or high frequency clones is noted in comparing the metaplastic versus non-metaplastic cases, with Cases 2 and 4 appearing to have more high frequency clones, and Cases 1 and 5 having less. Cases 1 and 2 were responders while Cases 4 and 5 did not respond to therapy.

## Immunoprofiling of MBC

In addition to the above, in this series we also demonstrated a method of interrogating for unique immunologic and/or genomic features of individual tumor cases, relative to a parent cohort. While limited due to the small number of MBCs in this case series, we found no consistent or extreme differences in evaluation of immune cells, PD-L1 expression, RNA sequencing, or TCR sequencing in our MBC cases compared to non-metaplastic TNBC. Of note, a recent study evaluating 44 cases of MBC versus 174 cases of TNBC found more CD163+ cells in the stroma and less CD8+ cells in the tumor of MBC cases (44). This study also found higher PD-L1 expression in tumor cells of MBC (44). In contrast, MBC cases had low PD-L1 expression (**Figure 7**), with 4/5 cases as positive by a CPS ≥1, but only 1 of 5 positive with a threshold of CPS ≥10. RNA and TCR sequencing may additionally provide further insight into the biology of MBC, and while this series was too small to evaluate for distinguishing features of MBC, this framework of reporting Z-scores of cases relative to a parent cohort may be helpful in future case series of rare tumor types such as MBC. For example, evaluation of gene pathways of interest could help identify targeted treatments that may be more effective for individual cases of MBC given the heterogeneity of this disease process.

## CONCLUSION

Three patients demonstrated a response to therapy, albeit limited in duration. One responding patient exhibited low-level ER expression and pleomorphic lobular features, whereas the other cases were triple negative breast cancer. Responses were observed in tumors with intermediate PD-L1 expression (CPS 1-10). The

**TABLE 6 |** Patient Characteristics.

| Patient | WHO Subtype | ER/PR/HER2 Status | Clinically relevant mutations | PD-L1 (TC) | PD-L1 (IC) | CPS | TILs | Prior chemotherapy | Outcome |
|---|---|---|---|---|---|---|---|---|---|
| Case 1 | Metaplastic squamous carcinoma | ER-/PR-/HER2- | PIK3CA, TP53, PTEN, CDKN2A | Pre: 0% Post: 0% | Pre: 10% Post: 40% | Pre: 5 Post:1.5 | Pre: 20% Post: 1% | Neoadjuvant: paclitaxel + ganetespib, doxorubicin + cyclophosphamide | Partial response, PFS: 5.3 months |
| Case 2 | Metaplastic carcinoma with heterologous mesenchymal differentiation (chondroid) | ER-/PR-/HER2- | TP53, MYC, DICER1 | 0% | 10% | 5 | 15% | Neoadjuvant: doxorubicin + cyclophosphamide,paclitaxel | Partial response, PFS: 5.7 months |
| Case 3 | Mixed metaplastic squamous carcinoma and pleomorphic invasive lobular carcinoma | ER+/PR-/HER2- | PIK3CA, TP53, AKT1, CDH1, KMT2D | Pre: 2% Post: 0% | Pre: 50% Post:50% | Pre: 10 Post:3 | Pre: 30% Post: 15% | Adjuvant: Right breast: cyclophosphamide + epirubicin + 5-FU, tamoxifen, letrozole, exemestane Left breast: cyclophosphamide + methotrexate + 5-FU Metastatic 1st line: fulvestrant + palbociclib Metastatic 2nd line: exemestane + everolimus | Complete response, PFS: 8.0 months |
| Case 4 | Metaplastic squamous carcinoma | ER-/PR-/HER2- | PIK3R1, CHEK2, NF1, NCOR1 | 0% | 2% | 0.5 | 2% | Neoadjuvant: doxorubicin + cyclophosphamide, carboplatin + paclitaxel | Progressive disease |
| Case 5 | Metaplastic carcinoma with heterologous mesenchymal differentiation (chondroid) | ER-/PR-/HER2- | TP53, MYC | 0% | 10% | 2 | 5% | None | Progressive disease |

*ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; PD-L1, Programmed death-ligand 1; TC, tumor cells; IC, immune cells; CPS, combined positive score (# of PD-L1+ cells/total # of viable tumor cells x100), TIL (H&E): TIL scoring per the guidelines of the International TILs working group (20). PFS, progression free survival. Both pre- and post-treatment biopsies were available for Cases 1 and 3. Cases 2, 4, 5, only had pre-treatment biopsies available.*

aggressive nature of MBC and unmet need for effective palliative options, support further investigation of the role of anti-PD-1/L1 in PD-L1-intermediate MBC is warranted.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Providence Portland IRB and Cedars IRB. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

IK – data analysis, manuscript writing. VR – data analysis. BB – data analysis. BC – data analysis. YW – data analysis. MM – data analysis. ZS – data analysis. WR – data analysis. KS – data analysis. RB – treatment of study subjects. HM – treatment of study subjects. DP – treatment of study subjects, manuscript writing and review. All authors contributed to the article and approved the submitted version.

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