Immunosuppression in breast cancer: a closer look at regulatory T cells

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The multifaceted role of regulatory T cells in breast cancer

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ABSTRACT

The microenvironment of breast cancer hosts a dynamic cross-talk between diverse players of the immune system. While cytotoxic immune cells are equipped to control tumor growth and metastasis, tumor-corrupted immunosuppressive immune cells strive to impair effective immunity and promote tumor progression. Of these, T_{regs}, the gatekeepers of immune homeostasis, emerge as multifaceted players involved in breast cancer. Intriguingly, clinical observations suggest that blood and intratumoral T_{regs} can have strong prognostic value, dictated by breast cancer subtype. In line, emerging preclinical evidence shows that T_{regs} occupy a central role in breast cancer initiation and progression, and provide critical support to metastasis formation. Here, T_{regs} are not only important for immune escape, but also promote tumor progression independent of their immune regulatory capacity. Combining insights into T_{reg} biology with advances made across the rapidly growing field of immuno-oncology is expected to set the stage for the design of more effective immunotherapy strategies.

THE IMMUNE SYSTEM: A DOUBLE-EDGED SWORD IN CANCER

Tumors are complex entities consisting of not just cancer cells, but also a variety of non-malignant cell types. The local niche in and surrounding tumors is collectively described as the tumor microenvironment (TME), which can profoundly impact the development and progression of cancer[1–3]. It is now clear that the TME is not a static element of tumors, but its composition and functional state is highly diverse between cancer types, subtypes, and even individual tumors. In the past decades, particularly the immunological component of the TME has been studied extensively, with a focus on answering the central question: how can tumors develop in the context of a functional immune system? Addressing this fundamental question is essential to fully exploit the immune system for the treatment of cancer.

Breast cancer is perhaps one of the most studied cancer types in the context of the TME. Although survival rates for breast cancer patients are steadily increasing, it is still the leading cause of cancer-related deaths in women worldwide[4,5]. The vast majority of breast cancer-related mortality is due to the incurable metastatic stage of the disease. Clearly, understanding, preventing and treating metastatic breast cancer is an unmet need. As such, mechanistic insights into the complex interactions of key players in the TME could pave the way for novel innovative treatments and improved patient stratification.

Clinical studies have exposed a dual role of the immune system in breast cancer. For example, tumor-associated macrophages (TAMs) are associated with invasion, metastasis and a worse prognosis[6], while tumor infiltrating lymphocytes (TILs) are associated with a favorable prognosis[7]. To understand this duality, it is important to realise that cancers host a plethora of immune cell subsets, such as lymphocytes, various myeloid cells and innate lymphoid cells to which both pro- and anti-tumorigenic functions have been attributed[2]. Although immune cells such as $CD8^+$ T cells and NK cells have the molecular gear to recognize and eradicate malignant cells, they often encounter a highly immunosuppressive environment in tumors which blunts effective anti-tumor immunity. This milieu is characterized by widespread expression of immune checkpoint receptors, inhibitory cytokines, hypoxia and low levels of nutrients, all of which restrain the recruitment and function of cytotoxic immune cells[8]. Importantly, lymphocytes and tumor-associated myeloid cells including macrophages, neutrophils and monocytes profoundly contribute to the creation of this immune suppressive environment as well as to systemic immunosuppression that often accompanies primary tumor growth and which further potentiates cancer progression by facilitating immune escape[3].

A key orchestrator of immunosuppression is the $CD4^+$ regulatory T cell (T_{reg}), which has since its discovery been in the crosshairs of cancer immunology research[9,10]. T_{regs} can be abundantly

present in primary breast tumors and metastases[11]. Still, their exact impact and relevance to breast cancer progression has proven challenging to uncover, due to the complexities of immune cell cross-talk and metastatic disease. Recently, fundamental and preclinical research has provided exciting new insights into the biology of T_{regs} in breast cancer. This comes at an important time, as initial results of immune checkpoint inhibitors in breast cancer have been relatively disappointing[12]. The expanding use of these drugs for the treatment of breast cancer therefore necessitates a comprehensive understanding of immunosuppressive T_{regs}; are we pulling the right strings? In this review, we will therefore explore and discuss the current knowledge, challenges and clinical use of T_{regs} in breast cancer.

REGULATORY T CELLS: GATEKEEPERS OF IMMUNE HOMEOSTASIS

The immune system is a sophisticated defense network, evolved to withstand innumerable pathogenic challenges at any anatomical location. To do so, complex cellular interactions coordinate pathogen recognition, immune cell activation and the execution of effector programs. In order to return to, or maintain homeostasis, immunosuppressive signals are essential to dampen immune responses to prevent pathological immune responses such as chronic inflammation or auto-immunity. A key cell type involved in this process is the T_{reg}. The importance of T_{regs} in immune tolerance has become evident through characterization of so-called “scurfy mice” that suffer from a severe lethal auto-immune syndrome, characterized by inflamed skin, red eyes, enlarged lymphoid organs and early death[13]. Scurfy mice were first reported in 1949, but it was not until the early 2000’s that a mutation in the *Foxp3* gene, and consequential loss of T_{regs}, was identified as a direct cause for the severe immune pathology[14]. Further research showed that FOXP3 is the master transcription factor for the previously identified specialized immunosuppressive $CD4^+$ $CD25^+$ T lymphocytes, now known as T_{regs}[15,16]. Since then, it has become clear that reduced T_{regs} numbers and/or impaired T_{reg} functionality stands at the basis of autoimmune and inflammatory diseases, such as diabetes, multiple sclerosis and inflammatory bowel disease[17,18]. In contrast, their activation and accumulation in tumors is considered detrimental, as we will explore in depth.

T_{regs} utilize several strategies to antagonize both adaptive and innate immunity. Among these, the release of immunosuppressive mediators as IL-10, TGF-β and adenosine, and high expression of immunomodulatory receptors as CTLA-4, PD-L1 and LAG-3 are well established aspects of T_{reg} functionality which can interfere with the propagation of immune responses[9,19,20]. Scavenging of IL-2 from the environment and killing of effector T cells by the release of granzymes additionally contributes to immunosuppression[21,22]. Combined, these mechanisms can be employed to restrain dendritic cell (DC) function, or directly inhibit cytotoxic cells[21]. The exact effector program that is engaged is highly dependent on the tissue

and nature of the immune response[19,23]. Emerging evidence shows that T_{regs} can acquire expression of T_{helper} subset transcription factors (TFs), such as T-bet, GATA3 and RORγT which directs their function towards suppression of T_{helper} cells of that particular subset[19,23]. For example, T_{regs} expressing the Th1 TF T-bet are important for suppressing Th1 mediated inflammation, but cannot suppress Th2 or Th17 responses[24].

Two flavors of FOXP3$^+$ T_{regs}

In vivo*,* two distinct populations of FOXP3$^+$ T_{regs} are defined, based on their ontogeny and stability: thymically developed (natural) T_{regs} and extrathymically developed (peripheral or induced) T_{regs}. Thymic T_{regs} (tT_{regs}) represent a dedicated lineage with stable expression of FOXP3 and affinity for self-antigen. The generation of tT_{regs} occurs through a unique developmental program in the thymus, based on a delicate balance of T cell receptor (TCR) affinity and antigen specificity of CD4$^+$ progenitor cells[25–27]. Through this program, tT_{regs} are equipped with T cell receptors biased towards recognition of tissue restricted self-antigens, which enables the suppression of immune responses directed towards host peptides upon activation via their TCR[28–30].

Unlike tT_{regs}, peripheral T_{regs} (pT_{regs}) are extrathymically generated in the periphery from non-regulatory FOXP3$^-$ CD4$^+$ T cells. A crucial element of pT_{reg} differentiation is its dependence on TGF-β signalling, which in FOXP3$^-$ CD4$^+$ T cells, induces the interaction of SMAD2/3 with an intronic enhancer in the *Foxp3* locus, CNS1[31–33]. pT_{regs} have unstable FOXP3 expression, and miss the characteristic demethylation of the intronic element CNS2 observed in tT_{regs}, which is essential for T_{reg} stability during proliferation[31,34]. In addition, pT_{regs} display a TCR repertoire that recognizes foreign antigens, parallel to conventional CD4$^+$ T cells[35]. As such, pT_{regs} have been found to play important roles at barrier sites, including the gut, lungs and placenta to mitigate inflammatory responses in response to foreign, but harmless, environmental, dietary and microbial antigens[36–39].

The specific contributions of either tT_{regs} or pT_{regs} in cancer remain elusive, as to date no genuine phenotypic or functional marker has been discovered to distinguish both T_{reg} subtypes in vivo[40]. Instead, the ontogeny of T_{regs} in human cancer samples can be assessed ex vivo either via TCR repertoire sequencing, or via epigenetic analysis of the CNS2 element in the *Foxp3* gene, which is demethylated in tT_{regs}, but mostly methylated in pT_{regs}. As most studies on T_{regs} do not distinguish between tT_{regs} or pT_{regs}, we will refer to these cells as T_{regs}, unless stated otherwise.

Now, nearly two decades after their discovery, the extent of T_{reg} functionality appears astonishingly diverse. T_{regs} play critical roles in tissue regeneration and repair, intestinal regulation of the microbiome, hair morphogenesis, metabolic homeostasis, pregnancy and cancer[19,41]. However, it is less clear which mechanisms are engaged in the context of breast

cancer progression and metastasis. Therefore, we will first review the current clinical literature; what evidence exists that forms the basis for their clinical relevance in breast cancer?

CLINICAL SIGNIFICANCE OF T_{REGS} IN BREAST CANCER

The discovery in 2001 that $CD4^+$ $CD25^+$ immunosuppressive cells can be found in the blood of healthy individuals[42] kick-started research into the presence and behavior of these cells in cancer patients. In the following years, it was reported that $CD4^+$ $CD25^+$ T cells are increased in blood and tumors of patients with a variety of cancers, including breast-, pancreatic-, ovarian- and non-small cell lung cancer[43]. However, as CD25 expression is not restricted to T_{regs}, but can also be expressed by effector T cells, it was not until the discovery of FOXP3 as a unique marker of T_{regs}[15,16] and the development of reliable monoclonal antibodies that the presence of T_{regs} could be convincingly demonstrated in human cancers[44,45]. Since then, many studies have investigated the association between the presence of intratumoral T_{regs} and patient survival and therapy response in breast cancer (Table 1).

Despite an extensive body of literature, the clinical significance of T_{regs} in breast cancer remains controversial due to contrasting results between studies (Table 1). A key challenge in interpreting these studies is that the prognostic value of T_{regs} seems to differ per molecular breast cancer subtype. These subtypes are broadly defined on the basis of tumoral expression of the estrogen and progesterone hormone receptors (HR^+), the growth factor receptor HER2, or absence of these (triple-negative breast cancer, TNBC)[4]. Several meta-analyses published over the last few years showed that high FOXP3 TILs in HR^+ breast tumors correlate with poor survival, high grade and lymph node involvement[46–48]. However, multivariate Cox regression on patient outcome including adjustment for tumor size, grade and lymph node stage revealed that FOXP3 TILs are not an independent prognostic factor in HR^+ breast tumors[49,50]. Whether T_{regs} are causally involved in the differentiation of high grade tumors, lymph node metastasis and poor prognosis cannot be concluded from these descriptive analyses. In contrast to HR^+ breast cancer, FOXP3 TILs strongly correlate with a favorable prognosis in HR^- and TNBC subtypes[46,49,51,52]. Here, T_{reg} infiltration is strongly associated with high $CD8^+$- and T_{helper} cell infiltration, perhaps reflecting a T cell permissive environment[53]. This is further supported by the observation that T_{regs} are not associated with prognosis in triple-negative tumors with low CD8 infiltration[51]. In conclusion, T_{regs} correlate with disease outcome, in a subtype dependent manner, but future preclinical research is necessary to uncover the mechanistic link between T_{regs} and breast cancer subtypes.

TABLE 1. Prognostic significance of FOXP3 TILs across breast cancer subtypes

Subtype analysed	Patients (n)	Correlations with high FOXP3 TILs:			Reference
		Prognosis	Subtype	Clinical features	
DCIS	62	Poor (univariate)	DCIS[d]	ND[e]	(Bates et al. 2006)
ER-	77	No effect	ER-	High grade, LN met+[g]	
ER+	148	Poor (univariate)			
ER-	364	No effect	ER-; HER2+; basal	High grade, LN met+[g], large tumor size	(Mahmoud et al. 2011)
ER+	982	Poor (univariate)[a]			
MIXED	398	Poor (multivariate)	ER-; HER2+;basal	High grade	(Yan et al. 2011)
MIXED	1270	Poor (multivariate)	ER-; PR-; HER2+	High grade	(Liu et al. 2011)
MIXED	72	Poor (univariate)[a]	NS[f]	LN met+ [g], p53+, Ki67+	(Kim et al. 2013)
MIXED	90	Poor (multivariate)	ER-; HER2+	High grade	(Takenaka et al. 2013)
MIXED	90	Poor (univariate)[a]	HER2+	High grade, LN met+[g], large tumor size	(Maeda et al. 2014)
MIXED	498	Poor (univariate)[a]	HER2+; TNBC	High γδ T cell	(Allaoui et al. 2017)
MIXED	118	Poor (univariate)	ND[e]	High grade,LN met+[g], Ki67+, tumor nest	(Peng et al. 2019)
TNBC	86	Favorable (multivariate)	ND[e]	LN met+ [g]	(Lee et al. 2013)
ER- HER2-	175	Favorable (univariate)	NS[f]	High grade, high CD8+, young age	(West et al. 2013)
ER- HER2+		No effect			
ER+	2166	No effect (multivariate)[b]	ER-; HER2+; basal	High grade, LN met+[g], High CD8+, young age	(Liu et al. 2014)
ER- HER2+	250	No effect (multivariate)[c]			
BASAL	330	Favorable (multivariate)			
ER+	554	ND[e]	ER+	ND[e]	(Tsang et al. 2014)
ER- HER2+					
MIXED	218	No effect	ND[e]	High grade, high CD8+, high PD1+	(Sun et al. 2014)
TNBC	101	No effect	ND[e]	High CD8+	(Miyashita et al. 2015)
MIXED	207	No effect	ER-, HER2+; TNBC	High grade, Ki67+	(Papaioannou et al. 2019)

a. not significant in multivariate analysis
b. Poor prognosis in low $CD8^+$ tumors
c. favorable prognosis in high $CD8^+$ tumors
d. compared to normal breast
e. Abbreviation; ND, not determined
f. Abbreviation; NS, not significant differences
g. Abbreviation; LN met+; Lymph node involvement
Table references: [45], [49], [129], [130], [131], [132], [133], [134], [135] [136], [51], [50], [137], [138], [139], [140]

Predictive value of T_{regs} in cancer immunotherapy

Novel therapeutics targeting immune checkpoints as PD-1/PD-L1 and CTLA-4 are transforming the treatment landscape across cancer types[54]. In order to maximize efficacy, numerous studies are currently evaluating predictive biomarkers and novel treatment combinations[55]. Importantly, T_{regs} can be direct targets of these treatments, due to their high expression of immune checkpoint molecules[56]. While the use of immunotherapy in breast cancer is still in its infancy, research in other cancer types has revealed the potential predictive significance of T_{regs} in the context of PD-1/PD-L1 blockade. For example, PD-1 blockade has been associated with disease progression in gastric cancer patients, via the activation and expansion of intratumoral PD-1^+ T_{regs}[57]. Likewise, high intratumoral T_{reg} proliferation in response to anti-PD-1 has been linked to recurrence in melanoma[58]. Finally, PD-L1 mediated expansion of pT_{regs} is an important immune-suppressive axis in glioblastoma[59]. In recent years, the first trials investigating the efficacy of immune checkpoint blockade (ICB) in metastatic TNBC have been published, with a strong focus on PD-1/PD-L1 blockade[12,60–65]. Although clinical benefit is observed for a small proportion (approximately 5-20%) of breast cancer patients, emerging evidence shows that selecting patients based on immune parameters such as a high TIL score and high PD-L1 expression may modestly improve response rates[66]. Up until now, T_{regs} have not been specifically reported to be correlated with efficacy in these early studies. As such, research in the coming years should clarify whether T_{regs} are predictive for PD-1/PD-L1 based treatments in breast cancer.

Qualitative clinical assessment of T_{regs} in breast cancer

Besides quantification of intratumoral T_{regs}, there is a growing body of evidence indicating that a more in-depth qualitative assessment of T_{regs}, including information on phenotype, functional state and immune-cell crosstalk, may be important for disease outcome. For example, recent reports have shown that intratumoral T_{regs} from breast cancer patients display an activated phenotype with high expression of CD25, CTLA-4 and PD-1, and exert immunosuppressive activity[11,67,68]. In one of these studies, the transcriptome of T_{regs} from 105 treatment-naïve breast cancer patients was analysed[67]. The chemokine receptor CCR8 was identified to be uniquely expressed by intratumoral T_{regs}, but not by T_{regs} isolated from breast tissue and blood from healthy donors. CCR8^+ T_{regs} were found to be highly proliferative and enriched in high grade tumors. Strikingly, while intratumoral T_{reg} abundance based on *FOXP3* mRNA expression did not correlate with clinical features, stratifying patients based on the CCR8:*FOXP3* ratio in the tumor strongly correlated with poor survival in patients[67]. These findings illustrate that in-depth analysis of intratumoral T_{regs} provides important information. As the patients in this cohort predominantly had HR^+ tumors (74.3%), an important next step would be to validate these findings in $HER2^+$ and TNBC subtypes, in which T_{regs} are associated with good prognosis[51].

Many studies have reported increased frequencies of T_{regs} in the peripheral blood of breast cancer patients across subtypes[69–73], indicating that breast tumors can systemically engage T_{regs}. Still, their significance has remained elusive for a long time, until a recent report performed in-depth analyses on T_{regs} isolated from the blood and tumors of breast cancer patients[74]. It was found that a subpopulation of T_{regs} (Foxp3^{hi} CD45RA^{neg})[75], comprising approximately 19% of the total T_{reg} population in the peripheral blood of patients strongly resembles intratumoral T_{regs}, based on phenotype, TCR repertoire and CCR8 expression. This may suggest that intratumoral T_{regs} derive from Foxp3^{hi} CD45RA^{neg} T_{regs} in peripheral blood, or *vice versa*. These T_{regs} from blood had superior suppressive potential in vitro, compared to Foxp3^{low} CD45RA$^{pos/neg}$ T_{regs}. Foxp3^{hi} CD45RA^{neg} T_{regs} were found to be heterogeneous between patients in their signaling response to both immunosuppressive and inflammatory cytokines. High T_{reg} responsiveness to immunosuppressive cytokines correlated with poor survival, whereas high responsiveness to inflammatory cytokines had the opposite effect[74]. This exposes the potential clinical significance of T_{regs} in peripheral blood of breast cancer patients, but also highlights how T_{reg} heterogeneity may potentially influence disease outcome.

Over recent years, studies focusing on FOXP3 TILs are moving from basic quantification analyses towards sophisticated in-depth characterization, yielding exciting new insights with prognostic and potential therapeutic implications. As we are starting to discover the characteristics of T_{regs} with tumor-promoting capabilities, mechanistic studies should investigate their functional role in breast cancer progression, and whether their emergence can be therapeutically halted.

THE FUNCTIONAL ROLE OF T_{REGS} IN BREAST CANCER PROGRESSION AND METASTASIS

Preclinical animal models are key to mechanistically understand how T_{regs} impact breast cancer progression. An important tool to dissect T_{reg} function in these models is through their systemic depletion, which can be achieved via two strategies. Firstly, antibody-based approaches deplete T_{regs} through targeting of cell-surface receptors which are highly expressed on T_{regs}, including CD25, GITR and FR4[76–78]. Secondly, the development of transgenic mice that express the diphteria toxin receptor (DTR) under control of *Foxp3* either via direct knock-in (*Foxp3*^{DTR} mice), or by its introduction using a bacterial artificial chromosome (DEREG mice) has allowed for short-term inducible depletion upon injection of diphteria toxin (DT)[79,80]. A transgenic mouse model for mammary tumorigenesis that is regularly used to study the biology of T_{regs} in breast cancer is the MMTV-PyMT mouse model. T_{regs} have been shown to highly infiltrate mammary tumors of MMTV-PyMT mice, which is in part dependent on CCR2 expression on T_{regs}[81]. Ablation of T_{regs} in *Foxp3*^{DTR} mice with orthotopically transplanted MMTV-PyMT tumors drastically reduced tumor growth and

pulmonary metastases[82]. Mechanistically, IFNγ and $CD4^+$ conventional T cells were required for the observed anti-tumor effect, which was independent of $CD8^+$ T cells or NK cells. As pro-inflammatory signaling by myeloid cells was increased upon T_{reg} depletion, the authors speculated that IFNγ-activated macrophages may contribute to anti-tumoral inflammation[82]. The observation that T_{regs} constrain anti-tumor immunity in tumors has been reported by others. For example, anti-CD25 treatment in mice inoculated with 4T1 cancer cells strongly reduced tumor growth, which correlated with an increase in DCs and effector $CD8^+$ T cells in tumor draining lymph nodes, suggesting that T_{regs} modulate DC function[83]. Indeed, it has been reported that T_{regs} can inhibit the expression of co-stimulatory ligands on DCs thereby restraining CD8 activation and tumor clearance in a KRAS mutant model for pancreatic cancer[84]. It would be of interest to investigate whether similar mechanisms are at play in breast cancer. Elimination of T_{regs} is not always sufficient to drive strong anti-tumor responses. For example, immunosuppressive T_{regs} were found to be highly enriched in inoculated TNBC T-11 tumors, but DT-based T_{reg} ablation only slightly slowed tumor growth. T_{reg} ablation did potentiate PD-1/CTLA4 based immunotherapy which correlated with an increase in $IFN\gamma^+$ $CD8^+$ T cells[85]. These findings suggest that T_{regs} can form an important barrier for immunotherapy-induced anti-tumor immunity which has been reported before in preclinical inoculated melanoma and colon carcinoma tumors[76].

The studies above suggest that targeting T_{regs} in (breast) cancer models induces anti-tumoral inflammation which, sometimes in combination with immunotherapy, may have the potential to unleash anti-tumor immune responses. However, therapeutic elimination of T_{regs} may trigger auto-immunity in cancer patients, in particular in combination with ICB. Thus, an important next step would be to define the context-dependent molecular mechanisms engaged by T_{regs}, to enable precise targeting of relevant effector programs instead. A key challenge here is the apparent variability of the clinical significance of T_{regs} per breast cancer subtype, which necessitates the need to study these cells in clinically relevant mouse tumor models. Currently, the vast majority of murine breast cancer cell lines used for inoculation into mice and genetically engineered mouse models (GEMMs) for breast cancer give rise to ER^- mammary tumors[86], whereas ~75% of human invasive breast cancers are ER^+ [87]. As T_{regs} have been associated with a detrimental role particularly in HR^+ breast cancers, future research should ideally focus on the development and use of HR^+ breast tumor models to uncover the subtype dependent role of T_{regs} in breast cancer.

While in the context of established tumors, T_{regs} can interfere with anti-tumor immunity (Figure 1), recent findings in spontaneously developing tumor models suggest that at the onset of neoplastic progression T_{regs} may unexpectedly constrain pro-tumoral inflammation which promotes tumor initiation. One study reported that DT-based ablation of T_{regs} during the early, non-invasive neoplastic phase in the MMTV-PyMT model accelerated the progression of non-invasive lesions into invasive tumors[88]. The elimination of T_{regs} resulted in the

accumulation of macrophages in mammary glands and an induction of the Th2 cytokines IL-4 and IL-5, which have been reported to induce tumorigenic functions in macrophages[89]. The $CD44^+$ $CD24^-$ mammary stem cell compartment was also found to be expanded, with increased colony forming capacity in vitro. Whether T_{regs} directly control mammary stem cell proliferation or indirectly via the micro-environment remains to be addressed. In line with these findings, T_{regs} have also been reported to inhibit pancreatic carcinogenesis of neoplastic lesions in a KRAS mutant GEMM by repressing the recruitment of immunosuppressive myeloid cells[90]. These findings reinforce that T_{regs} are potent suppressors of inflammation in early stages of tumorigenesis, which has context dependent effects on tumor progression. As T_{regs} have been found to expand in ductal carcinoma in situ (DCIS)[45], more research is needed to uncover whether these cells play a protective or detrimental role in pre-cancerous breast cancer lesions.

Research on T_{regs} in other cancer types has revealed the versatile nature of these cells, and has uncovered novel mechanisms of immune cell crosstalk[84]. For example, T_{reg} derived IL-10 and IL-35 can promote $CD8^+$ T cell exhaustion in melanoma[91]. It is also becoming increasingly clear that T_{regs} can interact with a variety of myeloid cells including eosinophils, mast cells, macrophages, neutrophils and basophils, to hamper anti-tumor immunity[92,93]. T_{regs} were found to control intratumoral eosinophil and basophil infiltration, both of which can promote recruitment of $CD8^+$ T cells, leading to tumor rejection of melanoma cell lines[94,95]. In addition, T_{regs} indirectly maintain an immunosuppressive phenotype in TAMs by inhibiting the release of IFNγ in the TMEs of inoculated B16 and MC38 tumors[96]. Up until now, these interactions have not been investigated in the context of breast cancer, illustrating that we have perhaps only scratched the surface on T_{reg} effector functions in breast cancer. Promisingly, a transcriptional signature specific for tumor infiltrating T_{regs} has revealed remarkable similarity across tumor types in both human and mouse[97], suggesting that effector mechanisms may be shared across tumor types. In line, the chemokine receptor CCR8 was identified as part of this signature, endorsing previously discussed findings in human breast cancer[67].

Mechanisms of intratumoral accumulation of T_{regs} in breast tumors

Three main hypotheses have been postulated to explain the accumulation of T_{regs} in breast tumors. Firstly, T_{regs} that circulate in peripheral blood and lymph nodes may migrate into the TME following chemokine gradients upon activation. Secondly, it has been hypothesized that tissue-resident T_{regs} locally expand in the TME. Finally, intratumoral conversion of conventional $CD4^+$ T cells into T_{regs} may represent an important mechanism for T_{reg} accumulation. Although these hypotheses are non-mutually exclusive and may all contribute to T_{reg} accumulation, in particular the migration hypothesis has been supported with experimental evidence. Studies in human and mice have shown that T_{regs} express a wide range of chemokine receptors which may facilitate intratumoral homing, of which CCR2, CCR4, CCR5, CCR8, CXCR3 and

CXCR6 have been associated with breast cancer[9,67]. For example, $CCR2^+$ T_{regs} accumulate in multiple tumor models including the PyMT-MMTV model[81]. These cells display an activated phenotype, and were found to be tumor-antigen specific in an OVA-expressing sarcoma cell line inoculation model. Specific ablation of CCR2 on T_{regs} strongly reduced intratumoral T_{reg} accumulation[81]. CCR2 was also found to be expressed by intratumoral T_{regs} in human breast tumors[67]. Others have reported high expression of CCR4 by T_{regs} in the blood of breast cancer patients, with migratory capabilities to CCL22 and CCL17[68]. As discussed above, CCR8 has emerged as a chemokine receptor expressed uniquely by intratumoral T_{regs}[67,74], and has therefore gained attention as a potential therapeutic target. Anti-CCR8 mAb treatment of mice inoculated with CT26 colon carcinoma cells significantly reduced T_{regs} in tumors and enhanced intratumoral IFNγ expression[98]. In contrast, others have shown that CCR8 may be redundant for intratumoral T_{reg} homing, as injection of $CCR8^{KO}$ T_{regs} in mice inoculated with MC38 colon carcinoma cells did not interfere with their migration into tumors[97]. It has also been reported that autocrine production of CCL1, the ligand for CCR8, potentiates both T_{reg} proliferation and suppressive potential[99], suggesting that CCR8 may play an important role in maintaining T_{reg}-mediated immunosuppression, in addition to its chemotactic properties.

Accumulating evidence shows that intratumoral T_{regs} in breast cancer are transcriptionally distinct from T_{regs} in peripheral blood and lymph nodes, and share gene expression profiles with mammary tissue resident T_{regs}[67,100,101]. This suggests that either tissue resident cells expand in tumors, or that the local micro-environment drives transcriptional adaption of cells migrating into the TME. It has been reported that intratumoral and healthy breast T_{regs} within patients showed relatively little overlap of their TCR repertoire, suggesting that intratumoral T_{regs} do not derive from resident cells[67]. In addition, Ki67 expression in T_{regs} of healthy breast tissue was found to be drastically lower than in T_{regs} from tumor or blood. In line with the second notion, scRNA-seq of murine T_{regs} of naïve mice revealed that T_{reg} migration from lymphoid to non-lymphoid tissues indeed induces a transcriptional program specifically tailored to the destined tissue[102]. Furthermore, scRNA-seq of $CD45^+$ cells sorted from human breast tumors, blood and lymph nodes uncovered that intratumoral immune cells can acquire diverse phenotypes that are not found in circulation or normal tissue[100]. Here, five different T_{reg} clusters unique to the TME were identified, which were highly activated and expressed anti-inflammatory, exhaustion-, hypoxia-, and metabolism-related gene sets. Together, these studies suggest that transcriptional adaptation of migratory T_{regs} in the TME may explain the transcriptomic resemblance between intratumoral and mammary tissue resident T_{regs}, although further TCR profiling and genetic tracing studies are needed to definitively confirm this.

Research on the accumulation of tT_{regs} versus pT_{regs} in cancer has been rather limited due to the complexities of distinguishing both T_{reg} subsets in vivo. Yet, local induction of pT_{regs}

in the TME may in fact be an important mechanism of immunosuppression, as TGF-β is abundantly expressed in cancers[103]. However, analysis of T_{regs} in human glioma, melanoma and lung cancer samples did not reveal a substantial contribution of pT_{regs} to the total intratumoral T_{reg} pool[67,104–106]. For example, one study found that the overlap between TCR clonotypes of FOXP3$^+$ and FOXP3$^-$ CD4$^+$ T cells obtained from six melanoma tumors was 0.5-13.2%, indicating a relatively small proportion of T_{regs} may have been pT_{regs}. Yet, others have attributed important roles to pT_{regs} in murine cancer models[107–110]. One of these reports provided indications for their presence in the TME of breast cancer patients[110]. TCR repertoire analysis on CD4$^+$ T cells from tumor, blood and lymph nodes of five breast cancer patients revealed that tumor infiltrating T_{regs} are most similar to naïve CD4$^+$ T cells from tumor and blood, suggesting intratumoral conversion. By using the MDA-MB-231 TNBC cell line in humanized mice, it was further shown that TAM-secreted CCL18 specifically recruits naïve CD4$^+$ T cells, but not T_{regs}, via PITPNM3 into the TME. Here, these naïve CD4$^+$ T cells were capable of converting into FOXP3$^+$ T_{regs}, via unknown mechanisms. Blocking CCL18 in tumor bearing mice reduced intratumoral T_{reg} numbers and inhibited tumor growth[110]. As data on the role of pT_{regs} in breast cancer is still limited, future studies should focus on expanding these findings in a larger cohort of patients.

It is now well established that T_{regs} have various ways to accumulate into primary tumors. However, breast cancer survival is largely dictated by the extent of metastatic disease. Thus far, we have mostly discussed research on T_{regs} in breast cancer in the context of primary tumors, raising questions on the link between primary tumors and metastasis. Can T_{regs} impact metastasis formation from within the primary tumor? Or do circulating and/or tissue resident T_{regs} induce a systemic immunosuppressive axis which impacts metastasis formation?

Impact of T_{regs} on metastatic progression

Primary cancer cells have to progress through a multi-step process in order to successfully metastasize. This so-called metastatic cascade consists of tumor cell invasion, intravasation, survival in the circulation, extravasation, and outgrowth in a foreign, hostile environment, all while evading destruction by the immune system[2]. Prior to metastatic spread, tumor-derived systemic factors can even further potentiate metastasis by instructing (immature) myeloid cells to establish a pre-metastatic niche[111]. T_{regs} may potentially be involved in all steps of the metastatic cascade, through mechanisms both dependent and independent of their immune-regulatory function. However, progress into understanding their impact on the metastatic cascade is hampered by the limited availability of preclinical models that realistically recapitulate metastasis[112]. Cancer cell line-based mouse models fail to fully recapitulate the chronic and systemic inflammation that underlies *de novo* tumor development, progression and metastasis[113]. In addition, research in both 4T1 and PyMT models has shown that T_{reg} depletion reduces primary tumor growth which may obscure mechanisms at play during the

metastatic cascade[82,114]. Despite these shortcomings, several studies have revealed that tumor-induced (systemic) activation of T_{regs} can contribute to metastatic progression (Figure 1). This activation can be mediated via the release of various tumor-derived soluble factors, such as prostaglandins, complement factors and beta-galactoside-binding proteins[115–117]. For example, tumor-secreted galectin-1 was reported to enhance systemic T_{reg} expansion and their suppressive potential resulting in increased lung metastases in mice bearing inoculated 4T1 mammary tumors[115]. Others showed that overexpression of COX2 in inoculated TM40D mammary tumors enhanced bone metastasis, which correlated with increased recruitment of T_{regs} into the primary tumor[116]. In addition to factors released by the primary tumor, the local (pre)metastatic niche can also play an important role in the activation and recruitment of T_{regs}. For example, IL-33 and CCL17 have both been reported to be released in metastatic foci in the lungs of 4T1 tumor-bearing mice, leading to the recruitment of T_{regs} that express the receptor for these molecules, thereby promoting metastasis[118,119].

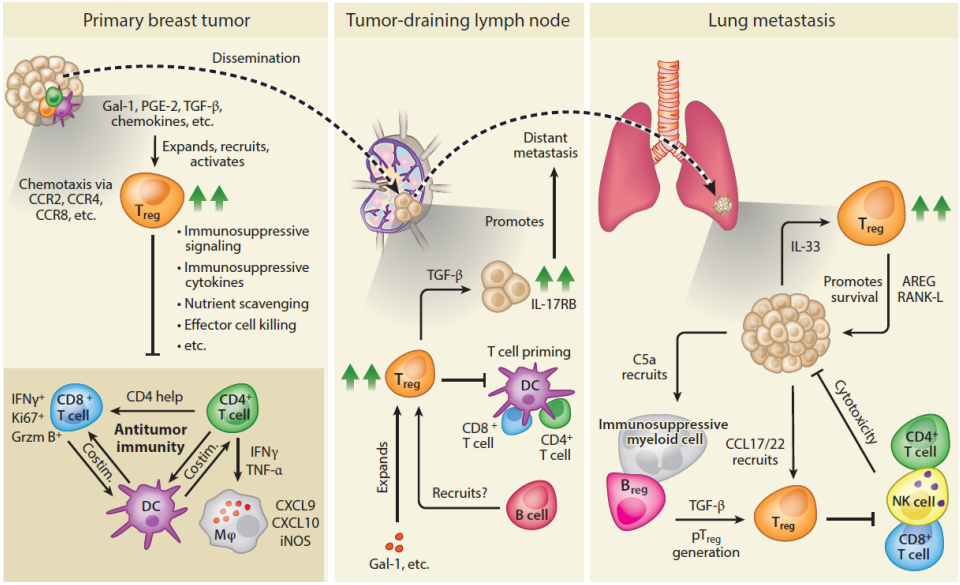

FIGURE 1. T_{regs} modulate their local environment to promote breast cancer progression. Tumor derived factors such as chemokines, cytokines and other mediators promote the accumulation and expansion of T_{regs} in primary breast tumors and metastatic niches. In the TME, T_{regs} constrain both innate and adaptive immune responses to counteract anti-tumor immunity. Mechanistically, T_{regs} can (among others) suppress the expression of co-stimulatory ligands on DCs, release inhibitory modulators that interfere with T cell activation, but are also equipped to induce apoptosis in effector cells. However, the effector mechanisms that are engaged in the context of the breast TME remain largely unknown. In addition, T_{regs} can enhance metastatic progression by promoting tumor cell survival and migration via secretion of TGF-β, AREG and RANK-L or by inhibition of cytotoxic effector cells. Abbreviations: Co-stim, co-stimulation; Gal-1, galectin-1; Grzm B, Granzyme B; Imm. suppr., immunosuppressive; Mϕ, macrophage.

Various tumor-driven pathways exist to systemically engage T_{regs} to the benefit of metastatic spread. An underlying question remains how T_{regs} mechanistically contribute to metastasis. Interestingly, T_{regs} have been found to directly contribute to metastasis of the 4T1 and MT2 cell lines in mice by promoting tumor cell survival via the release of RANK-L and AREG[118,120]. In addition, in line with their immunomodulatory properties, the pro-metastatic function of T_{regs} has been linked to inhibition of cytotoxic immune cells. To this regard, T_{reg} mediated inhibition of NK cells has been associated with increased pulmonary metastasis in the 4T1 model[121]. Others found that neoadjuvant ablation of T_{regs} in 4T1 bearing *Foxp3*^{DTR} mice almost completely abolished the formation of lung metastases, which was dependent on both $CD4^+$ and $CD8^+$ T cells, but not NK cells[114]. Of note, only neoadjuvant and not adjuvant T_{reg} depletion increased the systemic frequency and activation of tumor specific $CD8^+$ T cells[114]. It has not been addressed whether $CD4^+$ T cells directly engage in tumor-cell killing in the absence of T_{regs}, or perhaps provide essential help for $CD8^+$ T cell activation. The superiority of neoadjuvant over adjuvant targeting of T_{regs} suggests a role for T_{regs} in early stages of metastasis, which is supported by the clinical finding that T_{regs} associate with lymph node involvement[46].

Multiple clinical studies have reported associations between high T_{reg} infiltration in primary tumors and sentinel lymph nodes with lymph node (Table 1), but mechanistic data are limited. So far, one study has linked intranodal T_{regs} to breast cancer progression in mice. Here, using the 4T1 model, T_{reg}-derived TGF-β1 induced IL17RB in cancer cells in tumor draining lymph nodes (TDLN)[122]. IL17RB was found to potentiate the metastatic- and colony forming potential of cancer cells via NF-κb, which enhanced distant metastasis. Interestingly, analysis of IL17RB expression in lymph node metastasis and matched tumors of breast cancer patients confirmed that IL17RB is increased in lymph nodes, and correlates with FOXP3 frequency[122]. This study revealed that the TDLNs in breast cancer can function as a gateway to distant metastasis, with T_{regs} corrupted by the primary tumor. These findings raise the question whether T_{regs} are also involved in cancer cell dissemination to the draining lymph node. It has recently been reported that B cells promote metastasis to draining lymph nodes in the 4T1 and MMTV-PyMT models via the release of HSP4A-binding antibodies which directly promote tumor cell migration[123]. Interestingly, B cell depletion did significantly reduce tumor-induced T_{reg} accumulation in TDLNs. In line with these findings, it has previously been reported that regulatory B cells that accumulate in 4T1 tumor-bearing mice can induce pT_{regs} in a TGF-β dependent manner[109], revealing an interesting crosstalk between T_{regs} and B cells in breast cancer metastasis.

FUTURE PROSPECTS

T_{regs} have taken an increasingly important position in our understanding of the immune system in breast cancer. Preclinical research has revealed ingenious mechanisms employed by breast tumors to seize control of T_{regs} for their own benefit. In parallel, in-depth characterization of T_{regs} beyond traditional FOXP3 scoring in human samples is paving the way to advance their prognostic and predictive value in the clinic. Here, future efforts should focus on further defining the heterogeneity of T_{regs} and evaluate which features of T_{regs} are instrumental for disease progression, while also expanding current findings to HR^- subtypes of breast cancer where T_{regs} are associated with a good prognosis. As the use of immunomodulatory drugs is gaining momentum in the clinic, interrogating these observations in the context of immunotherapy is also an important next step.

The context dependency under which T_{regs} operate should also be increasingly taken into account in preclinical research. Until now the majority of research has been performed in a limited number of (cell line-based) breast cancer models, often with unclear translatability to human disease. An important challenge to address here is that breast cancer patients suffer from metastatic spread to a broad spectrum of anatomical locations, while experimental metastasis in animal models is often limited to the lungs. A crucial next step is therefore to validate preclinical findings in murine models that have increased translatability, both in terms of cancer subtype and metastasis formation. To achieve this, it is important to realize that the interaction between the immune system and cancer may even be more complex than initially assumed. We are only now beginning to understand that the genetic make-up of tumors may profoundly impact their accompanying micro-environment[124]. In addition, in-depth analyses of 168 metastatic and primary tumor samples from 10 breast cancer patients revealed that the composition of metastatic TMEs within patients was heterogeneous, even within particular organs. Moreover, the expression of immunomodulatory genes such as PD-1 and PD-L1 differed across metastases within individual patients[125]. These complexities of human metastatic disease illustrate the need for accurate models of metastasis.

Ultimately, these fundamental insights into the role of T_{regs} in breast cancer progression could form the basis for therapeutic intervention. As such, several early phase clinical trials have evaluated the FDA approved mAb daclizumab (anti-CD25) in combination with cancer vaccines in metastatic melanoma and breast cancer[126,127]. $FOXP3^+CD4^+$ T cells in peripheral blood were found to be reduced upon daclizumab treatment, but no significant clinical benefit was observed. However, daclizumab does not induce antibody-dependent cytotoxicity (ADCC), which others have suggested to be essential for intratumoral T_{reg} depletion and therapeutic efficacy[76,127]. Recently, an optimized ADCC inducing anti-CD25 antibody showed superior intratumoral T_{reg} depletion, and induced CD8-mediated tumor rejection in combination with anti-PD-1 in preclinical models. Alternatively, intratumoral injection of CD25 targeting immunotoxins also potently depletes intratumoral T_{regs}, leading to $CD8^+$ T

cell mediated tumor regression of inoculated 66c14 breast cancer tumors[128]. Importantly, these preclinical results suggest that effector T cell responses are not necessarily negatively impacted by CD25-based depletion, which may set the stage for clinical trials evaluating this new generation of T_{reg} targeting strategies. In addition to T_{reg} depletion, blocking of their intratumoral recruitment, conversion, or important effector mechanisms may be alternative future approaches to interfere with T_{reg}-mediated modulation of breast cancer[10].

In conclusion, recent research has exposed T_{regs} as important modulators of breast cancer progression and metastasis, while exciting advancements in clinical analysis improves the prognostic and predictive significance and potentially therapeutic targeting of these cells. The use of GEMMs that closely mimic the diversity and the step-wise progression of human breast cancer subtypes will propel our understanding of T_{reg} biology to a higher level and deepen our knowledge of underlying mechanisms. This knowledge could help to take full advantage of novel immunomodulatory drugs that may take the stage in breast cancer treatment.

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