THE UNIVERSITY OF CHICAGO

THE ROLE OF TUMOR-ASSOCIATED LYMPHATICS IN MODULATING RESPONSIVENESS TO CANCER IMMUNOTHERAPIES IN TRIPLE-NEGATIVE BREAST CANCER

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I dedicate this dissertation to my family, who have supported me throughout my academic journey. Being away from home and not seeing you for the past 6 years was one of the most difficult experiences in my life and it would not have been bearable without your love.

I am forever indebted to you and I look forward to the bright days before us!

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ABSTRACT

Triple-negative breast cancer (TNBC) has a higher relapse rate and fewer treatment options compared to other molecular subtypes of breast cancer. Cancer immunotherapies are currently being investigated as a new treatment modality in TNBC to improve survival rates. However, a significant subset of patients still does not respond to these therapies. A better understanding of the tumor microenvironment in breast cancer is needed to identify new biomarkers to stratify patients who would benefit from cancer immunotherapies. As a critical component of the immune system, the lymphatics have been shown to regulate anti-tumor immune responses in multiple tumor types, including in melanoma and glioblastoma. Here, I investigated the role of tumor lymphangiogenesis - the process of lymphatic vessel remodeling and expansion - in shaping anti-tumor immunity in breast cancer. In order to model tumor lymphangiogenesis *in vivo,* we transduced murine models of triple-negative mammary carcinoma to over-express the pro-lymphangiogenic vascular endothelial growth factor-C (VEGF-C). We show that tumor lymphangiogenesis increases recruitment of $CD4^+$ T cells and macrophages into the tumor microenvironment. Upon treatment with cancer immunotherapies, namely checkpoint inhibitors (CPI) and an agonist of Stimulator of Interferon Genes (STING) pathway, lymphangiogenic 4T1 tumors demonstrate improved responsiveness to the treatment and prolonged survival. The increase in the levels of various chemokines after immunotherapy treatment leads to recruitment of various immune cells, including cross-presenting dendritic cells (DCs) and interferon-γ (IFNγ) secreting $CD8^+$ T cells. Furthermore, after treatment with a combination of CPI and STING agonist, the potent systemic anti-tumor immunity in the lymphangiogenic 4T1 tumor-bearing mice reduces the spontaneous lung metastasis in 4T1 tumor-bearing mice. The beneficial effect of tumor lymphangiogenesis is dependent on $CD4^+$ T cells and macrophages, as their depletion abrogates

the observed phenotype. Consistent with the murine studies, expression of VEGF-C positively correlates with CD4 and macrophage gene expressions in human triple-negative breast tumors. These results reveal that tumor lymphangiogenesis improves responsiveness to cancer immunotherapies in mice and suggests that patients with increased tumor lymphatics may further benefit from these therapies.

CHAPTER 1: INTRODUCTION

1.1 Abstract

Cancer immunotherapies have revolutionized cancer treatment in the last two decades. Despite their success across multiple cancer types, they have had limited efficacy in certain non-immunogenic cancers, including breast cancer. Lymphatic vessels connect peripheral tissues to lymph nodes and have been known to be crucial in shaping immune responses. However, their role in anti-tumor immunity has not been explored until recently. In this chapter, I give an overview of the lymphatic system and breast cancer together with the limitations of cancer immunotherapies, specifically in breast cancer patients.

1.2 Lymphatic vessels

The network of lymphatic vessels is mainly known to be responsible for draining the interstitial fluid and returning it to the bloodstream through the lymph nodes and thoracic duct, thus completing the circulation system [1]. Moreover, lymphatic vessels transport small molecules (such as proteins and exosomes) and immune cells to the lymph nodes where the adaptive immune response is mounted [2]. The initial lymphatics in the periphery, lined by lymphatic endothelial cells (LECs), drain the interstitial fluid and transport it to the lymph nodes after converging into collecting lymphatics [1, 2] (**Fig 1.1**). The unidirectional valves and smooth muscles in the walls of collecting lymphatics allow the drained fluid, termed lymph, to be pumped by afferent lymphatics into the lymph node. After the surveillance of the antigens in the lymph by the leukocytes, the lymph carrying the activated lymphocytes exits the lymph node through efferent lymphatics and joins the circulation where the cells enter peripheral tissues to patrol for their cognate antigens [2].

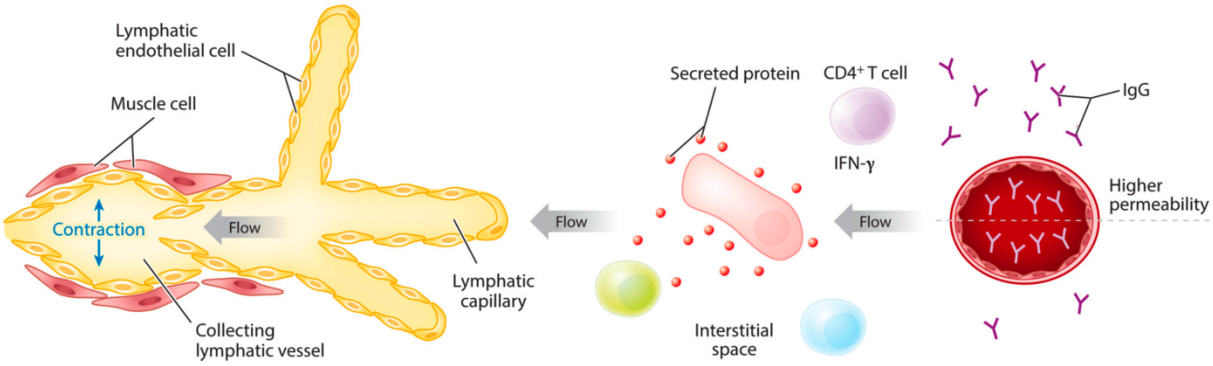

Figure 1.1. Transport of macromolecules and leukocytes through lymphatic capillaries and collecting vessels. The interstitial fluid is drained from the interstitial space by the lymphatic capillaries. The muscle layer around the collecting lymphatic vessels allows them to contract and unidirectionally drain the lymph to the lymph node. The lymph contains different macromolecules and cells including secreted proteins, CD4+ T cells and IgGs. Adapted from Randolph et al. [2].

1.3 Breast Cancer

Breast cancer is the second most common cancer among women in the US and will be diagnosed in 12% of all women in the US over their lifetime [3]. Physical examination, magnetic resonance imaging (MRI), ultrasound and mammography of the breast and biopsy of the lymph nodes are the diagnostic techniques used for diagnosis and staging of breast cnacer. At the time of diagnosis, more than 60% of breast cancers are confined to the breast, while about 30% have spread to the draining lymph nodes and the rest are metastatic [3]. Based on the histology diagnosis, most common breast cancers are invasive ductal carcinoma and invasive lobular carcinoma [4]. On the molecular level, there are three major breast cancer subtypes, namely hormone receptor positive (HR^+) (70%), human epidermal growth factor 2 positive ($HER2^+$) (15%) and the triple-negative subtype (15%), which lacks the over-expression of the estrogen receptor (ER), progestrone receptor (PR) and HER2 [3]. Triple-negative breast cancer (TNBC) is more common in youger women and women of color, whereas HR^+ breast cancer is more prevalent amongst older women [5]. The standard-of-care for non-metastatic breast cancer is surgical resection of the tumor and biopsy of sentinal lymph nodes with possible adjuvant radiotherapy [3]. However, local therapies in metastatic breast cancer are used mostly as a palliative strategy. The adjuvant or neoadjuvant systemic therapy in both metastatic and non-metastatic breast cancer depends on the molecular subtype, as described below.

Estrogen receptor is a steroid hormone receptor that upon binding of estrogen leads to activation of growth pathways, thus promoting growth of tumor cells when over-expressed on breast cancer cells [3]. Progestrone receptor signaling also induces the expression of mutual transcription factors with the ER signaling pathway [6]. Endocrine treatment, which inhibits estrogen signaling, is the primary systemic therapy for hormone receptor positive breast cancer

[3]. As an estrogen inhibitor, tamoxifen is effective in pre- and postmenopausal women. However, aromatase inhibitors, which inhibit conversion of androgens to estrogen, are only effective in postmenopausal women [6]. Longer-term studies have shown that a longer 10-year course of treatment, compared to the standard 5-year regimen, with either tamoxifen or aromatase inhibitors provides small improvement in clinical outcome, but increases toxicity and adverse events [7, 8]. Moreover, another trial demonstrated that a 5-year regimen of tamoxifen followed by a 5-year course of aromatase inhibitors reduces the risk of breast cancer recurrance by 40% [9]. In $HER2^+$ breast cancer patients, anti-HER2 antibodies and small molecule tyrosine kinase inhibitors in combination with chemotherapy are the standard systemic treatment regimen.

Compared with other subtypes, TNBC has the most limited treatment options and a higher rate of visceral metastasis, involving the brain and lungs [10]. Chemotherapy is the current standard-of-care for treatment of non-metastatic TNBC patients, with a pathologic complete response (pCR) of about 30% in the neoadjuvant setting [11, 12]. Based on mutations in DNA damage repair mechanisms in some TNBC tumors [13], DNA-crosslinking platinum chemotherapies are currently under investigation in clinical trials in TNBC [14, 15]. Compared to other breast cancer subtypes, TNBC has a higher replase rate in the first 3 to 5 years post-diagnosis [16]. Historic data shows that stage IV (distant metastatic disease) TNBC has a median overall survival of only 1 year, compared to 5 years for stage IV $HER2^+$ or HR^+ breast cancer [17]. Due to the substantial portion of metastatic TNBC patients that fail to respond to chemotherapy there is a critical need to improve and expand treatment regimens for TNBC patients.

1.4 Anti-tumor immunity

During cancer development, the genetic instability of cancer cells leads to expression of neoantigens which are then presented through binding to major histocompatibility complex class I (MHCI) molecules expressed on cancer cells and antigen-presenting cells (APCs) and recognized by T cells in the immune system [18]. Elimination of cancer cells by the immune system requires occurrence of a series of events termed cancer-immunity cycle [19] (**Fig. 1.2**). In brief, first the neoantigens expressed by cancer cells are taken up by professional APCs such as dendritic cells (DCs) in the presence of proinflammatory signals in the tumor microenvironment (TME). Next, APCs traffic to the draining lymph nodes and present the antigens on MHCI or MHCII molecules to T lymphocytes and activate them by expression of co-stimulatory molecules. Then, effector T cells exit the lymph nodes through efferent lymphatics and traffic to the TME by using the blood vasculature. In the TME, the antigen-specific T cells recognize the cancer cells by binding of their T cell receptors (TCRs) to the MHC-peptide complexes and kill the cell through secretion of cytolytic molecules. However, as the tumor grows and mutates, cancer cells evolve mechanisms to evade or dampen the anti-tumor immunity [20]. Downregulation of MHC molecules, antigen processing machinery, alteration in interferon-γ (IFN-γ) signaling, disruption of T cell infiltration and up-regulation of inhibitory molecules, such as programmed death ligand 1 (PD-L1), are amongst these strategies [20].

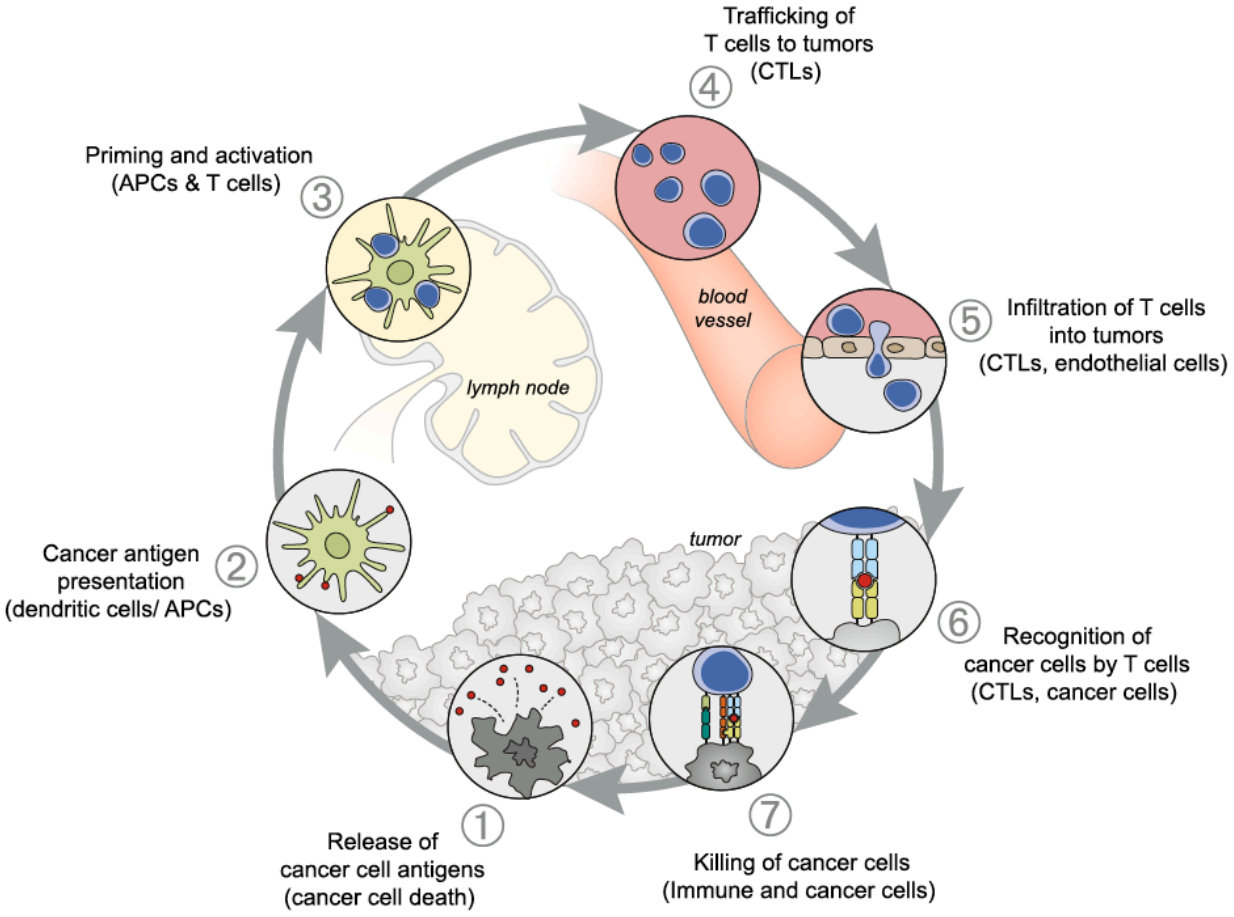

Figure 1.2. The different stages of the cancer-immunity cycle. Anti-tumor immunity against cancer cells is generated through propagation of the different stages of cancer-immunity cycle, as depicted here. Adapted from Chen & Mellman [19].

Various immune cells are involved in mediating the recognition and elimination of cancer cells. Here, I will briefly describe the role of some of these cell types in anti-tumor immunity.

1.4.1 Dendritic cells (DCs)

DCs, as one of the professional APC cell types, are critical in generation of antigen-specific immunity in different pathological conditions, including viral infections and cancer [21]. They present both endogenous and exogenous antigens to $CD4^+$ and $CD8^+$ T cells by loading them onto MHC class I and II molecules, which depending on the immunomodulatory signals can lead to

immunity or tolerance [22]. These immunomodulatory signals are determined by sensing the environmental cues using various receptors, including cytokine receptors and pattern-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [23]. Upon detection of the environmental cues, DCs express co-stimulatory molecules, including CD80 and CD86, and various cytokines, such as IL-12 and IL-10 to dictate T cell phenotypes [24, 25]. DCs are also an important source of chemokines in the TME [21]. Various DC subsets exist in mice and humans with specific roles in anti-tumor immunity [22, 26]. The two major DC subsets in mice are conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [21]. cDCs are derived from common DC precursors in the bone marrow and differentiate into two major categories of cDC1s, which includes resident $CD8\alpha^+$ DCs and migratory $CD103^+$ DCs, and $CD11b^+$ cDC2s [27]. It has been shown that cDC1s are potent inducers of immunity against intracellular pathogens and cancer cells due to their superior capability in cross-presenting exogenous antigens and priming $CD8^+$ T cells [27-29]. They are also the main producers of CXC-chemokine ligand 9 (CXCL9) and CXCL10 in the TME, which recruit effector $CD8^+$ T cells [30]. cDC2s on the other hand, have been demonstrated to be critical in priming $CD4^+$ T cells for anti-tumor immunity [31].

1.4.2 Macrophages

Tissue-associated macrophages (TAMs) are mostly differentiated from circulating monocytic precursors that are recruited to the TME both in the primary tumor and metastatic site [32, 33]. $Ly6C^+CCR2^+$ inflammatory monocytes have been shown to promote TAM accumulation in the TME [34] and distant organ metastasis in breast cancer [33]. However, a “non-classical” subset of monocytes, which patrol the lung tissue in healthy conditions, were demonstrated to inhibit lung metastases by recruitment and activation of natural killer (NK) cells [35]. Moreover,

recent evidence suggests that a proportion of tissue-resident macrophages in certain tissues are derived from precursors seeded during fetal and embryonic development [36]. TAMs are classically divided into pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages [37]. M1 macrophages are driven by IFNγ and IL-12 signaling, produced during type I immune responses, and mediate tumor cell killing [38]. However, type II associated cytokines, such as IL-4 and IL-13, induce M2 macrophages that are involved in tissue repair and immunity against parasites [39]. M2 macrophages have been shown to suppress T cell responses through different mechanisms, including metabolizing L-arginine through expression of arginase [40], which is essential for T cell anti-tumor activity [41]. Various chemokines and cytokines are involved in the recruitment and differentiation of TAMs. For instance, colony-stimulating factor-1 (CSF-1) attracts monocytes, provides survival signals for $CSF1R^+$ macrophages and promotes differentiation of M2 macrophages [42], whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) drives M1 differentiation [39]. Even though TAMs have traditionally been associated with adverse patient prognosis across various malignancies [43], recent evidence suggests that TAMs can play an inhibitory role on tumor growth rate, depending on the TME, disease stage and the involved tissue [38].

1.4.3 Neutrophils

Neutrophils are one of the major circulating leukocytes and among the first cells being recruited to inflammation sites and provide immunity against pathogens through phagocytosis and deposition of neutrophil extracellular traps (NETs) [44, 45]. In various cancer types, high levels of tumor-associated neutrophils (TANs) have been associated with poor prognosis [46]. For instance, levels of circulating neutrophils and neutrophil-to-leukocyte ratio have been correlated with worse clinical outcome to cancer immunotherapies [47]. Moreover, NETs have been shown

to promote tumor growth and metastasis in colorectal cancer patients [48, 49], and their inhibition with DNase I-coated particles has been demonstrated to reduce metastasis in the 4T1 triple-negative breast cancer model [50]. In support of their possible anti-tumorigenic roles, TANs have been demonstrated to promote $CD8^+$ T cell responses and improve survival in colorectal cancer patients [51]. Moreover, in the context of radiotherapy, TANs have been shown to promote the induced anti-tumor immunity [52]. TANs are mostly recruited to the TME through CXC chemokine receptor 2 (CXCR2) signaling by its ligands, namely CXCL1, CXCL2 and CXCL5 [53]. Similarly to macrophages, neutrophils have also been proposed to have two subpopulations, namely the anti-tumorigenic N1 TANs and pro-tumorigenic N2 TANs, despite lack of clear markers to identify them [54]. Transforming growth factor-β, secreted by cancer-associated fibroblasts (CAFs) and macrophages, has been shown to promote recruitment and differentiation of N2 TANs [55].

1.4.4 T cells

T lymphocytes are the main effector cells of the adaptive immunity arm in anti-tumor immune responses. High levels of tumor-infiltrating lymphocytes (TILs) have been correlated with improved survival and better prognosis in various malignancies [56]. The elimination of cancer cells recognized by T cells during tumor progression leads to growth of less immunogenic cancer cells, a processed termed immune-editing [57]. Naïve $CD8^+$ T cells are educated by professional APC and the resulting activated cells induce apoptosis in cancer cells once they recognize their cognate antigen presented on MHCI molecules [58]. Apoptosis is induced by ligation of death receptors on cancer cells, through Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL), or through secretion of cytolytic molecules, such as perforin and granzymes [58]. In order to maintain tolerance and prevent unwanted damage to self, effector T cells have evolved to

express inhibitory molecules such as CTLA-4, PD-1, TIM3 and LAG-3, activation of which induces exhaustion and anergy [59]. These molecules are over-expressed upon activation of T cells and cancer cells hijack these "checkpoint" mechanisms to evade the anti-tumor immunity [60, 61].

Upon antigen presentation, naïve $CD4^+$ T cells differentiate into various subsets, including T helper type I (T_H1) cells, T_H2, T_H9, T_H17, T_{FH} and T_{reg} cells [62]. Higher levels of T_H1 cells correlate with better clinical outcome in colorectal cancer [63] and breast cancer [64]. Notably, T_H1 cells were shown to correlate with responsiveness to anti-PD-1 therapy in melanoma [31].T_H1 cells have been shown to mediate tumor cell killing directly through secretion of IFNγ [31, 65] and improve the $CD8^+$ T cell response against cancer cells [66]. T_H2 cells, however, are generally considered pro-tumorigenic and promote tumor growth by production of IL-4, IL-5 and IL-13 cytokines [67]. The role of T_H17 cells in anti-tumor immunity is ambiguous and current evidence suggests they can have both pro-tumorigenic and anti-tumorigenic effects depending on the TME and cancer type [68, 69]. T_{reg} cells mediate antigen-specific tolerance and can dampen anti-tumor immunity, although recent evidence suggests they play an ambiguous role in tumor progression [70]. Finally, T_H9 cells, as a possible new subset of $CD4^+$ T cells [71], have been demonstrated to improve anti-tumor immunity and mediate cytolytic activity against cancer cells through secretion of granzymes.

1.5 Cancer immunotherapies

Cancer immunotherapies seek to eradicate the tumor cells by boosting the anti-tumor immunity or overcoming the immune-suppressive mechanisms in the tumor microenvironment (TME) that inhibit the cancer immunity cycle. Upon treatment, the cytotoxic cells in the body's immune system or adoptively transferred cells, including adoptively transferred T cells, recognize and eliminate the cancer cells [72]. Cancer immunotherapies, either as monotherapy or in combination with other treatment modalities, have revolutionized the clinical practice of cancer treatment for more than a decade [73] (**Fig. 1.3**). However, most of this success has been in cancer types with more immunogenic tumors, such as melanoma and microsatellite instable cancers [74]. Currently approved immunotherapies have had limited success in non-immunogenic tumors with low T cells infiltrations, such as breast cancer patients [73]. Moreover, even among the immunogenic cancer types, only a subset of patients responds to therapies such as checkpoint inhibitors (CPI) [75], thus emphasizing the importance of identifying new biomarkers to stratify breast cancer patients before treatment. This underscores the need to better understand the interplay between different cell types in the breast tumor microenvironment and the anti-tumor immunity in order to improve responsiveness to immunotherapies. There are various cancer immunotherapies that are FDA approved or currently under clinical development. Here, I describe some of the most important classes of these therapies:

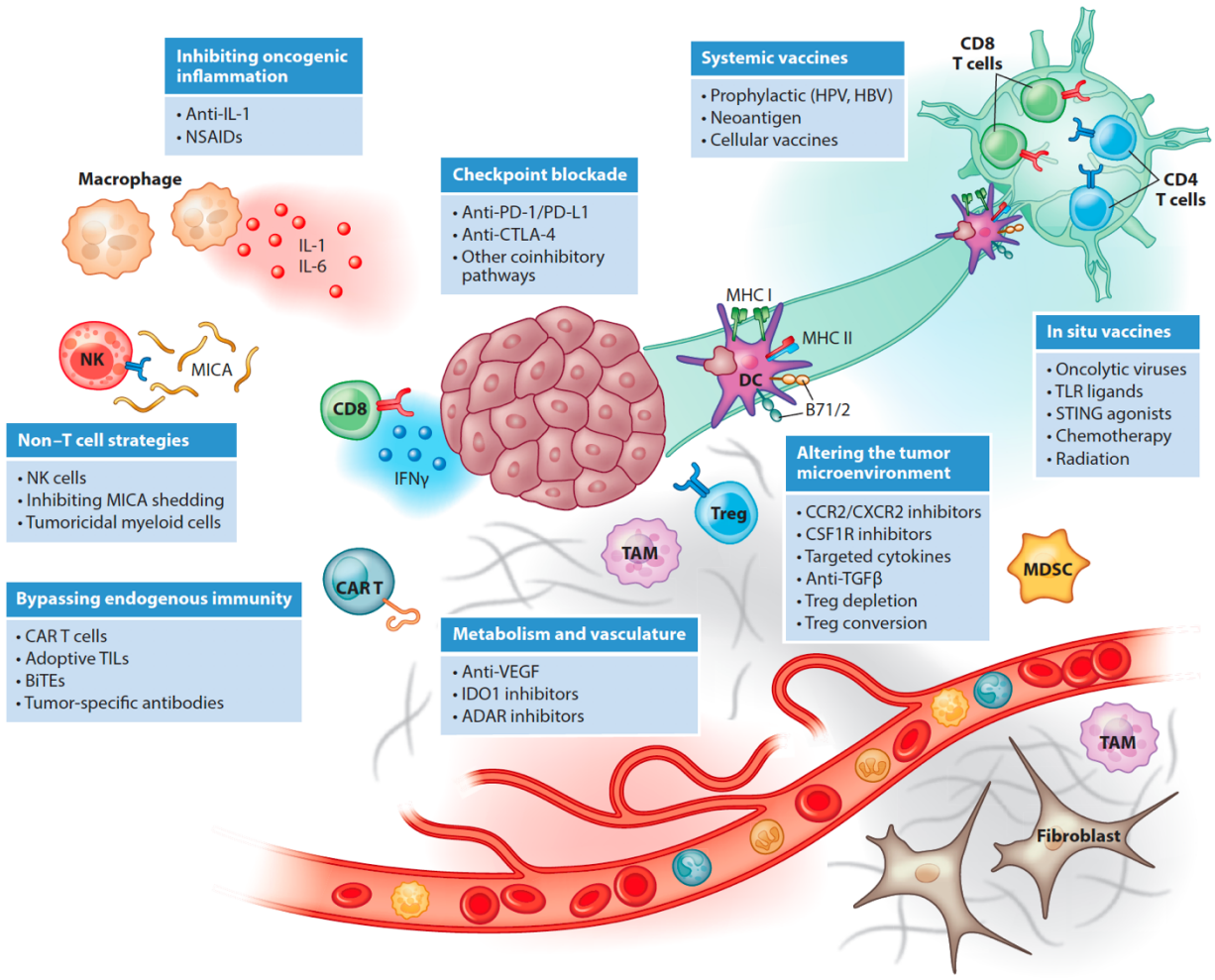

Figure 1.3. Different immunotherapeutic strategies to overcome immune evasion. These immunotherapies include checkpoint blockade inhibition, agents to alter the TME, cancer vaccines and adoptive cell therapies. Adapted from Dougan et al. [76].

1.5.1 Checkpoint inhibitors

T cells have evolved with negative regulators as "checkpoints" to dampen their activity to avoid self-damage due to hyperactivity. The two most potent of these mechanisms are mediated through the cytotoxic T lymphocyte-associated protein-4 (CTLA-4) and programmed cell death-1 (PD-1) molecules [77], which are also critical in establishing central tolerance and preventing autoimmunity in peripheral tissues [78]. CTLA-4 was discovered as a receptor with structural

similarity with CD28 and higher avidity for its ligands [79], the co-stimulatory molecules CD80 and CD86 [80]. Activation of conventional T cells induces CTLA-4 expression, whereas T_{reg} cells express it constitutively [72]. CTLA-4 regulates T activation through various mechanisms, including directly antagonizing CD80 and CD86 expressed on professional APCs and preventing their binding to CD28 [81]. It was demonstrated in murine tumor models that blocking CTLA-4 with neutralizing antibodies promotes antitumor immunity with a long-term memory response [82]. Following the evidence from clinical trials that treatment with monoclonal anti-CTLA-4 antibody ipilimumab improves survival in melanoma [83], it gained FDA approval for non-resectable late-stage melanoma patients in 2011 [84].

PD-1, identified as a new member of the immunoglobulin gene superfamily in 1992 [85], is expressed on T cells after activation and dampens their activity upon binding to its ligands, PD-L1 and PD-L2, which are expressed on APCs and can be upregulated on other cells types by pro-inflammatory cytokines [86, 87]. Studies in PD-1 knockout (KO) and CTLA-4 KO mice demonstrated distinct spatial and temporal roles for these two receptors in inducing tolerance and regulating auto-immunity [78]. Once PD-1 engages its ligands, it leads to T cell exhaustion or induction of apoptosis [88]. Cancer cells hijack this mechanism and evade the anti-tumor T cell response by inducing expression of PD-L1 or PD-L2 [89]. Clinically, expression of PD-1 on TILs, or its ligands on tumor cells, has been correlated with poor prognosis in multiple malignancies [90-92]. These studies encouraged the assessment of inhibition of PD-1 pathway for cancer treatment. In preclinical murine cancer models, neutralizing PD-1 using monoclonal antibodies demonstrated enhanced anti-tumor T cells responses [93, 94] and reduction of metastatic burden [95]. In clinical trials, humanized anti-PD-1 antibodies pembrolizumab and nivolumab exhibited marked improvement in anti-tumor immunity and patient survival, which led to their FDA approval in

2014 for refractory and resectable melanoma [96, 97]. Success in further clinical trials has culminated in the FDA approval of both anti-PD-1 and anti-PD-L1 antibodies for various other indications, including head-and-neck squamous cell carcinoma [98], advanced non-small-cell lung cancer [99, 100], advanced renal cell carcinoma [101]. Notably, patients treated with anti-PD-1 antibodies experience less sever immune-related adverse events compared to anti-CTLA-4 treated patients [102].

1.5.2 Innate immunity agonists

The prerequisite of a robust innate immunity response for adaptive anti-tumor responses has encouraged the exploration of innate immunity agonists as monotherapies or in combination with other immunotherapies, such as checkpoint inhibitors [103, 104]. DCs, as the main professional APC in the TME, have been targeted using various strategies to boost antigen presentation and subsequent T cell activation and infiltration [104]. One strategy is to induce expression of type I interferons (IFNs) in DCs, which promote cross-presentation of tumor-antigens and are required for $CD8^+$ T cell responses [105, 106], by activating toll-like receptors (TLRs). Multiple TLR agonists, including TLR9, TLR3 and TLR7/8, are in clinical trials for different cancer types [104]. Activation of the stimulator of interferon genes (STING) pathway, which detects cytosolic DNA, is another approach for induction of type I IFNs. The STING pathway has been shown to be critical in induction of anti-tumor $CD8^+$ T cell responses [107], and the anti-tumor efficacy of various human STING agonists is currently being evaluated in clinical trials [108, 109].

1.5.3 Cancer vaccines

Cancer vaccines aim to eliminate cancer cells by mounting an immune response against tumor-specific antigens. Prophylactic vaccines have proved to be successful in preventing

hepatocellular and cervical cancers by targeting the oncogenic viruses hepatitis B and human papillomavirus, respectively [110]. On the other hand, therapeutic vaccines for elimination of already proliferating cancer cells are further behind in development due to several challenges. Early studies in pre-clinical melanoma models demonstrated anti-tumor efficacy of autologous tumor cells, when virally infected with Newcastle disease [111, 112], and various other autologous tumor vaccines are currently in late-stage clinical trials [110]. However, this approach faces numerous obstacles, including the challenge of isolating patient-derived tumor cells in multiple cancers [110]. These limitations, in addition to the advent of next-generation sequencing, drove the development of personalized cancer vaccines. In this approach, the patient's tumor DNA is sequenced and algorithms are used to predict tumor neoantigen that would elicit immune responses when presented on the patient's MHC alleles [72]. The use of tumor-derived neoantigens in personalized vaccines compared to tumor-associated antigens, which are expressed at lower levels in normal tissues, reduces the chance of auto-immune related toxicities [72]. Even though personalized cancer vaccines have demonstrated promising results in early clinical trials [113, 114], challenges such as the genomic instability in cancer cells, computational limitations and production timeline remain before their clinical adoption [115].

1.5.4 Adoptive T cell transfer therapies

The first studies assessing the clinical benefit of adoptive transfer therapies for cancer treatment demonstrated that transplantation of patient-derived leukocytes and allogeneic hematopoietic stem cells resulted in tumor regression [116, 117]. These studies led to the investigation of transfer of expanded autologous or allogeneic TILs with IL-2 in melanoma patients, which showed modest response rates [118], with some patients experiencing complete remission of their tumors when undergoing lymphodepletion prior to the treatment [119].

However, expansion of antigen-specific TILs *ex vivo* proved to be challenging and inspired the development of chimeric antigen receptor (CAR) T cells [72]. One of the advantages of CAR T cells is that they are not restricted by MHC presentation of tumor antigens, which tumor cells downregulate [120], and bind directly to their target antigens on cancer cells. CAR T cells are generated by genetically engineering autologous or allogeneic T cells isolated from the patient to express a fusion protein made of the antigen-binding domain of the variable regions of an antibody and the signaling domains of the TCR [72]. Due to limited efficacy of this design in clinical trials [121], newer generation of CARs included co-stimulatory domains, such as CD28 or 4-1BB, to enhance T cell activation and proliferation [122, 123]. CARs T cells have demonstrated significant clinical benefit in the treatment of several B cell malignancies [124-126] and were approved by the FDA in 2017. One of the main drawbacks of the current CAR T cell technologies is the limitation of their targeting to a specific antigen expressed on the tumor cells, for instance CD19 in B cell lymphoma, which allows for a greater chance for cancer cells to evade the CARs by mutations in the target antigen [72].

1.6 Hypothesis and objectives

In this thesis, I investigated how tumor lymphangiogenesis affects the anti-tumor immunity when treating mammary tumors with cancer immunotherapies. This can lead to a better understanding of the tumor microenvironment and inform future decisions when selecting breast cancer patients for treatment with immunotherapies. The overall hypothesis of this thesis is that: "Tumor lymphangiogenesis improves responsiveness to immunotherapies in triple-negative breast cancer".

In order to test this hypothesis, my thesis was divided into the following two objectives:

1.6.1 To evaluate if tumor lymphangiogenesis in murine mammary tumors increases responsiveness to immunotherapies

In this section, in order to model tumor lymphangiogenesis *in vivo,* I transduced murine cell lines of triple-negative mammary carcinoma to over-express the pro-lymphangiogenic vascular endothelial growth factor-C (VEGF-C). I demonstrated that tumor lymphangiogenesis increases recruitment of $CD4^+$ T cells and macrophages into the tumor microenvironment of 4T1 triple-negative tumors. Upon treatment with cancer immunotherapies, namely checkpoint inhibitors (CPI) and an agonist of Stimulator of Interferon Genes (STING) pathway, lymphangiogenic E0771 and 4T1 tumors exhibited improved responsiveness to the treatment and mice showed prolonged survival. Furthermore, after treatment with a combination of CPI and STING agonist, the potent systemic anti-tumor immunity in the lymphangiogenic tumors reduced the spontaneous lung metastasis in 4T1 tumor-bearing mice. The beneficial effect of tumor lymphangiogenesis was dependent on $CD4^+$ T cells and macrophages, as their depletion abrogated the observed phenotype.

1.6.2 To identify if the expression of VEGF-C correlates with gene signatures of immune cells in human breast cancer

To validate the translational relevance of my findings, using The Cancer Genome Atlas (TCGA) dataset, I showed that in human triple-negative breast cancer expression of VEGF-C positively correlates with CD4 and CD68 gene expression in the tumor. These results reveal that tumor lymphangiogenesis has the potential to improve responsiveness to cancer immunotherapies due to the enrichment of the TME by immune cells such as macrophages and T cells.

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CHAPTER 2: EFFECT OF TUMOR LYMPHANGIOGENESIS ON CANCER IMMUNOTHERAPIES IN MURINE MAMMARY TUMORS

2.1 Abstract

Tumor lymphangiogenesis has recently been shown to improve responsiveness to immunotherapies in melanoma and glioblastoma. However, its role in affecting anti-tumor immunity in breast cancer has remained unexplored. Here, I investigated whether induction of tumor lymphangiogenesis modulates the response to cancer immunotherapies in murine triple-negative mammary tumors. First, I demonstrated that in the 4T1 triple-negative model, induction of tumor lymphangiogenesis leads to increased recruitment of $CD4^+$ T cells and macrophages into the TME. Second, I showed that upon treatment with CPI and an agonist of Stimulator of the Interferon Genes (STING) the lymphangiogenic 4T1 tumors respond more favorably to the treatments. Third, I found that despite the increased distant organ metastasis in the lymphangiogenic 4T1 tumor-bearing mice, treatment with a systemic immunotherapy is able to overcome metastasis to the lung. Finally, I demonstrated that the lymphangiogenic potentiation is dependent on CD4+ T cells and macrophages as their depletion abrogates the benefit. These data exhibit that tumor lymphangiogenesis in murine triple-negative breast cancer improves responsiveness to immunotherapies.

2.2 Introduction

2.2.1 Lymphangiogenesis

In the embryonic phase, the lymphatic system starts developing by sprouting from the blood vasculature and generating the lymph sacs. Next, the peripheral lymphatics are formed from the lymph sacs, which then is followed by maturation of lymphatic capillaries [1]. The lymphatic capillaries lack basement membranes and have "button-like" junctions, as opposed to the continuous junctions in blood vessels, which allows them to transport the leukocytes and macromolecules in the lymph [2, 3]. Once differentiated from blood endothelial cells (BECs), LECs display expression of distinct markers including prospero-related homeodomain transcription factor Prox1, podoplanin (gp38), and lymphatic vessel hyaluronan receptor-1 (LYVE-1) [4].

The proliferation of the LECs lining the lymphatic vessels, termed lymphangiogenesis, is driven by the signaling of vascular endothelial growth factor receptor-3 (VEGFR-3) [5] (**Fig. 2.1**). Lymphangiogenesis is induced upon stimulation of VEGFR-3 on LECs by vascular endothelial growth factors-C and-D (VEGF-C, VEGF-D) [6]. Post-embryonic development, lymphangiogenesis occurs only during pathologic conditions such as tumor growth and wound healing [7]. The cellular sources of VEGF-C in the tumor microenvironment include macrophages and tumor cells [8].

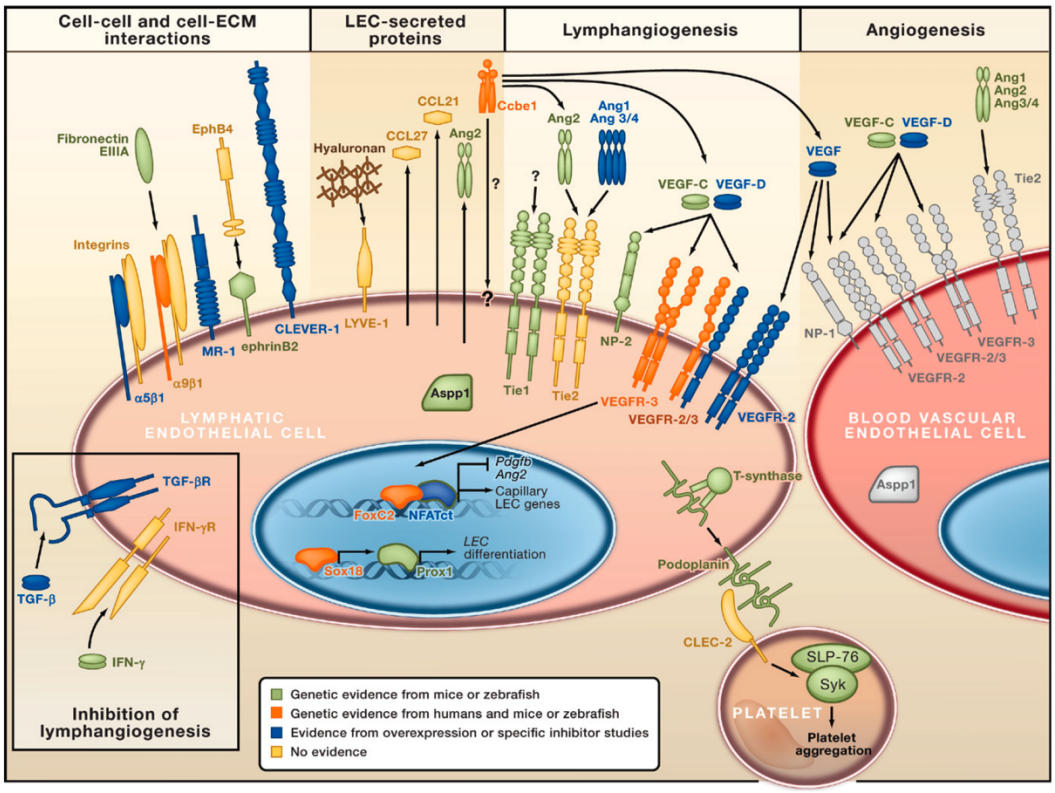

Figure 2.1. Molecular overview of lymphangiogenesis. Activation of VEGFR3 by VEGF-C/D growth factors drives lymphangiogenesis. Adapted from Tammela et al. [5].

2.2.2 Cancer immunotherapies in TNBC

Tumors with a strong pre-existing anti-tumor immunity have high numbers of tumor-infiltrating lymphocytes (TILs), high expression of PD-L1, and are called “T cell-inflamed” or simply “inflamed” [9]. On the other hand, some tumors either lack immune infiltrates or the infiltration is limited to the stroma around the tumor. These tumors are termed “immune-desert” or “immune-excluded”, respectively [10]. Amongst breast cancer subtypes, TNBC and HER2^{+} tumors are more inflamed than hormone-sensitive breast cancer [11, 12]. Increased numbers of TILs in TNBC patients is considered a positive prognosis, since it leads to better overall survival (OS), even in the absence of adjuvant therapy [13, 14]. When treated with adjuvant chemotherapy, TIL counts positively correlate with disease-free survival [15]. Moreover, TNBC patients with an

"inflamed" signature treated with neoadjuvant chemotherapy had higher pathologic complete response (pCR) rates than "non-inflamed" patients [16-18]. Higher TILs also correlate with better clinical outcome in $HER2^+$ patients, regardless of systemic adjuvant therapy [19]. The presence of TILs at diagnosis was a positive prognostic factor among 16,000 patients with TNBC and $HER2^+$ breast cancers [20]. These studies demonstrate that higher immune infiltration in the TME in breast cancer is a positive prognostic factor in TNBC patients. Furthermore, it has been shown that TNBC tumors have higher levels of nonsynonymous mutations [21], which could give rise to tumor-specific neoantigens and a stronger anti-tumor T cell response [22]. These data, together with the existing correlation between TILs and responsiveness to immunotherapies in other cancer types [23], have driven the evaluation of various immunotherapies in TNBC patients in different clinical trials [24, 25].

In the case of immune checkpoint inhibitor monotherapy regimens, the efficacy of response in TNBC is relatively low. In a phase I trial assessing the safety and efficacy of pembrolizumab (αPD-1), advanced TNBC patients had objective response rates (ORR) of 18.5%, with some patients experiencing durable responses [26]. In a phase II study (KEYNOTE-086) evaluating the efficacy and safety of pembrolizumab, patients receiving the treatment as first-line therapy responded better than the patients who had prior chemotherapy treatment [27, 28]. However, the median OS or progression-free survival (PFS) in TNBC patients undergoing monotherapy of checkpoint inhibitor was not longer than historical data from patients treated with chemotherapy, which suggests defects in the early stages of cancer immunity cycle. These results were confirmed in the following phase III trial (KEYNOTE-119), in which monotherapy of pembrolizumab did not provide any benefit in OS or PFS of pretreated TNBC patients compared to standard chemotherapy [29]. Furthermore, single-agent trials of PD-L1 inhibitors in pretreated metastatic

TNBC have also shown limited efficacy [30, 31]. These results encouraged the trial of other treatment modalities, such as chemotherapy, targeted therapy or radiation, in combination with checkpoint inhibitors to boost the immunogenicity in the tumor microenvironment [32].

Due to the enhancement of the immunogenic cell death by several chemotherapeutic agents [33], there are various trials combining chemotherapy with checkpoint inhibitors [24]. The phase III IMpassion130 trial, assessing nab-paclitaxel in combination with atezolizumab (αPD-L1) in untreated metastatic TNBC patients, demonstrated an increase in PFS in the combination arm, compared to the nab-paclitaxel/placebo arm (7.2 months vs. 5.5 months, P=.002) [34]. This study led to the FDA approval of this combination for metastatic TNBC, the first immunotherapy approval in breast cancer. Combination of immunotherapy and chemotherapy has shown promise in early stage TNBC patients. In a phase II trial (I-SPY2) pembrolizumab plus chemotherapy more than doubled the pCR compared to chemotherapy alone in early stage $HER2^-$ breast cancer in the neoadjuvant setting [35].

Combination of targeted therapies and immunotherapies is also being explored in TNBC. Poly (ADP-ribose) polymerase (PARP) inhibitors have been shown to activate the STING pathway and induce intratumoral T cell infiltration in BRCA-deficient murine models of TNBC [36]. The phase II TOPACIO trial found that the combination of PARP inhibitor niraparib with pembrolizumab has an ORR of 47% in advanced or metastatic TNBC with BRCA mutations [37], which is slightly lower than ORRs associated with single-agent PARP inhibitors [38]. Larger randomized trials of PARP inhibitor olaparib with atezolizumab (ETCTN) or durvalumab (DORA) are currently underway. The efficacy of AKT inhibitors is also being investigated in combination with checkpoint inhibitors. As a negative regulator of AKT, loss of tumor suppressor protein PTEN has been associated with anti-PD-1 resistance [39] and AKT inhibition has been shown to promote

expansion of tumor-specific memory T cells [40]. A phase I trial of AKT inhibitor ipatasertib in combination with chemotherapy and anti-PD-L1 exhibited an ORR of 73% in a small patient cohort [41]. These results have led to current evaluation of AKT inhibitors in combination with checkpoint inhibitors in larger trials IPATunity130 and BEGONIA. Finally, inhibition of mitogen-activated protein kinase (MEK) in preclinical models has been shown to promote responsiveness to checkpoint inhibitors through upregulation of PD-L1 and MHC expression [42]. Current and future trials will determine if MEK inhibitors can synergize with checkpoint inhibitors to improve the clinical outcome of TNBC patients to immunotherapies.

Novel immunotherapies other than checkpoint inhibitors are also currently in clinical development. The efficacy of toll-like receptor 9 (TLR9) agonist SD-101, shown to induce type I interferon expression in APCs and boost $CD8^+$ T cell activation [43], is being evaluated in the I-SPY2 trial in combination with chemotherapy and pembrolizumab in early-stage TNBC patients. Cancer vaccines including vaccines targeting over-expressed antigens in TNBC, such as PVX-410 vaccine targeting XBP1 and CD138 peptides [44], or neoantigen vaccines unique to each patient's tumor by predicting tumor-specific antigens using next-generation sequencing and epitope prediction are being explored in combination with checkpoint inhibitors [45]. Lastly, adoptive cell therapies, using expanded autologous TILs [46] or engineered chimeric antigen receptor (CAR) T cells [47], are also in clinical trials for treatment of TNBC.

2.2.3 Role of tumor-associated lymphatics in anti-tumor immunity

It has been known that during cancer development, lymphatics in the TME proliferate and remodel together with the corresponding angiogenesis of blood vasculature [48]. Even though tumor angiogenesis has been demonstrated to exacerbate the anti-tumor immunity [49], the role of tumor-associated lymphatics is still being investigated. Tumor-associated lymphatics can

modulate the anti-tumor immunity and cancer development both actively and passively. Tumor lymphatics connect the TME to the draining LNs, where the anti-tumor immunity is elicited, and thus are vital for mounting the adaptive immune response against tumor cells [50]. On the other hand, cancer cells metastasize to the lymph node by utilizing the tumor-associated lymphatics or inducing tumor lymphangiogenesis [51] (**Fig. 2.2**). In breast cancer patients, tumor lymphangiogensis has been correlated with increased metastasis rate and poor patient prognosis [52, 53].

LECs also actively regulate the anti-tumor immune response through secretion of chemokines and immunomodulatory molecules, which can recruit and modulate the activation status of immune cells [54]. For instance, LECs secrete the chemokine CCL21 which recruits $CCR7^{+}$ cells to the lymph nodes, including leukocytes and some cancer cells [55, 56]. Moreover, tumor lymphatics are associated with immune suppression in the draining lymph nodes and metastasis by providing an escape route for immune-suppressive factors and tumor cells [57, 58].

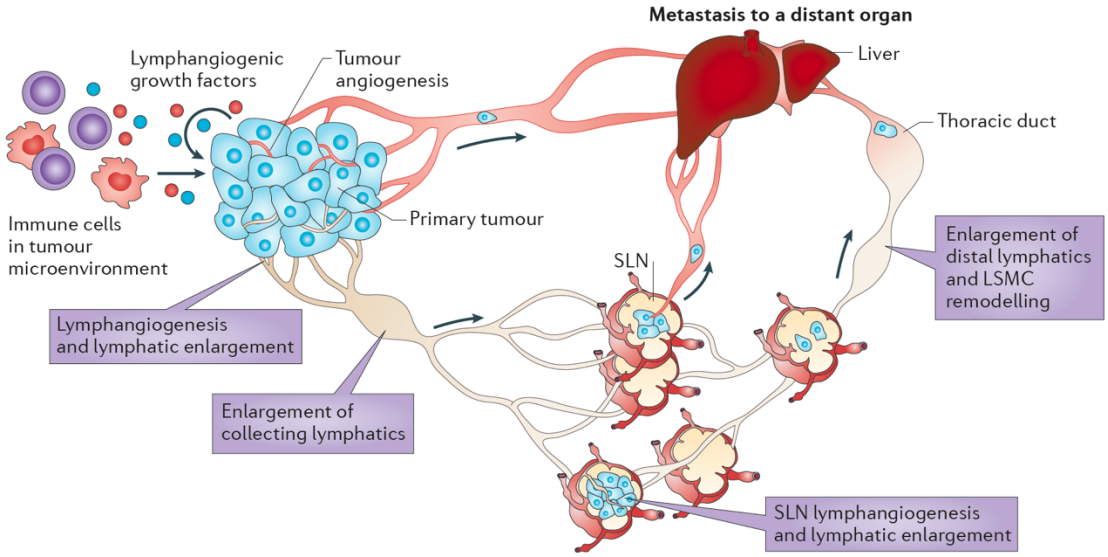

Figure 2.2. Tumor-associated lymphatics and their involvement in metastasis. Secretion of lymphangiogenic growth factors in the TME leads to tumor lymphangiogenesis or lymphatic enlargement. These changes to the tumor-associated lymphatics promotes metastasis to the tumor-draining lymph nodes and distant organs. Adapted from Stacker et al. [48].

Despite the existing knowledge on the contribution of tumor-associated lymphatics to trafficking of immune cells, mounting of anti-tumor immunity and their involvement in distant organ metastasis, how tumor lymphangiogenesis affects immune responses in the context of cancer immunotherapies was not investigated until recently. It was demonstrated that tumor lymphangiogenesis in murine and human melanoma renders them more responsive to immunotherapies, an effect termed lymphangiogenic potentiation of immunotherapies [59]. This potentiation was not specific to the type of immunotherapy administered, as lymphangiogenic tumors responded more favorably to a cancer vaccine, checkpoint inhibition in combination with adjuvants and adoptive cell therapy. The mechanism was, at least partially, dependent on the recruitment of naïve T cells to the tumor microenvironment through the CCL21-CCR7 chemokine axis and their subsequent *in situ* education [59]. A recent study in our group also demonstrated that a lymphangiogenic cell-based cancer vaccine is able to promote anti-tumor immunity in murine melanoma models [60]. Lymphangiogenic potentiation has also been observed in glioblastoma, where an increase in VEGF-C signaling led to the recruitment of $CD8^+$ T cells, clearance of tumors and a long-lasting anti-tumor immunity [61]. The role of tumor lymphangiogenesis in shaping anti-tumor immune responses upon treatment with immunotherapies in breast cancer, however, remains unexplored.

2.2. Materials and Methods

2.2.1 Mice

Wild-type (WT) C57BL/6 were purchased from The Charles River Laboratories. FVB/NJ and BALB/cJ mice were purchased from The Jackson Laboratory. Homozygous B6.129S7-Rag^{1tm1Mom}/J mice were obtained from The Jackson Laboratory. The mice were crossed with WT C57BL/6 mice to generate heterozygous offspring and then the line was maintained by crossing homozygous mice with heterozygous ones. The heterozygous littermates were used as control mice for *in vivo* experiments. All the mice used in the experiments were female and 8-12 weeks old at the start of experiments. All animal experimentations were approved by the Institutional Animal Care and Use Committee of the University of Chicago protocol #72551.

2.2.2 Genotyping

Tail clips or ear punch biopsies were used for DNA extraction. PCR was performed for WT Rag1 gene using forward primer TCT GGA CTT GCC TCC TCT GT and reverse primer CAT TCC ATC GCA AGA CTC CT.

2.2.3 Cell lines

EMT6, E0771 and 4T1 cell lines were obtained from ATCC and maintained in RPMI L-glutamate (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen). MMTV-PyMT (PyMT) cells were generated from spontaneously developed breast tumors in FVB-Tg (MMTV-PyVT) transgenic mice and cultured in DMEM (Gibco) supplemented with 10% FBS. Cell lines were routinely tested for mycoplasma contamination.

2.2.4 Plasmid DNA amplification and purification

The pD2109-mVEGFC vector, which contains the sequence for murine VEGFC cDNA (GeneID 22341), was purchased from DNA2.0 (Newark, California) and the corresponding control

lentiviral vector, designated as pD2109-control, was generated by excision of the VEGF-C sequence from the vector using restriction enzyme digestion. For plasmid amplification, E. coli were transformed at 42°C for 45 seconds and plated on LB-agar plates with 50 μg/ml kanamycin to obtain single colonies. After overnight incubation at 37°C, a few antibiotic-resistant colonies were selected and amplified in 500 ml of LB-kanamycin at 37°C and 225 rpm for 16-20 hours. The plasmids were then isolated using NucleoBond Xtra Plasmid Maxiprep Kit (Machery-Nagel) and the sequence of the plasmids were confirmed using DNA sequencing prior to lentiviral vector production.

2.2.5 Production of lentiviral vectors

Human embryonic kidney (HEK) 293T cells (ATCC) were co-transfected in the presence of 125 mM calcium chloride with 32 μg of either transfer plasmid pD2109-mVEGFC or pD2109-control, respectively, as well as 12.5 μg and 6.25 μg packaging plasmids pMDL9/pRRE and pRSV-Rev (gifts of D. Trono, Addgene plasmids #12251 and #12253), and 9 μg envelope plasmid pMD2 (gift of D. Trono, Addgene plasmid #12259) to yield third-generation pseudo-lentiviral particles. To increase the efficiency of lentiviral vector production, 15 μg of pAdvantage vector (Promega) was added during the procedure.

2.2.6 Transduction and selection of murine breast cancer cell lines

To generate VEGF-C overexpressing and control cell lines, cells were transduced using lentiviruses carrying pD2109-EFs_mVEGF-C and pD2109-EFs empty vectors, respectively (ATUM). The transduced cells were then selected based on the expression of puromycin resistance. The expression of VEGF-C by each cell line was assessed *in vitro*. Briefly, 100,000 cells were seeded in a tissue-cultured 12-well flat-bottom plate (Corning) in 2 ml media for 24 hrs and the secreted VEGF-C was measured using ELISA (R&D Systems).

2.2.7 Tumor inoculation and measurements

After culturing, cells were detached using Trypsin (Invitrogen) and resuspended in sterile PBS prior to orthotopic inoculation into the right 4th mammary fat pad (MFP) of the female mice (40 µl/injection). Number of cells injected for each cell line was: 2×10^5 for each of 4T1 and EMT6 cells in BALB/cJ mice, 3×10^5 E0771 cells in C57BL/6 mice and 1×10^6 PyMT cells in FVB/NJ mice. Tumors were measured using a digital caliper and the volume (V) was calculated using the following formula: $V = (\pi/6) \times \text{length} \times \text{height} \times \text{width}$. Mice were euthanized when the tumor volume reached 500 mm^3 or based on the humane endpoint criteria.

2.2.8 Immunotherapies

Treatment of mice for each group was started when the average size of tumors in that group was ~100 mm^3, unless otherwise stated. For experiments when an endpoint analysis was performed, in order to administer and euthanize the mice on the same day for both tumor types, 4T1-Ctrl tumors (due to their slower tumor growth rate) were inoculated 2 days earlier than 4T1-VC tumors. When treating mice with checkpoint inhibitors, sterile PBS or a combination of 100 µg αPD-1 (clone 29F.1A12, Bio X Cell) and 100 µg αCTLA-4 (clone 9H10, Bio X Cell) with PBS were administered intraperitoneally (i.p.) in a volume of 100 µl according to the schedules described in the figure legends. When treating mice with STING agonist, sterile PBS or 25 ug of ADU-S100 (ChemieTek) with PBS was administered intratumorally (i.t.) in a volume of 15 µl according to the schedules described in the figure legends.

2.2.9 Depletion and blocking antibodies

For the depletion study, the following anti-mouse antibodies were administered intraperitoneally every 3 days from the day of tumor inoculation for a total of 5 doses: 200 ug αCD4 (clone GK1.5, Bio X Cell) and 300 ug αCSF1R (clone AFS98, Bio X Cell). Mice were then

treated with STING agonist as shown in the corresponding figure. Mice were bled at day 14 post tumor inoculation, and the frequency of the circulating immune cells was assessed by flow cytometry. For blocking of TGF-β, 200 μg of αTGF-β antibody (clone 1D11, Bio X Cell) was administered every other day to tumor-bearing mice.

2.2.10 Production and purification of A3-VEGF-C (A3-VC)

The sequence encoding the fusion of human von Willebrand factor (VWF) A3 domain and mouse VEGF-C (A3-VC) (Table 2.1) was synthesized and subcloned into the mammalian expression vector pcDNA3.1(+) by GenScript. A sequence encoding 6×His was added at the C terminus for further purification of the recombinant protein. Suspension-adapted human embryonic kidney– 293F cells were routinely maintained in serum-free FreeStyle 293 Expression Medium (Gibco). On the day of transfection, cells were inoculated into fresh medium at a density of 1×10^6 cells/ml. Plasmid DNA (1 μg/ml), linear 25-kDa polyethylenimine (2 μg/ml; Polysciences), and OptiPRO SFM medium (4% final concentration; Thermo Fisher Scientific) were sequentially added. The culture flask was agitated by orbital shaking at 135 rpm at 37°C in the presence of 5% CO2. Six days after transfection, the cell culture medium was collected by centrifugation and filtered through a 0.22-μm filter. Culture medium was loaded into a HisTrap HP 5 ml column (GE Healthcare), using an ÄKTA pure 25 (GE Healthcare). After washing the column with wash buffer [20 mM imidazole, 20 mM NaH2PO4, and 0.5 M NaCl (pH 7.4)], protein was eluted with a gradient of 500 mM imidazole [in 20 mM NaH2PO4 and 0.5 M NaCl (pH 7.4)]. The eluate was further purified with size exclusion chromatography using a HiLoad Superdex 200 pg column (GE Healthcare). All purification steps were carried out at 4°C. After the purification, SDS-PAGE was carried out on 4-20% gradient gels (Bio-Rad) by loading the gel with proteins in non-reducing conditions. After running electrophoresis, gels were stained with SimplyBlue

SafeStain (Thermo Fisher Scientific) according to the manufacturer's instruction. The ChemiDoc XRS+ system (Bio-Rad) was used to acquire gel images.

2.2.11 Characterization and administration of A3-VC

After purification, the binding affinity of A3-VC to collagen and its bioactivity was assessed as described here. Briefly, medium binding 96-well plates were coated with 10 μg/ml of collagen I or collagen III (EMD Millipore) in PBS at 37°C overnight. Wells were then blocked with 1% BSA in PBS for 2 hrs, washed with PBS with 0.05% Tween 20 (PBS-T), followed by incubation with A3-VC or wildtype VEGF-C (WT-VC) in PBS for 1 hr at room temperature (RT). After washing with PBS-T, wells were incubated with biotinylated antibody against VEGF-C (R&D Systems) at 100 ng/ml for 1 hr at RT. Then, wells were incubated with horseradish peroxide (HRP)-conjugated streptavidin (R&D Systems) for 1 hr at RT, further washed with PBS-T and then bound A3-VC or WT-VC were detected using tetramethylbenzidine substrate by measurement of the absorbance at 450 nm with subtraction of the measurement at 570 nm. The apparent dissociation constant (K_D) values were obtained by nonlinear regression analysis in Prism software (v8, GraphPad Software) assuming one-site specific binding.

To assess the bioactivity of A3-VC, human LECs (hLECs) isolated from foreskin were cultured in dishes coated with 50 μg/ml rat tail collagen and in endothelial cell growth basal medium (EBM; Lonza), supplemented with 10% FBS, 1% penicillin/streptomycin-amphotericin B, 50 μM dibutyryl-cAMP, 1μg/mL hydrocortisone acetate (Sigma Aldrich), and 2 mM L-glutamine. hLECs were then starver in 1% BSA overnight and treated with 200 ng/ml of WT-VC (Abcam), A3-VC or PBS for 20 mins. Cells were then immediately washed, lysed and phosphorylation of VEGFR-3 was assessed by ELISA (R&D Systems). For induction of tumor

lymphangiogenesis *in vivo,* 3 ug of A3-VC per dose was administrated intratumorally in PBS according to the schedules in the figures.

2.2.12 Tissue digestion

At the experimental endpoint mice were euthanized, and different organs were isolated and digested as described here. Briefly, tumors were weighed and cut into small pieces with a scissor and then digested in 750 µl DMEM with 2% FBS, 1 mg/ml Collagenase IV (Worthington Biochemical), 40 µg/ml DNAse I (Worthington Biochemical) and 1.2 mM $CaCl_2$ for 30 mins at 37°C with magnetic stirring. Then the samples were pipetted 10 times and 750 µl of 3.3 mg/ml Collagenase D (Sigma), 40 µg/ml DNAse I and 1.2 mM $CaCl_2$ in DMEM with 2% FBS was added to each sample. They were then digested for another 15 mins. The samples were pipetted another 10 times and EDTA was added to a final concentration of 5 mM. Finally, single cell suspensions were prepared by filtering through a 70 µm cell strainer (Thermo Fisher Scientific). For lung analysis, mice were euthanized and perfused with 10 ml PBS through the left ventricle of the heart. The lung lobes were then isolated and cut by a scissor into small pieces. The pieces were then digested in 5 ml DMEM with 5% FBS, 3.3 mg/ml Collagenase D, 1 mg/ml Collagenase IV, 20 µg/ml DNAse I and 1.2 mM $CaCl_2$ for 1 hour at 37°C on a shaking stand. After using 5mM EDTA to quench the media, single cell suspensions were prepared using a 70 µm cell strainer. Red blood cells were lysed with 1 ml ACK buffer for 90 seconds and neutralized with 10 ml DMEM media with 5% FBS. For analyzing blood, 50 µl of blood was collected from the saphenous vein into EDTA-coated tubes (Eppendorf). Samples were washed with PBS and centrifuged at 300 g for 5 mins and then red blood cells in the samples were lysed using ACK lysis buffer (three 90-second incubations). For splenocyte analysis, single cell suspensions were prepared by mashing the spleens through a 70 µm strainer with a plunger and washing with DMEM with 2% FBS.

2.2.13 Flow cytometry

After preparing single cell suspensions, cells were counted, and 1 to 2 million cells were stained for each panel. The staining was done on ice in 96-well round bottom plates (Corning) and Fc receptor blocking was performed using CD16/32 antibody for 15 mins. The staining for antibodies against surface markers was done in PBS buffer with 2% FBS for 20 mins. For intranuclear/intracellular staining, cells were stained using the an intranuclear kit according to the manufacturer's protocol (00-5523-00, Thermo Fisher Scientific). The following anti-mouse antibodies were used for staining: CCR7 APC (clone 4B12, BioLegend), CD45 APC-Cy7 (clone 30-F11, BioLegend), CD45 BV785 (clone 30-F11, BioLegend), CD3e BUV395 (clone 145-2C11, BD Biosciences), Foxp3 AF488 (clone MF23, BD Biosciences), CD25 APC-Cy7 (clone PC61, BioLegend), CD62L BUV737 (clone MEL-14, BD Biosciences), CD69 PE-Cy7 (clone H1.2F3, BioLegend), CD103 PE (clone 2E7, BioLegend), CD31 BV421 (clone 390, BioLegend), gp38 AF488 (clone 8.1.1, BioLegend), CD8α BV510 (clone 53-6.7, BioLegend), CD4 BV421 (clone RM4-5, BioLegend), CD4 BV785 (clone RM4-5, BioLegend), CD44 PerCP-Cy5.5 (clone IM7, BioLegend), PD-1 BV605 (clone 29F.1A12, BioLegend), Perforin PE (cloneS16009B, BioLegend), KLRG1 APC (clone 2F1, BioLegend), CD86 BUV396 (clone GL-1, BD Biosciences), F4/80 FITC (clone MCA497F, AbD Serotec), CD206 PE (cloneC068C2, BioLegend), CD11c PE-Cy7 (clone N418, BioLegend), CD11b BUV737 (clone M1/70, BD Biosciences), CD80 BUV737 (clone 16-10A1, BD Biosciences), CD19 BUV395 (clone 1D3, BD Biosciences), Ly6G BV510 (clone 1A8, BioLegend), Ly6C BV605 (clone HK1.4, BioLegend), MHCII PerCP-Cy5.5 (clone, M5/114.15.2, BioLegend), IL-2 PE (clone JES6-5H4, BioLegend), GrB APC/Fire 750 (clone QA16A02, BioLegend), IFNγ APC (clone XMG1.2, BioLegend), TNFα FITC (clone MP6-XT22, BioLegend). The fixable viability dye eFluor 455UV dye (65-0868-14,

eBioscience) was used for determining cell viability. Flow cytometry data was acquired on LSR Fortessa X-20 (BD Biosciences) and analyzed using FlowJo software (Tree Star).

2.2.14 Intratumoral cytokine-producing T cells

4T1-Ctrl tumor cells were inoculated into WT mice two days prior to 4T1-VC tumor cells. Once tumors were established, mice were treated with two i.p. doses of PBS or CPI every 3 days and the mice were euthanized at day 6 after the start of treatment and their tumors were isolated for analysis. Tumor single cell suspensions were prepared and seeded at 5×10^5 per well in 96-well round bottom plates with the addition of AH1 peptide (SPSYVYHQF) at 1 μg/ml and incubated at 37°C for 3 hrs. Then, brefeldin A at 5 μg/ml was added to each well and the samples were incubated at 37°C for an additional 9 hrs. The samples were then washed with PBS and the frequency of intratumoral cytokine-producing T cells was assessed by flow cytometry.

2.2.15 Antigen-specific re-stimulation

4T1-Ctrl tumor cells were inoculated into WT mice two days prior to 4T1-VC tumor cells. Once tumors were established, mice were treated with two doses of PBS (i.p.), CPI (i.p.) or a combination of CPI (i.p.) and STING agonist (i.t.) every 3 days and the mice were euthanized at day 6 after the start of treatment and their spleens were isolated for analysis. Splenocytes were isolated and seeded at 5×10^5 per well in 96-well round bottom plates and cultured at 37°C for 3 hrs in the presence and absence of AH1 peptide (SPSYVYHQF) at 1 μg/ml. Then, brefeldin A at 5 μg/ml was added to each well and the samples were incubated at 37°C for an additional 9 hrs. The samples were then washed with PBS and intracellular cytokine expression by T cells was assessed using flow cytometry.

2.2.16 Immunofluorescence staining

Tissues were fixed in formalin-free zinc fixative (Bd Biosciences) for 2 days and then incubated in 15% and 30% sucrose solutions in Tris-buffered saline (TBS), each for 2 days. The samples were then embedded in Optimum Cutting Temperature compound and frozen on dry ice. A cryostat (Thermo Fisher Scientific) was used to obtain 7 μm sections. The sections were then rinsed with TBS and blocked with 0.5% casein in TBS for 30 mins at room temperature. They were then incubated with primary antibodies (1:200 dilution) in blocking solution for 1 hr at room temperature, followed by washing twice with TBS + 0.1% Tween20 (TBS-T) and once with TBS. Next, the sections were stained with secondary antibodies (1:400 dilution) for 1 hr at RT, followed washing twice with TBS-T and once with TBS. The sections were then covered with Prolong Gold Antifade reagent with DAPI (Thermo Fisher Scientific) and sealed with a coverslip. The following primary and secondary antibodies were used for immunostaining: rabbit anti-mouse LYVE-1 (Abcam), rat anti-mouse CD3 (Bio-Rad), Armenian hamster anti-mouse CD11c (Bio-Rad), goat anti-Armenian hamster AF647 (Jackson ImmunoResearch Inc.), donkey anti-rat AF594 (Jackson ImmunoResearch Inc.), goat anti-rabbit AF488 (Jackson ImmunoResearch Inc.). An IX83 microscope (Olympus) was used for imaging with a 10X magnification objective. Image processing was performed using ImageJ software (NIH).

2.2.17 Intratumoral cytokine/chemokine analysis

4T1-Ctrl tumor cells were inoculated into WT mice two days prior to 4T1-VC tumor cells. Once tumors were established, mice were treated with one dose of PBS (i.t.) or STING agonist (i.t.) mice were euthanized 2 days later and their tumors were isolated for analysis. After isolation, tumors were snap frozen in liquid nitrogen. Tumors were then resuspended in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with Halt Protease and

Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) and cut into smaller pieces with a scissor. Samples were then homogenized, and lysates were spun down at 14,000 g for 10 mins at 4°C. The protein content in the supernatants were then measured by BCA (Thermo Fisher Scientific). Intratumoral cytokines and chemokines were then quantified (as pg per μg of protein in each sample) using ELISA (PBL Assay Science) or LEGENDPlex kits (BioLegend) according to the manufacturer's protocol.

2.2.18 Quantification of metastatic nodules

4T1-Ctrl tumor cells were inoculated into WT mice two days prior to 4T1-VC tumor cells. Once tumors were established, mice were treated with PBS (3 doses, i.t.) or a combination of CPI (5 doses, i.p.) and STING agonist (3 doses, i.t.) every 3 days and the mice were euthanized at day 15 after the start of treatment and their lungs were isolated for analysis. Mice were perfused with 10 ml PBS and the lung lobes were excised and fixed in Bouin's fixative (Sigma-Aldrich) overnight. The samples were then washed, and the metastatic nodules were counted by eye.

2.2.19 Statistical analysis

Statistical analyses were performed using Prism v.8 (GraphPad). Data on tumor growth curves are shown as mean ± SEM and on bar graphs as mean ± SD. Unless otherwise specified, the following statistical tests were used: two-tailed Student's t-test with Welch's correction for comparison of 2 groups with parametric data, two-tailed Mann-Whitney U-test for comparison of 2 groups with non-parametric data, one-way ANOVA with Dunnett's multiple comparison test for analysis of multiple groups with parametric data and Kruskal–Wallis test followed by Dunn's multiple comparison test for analysis of multiple groups with non-parametric data. Survival curves were analyzed using log-rank (Mantel-Cox) tests. P values were reported as: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

2.3 Results

2.3.1 Tumor lymphangiogenesis increases immune infiltration into triple-negative mammary tumors

In order to study the role of tumor lymphangiogenesis on modulating the responsiveness to cancer immunotherapies, first I had to model lymphangiogenic tumors *in vivo* and characterize their tumor microenvironment. Thus, to induce lymphangiogenesis, I transduced the triple-negative 4T1 cell line with a lentiviral VEGF-C or control vector, termed 4T1-VC or 4T1-Ctrl, respectively. I confirmed the increased expression of VEGF-C in 4T1-VC tumor cells *in vitro* (**Fig. 2.3 A**). Then, I inoculated the 4T1-Ctrl and 4T1-VC cells orthotopically into mammary fatpads (MFP) of WT BALB/cJ mice. Analysis of the tumors at day 21 showed that over-expression of VEGF-C in 4T1 cells also led to increased VEGF-C levels *in vivo* and subsequent induction of lymphatic vessels in the tumor, as assed by ELISA and immunostaining for lymphatic marker LYVE-1 (**Fig. 2.3 B-C**). Next, I quantified the frequency of stromal cells in the tumor microenvironment by flow cytometry. The analysis confirmed an increase in the frequency of LECs, together with an increase in blood endothelial cells (BECs) (**Fig. 2.3 D-E**). However, there was no difference in the frequency of $gp38^{+}CD31^{-}$ cells, which include cancer-associated fibroblasts. These data confirm that over-expression of VEGF-C in the tumor cells results in tumor lymphangiogenesis *in vivo*.

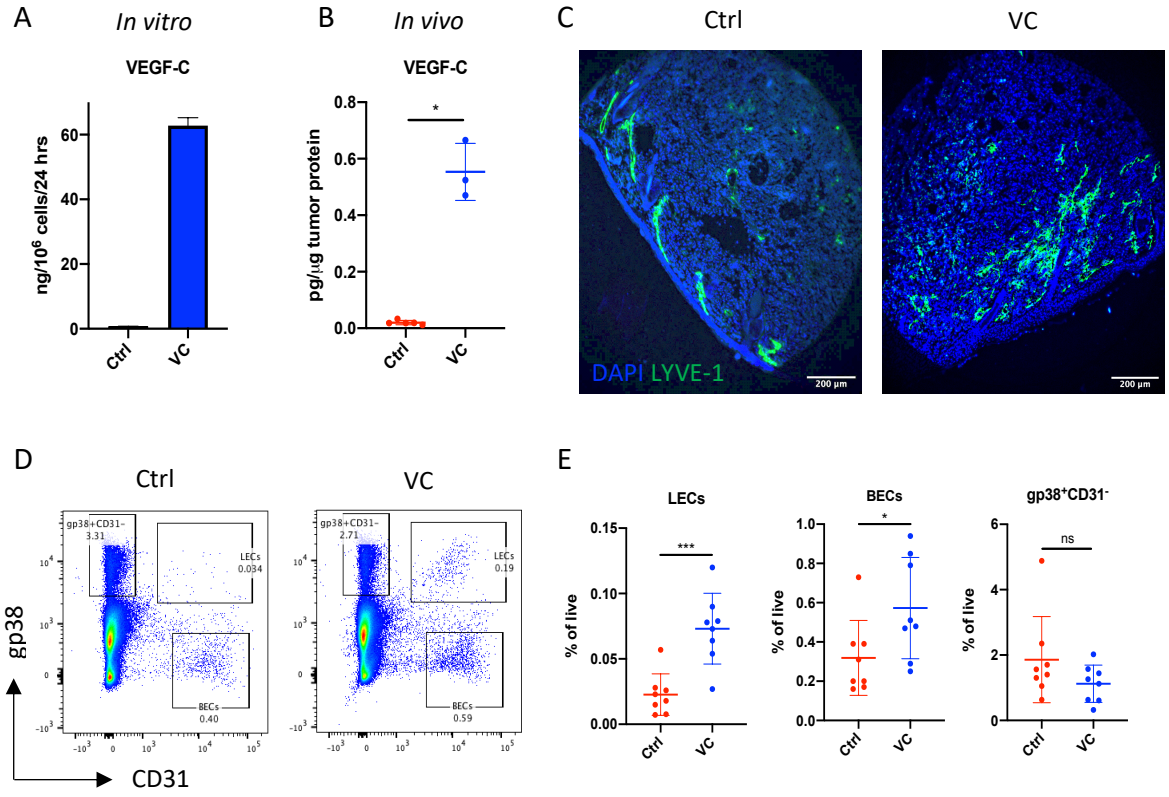

Figure 2.3. Over-expression of VEGF-C in triple-negative mammary tumors induces tumor lymphangiogenesis. 4T1 tumors cells were virally transduced with a VEGF-C or control vector and orthotopically inoculated into MFP of WT mice. Mice were euthanized for analysis at day 21. (A) Quantification of *in vitro* VEGF-C expression by tumors cells assayed by ELISA. (B) Intratumoral expression of VEGF-C *in vivo* assayed by ELISA. (C) Representative immunostaining images of intratumoral lymphatic vessels. Blue: DAPI, Green: LYVE-1. Scale bar: 200 µm. (D) Representative distribution of stromal cells in the TME using flow cytometry. (E) Flow cytometry analysis of intratumoral LECs ($CD45^-gp38^+CD31^+$), BECs ($CD45^-gp38^-CD31^+$) and $CD45^-gp38^+CD31^-$ cells as % of live cells (n=8). Data are mean ± SD (B-D). Flow cytometry analyses were performed twice with similar results. Statistical analyses were performed using unpaired two-tailed t-tests with Welch correction for parametric data or two-tailed Mann-Whitney test for nonparametric data. P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

Next, we characterized the immune infiltration into the tumors (**Fig. 2S1-2**). In the absence of any treatment, 4T1-VC tumors showed higher infiltration of tumor-associated macrophages (TAMs) than control tumors, and the ratio of M1 ($MHCII^+CD206^-$) to M2 ($CD206^+$) macrophages had decreased upon over-expression of VEGF-C (**Fig. 2.4 A**). The percentage of $CD86^+MHCII^+$ macrophages was also lower in the lymphangiogenic tumors. A lower M1/M2 ratio has been shown to be a negative prognostic factor and correlate with reduced survival in multiple cancers, including gastric cancer, ovarian cancer, advanced cervical cancer, and multiple myeloma [62-65]. Lymphangiogenic tumors also exhibited higher quantities of the immunosuppressive cytokine IL-10 (**Fig. 2.4 B**). However, both tumor types had similar numbers of dendritic cell (DC) infiltration, monocytic myeloid cells and granulocytic myeloid cells (**Fig. 2.4 C**). Characterization of T cell infiltration showed that 4T1-VC tumors had higher infiltration of conventional (conv) $CD4^+$ T cells and regulatory T (T_{reg}) cells (**Fig. 2.4 D**). However, both groups exhibited similar levels of $CD8^+$ T cell infiltration in the tumor, which led to a higher ratio of effector $CD8^+$ T cells, usually considered cytotoxic T cells (CTLs), to T_{reg} cells in the 4T1-Ctrl tumors compared to 4T1-VC tumors (**Fig. 2.4 E**). These results show that lymphangiogenic 4T1 tumors are more enriched in conv $CD4^+$ T cells and macrophages. Furthermore, the higher number of T_{reg} cells, a lower M1/M2 ratio, and a higher concentration of IL-10 in the lymphangiogenic tumors suggests a more immunosuppressed TME, which is consistent with previous studies in murine models of melanoma [57, 58].

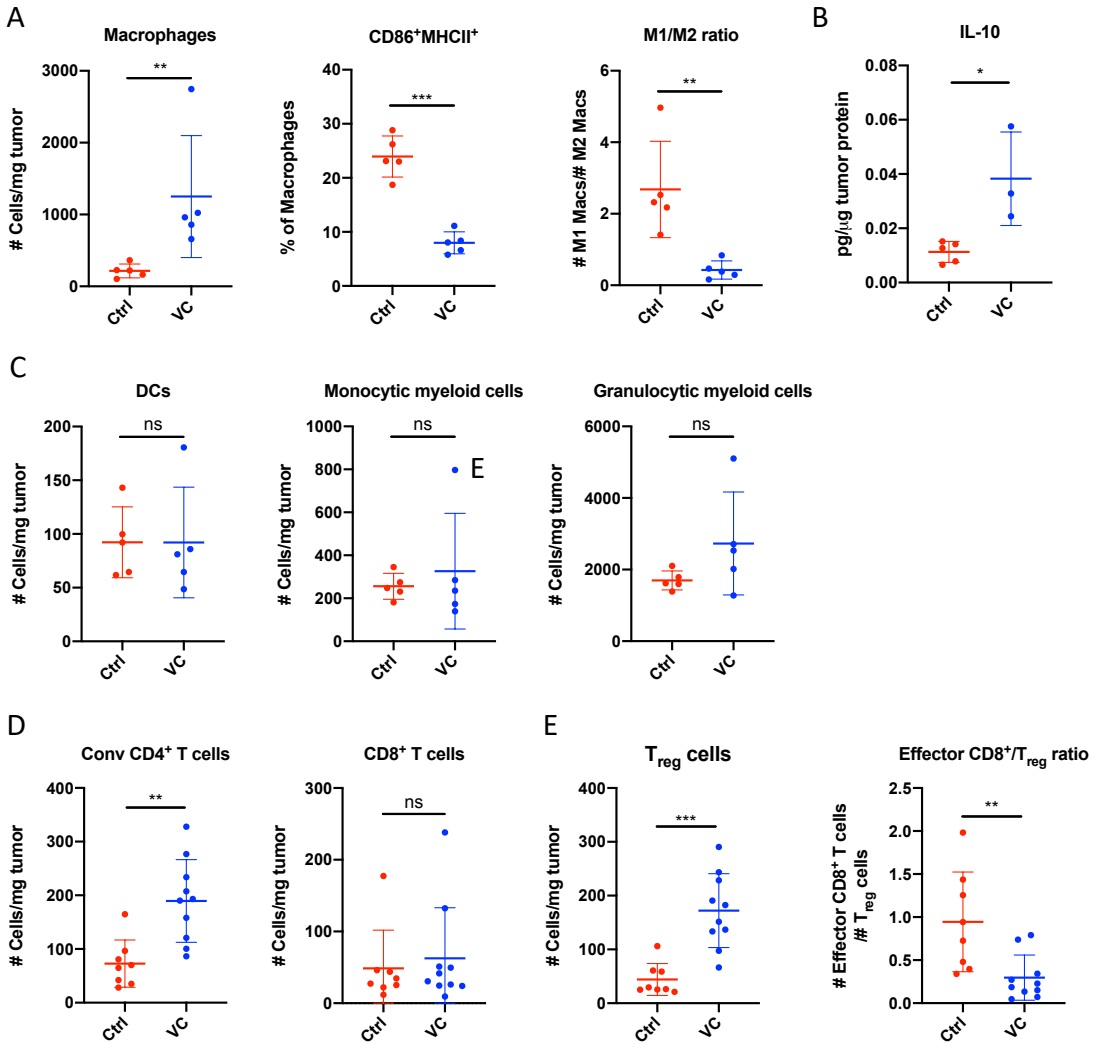

Figure 2.4. Lymphangiogenic triple-negative mammary tumors are enriched with immunosuppressive macrophages and CD4⁺ T cells. 4T1-Ctrl and 4T1-VC were orthotopically inoculated into MFP of WT mice. Mice were euthanized and tumors were isolated for analysis at day 15. (A) Flow cytometry analysis of macrophages ($CD45^+Ly6G^-Ly6C^-CD11b^+F4/80^+$), frequency of $CD86^+MHCII^+$ macrophages, and ratio of M1 ($MHCII^+CD206^-$) to M2 ($CD206^+$) macrophages (n=5). (B) Quantification of intratumoral IL-10 as assayed by ELISA (n=3-5). (C) Flow cytometry analysis of DCs ($CD45^+Ly6G^-Ly6C^-CD11c^+MHCII^+$), monocytic myeloid cells ($CD45^+CD11b^+Ly6G^-Ly6C^+$) and granulocytic myeloid cells ($CD45^+CD11b^+Ly6G^+$) (n=5). (D) Flow cytometry analysis of conv $CD4^+$ T cells ($CD45^+CD3^+CD8^-CD4^+Foxp3^-CD25^-$) and $CD8^+$ T cells ($CD45^+CD3^+CD8^+CD4^-$) (n=8-10). (E) Flow cytometry analysis of T_{reg} cells ($CD45^+CD3^+CD8^-CD4^+Foxp3^+CD25^+$) and ratio of effector $CD8^+$ T cells ($CD44^+CD62L^-$) to T_{reg} cells (n=8-10). Data are mean ± SD. Flow cytometry analyses were either performed twice with similar results (A, C), or data were compiled from two independent experiments (D-E). Statistical analyses were performed using unpaired two-tailed t-tests with Welch correction for parametric data or two-tailed Mann-Whitney test for nonparametric data. P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

Further characterization of the T cells showed that lymphangiogenic 4T1 tumors have higher percentages of naïve cells than their control counterparts amongst both conv $CD4^+$ T cells and $CD8^+$ T cells (**Fig 2.5 A-B**). This can be attributed to the recruitment of $CCR7^+$ naïve T cells to the TME due to increased expression of chemokine CCL21 by LECs, which was previously observed in murine melanoma models [56, 59].

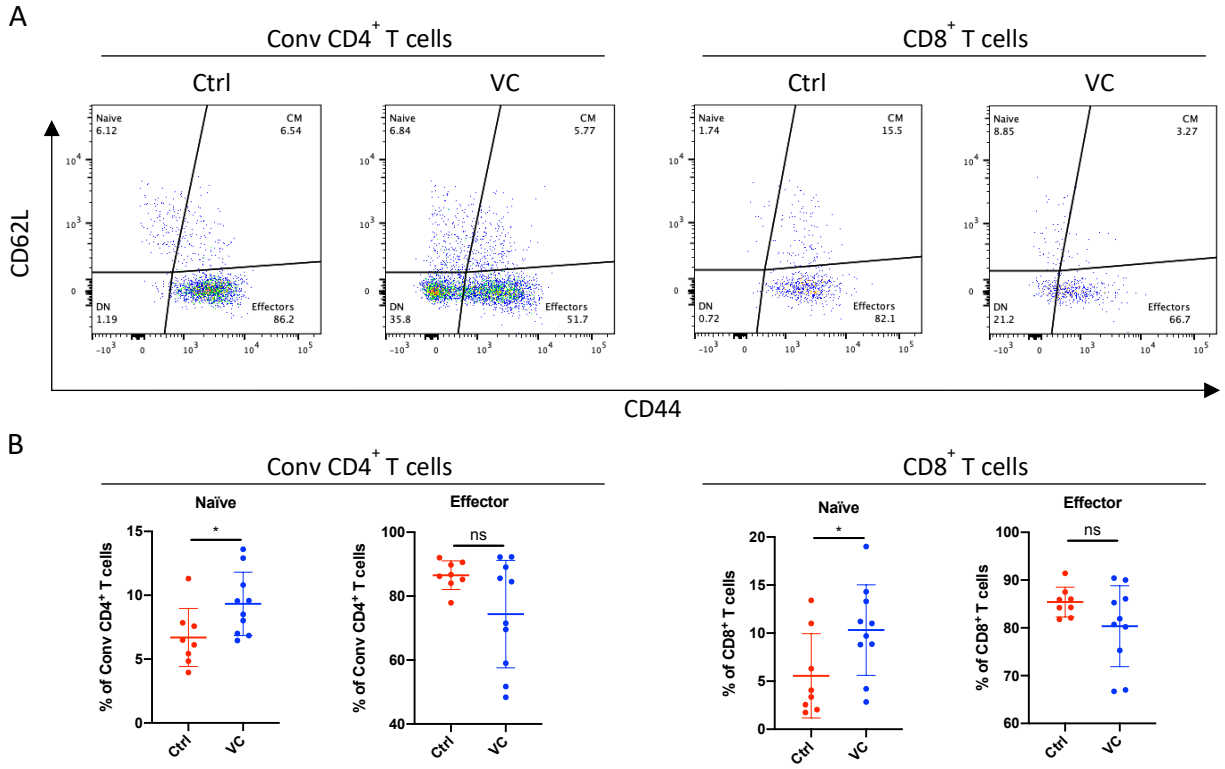

Figure 2.5. Lymphangiogenic triple-negative mammary tumors have higher percentages of naïve T cells. 4T1-Ctrl and 4T1-VC were orthotopically inoculated into MFP of WT mice. Mice were euthanized for analysis at day 15. (A) Representative distribution of T cell phenotypes in the TME using flow cytometry: naïve ($CD62L^+CD44^-$), effector ($CD62L^-CD44^+$), central memory (CM) ($CD62L^+CD44^+$) and double-negative (DN) ($CD62L^-CD44^-$). (B) Phenotype quantification of conv $CD4^+$ and $CD8^+$ T cells in the TME using flow cytometry (n=8-10). Data are mean ± SD. Flow cytometry data were compiled from two independent experiments (B). Statistical analyses were performed using unpaired two-tailed t-tests with Welch correction for parametric data or two-tailed Mann-Whitney test for nonparametric data. P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.3.2 Lymphangiogenic triple-negative mammary tumors respond more favorably to cancer immunotherapies

Since 4T1-VC tumors had higher infiltration of conv $CD4^+$ T cells and macrophages than their control counterparts, we investigated if treating them with immunotherapies would elicit a stronger immune response and improve the efficacy of the treatment. Despite the pro-tumorigenic phenotype of these macrophages in the lymphangiogenic tumors, we hypothesized that treatment with an innate immunity agonist would be able to repolarize those macrophages into a pro-inflammatory phenotype [66]. We first treated the tumors with ML-RR-S2 CDA (also known as ADU-S100), an agonist of the Stimulator of the Interferon Genes (STING) previously described to induce a strong anti-tumor immunity through expression of interferon-β (IFN-β) by antigen-presenting cells (including macrophages and DCs) that is currently in clinical trials [67, 68]. Treatment of the 4T1-VC tumors with STING agonist resulted in a stronger response demonstrated both by a four-fold reduction in tumor size and longer survival than 4T1-Ctrl tumor-bearing mice (**Fig. 2.6 A**). We next asked if lymphangiogenesis would improve responsiveness to the clinically used CPI combination of αCTLA-4 and αPD-1. Consistent with previous reports, 4T1 control tumors did not respond to CPI treatment, whereas CPI reduced tumor growth and increased survival in the lymphangiogenic 4T1 tumors [69] (**Fig. 2.6 B**). These results demonstrate that the induction of lymphangiogenesis in the TME of 4T1-VC tumors renders them more responsive to different cancer immunotherapies.

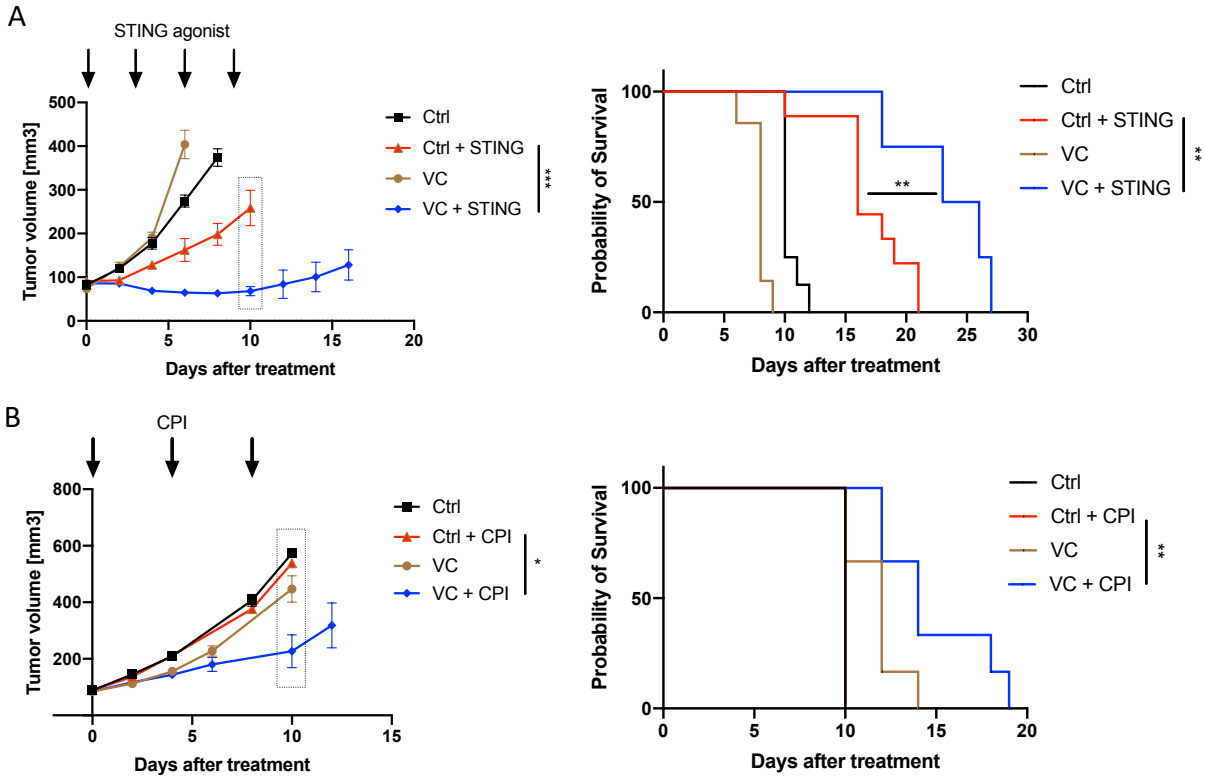

Figure 2.6. Induction of tumor lymphangiogenesis improves responsiveness of triple-negative mammary tumors to CPI and STING agonist treatment. 4T1-Ctrl and 4T1-VC tumor-bearing mice were treated with different immunotherapies or PBS once the tumors in each group reached an average size of 100 mm^3. (A) Tumor growth and survival curves of mice treated with STING agonist (n=7-9). (B) Tumor growth and survival curves of mice treated with CPI (n=5-6). Data are mean ± SEM. Experiments were performed twice with similar results. Statistical analyses were performed using two-tailed Mann-Whitney test (A), Kruskal–Wallis test followed by Dunn's multiple comparison test (B) and log-rank (Mantel–Cox) test for survival curves (A-B). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.3.3 Tumor lymphangiogenesis increases conv $CD4^+$ T cell and cross-presenting dendritic cell infiltration into the tumor microenvironment after immunotherapy treatment

In order to identify the key changes in the TME after immunotherapy treatment in control vs. lymphangiogenic tumors, we characterized the intratumoral immune infiltrates after treatment with STING agonist by flow cytometry. To ensure that the observed differences were mediated

through the STING pathway, we measured the levels of intratumoral IFN-β two days after treatment with STING agonist. The three-fold increase in IFN-β expression in the 4T1-VC tumors indeed demonstrated that the enhanced immunity in these tumors is mediated by the STING signaling pathway (**Fig. 2.7 A**). Over-expression of VEGF-C in 4T1 tumors elicited a stronger immune response demonstrated by pronounced activation of both the innate and adaptive arms of immunity. In contrast to macrophage infiltration in the absence of immunotherapy, VEGF-C over-expressing 4T1 tumors exhibited similar levels of pro-inflammatory $CD86^{+}MHCII^{+}$ macrophages and ratio of M1/M2 macrophages as the control tumors (**Fig. 2.7 B**). Importantly, 4T1-VC tumors were infiltrated with more DCs and had higher percentage of cross-presenting $CD103^{+}$ DCs, shown to play a critical role in the priming of $CD8^{+}$ T cells [70] (**Fig. 2.7 C**). 4T1-VC tumors also showed a trend towards fewer monocytic myeloid cells, and an increase towards more granulocytic myeloid cells (**Fig. 2.7 D**).

Analysis of the adaptive immune response showed that 4T1-VC tumors had higher infiltration of T cells, including more conv $CD4^{+}$ T cells and a trend towards more $CD8^{+}$ T cells (**Fig. 2.7 E-F**). With similar levels of T_{reg} cell infiltration, 4T1-VC tumors had higher ratio of effector $CD8^{+}$ T cells to T_{reg} cells (**Fig. 2.7 G**), which has been reported to act as a favorable prognostic factor in breast cancer [71]. Notably, the effector $CD8^{+}$ T cells had lower expression of PD-1 in the 4T1-VC tumors than control tumors (**Fig. 2.7 G**), demonstrating a less exhausted state [72]. These results show that upon treatment with STING agonist, the TME in lymphangiogenic 4T1 tumors switches from an immunosuppressed state to an immunogenic environment, which enhances responsiveness to the therapy.

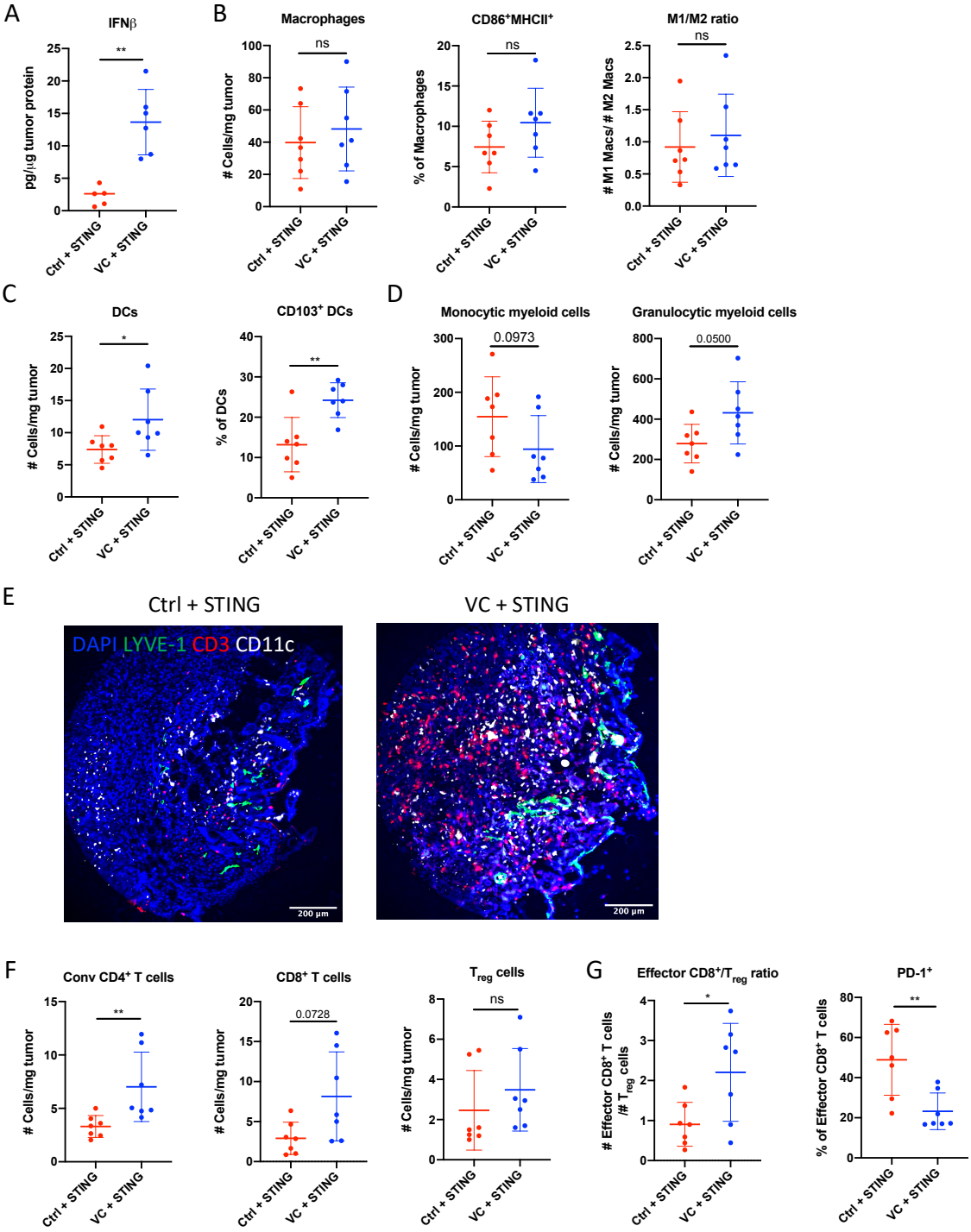

Figure 2.7. Lymphangiogenic tumors have higher tumor infiltration of CD4$^+$ T cells and dendritic cells after STING agonist treatment. Lymphangiogenic and control 4T1 tumor-bearing mice were treated with either one dose (A; n=5-6) or two doses of STING agonist and euthanized for analysis at day 15 post tumor inoculation (B-G; n=7). (A) Tumor-bearing mice were euthanized 2 days after one does of STING agonist treatment and IFN-β expression was assessed by ELISA.

Figure 2.7, continued. (B) Flow cytometry analysis of macrophages, frequency of $CD86^+MHCII^+$ macrophages and ratio of M1 to M2 macrophages. (C) Flow cytometry analysis of DCs and percentage of cross-presenting DCs ($CD103^+CD11b^-$). (D) Flow cytometry analysis of monocytic and granulocytic myeloid cells. (E) Representative immunostaining images of intratumoral $CD3^+$ and $CD11c^+$ cells. blue: DAPI, green: LYVE-1, red: CD3, gray: CD11c. Scale bar: 200 μm. (F) Flow cytometry analysis of conv $CD4^+$ T cells, $CD8^+$ T cells and T_{reg} cells. (G) Flow cytometry analysis of ratio of effector $CD8^+$ T cells to T_{reg} cells and frequency of PD-1^+ effector $CD8^+$ T cells. Data are mean ± SD. Flow cytometry analyses were performed twice with similar results. Statistical analyses were performed using unpaired two-tailed t-tests with Welch correction for parametric data or two-tailed Mann-Whitney test for nonparametric data. P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

I also characterized the intratumoral immune infiltrates after treatment of the tumors with CPI. Similarly to the results in the absence of treatment, lymphangiogenic 4T1 tumors had more conv $CD4^+$ T cells and T_{reg} cells than control tumors, but there was no significant difference in $CD8^+$ T cell numbers (**Fig. 2.8A**). However, the percentage of perforin$^+$ $CD8^+$ T cells was higher in 4T1-VC tumors than in 4T1-Ctrl tumors (**Fig. 2.8B**). Lymphangiogenic 4T1 tumors had higher levels of macrophages than control tumors, most of which showing an M2 phenotype (**Fig. 2.8C**). Despite similar numbers of DC infiltration, the percentage of cross-presenting $CD103^+$ DCs was higher in 4T1-VC tumors (**Fig. 2.8D**). Finally, there were no significant difference in the numbers of monocytic myeloid cells between the two groups, but lymphangiogenic tumors contained more granulocytic myeloid cells after CPI treatment (**Fig. 2.8E**).

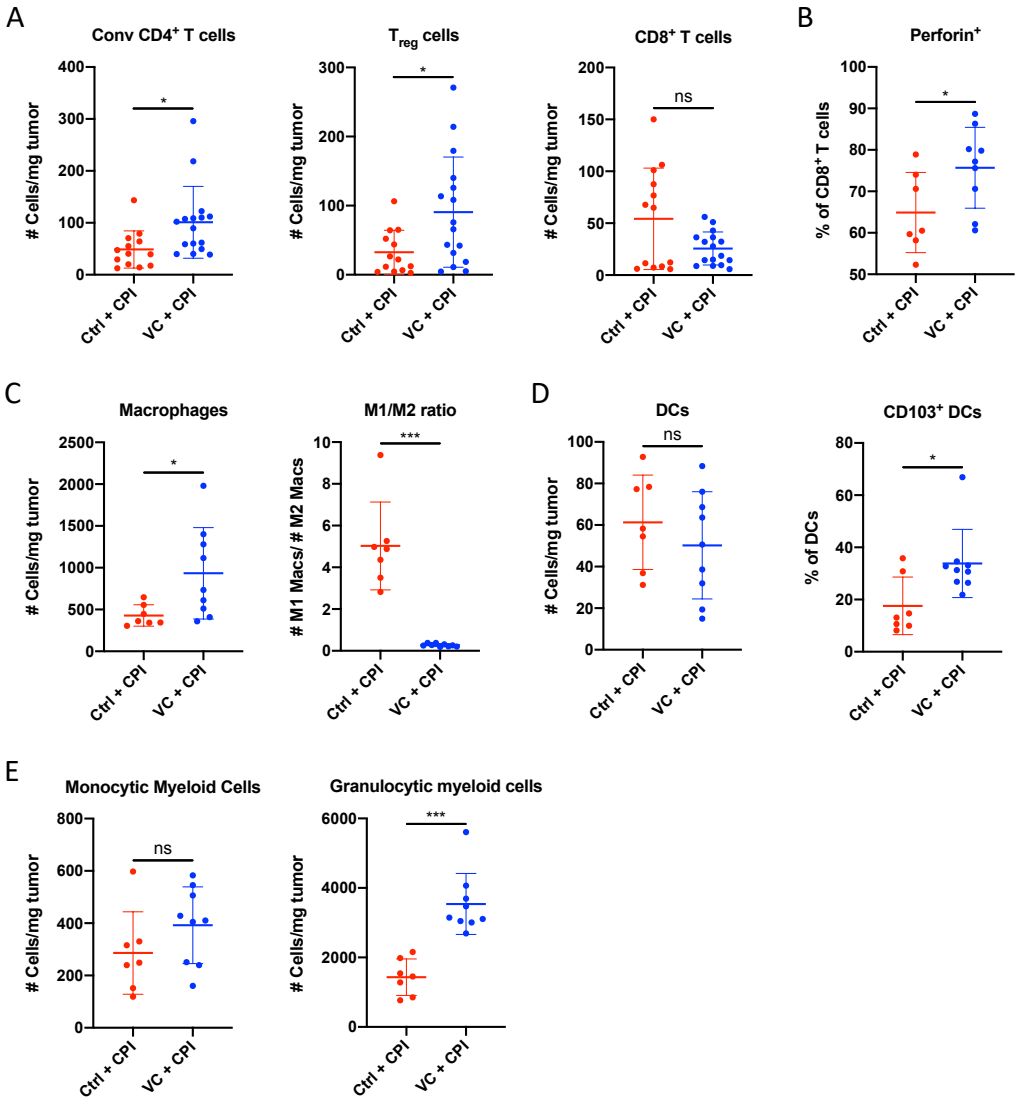

Figure 2.8. Lymphangiogenic tumors have higher tumor infiltration of CD4$^+$ T cells and cross-presenting CD103$^+$ dendritic cells after CPI treatment. Lymphangiogenic and control 4T1 tumor-bearing mice were treated with two doses of CPI and euthanized for analysis 6 days after the start of treatment. (A) Flow cytometry analysis of conv CD4$^+$ T cells, T_{reg} cells and CD8$^+$ T cells (n=13-16). (B) Percentage of perforin-expressing CD8$^+$ T cells (n=13-16). (C) Flow cytometry analysis of macrophages and ratio of M1 to M2 macrophages (n=7-9). (D) Flow cytometry analysis of DCs and percentage of cross-presenting DCs (CD103$^+$CD11b$^-$) (n=7-9). (E) Flow cytometry analysis of monocytic and granulocytic myeloid cells (n=7-9). Data are mean ± SD. Flow cytometry analyses were either pooled from two independent experiments (A) or performed twice with similar results (B-E). Statistical analyses were performed using unpaired two-tailed t-tests with Welch correction for parametric data or two-tailed Mann-Whitney test for nonparametric data. P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.3.4 Treatment with immunotherapy repolarizes the TME in lymphangiogenic mammary tumors from an immunosuppressive state into an immunogenic one

In order to further understand the changes in the TME in lymphangiogenic mammary tumors after treatment with immunotherapies, I characterized the intratumoral chemokine profile, which is critical in orchestrating a potent anti-tumor immunity [73, 74]. I treated the 4T1-Ctrl and 4T1-VC tumors with STING agonist and euthanized the mice for analysis of the intratumoral chemokines. Lymphangiogenic 4T1 tumors expressed higher levels of CCL2, CCL3 and CCL5, all of which have been shown to be important in recruiting monocytes and macrophages [73, 75, 76], than control tumors after STING treatment (**Fig. 2.9 A**). Notably, after STING treatment 4T1-VC tumors contained more CCL4 than 4T1-Ctrl tumors, a chemokine that plays a major role in recruiting cross-presenting DCs to the TME [77] (**Fig. 2.9 B**). CCL22, the chemokine responsible for recruiting T_{reg} cells [78, 79], was reduced in both tumor types after STING treatment (**Fig. 2.9 C**).

Importantly, expression of CXCL1 was reduced after treatment with STING agonist in lymphangiogenic 4T1 tumors and was lower than in control tumors (**Fig. 2.9 D**). Previous studies have demonstrated that CXCL1, mainly produced by TAMs, promotes metastasis in colorectal and breast cancer models [80, 81]. Lymphangiogenic tumors showed higher levels of CXCL5 after therapy, known to recruit myeloid-derived cells to the TME (**Fig. 2.9 E**) [82]. Strikingly, 4T1-VC tumors contained less CXCL10, which is correlated with potent anti-tumor T cell response [83], than control tumors in the absence of treatment, but exhibited a significant increase upon treatment with STING agonist (**Fig. 2.9 F**). Lastly, it has been shown that CXCL13 is over-expressed in breast tumors and positively correlates with metastasis [84]. Interestingly, lymphangiogenic 4T1

tumors exhibited lower levels of CXCL13 after STING treatment compared to non-lymphangiogenic tumors (**Fig. 2.9 G**).

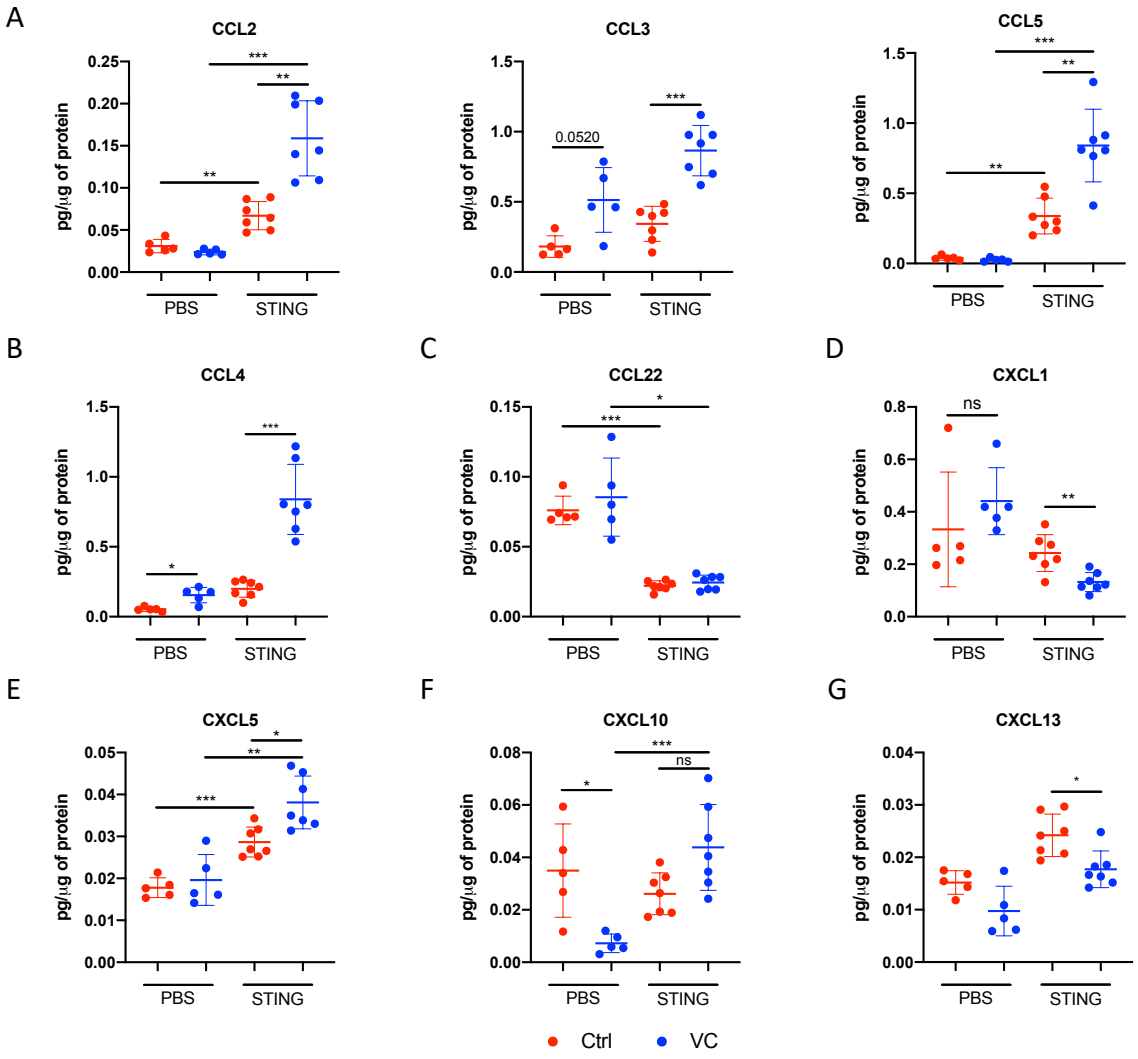

Figure 2.9. Induction of tumor lymphangiogenesis in triple-negative mammary tumors alters the chemokine expression profile in the tumor microenvironment after immunotherapy. 4T1-Ctrl and 4T1-VC tumor-bearing mice were treated with one dose of STING agonist and euthanized 2 days later for analysis (n=5-7). Intratumoral expression of (A) CCL2, CCL3, CCL4, (B) CCL5, (C) CCL22, (D) CXCL1, (E) CXCL5, (F) CXCL10 and (G) CXCL13 using LegendPlex. Data are mean ± SD. Experiment was performed once on independent biological samples. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test for parametric data and Kruskal–Wallis test followed by Dunn's multiple comparison test for nonparametric data. P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.3.5 Treatment with immunotherapy leads to enrichment of IFNγ$^+$ CD8$^+$ T cells in lymphangiogenic mammary tumors

Next, I characterized the cytokine profile in the TME to further delineate the mechanism behind lymphangiogenic potentiation in 4T1 triple-negative tumors. Due to the critical role of interferon-γ (IFNγ) in T cell-mediated cytotoxicity and its correlation with clinical response to CPI treatment [83, 85], I first treated 4T1-VC and 4T1-Ctrl tumor-bearing mice with CPI and analyzed cytokine production by intratumoral T cells. Lymphangiogenic 4T1 tumors contained a higher percentage of IFNγ$^+$ cells in the CD8$^+$ T cell compartment than their control counterparts post STING therapy (**Fig 2.10 A-B**). This is of significance due to the positive correlation between recruitment of T cells to the TME and IFNγ with patient survival [86]. I also assessed the production of Interleukin-2 (IL-2) by CD4$^+$ T cells. 4T1-VC tumors had significantly higher percentage of IL-2$^+$ CD4$^+$ T cells than control tumors when treated with PBS and had a trend towards more IL-2$^+$ cells after CPI therapy (**Fig. 2.10 C**). This is of significance since T helper type 1 (T_H1) cells secrete IL-2, which in turn activates CD8$^+$ T cells [87, 88].

In order to further characterize the cytokine milieu in the TME, I assessed the intratumoral cytokine levels after treatment with STING agonist. Non-lymphangiogenic tumors had higher levels of IL-2 after STING treatment (**Fig. 2.10 D**). 4T1-VC tumors had significantly higher levels of IL-4 than control tumors, undergoing PBS or STING therapy (**Fig. 2.10 E**). There was also a significant increase in IL-6 levels in STING-treated lymphangiogenic tumors than control tumors (**Fig. 2.10 F**). Lastly, there was a strong trend towards more tumor necrosis factor-alpha (TNFα) and IFNγ, both important in mediating tumor cell cytotoxicity [83, 89], in lymphangiogenic tumors than control tumors after STING therapy (**Fig. 2.10 G**).

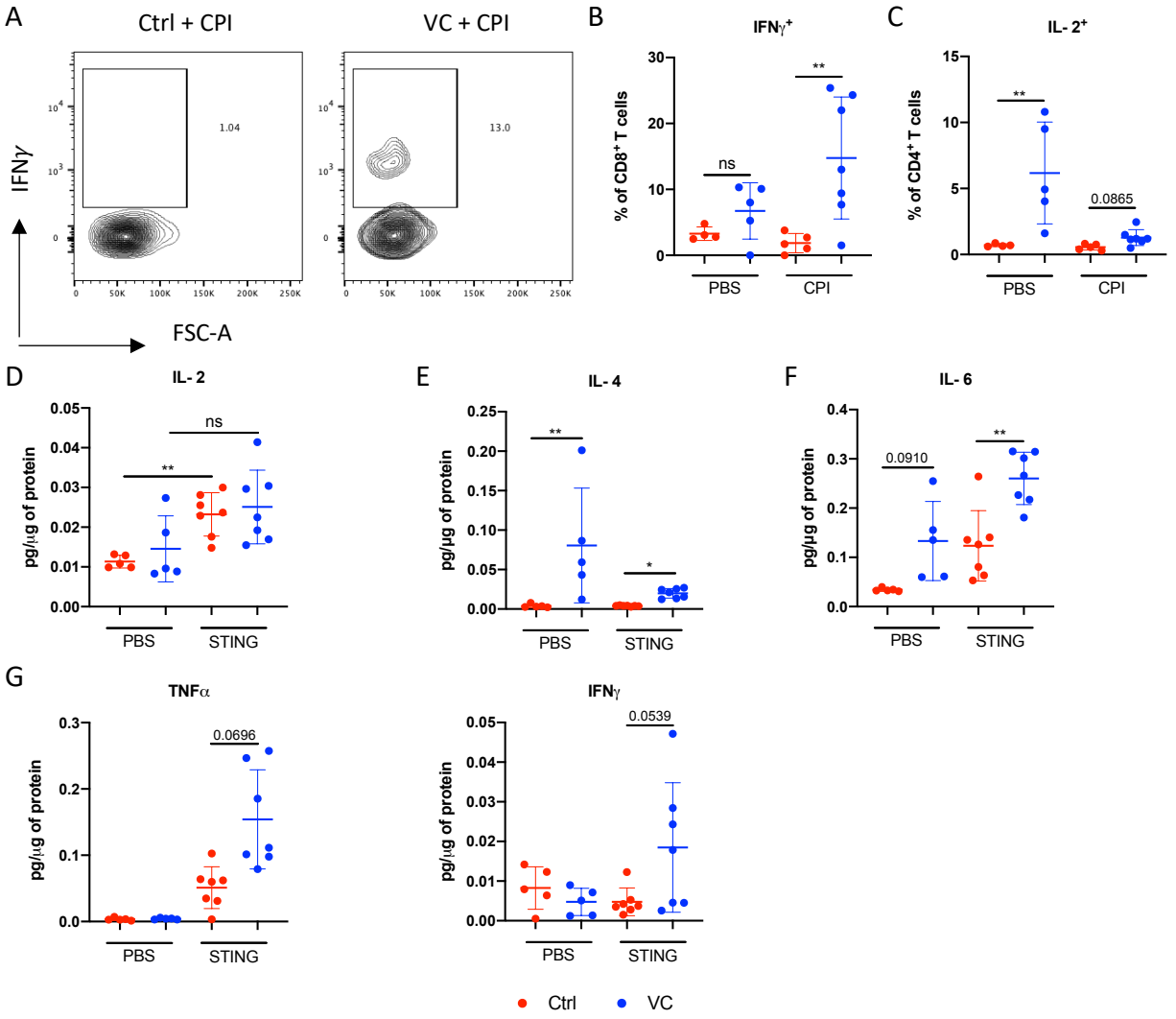

Figure 2.10. Lymphangiogenic mammary tumors have higher percentage of IFNγ-expressing CD8⁺ T cells upon treatment with immunotherapy. 4T1-Ctrl and 4T1-VC tumor-bearing mice were treated with either (A-B) two doses of CPI or PBS and euthanized for analysis at day 6 after start of treatment (n=4-7), or (D-G) one dose of STING agonist or PBS and euthanized at day 2 post treatment for analysis (n=5-7). (A) Representative intracellular expression of IFNγ in intratumoral $CD8^+$ T cells using flow cytometry. Quantification of (B) IFNγ expression in $CD8^+$ T cells and (C) IL-2 expression in $CD4^+$ T cells in the tumor as assayed by flow cytometry. Intratumoral expression of (D) IL-2, (E) IL-4, (F) IL-6, and (G) TNFα and IFNγ using LegendPlex. Data are mean ± SD (B-G). Experiments were performed once on independent biological samples. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test for parametric data and Kruskal–Wallis test followed by Dunn's multiple comparison test for nonparametric data. P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.3.6 Combination therapy synergizes with lymphangiogenic potentiation to induce a systemic antigen-specific response and overcome spontaneous lung metastasis

As previously mentioned, tumor lymphangiogenesis has long been known to positively correlate with increased metastasis in murine and human breast cancer [52, 53, 58]. The spontaneous metastatic nature of the 4T1 tumor model allowed us to assess whether lymphangiogenic potentiation is able to elicit a systemic anti-tumor immunity that would delay morbidity due to lymphangiogenic-associated metastasis. In order to induce an effective systemic immune response I treated the tumors with a combination of STING agonist and CPI, which has been shown to result in a potent systemic response [90]. Lymphangiogenic 4T1 tumor-bearing mice not only had a lower primary tumor burden than the control mice, but they also exhibited longer survival (**Fig. 2.11 A-C**). This increase in median survival, which is more than 50% longer than when treating the tumors with STING agonist only, suggests a reduced metastatic burden in the lungs.

In order to confirm if the metastatic burden was indeed reduced upon combination therapy, I quantified lung metastatic nodules (Mets) in tumor-bearing mice. The analysis showed a reduction in metastatic burden in lymphangiogenic tumor-bearing mice treated with the combination therapy compared to untreated 4T1-VC tumor-bearing mice and the treated control tumor-bearing mice (**Fig. 2.11 D-E**). Strikingly, only 3 out of the 8 mice with 4T1-VC tumors and treated with CPI and STING agonist had detectable Mets, whereas all the mice in the other groups had at least one metastatic nodule in the lungs (**Fig. 2.11 D**).

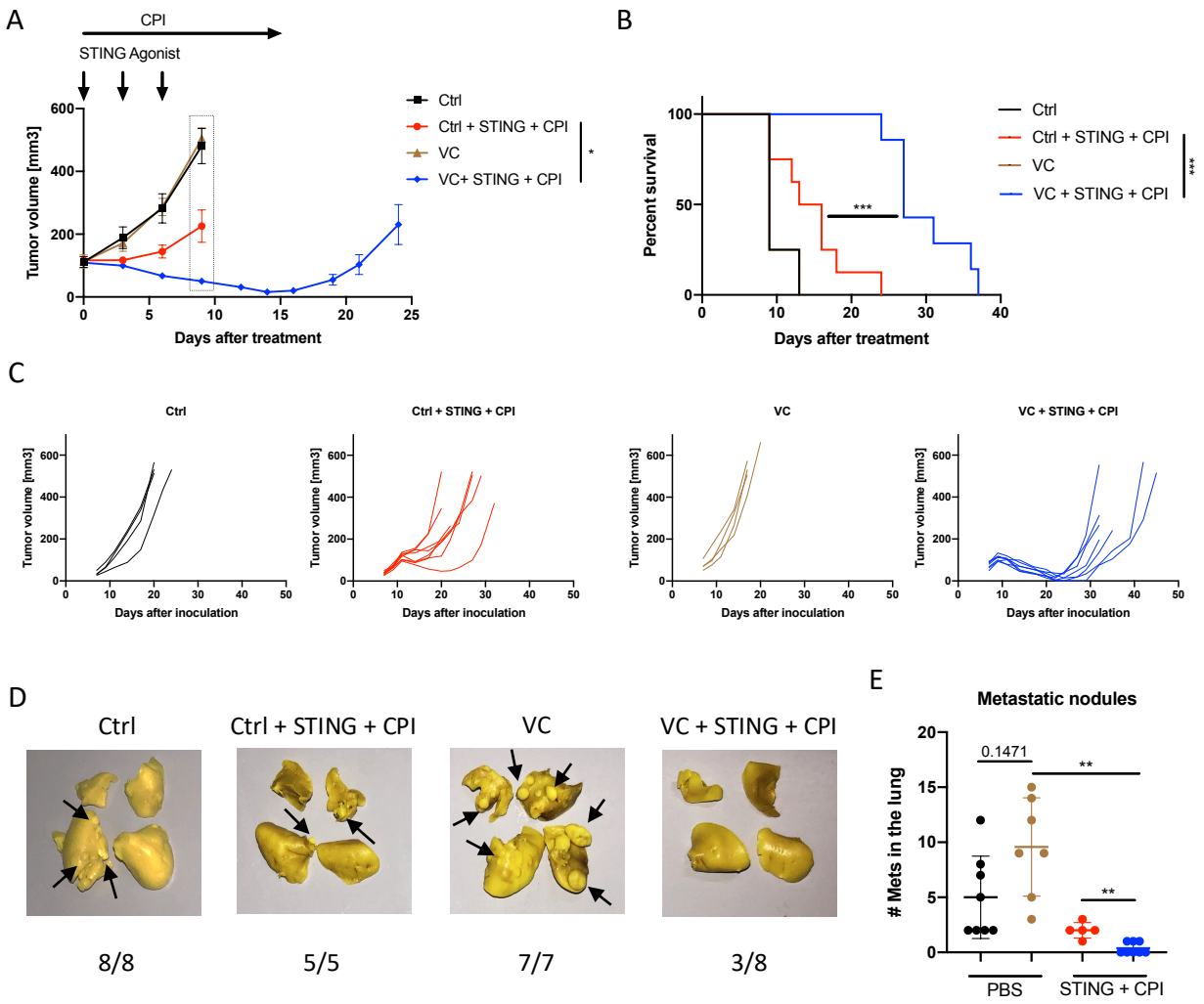

Figure 2.11. Tumor lymphangiogenesis synergizes with CPI and STING agonist combination therapy, leading to prolonged survival and delayed spontaneous lung metastasis. 4T1-VC and 4T1-Ctrl tumor-bearing mice were treated with PBS or CPI and STING agonist combination and were monitored for tumor growth and survival (A-C) or euthanized at day 24 for analysis (D-E). (A) Overall tumor growth curves, (B) survival curves and (C) individual growth curves of the treated mice (n=4-8). (D) Representative images of metastatic nodules in the lungs of tumor-bearing mice, with the number of mice with nodules in each group in the bottom row. Data are mean ± SEM (A) and mean ± SD (E). Experiments were either performed twice (A-C) or done once on distinct biological samples (D-E). Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test (A, E) and log-rank (Mantel–Cox) test (B). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

Next, I asked whether the reduction in lung metastatic burden of 4T1-VC tumor-bearing mice treated with combination therapy is due to induction of a systemic anti-tumor immunity. I restimulated splenocytes from tumor-bearing mice *ex vivo* with the AH1 peptide that has been reported to be over-expressed in different tumor cell lines, including the 4T1 model, but not in normal tissues [91]. When treated with CPI, splenic $CD8^+$ T cells in lymphangiogenic tumor-bearing mice indeed showed a higher expression of intracellular IFNγ, TNFα and granzyme B (GrB) than the ones in control mice, suggesting a more potent systemic antigen-specific immune response (**Fig. 2.12 A**). Furthermore, flow cytometry characterization of immune infiltrations in the lung of tumor-bearing mice showed increased infiltration of DCs, macrophages and B cells in mice with lymphangiogenic tumors after treatment with the combination immunotherapy (**Fig. 2.12 B**). Interestingly, there was a trend towards fewer neutrophils in the lungs of treated 4T1-VC tumor-bearing mice, compared to control mice (**Fig. 2.12 B**). Moreover, these mice exhibited enrichment of $CD4^+$ T cells, $CD8^+$ T cells and a higher percentage of $CD8^+$ T cells expressing KLRG1 in their lungs (**Fig. 2.12 C**). Further analysis shows that the percentage of T cells infiltrating the lungs negatively correlates with primary tumor size and number of Mets in the lungs (**Fig. 2.12 D**). Together, these results demonstrate that lymphangiogenesis not only potentiates immunotherapy in the context of primary tumor growth, but also promotes a systemic immune response reducing metastatic burden in distant sites.

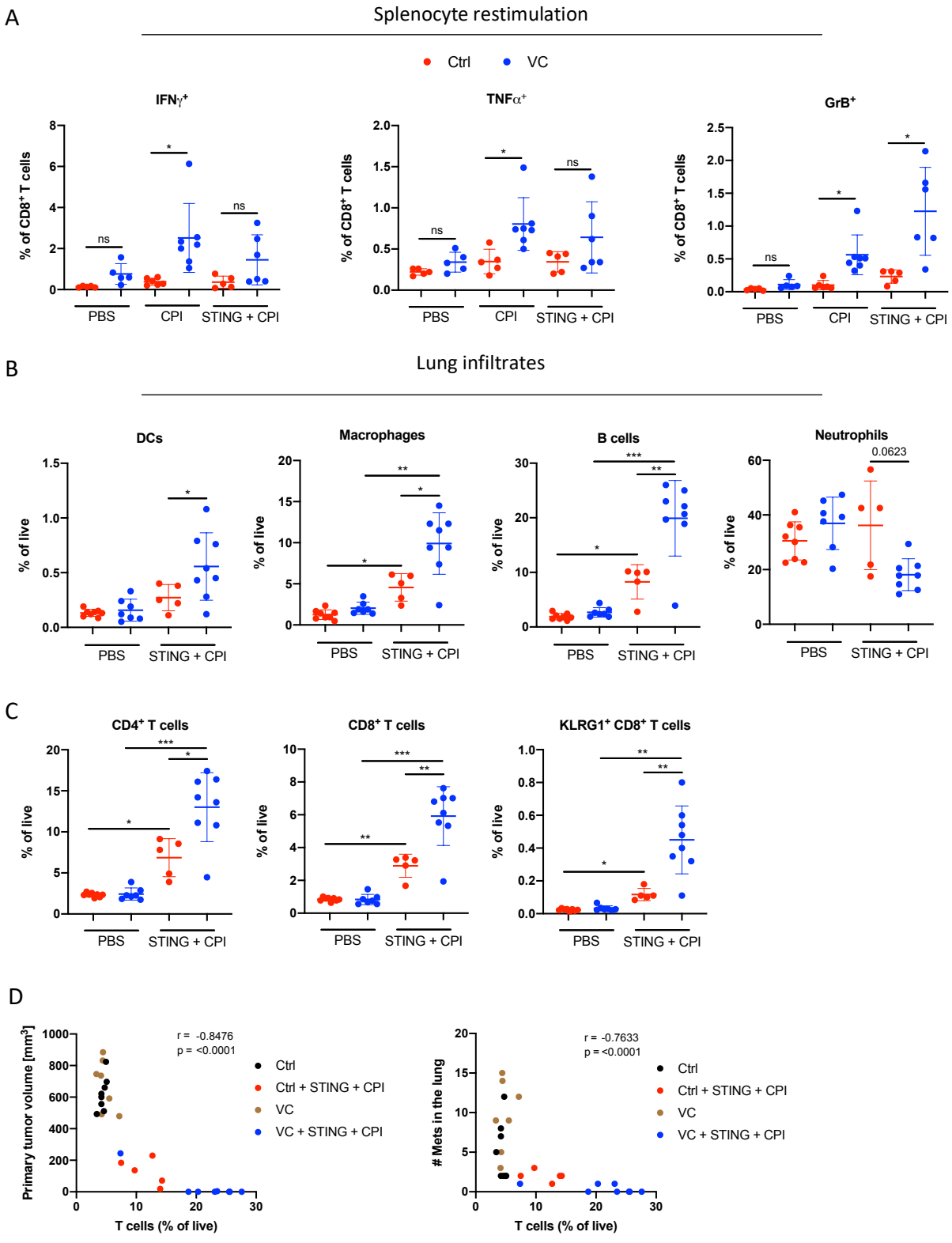
A
Splenocyte restimulation
Ctrl
VC
IFNγ+
TNFα+
GrB+
% of CD8+ T cells
ns
*
PBS
CPI
STING + CPI
B
Lung infiltrates
DCs
Macrophages
B cells
Neutrophils
% of live
0.0623
C
CD4+ T cells
CD8+ T cells
KLRG1+ CD8+ T cells
**
***
D
Primary tumor volume [mm3]
T cells (% of live)
r = -0.8476
p = <0.0001
Ctrl
Ctrl + STING + CPI
VC
VC + STING + CPI
# Mets in the lung
r = -0.7633
p = <0.0001

Figure 2.12 Treatment of lymphangiogenic mammary tumors with immunotherapy induces a systemic antigen-specific immune response and increases immune infiltrates in the metastatic lesions. 4T1-VC and 4T1-Ctrl tumor-bearing mice were treated with either (A) PBS, two doses of CPI or one dose of STING agonist in combination with two doses of CPI and euthanized at day 6 after start of treatment for analysis of splenocytes or (B-D) with PBS or CPI and STING agonist combination and euthanized at day 24 post inoculation for analysis of lung lobes. (A) Percentage of $CD8^+$ T cells expressing intracellular IFNγ, TNFα and GrB after *ex vivo* antigen-specific restimulation of splenocytes with AH1 peptide as assayed by flow cytometry. Lung infiltrate quantification (% of live cells) of (B) DCs, macrophages, B cells and neutrophils, and (C) $CD4^+$ T cells, $CD8^+$ T cells and $KLRG1^+$ $CD8^+$ T cells using flow cytometry (n=5-7). (D) Correlations of primary tumor volume and lung Mets with T cell infiltrates in the lung. Data are mean ± SD (A-C). Experiments were performed once on distinct biological samples. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test (A-C) and nonparametric Spearman correlation analysis (D). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.3.7 The improved efficacy of immunotherapies in lymphangiogenic tumors is mediated by $CD4^+$ T cells and macrophages

The higher infiltration of conv $CD4^+$ T cells and macrophages in 4T1-VC tumors compared to control mice in the absence of immunotherapy may explain the lymphangiogenic potentiation of immunotherapies in triple-negative breast cancer. In order to test this hypothesis, I administered depleting antibodies against CD4 and CSF1R starting at day 0 to inhibit the enrichment of $CD4^+$ T cells and macrophages in the tumor microenvironment prior to treatment with STING agonist. First, I confirmed the depletion of targeted populations in the blood at day 21 post tumor inoculation (**Appendix Fig. 2S3. A**). $CD4^+$ T cells were not detectable in the blood of mice treated with αCD4 and αCSF1R treatment led to the reduction of $Ly6C^-F4/80^+$ cells in the bloodstream.

Notably, antibody depletion of $CD4^+$ T cells negatively affected the efficacy of STING treatment and reduced survival in the lymphangiogenic tumor-bearing mice, whereas it improved both in the control mice (**Fig. 2.13 A-B, E**). Analysis of the blood of tumor-bearing mice revealed that CD4 depletion increased the percentage of $CD8^+$ T cells in the mice in the control group,

suggesting a mechanism for the observed improved efficacy in the control group (**Appendix Fig. 2S3. B**).

The depletion of macrophages increased the tumor burden and reduced the survival in both tumor types (**Fig. 2.13 C-D**), possibly due to the mechanism of action of the STING agonist [67]. However, the tumor burden increase was more drastic in the 4T1-VC tumor-bearing mice, i.e. five-fold increase in the VC group compared to less than two-fold increase in the Ctrl group (**Fig. 2.13 F**). Moreover, macrophage depletion reduced the percentage of $CD8^+$ T cells only in the lymphangiogenic tumor-bearing mice, attesting to their importance in inducing the anti-tumor immunity in the lymphangiogenic tumors (**Appendix Fig. 2S3. B**). These data show that the beneficial effect of tumor lymphangiogenesis in triple-negative mammary tumors, when treated with immunotherapies, is dependent on $CD4^+$ T cells and macrophages.

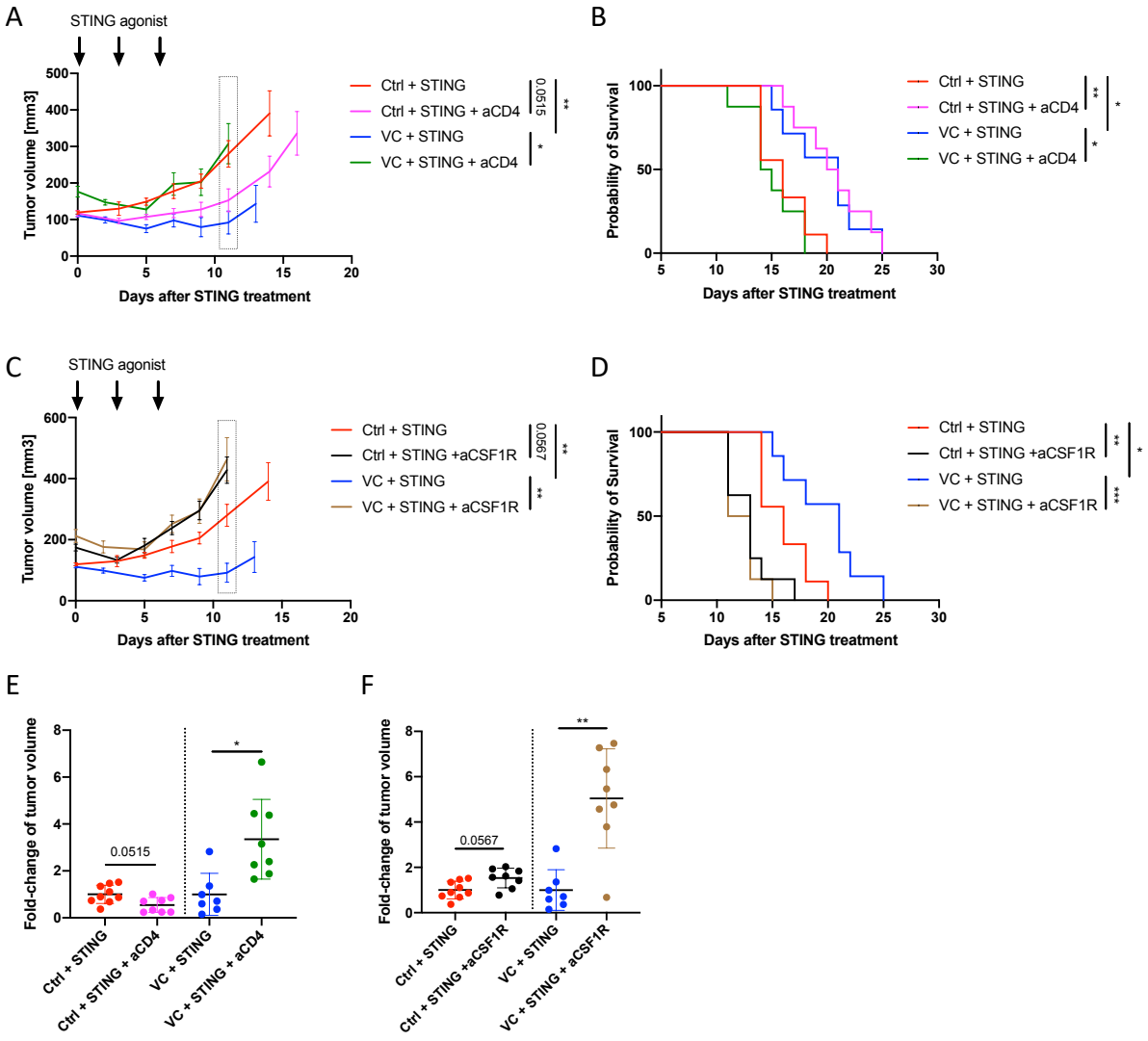

Figure 2.13. Lymphangiogenic potentiation in triple-negative mammary tumors is dependent on CD4 T cells and macrophages. Lymphangiogenic and control 4T1 tumor-bearing mice (n=7-9) were administered depletion antibodies (αCD4 and αCSF1R) from the day of tumor inoculation and were treated with 3 doses of STING agonist once tumors were established. (A) Tumor growth curves and (B) survival curves of mice treated with STING agonist and either PBS or αCD4 antibodies. (C) Tumor growth curves and (D) survival curves of mice treated with STING agonist and either PBS or α CSF1R antibodies. (E-F) Fold-change of tumor volume for each group compared to the non-depleted group of the same tumor type at day 11 after the start of STING agonist treatment. Data are mean ± SEM (A, C) and mean ± SD (E-F). The experiment was done once on distinct biological samples. Statistical analyses were performed using log-rank (Mantel–Cox) test (B, D) and one-way ANOVA followed by Dunnett's multiple comparison test (A, C, E-F). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.3.8 STING agonist treatment does not synergize with TGF-β blocking in 4T1 triple-negative mammary tumors

It has been shown that LECs secrete multiple immunosuppressive chemokines and molecules, including TGF-β [54]. These inhibitory molecules dampen the anti-tumor immune response and reduce the efficacy of cancer immunotherapies through different mechanisms, such as exclusion of T cells from the TME [92, 93]. Quantification of intratumoral TGF-β levels revealed that there is a trend towards more TGF-β in the lymphangiogenic 4T1 tumors after treatment with STING agonist (**Fig. 2.14 A**). To assess if inhibiting TGF-β would improve the efficacy of STING therapy in the 4T1-VC tumor-bearing mice, I treated both tumor types with STING agonist in combination with either PBS or TGF-β blocking antibody. Surprisingly, αTGF-β blocking did not improve the respective response to STING therapy in either tumor type in terms of tumor growth rate or survival (**Fig. 2.14 B-D**). These results show that blocking TGF-β does not synergize with STING agonist therapy in 4T1 triple-negative mammary tumors.

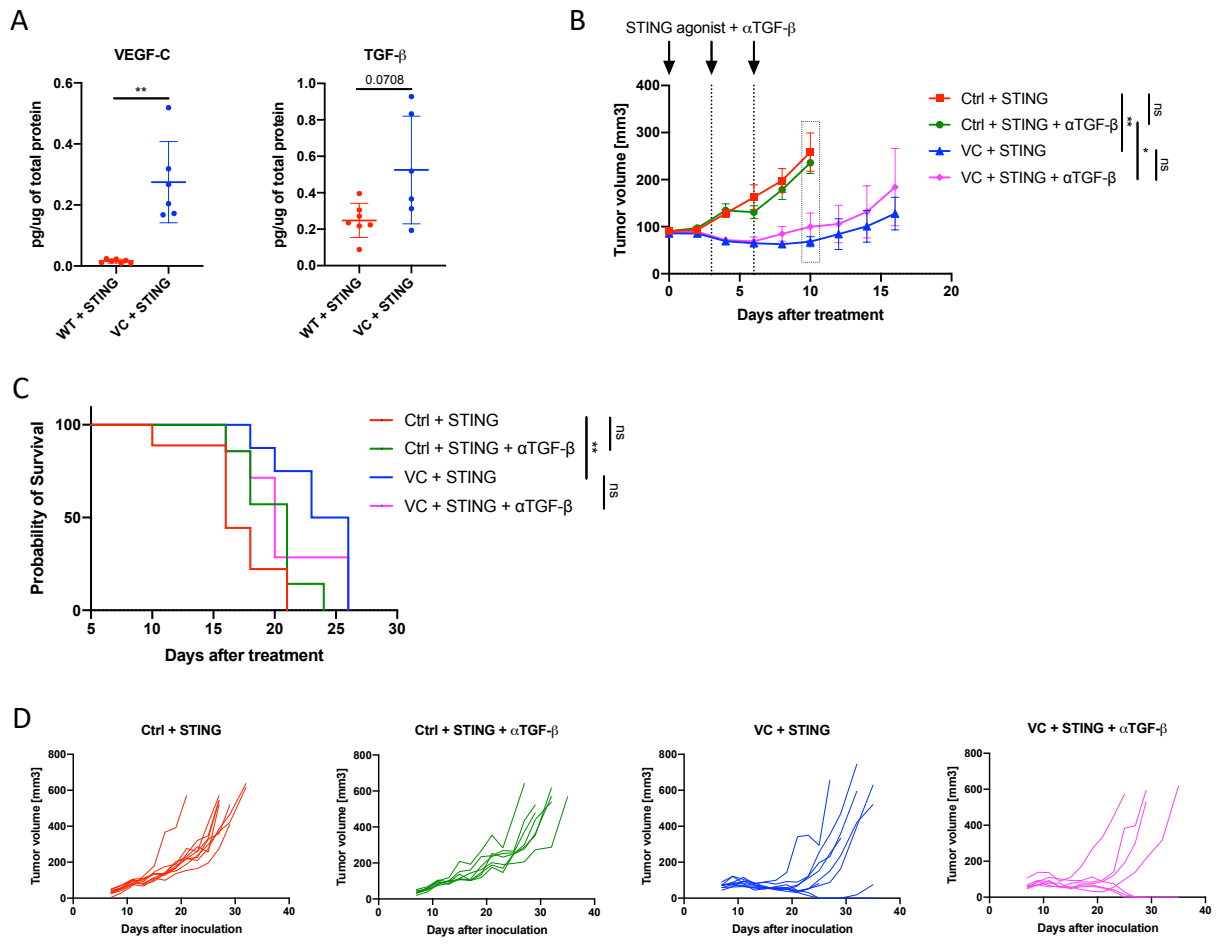

Figure 2.14. Blocking of TGF-β does not improve the responsiveness of 4T1 mammary tumors to STING agonist treatment. 4T1-Ctrl and 4T1-VC tumor-bearing mice were treated with STING agonist in combination with either αTGF-β antibody or PBS after tumors had reached an average size of ~100 mm^3. (A) Intratumoral VEGF-C and TGF-β quantification 6 days after STING treatment, as assayed by ELISA. (B) Overall tumor growth curves, (C) survival curves and (D) individual growth curves of the mice (n=7-9). Data are mean ± SEM. Experiment was performed twice with similar results. Statistical analyses were performed using unpaired two-tailed t-tests with Welch correction (A), one-way ANOVA followed by Dunnett's multiple comparison test (B) and log-rank (Mantel–Cox) test for survival curves (C). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.3.9 Over-expression of VEGF-C does not potentiate responsiveness to immunotherapies in a HER2$^+$ mammary carcinoma model

Next, I asked if the lymphangiogenic potentiation that I observed in the 4T1 triple-negative mammary carcinoma also occurs in other breast cancer models with a different molecular subtype.

I used the polyoma middle T antigen (PyMT) tumor cell line that we had isolated from MMTV-PyMT transgenic mice, in which the expression of the oncogene is driven by the Mouse Mammary Tumor Virus promoter (MMTV). The PyMT tumors are negative for expression of ER and PR, but express HER2 particularly in the later stages of tumor development [94, 95]. We lentivirally transduced these cells with our Ctrl vector or VEGF-C vector and assessed the VEGF-C expression *in vitro*. There was an ~ 8-fold increase in the expression level of VEGF-C by the PyMT-VC tumor cells compared to PyMT-Ctrl cells (**Fig. 2.15 A).** Next, to assess if over-expression of VEGF-C affected the responsiveness of lymphangiogenic PyMT tumors to immunotherapies, I inoculated FvB/NJ with both tumor cell types and treated them with checkpoint inhibitors. Even though both tumor types responded to the treatment, there was no difference between the lymphangiogenic tumor-bearing mice and control mice, both in terms of tumor growth rate and survival (**Fig. 2.15 B-D).** These data exhibit that lymphangiogenic potentiation does not occur in HER2^{+} PyMT mammary tumors.

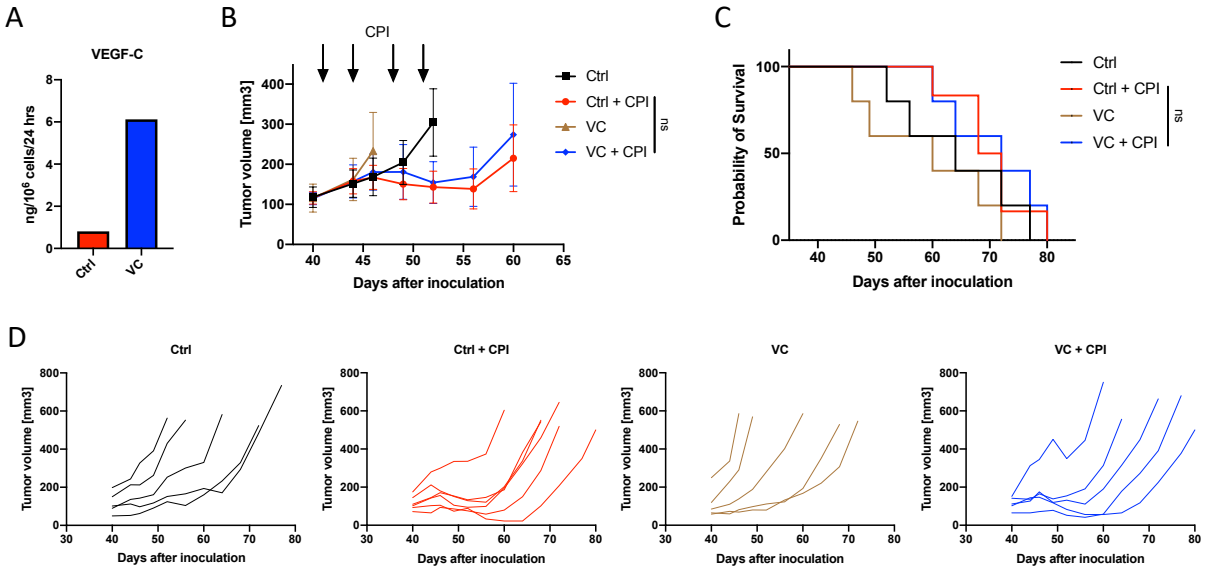

Figure 2.15. Tumor Lymphangiogenesis does not improve the responsiveness of PyMT mammary tumors to immunotherapies. Mice were inoculated with PyMT-VC and PyMT-Ctrl tumors and treated with PBS or CPI at day 41, 44, 48 and 51 post tumor inoculation. (A) Quantification of VEGF-C secretion by PyMT-VC and PyMT-Ctrl cells *in vitro* using ELISA. (B) Overall tumor growth curves, (C) survival curves and (D) individual growth curves (n=5-6). Data are mean ± SEM (B). Experiment was performed once on distinct biological samples. Statistical analyses were performed using unpaired two-tailed Mann-Whitney test (B) and log-rank (Mantel–Cox) test for survival curves (C). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.3.10 Tumor lymphangiogenesis renders E0771 triple-negative mammary tumors more immunogenic in the absence of immunotherapies in a leukocyte-dependent manner

Based on the results obtained in the PyMT tumor model, I sought to assess if tumor lymphangiogenesis would improve responsiveness to immunotherapies in a triple-negative mammary tumor model other than 4T1. I transduced triple-negative E0771 tumor cells with VC vectors and compared its VEGF-C secretion *in vitro* to the parental WT cell line (**Fig. 2.16 A).** Then, I inoculated C57BL/6 mice with WT tumor cells or a mixture of WT cells and VC-transduced cells (3:1 ratio). When treated with CPI, there was no significant difference between the efficacy of treatment in wild-type tumor-bearing mice and mice bearing tumors containing VC over-expressing cells (**Fig. 2.16 B-D)**. However, I observed that the VC over-expressing cells caused the tumors to grow at a slower rate than the WT tumors (data not shown). In order to assess if this slower growth rate is due to the higher immunogenicity in this tumor model [96] and dependent on T cells, I inoculated B6.129S7-Rag^{1tm1Mom}/J mice (known as Rag1 KO) and their control littermates with E0771-WT or E0771-VC tumor cells. The mice homozygous for the Rag^{1tm1Mom} mutation lack mature B cells and T cells. Notably, there was a trend towards faster tumor growth in the KO mice for both tumor types, but it only reached statistical significance for the E0771-VC tumor-bearing mice (**Fig. 2.16 E**). More importantly, all the VC tumors in the control littermates regressed after growing slightly and 4/4 mice remained tumor-free at day 50

post tumor inoculation, whereas only 2/4 mice bearing WT tumors survived (**Fig. 2.16 F**). These results demonstrate that over-expression of VEGF-C in the immunogenic E0771 triple-negative mammary tumors leads to their regression in the absence of any treatment; an effect that is dependent on mature T and B cells.

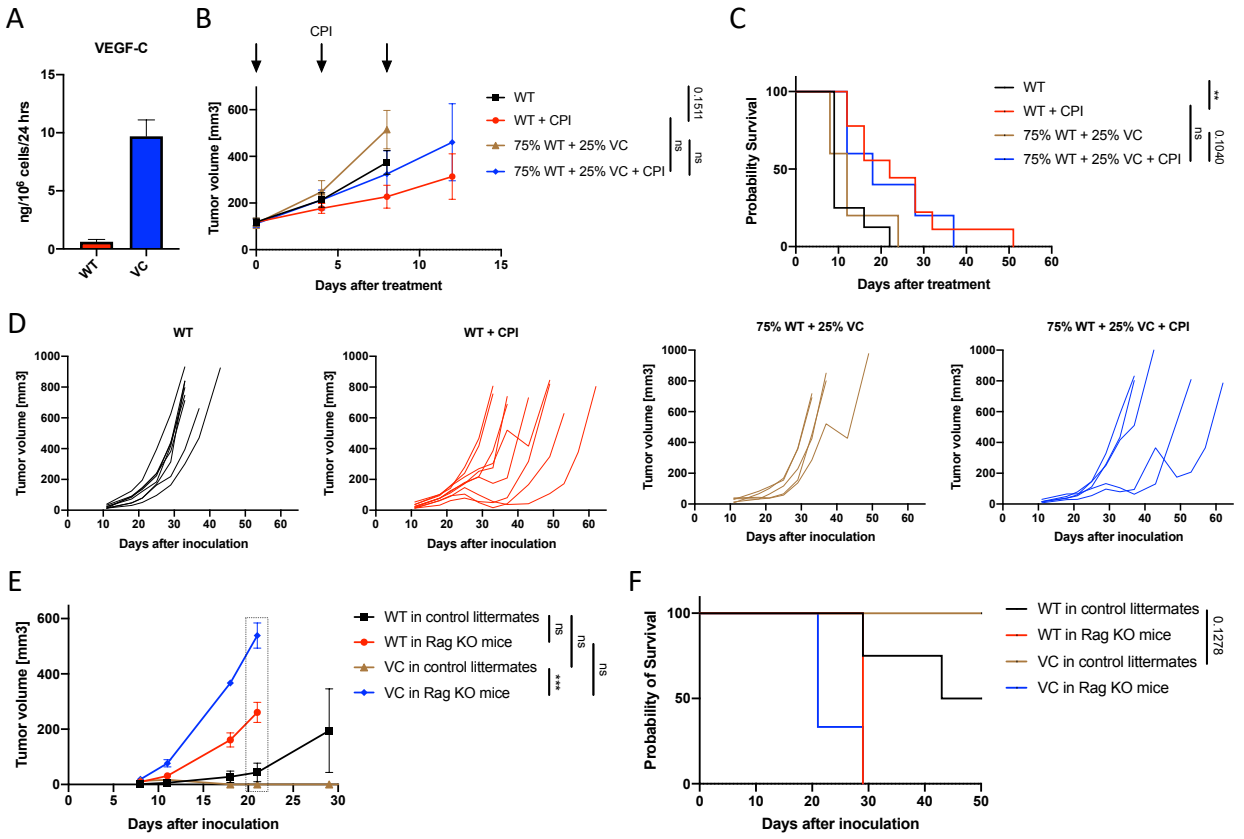

Figure 2.16. The complete remission of lymphangiogenic E0771 triple-negative mammary tumors in the absence of treatment is abrogated in Rag1 KO mice. Wildtype mice (B-D) or B6.129S7-Rag^{1tm1Mom}/J mice and their heterozygous littermates (E-F) were inoculated with WT E0771 cells, E0771-VC cells or a mixture of both and were either treated with PBS or CPI (B-D) or left untreated (E-F). (A) Quantification of VEGF-C secretion by WT E0771 and E0771-VC cells *in vitro* using ELISA. (B) Overall tumor growth curves, (C) survival curves and (D) individual growth curves of tumors in WT mice (n=5-9). (E) Tumor growth and (F) survival curves of tumors in Rag1 KO mice and their control littermates (n=4-6). Data are mean ± SEM (B, E). Experiment was performed either twice with similar results (B-D) or once on distinct biological samples (E-F). Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test (B), Kruskal–Wallis test followed by Dunn's multiple comparison test (E) and log-rank (Mantel–Cox) test for survival curves (C, F). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

Since the VC over-expressing E0771 cells were driving tumor remission in the absence of any treatment, I aimed to titer down the tumor lymphangiogenesis by using a protein delivery technology, similar to previous studies that used a fibrin-binding VEGF-C variant [97]. I designed and produced a collagen-binding VEGF-C variant. Briefly, the A3 domain of human von Willebrand factor (VWF) protein, which has high binding affinity for collagen [98], was recombinantly fused to the C-terminus of murine VEGF-C, similarly to previously published [99]. The binding affinity of the A3 domain to collagen in the extracellular matrix leads to the retention of the recombinant protein in the TME and induction of local lymphangiogenesis [99]. First, I confirmed the molecular weight and purity of our A3-VC variant using SDS-PAGE (**Fig. 2.17 A**). Notably, a band was observed at ~70 kDa for A3-VC in non-reducing condition, which confirmed its proper dimerization capability that is required for phosphorylation of VEGFR3 receptor [5]. I also confirmed the binding affinity of A3-VC for collagen I and III using ELISA (**Fig. 2.17 B**). Next, I assessed the bioactivity of our A3-VC variant by stimulating human LECs (hLECs) and quantifying the phosphorylation of VEGFR3 (p-VEGFR3) receptor. After *in vitro* stimulation of hLECs for 20 mins, both WT-VC and A3-VC had increased p-VEGFR3 over the unstimulated condition, whereas there was no significant difference between the two VC variants (**Fig. 2.17 C-D**). Next, I assessed whether induction of tumor lymphangiogenesis using A3-VC would render E0771 more responsive to CPI, without their spontaneous rejection. I intratumorally treated mice with WT E0771 tumors using A3-VC and further treated them with PBS or CPI. Even though both groups responded to CPI treatment, there was no difference in terms of tumor growth rate and survival between A3-VC treated mice and control mice receiving CPI (**Fig. 2.17 E-F**).

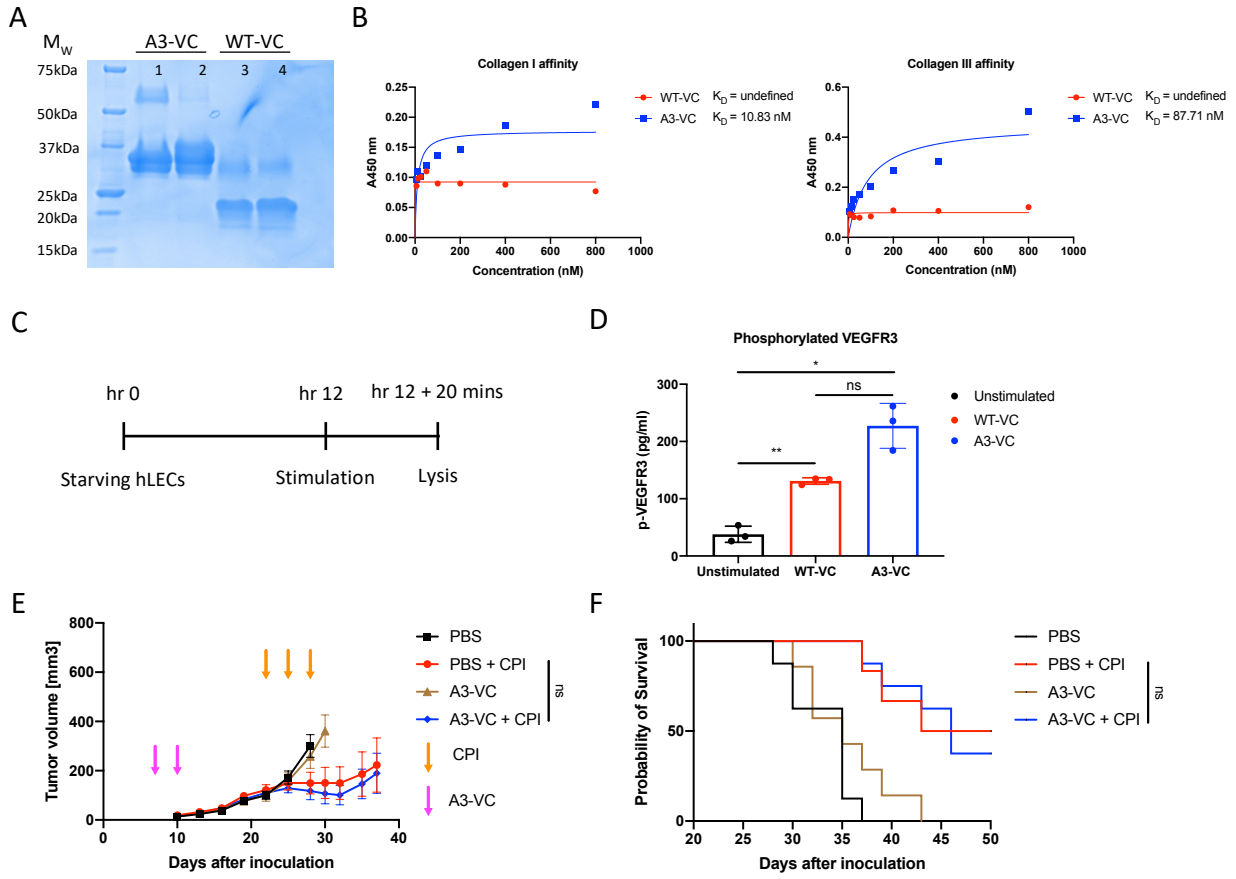

Figure 2.17. Treatment with A3-VC does not render E0771 tumor-bearing mice more responsive to CPI therapy compared to control-treated mice. Wildtype mice were inoculated with WT E0771 cells and were either treated with PBS or A3-VC prior to treatment with CPI or PBS according to the shown schedule (E-F). (A) Purity and molecular weight (M_W) assessment of A3-VC and WT-VC in reducing (2,4) and non-reducing (1,3) conditions using SDS-PAGE. (B) Affinities of VC variants against collagen I and III using ELISA. (C) Experimental schedule of hLECs stimulation assay. (D) Quantification of p-VEGFR3 in hLECs using ELISA. (E) Overall tumor growth curves and (F) survival curves of tumor-bearing mice (n=6-8). Data are mean ± SD (D) and mean ± SEM (E). Experiments was performed either twice with similar results (A-D) or once on distinct biological samples (E-F). Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test (D), unpaired two-tailed t-test with Welch correction (E) and log-rank (Mantel–Cox) test for survival curves (F). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

In order to assess if the lack of difference in the efficacy of CPI therapy post treatment with A3-VC was due to similar immune landscape in the TME, I performed flow cytometry characterization of tumor immune infiltration after treating E0771 tumor-bearing mice with PBS

or A3-VC. A3-VC treated mice did not show an increase in tumor LECs or BECs, which suggests that the dosing of the treatment needs to be altered (**Fig. 2.18 A**). Characterization of the immune cell subtypes also showed lack of any difference between the two groups, except a trend towards fewer T_{reg} cells in the A3-VC treated tumors compared to control tumors (**Fig. 2.18 A-B**). These results show that inducing tumor lymphangiogenesis using a protein delivery approach requires optimization of dosing schedule, or other approaches such as viral or mRNA delivery need to be pursued [61].

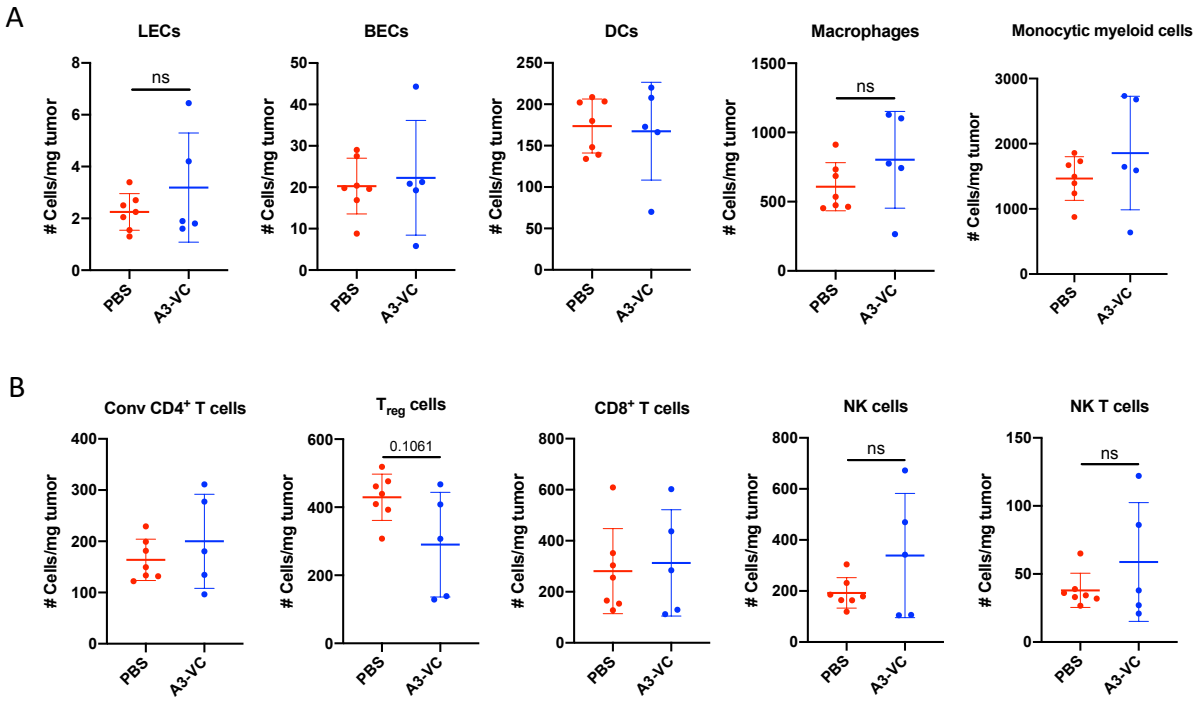

Figure 2.18. A3-VC treatment does not alter the tumor immune infiltrates in E0771 tumor-bearing mice. Wildtype mice were inoculated with E0771 tumor cells and were treated with PBS or A3-VC intratumorally at day 7, 10 and 13, and mice were euthanized at day 20 for analysis (n=5-7). (A) Flow cytometry analysis of LECs, BECs, DCs, macrophages and monocytic myeloid cells. (B) Flow cytometry analysis of conv $CD4^+$ T cells, T_{reg} cells, $CD8^+$ T cells, NK cells ($CD45^+NK1.1^+$) and NK T cells ($CD45^+NK1.1^+CD3^+$). Data are mean ± SD. Flow cytometry analyses were performed once on distinct biological samples. Statistical analyses were performed using unpaired two-tailed t-tests with Welch correction for parametric data or two-tailed Mann-Whitney test for nonparametric data. P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

2.4 Discussion

In this chapter, I studied the role of tumor lymphangiogenesis in modulating the anti-tumor immunity in mammary tumors upon treatment with different cancer immunotherapies. Although the effects of tumor lymphangiogenesis on responsiveness to immunotherapies has been studied in other cancers [59, 61], its role in breast cancer is unexplored. I used various murine breast cancer models for *in vivo* studies, including the 4T1, PyMT and E0771 tumor cells. I demonstrated that upon over-expression of VEGF-C in 4T1 tumors cells, there is an increased VEGF-C expression in the TME accompanied by an expansion in the number of LECs and also BECs due to the lower affinity of VEGFR2 expressed on BECs for VEGF-C (**Fig. 2.3**) [5].

Flow cytometry characterization of intratumoral myeloid cells revealed an increase in intratumoral macrophages with an immune-suppressive profile (lower M1/M2 ratio) in the 4T1-VC tumors. This increase could be mostly driven by recruitment of $VEGFR3^+$ macrophages to the TME [100]. In murine colorectal cancer models, it has been shown that VEGFC signaling promotes M2-associated gene expression in macrophages [101]. Moreover, VEGFR3 signaling in macrophages inhibits TLR4 signaling and reduces production of pro-inflammatory cytokines [102]. This increase of macrophages was also accompanied by an increase in the immune-suppressive cytokine IL-10. Moreover, quantification of intratumoral T cells showed that lymphangiogenic 4T1 tumors had higher levels of conv $CD4^+$ T cells and T_{reg} cells, but similar numbers of $CD8^+$ T cells. This led to a decrease in the ratio of $CD8^+$ T cells/T_{reg} cells in the 4T1-VC tumors (**Fig. 2.4)**. Given that increased TAM infiltration in breast cancer has been associated with reduced OS and more aggressive disease [103, 104] and increased metastasis [105], these results are consistent with previous studies reporting that tumor lymphangiogenesis promotes immune suppression and is a negative prognostic factor in different cancers types [48, 53, 57].

We hypothesized that upon treatment with cancer immunotherapies, the increased tumor infiltration of macrophages and conv $CD4^+$ T cells in the lymphangiogenic 4T1 tumors might render them more responsive compared to non-lymphangiogenic tumors. Indeed, 4T1-VC tumor-bearing mice showed improved efficacy in response to STING agonist and CPI treatment, both in terms of tumor growth rate and survival (**Fig. 2.6**). The higher levels of IFN-β in the TME of lymphangiogenic tumors in response to STING agonist could be due to increased type I IFN expression by not only APCs, but also endothelial cells [106]. Characterization of myeloid cells after STING agonist treatment showed a repolarization of macrophages, as the M1/M2 ratio was no longer higher in the 4T1-VC tumors (**Fig. 2.7**). Suggesting a stronger adaptive immune response, lymphangiogenic 4T1 tumors contained higher levels of DCs, particularly cross-presenting $CD103^+$ DCs. They also contained more conv $CD4^+$ T cells and a trend towards more $CD8^+$ T cells. Similar levels of T_{reg} cells in both tumor types led to higher $CD8^+$ T cells/T_{reg} cells ratio in the 4T1-VC tumors (**Fig. 2.7**). These results are of significance since a higher M1/M2 ratio has been correlated with a higher $CD8^+$ T cells/T_{reg} cells ratio in the TME and better OS in pediatric Hodgkin lymphoma [74] and $CD8^+$ T cells/T_{reg} cells ratio is also a positive prognostic factor in ovarian cancer [107]. Analysis of intratumoral immune infiltrates after CPI treatment revealed that despite similar levels of $CD8^+$ T cells, the percentage of $perforin^+$ $CD8^+$ T cells was higher in the 4T1-VC tumors, which suggests a more potent $CD8^+$ T cell response. The lymphangiogenic tumors also had higher levels of cross-presenting $CD103^+$ DCs (**Fig. 2.8**).

Analysis of the chemokine landscape in the TME revealed an increase in expression of CCL2, CCL3 and CCL5, partially by secreted by macrophages (**Fig. 2.9**) [108]. These chemokines recruit macrophages and monocytic myeloid cells into the tumor [73], which can explain our findings here. However, it should be noted that these chemokines have been shown to have

ambiguous roles in affecting tumor growth and survival, through direct and indirect mechanisms. For instance, CCL2 and CCL5 have been reported to cause tumor cell proliferation and survival and promote invasiveness of cancer cells [109, 110]. CCL2 has also been shown to promote metastasis in breast cancer by recruitment of monocytes and macrophages [75, 111]. Moreover, I observed higher levels of CXCL5 in the 4T1-VC tumors (**Fig. 2.9**), which also has been reported to associate with metastasis in breast cancer and gastric cancer [112, 113]. Altogether, these studies suggest that further characterization is needed to delineate the phenotype and functionality of the increased macrophages we observed in our experiments to confirm their anti-tumorigenic activity.

Furthermore, I observed higher levels of CXCL10 in the lymphangiogenic 4T1 tumors than control tumors after STING agonist treatment (**Fig. 2.9**). It has been shown that macrophages and cross-presenting DCs can produce CXCL10 [114, 115], which drives recruitment of $CD8^+$ T cells and T_H1 cells and correlates with improved survival [86, 116]. CXCL10 can also directly inhibit tumor angiogenesis by directly acting on endothelial cells [117]. Interestingly, CXCL13 expression was lower in the 4T1-VC tumors after immunotherapy (**Fig. 2.9**). Previous studies have shown that inhibiting CXCL13 reduces the growth rate of 4T1 tumors [118], which provides another insight into the mechanism of action of lymphangiogenic potentiation.

$CD4^+$ T cells play a critical role in the cancer-immunity cycle by inhibiting tumor growth through various ways [119]. It has been shown that T_H1 cytokines can induce cancer cell senescence and mediate direct cytolytic activity against tumor cells by producing IFNγ and TNFα [119-121]. Critically, $CD4^+$ T cells provide help for $CD8^+$ T cells by engaging DCs in an antigen-specific manner and promote their activation (expression of co-stimulatory molecules and IL-12) [122], resulting in a potent CTL response [123, 124]. Based on the increased levels of conv $CD4^+$ T cells in the 4T1-VC tumors, we hypothesized they may play a role in mediating the

lymphangiogenic potentiation. Strong trends towards higher levels of IFNγ and TNFα in the 4T1-VC tumors after STING treatment and the elevated levels of IL-4 in these tumors in the absence of therapy suggested a T_H2 to T_H1 shift in $CD4^+$ T cell phenotype upon treatment (**Fig. 2.10**). Furthermore, the observed increased IL-6 expression in the lymphangiogenic tumors could drive the differentiation of $CD4^+$ T cells into T_H17 cells, which have been shown to mediate direct cytotoxicity in melanoma [125]. However, future work is required to further characterize the transcriptomic landscape in the $CD4^+$ T cell compartment and assess their phenotype using functional assays.

Since tumor lymphangiogenesis has been associated with metastasis in breast cancer [48], I investigated if the lymphangiogenic potentiation was able to overcome distant tumor metastasis. Strikingly, upon treatment with a systemic immunotherapy, lymphangiogenic 4T1 tumor-bearing mice showed prolonged survival and had significantly fewer metastatic nodules in their lungs than control-treated mice (**Fig. 2.11)**. Notably, there was a strong trend towards fewer neutrophils in the lungs of 4T1-VC tumor-bearing mice treated with STING agonist and CPI than control mice (**Fig. 2.12)**. This is consistent with prior studies in murine mammary tumor models that showed bone-marrow derived neutrophils promoted lung metastasis [126, 127].

Depletion of macrophages and $CD4^+$ T cells abrogated the benefit of tumor lymphangiogenesis in response to STING agonist (**Fig. 2.13)**. Strikingly, in the mice with control tumors, CD4 depletion improved survival and almost significantly reduced tumor growth rate when combined with STING treatment. This could be due to the depletion of T_{reg} cells, although further characterization is required to confirm this hypothesis, possibly by depletion of T_{reg} cells using αCD25 antibodies. It should be noted that macrophage depletion also reduced survival and increased tumor growth in the control tumor-bearing mice, although not to the same extent as mice

with the lymphangiogenic tumors. The underlying cause may be the mechanism of action of STING agonist, which is increasing IFN-β expression through activating DCs and macrophages [67].

Despite the benefit of tumor lymphangiogenesis that I observed in the 4T1 triple-negative mammary tumors, this effect was not recapitulated in the $HER2^+$ PyMT tumors (**Fig. 2.15**). This may be due to the higher immunogenicity in the triple-negative tumors, compared to the other molecular subtypes [14]. However, in the immunogenic triple-negative E0771 breast tumors, VC over-expression led to the spontaneous eradication of the tumors in the absence of treatment (data not shown). This phenomenon was dependent on mature T and B cells, as the effect was abrogated in Rag1 KO mice (**Fig. 2.16**). Thus, I hypothesize that the role of tumor lymphangiogenesis and VEFG-C signaling in enhancing responsiveness to immunotherapies in breast cancer, by recruitment of macrophages and T cells, is dependent on the condition of TME and its immunogenic/immunosuppressive status prior to treatment.

As an alternative to over-expression of VEGF-C for induction of tumor lymphangiogenesis, I employed protein delivery using a collagen-binding variant of VEGF-C I produced in-house, called A3-VC (**Fig. 2.17**). Despite the bioactivity of A3-VC and its affinity of collagen, *in vivo* delivery failed to induce a detectable increase in the number of LECs in TME, or any significant changes in the intratumoral immune infiltration (**Fig. 2.18**). Other than the possibility of non-optimized dosing schedule, the cause may be the lower efficacy of protein delivery compared to other delivery methods, such as viral or mRNA delivery, in inducing tumor lymphangiogenesis [61].

In summary, tumor lymphangiogenesis can enhance the responsiveness of triple-negative mammary tumors to cancer immunotherapies through the recruitment of macrophages and $CD4^+$

T cells. Importantly, when treated with a potent immunotherapy, mice with lymphangiogenic tumors had a stronger systemic anti-tumor immunity which prolonged their survival despite increased metastasis associated with tumor lymphangiogenesis.

2.5 Appendix

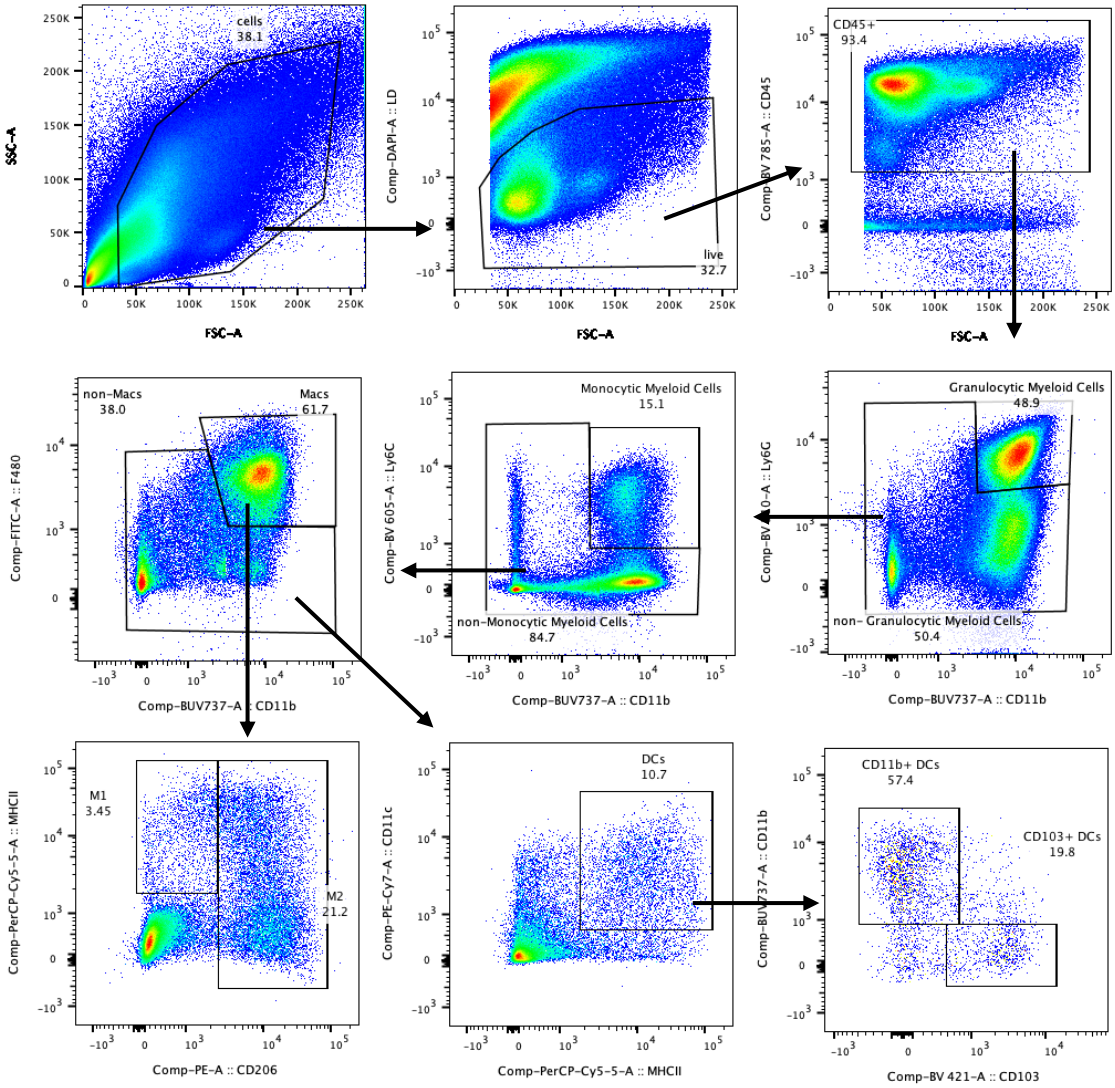

Figure 2.S1. Representative gating strategy for identifying myeloid cells in the 4T1 tumor microenvironment.

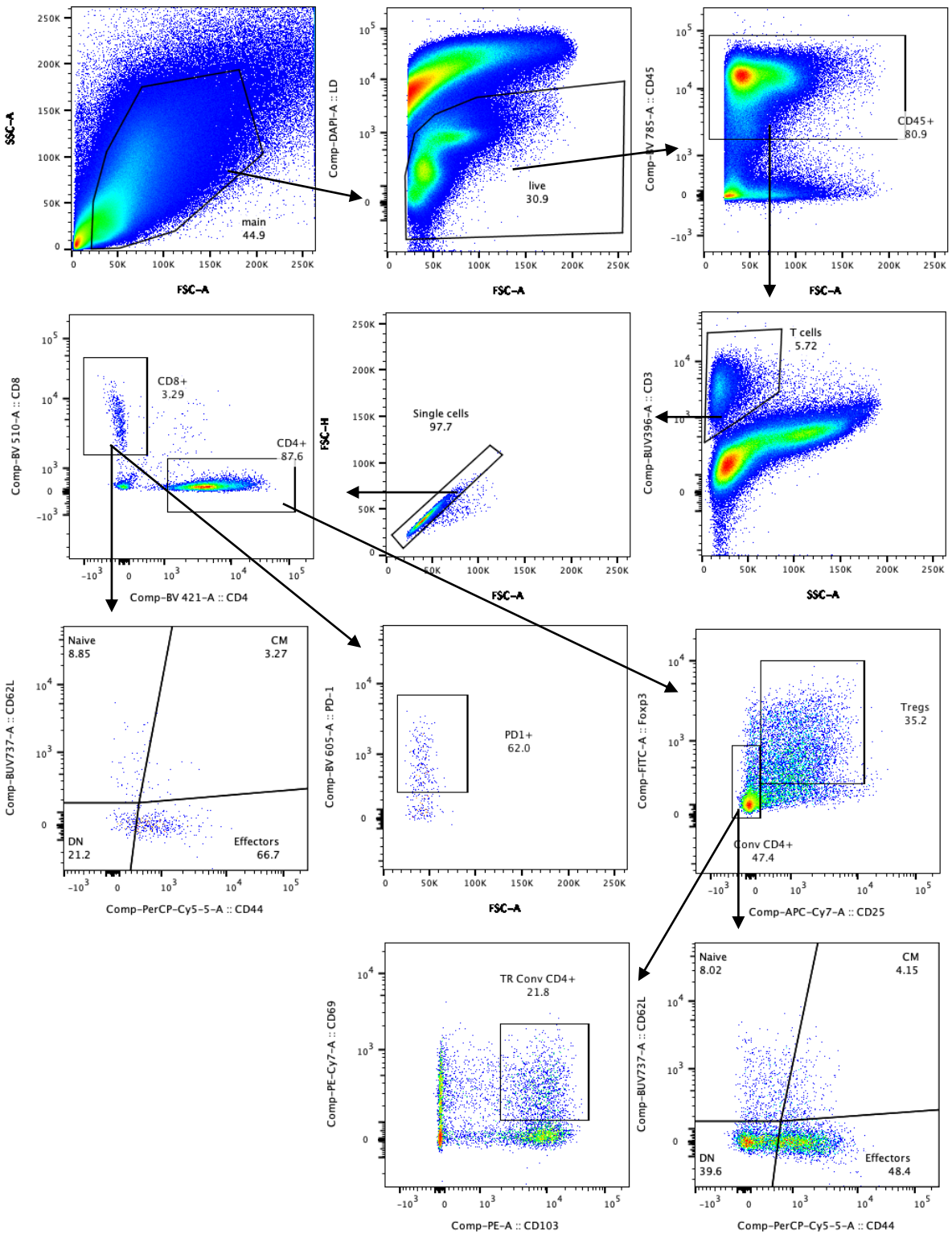

Figure 2.S2. Representative gating strategy for identifying T cells in the 4T1 tumor microenvironment.

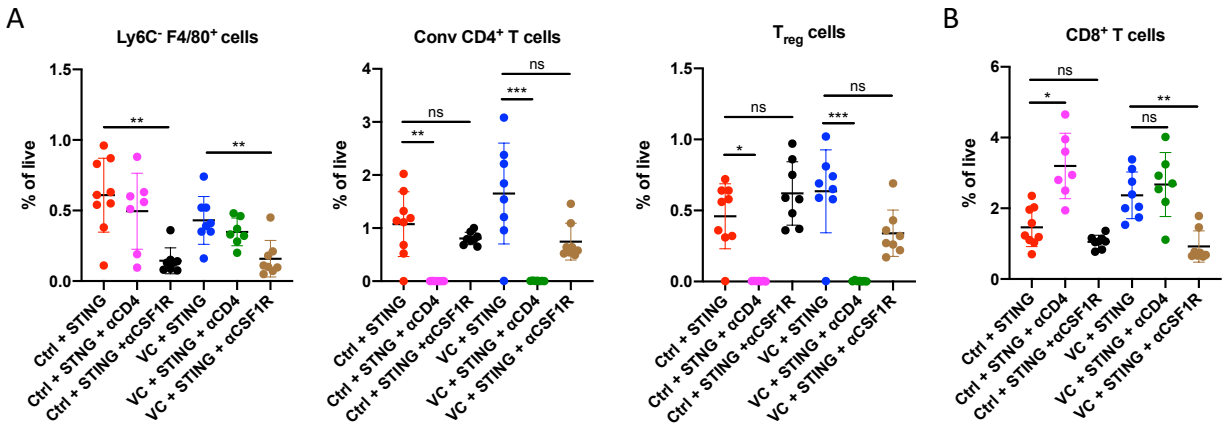

Figure 2.S3. Assessment of circulating immune cells in the blood of 4T1 tumor-bearing mice after administration of depleting antibodies. Lymphangiogenic and control 4T1 tumor-bearing mice (n=7-9) were administered depletion antibodies (αCD4 and αCSF1R) from the day of tumor inoculation and were treated with 2 doses of STING agonist once tumors were established. Mice were bled at day 14 after tumor inoculation and percentage of immune cells in the blood was assessed by flow cytometry. Percentage of (A) Ly6C$^-$F4/80$^+$ cells, (B) conv CD4$^+$ T cells, (C) T_{reg} cells and (D) CD8$^+$ T cells amongst live cells. Data are mean ± SD. The experiment was done once on distinct biological samples. Statistical analyses were performed using Kruskal–Wallis test followed by Dunn's multiple comparison test. P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

Sequence of Human VWF A3 and mouse VEGF-C fusion protein	CSQPLDVILLLDGSSSFPASYFDEMKSFAKAFISKANIGPRLTQV SVLQYGSITTIDVPWNVVPEKAHLLSLVDVMQREGGPSQIGDA LGFAVRYLTSEMHGARPGASKAVVILVTDVSVDSVDAAADAA RSNRVTVFPIGIGDRYDAAQLRILAGPAGDSNVVKLQRIEDLPT MVTLGNSFLHKLCSGFVRIGGGGSGGGGSAHYNTEILKSIDNE WRKTQCMPREVCIDVGKEFGAATNTFFKPPCVSVYRCGGCCNS EGLQCMNTSTGYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMS KLDVYRQVHSIIRRHHHHHH

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CHAPTER 3: THE RELATION BETWEEN TUMOR LYMPHANGIOGENESIS AND IMMUNE GENE SIGNATURES IN HUMAN BREAST CANCER

3.1 Abstract

Tumor-associated lymphatics and lymphangiogenesis have long been correlated with metastasis and poor patient survival in various cancers. Their role in shaping the anti-tumor immunity, particularly in the context of cancer immunotherapies had been underappreciated until recently. Our group has shown that melanoma patients with higher levels of VEGF-C in their serum had improved response to checkpoint inhibitors. In this chapter, I characterized the relation between VEGF-C expression, together with other lymphatic markers, with the expression of various immune cells in human breast cancer patients using The Cancer Genome Atlas (TCGA) database. This analysis was performed for each molecular subtype to reveal the differences between each group. Moreover, I identified the genes whose expression was altered the most in relation with VEGF-C expression in the tumors.

3.2 Introduction

3.2.1 Biomarkers for patient stratification in breast cancer

Similar to other cancer types, identification of biomarkers for stratification of breast cancer patients for various immunotherapies has been an ongoing effort [1]. In the case of checkpoint inhibitors, which target the PD-1/PD-L1 pathway, the expression of PD-L1 on tumor cells or immune cells has been investigated as a prognostic biomarker for responsiveness to checkpoint inhibitors across different cancer types [2]. For instance, atezolizumab (in combination with paclitaxel) has been approved for untreated metastatic TNBC patients with PD-L1 positive status based on the expression on immune cells [3]. However, only 20% of breast cancers express detectable PD-L1, with the expression levels being higher in TNBC and $HER2^+$ breast cancer (33% and 11%, respectively) [4]. Moreover, the use of PD-L1 expression as a biomarker for immunotherapy response in breast cancer has been controversial [4]. In a phase III randomized trial of previously-treated metastatic TNBC patients, single-agent pembrolizumab did not improve median overall survival compared to chemotherapy, regardless of PD-L1 status [5]. However, in another randomized phase III trial where the efficacy of pembrolizumab in combination with chemotherapy was evaluated against placebo and chemotherapy in untreated TNBC, in patients with a high PD-L1 score (on tumor cells and immune cells combined) the pembrolizumab-chemotherapy combination significantly improved PFS as compared to placebo-chemotherapy [6]. Furthermore, uncertainty over positivity cut-off rates, existence of multiple assays (using different antibodies) for the scoring of PD-L1, its varying expression over time and the response of some PD-L1 negative patients to checkpoint inhibitors has prevented its clinical adoption as a broad predictive biomarker [7].

TILs have been explored to be employed as biomarkers for response to immunotherapies, particularly since they are a major indication of pre-existing adaptive immunity against tumors cells [8]. Overall, higher $CD8^+$ T cells are associated with better prognosis in TNBC and $HER2^+$ cancer [9]. In a phase I study evaluating the safety of atezolizumab, TILs and $CD8^+$ cells at the baseline were associated with higher response rates [10]. Moreover, in a phase II trial, metastatic TNBC patients with higher TILs had improved ORRs to pembrolizumab, with PD-L1 expression correlating with TIL levels [11]. Interestingly, serum lactate dehydrogenase (LDH) was a predictor for worse response to pembrolizumab in this study [11]. Despite these studies demonstrating promising results on the use of TILs as a predictive biomarker, it still has not been FDA approved as a patient stratification tool in breast cancer.

3.2.2 VEGF-C as a biomarker in cancer

Tumor-associated lymphatics and tumor lymphangiogenesis have been associated with metastasis and poor patient prognosis in multiple cancer types [12, 13]. As the main growth factor driving lymphangiogenesis, VEGF-C can be used as a proxy for quantifying tumor-associated lymphatics as it has been shown to correlate with intratumoral lymphatics [14, 15]. Moreover, our group has demonstrated that dermal lymphatics are required for the initiation of anti-tumor immune responses against cancer cells in melanoma [16] and that tumor lymphangiogenesis improves responsiveness to immunotherapies in murine melanoma models [17]. Importantly, in human melanoma, intratumoral VEGF-C expression correlated with T cell gene signatures and pre-treatment serum VEGF-C levels positively correlated with responsiveness to checkpoint inhibitors in a clinical trial [17]. These findings suggest that intratumoral and serum VEGF-C levels can be candidates for predicting responsiveness to immunotherapies.

Given that a considerable portion of breast cancer patients still do not respond to cancer immunotherapies, for instance more than 40% non-responders to combination of chemotherapy and atezolizumab in TNBC patients [3], there is a critical need for identification of a predictive biomarker to select patients who would benefit the most from immunotherapy treatment. In order to assess the potential use of tumor-associated lymphatics as a predictive biomarker, I used the TCGA dataset to characterize the relation between lymphatic markers (including VEGF-C) and immune gene signatures in different molecular subtypes of breast cancer. This analysis is important since the pre-existing anti-tumor immunity and TILs have been shown to associate with responsiveness to immunotherapies in various cancer types [18, 19].

3.3 Materials and methods

Normalized RSEM data generated by mRNA sequencing of human tumor samples by the TCGA Research Network were downloaded from http://firebrowse.org. The data were analyzed using Microsoft Excel and RStudio. After extracting the data from primary tumors only, Pearson's r correlations between VEGF-C/VEGF-D and selected genes were computed using logarithm of RSEM values in base 2. For analysis of macrophages, a breast cancer TAM gene signature was used that employs 37 macrophage-associated genes described previously [20].

Principal component analysis was performed on patients from each molecular subtype using the R function prcomp with zero-centered and unit-scaled RSEM gene variables. In all analyses, the patient quartile with highest VEGF-C expression was defined as VEGF-C^{high}, the patient quartile with lowest VEGF-C expression was defined as VEGF-C^{low}, and the remaining patients were defined as VEGF-C^{mid}. For volcano plots, fold increases in gene expression were defined as the average gene expression in VEGF-C^{high} patients over the average gene expression in VEGF-C^{low} patients. Expression of VEGFC, PDPN and LYVE1 were normalized among the patients (such that the average expression of each gene among the patients is zero and the standard deviation is one) and the lymphatic score was defined as the sum of normalized values for each patient. for P-values were computed using two-tailed, unpaired, equal variance t-tests, unless otherwise stated.

3.4 Results

3.4.1 VEGF-C expression level in human TNBC tumors leads to distinctive gene expression profile among patients

We assessed the relevance of our findings in mice in the previous chapter to humans by analyzing the mRNA sequencing data from The Cancer Genome Atlas (TCGA) database to characterize the gene expression in the primary tumors of breast cancer patients. First, we focused on TNBC patients since we had observed the lymphangiogenic potentiation benefit in the triple-negative 4T1 tumors. After confirming the Gaussian distribution of VEGF-C expression in the tumors (**Fig. 3.1A**), we stratified the patients based on their VEGF-C expression levels into VEGF-C^{high}, VEGF-C^{mid} and VEGF-C^{low} subsets (**Fig. 3.1B**). Next, we looked at the fold-changes of gene expressions comparing the VEGF-C^{high} and VEGF-C^{low} groups. Among the genes most up-regulated were lymphatic-associated genes, including CCL21 and fatty acid binding protein 4 (FABP4) [21, 22] (**Fig. 3.1C**). Notably, FABP4, which is critical in glucose regulation and lipid hemostasis, has been shown to be a prognostic factor in breast cancer patients [23]. Moreover, complement component 7 (C7), expression of which is associated with better clinical outcome in different cancers [24], was also among the most up-regulated genes in the VEGF-C^{high} subset (**Fig. 3.1C**). Importantly, CCL14 which in hepatocellular carcinoma has been shown to act a tumor suppressor by inhibition of Wnt/β-catenin signaling [25] and correlate with B cells and T cells infiltration and a positive clinical outcome [26], was also highly expressed in the VEGF-C^{high} population (**Fig. 3.1C)**.

Amongst the genes that were most down-regulated in the VEGF-C^{high} group, we observed mitogen-activated protein kinase 4 (MAPK4), an enzyme whose over-expression has been associated with poor patient survival in multiple cancers due to the activation of AKT/mTOR

signaling pathway [27]. Tumor-associated antigen preferentially expressed in melanoma (PRAME) was also down-regulated in VEGF-C^{high} group (**Fig. 3.1C**). It has been demonstrated that PRAME-induced epithelial-to-mesenchymal transition in triple-negative breast cancer cells and its expression is correlated with reduced survival in TNBC patients [28]. Moreover, microtubule-associated serine/threonine kinase 1 (MAST1) and GDNF family-receptor alpha-3 (GFRα3) were also among the most down-regulated genes when comparing VEGFC^{high} to VEGFC^{low} patients (**Fig. 3.1C**). MAST1 has been shown to cause cisplatin-resistance in various cancers [29] and GFRα3 expression has been associated with lymph node metastasis and poor patient outcome in mammary carcinomas [30]. Finally, principal component analysis of the TNBC patients revealed distinct genetic profiles between the patients based on the VEGFC expression in the tumors (**Fig. 3.1D**). These results demonstrate that intratumoral VEGFC levels in TNBC patients correlate with expression of various genes critical in clinical outcome in breast cancer and lead to a distinct gene signature among the patients.

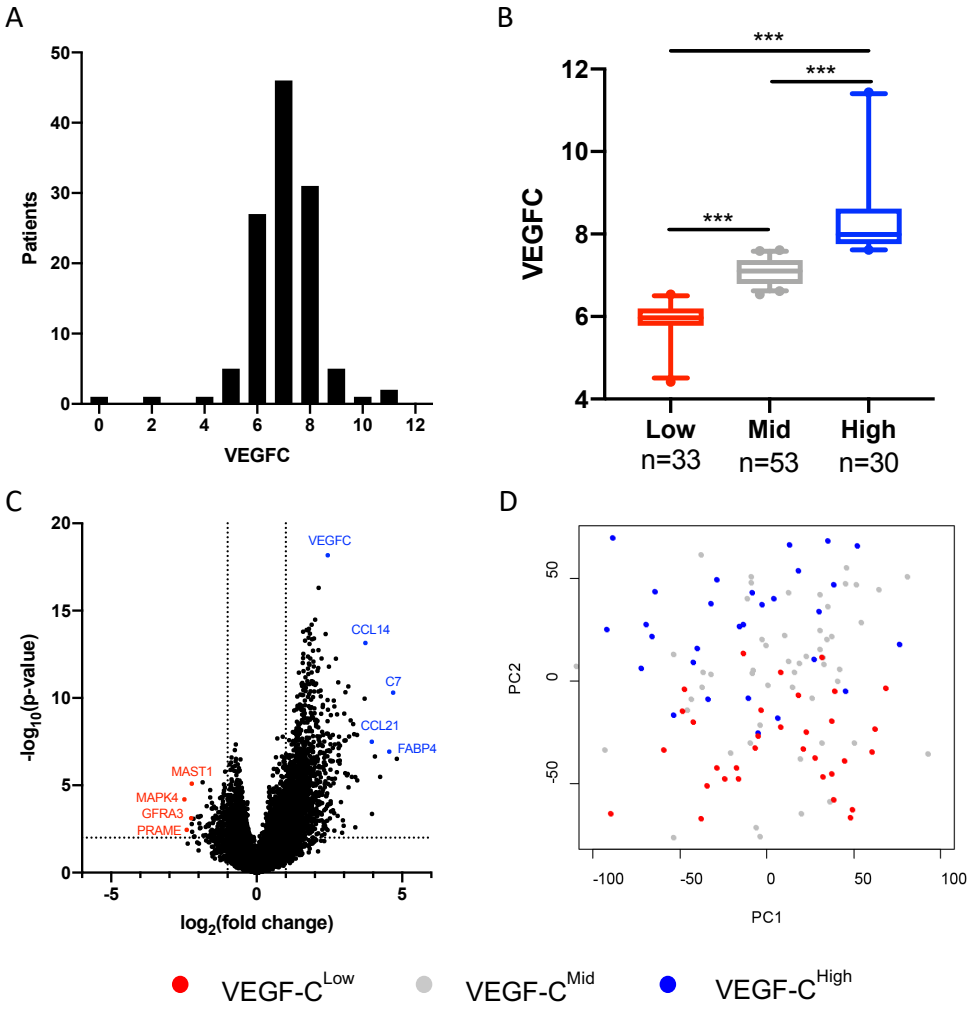
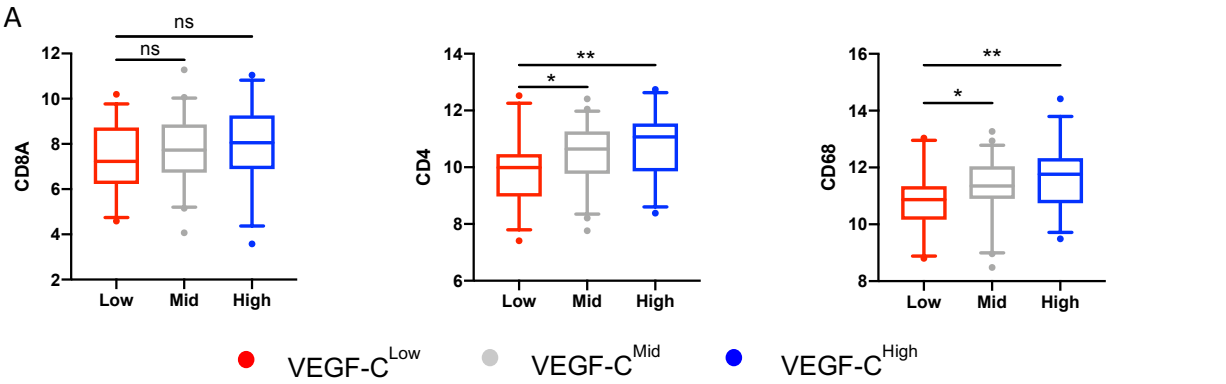


Figure 3.1. Principal component analysis of human TNBC tumors based on VEGF-C expression reveals a distinct gene signature. Human TNBC tumor mRNA expression data was extracted from TCGA dataset. (A) Distribution of patients based on VEGF-C expression levels (n=116). (B) Volcano plot of gene expression fold-changes based on VEGF-C^{high} over VEGF-C^{low} tumors. (C) VEGF-C expression levels in three patient subsets of VEGF-C^{high}, VEGF-C^{mid} and VEGF-C^{low}. (D) Principal component analysis of the three patient subsets. Genes labeled in blue are up-regulated and genes labeled in red are down-regulated (B). Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test (C). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

3.4.2 Expression of CD4 and macrophage-associated genes positively correlate with lymphatic score in TNBC tumors

Based on my results in murine triple-negative mammary tumors, we looked at the expression of CD4, CD8A and CD68 genes in the TNBC tumors based on VEGF-C expression levels. Consistent with my results in the 4T1 murine studies, there was a significant increase in CD4 and CD68 expression in VEGF-C^{mid} and VEGF-C^{high} tumors, whereas expression of CD8A was not different among the three groups (**Fig. 3.2A**). Next, we calculated a lymphatic score to better define the relation between gene signatures and intratumoral lymphatics. For this purpose, we normalized and summed the expression levels of three lymphatic associated genes, namely VEGFC, PDPN and LYVE1, as described previously [31]. As we hypothesized, the lymphatic score positively correlated with CD4 and CD68 gene expression, yet there was no correlation with CD8A expression (**Fig. 3.2B**). Notably, there was also a strong correlation between lymphatic score and macrophage markers MRC1 (CD206), CSF1R and a tumor-associated macrophage (TAM) score composed of 37 genes identified in breast cancer (**Fig. 3.2C**) [20]. Interestingly, we did not observe any significant correlation between the lymphangiogenic growth factor VEGF-D and CD68 expression and it showed only a mild correlation with CD4 gene expression ($p = 0.0423$) (**Fig. 3.2D**). These results demonstrate that similarly to our studies in mice, intratumoral VEGF-C levels in triple-negative mammary tumors correlate with CD4 and macrophage gene expression.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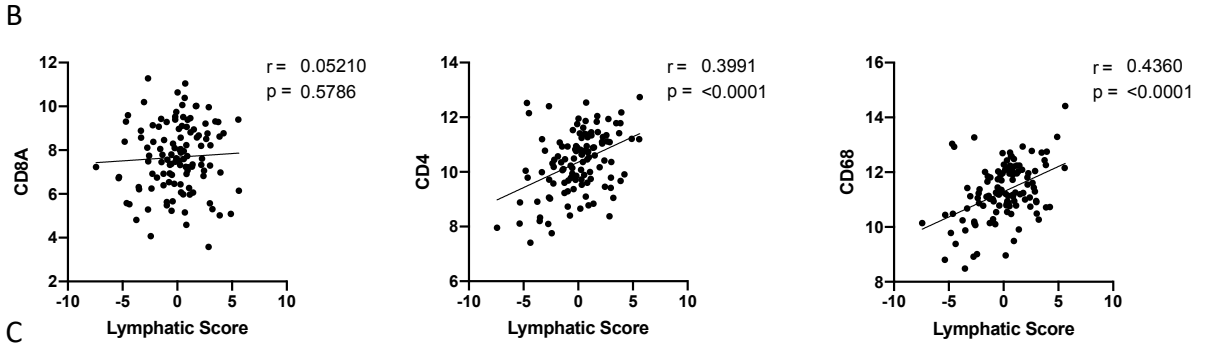
B
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p = 0.5786
CD8A
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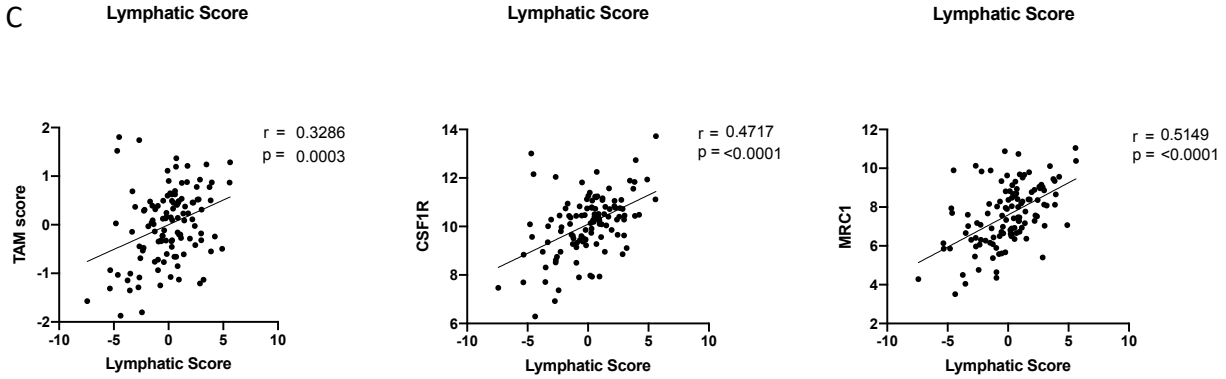
r = 0.3991
p = <0.0001
CD4
Lymphatic Score
r = 0.4360
p = <0.0001
CD68
Lymphatic Score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p = 0.0003
TAM score
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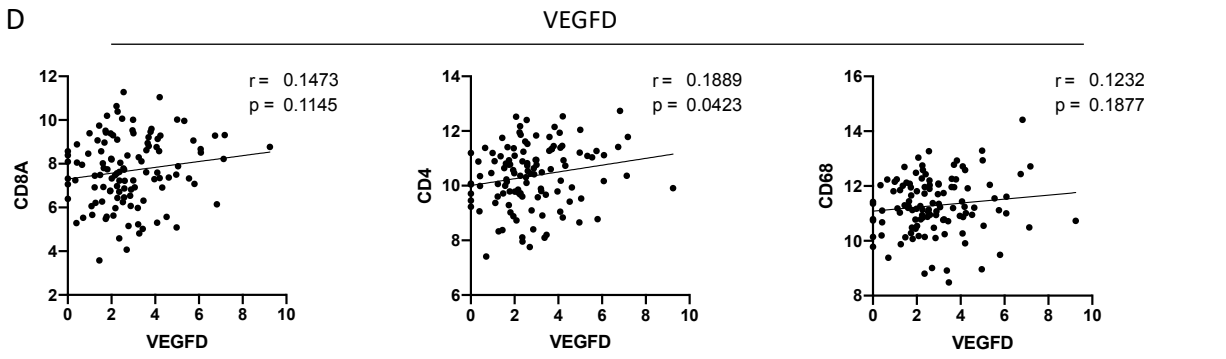
CSF1R
Lymphatic Score
r = 0.5149
p = <0.0001
MRC1
Lymphatic Score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VEGFD
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p = 0.1145
CD8A
VEGFD
r = 0.1889
p = 0.0423
CD4
VEGFD
r = 0.1232
p = 0.1877
CD68
VEGFD

Figure 3.2. Expression of VEGF-C positively correlates with CD4 and macrophage gene expression in human TNBC tumors. (A) Gene expression of CD8A, CD4 and CD68 amongst patient subsets based on VEGF-C expression levels. (B) Correlations of lymphatic score (composed of LYVE1, PDPN, VEGFC expression) with CD8A, CD4 and CD68 gene expression. (C) Correlations of lymphatic score with TAM score (37 genes), MRC1 and CSF1R gene expression. (D) Correlations of VEGF-D expression with CD8A, CD4 and CD68 gene expression. Dot plots are shown with linear regression correlations using Pearson's r test. Statistical analyses were performed using ordinary one-way ANOVA followed by Tukey's multiple comparison test (A). P values are reported as: *P < 0.05, **P <0.01, ***P < 0.001.

Next, we looked at the expression of lymphangiogenic growth factor VEGF-C amongst breast cancer molecular subtypes other than TNBC. Notably, the intratumoral expression of VEGF-C was lower in triple-negative tumors compared to the other molecular subtypes (data not shown). In the ER/PR^{+}HER2^{+} subtype, VEGF-C positively correlated with CD4 and CD68 gene expressions only, similarly to TNBC tumors (**Fig. 3.3A**). Among the ER/PR^{+}HER2^{+} tumors, VEGF-C positively correlated with all three genes (**Fig. 3.3B).** However, in the ER/PR^{-}HER2^{+} tumors, there was no significant correlation with any of CD4, CD8A or CD68 genes (**Fig. 3.3C**)**.** This could be due to the smaller patient pool in this subtype. Among these three molecular subtypes, only the triple-positive subtype exhibited the same correlations as the TNBC tumors.

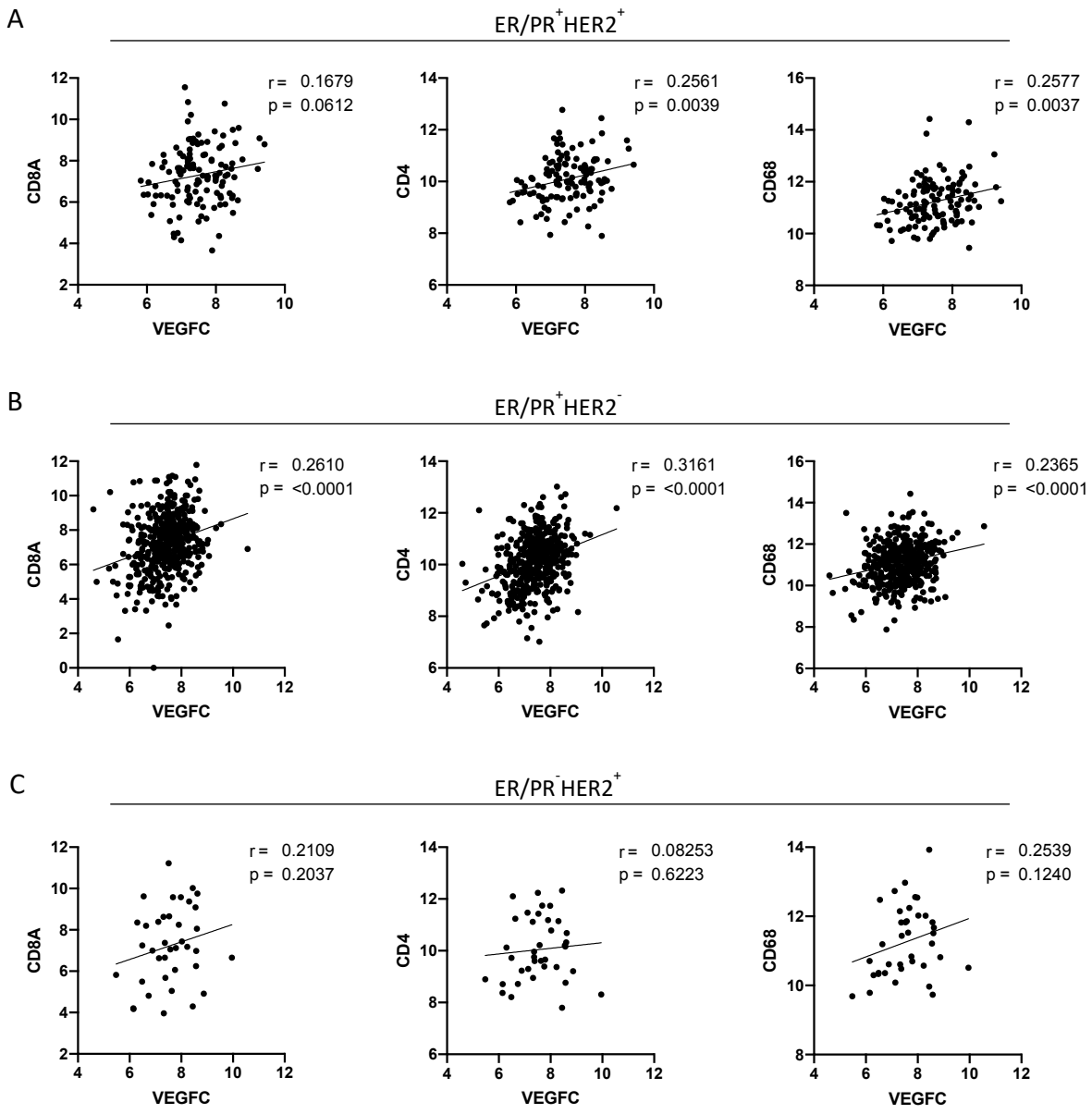

Figure 3.3. Correlations of intratumoral VEGF-C expression with the expression of T cell and macrophage genes amongst different breast cancer molecular subtypes. Correlations of intratumoral VEGF-C expression with the expression of CD8A, CD4 and CD68 in (A) ER/PR^{+}HER2^{+} (n=125), (B) ER/PR^{+}HER2^{-} (n=435) and (C) ER/PR^{-}HER2^{+} (n=38) patients. Dot plots are shown with linear regression correlations using Pearson's r test.

3.5 Discussion

There are currently various biomarkers established as predictive factors for responsiveness to immunotherapies in other malignancies that could be of value in breast cancer patients. Gene signatures associated with T cells and IFNγ positively correlated with better outcome in response to checkpoint inhibitors in melanoma and non-small cell lung cancer (NSCLC) patients [32, 33]. In addition, T cell receptor (TCR) diversity in the peripheral blood of melanoma patients at baseline was associated with improved clinical outcome to ipilimumab (αCTLA-4) therapy. Even though $HER2^+$ and TNBC tumors have higher mutational burdens compared to the other molecular subtypes [34], further research and clinical data are required to investigate if these putative biomarkers would be predictive of response to immunotherapy in breast cancer. Moreover, biomarkers such as microsatellite instability and tumor mutational burden are predictive markers in other malignancies [35, 36], but are not feasible candidates for assessing responsiveness to immunotherapies in breast cancer due to their infrequent occurrence [37], except in BRCA-deficient breast tumors [38].

Due to the non-invasive and cost-effective nature of liquid biopsies, such as circulating proteins, exosomes or tumor cells, there has been a significant effort in identifying liquid biopsy biomarkers for responsiveness to immunotherapies [39]. In a small study attempting to assess the use of PD-L1 expression on circulating immune cells as a predictive biomarker, it was shown that in NSCLC patients with PD-L1 negative tumors, the percentage of circulating PD-L1 myeloid cells was predictive of objective response to checkpoint inhibition [40]. The predictive role of PD-L1 expression on circulating immune cells in response to immunotherapies has not been investigated in breast cancer patients though.

Circulating tumor cells (CTCs) have been shown to have prognostic value in breast cancer [41], and are also being explored as predictive biomarkers for responsiveness to immunotherapies [1]. It was demonstrated that CTCs in breast cancer patients express PD-L1 [42] and their frequency is higher in metastatic patients [43]. These results show promise for use of CTCs as a predictive biomarker for immunotherapy response, however the correct challenges with reproducibility of CTC isolation techniques need to be addressed first before any potential clinical adoption. There has also been significant interest in the use of circulating tumor DNA (ctDNA) in predicting response to immunotherapies. A recent study showed that ctDNA was associated with PFS and OS when treating multiple solid tumors with pembrolizumab [44]. The use of ctDNA as an immunotherapy predictive biomarker in breast cancer remains unexplored.

On the promise of employing exosomes as biomarkers, PD-L1 expression on circulating exosomes was predictive of poor clinical outcome to anti-PD-1 therapy in melanoma patients [45]. Moreover, in another group of NSCLC patients treated with anit-PD-1, higher expression of a set of microRNAs was correlated with improved PFS and OS [46]. Despite the evidence that exosomal biomarkers (including microRNAs) could provide prognostic value in breast cancer [47, 48], there have not been large clinical trials assessing their predictive role in the context of predicting responsiveness to immunotherapies. Thus, identification of biomarkers for selection of breast cancer patients who would benefit from cancer immunotherapy is still a critical unmet need.

In this chapter, we showed that in human TNBC tumors expression of VEGF-C and the lymphatic score positively correlate with immune gene signatures of CD4 T cells and macrophages, which were constituent which I observed in the previous chapter using murine triple-negative mammary tumors. Moreover, principal component analysis of the patients based on VEGF-C expression levels showed a distinct genetic signature. These results suggest that tumor

lymphangiogenesis correlates with immune infiltrates, which might render the patients more responsive to appropriate cancer immunotherapies. As there is minimal lymphangiogenesis in healthy people [49],

It should be noted though that the TCGA dataset is obtained from patients who were either untreated or had undergone some form of clinical treatment (e.g. radiation). Moreover, it is unclear if the patients with higher VEGF-C expression levels would have indeed responded more favorably to immunotherapies. However, in a neoadjuvant phase III trial (KEYNOTE-522) in patients with early triple-negative breast cancer (stage II/III), who were treated with either a combination of pembrolizumab (aPD-1) plus chemotherapy or chemotherapy alone, the interim results showed that those with a node-positive status responded more favorably to the combination therapy than those with a negative nodal status (20.6% versus 6.3% in difference in pathological complete response) [50]. Although tumor lymphatics (or lymphangiogenesis) is not quantified in this study, the fact that tumor lymphangiogenesis correlates with increased metastasis and a positive nodal status [12, 51] suggests that patients with more tumor lymphangiogenesis could have a higher response rate to the combination therapy than those with less tumor lymphangiogenesis. Further studies are required to assess the relation between tumor lymphangiogenesis and clinical outcome to immunotherapies and investigate if serum VEGF-C levels can be used a predictive biomarker, similarly to melanoma [17].

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CHAPTER 4: DISCUSSION

4.1 Summary and impact

The role of tumor-associated lymphatics in promoting metastasis and immune-suppression in various cancer types has been known for a long time [1-3]. However, how tumor lymphangiogenesis affects anti-tumor immunity in the context of cancer immunotherapies was not studied until recently. It was demonstrated by our group and others that through promoting recruitment of T cells into the TME, tumor lymphangiogenesis increases responsiveness to immunotherapies, both in melanoma and glioblastoma [4, 5]. How tumor lymphangiogenesis modulates the response to cancer immunotherapies in breast cancer patients though, remained unexplored.

In chapter 2, I showed that induction of tumor lymphangiogenesis in triple-negative mammary tumors leads to increased numbers of immune-suppressive macrophages, together with higher levels of conv $CD4^+$ T cells and T_{reg} cells in the TME. However, I demonstrated that upon treatment with different immunotherapies, this immunosuppressive TME is transformed into a more immunogenic state, compared to non-lymphangiogenic tumors, and leads to improved responsiveness to the treatment. This is the first study showing that tumor lymphangiogenesis can be beneficial in promoting the responsiveness of murine TNBC tumors to cancer immunotherapies.

I also unraveled a new mechanism of how increased levels of tumor-associated lymphatics can affect anti-tumor immunity by showing the dependence of lymphangiogenic potentiation in TNBC tumors on $CD4^+$ T cells and macrophages. Previous studies had shown the importance of $CD8^+$ T cells in mediating lymphangiogenic potentiation [4, 6], but the role of $CD4^+$ T cells and macrophages and the underlying cytokine milieu in the TME affecting the M1/M2 and T_H1/T_H2 differentiation was underappreciated. This new mechanism further deepens our understanding of how tumor lymphangiogenesis, depending on the TME and cancer type, alters the immune

infiltrates together with the cytokine/chemokine profile and promotes responsiveness to immunotherapies.

Furthermore, I discovered that when lymphangiogenic tumor-bearing mice are treated with a potent immunotherapy, the induced systemic anti-tumor immunity overcomes distant organ metastasis. This finding can have implications in translational application of this study, as tumor lymphangiogenesis has been associated with increased metastasis [7, 8]. Even though lymphangiogenic tumors are more prone to metastasis, my findings suggest that the benefit of lymphangiogenic potentiation and the increased systemic immune response could outweigh increased trafficking of tumor cells to distant organs (**Fig. 4.1**).

The analysis in the third chapter of this thesis demonstrates that in human TNBC tumors expression of VEGF-C and lymphatic-associated genes positively correlate with $CD4^+$ T cell and macrophage-associated gene expression. These findings are consistent with my results in murine triple-negative mammary tumors, suggesting that human lymphangiogenic tumors might be more responsive to immunotherapies due to increased intratumoral immune infiltrates.

Despite the recent success of immunotherapies across many cancers, they have had limited efficacy in less immunogenic malignancies, such as breast cancer [9, 10]. Moreover, the majority of these patients do not respond to treatment [11], thus emphasizing the critical need to identify predictive biomarkers to select breast cancer patients mostly likely to benefit from cancer immunotherapies [12]. This study proposes that tumor lymphangiogenesis could be explored as a putative predictive biomarker for responsiveness to immunotherapies in TNBC patients.

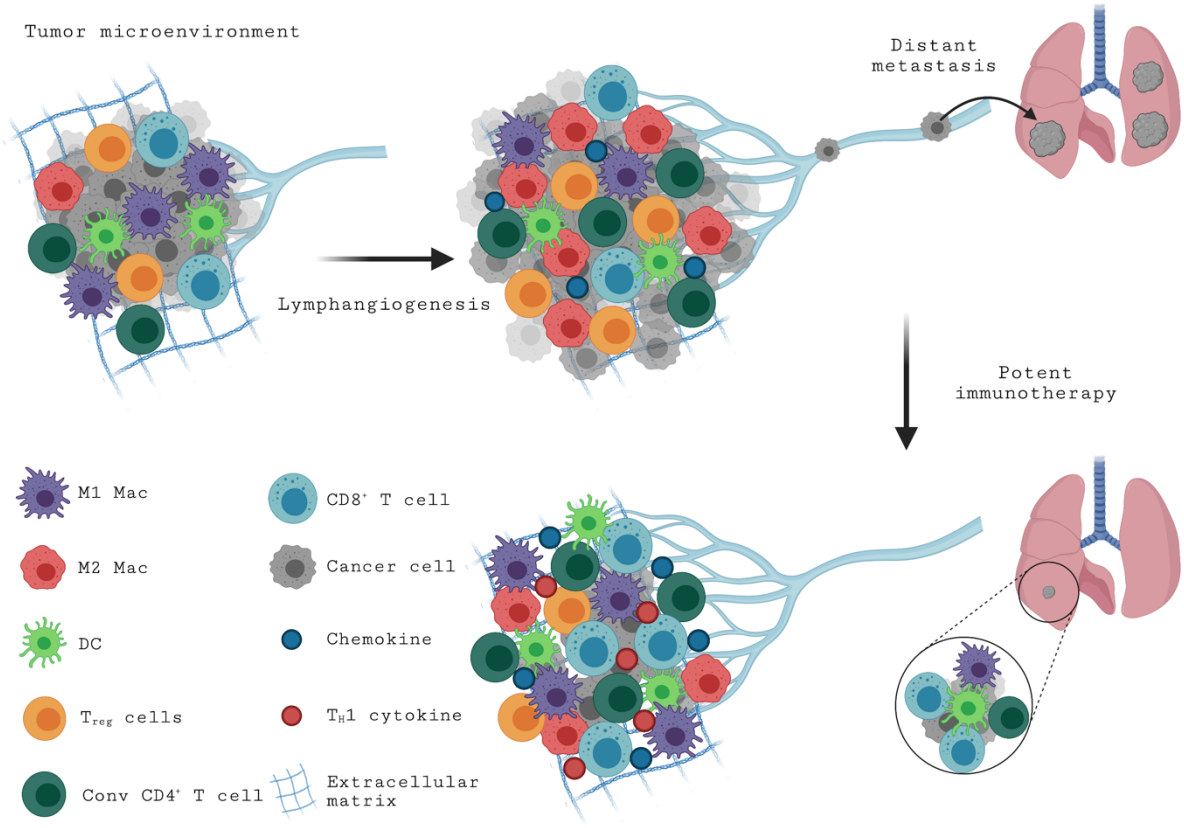

Figure 4.1. The effects of lymphangiogenesis on the immune infiltration in the tumor microenvironment and reduction of distant organ metastasis upon immunotherapy treatment. Tumor lymphangiogenesis leads to infiltration of immune-suppressive cells into the TME and increased metastasis. However, upon treatment with immunotherapies, the systemic anti-tumor immunity reduces both the primary tumor growth and the metastatic burden. Created with BioRender.com

4.2 Future directions

Most of the *in vivo* data in this dissertation was generated using the spontaneous metastatic triple-negative 4T1 mammary tumors in an orthotopic setting. Despite the relevance and widespread use of this model for studying TNBC in immuno-competent mice, our results need to be replicated in other murine tumor models. I showed that in other murine TNBC models, depending on the immunogenic state of the TME, the role of tumor lymphangiogenesis could be different than what we observed in our 4T1 studies. Moreover, our mechanistic studies showing the dependence of lymphangiogenic potentiation on $CD4^+$ T cells and macrophages can go a step further by investigating the functional roles of these cells using *ex vivo* activation assays with $CD8^+$ T cells. Also, future studies can delineate the changes in $CD4^+$ T cell differentiation in the TME by characterizing transcription factors in these cells. It should be noted that these mechanistic data are also limited in their scope, as the 4T1 tumors contain a relatively high $CD4^+$ T cell to $CD8^+$ T cell ratio, potentially skewing the relative importance of the conv $CD4^+$ T cell compartment.

It must be noted that our human TNBC analysis was limited to publicly available data from TCGA, which is obtained from samples of patients with various treatment schedules (chemotherapy or radiotherapy) prior to their biopsy. Moreover, our analysis only demonstrated correlations between higher VEGFC expression or lymphatic score and immune gene signature, not a causal relationship. More importantly, we do not know if the patients with higher lymphatic score would have been more responsive to cancer immunotherapies. Despite this, we speculate that the higher immune infiltration in these tumors could render them more responsive to immunotherapies than non-lymphangiogenic tumors; this needs to be verified in future clinical studies.

Liquid biopsies are convenient and efficient alternatives to traditional tumor biopsies and thus hold great promise as future predictive biomarkers in cancer immunotherapy [13]. Serum VEGF-C levels were previously shown to positively correlate with responsiveness to checkpoint inhibition in melanoma patients [4]. We speculate that serum VEGF-C levels in TNBC patients, by itself or used in combination with other markers as a predictive score, could be used as a biomarker for selection of patients for cancer immunotherapies. Future studies are required though to assess if serum VEGF-C levels correlate with tumor lymphangiogenesis and clinical response to immunotherapies in TNBC.

Overall, this study builds upon the current knowledge of how tumor microenvironment affects anti-tumor immunity in the context of cancer immunotherapies. We show that a proper characterization of the tumor microenvironment, including the status of tumor lymphatics [14], is critical prior to making clinical decisions for triple-negative breast cancer patients. In other words, the scientific community needs to take into account the tumor-associated lymphatics as an important component of the cancer-immunity cycle. Our data suggest that patients with more tumor lymphatics/lymphangiogenesis are more likely to benefit from immunotherapies in a neoadjuvant setting, i.e. before undergoing tumor/lymph node resection surgeries. Clinical studies, however, are required to delineate this further by investigating if tumor lymphatics in human primary tumors correlate with objective response to neoadjuvant cancer immunotherapies.

4.3 References

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