FACTORS SHED BY BREAST TUMOR CELLS, TUMOR NECROSIS FACTOR ALPHA CONVERTING ENZYME ACTIVITIES, AND THE GENERATION OF PRO-TUMOR MACROPHAGES

by

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A dissertation submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Biology

Charlotte

2013

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ABSTRACT

STEPHEN LEE REGO. Factors shed by breast tumor cells, tumor necrosis factor alpha converting enzyme activities, and the generation of pro-tumor macrophages. (Under the direction of Dr. DIDIER DRÉAU)

The role of the tumor microenvironment, especially of tumor associated macrophages (TAMs), in the progression and metastatic spread of breast cancer is well established. TAMs are activated in the breast tumor microenvironment to express primarily a M2 (wound-healing) phenotype with minimal cytotoxic activities. The factors involved in the activation of TAMs to display a pro-tumor phenotype are still debated although the key roles of immunomodulatory cytokines released by tumor cells including colony stimulating factor 1 (CSF1), tumor necrosis factor (TNF) and soluble TNF receptors 1 / 2, soluble vascular cell adhesion molecule 1 (sVCAM1), soluble interleukin 6 receptor (sIL6R) and amphiregulin (AREG) have been demonstrated. Notably, these factors are all released through the mechanism of ectodomain shedding by activities of tumor necrosis factor alpha converting enzyme (TACE, i.e., a disintegrin and metalloproteinase 17 (ADAM17)). The role of TACE activation leading to autocrine effects on tumor progression has been studied in detail. In contrast, limited information is available on the role of tumor cell TACE activities on TAM functions in breast cancer. TACE inhibitors, currently in development for clinical trials, may influence TAMs and subsequently treatment outcomes through the substrates TACE releases. However, the mechanisms altered in macrophages following exposure to tumor cell TACE-shed cytokines and/or cytokine receptors remain unclear. Therefore, we first outline (1) the current understanding of the roles of molecules released by TACE ectodomain shedding

from breast tumor cells on TAM phenotypes and functions. Next, we report (2) that tumor cell TACE activities specifically promote the shedding of TNFRs, which binds to and sequesters exogenous TNF, thereby preventing its pro-migratory effects on macrophages. These effects are shown to be mediated by the protein kinase B (AKT) signaling molecule, a common downstream target of TNFR2 but not of TNFR1. Further we detail (3) how tumor cell TACE-shed MCSF in combination with secreted chemokine (C-C motif) ligand 2 (CCL2) promote the secretion of vascular endothelial growth factor (VEGF) and subsequent angiogenesis. The pro-angiogenic abilities of these macrophages are shown to be dependent of nuclear factor kappa B (NFκB) signaling. Finally, (4) these new data are summarized and discussed in the larger perspective of future research and treatments, harnessing the tumor stroma as a target in breast cancer. Overall, this research highlights specific mechanisms mediated by mammary tumor cell TACE-shed substrates involved in macrophage migration and promotion of angiogenesis that provide useful insights in the use of TACE inhibitors for the treatment of cancer as well as other potential targets involved in tumor cell modulation of TAMs.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Didier Dréau, for providing outstanding support and guidance throughout my graduate studies. I would also like to thank the members of my committee, Dr. Mark Clemens, Dr. Pinku Mukherjee, Dr. Laura W. Schrum, and Dr. Jerry Troutman, for providing invaluable feedback and instruction in research and academics. I would like to thank every past and present members of Dr. Dréau's lab especially the exceptional technical support provided by Jill Eddy and Amanda Lance, the excellent mentoring delivered by post-doctoral fellows Dr. Muthulekha Swamydas and Dr. Danielle Van. I would also like to acknowledge the support of my fellow former graduate students Ashley Jewell, Adam Secrest, Krista Ricci, and former honors and undergraduate students Ronald Valencia, Alex De Piante, Rachel Helms, Jocsa Cortez, Mariah Andrews, Kai Coppage. Their support and the continuing support of the current graduate students in the Dréau lab Yas Maghdouri-White, Rachel Helms and Dr. Michelle Coleman along with their advice and friendship made my graduate studies at UNC Charlotte a wonderful experience. I would also like to thank the members of other labs that I've had the pleasure of working with including the Mukherjee lab, Schrum lab and the Clemens lab for their collaborations. I would like to acknowledge the Department of Defense (CRMDP), National Science Foundation (NSF) and Sigma Xi for funding this research. I would also like to thank the UNCC graduate school for the Graduate Assistant Support Plan for providing funding for my graduate education. Lastly, I would like to thank my parents, Madelaine and Ivan Rego, sister's Jane Langfitt and Maria Mobley, friends and future wife, Amritha Kidiyoor, whom have always been there to support, encourage and inspire me.

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LIST OF ABBREVIATIONS

ADAMs	a disintegrin and metalloproteinase
ADAM17	a disintegrin and metalloproteinase 17
AKT	protein kinase B
ANOVA	analysis of variance
AREG	amphiregulin
ARG1	arginase 1
BCL2	B-cell lymphoma 2
BSA	bovine serum albumin
CCL2	chemokine (C-C motif) ligand 2
CCL5	chemokine (C-C motif) ligand 5
CCR2	chemokine (C-C motif) receptor 2
CD62L	l selectin
CM	conditioned media
CONA	concavalin A
CSF1	colony stimulating factor 1
CSF1R	colony stimulating factor 1 receptor
CTL	circulating t lymphocyte
CTC	circulating tumor cell
CXCL12	chemokine (C-X-C motif) ligand 12
DMEM	Dulbecco's modified eagle medium
ECM	extracellular matrix
EC	endothelial cell

EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
FASL	fas ligand
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GFs	growth factors
H2SO4	sulfuric acid
HB-EGF	heparin binding epidermal growth factor
HIF1α	hypoxia inducible factor alpha
HIF1β	hypoxia inducible factor beta
HRP	horseradish peroxidase
HUVEC	human umbilical cord vein endothelial cells
IBC	inflammatory breast cancer
ICC	immunocytochemistry
ICAM1	intercellular adhesion molecule 1
IHC	immunohistochemistry
IL6	interleukin 6
IL8	interleukin 8
IL10	interleukin 10
IL6R	interleukin 6 receptor
INOS	inducible nitric oxide synthase
JAMA	juntional adhesion molecule A

JNK	c-Jun NH(2)-terminal kinase
LAG3	lymphocyte-activation gene 3
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MCSF	macrophage colony stimulating factor
MCSFR	macrophage colony stimulating factor receptor
MICA	MHC class I polypeptide-related sequence A
MMPs	matrix metalloproteinases
MUC1	mucin 1
ND	not detected
NFκB	nuclear factor kappa b
NMUMG	normal murine mammary gland
PBS	phosphate buffer solution
PERK	phosphorylated extracellular signal-regulated kinase
PFA	paraformaldehyde
PI3K	phosphoinositide-3-kinase
PJNK	phosphorylated c-Jun NH(2)-terminal kinase
PLCɤ2	phospholipase C gamma 2
PMCSFR	phosphorylated macrophage colony stimulating factor receptor
PNFκB	phosphorylated nuclear factor kappa b
RAS	rat sarcoma
RB	retinoblastoma
RCC	renal cell carcinoma

ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SDF1	stromal cell-derived factor 1
SEM	standard error of the mean
SIL6R	soluble interlukin 6 receptor
SIRNA	small interfering ribonucleic acid
SRC	sarcoma
STAT3	Signal transducer and activator of transcription 3
STNFR1	soluble tumor necrosis factor receptor 1
STNFR2	soluble tumor necrosis factor receptor 2
SVCAM1	soluble vascular cell adhesion molecule 1
TACE	tumor necrosis factor alpha converting enzyme
TAMs	tumor associated macropahges
TBST	tris buffered saline with Tween® 20
TDSF	tumor derived soluble factors
TEM	transendothelial migration
TGFβ	transforming growth factor beta
TMEM	tumor microenvironment of metastasis
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2
Tregs	regulatory T cells
TNF	tumor necrosis factor
TNFα	tumor necrosis factor alpha

VCAM1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VLA4	very late antigen-4

CHAPTER 1: INTRODUCTION

1.1 Clinical Relevance and General Features of Breast Cancer Progression

Breast cancer is the most common malignancy among women in developed countries accounting for a third of total cancers diagnosed in women [1]. The American Cancer Society estimates more than 230,000 new breast cancer cases will be detected and that nearly 40,000 breast cancer related deaths will occur in the United States in 2013 [2]. Most cancer related mortalities are associated with complications due to metastasis, i.e., the spread of cancer. An estimated 1 out of 3 women with breast cancer will develop metastases [1,2]. Treatments for primary breast tumors have greatly improved and the recurrence of primary early breast cancer at least in the 5-year following diagnosis can be prevented in most patients through combinations of surgery, chemotherapy, and radiation [3]. In contrast, treatments for patients with metastatic breast cancer remain overall ineffective. Although our understanding of breast cancer progression and metastasis including the numerous molecules and mechanisms involved continues to improve, detailed knowledge of the origin, concentration and interplay of these molecules within the breast cancer microenvironment is still lacking. Indeed, the identification of these interactions during breast cancer progression will provide additional therapeutic targets for disease treatment while limiting potential side effects.

The progression from a benign state to a malignant state is associated with the

acquisition by tumor cells of multiple characteristics including (1) the ability to promote uncontrolled growth irrespective of the microenvironment, (2) loss of dependence on growth signals essential to the proliferation of normal cells, (3) insensitivity to anti-growth signals, (4) ability to evade programmed cell death or apoptosis (5), induction of angiogenesis, and (6) activation of metastasis (Figure 1.1) [4].

More recently, two emerging hallmarks of cancer were added: chronic inflammation associated with abundant inflammatory cells, cytokines and gaseous mediators and the capacity to evade immune detection (Figure 1.2) [5]. Overall, within a tumor mass, the net growth is directly proportional to the ratio tumor cell proliferation and tumor cell apoptosis. These characteristics are acquired through both tumor intrinsic as well as extrinsic mechanisms mediated by genomic alterations in the tumor cells and alterations of the microenvironment, respectively.

Intrinsic mechanisms used by tumor cells to evade anti-proliferative and pro-apoptotic extracellular signals have been demonstrated in breast cancer cells [6]. For example, activating mutations leading to constitutive activation of epidermal growth factor receptor (EGFR) or the Ras family of proteins, commonly observed in cancers, promote uncontrolled tumor cell proliferation. Additionally, cancer cells often acquire inactivating mutations in pro-apoptotic genes such as B-cell lymphoma 2 (bcl-2) and the first discovered human tumor suppressor gene retinoblastoma (Rb). Furthermore, both the shedding of growth factors, transforming growth factor alpha (TGFα) and AREG, and death receptors, TNFR1, have been shown to activate autocrine growth factor signaling and inhibit apoptotic signaling, respectively [6,7]. These mechanisms are well defined and widely studied as driving forces in tumorigenesis. However, although required, these

intrinsic mechanisms are not sufficient for a tumor cell to become fully invasive [4,5]. Indeed, aside from tumor cell mutations, evidence of the involvement of the tumor microenvironment in promoting breast cancer progression (described below) is accumulating [8,5,9].

1.2 The Tumor Microenvironment

In addition to a dense network of proteins forming the extracellular matrix (ECM), the tumor microenvironment consists of different stromal cells, including endothelial cells (ECs), fibroblasts, adipocytes, and infiltrating immune cells [8,10] (Figure 1.2). The infiltrating immune cells comprise leukocytes, T-cells, and monocytes [8,11,12]. The phenotype of these immune cells is influenced by the tumor microenvironment leading to the recruitment and generation of both T regulatory cells (Tregs) and tumor associated macrophages (TAMs) [13-15]. In a subset of breast cancers, the inflammatory breast cancers (IBC) tumor masses are infiltrated by a large number of immune cells: in some cases, more than 50% of the tumor mass consists of macrophages [16]. This infiltration and activation of these macrophages contributes to the inflammatory environment associated with this aggressive form of breast cancer as well as the other subtypes of breast cancers [14,17].

The reciprocal interactions, mediated by soluble factors, between tumor cells and the surrounding stroma, especially immune cells, can lead to amplified pro-tumor effects in a tumor mass [18]. Indeed, tumor cells have been shown to influence the surrounding microenvironment through multiple mechanisms [5]. For example, tumor cells release chemotactic molecules, including macrophage colony stimulating factor (MCSF) and chemokine (C-C motif) ligand 2 (CCL2), which attract specific subsets of immune cells

to the tumor site, including macrophages [19-21]. In addition, tumor and immune cells secrete specific cytokines, growth factors (GFs), reactive oxygen species (ROS), and hydrolytic enzymes, all paracrine and autocrine modulators of the local immune response and tumor behavior [8,19,5,9]. Tumor cells have also been shown to shed surface receptors, through the activity of specific enzymes such as A Dis-integrin Metallo-proteinases (ADAMs), leading to inhibition of anti-tumor cytokine activities [6].

The interactions between stroma and tumor cells, mediated by soluble factors, are the subjects of intense research especially toward fully understanding the potential to alter the tumor stroma in preventing breast cancer progression [5,22]. Cytokines [e.g., interleukin 6 (IL-6), transforming growth factor β (TGFβ)], chemokines [e.g., Chemokine (C-X-C motif) ligand 8 (CXCL8), CXCL12, CCL2, CCL5], and other mediators such as prostaglandins and reactive oxygen species (ROS) have already been identified as molecules involved in the tumor cell / immune cell interactions [23-27].

TNF, primarily released by macrophages, is a key cytokine in inflammation with pleiotropic effects including control of cell proliferation, differentiation, and apoptosis in numerous cells [28]. Because of its key role in inflammation, TNF blocking treatments have been investigated and are used to treat rheumatoid arthritis [29]. However, the effectiveness of TNF-based treatments in cancer have been limited and hindered by significant side-effects [30]. Nevertheless, TNF-based treatments specifically targeting tumors led to tumor cell death and decreased vascularization [30].

Taken together, these observations strongly support a key role for tumor derived soluble factors in generating a microenvironment permissive to cancer progression and

the crucial importance of this tumor microenvironment in promoting cancer progression and metastasis.

1.3 Inflammation

More than 150 years ago, Dr. Rudolph Virchow observed that malignancies were accompanied by increased levels of immune cell infiltrate and postulated that cancer arose in sites of inflammation [31]. Data collected over the past decades further support for the role of inflammation in cancer [32-34,5,17]. Indeed, recently the inflammatory environment of the adipose tissue was linked to cancer initiation and progression [35]. Overall, while an acute inflammatory response may hinder cancer initiation, chronic inflammation leads to the development and progression of established breast cancer [31]. Although inflammatory breast cancer represents only 5% of the total of breast cancer cases it is one of the most aggressive forms [36]. Indeed, inflammation of the breast tumor is an indicator of poor prognosis [36]. The pro-tumor effects of chronic inflammation are mediated by inflammatory cytokines (Table 1), secreted by the tumor and the stromal cells present in the tumor microenvironment [37]. Macrophages have prominent roles in both the initiation and persistence of an inflammatory response [33].

Initiated by damage or stress signals, the onset of inflammation promotes the recruitment and infiltration of macrophages from adjacent tissue and/or the bone marrow [33,37]. Within the tumor mass, macrophages generate a sustained inflammatory microenvironment, which in turn secrete various cytokines, including TNF and CCL2 to further the recruitment of additional inflammatory cells. The signals present in the microenvironment influence the macrophage phenotype to sustain a classically activated inflammatory (M1) phenotype or an anti-inflammatory (M2) phenotype or any variation

in between. The M2 macrophages have been shown to function as immuno-suppressive cells [38]. The M1 and M2 phenotypes lie at the extremes of a continuum of macrophage activation states [39]. Interestingly, a mixed population of macrophages containing characteristics of both M1 and M2 macrophages were found in the adipose tissue adjacent to a growing tumor [35]. Typically, M2 macrophages are thought to be pro-tumor whereas M1 have anti-tumor properties [38]. However, inflammatory M1 activated macrophages have important roles in tumor initiation through release of ROS whereas later during tumor progression M2 macrophages are shown to promote invasion of tumor cells through the ECM towards nearby blood vessels [9,35,40]. These data and other [41,14,42,43] underline that pro-tumor functions shown in both macrophage phenotypes depends on their temporal/spatial localization, suggesting that the M1/M2 classification, although useful, may not be completely relevant to describe the tumor associated macrophages. Regardless of their phenotype, macrophages play a critical role in the promotion and maintenance of the vasculature and are extremely important in promoting the formation of new blood vessels or angiogenesis.

1.4 Angiogenesis

As tumors grow, cancer cells within the tumor mass further away from the blood vessels are oxygen and nutrient deprived leading to the accumulation of spent molecules during the early phase of this highly metabolic process [44]. Low oxygen concentration (pO_2) or hypoxia due to poor diffusion and distance from blood vessels affects cells in the center of a tumor mass as little as 250-1000 cells [44]. Intracellular hypoxia triggers a cascade of intracellular responses including the stabilization of hypoxia inducible factor 1 α (HIF1α) and its combination with the constitutively expressed HIF1ß forming a

transcription factor that promote the expression of multiple molecules promoting angiogenesis including, VEGF [45,46] (Figure 1.3). Angiogenesis promotes tumor progression toward a malignant state. The "angiogenic switch" defines the change of endothelial cells from dormant to rapidly growing resulting in the development of a dense microvasculature that connects to the host circulation to the tumor mass and is thought to be controlled by macrophages in many settings including cancer [41]. In contrast with normal vasculature, this newly formed tumor vasculature is much less organized and lacks most pericytes [47]. In addition to alterations in HIF1α expression, preclinical and clinical observations indicate that molecules expressed by tumor infiltrating immune cells especially macrophages also activate tumor angiogenesis [41]. For example, inhibition of macrophage maturation led to a decrease in angiogenesis, whereas the presence of high number of macrophages within the tumor mass in transgenic mouse models caused increased tumor vasculature and subsequent malignant progression [41].

Specifically, inflammatory molecules secreted by macrophages including VEGF and matrix metalloproteinase (MMPs) are both promoters of angiogenesis [41,48,49]. VEGF is a potent angiogenic factor released by both tumor cells and macrophages and receptors stimulated by VEGF (VEGFRs) have been successfully targeted in many cancers [45,46]. Targeting VEGFRs for the treatment of breast cancer has been ineffective [50], indicating the need to gain a fuller understanding of the molecules and mechanisms in breast cancer angiogenesis to provide further targets for inhibiting this pathway. The multiple interactions between tumor cells and macrophages associated macrophages leading to the promotion of angiogenesis and cancer progression remain to be fully assessed.

1.5 Modeling Cell-Cell Interactions to Study Cancer

The complexities of the tumor microenvironment *in vivo* render analyses of specific cell signaling and molecular interactions challenging [51,40]. Therefore, multiple approaches, especially *in vitro,* have been developed [52,51,10,53]. To study the effects of hypoxia within a growing tumor mass 3D modeling systems with tumor spheroids / colonies have been developed which possess a characteristic necrotic core due to decreased oxygen / nutrient diffusion [54,10]. Numerous co-culture systems have been engineered to study the effects of one cell type on another including direct, transwell and conditioned media (CM). For the study of interactions mediated by direct cell-cell effects the use of the direct co-culture method is appropriate [55,56]. This system has led to the discovery of many adhesive interactions of tumor cells and macrophages mediated by integrins as well as providing a simplified way to observe the process of tumor cell-macrophage co-invasion observed in the process of tumor cell streaming [40]. To analyze the reciprocal crosstalks between tumor cells and macrophages mediated by soluble factors the transwell co-culture systems are suitable and have been used in many studies revealing the soluble factors involved in TAM promotion of tumor cell migration, invasion and streaming [40,57]. Finally, to analyze the effects of one cell type on another the use of CM provides simplified and fitting approach. This method minimizes the complexities of multiple cell co-culture systems, allows one to have improved control over culture conditions and makes the effector cell type easier to identify [53,26,58,59]. Furthermore, this system allows the specific targeting of factors only on one cell type whereas other co-culture methods make this task much more challenging. In the studies performed here CM was used to make many of the observations, providing a useful way

to isolate the effects of specific tumor associated environmental factors on macrophage phenotype *in vitro* and gain a clearer understanding of the mechanisms and pathways involved in these interactions.

1.6 Tumor Cell TACE Shedding in Stimulating Pro-tumor TAMs

The tumor derived soluble factors associated with pro-tumor activities and involved in the modulation of macrophages include MCSF, TNF and TNFRs [38]. These factors are released from cells through ectodomain shedding by the enzyme TACE [60]. Levels of TACE and the TACE substrate TGFα are correlated to decreased survival in breast cancer patients [7]. One mechanism to explain this observation was shown through TACE shedding of EGFR ligands stimulated autocrine activation of EGFR and downstream growth factor signaling pathways in breast cancer cells [7].

To date, no studies have investigated the paracrine roles of tumor cell TACE shedding on stromal cells within the breast tumor microenvironment. Based on the immune-modulatory factors shed by TACE, including MCSF, TNF and TNFRs, TACE activities may significantly modulates the behavior of various stromal cells within the breast tissue, especially macrophages. Both TNF and MCSF have positive effects on macrophage migration, however, these two molecules lead to opposite effects on macrophage polarization [38]. Indeed, TNF promotes the activation of M1 cytotoxic macrophages whereas MCSF stimulates M2 wound-healing macrophages [38]. Whether tumor cell TACE shedding influences pro-tumor macrophage activities is still unknown (outlined in Figure 1.4).

1.7 Objectives

Therefore, here we first outlined the known pro-tumor activities of TACE substrates in mammary tumorigenesis and the effects of TACE substrates on macrophage functions through an extensive literature review (Chapter 2). Next, we demonstrated the effects of tumor shed TNFRs on the migration of macrophages towards TNF (Chapter 3). In Chapter 4, we investigated the role of tumor cell TACE-shed MCSF in promoting pro-angiogenic macrophages. Since, in breast cancer, MCSF alone has not been implicated in stimulating TAM angiogenesis we determined whether other factors, such as CCL2, were involved in promoting angiogenic macrophages. Finally, the importance of these findings, in furthering our understanding of tumor associated macrophages and shaping the future of breast cancer treatments, is discussed.

Table 1.1. Cytokines involved in breast cancer [61]. The effects of numerous cytokines on tumor growth and invasion in human patients are outlined. Many of these cytokines are shown to have an impact on the prognosis of patients.

Table 1
Main recent data on cytokines involved in growth and prognosis of human breast cancer

Cytokine (s, t)[Ref]	Tumour growth		Prognostic role
Type	Proliferation	Invasion	
t IL-1 (α and β) [3–5,7]	+	+	
t IL-1α, s IL-1β [3,5,18]			−
Soluble IL-2R [19]			+
t IL-6 [7,8,13,27,28]	+	+	c
s IL-6, t IL-8, s IL-8, s IL-10 [18,20–25,31,32]			−
t IL-11 [14]	i	+	i
t IL-12 [16]	−	−	i
i.p. IL-18 [17]		−	i
s IL-18 [33,34]			−
t IFNα (2a, 2b), t IFNβ, t IFNγ [2,40,41,46–51]	−	−	i
t IFNγ p [61,62,69]	+	+	−
t TGFβ [75]	+	+	i
t TGFβp [28]			−
t gp130 [27,78]	+	+	+
s gp130 [10]			−

For the details, see text. Abbreviations: Ref = reference; s = serum; t = tissue; i.p. = intraperitoneal; p = polymorphism; *tumour growth*: + = favoured; − = inhibited; *prognostic role*: + = favourable; − = unfavourable; i = indefinite; c = controversial.

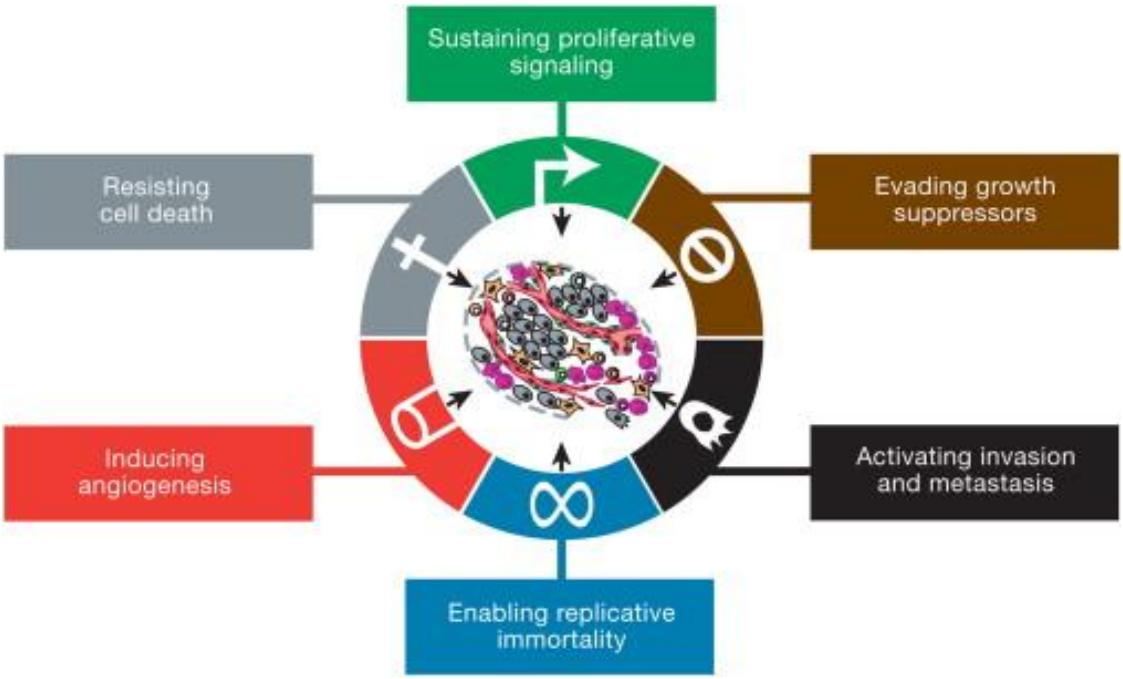

Figure 1.1. The hallmarks of cancer [5] outlines six critical characteristics needed for a cell to become malignant including sustained proliferation, decreased apoptosis, inhibition of growth suppression, angiogenesis, decreased senescence and implementing metastasis. All of these characteristics are required for a tumor to become malignant.

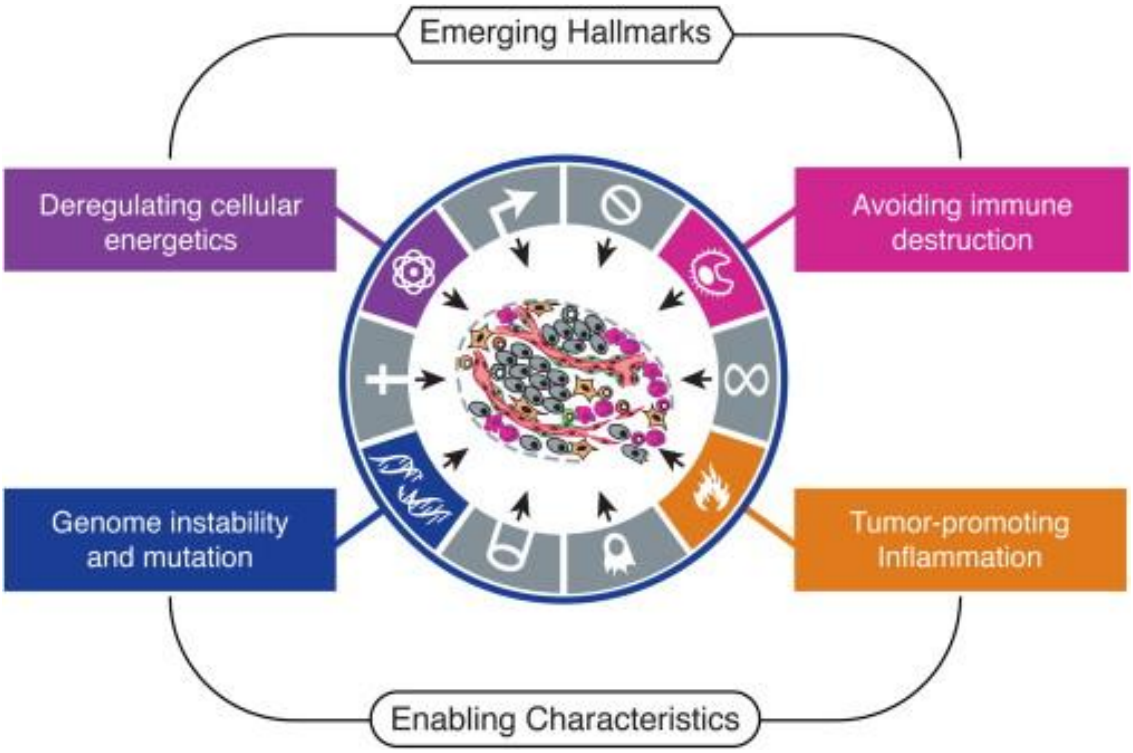

Figure 1.2. Recent additions to the hallmarks of cancer [5]. Along with the six characteristics of cancer cells described previously, studies over the last ten years reveal four more properties of malignant cells including unregulated metabolism, genome instability, evade immune detection and inflammation.

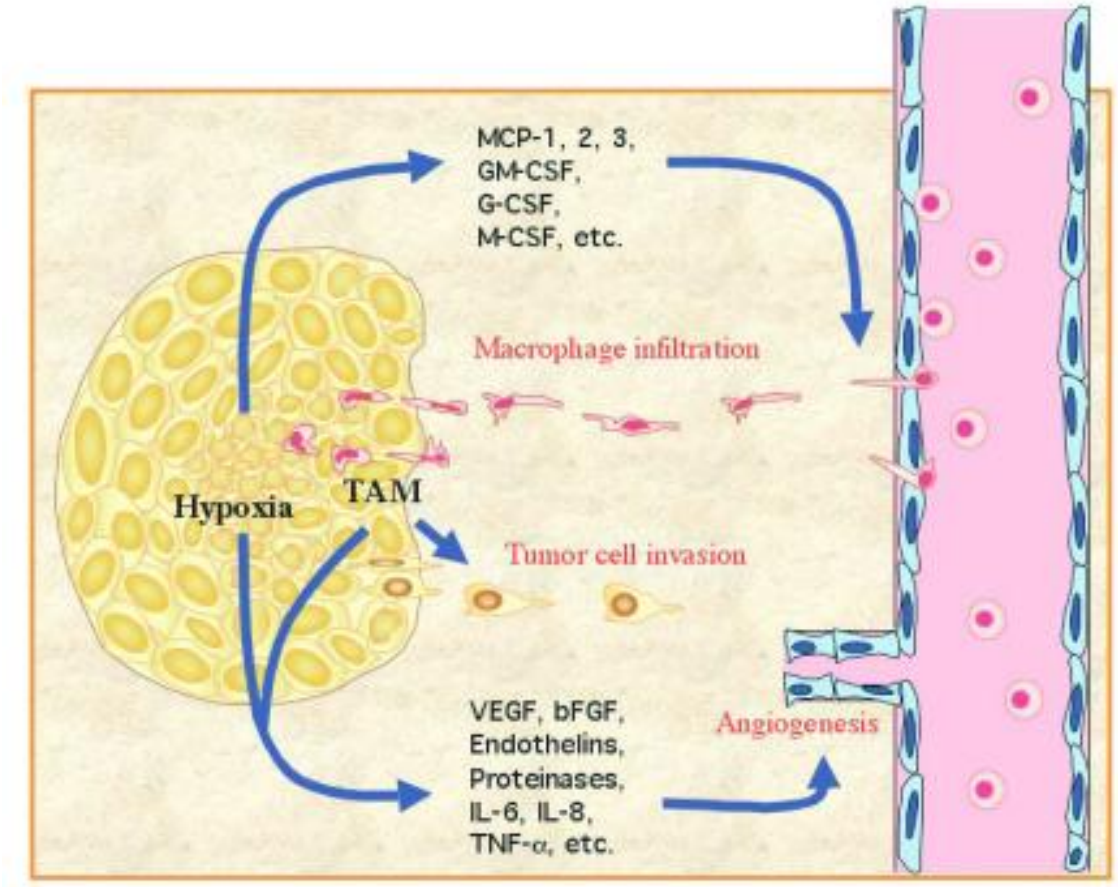

Figure 1.3. Linking hypoxia to angiogenesis through tumor cells and macrophages [62]. Critical properties of the tumor microenvironment, hypoxia and inflammation, are shown to exert tumor promoting effects on one another such that these conditions promote the invasion of tumor cells towards the vasculature.

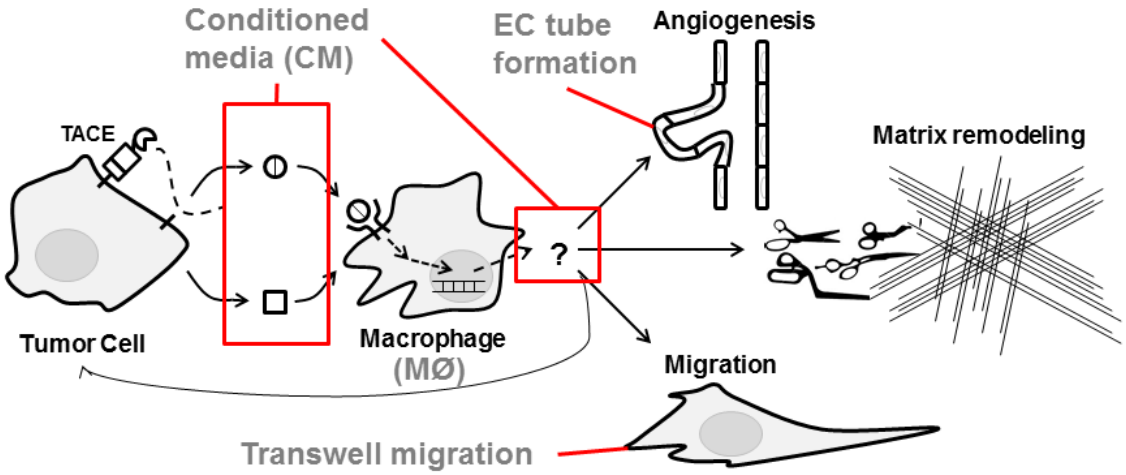

Figure 1.4. Proposed effects of tumor cell TACE shedding of substrates on various tumor associated macrophage functions. The studies presented investigate the paracrine effects of TACE shedding by tumor cells on macrophage migration, angiogenesis and matrix remodeling. The detailed pathways involved in these interactions are critical to the understanding of tumor cell interactions with their microenvironment. These studies utilize different in vitro approaches including conditioned media, transwell migration and endothelial cell tube formation assays.

CHAPTER 2: TUMOR NECROSIS FACTOR ALPHA CONVERTING ENZYME ACTIVITES AND TUMOR-ASSOCIATED MACROPHAGES IN BRESAT CANCER

2.1 Abstract

The role of the tumor microenvironment especially of TAMs in the progression and metastatic spread of breast cancer is well established. TAMs have primarily a M2 (wound-healing) phenotype with minimal cytotoxic activities. The mechanisms by which tumor cells influence TAMs to display a pro-tumor phenotype are still debated although the key roles of immuno-modulatory cytokines released by tumor cells including CSF1, TNF and sTNFR1 / 2, sVCAM1, sIL6R and AREG have been demonstrated. Importantly, these factors are released through ectodomain shedding by the activities of the TACE. The role of TACE activation leading to autocrine effects on tumor progression has been extensively studied. In contrast, limited information is available on the role of tumor cell TACE activities on TAMs in breast cancer. TACE inhibitors, currently in clinical trials, will certainly affect TAMs and subsequently treatment outcomes based on the substrates it releases. Furthermore, whether targeting a subset of the molecules shed by TACE, specifically those leading to TAMs with altered functions and phenotype, hold greater therapeutic promises than past clinical trials of TACE antagonists remain to be determined. Here, the potential roles of TACE ectodomain shedding in the breast tumor microenvironment is reviewed with a focus on the release of tumor-derived immuno-modulatory factors shed by TACE that direct TAM phenotypes and functions.

2.2 Pro-tumor Functions of TAMs Modulated by TACE

In addition to their intrinsic properties, breast tumor cell growth and ability to form metastases rely heavily on interactions with stromal cells in the breast tumor microenvironment [5,63]. Indeed, within the breast tumor microenvironment structural proteins from the ECM and both soluble and insoluble factors generated by tumor cells and stroma cells including immune cells, fibroblasts, endothelial cells and adipocytes modulate cancer progression. Although each of these stroma cells influences breast cancer progression, TAMs in particular have been associated with worsened clinical outcome in breast cancer patients [64,65].

Macrophages located in and around the tumor i.e., the TAMs have both anti- and pro-tumor activities [66,20,67-69] which have been detailed thoroughly in breast cancer [64,8,70-73,20,57]. TAMs display diverse functions and lie on a phenotype continuum from M1 macrophages with anti-tumor properties (classically activated) to M2 macrophages with pro-tumor properties (alternatively activated) [74]. It should be emphasized that this classification system was developed to highlight the markers and functions of M1 and M2 macrophages in different contexts and may not precisely represent the anti-tumor and pro-tumor TAMs, respectively. Indeed, other distinct populations of monocyte-derived cells isolated within the breast tumor microenvironment have been identified including the Tie2 expressing monocytes (TEM) [17]. Furthermore, macrophages expressing the canonical M1 marker inducible nitric oxide synthase (iNOS) had anti- and pro-tumor effects on the cytotoxicity and tumor cell invasion, respectively [73]. Nonetheless, the M1 / M2 nomenclature system provides a useful framework to group functionally distinct macrophage subsets in cancer.

Macrophages express distinct phenotypes based on signaling molecules present in their local microenvironment [74]. This macrophage plasticity will vary depending on cytokine exposure, ECM composition and oxygen availability [74,38]. In the breast tumor microenvironment, the pro-tumor activities of TAMs are modulated by numerous tumor derived soluble factors (TDSFs). Many TDSFs are released from tumor cells by ectodomain shedding mediated mainly by the sheddase TACE; i.e., ADAM17 [75,76]. TACE-shed TDSFs essential in the recruitment and activation of pro-tumor TAMs include; CSF1, TNF, intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule (VCAM1), sIL6R, AREG and TGFα [7,77,66].

In the present review, we detail the mechanisms associated with the TACE shedding by tumor cells within the tumor microenvironment focusing on its effects on macrophage activity and tumor progression and briefly summarize the well-defined pro-tumor effects of TACE activities. Additionally, the role of TACE in paracrine cell signaling involved in normal mammary tissue homeostasis is addressed. The activities of TACE in epithelial cell-stromal cell interactions during normal mammary gland development and breast cancer have been studied and will be discussed here. Further, these interactions are relevant to the growth of any tumor mass in which TACE-shed factors and macrophages are present, however, few studies investigate this process outside of the mammary gland. Here, the impact of tumor cell TACE-shed factors in the promotion of pro-tumor macrophages and in the compartmentalization of specific macrophage subsets to distinct areas of the tumor is discussed. The main thrust of the present review is to define the influential role of TACE directed ectodomain shedding by

tumor cells on TAMs recruitment and phenotype. Other TACE activities have been reviewed elsewhere recently [78,75,79,80].

2.3 TAMs Phenotype and Function in Breast Cancer

TAMs exert pro-tumor effects through the promotion of angiogenesis, degradation of the ECM, suppression of antitumor immune responses and promotion of tumor cell invasion [81,13,20,9,42,82]. In many cancers, including breast cancer, the increased presence of TAMs is associated with increased aggressiveness of tumors and decreased patient's survival [17,20,83,63,84]. Furthermore, numerous studies suggest the pro-tumor activities of TAMs prevail in breast cancer [81,85,20,83,66]. However, the mechanisms by which the TAMs are skewed toward a pro-tumor (M2) phenotype remain unclear. TAMs are recruited to the tumor site from the bone marrow in response to signals released by both tumor and stromal cells [20,83,66]. The steps of macrophage trafficking to the tumor site include monocyte production in the bone marrow and transport within the blood stream, adhesion to the endothelium, diapedesis and invasion [86]. This process is guided by the presence of chemotactic and chemostatic molecules leading to the mobilization of macrophages in different regions of the breast tumor [17,20,86]. Interestingly, macrophages with different phenotypes i.e., different membrane-bound and excreted protein profiles, are located in distinct areas of a tumor [42,74]. Indeed, the immature population of monocytes (TEM) localize to the hypoxic core [17], whereas M2-like macrophage populations are found at the invasive edge of breast tumors [85]. The pathways activated in macrophages and molecules released by the tumor stroma leading to the generation of macrophage subsets in distinct tumor regions are under intense investigation [74,23,22,87,14,9,42].

2.4 Pro-Tumor Activities of TACE

Tumor cells release many pro-tumor factors that promote tumor cell growth and alter macrophage phenotype and migration in the microenvironment [39,20,57,88,70], including AREG, TGFα, CSF1, TNF, VCAM1, sIL6R, and ICAM1 respectively [7,77,66]. These molecules are released from tumor cells through ectodomain shedding by TACE [78,7,77]. This sheddase belongs to the ADAM family of transmembrane proteins, and structurally includes a metalloprotease domain, an integrin binding domain, and a cytoplasmic tail [78-80]. In addition to protein shedding, ADAMs are involved in integrin binding and intracellular signaling through their disintegrin and cytoplasmic domains, respectively [75,60]. During ectodomain shedding, proteins are proteolytically cleaved from the cell membrane. This process frees approximately 10% of all proteins released from cells into the extracellular space [89].

In vitro the pro-tumor activities of TACE include increases in tumor growth, proliferation, invasion, and maintenance of the malignant phenotype of tumor cells [7,90-93]. *In vivo* TACE activities also promote tumor growth, tumor formation and tissue invasion [90]. Additionally, increased protein expressions of TACE have been correlated to clinical parameters of tumor progression i.e., presence of lymph node metastases and decreased patient's overall survival [7,91]. Specifically, TACE pro-tumor activities initiate the shedding from breast tumor cells of the epidermal growth factor receptor (EGFR) ligands, TGFα and AREG, which through autocrine signaling promote the malignant phenotype [7,93]. Over expression of TACE and one of its substrates TGFα also correlated with decreased survival in breast cancer patients [7,94]. These

observations support the use of TACE as a therapeutic target for the treatment of breast cancer.

2.5 Inhibition of TACE

TACE inhibitors have been investigated as a means to prevent / limit inflammation by blocking the release of TNF in inflammatory diseases [78]. In past phase 2 clinical trials, the TACE inhibitors tested (TMI-005 and BMS-561392) were unsuccessful due to their lack of efficacy and systemic toxicities [78]. Both of these inhibitors displayed off-target inhibition of MMPs, including MMP1, MMP2 and MMP13, which may account for their observed liver toxicities [95]. However, more specific TACE inhibitors may be associated with limited liver toxicities. Indeed, a 10-fold reduction in liver toxicities was observed in mice treated with specific TACE inhibitors compared to those administered BMS-561392 [96]. Currently, TACE more specific inhibitors, which have shown great promise in anti-inflammatory preclinical studies, are under investigation [78]. These trials highlight the potentials and the challenges associated with the use of TACE inhibitors in treating breast cancer patients. In particular, they highlight the need for an in-depth understanding of the indirect effects of TACE and TACE inhibitors on stroma cells, specifically macrophages, as a key step toward the successful use of TACE inhibitors to treat breast cancer patients.

2.6 Expression and activities of TACE during normal breast tissue development

2.9.1 TACE Expression and Activities

The primary function of TACE is ectodomain shedding leading to the release of trans-membrane proteins from the cell surface (outlined in Figure 2.1). The ectodomain shedding resulting from TACE activities releases functional ligands including cytokines

and growth factors, which upon binding to their cognate receptors activate autocrine and / or paracrine signaling pathways [60]. TACE activities can also shed receptor-like molecules from the cell membrane, which once freed can sequester associated ligands within the microenvironment thereby preventing their signaling effects on cells [60]. In breast tissues, TACE sheds multiple growth factors and cytokines with essential roles in both normal mammary gland development and immune cell functions [97,98]. Indeed, the experimental deregulation of TACE activities, required for normal development, maintenance and function of the breast tissue, promotes breast tumorigenesis (Table 1) [60,7,97]. TACE is expressed in nearly all cell types with, however, variable expression and activity levels [76,99,100]. The expression and activities of TACE by various cells, including macrophages and chondrocytes, [101,102,60,103,104] endothelial and vascular smooth muscle cells [105,56], and astrocytes [106] have been reviewed earlier [56,60,101,105,106]. Human tissues with highest TACE expression include the heart and reproductive organs and cell types with relatively high expression levels include macrophages as measured by Western and Northern blot analysis [76,60].

2.9.2 TACE in Mammary Development

Normal mammary gland development occurs mainly during puberty and toward the end of pregnancy and includes steps of branching morphogenesis leading to complex ductal networks [107,108]. This process is tightly regulated in part through reciprocal cross-talks between epithelial and stromal cells [97,98]. Using tissue recombination methods with wild type and transgenic mice, Sternilicht et al. showed that the expression of TACE and AREG on epithelial cells and the expression of EGFR on stromal cells are required for normal mammary ductal morphogenesis [98]. In particular, TACE cleavage

of AREG from epithelial cells, allowed AREG to bind to and activate EGFR on stromal cells, thereby promoting ductal growth [97,98]. TACE activation also specifically affects stromal macrophages in mammary gland development, leading to activities that are critical to branching morphogenesis through mediating the formation of terminal end buds [109,110].

2.7 Expression and Activities of TACE During Cancer Progression

Microenvironmental signals modulate the activation status of each cell type within the tumor mass including the expression and activities of TACE. Indeed, increased TACE gene / protein expression is observed in both macrophages during inflammation and macrophages isolated from patients with triple negative breast cancer [102,111]. The TACE activities of macrophage at sites of inflammation lead to the release of several inflammatory cytokines, including TNF, a critical pleotropic cytokine involved in cell death, cell migration and inflammation [60,109,78,77,112]. Details on the importance of macrophage TACE activity and its consequences on the inflammatory process can be found in recent reviews [112,109,78].

Along with the role of TACE in normal mammary gland development and inflammation activities of TACE are pivotal in breast tumorigenesis and metastasis as demonstrated by observations that the experimental upregulation of TACE in mammary tissue promotes malignancy (Table 1) [60,7,97]. Increased TACE shedding led to the release and binding of EGFR ligands, AREG and TGFα to EGF receptor(s) on the breast cancer cells sustaining the malignant phenotype of breast tumors. Also, the activation of the EGFR on breast cancer cells led to the activation of the mitogen-activated protein kinase (MAPK) pathway which stimulated cancer cell proliferation and a loss of cell

polarity in 3D *in vitro* cultures [7]. In this study, both chemical inhibition of the TACE metalloproteinase activity and knockdown with TACE siRNA prevented the expression of the malignant phenotype [7]. This is one critical mechanism employed by tumor cells to decrease their reliance on external growth factors and an essential early step in tumorigenesis. Similar mechanisms have been demonstrated in colorectal cancer leading to increased resistance to chemotherapies [113].

TACE cleaves many EGFR ligands including AREG and TGFα as well as heparin binding EGF (HB-EGF) and epiregulin (EPR) [7,97,114]. The ErbB family of receptors has been implicated in breast cancer progression in particular through the ErbB1 / EGFR signaling pathway [115,7]. Indeed, the deregulation of EGFR contributes to the decreased reliance of tumor cells on external growth signals, a defining characteristic of tumorigenesis [7]. Growth factor receptor pathways are vulnerable to malignant transformations including mutations to the receptor or to downstream signaling molecules resulting in a constitutive activation [116]. Alternatively, continuous release of ligands stimulating autocrine signaling also disrupt the tumor cells EGFR pathway [7]. TACE shedding and the autocrine effects of EGFR ligands in breast cancer have been well studied [78,117,80].

2.8 The Effects of TACE Substrates on Macrophages in Cancer

In contrast with the extensively studied autocrine effects of tumor TACE shedding [78,117,80], data on the effects of tumor cell TACE shed factors on TAMs are limited. The TACE substrates involved in the tumor cell / macrophage interactions include CSF1, TNF and TNFRs, ICAM1, VCAM and EGFR ligands. Each of these molecules modulates one or more steps of macrophage recruitment and / or phenotype

activation. Thus, these TACE-shed molecules participate in the formation of specific macrophages along the M1 - M2 continuum in the breast tissue. Furthermore, TNF and EGFR ligands also dramatically affect the survival of macrophages once in the target tissue.

The primary effects of TACE-shed molecules on macrophages are presented in Table 2. The substrates of TACE involved in regulating macrophage activities include CSF1, CSF receptor 1 (CSFR1), TNF, TNFR1/2, TGFα, AREG, interleukin 1Rα (IL-1Rα), mucin 1 (MUC1), VCAM1, ICAM1, sIL6R, L-selectin (CD62L), lymphocyte activation gene 3 (LAG-3), CD30, CD40, IL-6R, chemokines (CX3CL1 and CXCL16), junctional adhesion molecules A (JAM-A), and the MHC class I polypeptide-related sequence A (MICA) [28,74,70,78,20,38,39,86,118,117]. Of these molecules CSF1, TNF, TNFRs, VCAM1 and ICAM1 are released by tumor cells and critically regulate pro-tumor macrophage phenotype and functions.

2.9.1 Tumor Cell TACE-shed CSF1 and TAMs

CSF1, TNF, TNFRs, VCAM1 and ICAM1 play essential role in the interactions of breast cancer cells and macrophages as they promote changes in macrophage phenotype, migration and apoptosis. The presence and binding of CSF1, shed by breast tumor cells through TACE activities, to CSFR1 highly expressed by macrophages within the tumor mass correlates with poor prognosis [119]. In a CSF1 null transgenic mouse model that spontaneously developed mammary tumors, the presence of the CSF1 protein was shown to significantly increase the number of lung metastasis while having no effect on primary tumor growth [20]. More recently, a paracrine feedback loop between tumor cells secreting CSF1 to recruit and activate TAMs and TAMs stimulating production of

EGF, which in turn promotes the invasion of tumor cells, was identified using an *in vivo* murine model [57,71]. This feedback loop is also essential in directing tumor cell / macrophage streaming to the tumor microenvironment of metastasis (TMEM) leading to dissemination of tumor cells from the primary tumor into the circulation. Others have shown a similar mechanism of tumor derived CSF1 stimulating TAMs to produce stromal cell-derived factor 1 (SDF1) and VEGF leading to increased angiogenesis and tumor cell invasion [19]. Additional research is required to define whether the breast tumor / macrophage feedback loop directed by CSF1 primarily stimulates the production of EGF or other molecules such as SDF1 and VEGF to determine the relative role of each of those factors in tumor progression.

CSF1 activation of CSF1R on macrophages has been shown to alter every aspect of macrophage functions including proliferation / survival, differentiation and migration through stimulation of the phosphatidylinositide 3-kinases / rat sarcoma (PI3K/Ras), Phospholipase Cγ2 / PI3K (PLCγ2/PI3K) and sarcoma / PI3K (Src/PI3K) pathways, respectively [120]. Additionally, macrophages stimulated by CSF1 support the promotion and maintenance of angiogenesis in mammary tumors [41]. Moreover, our recent data indicates that macrophages stimulated by tumor cell TACE-shed CSF1 secrete higher levels of the angiogenic factor VEGF [121]. Furthermore, knocking down the expression of CSF1 by breast tumor cells prevented both the colonization and activation of TAMs at the tumor site and also significantly limited the ability of tumor cells to form metastases [20]. These observations strongly support the involvement of CSF1-activated TAMs in breast cancer promotion and metastasis [122,57,71].

2.9.2 Tumor Cell TACE-Shed TNF / TNFRs and Macrophages

The pleiotropic cytokine TNF is also a key substrate of TACE critical in the promotion of inflammatory responses. Both stroma cells and macrophages release TNF to promote inflammation, which can either lead to apoptosis of tumor cells or tumor promotion [123-126]. TNF promotes the chemotaxis of macrophages to sites of inflammation, where additional TNF activation stimulates macrophages toward the classically activated phenotype [38,127]. The receptors for TNF (TNFR1 and TNFR2) are expressed by most cells of the body and are also susceptible to ectodomain shedding [124,128,129]. Once shed by TACE sTNFRs, bind to TNF, thereby blocking its signaling effects including apoptosis of tumor cells and chemotaxis and activation of macrophages [124]. Indeed, sTNFRs shed by tumor cells through TACE activities inhibited macrophage activation of AKT and subsequent chemotaxis toward TNF [130]. As with EGF and EGFR shedding, tumor cells may use TNF - sTNFR interactions to modulate the number and phenotype of macrophages within the tumor or specific tumor locations.

2.9.3 TACE Shed VCAM1 / ICAM1 and TAMs

Serum levels of the adhesion molecules and TACE substrates, sVCAM1 and sICAM1, have been correlated with breast cancer staging [131] and metastasis [132-134], respectively. Further, sVCAM1 serum concentrations correlated with microvasculature density in tumors, the presence of circulating tumor cells (CTLs) [135,131] and decreased tumor cell killing [70]. VCAM1 expression by breast tumor cells has been shown to promote metastasis to the lungs [136,70,131]. Mechanistically, VCAM1 on tumor cells interacts with integrins on macrophages leading to tumor cell survival and invasion through PI3K signaling [70]. Moreover, through these interactions and in conjunction with macrophages, breast tumor cells metastasize to the bone [137]. Since VCAM1 is

also involved in the accumulation of monocytes at atherosclerotic lesions, VCAM1 also likely plays an essential role in generation of the metastatic niches for breast tumor cells [138].

ICAM1 correlates to TAM abundance and is involved in macrophage infiltration to the tumor site [139-141]. Furthermore, ICAM1 mediates tumor cell / macrophage adhesion through heterotypic binding to MUC1 [139,142]. Macrophage adhesion to the endothelium is also mediated in part through interactions with ICAM1 further indicating the role of ICAM1 in macrophage infiltration into the tumor site [143]. ICAM1, through interactions with other selectins / integrins supports the adhesion of tumor cells to the endothelium during the metastatic process [134,144,145]. Interestingly, macrophages play an essential role in the adhesion of tumor cells to the endothelium and extravasation during the process of metastasis through interactions with ICAM1 as well [146,140,147]. Furthermore, our recent data that indicates molecules shed through TACE activities, which includes ICAM1, by tumor cells differentially regulate macrophage subset chemotaxis, having stronger effects on M2 macrophages while minimally affecting M1s [148]. Altogether, the shedding by tumor cells of CSF1, TNF, TNFRs, VCAM1 and ICAM1 through TACE activities greatly impacts tumor cell progression and metastasis in part through the modulation of tumor cell / macrophage interactions at both primary and metastatic sites.

2.9.4 TACE-Shed IL6R and IL6 Trans-signaling and TAMs

Canonical IL6 signaling is engaged in cancer progression. However, IL6 trans-signaling, which involves the shedding of IL6R by TACE, has only recently been identified in cancer. Indeed, high levels of IL6 and sIL6R correlate to decreased

survival/increased metastasis and adverse outcomes in breast cancer patients, respectively [149-151]. The mechanism for IL6 trans-signaling involves the shedding of IL6R from the surface of IL6R positive cells, the binding of soluble IL6R to soluble IL6 forming a complex that activates IL6 signaling in IL6R positive or negative cells through binding to the ubiquitously expressed GP130 receptor (Figure 2.2) [152]. IL6 trans-signaling leads to downstream activation of janus kinase / signal transducer and activator of transcription 3 (JAK/STAT3) [152] and of nuclear factor kappa B (NFκB) [153], two pathways linked to cancer progression [154-156]. Moreover, JAK/STAT3 signaling in macrophages leads to cell polarization and an M2 pro-tumor phenotype [74] with enhanced secretion of cancer stem cell promoting factor, IL1β and IL6 [157], angiogenic molecules, VEGF and bFGF [158], and the immunosuppressive enzyme, arginase 1 (arg1) [159]. Recent studies on trans-signaling demonstrate that sIL6R acts as a reservoir for IL6 by extending its half life as well as a mechanism for amplifying IL6 signaling on both IL6R expressing and non-expressing cells [152]. Therefore, TACE activation may be one of the mechanisms used by tumor cells to amplify IL6 signaling, the activation of which promotes the display of primarily pro-tumor functions by macrophages.

In addition to the effects of tumor cell TACE-shed substrates on TAMs, these substrates also affect other cell types present in the tumor microenvironment involved in breast cancer progression. For example, TACE-shed substrates affect the activities of endothelial cells leading to alterations in endothelial cell morphology, proliferation and invasion *in vitro* and neo-vascularization *in vivo* [160,161]. Since the adhesion to endothelial cells within the tumor mass is a key step in macrophage recruitment to the tumor site, the critical role of the interactions between tumor cells and endothelial cells

mediated by TACE in the process of macrophage recruitment cannot be overlooked. Indeed, VCAM1, ICAM1, L-selectin and very late antigen 4 (VLA4), all targets of TACE, are key mediators of leukocyte adhesion to the vascular endothelium [162,145]. Thus, a better knowledge of the multiple TACE activities in the tumor microenvironment, especially its effects on the stromal components may further our understanding of TACE activities within the tumor microenvironment and translate into improved therapeutic use of current and future TACE inhibitors.

2.9 New Therapeutic Avenues and Future Research Axes

2.9.1 TACE Inhibition

TACE expression and activities have shown promise as prognostic indicators in breast cancer. Indeed, elevated plasma TACE concentrations were an independent predictor of decreased breast cancer patients' overall survival [163,91]. Additionally, increased TACE mRNA concentrations in the tumor mass were predictive of poor prognosis in breast cancer [7]. These observations and others [93] strongly imply the involvement of TACE activities in cancer progression [75,78,60,7] and support the clinical targeting of TACE using either small molecule chemical inhibitors [164,165], pro-domain analogs [166], or monoclonal antibodies [94]. The currently available and studied TACE inhibitors are presented (Table 3). Thus far, INCB7839 is the sole TACE inhibitor clinically tested on a cohort of breast cancer patients [167]. In that phase II clinical trial, patients receiving INCB7839 treatment exhibited moderate stabilization and decreased levels of EGF ligands [167]. Many other TACE inhibitors have been tested for the treatment of autoimmune diseases, and were shown to be safe at the dose tested but the treatment efficacy was limited [168,169]. In these clinical studies, the effects of

TACE inhibition on TAMs including altered macrophage phenotype, infiltration and activities, however, were not addressed. Thus, based on the substrate released by TACE, inhibitors of this enzyme may decrease both recruitment and activation of pro-tumor TAMs within the tumor microenvironment, potentially altering the observed clinical responses. For example, inhibiting TACE on tumor cells decreases the concentrations of CSF1-shed in the tumor microenvironment, thereby reducing the chemotaxis, infiltration and activation of pro-tumor TAMs [71,20,57].

2.9.2 TAM Inhibition

Ongoing approaches include the direct targeting of TAMs (detailed in Table 3). Methods modulating the presence and activities of macrophages at the tumor site through direct targeting of the pro-tumor effects of TAMs are under investigation [170,87,171,172]. For instance, the well-tolerated macrophage inhibitor PLX-3397 combined with other therapies was tested in the phase II clinical trial in patients with Hodgkin lymphoma [173]. In that trial, PLX-3397 led to significant decreases in both circulating monocytes and CTLs [173]. A similar treatment strategy with PLX-3397 in combination with chemotherapy for the treatment of triple negative metastatic breast cancer provided supportive preclinical data [12]. Currently patients with metastatic breast cancer are being recruited for a phase II clinical trial to determine the efficacy of PLX-3397 for treatment of metastatic breast cancer [174]. Oral administration of the macrophage inhibitor Clodronate for the treatment of early stage breast cancer led to decreased recurrence and metastasis in women over the age of 50 [175]. By decreasing pro-tumor TAM abundance in breast tumors, TACE inhibitors may have similar effects to macrophage inhibitors. Thus far, the data available supports the use of TACE

inhibitors as an additional approach to prevent pro-tumor macrophages, thereby limiting breast cancer progression.

2.10 Future Prospects and Challenges

The pro-tumor effects of TACE activation have been demonstrated in multiple solid tumors including breast cancer. The data strongly suggest that either targeted TACE inhibitors or inhibitors of TACE substrates that focus on the tumor / stroma cell interactions may have therapeutic benefits. Indeed, in addition to the autocrine effects of tumor cell TACE activities on tumor progression, data collected in the past decade indicate a clear link between TACE activities / the distribution of immuno-modulatory TACE substrates and TAMs infiltration, phenotype and functions. TACE inhibition prevents autocrine tumor growth factor signaling and may hinder pro-tumor macrophages within the breast tumor microenvironment. Specifically, targeted TACE inhibition may decrease the abundance and activation of pro-tumor TAMs within the breast tumor microenvironment.

Given the multiple effects of TACE inhibitors on both tumor and stroma cells, such a therapeutic approach, depending on the route, dose and schedule, will likely modulate both macrophage trafficking and activation. To improve TACE-derived therapeutic approaches, a better understanding of the effects of TACE inhibition on macrophages and other stromal cells is needed. Indeed, successful targeted therapies are always initiated by concrete and encompassing basic research. Furthermore, the transition from thinking of tumors as populations of transformed cells towards a complex organ involving multiple cell-cell interactions supporting malignancy makes understanding a therapies effect on the microenvironment of great interest. Options including targeting

TACE on specific cell types or specific areas of the tumor to minimize undesired side effects on the tumor microenvironment may prove more efficacious than systemic inhibition. Furthermore, defined subsets of breast cancer patients may benefit from TACE inhibitor treatments, based on the heterogeneity of their tumors, especially those patients with increased TAM abundance.

Such approaches as targeting specific substrates of TACE, modulation of TACE activities and TAM infiltration within the tumor mass will provide new targeted therapeutic approaches to treat breast cancer patients but come with their own sets of challenges. First, mutations in TACE, although rare, may generate tumors which are unresponsive to TACE inhibition. In this case downstream substrates may provide better targeting options. Next, deciding which downstream TACE substrates are most appropriate to target will vary patient to patient depending on the makeup and composition of the breast tumor microenvironment. For example, CSF1 may be a suitable target in tumors with increased TAM abundance whereas targeting ICAM1 or L-selectin targeting would benefit patients with increased vasculature or lymphocyte abundance, respectively. Finally, developing chemical inhibitors that specifically inhibit the catalytically active conserved metalloproteinase domain of TACE thus far have proven difficult. Although, more specific chemical inhibitors are being developed the use of monoclonal antibodies or pro-domain analogs may be more suitable to target TACE.

In conclusion, a full understanding of the effects TACE inhibition on both tumor cells and stoma cells will improve our ability to appropriately and proficiently treat breast cancer patients.

2.11 Tables and Figures

TABLE 2.1. TACE substrates expressed during mammary development and / or breast tumorigenesis

Classification & Substrates	Mammary development		References
	Normal	Cancer	
Growth factors			
AREG	√	√	[97] [114] [7]
HB-EGF	√	X	[176] [18] [177]
TGFα	√	√	[97] [111] [7]
Cytokines			
TNFα	√	√	[178] [179] [180] [181]
Fractalkine	X	√	[182] [183] [184]
Receptors			
TNFR1	√	√	[185] [123]
TNFR2	√	X	[186]
M-CSFR	√	√	[187] [188]
NOTCH	√	√	[189] [190]
Adhesion molecules			
ICAM1	X	√	[134] [132]
VCAM1	X	√	[70] [136] [131]
L-selectin	X	√	[55] [191]
Other			
APP	√	√	[192] [15]
MUC1	X	√	[193] [194]

√ - denotes the molecules involvement in either normal mammary development or mammary cancer progression.

X - denotes unknown / untested effects on normal mammary development or mammary cancer progression.

TABLE 2.2. TACE substrates involved in macrophage activation, migration and apoptosis

Substrates	**Macrophage functions**			**References**
	Activation (alternative)	Migration	Apoptosis	
CSF1	+	+	-	[20] [42] [57]
CSFR1	+	+	-	[20] [42]
TNFα	-	+	-	[195] [196]
TNFR1	+	-	+	[197] [196]
TNFR2	+	-	+	[197]
TGFα	+	NA	+	[198] [199]
AREG	+	-	-	[200]
IL1Rα	+	-	-	[201]
MUC1	+	+	-	[139] [13] [202]
VCAM1	+	+	-	[70] [137]
ICAM1	-	+	-	[203] [204]
L-selectin	-	+	-	[205] [206]
LAG3	-	NA	NA	[207]
CD30	-	+	NA	[208] [209]
CD40	-	NA	-	[210] [211]
IL6R	-	NA	-	[212] [23]
Fractalkine (CX3CL1)	NA	+	-	[213] [214]
CXCL16	+	+	NA	[215] [216]
JAM-A	-	+	NA	[217]
MICA	-	NA	NA	[218] [218]

+ and – denote whether the molecule positively or negatively affects the alternative (M2) activation, migration and/or apoptosis of macrophages, respectively. NA indicates that the molecule has no known effect on that specific macrophage function.

TABLE 2.3. TACE inhibitors and macrophage targeted treatments currently available (and / or under investigation)

Classification & Name	**Targets(s)**	**Manufacturer**	**References**
TACE inhibitors			
WAY-022	TACE	Wyeth-Aherst	[164]
TMI-2	TACE	Pfizer	[165]
INCB3619	TACE, ADAM10	Incyte	[219]
INCB7839	TACE, ADAM10	Incyte	[220]
GW280264X	TACE, ADAM10	Glaxo Smith Kline	[221]
TAM inhibition approaches			
Anti-CPG/IL-10	M2s		[170]
Bindarit	CCL2, MCSF		[87]
PLX3397	Macrophages / mast cells	Plexxikon	[173]
Type 1 IFNα	TAMs		[222]
Trabectedin	TAMs	Johnson and Johnson	[223]
Clodronate liposomes	TAMs		[85]
Liposome	TAMs		[224]
HRG	PDGF		[171]
Silibinin	NFκB, STAT3		[172]
Legumain	TAMs		[225]
LCL-PLP	TAMs		[226]

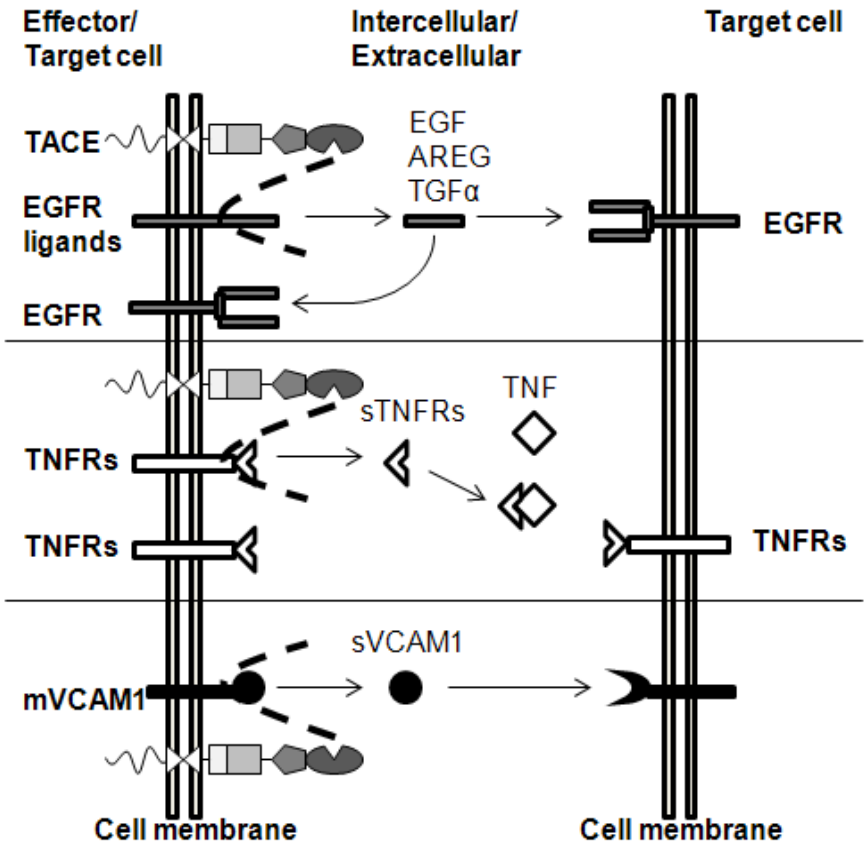

Figure 2.1. The different outcomes of TACE / ADAM17 shedding in autocrine / paracrine cell signaling. TACE is involved in proteolytic ectodomain shedding of membrane-bound ligands. The released ligands i.e., EGF, AREG, TGF□, TNF, sTNFRs and sVCAM1 are free to bind to and activate EGFR, TNFRs and α4β receptors (VLA4/α4β1 and α4β7). This ligand / receptor interaction can be either autocrine or paracrine leading to downstream signaling in both effectors and target cells.

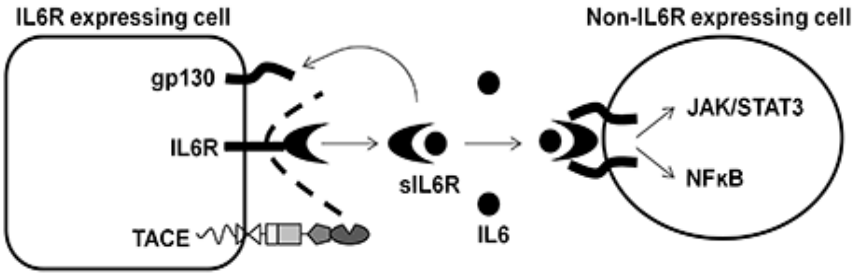

Figure 2.2. TACE activation and shedding of sIL6R in IL6 trans-signaling. Outline of IL6 trans-signaling mediated by TACE. TACE shedding of IL6R produces sIL6R, the initial step in IL6 trans-signaling. Next, sIL6R binds to free IL6 in the microenvironment and this complex binds to the universally expressed signal transducer, gp130. The sIL6R/IL6 complex has a longer half life than IL6 alone. This process stimulates downstream activation of IL6 signaling pathways including JAK/STAT3 and NFκB.

CHAPTER 3: SOLUBLE TUMOR NECROSIS FACTOR RECEPTORS SHED BY BREAST TUMOR CELLS INHIBIT MACROPHAGE CHEMOTAXIS

3.1 Abstract

Breast tumor cells alter their microenvironment in part through the expression of pro-tumor molecules that influence macrophages during tumor progression and metastasis. Macrophage recruitment is stimulated by chemotactic factors including tumor necrosis factor alpha (TNFα) which also stimulates the cytotoxic/tumor cell killing macrophage phenotype. Through TNFα converting enzyme (TACE/ADAM17) activities, breast tumor cells shed membrane-bound proteins including their TNF receptors (sTNFR1/2), which serve as decoys sequestering TNFα and preventing TNFα-driven apoptosis of tumor cells, thereby decreasing TNFα bioavailability. Here, we investigated the levels of sTNFRs shed by breast tumor cells and determined the effects of shed sTNFRs on macrophage migration toward TNFα. TNFα and sTNFRs concentrations were measured in murine normal epithelial, stromal, and mammary tumor cells. The migration of murine macrophages towards TNFα in the presence of tumor derived soluble factors (TDSFs) shed by TACE was determined. TNFα concentrations secreted by tumor and normal epithelial cells were below the detection limit contrasting with stromal cells, especially macrophages, which expressed higher levels of TNFα ($p<0.001$). Regardless of the cell tested, treatment with the TACE inhibitor TAPI-0 led to a significant decrease in sTNFR2 shed ($p<0.05$). The dose-dependent macrophage migration toward TNFα prevented by incubation with TDSFs was not observed with

TDSFs collected following TAPI-0 treatment ($p<0.05$). Furthermore, the TNFα -driven increased pAkt expression in macrophage was inhibited by TACE shed TDSFs ($p<0.05$). These results highlight the role of tumor-shed sTNFRs in TNFα -driven macrophage chemotaxis.

3.2 Introduction

Breast cancer is a common malignancy among women with significant mortality associated with the development of metastasis, and a 5-year survival rate of 23% for women with metastatic breast cancer at diagnosis [227,1,2]. The invasion and metastatic spread of breast cancer is greatly influenced by the tumor microenvironment [5]. Furthermore, the effectiveness and outcome of the standard of care for late stage breast cancer, which includes radiation and surgical resection and/or systemic chemotherapy and hormone therapy [228] heavily relies on the composition and expression profile of the microenvironment [229].

The breast tumor microenvironment consists of non-malignant cells that infiltrate the developing tumor including fibroblasts, adipocytes, endothelial cells and immune cells, all of which may enhance cancer progression [230]. Tumor associated macrophages (TAMs) account for a large fraction of the infiltrating immune cells within most breast tumor masses and their presence has been linked to poor prognosis [14,231]. The phenotype of TAMs is similar to M2 macrophages, which are associated with wound-healing properties [43]. In contrast to M1 macrophages which promote cytotoxicity, M2 macrophages through their cytokine and chemokine expression promote tumor growth and invasiveness [43,38]. TAMs contribute to tumor progression and invasion through remodeling of the extracellular matrix (ECM), release of growth and angiogenic factors and suppression of antitumor immune responses [22,14].

At the tumor site, the macrophages are influenced by various physical and chemical interactions with the tumor and surrounding microenvironment. Some early inflammatory cytokines expressed in the tumor mass include tumor necrosis factor alpha

(TNFα) and colony stimulating factor 1 (CSF-1) [232,233]. TNFα is expressed by multiple cell types including tumor cells, macrophages, and adipocytes [234,59,235]. The binding of TNFα to either one of its receptors, TNF Receptor 1 (TNFR1) or TNFR2 promotes tumor cell apoptosis or survival respectively, but also stimulates macrophage migration and secretion of pro-inflammatory molecules further promoting macrophage infiltration [8,236]. The TNFα binding to TNFR1 activates c-Jun N-terminal kinases (JNK) and c-Jun, whereas TNFα binding to TNFR2 led to the activation of Akt in various cells including macrophages [237,49]. In particular, TNFα promotes monocyte/macrophage invasion through positive chemotaxis and has been associated with increased metastasis [234,59]. The TNFα signaling pathway is modulated by various factors including lipopolysaccharide (LPS), transforming growth factor β (TGF-β) and interleukin 10 (IL-10) [238,24]. This pathway is also modulated by the bioavailability of both TNFα and TNFRs within the tumor microenvironment. Indeed, cells through ectodomain shedding by TNFα converting enzymes (TACE) release both TNFα and soluble (sTNFRs) which can neutralize the response to shed TNFα thereby preventing TNFα signaling [239]. Increased expression of TACE in breast tumor cells is linked to poor prognosis [7]. Although the general mechanisms of the shedding of sTNFRs and TNFα is well understood [109,78], the role of the TACE activity of breast tumor cells on the migration of macrophages has yet to be fully investigated.

Increased local and systemic concentrations of shed molecules including TNFRs, CSF-1 and CSF-1R have been implicated in inflammatory/autoimmune diseases and some malignancies [240,64]. A link between serum concentrations of sTNFR in breast cancer patients and poor prognosis has not been demonstrated [241], possibly because the

serum sTNFR concentrations significantly differ from the sTNFR concentrations within the tumor [242]. Indeed, in addition to sTNFRs expression that has primarily been assessed in immune cells [243,244], both adipocytes and breast tumor cells also shed sTNFRs and CSF-1 [64,20].

Whether sTNFRs shed by tumor cells through TACE activities modulate the chemotaxis of macrophages toward TNFα is unknown. Here we investigated the chemotaxis and signaling of macrophages toward TNFα in the presence of tumor derived soluble factors (TDSFs) collected following treatments with or without a sheddase inhibitor. Results underline the role of sTNFRs in modulating macrophage chemotaxis.

3.3 Materials and Methods

Cell culture conditions

Murine mammary epithelial cells NMuMG, carcinoma cells 4T1, endothelial cells 2H11, and mesenchymal stem cells D1 were obtained from ATCC (Manassas, VA). Murine mammary cells 67NR and 4T07 were a generous gift from Dr. Miller (Karmanos Cancer Institute, Detroit, MI). Media supplies were obtained from Mediatech (Herndon, VA). Epithelial and endothelial cells were cultured at 37°C and 5% CO_2 in DMEM media supplemented with 10% FBS, gentamycin, and amphotericin B. For NMuMG and D1 cells, media was also supplemented with 10µg/ml of insulin and 4 mM glutamine (SigmaAldrich, St. Louis MO), respectively. Adipocytes were derived from D1 cells following incubation with a differentiation treatment composed of 100 µg/ml insulin, 0.5 µM dexamethasone (SigmaAldrich), and 0.5 mM isobutylmethylxanthine (SigmaAldrich) for 48 hours [58]. To block the shedding of TNFRs, 4T1 and NMuMG cells were treated with the TACE inhibitor TAPI-0 diluted in DMSO (250 nM; CalBiochem, Rockland, MA) for 24 hours.

The macrophage J774.2 and RAW264.7 (here on referred to as J774 and RAW, respectively) cells were obtained from ATCC. These cells were cultured in DMEM supplemented with 10% FBS, 1.5g/l $NaCO_3$, 4.5g/l glucose, 4 mM glutamine amphotericin B and gentamycin (all reagents were obtained from Mediatech).

Collection of conditioned media

Conditioned media (CM) was obtained as described previously [58,245]. The collection time was optimized through a time curve and 48 hour incubations were optimal. Briefly, epithelial (NMuMG, 4T1), endothelial (2H11), pre-adipocyte (D1),

differentiated adipocyte and monocyte (J774, RAW2674.7) cells were cultured in media described above at 37°C and 5% CO_2. For tumor cells, conditioned media (CM) contains tumor-derived soluble factors (TSDFs), and thus TDSFs is used to refer to 4T1 CM. Once cells reached 90% confluence serum free media with TACE inhibitor treatment (TAPI-0) or vehicle control (DMSO) was added for 24 hours. Treatments were removed by washing twice with phosphate buffer saline (PBS) and cells were incubated in 7 ml RPMI media depleted of serum and phenol red. Following a 48 hour incubation, the CM was collected, filtered (0.2 µm) and stored at -20°C. The volume of each conditioned medium was adjusted to 1 ml/10^6 cells based on the number of cells present in the culture vessel as determined by Trypan blue cell counting at collection time.

Immunocytochemistry Analyses

J774 and RAW cells (50,000 cells/well) were seeded in 8 well chamber slides and allowed to grow for 24 hours until confluent. Cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes at 37ºC and blocked with 1% bovine serum albumin (BSA) for 30 minutes and then incubated with either anti-TNFR1 or anti-TNFR2 antibodies for 1 hour at room temperature. After washing, cells were incubated with a fluorophore (Texas red) - conjugated secondary anti-rabbit antibody for 1 hour at room temperature. Cells were stained with the vital dye Hoechst and mounted with VectaShield (Burlingame, CA). The presence of either TNFR1 or TNFR2 was visualized using a IX71 fluorescent microscope (Olympus) and microphotographs were taken using similar conditions of fluorescence illumination for a given set of immune-stained samples and similar conditions of magnification (Size is denoted by a bar on microphotographs) using a DP70 camera (Olympus).

Transwell Chemotaxis Assays

J774 and RAW macrophage cells (60,000 cells/well) were seeded in serum-free media supplemented with the vital nuclear dye Hoechst (1:2000 dilution) in the top chamber of transwell migration chambers in 24 well plates. The lower chambers were filled with 500 µL (1:2 dilution) of either TDSFs collected after treatment with or without TAPI-0 (250 nM) in the presence or in the absence of TNFα (0.5ng-15ng) or control media (0% FBS, 10% FBS for negative and positive controls, respectively). After 6 hours, cells were removed from the upper side of the transwell membrane using a cotton swab and microphotographs of the cells attached to lower side of the membranes were taken (at least 5 random fields; 200x magnification), counted and the number of macrophages that migrated was normalized to the total transwell membrane surface area.

TNFα and sTNFRs ELISAs

TNFα and sTNFR levels were assessed using ELISAs conducted following the manufacturer's recommendations (R&D Systems, Minneapolis, IN) with all steps conducted at room temperature. Briefly, 96-well plates were coated with the capture antibody and incubated overnight. Following blocking with 1% bovine serum albumin for 1 hour, CM samples were added, and the plates were incubated for 2 hours. In the subsequent incubations, a biotin conjugated detection antibody and streptavadin-Horseradish Peroxidase (HRP) were added for 60 and 20 minutes, respectively. The presence of HRP-conjugated complexes was determined following the addition of the substrate solution (TMB, Pierce Inc. Rockford, IL) and the enzymatic reaction stopped by the addition of H_2SO_4 (2N). Optical densities (450 nm) resulting from HRP activities were measured using a microplate reader (Biotek, Winooski, VT) and based on standard

curves ran along with the samples, TNFα and sTNFRs were expressed in pg/ml per 10^6 cells.

Western Blots

Protein lysate immunoblotting was conducted as described earlier [246]. Briefly, 25 µg of total protein per well were loaded on 8% polyacrylamide gels and run in SDS-PAGE denaturing conditions and the proteins were then transferred onto nitrocellulose membranes. Loading of equal protein amounts was assessed by staining membrane with 0.1% Ponceau S (Sigma) in 5% acetic acid and further assessed by evaluating the presence of ß-actin by immunoblots. After a 1-hr incubation with TBS-T (0.1% Tween 20) containing 5% nonfat milk to block non-specific binding, membranes were incubated with antibodies specific for Akt and pAkt (Santa Cruz biotechnology), pJNK and cJun (Cell signaling, Danvers, MA) or β-actin (SigmaAldrich, St Louis, MO). Following a one-hour incubation with the appropriate HRP-conjugated secondary antibody and the addition of a chemiluminescent substrate (Pierce, Rockford, IL), the presence of protein was detected using a biochemiluminescent imaging system and the VisionWork software (UVP, Upland, CA). Differences in protein expression were evaluated by densitometry using Quantity One software (Biorad, Hercules, CA) following normalization to ß-actin expression.

Flow Cytometry

Following cell collection using trypsin (epithelial cells) or scraping (macrophages), cells were fixed with 1% PFA for 30 minutes at room temperature and resuspended in PBS supplemented with 1% BSA. The presence of TNFR1 and TNFR2 surface receptors was determined by cell surface staining using antibodies specific for

TNFR1 and TNFR2, respectively. Briefly, resuspended cells were incubated with either anti-TNFR1 or anti-TNFR2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 minutes at 4ºC. Cells were then washed twice with PBS and incubated with appropriate FITC-conjugated secondary antibody (Invitrogen, Grand Island, NY). Control stain included the secondary antibody alone. Following additional washes, the presence of cell surface TNFR1 and of TNFR2 cells was monitored by flow-cytometry (Fortessa cytometer, Becton Dickinson, San Jose, CA, USA). Analyses were conducted using the CellQuest software and graphical representation was obtained using FlowJo software. Data are presented as percentage of positive cells for either TNFR1 or TNFR2. The control corresponding the background stain associated with the secondary antibody is displayed on each histogram.

Statistical Analyses

All data were expressed as mean ± SEM. Statistical analyses were conducted using one-way ANOVAs and Newmann-Keul post-hoc tests (Prism, Graphpad Software, Inc., La Jolla, CA). Significance was set a priori to p value below 0.05. A correlation between concentration of TAPI-0 and sTNFR2 excretions was determined using a linear regression analysis of log transformed values.

3.4 Results

TNFα is secreted by mammary stromal cells, in particular macrophages but not epithelial or tumor cells

First, we determined whether TNFα, which is mainly produced by stromal cells, and often found at high levels in breast carcinomas, was secreted by the murine cells investigated here by ELISA in conditioned media (CM) from each cell type. TNFα was not detected (ND or below the detection limit) in the CMs collected from the non-invasive (67NR), non-metastatic (4T07) and metastatic (4T1) murine mammary carcinoma cells or murine epithelial cells (NMuMG) (Fig. 3.1A). In contrast, TNFα was present in the culture media of murine mesenchymal D1 stem cells, differentiated adipocytes and 2H11 endothelial cells (Fig. 3.1A). The J774 and RAW macrophage cells secreted between 10-fold and 40-fold higher concentrations of TNFα than other stromal cells ($p<-.005$, Fig. 3.1B). Furthermore, the expression of TNFα varied among the macrophage cells tested with much higher levels of TNFα in the CMs collected from RAW cells ($p<0.001$).

TNFR1 and TNFR2 are expressed on the cell surface of macrophages and tumor cells

As TNFα signaling is initiated through binding of TNFα to one of its two cognate receptors TNFR1 or TNFR2 bound to the cell membrane, using immunocytochemistry (ICC), Western blots and flow cytometry, we investigated TNFR1 and TNFR2 expressions on murine macrophages. TNFRs were expressed by both J774 and RAW macrophages as assessed by ICC (data not shown), Western blots (data not shown) and flow cytometry (Fig 2A, B). The tumor cells tested by flow cytometry expressed TNFRs regardless of their metastatic potential (Fig. 3.2A, B). Interestingly, whereas the

expression of TNFR1was comparable between the cells tested, the expression of TNFR2 was consistently higher in the metastatic 4T1 cells as compared to the non-invasive 67NR and non-metastatic 4T07 cells (Fig. 3.2B).

sTNFR1 and sTNFR2 are shed by mammary epithelial and cancer cells and this shedding can be blocked through incubation with the TACE inhibitor TAPI-0

To determine whether TNFR1 and TNFR2 are shed through ectodomain shedding, the soluble forms of TNFR1 (sTNFR1) and TNFR2 (sTNFR2) were measured by ELISA in secretions from 4T1 and NMuMG cells. Both sTNFR1 and sTNFR2 were present in CMs from 4T1 and NMuMG cells with sTNFR1 concentrations similar between 4T1 and NMuMG cells (Fig. 3.3A). Concentrations of sTNFR2 in 4T1 CM was significantly higher than concentrations of sTNFR1 in 4T1 CM and sTNFR2 in NMuMG (5.6 fold and 1.8-fold, respectively, $p<0.001$, Fig. 3.3A).

The chemical inhibition of TACE through incubation with the TACE/ADAM-17 specific inhibitor TAPI-0 led to a dose-dependent decrease in sTNFR2 concentrations in 4T1 CM ($r^2=0.9634$, $p=0.0185$, Fig. 3.3B). Compared to vehicle treatment, the incubation with the TACE/ADAM-17 specific inhibitor TAPI-0 at 250 nM led to significant decreases in sTNFR2 concentrations in both NMuMG and 4T1 CMs ($p<0.05$, Fig. 3.3B).

TNFα-driven macrophage chemotaxis is inhibited by 4T1 TDSFs but not by 4T1 TDSFs collected following treatment with the TACE inhibitor TAPI-0

Next, we investigated whether sTNFRs present in 4T1 tumor CMs modulated TNFα-driven macrophage chemotaxis, by sequestering TNFα. Increasing concentrations of TNFα led to a dose-dependent increase in the chemotaxis of J774 macrophages (Fig. 3.4A, B) and RAW macrophages (Fig. 3.4C, D). The addition of 4T1 CMs inhibited the

chemotaxis of both J774 and RAW macrophages towards either 1.5 or 15 ng/ml concentrations of TNFα (Fig. 3.4A-D).

In contrast, the 4T1 CMs collected following treatment with the TACE/ADAM17 inhibitor, TAPI-0 and thus containing lower sTNFR concentrations did not inhibit the TNFα-driven macrophage chemotaxis ($p < 0.001$, Fig. 3.5).

The TNFα-driven macrophage chemotaxis is in part mediated through the Akt pathway and blocked by tumor TACE-shed molecules

To ascertain whether specific pathways downstream of TNFα/TNFR signaling were involved in the observed alterations of macrophage chemotaxis led by 4T1 TDSFs, the Akt and JNK pathways were analyzed in both J774 and RAW macrophages following activation and inactivation by TDSFs of the TNFα/TNFR signaling pathway. No significant differences were observed in total Akt, cJun, pJNK and β actin regardless of treatment (n.s., Fig. 3.6 A, D). However, the ratio of pAkt/total Akt protein expression in J774 and RAW macrophages was significantly increased following incubation with increasing TNFα concentrations ($p<0.05$, Fig. 3.6 B, C). This increase in pAkt/total Akt was inhibited by concomitant 4T1 TDSFs and TNFα treatments but not by concomitant treatments with TNFα and 4T1 TDSFs collected following TAPI-0 treatment ($p<0.05$, Fig. 3.6 A-C).

3.5 Discussion

The pleitropic cytokine TNFα is expressed in breast cancer tissue and stimulates macrophage migration and activates cytotoxic macrophages (i.e., M1 macrophages); however, M1 macrophages are mostly absent within the breast tumor [59,38,247,195]. TNFα has been shown to promote chemotaxis of macrophages in various pathologies; however, its role in macrophage trafficking to the breast tumor site is unclear [247,248,8]. The primary mechanisms by which tumor cells alter macrophages include release of immunomodulatory factors (CSF1, CCL2, CCL5) leading to increased recruitment and stimulation of alternatively activated macrophages (i.e., M2) [8,119,25]. Importantly, the shedding activities of TACE/ADAM17, highly expressed by cancer cells [7], leads to the release of TNFRs. However, the role of TACE activities and tumor shed TDSFs on TNFα-driven macrophage chemotaxis remains to be addressed. Furthermore, whether the observed TNFα-driven recruitment of macrophage to inflamed tissues is through either activation of TNFR1 or TNFR2 which stimulate the JNK and Akt signaling pathways, respectively, in breast cancer is unclear [248,247,237,8]. Our data show that stromal cells including macrophages shown to be present in the breast tumor microenvironment secrete TNFα [8]. Furthermore, the results indicate that TNFα-driven macrophage chemotaxis is dose-dependent. More interestingly, our data highlight a mechanism by which mammary tumor cells alter the response of macrophages to TNFα by shedding their TNFRs through TACE activities leading to an inhibition of TNFα-driven macrophage chemotaxis. In addition, our results suggest a role for the Akt pathway in the TNFα-driven macrophage chemotaxis.

The *in vitro* models used here to investigate the TNFα-TNFR signaling between tumor cells and macrophages have been utilized extensively to further our understanding of the pro-tumor microenvironment [19,249]. Despite their limitations, in vitro 2D and 3D models of mammary tissues and breast cancer progression have proven invaluable in the assessment of the mammary microenvironment including the cell-cell interactions between tumor and stroma cells [19,249].

The results presented here confirm that the TNFα pathway is active in the tumor microenvironment through TNFα mainly secreted by stromal cells including macrophages and by signaling via the membrane-bound TNFR1 and TNFR2 expressed on the macrophage cells [250,251]. TNFα activates TNFR1 or TNFR2, the latter of which lacks a death domain, leading to either cell death or cell survival, respectively [252-254]. In line with a previous study [251], we show TNFR1 and TNFR2 are expressed by both malignant and stromal cells in the breast tumor including macrophages. Interestingly, here we further demonstrate TNFR2 levels are relatively higher in the metastatic tumor cells (4T1) as compared to non-metastatic tumor cells (4T07 and 67NR) and macrophages (J774 and RAW) (Figure 3.2B).

The increased expression of TNFR2 may be one of the mechanisms by which breast tumor cells subvert apoptosis and promote their survival, as observed in colon cancer [255]. Alternatively, the pro-apoptotic effects of TNFα/TNFR signaling are diminished through the sequestration of TNFα by soluble forms of TNFR1 and TNFR2 [256]. Our data highlight the shedding of TNFR especially TNFR2 through TACE/ADAM17 activities. The increase in sTNFR2 shed by tumor cells observed here may be associated with a higher cell surface expression and/or a preferential shedding of

TNFR2 by TACE/ADAM17. In addition, increased internalization of the receptors by normal cells may also be involved [257].

TNFα is present in the breast tumor microenvironment, however, its stimulation of tumor cell apoptosis has been shown to be prevented by tumor cells shedding TNFRs [256]. Macrophages also respond to TNFα concentrations present in the breast tumor microenvironment through both paracrine and autocrine signaling leading to prolonged inflammation caused by a positive feedback loop with TNFα [258]. The effects of TNFα on macrophage migration have been seldom studied in breast tumors. Our data indicate that tumor cells through the shedding of TNFR2 significantly inhibit the macrophage chemotaxis toward TNFα in part through inhibition of the Akt pathway. The inhibition of the TNFα stimulated Akt pathway downstream of TNFR2, but not of the JNK/c-Jun pathway, in macrophages by TACE-shed TDSFs strongly support the modulation of the infiltration and cytotoxic activities of the macrophage subsets within the tumor microenvironment. The inhibition of the TNFα-driven macrophage chemotaxis by tumor conditioned media and especially by TACE tumor-shed molecules highlight the importance of this mechanism. Furthermore, although the data presented do not address directly the macrophage infiltration of the tumor mass, the strong modulation of the macrophage chemotaxis and invasion observed here may interfere with the recruitment and or differentiation of cytotoxic macrophages within the tumor mass [247,8].

Our observations, that sTNFRs shed by tumor cell TACE activity negatively impact the ability of TNFα to stimulate macrophage chemotaxis further underscore the pro-tumor role of TACE/ADAM17 [7,163]. Our results (not shown) and others demonstrate the presence and activities of TACE in all the epithelial and tumor cell tested

[7]. Tumor cell TACE activities shed many growth factors and immunomodulatory cytokines that play key roles in tumor progression [60]. Indeed, TACE inhibitors are currently in phase II clinical trials for a subset of metastatic breast cancer patients [167,259]. To date, clinical trials using TACE inhibitors have been unsuccessful partly because of the lack of specificity exhibited by inhibitors tested [75,60]. The data presented here suggest that the testing of more specific inhibitors may be more successful. Furthermore the targeting of sTNFRs especially sTNFR2 may also modulate both the infiltration of cytotoxic macrophages and/or the activation of cytotoxic macrophages within the breast tumor microenvironment that in turn may promote tumor regression.

Taken together, our findings along with previous studies support a mechanism by which mammary tumor cells abrogate the TNFα signaling response in macrophages by shedding their TNFRs through TACE enzyme activities. This pathway offers many potential targets to promote the cytotoxicity of macrophages in the breast tumor beyond the direct actions on TNFα or TACE activities including interferences with the breast tumor concentrations of sTNFR1 and sTNFR2, respectively. Further validation of these observations may provide new avenues with more targeted approaches promoting the stimulation of the patient's own immune system especially macrophages leading to the therapeutic benefit of the destruction of breast tumor cells.

3.6 Figures

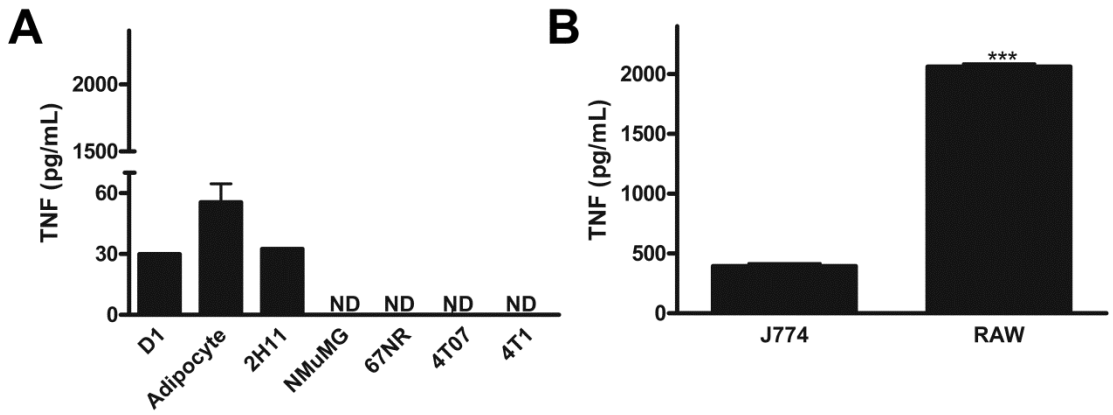

Figure 3.1. Mammary stromal cells but not epithelial or tumor cells secrete TNFα. CMs were harvested following a 24-hour serum starvation period and a 48-hour incubation with phenol red free RPMI of the following murine cells: (A) D1 mesenchymal stem cells, D1-derived adipocytes, 2H11 endothelial cells, NMuMG mammary gland epithelial cells, 67NR, 4T07 and 4T1 mammary tumor cells and (B) J774 and RAW macrophage cells. The volume of each CM solution was adjusted to 1 ml per 10^6 cells counted at collection time. TNFα concentrations (pg/ml, mean ± SEM) were determined by ELISA. ***$p<0.001$.

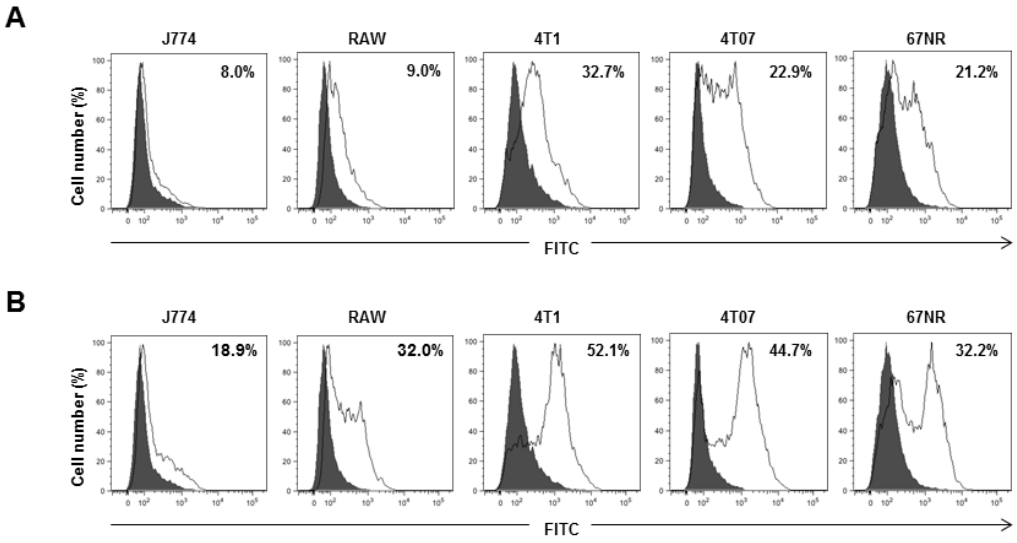

Figure 3.2. Both TNFR1 and TNFR2 are expressed by mammary tumor cells and macrophages. (A) TNFR1 and (B) TNFR2 surface receptor expression (white area) represented as percent positively stained cells assessed by flow cytometry. The background is measured by only staining cells with fluorescently labeled secondary antibody (grey area).

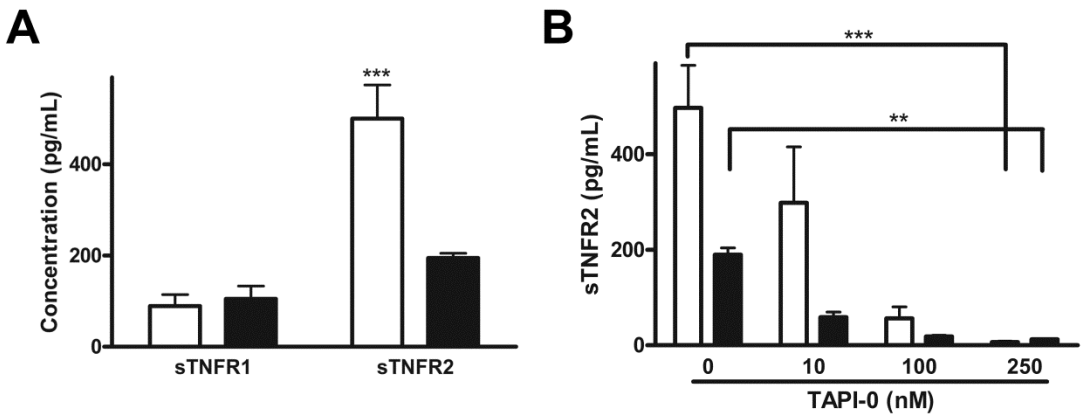

Figure 3.3. 4T1 mammary tumor cells shed more sTNFR2 than sTNFR1 and treatment with the TACE inhibitor TAPI-0 prevented sTNFR2 shedding. (A) Both CM from NMuMG (open bars) and 4T1 (darkened bars) cells contained sTNFR1 and sTNFR2 (pg/ml per10^6 cells ± SEM) as determined by ELISA. sTNFR1 concentrations present in CMs from both cells were not significantly different. The sTNFR2 concentration in 4T1 CM was significantly higher than the concentration of sTNFR1 (5.6-fold for 4T1, $p<0.05$). Furthermore, the concentration of sTNFR2 was much higher in 4T1 TDSFs than sTNFR2 in NMuMG CM ($p<0.001$). (B) Incubation with increasing doses of the TACE inhibitor TAPI-0 was associated with a significant decrease in sTNFR2 present within 4T1 (open bar) and NMuMG (darkened bar) CMs. The decrease in sTNFR2 was dose-dependent from 0 to 250 nM of TAPI-0 (physiologic dose – 50 mg/kg (Han and others 2010)). The treatment with 250nM of TAPI-0 led to a significant decrease in sTNFR2 present in 4T1 TSDFs and NMuMG CM. **$p<0.01$, ***$p<0.001$.

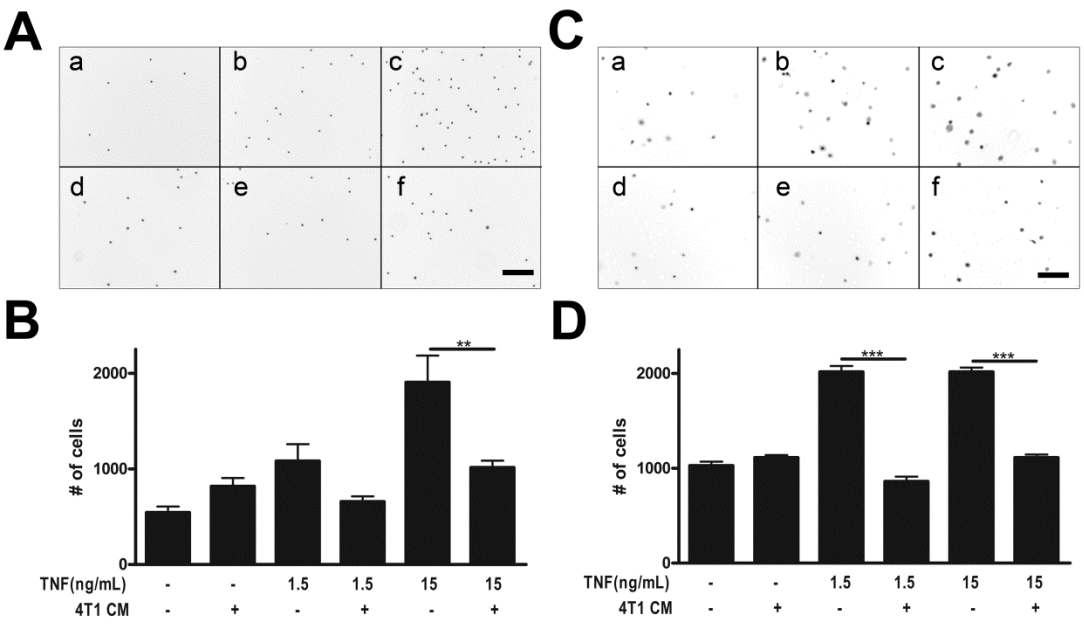

Figure 3.4. TNFα-driven macrophage chemotaxis is inhibited by factors in 4T1 TDSFs. J774 (A, B) and RAW (C, D) macrophage chemotaxis towards increasing concentrations of TNFα with or without TDSFs was assessed using transwell assays (see materials and methods for details). (A, C) Representative microphotographs (bar = 100 μm) of the chemotaxis of J774 (A) and RAW (C) cells. All microphotographs were taken at the same magnification and inverted. Briefly, macrophages stained with the vital dye Hoechst were resuspended in serum free media and placed in the top wells of transwell inserts and allowed to migrate toward (a) serum free media, (b) 1.5 and (c) 15 ng/mL TNFα or (d) 4T1 TDSFs (1:2 dilution) alone or 4T1 TDSFs (1:2 dilution) and (e) 1.5 or (f) and 15 ng/ml of TNFα for 6 hours. (B, D) Quantifications of J774 (B) and RAW (D) macrophage chemotaxis. Following chemotaxis, cells were counted and expressed as total number of migrated cells per well (average ± SEM). The data presented are representative of at least three independent experiments. ***$p<0.001$ and **$p<0.01$.

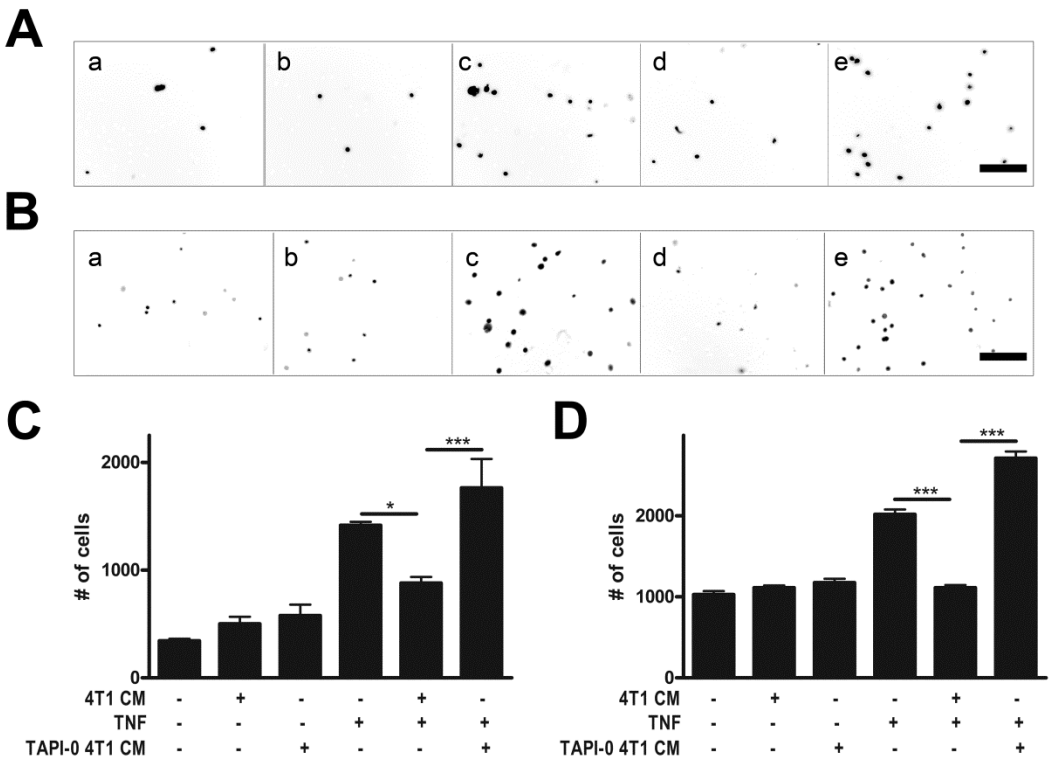

Figure 3.5. TACE-shed TDSFs decrease TNFα-driven macrophage chemotaxis. (A, B) representative microphotographs (bar = 100 μm) of J774 (A) and RAW (B) macrophage chemotaxis. Briefly, the TNFα-driven chemotaxis of J774 and RAW cells with or without TDSFs or with TDSFs collected following TAPI-0 treatment was assessed using transwell assays. All the microphotographs were taken at the same magnification and inverted. Macrophages stained with the vital dye Hoechst were resuspended in serum free media and placed in the top wells of transwell inserts and allowed to migrate toward (a) serum free media, (b) 4T1 TSDF (1:2 dilution) (c) 15 ng/mL TNFα or (d) 4T1 TDSFs collected following TAPI-0 treatment (TAPI-0 4T1 TSDFs) or (e) the combination TAPI-0 4T1 TSDFs and TNFα for 6 hours. (C, D) Quantifications of the chemotaxis of J774 (C) and RAW (D) macrophages. Following chemotaxis, cells were counted and expressed as total number of migrated cells per well (average ± SEM). The data presented are representative of at least three independent experiments. *** $p<0.001$ and ** $p<0.01$.

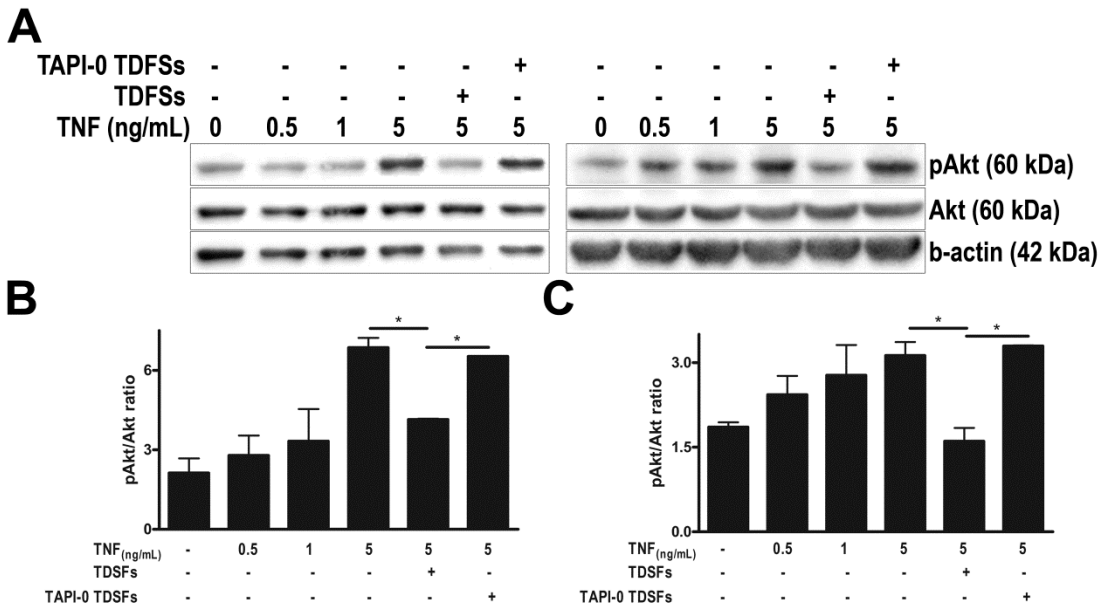

Figure 3.6. The expression of pAkt is increased in TNFα-stimulated J774 and RAW macrophages incubated with 4T1 TDSFs collected following TAPI-0 treatment. (A) Representative immunoblots of pAkt, Akt and β actin expressions by J774 (A-left panel) and RAW (A-right panel) macrophages following treatment with TNFα, 4T1 TDSFs and/or TDSFs collected following TAPI-0 treatment. The ß-actin expression served as loading control (see material and methods for details). (B, C) Quantification of the expression of key downstream molecules involved in TNFR signaling. The ratio of pAkt/total Akt protein expression in J774 (B) and RAW (C) macrophages were significantly increased following incubation with TNFα. This increase was inhibited by concomitant 4T1 TDSFs treatment but not by but not by 4T1 TDSFs collected following TAPI-0 treatment. Data are presented as pAkt/total Akt intensity ratio (average ± SEM, no unit).

CHAPTER 4: BREAST TUMOR CELL TACE-SHED MCSF PROMOTES PRO-ANGIOGENIC MACROPHAGES THROUGH NF-κB SIGNALING

4.1 Abstract

Most deaths associated with breast cancer, the most common malignancy in women, are caused by metastasis. Tumor associated macrophages (TAMs) significantly contribute to breast cancer progression and development of metastasis through the promotion of angiogenesis which involves a central regulator of macrophage functions: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Macrophages are activated by macrophage colony stimulating factor (MCSF) and chemokine (C-C motif) ligand 2 (CCL2) to secrete angiogenic factors including vascular endothelial growth factor (VEGF). The release of macrophage colony stimulating factor from tumor cells is mediated by ectodomain shedding through tumor necrosis factor alpha converting enzyme activation (TACE). Here we determined whether tumor cells TACE-shed MCSF promotes angiogenesis through the activation of the NF-κB pathway in macrophages and the subsequent release of VEGF. These interactions were modeled *in vitro* using a panel of mammary cells mimicking the breast cancer progression from normal murine mammary gland (NMuMG) cells to metastatic 4T1 cells along with J774 macrophages, all derived from BALB/c mice. TACE and MCSF expressions were higher in metastatic cells compared to epithelial cells ($p<0.05$). Tumor conditioned medias (CMs) activated the expression of VEGF by macrophages through the stimulation of NF-κB pathway and the resulting macrophage secretions that promoted high levels of endothelial cell tubes.

Furthermore, the combinations of CCL2, also highly expressed by tumor cells, and MCSF promoted pro-angiogenic macrophages. These results highlight the key role of tumor cell TACE-shed MCSF and secreted CCL2 in stimulating pro-angiogenic macrophages.

4.2 Introduction

Breast cancer is the most commonly diagnosed malignancy among women with significant mortalities associated with the development of metastasis [1,2]. The progression and metastatic spread of breast cancer is greatly influenced by the tumor microenvironment [5]. Tumor associated macrophages (TAMs), in particular, are vital for both breast cancer progression and the successful dissemination of metastasis [14]. Indeed, TAM abundance correlates with both decreased survival and increased metastasis in breast cancer patients [14,231].

Macrophages can be activated to recognize and destroy malignant cells; however, the tumor promoting macrophage phenotypes are prevalent in the breast tumor microenvironment. Pro-tumor TAMs are characterized by secretion of factors that promote growth, chemotaxis, angiogenesis and remodeling of the extracellular matrix (ECM) [38,42]. The phenotype expressed by TAMs is stimulated by cues present in the breast tumor microenvironment including soluble immuno-modulatory factors produced by tumor cells [260]. Many of these soluble factors are released through tumor cell ectodomain shedding mediated by tumor necrosis factor alpha converting enzyme (TACE) [75], whose expression in breast tumors correlates with decreased patient survival [7]. TACE-shed molecules involved in tumor cell - macrophage interactions include macrophage colony stimulating factor (MCSF) [261], tumor necrosis factor (TNF) [76], epidermal growth factor receptor (EGFR) ligands [262] and intercellular adhesion molecule 1 (ICAM1) [75,263]. Of these TACE-shed molecules, MCSF regulates many pro-tumor functions of TAMs especially the promotion of tumor cell invasion and angiogenesis [14,42]. Furthermore, tumor cell - macrophage interactions

directed by MCSF also leads to cell streaming and the subsequent generation of the tumor microenvironment of metastasis [40]. The role of TACE activities in promoting pro-tumor macrophages at the breast tumor site remains unclear.

Macrophages infiltrate the breast tissue through chemotaxis stimulated mainly by cytokines including MCSF and chemokine (C-C motif) ligand 2 (CCL2) [21,264]. Furthermore, within the breast tissue, macrophages are activated by multiple factors of both stroma and / or tumor cell origin including MCSF and CCL2 [9,42]. Expressions of MCSF and its corresponding receptor MCSF receptor (MCSFR) have been linked to poor prognosis in breast cancer patients, mainly due to their roles in macrophage recruitment and activation [72,265]. Indeed, MCSF shed by tumor cells stimulates EGF production in macrophages leading to a paracrine feedback loop essential to tumor cell invasion and extravasation [122]. The roles of MCSF in the earlier steps of metastasis, including angiogenesis, have been demonstrated in many cancers [266]. In breast cancer, however, the role(s) and the mechanisms of MCSF in the early steps of cancer progression remain to be fully defined.

MCSF stimulates macrophage production and secretion of the potent angiogenic factor vascular endothelial growth factor (VEGF). TAM release of VEGF is a major contributor to angiogenesis in tumors which is a rate-limiting step essential to tumor progression and metastasis [41,267,268]. As angiogenesis supports progression from pre-malignant to malignant tumors, blocking vessel growth alone or in combination with other treatments has demonstrated significant benefits in patients with colon lung, kidney and brain cancers [269,270]. For example, the use of bevacizumab®, an inhibitor of VEGF-A, has been clinically successful [270]. However, this anti-angiogenic therapy had

no significant overall survival benefit for the treatment of patients with breast cancer [270,271]. The disparities between the benefits of anti-VEGF therapies in some cancers compared to breast cancer highlights the need for further understanding of breast tumor angiogenesis.

In macrophages, VEGF expression and secretion are stimulated through multiple intracellular pathways especially the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling [48]. Indeed, both MCSF and CCL2 activate NF-κB signaling pathways in macrophages leading to increased survival and the release of factors that promote tumor cell invasion [272]. Typically upon activation of NF-κB signaling, IκB the inhibitor of κB is phosphorylated leading to its ubiquitination and proteasomal degradation allowing active NF-κB dimers to translocate to the nucleus and initiate transcription [273,274]. These signaling events are critical for development / progression of cancer and macrophage VEGF release, but the characterization of the tumor cell macrophage interactions involved in promoting these events remains to be clearly understood in breast cancer [48,275].

Thus, we investigated whether breast tumor cell TACE-shed MCSF activated NF-κB in macrophages leading to the production of VEGF and subsequent angiogenesis. The results indicate that tumor cell TACE-shed MCSF along with CCL2 stimulates pro-angiogenic macrophages through the NF-κB signaling pathway.

4.3 Materials and Methods

Cell lines and culture conditions

Murine mammary carcinoma cells 4T1, mammary epithelial cells NMuMG, endothelial cells 2H11 and macrophages J774.2 (hereto forth referred to as J774) were purchased from ATCC (Manassas, VA, USA). 4T07 and 67NR cells were a generous gift of Dr. Miller (Karmanos Cancer Center Detroit MI). All cells (except J774 and NMuMG) were cultured at 37ºC and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), gentamycin, and amphotericin B obtained from Mediatech (Herndon, VA, USA). NMuMG cells were cultured in the same media supplemented with 10 µg/mL insulin. J774 cells were cultured in DMEM supplemented with 10% FBS, 1.5g/l $NaCO_3$, 4.5g/l glucose, 4 mM glutamine amphotericin B and gentamycin (all media reagents obtained from Mediatech).

Collection of conditioned media

Conditioned media (CM) were obtained as described previously [58,245]. Briefly, 4T1 carcinoma cells were cultured in media described above at 37°C and 5% CO_2 until 90% confluent. Media was then replaced with serum-free media supplemented with either 250 nM of the TACE inhibitor (TAPI-0) or the vehicle control (dimethyl sulfoxide (DMSO)) and cells were cultured for an additional 24 hours. Following washes with phosphate buffered saline (PBS) to remove treatments and an additional 48 - hour incubation in serum-free and phenol red-free RPMI media (7 ml), CMs were collected, filtered (0.2 µm) and stored at -20°C. Volumes of each CM were adjusted to 1 ml per 10^6 cells based on the number of cells present in the culture vessel as determined by Trypan

blue exclusion cell counting at collection time. Macrophage CMs were collected similarly following treatments with MCSF, CCL2 and/or specified 4T1 CMs.

Inhibition of tumor cell TACE activities

Carcinoma cells' TACE shedding activities were inhibited through either incubation with the TACE specific inhibitor TAPI-0 (CalBiochem, Rockland, MA, USA) or specific TACE small interfering RNA (siRNA) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were incubated with the TACE inhibitor TAPI-0 (250 nM in DMSO) in 0% FBS media for 24 hours. Knockdown of TACE expression in cells was achieved through treatments with TACE siRNA (100 nM; Santa Cruz Biotechnology, Santa Cruz, CA) for 48 hours according to manufacturer's protocol. Briefly, 400,000 4T1 cells were seeded in 6-well plates and incubated for 24 hours in 10% FBS media free of antibiotics. Cells were then incubated for an additional 24 hours with TACE siRNA (100 nM) complexed with Lipofectamine® 2000 (Life Technologies, Grand Island, NY). Following washes, cells were incubated with fresh media for an additional 48-hours before both cell lysates and supernatants were collected and stored at -20º C.

Macrophage treatments

J774 macrophages were incubated with 4T1 tumor cell conditioned media (4T1 CMs) and their pro-angiogenic properties including the concentrations of VEGF secreted determined. Additionally, the role of the NF-κB pathway in the pro-angiogenic macrophage activities was assessed through pretreatments with the NF-kB inhibitor BAY 11-7083 (20 μM; Minneapolis, MN, USA) for 30 minutes before stimulation with 4T1 CMs. Macrophages were also treated with increasing doses of murine MCSF (0-1000

ng/mL, BioVision, Milpitas, California, USA) and / or CCL2 (100 ng/mL) for 24 hours where indicated.

Immunoblot analyses

Protein lysates obtained from tumor cells and macrophages following treatments were collected and prepared for immunodetection as described earlier [246,130]. Briefly, 25 µg of total protein per well were loaded on 8% polyacrylamide gels and separated with SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes using a semi-dry transfer method. Ponceau S (0.1%, Sigma) staining was performed to determine even loading. Membranes were blocked with TBS-T (Tris-buffered saline-0.1% Tween 20) containing 5% nonfat milk to minimize non-specific binding, then incubated with antibodies specific for MCSFR, p-MCSFR, NF-κB p65, p-NF-κB p65 (Cell signaling, Danvers, MA, USA) or β-actin (SigmaAldrich, St Louis, MO, USA). After several washes in TBS-T, membranes were incubated with a species-specific horseradish peroxidase (HRP) conjugated secondary antibody. Following subsequent washes, a chemiluminescent HRP substrate (Pierce, Rockford, IL, USA) was added. Antibody-bound proteins were detected using a biochemiluminescent imaging system and the VisionWork software (UVP, Upland, CA, USA). Differences in protein expressions were evaluated by densitometry using Quantity One software (Biorad, Hercules, CA, USA) following normalization to ß-actin expression or to the total unphosphorylated protein where indicated.

Flow-cytometry analyses

Following detachment using trypsin or scrapping, cells were fixed in 1% paraformaldehyde (PFA) and then resuspended in PBS before staining for flow-

cytometry analyses. TACE surface protein expression was determined as previously described [130]. Briefly, after a 10-minunte incubation in 1% bovine serum albumin (BSA) solution to prevent non-specific binding, cells were incubated with a TACE specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 minutes at room temperature. Following washes, cells were then incubated with a fluorescein (FITC) conjugated secondary antibody (Life Technologies, Grand Island, NY, USA). Background staining was determined following incubation with the secondary antibody alone. Following additional washes, the presence of TACE on the cells was determined by flow-cytometry analyses (Fortessa cytometer, Becton Dickinson, San Jose, CA, USA). Analyses and graphical representation were obtained using the FlowJo software (Ashland, OR, USA). The background associated with the secondary antibody alone is displayed on each histogram and data representative of at least three independent repeats are presented as percentage of TACE positive cells.

MCSF and VEGF ELISAs

MCSF and VEGF levels were assessed using ELISAs conducted following the manufacturer‘s recommendations (R&D Systems, Minneapolis, IN, USA). All steps were conducted at room temperature. Briefly, plates were coated with either a MCSF or a VEGF - specific capture antibody diluted in phosphate buffer saline (PBS) and incubated overnight. Following blocking with 1% BSA for 1 hour, CM samples were added and the plates were incubated for 2 hours. In a subsequent 2 - hour incubation, MCSF or VEGF - specific biotin conjugated detection antibodies were added. Following 20 minute incubations with the complex streptavidin - HRP, the amount of bound detection antibody was detected through the addition of the HRP substrate solution (TMB, Pierce

Inc. Rockford, IL, USA). After stopping the reaction with a 2N sulfuric acid (H_2SO_4) solution, the absorbance (450 nm) of each well was determined using a microplate reader (Biotek, Winooski, VT, USA) and the concentrations (pg/mL) of MCSF and VEGF present in each sample were derived from standards ran along the samples.

Tube formation assays

In vitro tube formation assays were performed as described previously [51,52] with the following modifications. Briefly, 2H11 cells were seeded at a density of 40,000 cells per well onto Matrigel™ (San Jose, California, USA) coated 96 well plates. These 2H11 cell cultures were incubated with specific angiogenic molecules including VEGF and / or macrophage CMs. 2H11 cell cultures were incubated at 37°C and 5% CO_2 for 9 hours and the tube formation determined. The presence of tubes was recorded through microphotographs (40X magnification) using an IX71 fluorescent microscope equipped with a DP70 camera (Olympus, Center Valley, Pennsylvania, USA). The numbers of tubes with lengths greater than 100 µm were counted as described earlier and averages from at least 3 separate experiments are presented.

Cytokine arrays

Cytokine arrays of 4T1 CMs were performed following the manufacturer's recommendations (RayBiotech, Norcross, GA, USA). All steps were performed at room temperature. Briefly, membranes were incubated in blocking buffer for 30 minutes. Next, following 2 hour incubations with 4T1 CMs, washes, another 2 hour incubation with a cocktail of specific biotin-conjugated antibodies, cytokine array membranes were washed and incubated with streptavidin - HRP. The HRP activity was detected using a chemiluminescent substrate and signal detected using a biochemiluminescent imaging

system and the VisionWork software (UVP, Upland, CA, USA). Relative differences in protein expressions were evaluated by densitometry using Quantity One software (Biorad, Hercules, CA, USA).

Statistical analyses

All data were expressed as mean ± SEM unless noted otherwise. Differences between parameters tested were determined using one-way ANOVAs and Newmann-Keul post-hoc tests (Prism, Graphpad Software, Inc., La Jolla, CA, USA). Significance was set *a priori* to p value below 0.05.

4.4 Results

TACE is expressed at higher levels by 4T1 metastatic mammary tumor cells than by less invasive carcinoma cells and normal mammary epithelial cells.

First, the expression of TACE was determined on a panel of murine mammary tumor cells and murine epithelial cells. Western blot analyses of protein lysates collected from cells demonstrated the presence of TACE. TACE expressions in epithelial cells (NMuMG) and non-invasive carcinoma cells (67NR) were lower than TACE expression detected in the invasive (4T07) and metastatic (4T1) mammary cells (Figure. 4.1A and B). Indeed, 4T1 cells expressed significantly higher levels of TACE compared to NMuMG cells ($p < 0.05$). Moreover, flow-cytometry analyses of the cell-surface expression of TACE on mammary tumor cells indicated that 4T1 and 4T07 mammary tumor cells were consistently more positive for cell surface TACE expression than 67NR cells (Figure. 4.1C).

MCSF shed through tumor cell TACE activity activates the MCSFR on J774 macrophages.

Presence of the TACE substrate MCSF in the supernatants of the mammary carcinoma cells tested was measured over time. The MCSF concentration in tumor cells peaked after a 48-hour incubation in serum-free media (Figure 4.2A). Following 48-hour incubations, as measured by ELISAs on CMs collected from epithelial and mammary tumor cells MCSF was not detected in NMuMG CMs. However, MCSF was shed / secreted at significantly lower concentrations in CMs from 67NR cells compared to CMs from 4T07 and 4T1 carcinoma cells ($p < 0.01$, Figure 4.2B). Furthermore, the inhibition of TACE activities using either treatments with the chemical TACE inhibitor TAPI-0 or

with a specific TACE siRNA led to significant decreases in MCSF concentrations present in the CMs of 4T1 cells compared to the MSCF concentrations detected in 4T1 CM under control conditions ($p < 0.05$, Figure 4.2C).

Whether tumor cell TACE-shed MCSF activated MCSFR on J774 macrophages was analyzed through Western blots. Macrophages treated with 4T1 CMs and/or MCSF (200ng/ml) displayed a significantly higher ratio of the activated MCSFR (pMCSFR) normalized to total MCSFR compared to those treated with 4T1 CMs collected after TACE inhibition (TAPI-0 4T1 CM) ($p < 0.01$, Figure 4.2D). Furthermore, macrophages treated with TAPI-0 4T1 CMs supplemented with MCSF produced similar levels of pMCSFR / MCSFR as those treated with 4T1CMs.

TACE-shed MCSF from 4T1 cells induces J774 macrophages to secrete VEGF and promote endothelial cell tube formation.

J774 macrophage CMs collected after treatment with tumor cell CM were assessed using ELISAs for VEGF and endothelial cell tube formation assays. Macrophage VEGF secretions induced by 4T1 CMs were significantly higher than the controls and TAPI-0 4T1 CM treatment ($p < 0.001$, Figure 4.3A). Macrophages treated with TAPI-0 4T1 CMs supplemented with MCSF secreted similar concentrations of VEGF compared to 4T1 CM treated macrophages. Treatment of macrophages with 100 ng/mL of MCSF alone stimulated higher VEGF secretions than control conditions ($p < 0.05$, Fig 4.3A). However, the concentrations of VEGF secreted were significantly lower than the concentrations of VEGF secreted following macrophage incubation with 4T1 CMs ($p < 0.05$, Figure 4.3A).

CMs collected from macrophages treated with both 4T1 CMs (b) and TAPI-0 4T1 CM plus MCSF (d) led to significantly higher numbers of tubes in 2H11 tube formation assays compared to CMs collected from macrophages incubated with control media (a) or TAPI-0 4T1 CM (c) alone ($p < 0.001$, Figure 4.3B). CMs obtained from macrophages treated with MCSF alone (e) did not promote 2H11 tube formation (Figure 4.3C) suggesting the presence of at least one additional factor in the tumor CMs that along with MCSF promote macrophages with pro-angiogenic properties. In contrast, addition of increasing VEGF concentrations to endothelial cells (f and g) led to a dose-dependent increase in 2H11 tube formation (Figure 4.3C).

The stimulation of macrophage NFkB signaling pathway by tumor cell TACE-shed MCSF is essential to the pro-angiogenic properties of macrophages.

Next, activation of the NFκB signaling pathway, critical in tumorigenesis, was investigated in macrophages. Macrophage pNFkB p65 / NFκB p65 ratios were significantly increased by treatment with total 4T1 CMs but not TAPI-0 treated 4T1 CMs as compared to control conditions ($p < 0.05$, Figure 4.4A). The increased pNFkB p65 / NFκB p65 levels were rescued by supplementing TAPI-0 4T1 CM treated macrophages with MCSF (Figure 4.4A). Additionally, treatment of macrophages with the NFκB pathway inhibitor BAY 11-7083 significantly diminished macrophage VEGF secretion following incubation with 4T1 CMs ($p < 0.001$, Figure 4.4B). Furthermore, the number of 2H11 tubes formed decreased following incubation of 2H11 cells with CM collected from BAY 11-7083 inhibited tumor cell CM treated macrophages ($p < 0.05$, Figure 4.4C). 2H11 tube formation was rescued by adding VEGF to BAY 11-7083 inhibited macrophage CMs (Figure 4.4C). Interestingly, whereas MCSF along with tumor

secretions promoted activation of NFκB, MCSF alone had no effect on NFκB activation. Taken together with observations that macrophage VEGF secretions and tube formation are significantly lower in MCSF stimulated macrophages compared to 4T1 CM stimulated macrophages (Figure 4.3) strongly suggest the presence of other factor(s) in the 4T1 CMs cooperating with MCSF to activate NFκB, stimulate VEGF production and promote angiogenesis.

Malignant mammary cells secrete significantly higher levels of CCL2 compared to mammary epithelial cells which is not affected by TACE inhibition.

High CCL2 expression was detected in 4T1 CMs as evaluated in cytokine arrays (Figure 4.5A). Furthermore, significantly higher CCL2 concentrations were secreted by malignant mammary cells (4T1, 4T07 and 67NR) compared to normal mammary epithelial cells (NMuMG) ($p < 0.05$, Figure 4.5B). Regardless of the cells tested, following treatment with TAPI-0, secreted CCL2 concentrations remained unchanged (n.s., Figure 4.5B).

TACE-shed MCSF and secreted CCL2 by tumor cells induce pro-angiogenic macrophages through the activation of MCSFR.

J774 macrophages treated with the combination of CCL2 and MCSF secreted significantly higher concentrations of VEGF than when treated with MCSF alone ($p < 0.05$, Figure 4.6A). Furthermore, compared to the CMs from macrophages treated with MCSF or CCL2 alone, the CMs collected from macrophages treated with the combination CCL2 and MCSF lead to significantly higher 2H11 tube formations ($p<0.001$, Figure 4.6B). In contrast, endothelial cells incubated with MCSF or CCL2 alone, at the same concentrations used for macrophage treatments, had no direct effects

on tube formation (n.s., data not shown). The number of 2H11 tubes formed by macrophage CMs collected following incubation with CCL2 was similar to the number of 2H11 tubes formed following incubation with macrophage CMs collected following incubation with TAPI-0 4T1 CMs (Figure 4.6B). Moreover, macrophage CMs collected following incubation with CCL2 and MCSF led to similar 2H11 tube formations as macrophage CMs collected following incubation with 4T1 CMs (Figure 4.6B). Macrophage pNFkB p65 / NFκB p65 protein expression ratios were significantly increased by treatment with MCSF and CCL2 alone compared to pNFkB p65 / NFκB p65 protein expression ratios in macrophage incubated in control conditions ($p < 0.05$, Figure 4.6C and D). Furthermore, following treatment with the combination MCSF and CCL2, macrophage pNFkB p65 / NFκB p65 protein expression ratios were significantly higher than when incubated with either cytokine alone ($p < 0.05$, Figure 4.6C and D**).**

Next, to determine whether CCL2 synergized with MCSF through increased activation of the MCSFR, levels of activated MCSFR in macrophages incubated with CCL2 and MSCF were assessed. Macrophages treated with MCSF alone but not with CCL2 alone expressed significantly higher pMCSFR / MCSFR protein ratios compared to macrophages incubated in control conditions ($p < 0.05$, Figure 4.7A and B). Moreover, macrophages incubated with the combination MCSF and CCL2 expressed significantly higher pMCSFR / MCSFR protein ratios compared to macrophage incubated with any other conditions tested ($p < 0.05$, Figure 4.7A and B). Furthermore, a dose-dependent increase of the pNFkB p65 / NFκB p65 protein expression ratio was observed in macrophages incubated with increasing MCSF (0-1000 ng/ml) concentrations (Figure 4.7C). However, since the concentrations of MCSF shed by macrophages treated with

increasing doses of CCL2 (0-1000 ng/ml) remained unchanged (Figure 4.7D), CCL2 likely does not promote an autocrine MCSF loop in macrophages.

4.5 Discussion

TAMs are critical regulators of the tumor vasculature [41] and in some areas of the tumor the principal producers of the potent angiogenic factor, VEGF [276]. Tumor-derived MCSF shed through TACE activities, and secreted CCL2 both stimulate macrophage production of VEGF [264,261,266]. The role of VEGF released from TAMs in promoting breast tumor angiogenesis is well established; however, the specific pathways involved remain elusive and anti-VEGF therapies lack effectiveness in the treatment of breast cancer [271]. Here using *in vitro* models, we demonstrate that (1) TACE, MCSF and CCL2 expressions are increased in invasive and metastatic mammary carcinoma cells and that (2) tumor cell TACE-shed MCSF activates the NF-κB associated signaling pathway in macrophages stimulating the secretion of VEGF which in turn leads to endothelial cell tube formation. Furthermore, our observations indicate that 4T1 cell TACE-shed MCSF together with secreted CCL2 promote the generation of pro-angiogenic macrophages. Taken together we propose a model in which breast tumor cell shed MCSF and secrete CCL2 stimulate pro-angiogenic macrophages through NF-κB (Figure 4.8).

In vitro models have been utilized extensively to advance our understanding of the complex cell-cell interactions occurring within the tumor microenvironment in both 2D and 3D cultures conditions [10,277]. In particular, previous works have revealed a critical interplay between tumor derived factors such as MCSF and CCL2, leading to the promotion of pro-tumor macrophages [73,130]. Additionally, the roles of stromal cells, including macrophages and mesenchymal stem cells, in the formation of mammary gland structures, such as blood vessels and acini, have been identified [277,11,54]. The

fundamental processes and pathways exposed using *in vitro* models has greatly enhanced our understanding of breast tumorigenesis [278].

In the present study, TACE was expressed at higher levels in invasive and metastatic murine mammary carcinoma cells supporting previous observations in human breast tumors [7,91]. Indeed, increased TACE expression in the tumors of breast cancer patients correlates to decreased survival [7]. Our observations further indicate that the TACE activities in tumor cells led to the shedding of multiple molecules including MSCF, which through paracrine interactions with macrophages promoted the stimulation of pro-tumor TAMs. This observation is in line with the previously identified role of tumor cell TACE shedding in blunting macrophage responses to the anti-tumor macrophage stimulator TNF [130]. In addition to paracrine signaling in tumors, TACE activities also strongly influence autocrine signaling. Indeed, tumor cell TACE shedding led to autocrine activation of EGFR and stimulated the mitogen-activated protein kinase (MAPK) signaling pathway resulting in a malignant phenotype of tumor cells [7]. Identifying the multiple TACE substrates cleaved from tumor cells involved in paracrine and autocrine pro-tumor activities may provide more specific targets for cancer treatment. Additionally, the non-shedding activities of TACE including adhesion through the disintegrin domain [279] and signaling through the cytoplasmic tail [280] may also be critical in breast cancer progression.

This study indicated that increases in soluble MCSF concentrations correlated to TACE expression and were higher in metastatic cancer cells. Others have shown MCSF response gene expressions to be associated with poor prognostic factors including high tumor grade, decreased expression of estrogen receptor / progesterone receptor and

increased p53 mutations in breast cancer patients [64]. Here, MCSF a key regulator of macrophages and of TAM functions [42,281] produced through tumor cell TACE shedding stimulated pro-angiogenic macrophages. Our observations indicated that MCSF, through macrophage MCSFR activation, led to the release of VEGF and to subsequent increases in endothelial cell tube formation highlighting the pivotal role of MCSF in promoting breast tumor angiogenesis through activation of macrophages [41,276]. These results confirm previous works that demonstrated MCSF induced release of VEGF from primary human monocytes [282] and that MCSF deficient mice have decreased TAMs abundance and subsequent diminished tumor vasculature [41].

The promotion of a pro-angiogenic macrophage phenotype through tumor cell TACE-shed MCSF was shown here to be dependent on NF-κB signaling. Indeed, NF-κB inhibition prevented the tumor stimulated macrophage release of VEGF and 2H11 tube formation. In macrophages, NF-κB is a central transcription factor involved in the activation of both pro- and anti-tumor genes [283-285]. MCSF derived from the metastatic human breast cancer MDA-MB-231 cells induced NF-κB signaling in RAW264.7 murine macrophages, which in turn caused release of nitric oxide to promote MDA-MB-231 tumor cell invasion [73]. Moreover, the stimulation with recombinant MCSF of macrophages led to VEGF production that was dependent on extracellular regulated kinase (ERK) signaling [266]. Additionally, the polarization of pro-tumor macrophages has been shown to be dependent on signal transducer and activator of transcription (STAT) signaling [283,286]. Together these data strongly suggest that multiple pathways, especially NF-κB, in TAMs likely cooperate to promote tumor angiogenesis.

Interestingly, the effects of 4T1 CM on the generation of pro-angiogenic macrophages could be reproduced using the combination of MCSF and CCL2. Indeed, here the formation of tubes by 2H11 murine endothelial cells was sensitive to VEGF released by macrophage stimulated with MCSF and CCL2. This is in contrast with human umbilical vein endothelial cells (HUVECs), which form tubes mainly in response to VEGF released by MCSF stimulated macrophages [282] and lack the ability to respond to other angiogenic factors, including interleukin 8 (IL-8) [287]. However, our findings are comparable to observations made in melanoma in which MCSF and CCL2 act together to promote macrophage VEGF release [288]. Whether the difference in sensitivity of macrophages and subsequent VEGF secretion may explain the ineffectiveness of anti-VEGF inhibitors in the treatment of melanoma [289] and breast cancer [271] remains to be fully investigated.

Taken together, these findings validate the role of MCSF shed from breast cancer through TACE activities and highlight the critical cell-cell interactions within the tumor microenvironment. The specific pathway identified here through which tumor cell TACE-shed MCSF activates NF-κB in macrophages leading to VEGF release and subsequent 2H11 tube formations (see figure 4.8) underlines potential targets including TACE, MCSF and CCL2 that if locally targeted and specifically inhibited alone or together may prevent TAM stimulated angiogenesis in breast cancer.

4.6 Figures

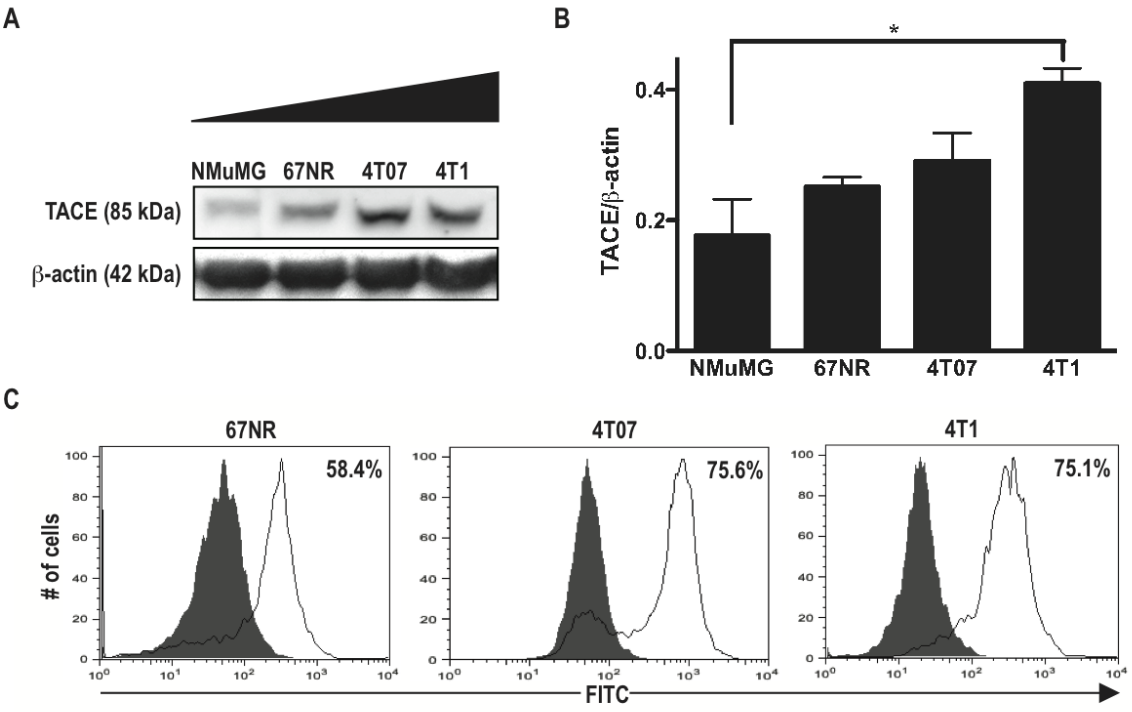

Figure 4.1. TACE expression is increased in metastatic mammary tumor cells. (A) Representative immunoblots of the TACE expressions in NMuMG epithelial cells, 67NR, 4T07 and 4T1 murine mammary carcinoma cells (level of aggressiveness denoted by gradient). ß-actin expression served as loading control. (B) Quantification of TACE expression normalized to ß-actin in epithelial and carcinoma cells indicates that metastatic 4T1 cells express significantly higher levels of TACE compared to NMuMG epithelial cells ($p < 0.05$). (C) TACE expression assessed by flow-cytometry. The background (shaded area) is defined by the non-specific binding associated with the use of the secondary antibody alone. The cell-surface expression of TACE (open area) by 4T07 and 4T1 murine mammary carcinoma cells was higher than the expression detected in 67NR cells. The observations presented are representative of 3 or more independent repeats.

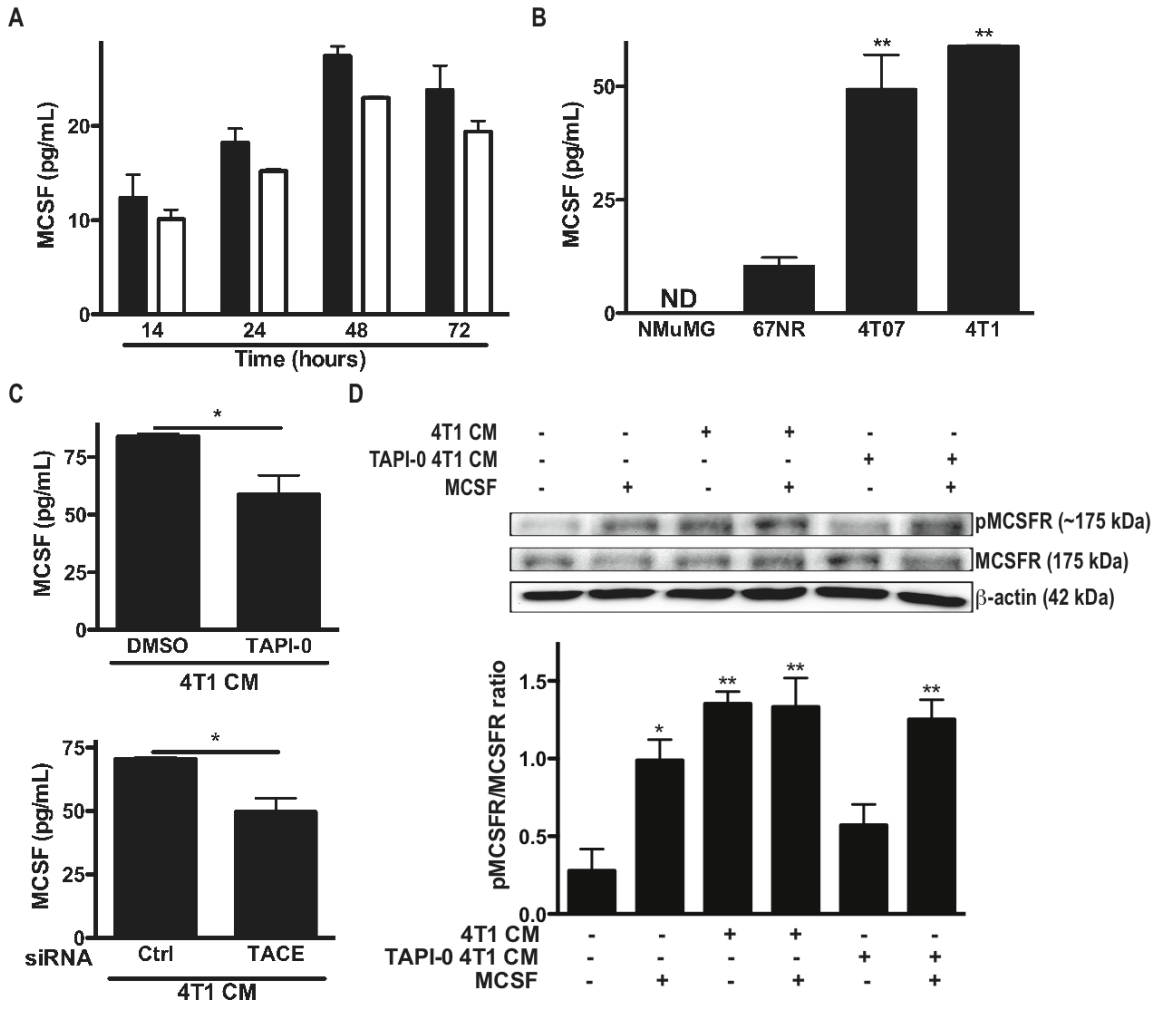

Figure 4.2. MCSF-shed through mammary tumor cell TACE activities is blocked by either a TACE inhibitor (TAPI-0) or TACE siRNA and promotes the activation of MCSFR on J774 macrophages. (A) By ELISAs, the highest concentrations (pg/ml) of MCSF were observed in supernatants collected over time from 4T1 (filled bars) and 4T07 cells (open bars) after 48-hour incubations. (B) Concentrations of MCSF determined by ELISA in CMs harvested following a 24-hour serum starvation / treatment period and a 48-hour incubation with phenol red free RPMI demonstrate that 4T07 and 4T1 cells shed significantly higher concentrations of MCSF than 67NR and NMuMG cells ($p < 0.01$). (C) Incubation of 4T1 cells with either the TACE inhibitor (TAPI-0; 250 nM) or a TACE specific siRNA (100 nM) lead to significant decreases of shed MCSF compared to control conditions ($p < 0.05$). (D) Immunoblots of protein lysates from J774 macrophages incubated with 4T1 tumor CMs and / or MCSF indicates that 4T1 CMs but not 4T1 incubated with TAPI-0 CMs promoted the activation of MCSFR leading to a higher pMCSFR / MCSFR ratio ($p < 0.01$). Moreover, the 4T1 incubated with TAPI-0 CMs supplemented with MCSF also increased pMCSFR / MCSFR ratio in J774 cell lysates ($p < 0.01$).

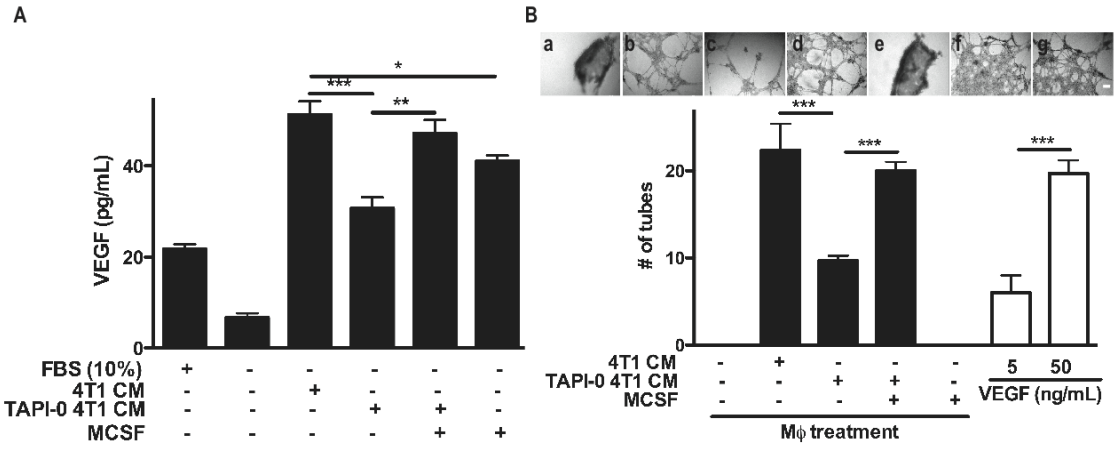

Figure 4.3. 4T1 mammary tumor cell TACE-shed MCSF stimulates the secretion of VEGF by J774 macrophage and induce endothelial cell tube formation. (A) Following incubation with tumor CMs and / or MCSF, concentrations of VEGF secreted by J774 macrophages were determined by ELISAs. Both 4T1 CMs and TAPI-0 4T1 CMs plus MCSF treatments promoted significantly higher VEGF secretions by macrophages compared to incubations with TAPI-0 4T1 CMs alone or control media alone ($p < 0.01$). Also, macrophages treated with 4T1 CMs secreted significantly higher concentrations of VEGF than those treated with MCSF only ($p < 0.05$). (B) The capacity of these macrophage CMs to promote 2H11 endothelial cell tube formation in Matrigel® was determined. Representative microphotographs of 2H11 tubes after treatments are provided (bar = 400 µm). CMs from macrophages treated with 4T1 CMs and TAPI-0 4T1 CMs plus MCSF promoted significantly higher numbers of tubes than TAPI-0 4T1 CMs and control media treated macrophages ($p < 0.001$). No tubes were observed with CM from macrophages treated with MCSF alone. VEGF promoted tube formation in a dose dependent manner.

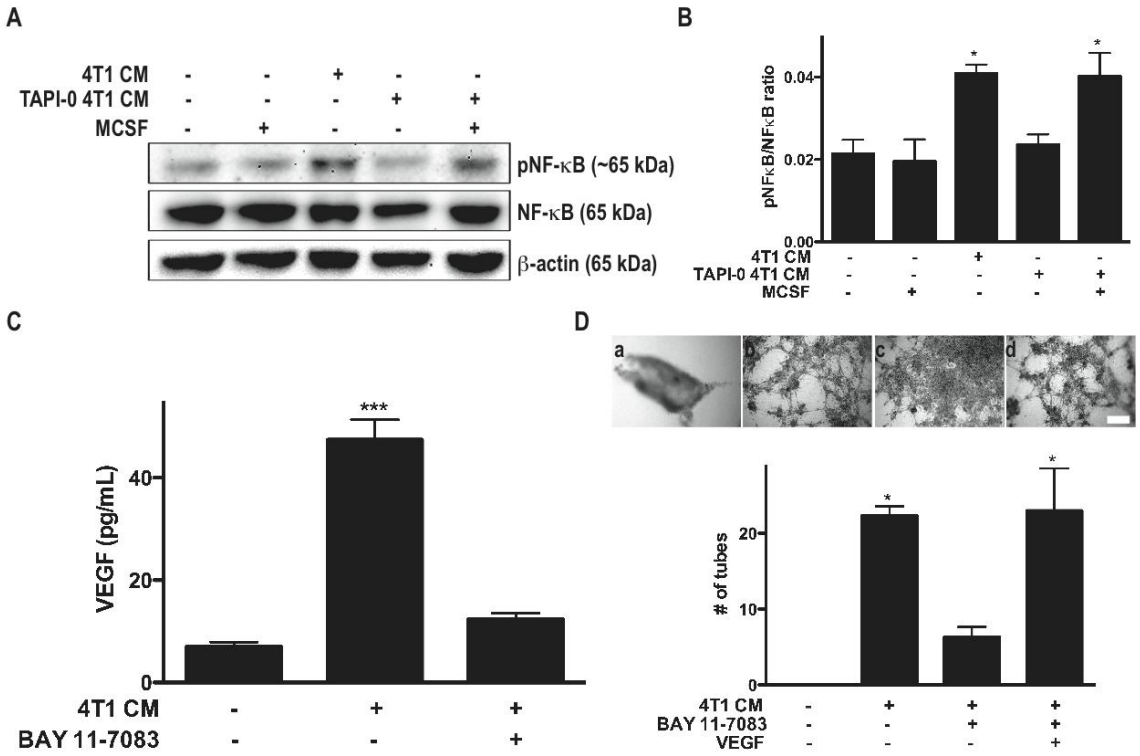

Figure 4.4. MCSF-shed by mammary tumor cell promotes NF-kB signaling in macrophages leading to the secretion of factors promoting endothelial cell tube formation. (A) Representative immunoblots of pNFκB p65, NFκB p65 and β actin expressions by J774 macrophages following incubations with MCSF, 4T1 CMs and / or 4T1 CMs collected following TAPI-0 treatments. (B) The ratio of pNF-κB p65 / total NF-κB p65 protein expression in J774 macrophages increased significantly following incubation with 4T1 CMs and TAPI-0 4T1 CMs plus MCSF treatments ($p < 0.05$). (C) VEGF concentrations present in the supernatants collected from J774 macrophages incubated with 4T1 tumor CMs were higher than in in the supernatants collected from J774 macrophages incubated without 4T1 tumor CMs ($p < 0.01$). Moreover, the supplementation of macrophages incubated with 4T1 tumor CMs with the NF-kB inhibitor (BAY 11-7083) inhibited that VEGF secretion. (D) Furthermore, secretions from macrophages treated with 4T1 CM promoted significantly higher 2H11 cell tube formation than control media treated macrophages ($p < 0.05$), whereas 4T1 treated macrophage CMs collected following NK-kB inhibitor treatment (BAY 11-7082) lead to significantly lower 2H11 tube formation. This decrease in 2H11 tube formation could be restored through VEGF supplementation ($p < 0.05$). Scale bar = 200 μm.

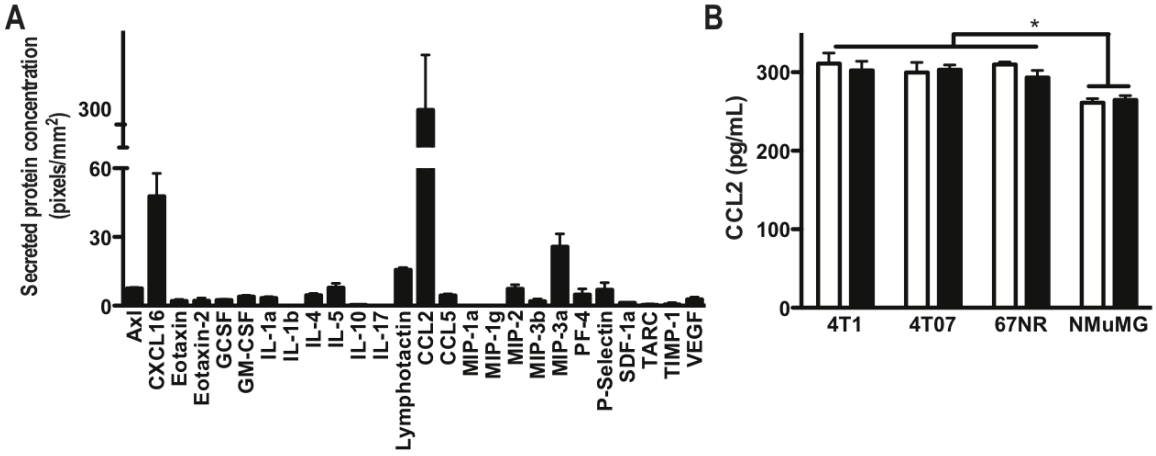

Figure 4.5. CCL2, highly expressed by 4T1 cells is secreted significantly more by murine tumor cells than by normal murine epithelial cells. (A) Among the inflammatory cytokines secreted by 4T1 cells as assessed using semi-quantitative protein arrays, CCL2 is highly expressed. (B) As determined by ELISA, concentrations of secreted CCL2 were significantly higher in the supernatants collected from malignant breast cells compared to the supernatants collected from normal breast epithelial cells (NMUMG). Following treatment with the TACE inhibitor TAPI-0 (filled bar) cells yielded similar CCL2 secretions regardless of the cells tested compared to cell incubated with the vehicle treatment (DMSO; open bar).

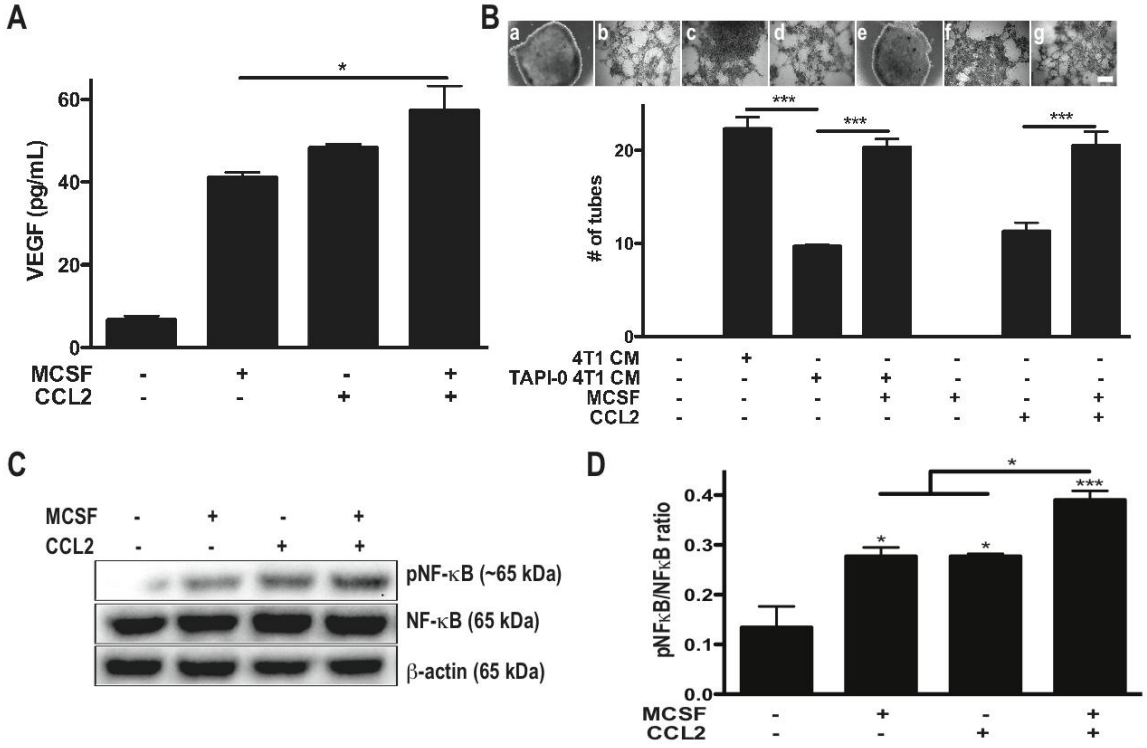

Figure 4.6. The combination of 4T1 tumor cell TACE-shed MCSF, 4T1 tumor cell secreted CCL2 promotes the macrophage secretion of VEGF and 2H11 endothelial cell tube formation. (A) J774 macrophages incubated with the combination MCSF (100 ng/ml) and CCL2 secreted higher VEGF concentrations than J774 cells incubated with MSCF alone. (B) Supernatants from macrophages treated with 4T1 CM, TAPI-0 4T1 CM plus MCSF, and MCSF plus CCL2 promoted significantly higher 2H11 endothelial cell tube formation than supernatants from macrophages treated with TAPI-0 4T1 CM, MCSF and CCL2 treatments alone ($p < 0.001$; Scale bar = 400 μm). (C) Representative immunoblots of pNFκB p65, NFκB p65 and β actin expressions by J774 macrophages following treatment with MCSF (200 ng/ml) and/or CCL2 (D) The ratio of pNF-κB p65 / total NF-κB p65 protein expression in J774 macrophages was significantly increased following incubation MCSF (200 ng/ml) or CCL2 alone ($p<0.05$). Furthermore, treatment with the combination MCSF and CCL2 led to an even higher pNF-κB p65 / total NF-κB p65 protein expression ratio in J774 macrophages ($p < 0.05$)

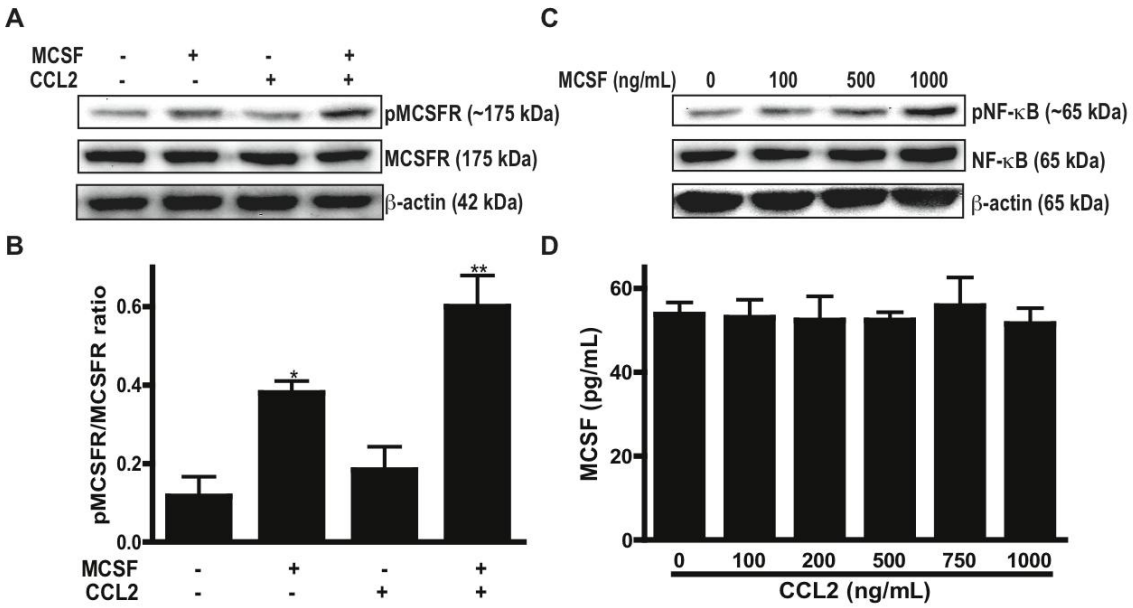

Figure 4.7. MCSF and CCL2 combined activation of J774 macrophage MCSFR. (A) Lysates from J774 macrophages incubated with MCSF or the combination MCSF and CCL2 expressed significantly higher pMCSFR / MCSFR ratios as shown in representative Western Blots. (B) Quantification of the pMCSFR / MCSFR ratios confirmed that J774 macrophages incubated with MCSF or the combination MCSF and CCL2 expressed significantly higher pMCSFR / MCSFR ratios compared to J774 cells incubated in control conditions ($p < 0.05$ and $p < 0.01$ respectively). (C) J774 macrophages incubated with increasing MCSF concentrations (0-1000 ng/ml) expressed dose-dependent increases in pNF-κB p65 / total NF-κB p65 ratios as shown in representative Western blots. (D) However, MCSF concentrations in the CM collected from J774 macrophages treated with increasing CCL2 concentrations (0-1000 ng/mL) remained unchanged

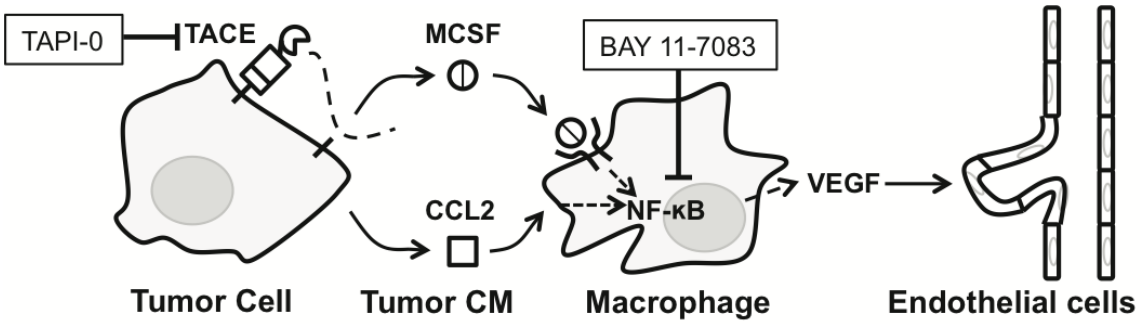

Figure 4.8. Proposed mechanism of the activation of pro-angiogenic macrophages by breast carcinoma cell TACE-shed MCSF. Tumor cells through TACE shedding of MCSF and secretion of CCL2 promote activation of the NF-κB signaling pathway in macrophages, which in turn secrete VEGF. The secretions of VEGF from these macrophages promote angiogenesis. Tumor CM stimulated release of VEGF and promotion of angiogenesis by macrophages can be blocked by inhibiting TACE (TAPI-0) on tumor cells or NF-κB (BAY 11-7083) in macrophages.

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

Breast cancer remains the most prevalent malignancy in women and among the most deadly [290]. The emergence of the tumor microenvironment as a major contributor to the progression and metastatic spread of cancer [5] highlights the need for a further understanding of the mechanisms and molecules involved in the promotion of a pro-tumor microenvironment. In particular, elucidating how TAMs are activated to express primarily pro-tumor characteristics rather than tumor cytotoxic activities may reveal factors and pathways that, through treatment, may skew TAMs towards an anti-tumor phenotype. At specific locations within tissues, the activation of the macrophage phenotype activation is defined by the presence and activities of immunomodulatory factors that promote changes in macrophage recruitment and macrophage protein expression. Therefore, the aim of my dissertation was to investigate the role of tumor cell TACE activities in modulating pro-tumor macrophage functions through the immunomodulatory substrates it releases, chiefly TNFRs and MCSF.

The primary downstream events activated by TNF and MCSF are migration and phenotype activation, respectively; therefore, we investigated the effects of TACE shedding on these macrophage activities. Our data show that the chemotaxis of macrophages towards the anti-tumor (M1) stimulus TNF was diminished by tumor-shed TNFRs. We also provide evidence that the ability of macrophages to promote angiogenesis, an M2 characteristic, is mediated in part by TACE-shed MCSF. Taken

together, these results highlight the importance of tumor cell TACE activities in the generation of pro-tumor macrophages and underline potential therapeutic targets for the treatment of cancer. Identifying additional TAM activities altered by tumor cell TACEshedding activities will deepen our understanding of pro-tumor macrophages functions. For example, shed VCAM1 alters monocyte adhesion to the endothelium, a critical step in TAM recruitment, in RCC whereas shed TGFα drastically modifies macrophage proliferation in leukemias [199,140]. Recently, shedding of IL6R has been shown to amplify IL6 signaling, a critical signaling pathway involved in macrophage recruitment and M2 phenotype activation in wound settings and numerous cancers [212,23,152]. Thus, a molecular understanding of the role of TACE-shedding and TACE shed molecules signaling alone or in combination [291] in modulating TAM activities at the tumor site is needed to improve the use of TACE inhibition to treat breast cancer. The observations made here participate in deciphering the effects of TACE shedding in modulating TAMs migration and angiogenesis. The investigated effects of TACE shedding on macrophage migration and phenotype and proposed effects on TAMs adhesion, apoptosis, matrix remodeling abilities are diagramed in Figure 5.1 and the specific effects that have been demonstrated are outlined in Table 5.1. The macrophage functions that are altered by TACE shedding will depend on the concentrations and activities of the factors (outlined in Table 2.2) present.

The cell-cell interactions studied here are complex and more suitably analyzed using *in vitro* models [305,306]. The 4T1 murine mammary cancer progression series includes the 4T1, 4T07 and 67NR cells which model metastatic, invasive and benign breast cancer, respectively (outlined in Table 5.2) [307]. This mammary cancer series

summarizes breast cancer progression stages and allows the comparison of the relative expression of TACE and its substrates during various stages of breast cancer progression [308]. The benefits and limitations of this approach have been detailed elsewhere [307,308]. The use of the 4T1 murine mammary series was dictated by the need for immunological compatibility with J774 and RAW macrophages also derived from Balb/c mice [309,310,311]. Whether the present results can be extended to other mammary tumor cells and breast cancer progression series remains to be determined. Although multiple human mammary cell lines are available to verify the observations made here in a murine mammary tumor progression series [312,313], analyses of macrophages tumor cell interactions remains limited by the available macrophage cells. Nevertheless, carrying out these experiments is essential to validate the role of tumor cell TACE-shed molecules in the generation of a macrophage driven pro-angiogenic environment.

Changes in macrophage functions, such as adhesion and apoptosis, were expected to result from tumor cell TACE shedding [292]. Our recent (unpublished) experiments using *in vitro* approaches tested whether tumor cell TACE shedding impacts the apoptosis of macrophages. The data indicates that tumor cell TACE shed factors significantly promote J774 and RAW macrophage apoptosis as shown through nuclear condensation and caspase activity assays (Figure 5.2). Although the TACE shed factor(s) responsible for promoting macrophage apoptosis remains to be identified, other studies have shown that shed Fas ligand (FasL) and / or sTNFRs promote macrophage apoptosis [293-295]. Therefore, future studies on the mechanisms involved in the apoptosis of macrophages observed here will assess the role of shed Fas ligand (FasL) and / or sTNFRs in macrophage apoptosis through studies using macrophages treated with CM

from tumor cells collected after siRNA knockdown or neutralizing antibodies for FasL or TNFRs. Studies determining that FasL and/or TNFRs recapitulates the effects of TACE inhibition and rescuing the apoptosis promotion through using supplementation of TACE inhibited CM with FasL and / or TNFRs would further indicate the key role of FasL or TNFRs in the regulation of macrophage apoptosis. Moreover, determining whether tumor induction of macrophage apoptosis is selective, i.e. specific to M1 macrophages, is critical and would provide an alternate explanation for the presence of primarily M2 pro-tumor population of TAMs.

Additionally, our data (unpublished) indicates that tumor cell TACE shedding is important in macrophage adhesion. The adhesion of macrophages to endothelial cells is an essential step in recruitment to cancerous or inflamed tissue [39]. Numerous tumor-shed TACE substrates, including VCAM1, MCSF and ICAM1, have dramatic effects on macrophage adhesion. Thus, our studies defined the effects of TACE shedding on macrophage adhesion to the endothelium. In experiments measuring the adhesion of macrophages to an endothelial cell monolayer under various conditions, macrophages treated with tumor-shed factor had a significantly higher adhesion to endothelial cells *in vitro* (Figure 5.3). Further, the macrophage adhesion to 2H11 endothelial cells stimulated by tumor cells TACE shedding was dependent on lectin-binding as indicated by the decreased adhesion observed following pretreatment with the α-D-mannosyl and α-D-glucosyl residue inhibitor, Concavalin A (Con A) (Figure 5.3, $p < 0.05$). Current experiments aim to identify the specific tumor cell TACE-shed substrate associated with increased macrophage adhesion and determine whether inhibiting TACE translates to decreased macrophage adhesion and infiltration *in vivo.* These observations along with

the results outlined in Chapters 3 and 4 provide strong evidence for the role of TACE activities on macrophage recruitment, survival and M2 activation.

Aside from anti-angiogenic treatments, no other approved therapies exist that effectively target the non-malignant cellular component of the tumor microenvironment [289,269]. Although effective in many cancers including glioblastoma [296], colorectal cancer [297], renal cell carcinoma [298] and non-small cell lung cancers [299] anti-angiogenic treatments have failed to extend overall survival in breast cancers patients [300]. This is likely due to other mechanisms supporting vasculogenesis especially vascular mimicry which has been shown to play a critical role in breast cancer and melanoma and appear to have no or a more limited effects in other cancers [314,315]. Our studies, along with others [85,70,41,20,43], provide evidence that targeting macrophages or pro-tumor macrophage functions could benefit breast cancer patients. TAMs support many pro-tumor functions including promotion of tumor cell invasion and matrix remodeling, immune-suppression, chemo-resistance and angiogenesis as confirmed here. Identifying methods to reduce the presence of TAMs at the tumor site or reverse their pro-tumor activation has the potential to inhibit tumor progression at numerous stages as well as improve the effectiveness of chemotherapies [301,15]. Our results indicate that TACE inhibition promotes macrophage response to anti-tumor stimuli and diminishes pro-tumor, angiogenic, TAM activation. Thus, inhibiting TACE at the tumor site to target TAMs may promote cytotoxic macrophages and diminish angiogenesis. Understandably, targeting a nonmalignant cells, such as macrophages, must be done with care as macrophages are vital to the normal physiologic processes, including response to pathogens, wound healing and organ development [38,302,118,66]. However, with

evidence that pro-tumor macrophages are observed in every subtype of breast cancer the use of TAM targeted therapies as a tool to overcome tumor heterogeneity remains extremely attractive. Therefore, rather than systemic depletion or inactivation of macrophages, approaches promoting tissue-specific reduction, depletion and / or redirecting the activation of breast tumor TAMs toward M1 like phenotype may lead to significant changes in breast cancer progression with limited side-effects. Current targeted cancer therapies using antibody conjugates or loaded nanoparticles [170,47,95] combined with specific TACE inhibitors to treat breast cancers may prove to be a clinically relevant approach.

TACE activation has been demonstrated previously to have pro-tumor effects in breast cancer through the stimulation of tumor cell growth and invasiveness following autocrine release of GFs [7]. Our data demonstrate some of the indirect effects of tumor-TACE-shed factors on pro-tumor TAMs (Figure 5.3). Taken together, these studies indicate the use of TACE inhibitors for the treatment of breast cancer may have beneficial effects by directly blocking GF signaling in tumors and diminishing pro-tumor TAM presence and functions. *In vivo s*tudies will assess the impact of TACE inhibitors on tumor growth as well as TAM abundance and phenotype and verify whether this treatment approach will translates into extended disease-free and overall survival. Little is known regarding the regulation of TACE. However, TACE activity is stimulated rapidly in response to inflammatory and growth factor signaling molecules through processes involving the extracellular catalytic domain of TACE [316]. Current inhibitors including TAPI-0 prevent TACE activities by irreversibly binding to the catalytic domain of TACE [316].

As shown here the inhibition of the tumor-shed TACE substrates, cytokine receptors TNFRs and cytokine MCSF, drastically altered macrophage functions. Thus approaches targeting TNFRs and MCSF signaling pathways will likely generate more tailored effects on macrophages than the direct inhibition of TACE activities. As indicated in chapter 2, tumor cell shedding of TNFRs is a mechanism by which tumors inhibit the response of macrophages to TNF [293]. Accordingly, if sTNFRs were sequestered or TACE activation inhibited, TNF may stimulate tumor cell death directly as well as indirectly through the activation of M1 cytotoxic macrophages. This likely would result in the presence of activated macrophages at the tumor site that could better engulf and destroy tumor cells. In the present studies, the respective contributions of the shedding of TNFR1 and of TNFR2 were not investigated as shedding of sTNFR2 was increasing with the aggressiveness of the mammary tumor and as blocking specifically the sTNFR2 strongly affected the TNF signaling. However, a contribution of the sTNFR1 cannot be excluded by the data presented here. Alternatively, one could potentially administer TNF into the breast tumor mass preventing its diffusion to other tissues, at a dose that ensures its effects on macrophage activation aren't hampered by sequestration with sTNFRs. Thus far, the use of TNF for the treatment of cancers has been largely ineffective due to tumor intrinsic mechanisms to prevent its apoptotic effects [236,28] such as the mechanisms presented here and the overall toxicity and side-effects of TNF treatments [28,303]. Nonetheless, it remains to be determined whether sequestration of sTNFRs leading to TNF induced M1 macrophages alone would promote tumor regression in breast cancer patients.

Targeting MCSF, the other TACE substrate identified in our studies, to promote pro-tumor macrophages may also have beneficial effects. Blocking MCSF at the tumor site would decrease multiple pro-tumor TAM activities. Here we demonstrate that MCSF in the context of other tumor-derived factors significantly contributes to the promotion of angiogenic macrophages. Others have demonstrated that TAMs are essential stimulators of angiogenesis in tumors going as far as to coin them the controllers of the "angiogenic switch" [41]. Therefore, inhibiting MCSF may decrease breast tumor angiogenesis through decreased TAM activation. It should be pointed out that the studies done here indicated that MCSF alone was not sufficient to promote angiogenesis alone. Whether blocking MCSF signaling in macrophage depleted versus control tumors leads to alteration in angiogenesis assessed through the number of blood vessels present remains to be determined. Others have demonstrated an important role of MCSF in reciprocal tumor cell-macrophage signaling which guides the process of tumor cell streaming and results in the invasion and intravasation of breast tumor cells into the blood stream [122]. Thus, inhibiting MCSF may also interfere with tumor cell streaming effectively disrupting the ability of breast tumor cells to metastasize.

The ability of tumor shed MCSF to stimulate macrophage angiogenesis was reliant on the presence of CCL2 in the tumor CM as demonstrated in chapter 3. Clinical studies have drawn attention to the fact that inhibiting a single factor is often never sufficient to successfully treat the advanced stages of breast cancer [304]. Although the use of combination therapies is increasing, very few targeted combination inhibitors are currently available in part due to lack of understanding of the cell and molecular combined effects of multiple molecules. Our studies indicated that blocking TACE

shedding of MCSF alone resulted in residual angiogenic activity in macrophages compared to controls, which exhibited no angiogenic activities. This residual angiogenic activity was associated with tumor secreted CCL2 activities. Further, the dual inhibition of MCSF and CCL2 prevented the generation of pro-angiogenic macrophages when compared to treatment inhibiting MCSF alone (Figure 5.1). In addition to the combined direct inhibition of MCSF and CCL2, other targets may be identified by further analyses of synergism mechanisms between these factors. Indeed, the cooperative effects of MCSF and CCL2 in stimulating angiogenic macrophages appear to be guided by crosstalk between the MCSFR and CCR2 on macrophages. Furthering our understanding of the interactions between CCL2 and MCSF in the activation of pro-angiogenic macrophages including defining whether the effects of MCSF and CCL2 are additive or synergistic is crucial for the development of effective and targeted inhibitors.

Just as the appreciation for the heterogeneity in breast cancers has led to divergent treatment strategies for the different cancer subtypes, therapies targeting TAMs for the treatment of breast cancer may follow a similar trend. Patients whom benefit the most from TAM targeted therapy, such as TACE inhibition, will likely be those with high levels of TAM infiltration and M2 phenotype markers. Since the majority of breast cancer patients exhibit high levels of TAMs the patient population eligible and potentially responsive to anti-TAM therapy is large [64,22]. Targeting the microenvironment is one approach to overcome tumor heterogeneity as increased angiogenesis and TAM abundance are observed in breast and other cancers [64,41]. However, the assessment of therapies directed against the nonmalignant tumor microenvironment remains difficult. Aside from the clinical parameters (disease-free and overall survival) and measuring the

tumor burden, measures of the treatment efficacy, the use of therapies targeting the tumor microenvironment either alone or in combination with tumor directed therapies may require additional assessment tools including the determination of TAM abundance and phenotype or vascular density within the tumor mass.

In summary, our results indicate that molecules shed by TACE from tumor cells significantly alter macrophage functions including recruitment, survival and activation through various mechanisms (Figure 5.1). These results obtained *in vitro* using a well-defined mammary tumor progression series need to be confirmed in human breast cancer specimens. Overall, our data highlight the key role of the tumor microenvironment in essential steps of breast tumor progression, further our understanding of the tumor cell – macrophage – endothelial cell interactions within the tumor mass and suggest new treatment strategies that may benefit breast cancer patients.

5.2 Figures

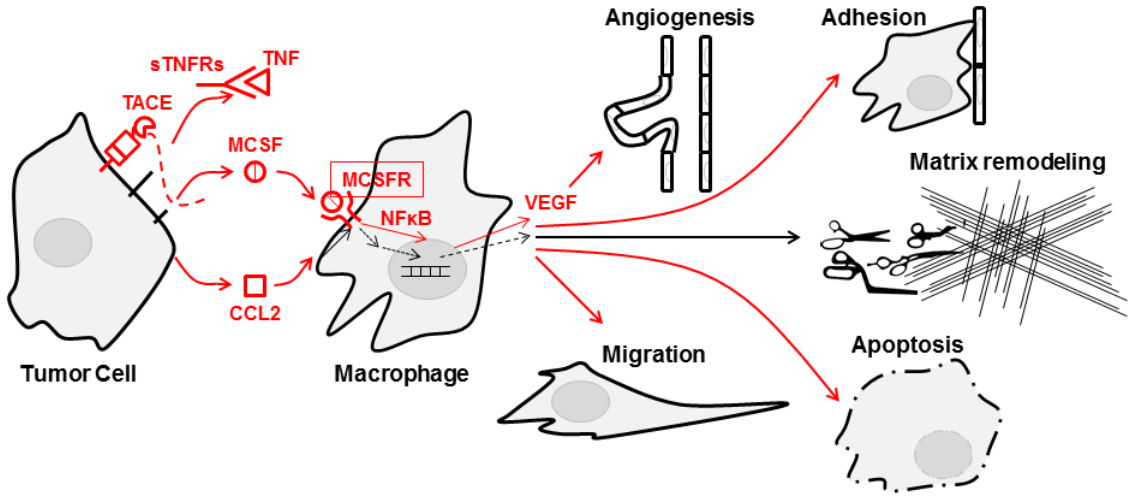

Figure 5.1. Multiple mechanisms associated with TACE shedding by tumor cells in modulating macrophage functions. The red highlights the contribution of the present work to our understanding of those mechanisms.

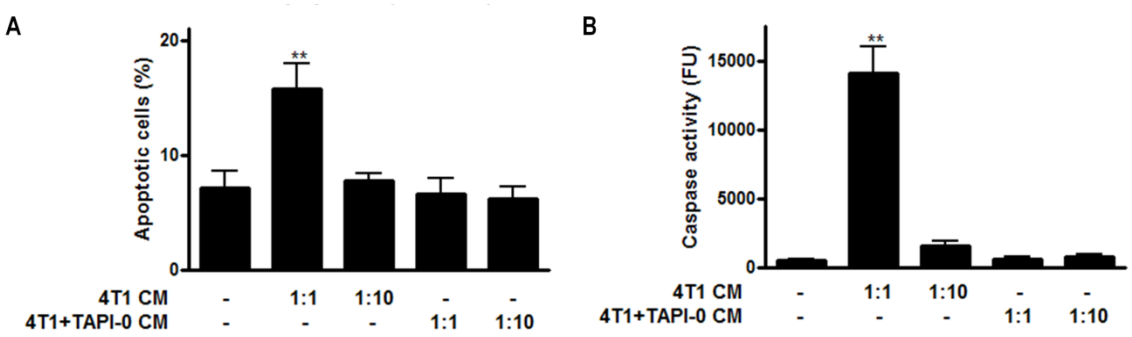

Figure 5.2. Macrophage apoptosis and caspase activation following treatment with tumor CMs. (A) RAW macrophages were treated with different concentrations of 4T1 tumor CMs collected following incubation in media (4T1 CM) or in media + TAPI-0 (TACE inhibitor; 4T1 + TAPI-0 CM) and the % of apoptosis determined. Briefly, cells were were stained with the vital nuclear dye (Hoerchst) and number of condensed nuclei counted and normalized to total number of nuclei. 4T1 CM (1:1) promoted significantly higher levels of macrophage apoptosis compared to all other treatment groups ($p < 0.01$). (B) Caspase 3/7 activities were assayed in lysates collected from RAW macrophages after treatment with varying concentrations of 4T1 tumor cell 4T1 CM or 4T1 + TAPI-0 CM. Similarly, macrophages treated with 4T1 tumor cell CMs (1:1) had at least a 15-fold higher caspase activity compared to all other treatment groups tested ($p < 0.01$).

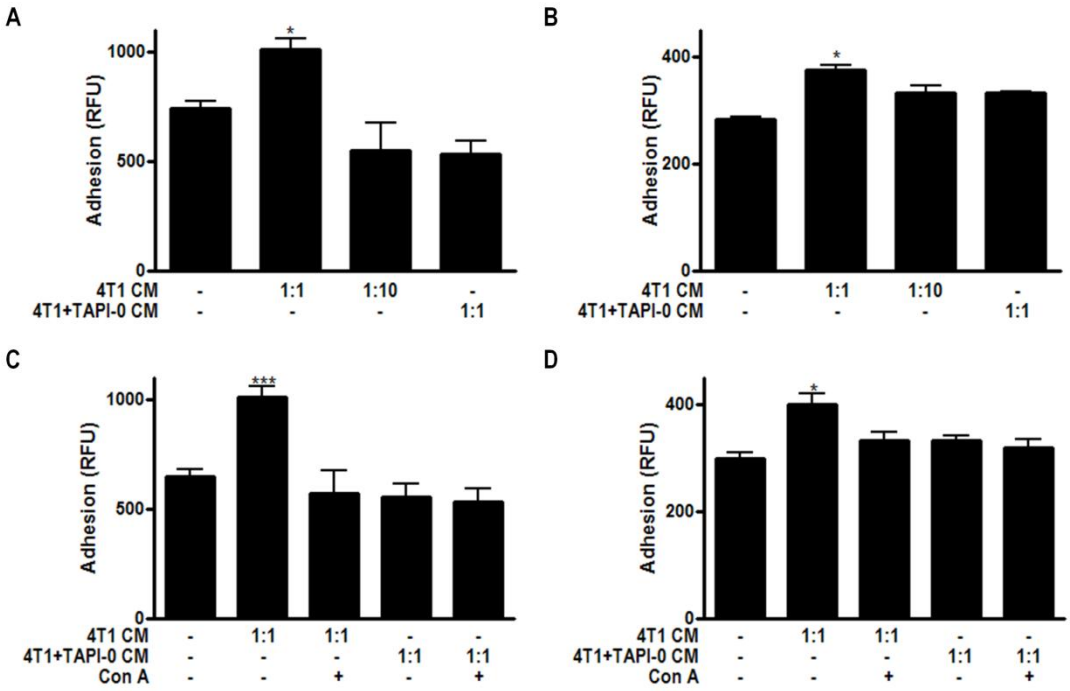

Figure 5.3. Macrophage adhesion to a 2H11 endothelial layer following treatments with 4T1 tumor CMs and / or lectin inhibitors. The adhesion of fluorescently labeled J774 (A) and RAW (B) macrophages to an endothelial cell monolayer in the presence of different 4T1 tumor cell CMs was measured after 1 hour. The adhesion is expressed in relative fluorescence unit [RFU arbitrary units] following removal of unattached cells. .Adhesion of J774 (C) and RAW (D) macrophages was measured following treatments with tumor CMs alone or with the lectin inhibitor concavalin A (Con A). In all experiments macrophage adhesion was significantly enhanced by total tumor CM compared to all other treatment groups ($p < 0.05$).

TABLE 5.1. Effects of TACE substrates on macrophage functions

Macrophage Function	**Effect**		**Substrate(s) involved**
	Increase	Decrease	
Recruitment			
chemotaxis	-	+	TNFR1/2
adhesion	+	-	unknown
M2 phenotype activation	+	-	MCSF
VEGF secretion	+	-	MCSF
EC tube formation	+	-	
NFκB activation	+	-	unknown
Arginase1expression/activity	+	-	unknown
Survival			
apoptosis			

A + denote in the increase column or the decrease column indicates whether the molecule increased or decreased a macrophage function, respectively. – denotes an opposite effect was observed.

TABLE 5.2. Cells used to investigate breast cancer cell / stromal cell interactions

Cell line	**Origin**	**Cancer Stage (if applicable)**			**Source**
		Benign	Invasive	Metastatic	
NMuMG	epithelial	-	-	-	ATCC*
67NR	epithelial	+	-	-	Dr. F. Miller**
4T07	epithelial		+	-	Dr. F. Miller
4T1	epithelial			+	ATCC
J774	monocyte	NA			ATCC
RAW	monocyte	NA			ATCC
D1	mesenchymal	NA			ATCC
2H11	endothelial	NA			ATCC

* http://www.atcc.org/

** Generously given by Dr. Miller (Karmanos Cancer Institute, Detroit, MI-http://www.karmanos.org/)

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