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Inflammatory Breast Cancer: The Immune Perspective

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by

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DEDICATION

This dissertation is dedicated to my parents, Stephen and Marin Cohen, and the patients of the Morgan Welch Inflammatory Breast Cancer Research Program and Clinic at the University of Texas MD Anderson Cancer Center.

In memory of Marilyn Kretzer

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Inflammatory Breast Cancer: The Immune Perspective

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ABSTRACT

Inflammatory breast cancer (IBC) is the most insidious form of locally advanced disease. Although rare and less than 2% of all breast cancer, IBC is responsible for up to 10% of all breast cancer deaths. Despite the name, very little is known about the role of inflammation or immune mediators in IBC. Therefore, we analyzed blood samples from IBC patients and non-IBC patients, as well as healthy donor controls to establish an IBC-specific profile of peripheral blood leukocyte phenotype and function of T cells and dendritic cells and serum inflammatory cytokines.

Emerging evidence suggests that host factors in the microenviromement may interact with underlying IBC genetics to promote the aggressive nature of the tumor. An integral part of the metastatic process involves epithelial to mesenchymal transition (EMT) where primary breast cancer cells gain motility and stem cell-like features that allow distant seeding. Interestingly, the IBC consortium microarray data found no clear evidence for EMT in IBC tumor tissues. It is becoming increasingly evident that inflammatory factors can induce EMT. However, it is unknown if EMT-inducing soluble factors secreted by activated immune cells in the IBC microenvironment canπ account for the absence of

EMT in studies of the tumor cells themselves. We hypothesized that soluble factors from immune cells are capable of inducing EMT in IBC.

We tested the ability of immune conditioned media to induce EMT in IBC cells. We found that soluble factors from activated immune cells are able to induce the expression of EMT-related factors in IBC cells along with increased migration and invasion. Specifically, the pro-inflammatory cytokines TNF-α, IL-6 and TGF-β were able to induce EMT and blocking these factors in conditioned media abated the induction of EMT. Surprisingly, unique to IBC cells, this process was related to increased levels of E-cadherin expression and adhesion, reminiscent of the characteristic tightly packed tumor emboli seen in IBC samples.

This data offers insight into the unique pathology of IBC by suggesting that tumor immune interactions in the tumor microenvironment contribute to the aggressive nature of IBC implying that immune induced inflammation can be a novel therapeutic target. Specifically, we showed that soluble factors secreted by activated immune cells are capable of inducing EMT in IBC cells and may mediate the persistent E-cadherin expression observed in IBC. This data suggests that immune mediated inflammation may contribute to the highly aggressive nature of IBC and represents a potential therapeutic target that warrants further investigation.

Table of Contents

List of Tables

List of Figures

List of Illustrations

ABBREVIATIONS

αCD3.....	anti-CD3 (TCR) or anti-CD3-stimulated conditioned media
AJCC.....	American Joint Committee on Cancer
ALDH	Aldehyde dehydrogenase
$ALDH^{br}$	Bright expression Aldefluour®
APC.....	Antigen presenting cell
APC.....	Allophycocyanin (a far-red dye for flow cytometry)
BCG	Bacillus Calmette-Guerin
BMI	Body Mass Index
CD	Cluster of Differentiation
CTC.....	Circulating tumor cell (Veridex – $CD326^{+}CD45^{-}CK19^{+}DAPI^{+}$)
Cox-2	Cyclooxygenase-2 (prostaglandin-endoperoxide synthase)
DAMPS.....	Damage-associated molecular pattern molecules
DC	Dendritic cell
DCIS	Ductal carcinoma *in situ*
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor (nuclear hormone)
FACS	Fluorescence activated cell sorter, a.k.a. “flow cytometry”

FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate (green fluorescent dye)
Her2/neu	Human epidermal growth factor receptor 2 (CD340)
HMGB1	high-mobility group box 1
IBC	Inflammatory Breast Cancer
IFN	Interferon
IL	Interleukin
IRB	Institutional Review Board
LABC	Locally advanced breast cancer (excluding IBC for most analyses)
LPS	Lipopolysaccharide or LPS-stimulated conditioned media
mDC	Monocytoid dendritic cell ($Lin^-HLA\text{-}DR^+CD11c^+CD123^-$)
MAP	MultiAnalyte Panel (Luminex beads)
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
mRNA	Messenger ribonucleic acid
Muc1	Mucin 1, cell surface associated
NF-$\varkappa$B	Nuclear factor kappa light chain enhancer of activated B cells
Non-IBC	Breast cancer without inflammatory features
PB	peripheral blood
PBC	primary breast cancer (non-metastatic)
pCR	Pathological complete response
PCR	Polymerase chain reaction

pDC	Plasmacytoid dendritic cell ($Lin^{-}HLA\text{-}DR^{+}CD11c^{-}CD123^{+}$)
PE	Phycoerythrin (a red dye for flow cytometry)
PEV	*poussée évolutive* (rapidly progressing breast cancer)
PR	Progesterone receptor
qPCR	Quantitative (reverse transcriptase) polymerase chain reaction
RhoC	Ras homolog gene family, member C GTPase
RT	reverse transcriptase or reverse transcription
RT-PCR	Reverse transcriptase polymerase chain reaction
SEER	Surveillance Epidemiology and End Results
S.E.M.	Standard error of the mean
STAT	Signal transducer and activator of transcription
TAP	transporter associated with antigen presentation
TC1	cytotoxic T lymphocyte class I
TCR	T-cell receptor
TH1	T helper cell (IFN-γ producing, cellular immunity)
TH2	T helper cell (IL-4 producing, humoral immunity)
TH17	T helper cell (Il-17 producing, extracellular pathogens)
TNF	Tumor necrosis factor
US	Unstimulated
VEGF	Vascular endothelial growth factor
Wisp3	WNT1-inducible-signaling pathway protein 3
ZEB	Zinc finger E-box-binding homeobox

Chapter 1: Introduction

Inflammatory Breast Cancer

THE DISEASE

Breast cancer claims the lives of more than 39,000 women in the United States every year and is the leading cause of death among American women aged 45-55. Over 232,000 women in the United States are diagnosed with breast cancer every year and for most women, cancers confined to the breast are highly survivable. As with most cancers, breast cancer mortality results from metastatic disease. Inflammatory breast cancer (IBC) is the most insidious form of locally advanced breast cancer and while defined as a local entity, it rapidly progresses to metastasis and has a very poor prognosis. However, the role of inflammation in inflammatory breast cancer is poorly understood.

IBC was first described in 1814 by Sir Charles Bell as "a purple color on the skin over the tumor accompanied by shooting pain" (1) although pain is infrequently associated with disease progression (2). Lee and Tannenbaum first published the term "inflammatory carcinoma of the breast" in 1924 (3). The first diagnostic criteria for IBC were published by Haagensem in 1956 (4). A similar disease, "rapidly progressing breast cancer" (poussée évolutive) has been described, particularly in Tunisia and northern Africa, and has formed the basis of much of the etiological studies of IBC (5). Unfortunately, recent reviews suggest that these cases may not be true IBC and therefore our understanding of IBC is limited.

IBC usually affects women 50-55 years old. This demographic is slightly younger than patients with non-IBC. In contrast to IBC, lactation associated mastitis, a common differential diagnosis, tends to afflict women of a slightly younger age. Common findings on mammography are skin thickening, diffusely increased breast density, and trabecular thickening but are often reported as negative contributing to delayed diagnosis (2).

The histologic hallmark of IBC is dermal lymphatic invasion (DLI) of tumor emboli that retract away from the endothelial lining (6). However, pathologic evidence of dermal lymphatic involvement is not required for diagnosis as negative results may be due to sampling errors. Additionally, lymphatic tumor emboli may be observed in non-Hodgkin's lymphoma (7) and non-inflammatory breast cancer (8). It is important to note that inflammatory breast cancer is primarily a clinical diagnosis according to the American Joint Committee on Cancer (AJCC) staging manual (9).

IBC is a separate entity from other non-inflammatory locally advanced breast cancers (LABC). According to the Surveillance, Epidemiology and End Results (SEER) Program database, IBC median survival is 2.9 years vs. 6.4 years for LABC (10). Suggesting a unique genetic background, blacks have at least 50% higher incidence than whites and are diagnosed at an earlier age (11). In contrast to non-IBC, women with aggressive breast cancer are more likely to have had a younger age of first parity (11). A high body mass index (BMI) is associated with IBC in both premenopausal and postmenopausal women. Interestingly, BMI is only associated with increased non-IBC breast cancer only in pre-menopausal women (11).

Three types of IBC are recognized: primary, secondary and occult (12). Primary IBC arises as a new malignant tumor. In contrast, inflammation that develops later in the course of cancer progression is referred to as secondary IBC. Occult was first defined by Salzstein in 4 patients (13). IBC is not limited to any specific histologic type. Pathologists usually report a poorly a differentiated ductal carcinoma, but IBC also can be lobular, medullary, colloid carcinoma or comedo-type ductal carcinoma *in situ* (DCIS). Notably, locally advanced breast cancer, which also involves dermal penetration but lacks dermal lymphatic involvement of IBC, is very often medullary or medullary-like (12).

According to the SEER database, IBC presents with more ER negative disease than non-IBC and is frequently Her2 amplified or mutated (10). In addition to the typical breast cancer prognostic markers, several other pathways have been implicated in IBC including E-cadherin, Muc1, and RhoC (14) and p53 is frequently mutated (15).

E-cadherin is a calcium dependent transmembrane glycoprotein that mediates homotypic adhesion between epithelial cells (16). Loss of E-cadherin expression is associated with the epithelial to mesenchymal transition (17) and is generally a poor prognostic factor in breast caner (18). In IBC, however, E-cadherin expression is maintained (19) and is up-regulated in IBC models (20). The clusters of tumor cells that comprise the lymphatic emboli characteristic of IBC are maintained through E-cadherin interactions. Sanford Barsky's lab showed that anti-E-cadherin would cause dissolution of tumor emboli in the Mary-X IBC mouse model (20). Therefore, E-cadherin is thought to be a unique factor in IBC.

STEM CELLS

The Marry-X model exhibits "florid lymphovascular invasion" (21) and expresses stem cell-like phenotypes in culture including stellar, rex-1, nestin, H19, oct-4, nanog, sox-2, CD44+CD24-/low, ALDH1, and CD133 but not CD34 (22). Notably, the authors claim that non-IBC breast cancer cells lines do not express CD133.

Aldehyde dehydrogenase (ALDH) activity is involved in the metabolism of retinoic acid which signals through the retinoic acid receptor to maintain cells in a stem cell-like undifferentiated state (23). ALDH activity may contribute to the therapy resistant properties (24) of stem cells. Furthermore, ALDH1 expression has recently been shown to be a poor prognostic factor in breast cancer (25). In this paper, the authors describe a fluorescence-based technology that allows easy identification of the aldehyde

expressing cells by a bright fluorescence of the proprietary agent Aldefluor (ALDHbr). Although ALDH1 is typically thought to be the most influential isoform of ALDH in stem cell maintenance, Charafe-Jauffret et al note that different isoforms of ALDH1 contribute to the ALDHbr population (26). In IBC, aldehyde and alcohol metabolism pathways were found to be upregulated in IBC vs. non-IBC in a pathway analysis of Affimetrix chip data from tumor epithelium laser capture microdissection (27). Therefore stem cell signaling through ALDH may play a critical role in the biology of IBC.

INFLAMMATION IN IBC

There is much debate over whether the inflammatory features observed in inflammatory breast cancer represent a true inflammatory process. Inflammation is traditionally defined by the Latin rubric: *calor, dolor, rubor and tumor*, meaning "heat, pain, redness, and swelling." Each of these is normally attributed to the action of local inflammatory cytokines released in response to a pathologic process (28). However, in IBC, current consensus contends that inflammation is caused by a continuous growth of tumor cells within the lymphatic (and occasionally venous) vessels that eventually blocks all drainage from the tissue resulting in the edema. Inflammatory cellular infiltrates are limited to lymphocytes and monocytes invading the perivascular area in response to the lymphatic blockage. Other leukocytes such as neutrophils, eosinophil and mast cells are not commonly seen in IBC (12). While these leukocytes are seen around the periphery, inflammatory cells are rarely reported in the tumor field (29). Most of the reviews of leukocytic infiltrates in IBC are anecdotal and a clear consensus is hard to establish. Unfortunately, although a thorough review of leukocytic infiltrates in IBC has not been published, it is beyond the scope of the current study.

As IBC presents with inflammatory features in the breast, it can be easily confused with mastitis, an acute inflammation of the breast that is almost exclusively associated with lactation. The skin overlying a mastitis-related abscess is typically smooth and shiny whereas IBC presents with the characteristic *peau d'orange* or orange-peel skin. Mastitis related inflammation quickly resolves or progresses to abscess and can be readily treated with antibiotics (12).

As secretion of cytokines is one of the defining hallmarks of inflammation, several studies have examined cytokines in model systems and patient material. Stephen Ethier's group in Michigan developed the SUM149 cell line from an aggressive inflammatory breast cancer (30) and showed that it produces IL-1α and IL-1β in response to autocrine amphiregulin stimulation of epidermal growth factor receptor (EGFR) (31). However, Sofia Merajver's group from the same instuition has unpublished data suggesting that IBC tumors produce "negligible levels" of IL-1 (8). Similarly, Bièche et al found similar expression levels of inflammatory cytokine mRNA in IBC and LABC (32). In this study, Bièche et al performed RT-PCR analysis of 538 genes in 36 IBC and 22 LABC samples collected over 7 years from one institution in France and found similar levels of interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-10, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. However, IL-6 expression was 9.3 times higher in IBC than LABC. Additionally, they reported increased expression of Cox-2. It is worth noting that this was a small study and although ER was found to be less frequently expressed in IBC, as expected, they study did not find expression differences in the other pathways most frequently associated with IBC including E-cadherin, Ras homolog gene family, member C GTPase (RhoC) or WNT1-inducible-signaling pathway protein 3 (Wisp3, as known as CCN6). Further reverse transcription-polymerase chain reaction (RT-PCR)

analysis of the same sample set found that 35 of 60 (58%) NF-κB pathway related genes were up-regulated in IBC (33).

The transcription factor NF-κB is a critical in regulator of inflammation. The IL-1 receptor is highly homologous to the NF-κB inducing Toll-like receptors (TLR) that are conserved from drosophila to humans (34) and there is a positive feedback loop between IL-1 and NF-κB activation where each induces activation of the other. Gene chip studies at MD Anderson have shown that expression of IL-1β mRNA is significantly higher in IBC cell lines than in non-IBC MCF-7. Additional NF-κB dependent genes such as CXCL1 were also unregulated in IBC. The inflammatory intermediary Cox-2 is highly expressed in the IBC cell line SUM149 relative to non-IBC cell lines (35).

Although the clinical indications of inflammation in IBC seem to be primarily related to blockage of dermal lymphatic vessels, this research has shown that sub-cellular inflammatory signaling including Cox-2 and NF-κB is highly prevalent in the SUM149 model cell line.

MODEL SYSTEMS

Inflammatory Breast Cancer is defined by its clinical and pathological presentation, there is no current molecular determinant. As such model systems are a bit limited in their ability to fully recapitulate the disease. A limited number of cell lines derived from patients with confirmed IBC are available and these serve as the basis for most in vitro work. Stephen Ethier's group in Michigan developed the SUM149PT and SUM190PT cell lines from the primary tumors of patients diagnosed with aggressive inflammatory breast cancer (30). The SUM190 cell line harbors a chromosomal amplification of the Her2/neu oncogene whereas the SUM149 is triple receptor negative and lacks expression of the three primary breast cancer prognostic markers: ER,

progesterone receptor (PR) and the epithelial growth factor receptor family member Her2. Such triple receptor negative breast cancers (TNBC) (sometimes referred to as basal-like) are often highly aggressive and it can be difficult to know if SUM149 is modeling IBC behavior or TNBC behavior. Several additional Her2+ IBC cell lines exist including KPL4 and a cell line recently developed at MD Anderson, IBC-3(36). The MARYX model is a human IBC cell line that is passaged through murine xenografts. The model recapitulates the tumor emboli so common in IBC, however the cell line is not readily available. van Golen's group has been describing an *in vitro* a model that can mimic the oscillating interstitial fluid pressure within the lymphatics (37).

IMMUNOLOGY

IMMUNOLOGY AND BREAST CANCER

Tumor Infiltrating Leukocytes

Most studies of tumor leukocytic infiltrates in breast cancer rely on single color stains. Ruffell et al compared malignant breast tissue, non-adjacent normal breast tissue and peripheral blood as a control using several multi-parameter techniques in a small series of 20 breast cancer patients. They reported that activated T-cells constitute the largest proportion of infiltrating leukocytes in the tumors of chemotherapy naïve patients. In these samples, macrophages are rare in the tumor but more common in the non-adjacent normal tissue. In contrast, myeloid cells are more common in the malignant tissue of patients, post-chemotherapy (38).

IMMUNOLOGY AND IBC

Immunological studies of inflammatory breast cancer are limited. After anecdotal reports of immune suppression in French breast cancer patients diagnosed with *poussée*

évolutive (PEV) or rapidly progressing breast cancer, Levin et al. used delayed hypersensitivity skin tests (5) and in vitro cellular immunity tests (39) to study immunity in PEV patients in Tunisia. In both studies, the authors found almost no difference between PEV+ and PEV- patients with respect to tumor antigen, recall antigen, and mitogen (phytohemaglutinin A or Concanavalin A) stimulation. Unfortunately, diagnostic criteria used in these studies were not based on the current definition of IBC, making interpretation of these data difficult (40).

About the same time, immunotherapy trials using Bacillus Calmette-Guerin (BCG) as an adjuvant were conducted in breast cancer patients (41). A long-term follow-up of IBC patients vaccinated with allogeneic tumor + BCG found 4 of 13 patients (31%) were still alive after 10 years (42). Interestingly, Pogo et al. have reported that components of a human virus very similar to mouse mammary tumor virus (MMTV) are present in IBC patients (43). This is highly intriguing for a number of reasons including 1) MMTV is a well known mammary oncogene that is frequently used as a model for breast cancer in mice and 2) it suggests an immune aspect to IBC. Unfortunately, the MMTV data are yet to be confirmed. Additionally, there is a single case report from 1994 that notes a reconstitution of immune parameters following administration of subcutaneous IL-2 (44). Recent anecdotal evidence suggested that there is a high prevalence of viral infection in IBC. Overall the literature is very thin and a basic understanding of immune competence in IBC is needed.

MOLECULAR SIGNALING

Recent research has suggested that several signaling pathways contribute to the unique aggressive nature of inflammatory breast cancer. Similar to other breast tumors, p53 and Her2 are frequently mutated in IBC (45). More unique to IBC is an increased

expression of the oncogene RhoC GTPase, the cell adhesion molecules E-cadherin and MUC-1 and loss of the tumor suppressor Wnt-1 induced secreted protein 3 (WISP-3, also known as "lost in IBC") (46, 47). Furthermore, IBC tends to be highly vascularized (47) with higher levels of vascular endothelial growth factor (VEGF) than non-inflammatory breast cancer (48).

The tumor suppressor p53 is often called the "guardian of the genome" due to its ability to prevent replication of damaged DNA (49). Moll et al. found that p53 dysfunction in IBC occurs by two major mechanisms. The authors found that in p53 positive samples with nuclear localization, the protein was impaired by a variety of missense mutations. In contrast, wild type p53 was found in IBC patients who were p53 negative or with cyoplasmically localized tumors. Therefore, the authors proposed that missense mutation and nuclear exclusion are the primary mechanisms of p53 alteration IBC (50).

Over-expression of E-cadherin is an interesting phenomenon in IBC. E cadherin helps anchor epithelial cells together. In epithelial cancers, E-cadherin is typically lost in malignant progression as its down-regulation is associated with the epithelial to mesenchymal transition and therefore invasion and metastasis. In contrast, E-cadherin is highly expressed in the lymphovascular emboli of IBC. β-catenin acts as an adapter protein binding E-cadherin to the actin cytoskeleton (20). This association of E-cadherin with β-catenin should sequester β-catenin in the cytoplasm and limit Wnt signaling. Similarly, WISP3 is typically lost in IBC (51). Interestingly, WISP3, or cystein-rich protein CCN6, seems to maintain cell adhesion and E-cadherin expression. Loss of CCN6 in non-IBC cells lead to a down-regulation of E-cadherin through induction of Snail and Zinc finger E-box-binding homeobox 1 (ZEB1) (52) which concurs with the increased expression of Snail in IBC (32).

Although IBC tumor cells clump together due to homotypic interactions of E-cadherin, the characteristic emboli retract from the endothelial wall. Sanford Barsky's lab (53) showed that although the adhesion molecule MUC1 is overexpressed in the MARY-X model, sialyl-Lewis^x/a carbohydrates are markedly decreased due to a loss of α3/4-fucosyltransferase activity. Without the sialyl-Lewis^x/a moieties, MUC1 and other adhesion molecules are unable to bind the E-selectin on the endothelial vessels (53).

RhoC GTPase is typically overexpressed in IBC (46). RhoC is a member of the ras with high homology to RhoA. The ras signaling pathway initiates cell motility and focal adhesions (14). As prenylation is required for ras function, a farnesyl transferase inhibitor may be useful in treating IBC (54).

SUMMARY

In conclusion, inflammatory breast cancer is a rare but highly aggressive form of breast cancer. A number of clinical, pathological, and molecular attributes make IBC a distinct entity from other locally advanced breast cancers. The disease is characterized clinically by erythema and edema of the breast and a rapid disease progression. Although not required for diagnosis, the pathological hallmark of IBC is the presence of tumor nests in the dermal lymphatics. These emboli are held together by E-cadherin as confirmed by the MARY-X mouse xenograft model. Additionally, IBC is typically ER- with altered Her2. RhoC GTPase is typically over-expressed and WISP3 is consistently lost. Although IBC is not a true inflammatory process with few infiltrating leukocytes and minimal production of inflammatory cytokines, the tumor is characterized by and high levels of angiogenic signaling and expression of the Cox2 and NF-κB inflammatory signaling pathways.

A diagnosis of inflammatory breast cancer has been associated with all intrinsic subtypes of breast cancer in frequencies fairly similar to breast cancer in general. Furthermore, no specific molecular event has been identified as a causative agent of IBC. As such, it is possible that the factors that make IBC such an aggressive disease may not be intrinsic to the tumor. We propose that host factors from the tumor microenvironment may play a pivotal role in tumor evolution. As IBC is defined by the presence of inflammatory features, later chapters of this thesis will examine the relationship between inflammatory factors and IBC.

RESEARCH OBJECTIVES

The overall goal of this project is to understand the role of immune system and immune mediated inflammation in inflammatory breast cancer. The central hypothesis is:

> **Inflammatory breast cancer (IBC) is distinguished from non-IBC by unique immunological characteristics that contribute to the rapid progression of the disease.**

To that end, four specific aims are offered:

Specific Aim 1: Establish that IBC patients have a serum cytokine profile that is unique from that of non-IBC and healthy donors

Specific Aim 2: Establish IBC patients have a hematologic profile that is different from that of non-IBC and healthy donors

Specific Aim 3: Establish that the peripheral blood cells of IBC patients have functional changes that are unique and distinguishable from those of non-IBC and healthy donors

Specific Aim 4: To establish that immune mediated inflammation induces aggressive changes in inflammatory breast cancer tumor cells

Chapter 2: Soluble Factors

POPULATION STUDIED

In order to establish a serum cytokine profile in inflammatory breast cancer, we analyzed peripheral blood samples obtained under several University of Texas MD Anderson Cancer Center IRB approved protocols including Lab08-0199 "Reactivation of Epstein Barr virus in patients with breast cancer" and 2006-1072 "Inflammatory Breast Cancer Registry." Lab08-0199 served as the basis for the remainder of the studies in this dissertation and is described in the following chapter. The Inflammatory Breast Cancer Registry protocol 2006-1072 was initially set up to capture all newly diagnosed IBC patients with (M1) or without (M0) *de novo* metastases at The University of Texas MD Anderson Cancer Center. The protocol was later expanded to include Cohort II consisting of IBC patients who had received systemic therapy. For serum cytokine profiling 125 treatment-naïve and 57 previously treated IBC patients were analyzed. Under protocol Lab08-0199, samples were collected at the start of a new line of therapy.

Table 2. 1

Patient characteristics for serum cytokine studies. Patients in this study were recruited under MD Anderson IRB-approved protocols Lab08-0199 and 2006-1072. Patients included locally advanced breast cancer patients with and without inflammatory features (IBC and LABC) and Stage IV metastatic patients with and without inflammatory features (MIBC and MBC). Tumor subtypes are derived from pathological report, TNBC tumors lacked expression of ER, PR and Her2; tumors were classified as Her2+ if they were IHC 3+ or FISH+; tumors were classified as luminal if they were positive for hormone receptors (ER and/or PR) staining but not Her2 amplified. Abbreviations: HR, Hormone receptor positive IBC Inflammatory Breast Cancer; LABC, Locally Advanced Breast Cancer; MBC, Metastatic Breast Cancer; MIBC, Metastatic breast cancer with inflammatory features; TNBC, triple receptor negative breast cancer. Healthy donors (HD) consisted of women without cancer and of age 24-60 recruited from MD Anderson Cancer Center.

		IBC	LABC	MBC	MIBC	HD
N		106	30	28	76	29
Age		52.2 (23 – 80)	51.6 (32 - 67)	53.3 (34 – 76)	51.7 (25 – 78)	39.4 (24 – 60)
Race	Asian/Pacific Islander	2	1	1	0	
	African American	10	3	3	7	
	Spanish, Hispanic	11	6	5	5	
	Caucasian	83	20	19	64	
Stage	II	-	14	-	-	
	III	106	16	-	-	
	IV	-	0	28	76	
Grade	1	1	3	0	1	
	2	25	8	8	17	
	3	68	19	14	51	
Treatment Status	Treatment Naive	79	29	6	46	
	Previously Treated	27	1	22	30	
Subtype	HR+	33	15	13	25	
	Her2 amplified	48	9	8	25	
	TNBC	24	6	7	26	
Lymphatic Invasion	No	24	16	5	14	
	Yes	44	7	10	35	

BACKGROUND

To establish a profile of systemic soluble inflammatory factors in inflammatory breast cancer, serum samples were analyzed in batch using Luminex multiplex bead technology. The Luminex cytokine assays are similar to a sandwich enzyme-linked immunosorbent assay (ELISA) consisting of an immobilized capture antibody and a reporter-conjugated detection antibody. In an ELISA the capture antibody is bound to a polystyrene plate; samples, standards and in some cases, analytes, are indexed to the physical location in the plate. Luminex assays conjugate the capture antibody to a polystyrene bead filled with a precise ratio of flourophores that give the bead a unique fluorescent signal that can be decoded by two fluorescent parameters on a flow cytometer (55). Different detection antibodies are conjugated to different colored beads and added to each sample in multiplex. The first generation Luminex technology allows up to 100 different beads to be read, but in practice, antibody interactions become problematic above multiplexes with 30 to 40 analytes.

In this study, 45 different factors were analyzed in 5 separate panels. The cytokines are listed in Table 2. 2, with a brief description of each. Cytokines and chemokines can be highly promiscuous, as most are derived from multiple sources and act on a variety of targets. The chemokines in particular (listed on the second page of Table 2. 2) can bind to multiple receptors. Furthmore, many of the inflammatory pathways are highly redundant with small functional differences between molecules. As such, an inflammatory profile can be different in different patients. Therefore, as a first pass, we looked at the sum of the profile (literally): the z-scores each for cytokine were summed to give an unbiased, equal weight to each factor amalgamated into a single number. For this total expression profile, there was no difference between IBC and non-

IBC ($p = 0.171$). However, IBC, MIBC and LABC had lower total expression of cytokines than HD ($p < 0.001$, $p = 0.003$, and $p = 0.001$) but MBC was not significantly different from HD ($p = 0.055$). This did not seem to be treatment related as the same relationships held when looking only at treatment naïve patients ($p = 0.001$, $p = 0.008$ and $p = 0.003$ with MBC vs. HD $p = 0.191$). It is possible that this is due to lower total protein levels, which might be possible to correct for using total serum albumin, but this was not analyzed.

Systemic inflammation can be estimated by looking at levels of C-reactive protein (CRP) that binds phosphocholine on the surface of damaged cells and some microbes to activate complement. In contrast to the other factors profiled here, CRP is produced almost exclusively by the liver in response to other inflammatory factors such as IL-6. Normal CRP levels are below 10 mg/L. Following an acute assault, CRP serum levels rises within 2 hours and can peak at around 200 mg/L within 2 days during an active bacterial infection. Mild inflammation, viral infections and late pregnancy generally have CRP levels between 10-40 mg/L. Chronically elevated levels have been associated with inflammatory conditions such as arthritis and with malignancies. Even levels in the high range of normal have been associated with an increased risk of cardiovascular disease. Interestingly, statins, which are currently of great interest for their possible protective effects in breast cancer (56), have been shown to reduce CRP levels (57).

Cytokines perform several basic functions. Table 2. 2 groups cytokines by the most common function for each cytokine. There are inflammatory factors, angiogenic factors, factors that support a type I cellular immune response, factors that drive a type II humoral response, anti-inflammatory factors, growth factors, hematopoietic growth factors, chemokines that act through G-protein coupled receptors that give directional

signals, and factors that are highly associated with bone metabolism (critical in breast cancer as bone is one of the most frequent metastatic sites).

These are general categories and most factors will fall into several different categories, and even show diametrically opposed functions under different conditions. For example, IL-6 is sometimes considered anti-inflammatory because it opposes the type I immune response which is usually initiated through cells expressing both the IL-6 receptor (IL-6R) glycoprotein 130 (GP130). While GP130 is ubiquitously expressed, IL-6R has a limited expression pattern. However, soluble forms of the IL-6R are able to interact with both soluble IL-6 and membrane-bound GP130 to initiate signaling in a wide variety of cells. This signaling complex initiates signal transducer and activator of transcription 3 (Stat3) (58). This dual role of IL-6 may indicate that IL-6 plays a key role in the transition from innate to adaptive immunity. Early stages of a typical acute immune response include the recruitment of neutrophils and then replaced by monocytes and T cells after 1 or 2 days. This early stage includes inflammatory factors TNF-α and IL-1β that activate neutrophils. Proteolytic cleavage of the IL-6R by the invading neutrophils can release sIL-6R to initiate trans-signaling and induce the switch away from neutrophil-attracting chmokines like (Groα, IL-8 and fractlakine) to monocyte-attracting chemokines (MCP-1, and MCP-2) (59, 60). IL-6 trans-signaling also recruits T-cells (61). Critically, IL-6 signaling also induces EMT and stem-like properties in breast cancer cells (62) Trastuzumab-induced cancer stem cell expansion with a concomitant increase in Twist or Vimentin and loss of EpCAM and E-cadherin is mediated by IL-6 (63). Such inflammatory signals can derive from the tumor, invading mesenchymal stromal cells (64), or hematopoietic cells. In addition to IL-6, IL-8 has also been shown to be critical in maintaining breast cells in a stem-like state (65, 66). As such, IL-6 and IL-8 levels can be critical regulators of metastatic potential in breast cancer.

As mentioned above, TNF-α and IL-1β are critical factors in acute inflammation. These two cytokines are potent inducers of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), one of primary inflammatory signaling pathways and a critical promoter of inflammation associated cancers (67). TNF-α activates NF-κB through IκBβ (68). Receptor activator of nuclear factor kappa-B ligand (RANKL), critical in bone metabolism through the activation of osteoclasts (69), is also critical in during mammary gland development (70) and tumorigenesis (71). During pregnancy, RANKL activates NF-κB through IκBα (70) to promote cyclin D1 dependent proliferation of epithelial cells. As such, dysregulated inflammatory signaling through NF-κB can drive proliferative cells.

In addition to NF-κB, cyclin D1 proliferation also requires AP1 signaling, often derived from growth factors such as epidermal growth factor receptors EGFR and Erbb2 (72). Epidermal growth factor (EGF) and Transforming growth factor alpha (TGF-α) are stong inducers of EGFR signaling. EGF is particularly important in TNBC (73) (Ueno and IBC (74).

The adaptive immune response has been divided into 2 broad classes: a type-I response driven by cytotoxic T-cells and a humoral type-II response driven by B-cells. $CD4^+$ helper T cells (T_H) produce cytokines that polarize these dichotomous responses. The type-I (cellular) response is typified by a class of cytokines, such as interferon gamma (IFN-γ), interleukin-2 (IL-2), and IL-12. These induce and are produced by type-1 polarized T-cells referred to as TH1. In contrast, the type-II (humoral) response favors the production of IL-4, IL-5, and IL-13. These are referred to as TH2 cytokines. As IL-10 and IL-6 promote B cell growth, these are typically considered TH2 cytokines, as well. As the TH2 cytokines inhibit the production of TH1 cytokines, they are sometimes considered anti-inflammatory. But more importantly, the TH1 response is generally

considered to control tumor growth through the activation of cytotoxic killing whereas the TH2 polarization is considered to promote tumor immune evasion and tumor growth. For example, TH2 polarized CD4+ cells have been shown to induce a type-II polarization in tumor infiltrating macrophages that produce EGF to promote tumor growth(74). Therefore, the level of expression of TH1 and TH2 cytokines can promote tumor control by the immune system or tumor progression.

Recently, a third type of adaptive response has been identified that controls extracellular bacteria, particularly at epithelia. Primed by IL-6, TGF-β, and IL-1β, the so called TH17 response is typified by production of IL-17 family members. This is considered to be a highly inflammatory response and is related to auto-immunity. In this study, we focused on the prototypical cytokine IL-17 and the IL-12p40 subunit that is involved in IL-12 (TH1) polarization and IL-23 TH17 polarization.

TGF-β is usually considered an anti-inflammatory agent, yet with the induction of TH17 is a very strong promoter of inflammation. In conjunction with IL-10, TGF-β produced by regulatory T cells (T_{Reg}) is a potent inhibitor of both TH1 and TH2 responses.

These cytokines are summarized in the tables on the following pages.

INFLAMMATORY BREAST CANCER SERUM SIGNATURE

To see if an IBC-specific pattern of inflammation exists, we performed an unsupervised hierarchical clustering of the 45 serum cytokines as seen in Figure 2. 1 on page 27. No clear IBC-specific pattern could be discerned.

Table 2. 2

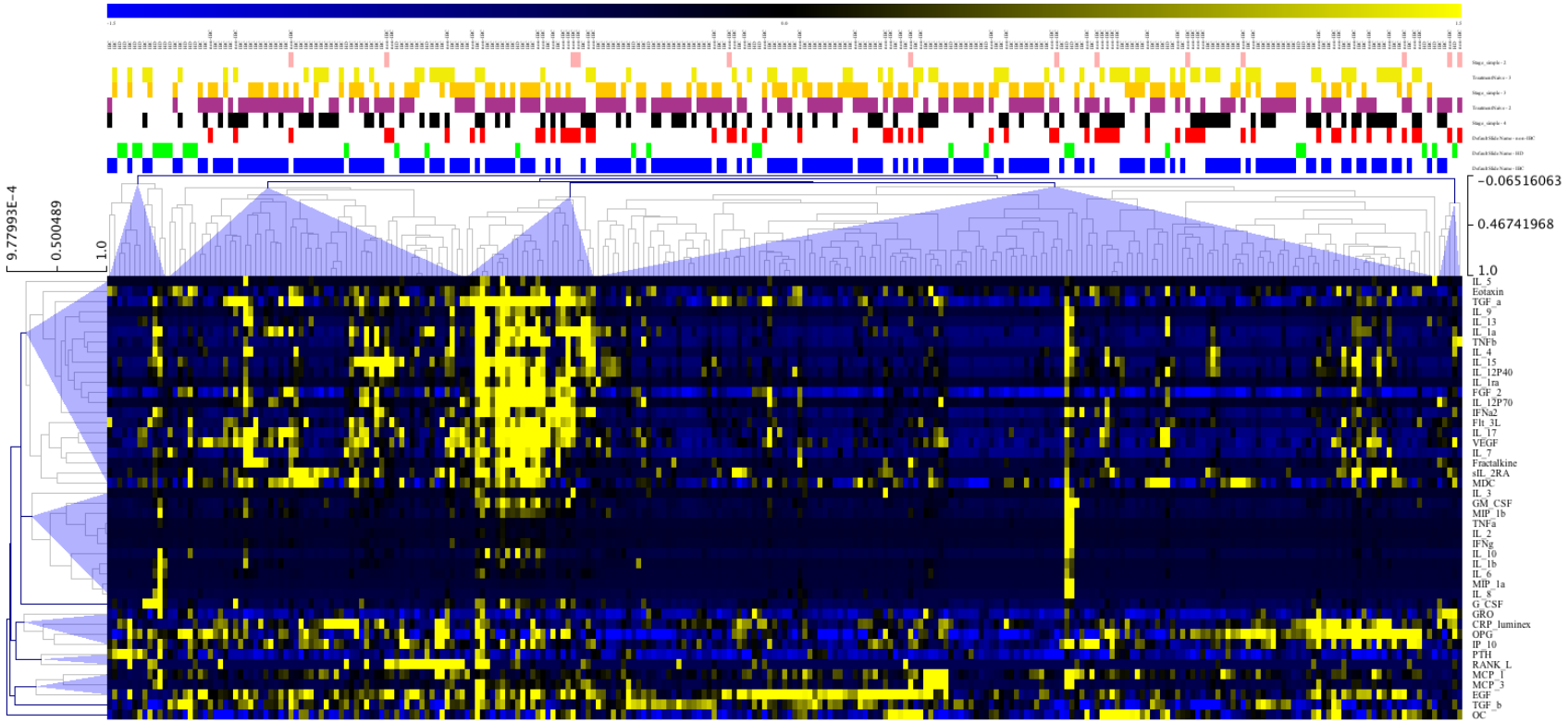
List of cytokines analyzed. Forty-five cytokines were analyzed by Luminex multiplex beads including inflammatory cytokines, angiogenesis-related factors, T-helper (TH)1-, TH2-, and TH17-related cytokines, growth factors, hematopoietic growth factors, chemokines and bone-related factors.

	Cytokine		Name	Function
Inflammatory	CRP		C-reactive protein	Acute systemic inflammation, produced by liver
Inflammatory	IL-1α		Interleukin 1 alpha	Expressed by mostly epithelial cells, important in maintaining epidermis
Inflammatory	IL-1β		Interleukin 1 beta	Pro-inflammatory, canonical inducer of NF-κB signaling
Inflammatory	IL-1RA		Interleukin 1 receptor antagonist	Prevents IL-1β signaling
Inflammatory	IL-6		Interleukin 6	Th2 and highly pro-inflammatory, increases with exercise, bone resorption, shown to induce EMT
Inflammatory	sCD40L	CD154	Soluble CD40 ligand	A TNF family member expressed on T cells that activates APC
Inflammatory	TNF-α		Tumor necrosis factor alpha	Pro-inflammatory, induces IL-1 and IL-6
Inflammatory	TNF-β		Tumor necrosis factor beta (lymphotoxin)	Functional homologue to TNF-α
Inflammatory	IL-8	CXCL8	Interleukin 8	Recruits neutrophils and granulocytes, angiogenic, supports breast cancer stem cells
Angiogenic	FGF-b		Basic fibroblast growth factor	Promotes angiogenesis
Angiogenic	VEGF		Vascular endothelial growth factor	Promotes angiogenesis
TH1	IFN-γ		Interfern gamma (class II interferon)	Primary TH1 cytokine, promotes macrophages and NK killing
TH1	IL-2		Interleukin 2	T-cell growth factor, a TH1 cytokine, critical for T_{Reg}
Inflammatory	IL-12P40		Interleukin 12 p40 subunit (beta)	Subunit sharted by IL-12 (promotes TH1, NK) and IL-23 (promotes TH17 and inflammation)
TH1	IL-12P70		Interleukin 12p70 (p40 + p35)	Promotes TH1
TH2	IL-13		Interleukin 13	Similar to IL-4, Promotes TH2, induces MMPs
TH2	IL-4		Interleukin 4	The primary TH2 cytokine, induces TH2, humoral immunity
TH2	IL-5		Interleukin 5	Produced by TH2 and mast cells
TH2	IL-9		Interleukin 9	T-cell growth factor, TH2-like
anti-viral	IFN-α2		Interferon alpha 2	Anti-viral
TH17	IL-17		Interleukin 17	Primary TH17 cytokine, highly pro-inflammatory
Anti-inflammatory	IL-10		Interleukin 10	Produced by monocytes, TH2, Treg; inhibits pro-inflammatory signaling, growth factor for B cells
Growth factor	TGF-β		Transforming growth factor beta	Highly pleiotropic, anti-inflammatory, anti-proliferative, strong inducer of epithelial mesenchymal transition (EMT)
Growth factor	EGF		Epidermal growth factor	Promotes growth, differentiation, and proliferation
Growth factor	TGF-α		Transforming growth factor alpha	Similar to EGF, can bind EGFR

	Cytokine		Name	Function
Hem. growth factor	Flt3L		Fms-related tyrosine kinase 3 ligand	Similar to stem cell factor, proliferation and differentiation of various blood cell progenitors including dendritic cells
Hem. growth factor	G-CSF		Granulocyte colony-stimulating factor	Stimulates proliferation and differentiation of granulocytes can also mobalize stem cells from bone marrow
Hem. growth factor	GM-CSF		Granulocyte-macrophage colony-stimulating factor	Stimulates proliferation and differentiation of granulocytes and monocytes
Hem. growth factor	IL-15		Interleukin 15	Similar to IL-2 promotes T and NK functions , usually trans-presented for juxtacrine signaling
Hem. growth factor	IL-3		Interleukin 3	Growth factor for myeloid and lymphoid progenitors, matures pDC
Hem. growth factor	IL-7		Interleukin 7	Lymphocyte homeostasis
Hem. growth factor	sIL-2Rα	sCD25	soluble IL-2 Receptor alpha (CD25)	Shed from activated T and T_{Reg}, can inhibit IL-2 signaling
Chemokine	GRO	CXCL1	Growth related oncogene	Neutrophil chemoattractant
Chemokine	IP-10	CXCL10	Interferon gamma-induced protein 10	Recruits hematopoetic cells
Chemokine	MCP-1	CCL2	Monocyte chemotactic protein-1	Recruits monocytes, dendritic cells and memory T cells
Chemokine	MIP-1α	CCL3	Macrophage inflammatory protein alpha	Activate granulocytes and induce inflammatory cytokines
Chemokine	MIP-1β	CCL4	Macrophage inflammatory protein beta	Activate granulocytes and induce inflammatory cytokines
Chemokine	MCP-3	CCL7	Monocyte chemotactic protein-3	Similar to MCP-1, attracts monocytes
Chemokine	Eotaxin	CCL11	Eotaxin	Binds CCR2, 3, 5, recruits eosinophils
Chemokine	MDC	CCL22	Macrophage-derived chemokine	Recruits mDC and IL-2 activated NK
Chemokine	Fractalkine	CX3CL1	Fractalkine	Chemoattractant for T cells and monocytes
Bone	OC		Osteocalcin	Promotes osteoblastic bone generation
Bone	OPG		Osteoprotegerin	Decoy for RANK-L
Bone	PTH		Parathyroid hormone	Bone resorption
Bone	RANKL		Receptor activator of nuclear factor kappa-B ligand	TNF family member activated osteoclasts

Figure 2. 1

Unsupervised hierarchical clustering of serum cytokines shows no IBC signature. 45 Serum cytokines were measured using Luminex multiplex beads. Each cytokine was normalized to the healthy donors using the formula $(x_{j,i} - \mu_{j,HD}) / (SD_{j,HD})$ where μ_{HD} is the mean of the healthy donors, SD_{HD} is the standard deviation of the healthy donors for each cytokine j and subjected to hierarchical clustering using the Person correlation as the distance metric with average linkage clustering. Black values are near the mean of the healthy donors, yellow represents measured serum concentrations higher than the mean of the healthy donors and blue represents lower levels. The sample classification color code is along the top: IBC, HD and non-IBC are shown in blue green and red, respectively. No clear IBC-unique signature is observed. Note that the cluster of upregualted inflammatory factors is not enriched for any subtype.



We next looked at median levels each cytokine in IBC, non-IBC and healthy donors. The data are summarized in heat-map form in Figure 2. 2 on page 31. In contrast to our hypothesis, IBC is not characterized by increased inflammation. Using CRP as a measure of general inflammation, median levels are slightly higher in IBC but not significantly ($p = 0.706$). However, patients with metastatic disease, both MBC and MIBC, have elevated levels of CRP compared to normal ($p = 0.050$ and 0.049, respectively). MBC was higher than MIBC, but not significantly ($p = 0.498$). We might expect inflammatory markers to increase with treatment as chemoradiation induces cell death. In MIBC, the elevated CRP is not a result of treatment, as treatment naïve patients have elevated levels of CRP as well ($p = 0.032$). In contrast, treatment naïve MBC patients ($n = 6$) did not have elevated CRP levels ($p = 0.965$). In fact, with a median CRP of 8.2 mg/L, MBC was only slightly higher than HD (median 5.6 mg/L) and with a trend to be lower than MIBC (median = 24.9 mg/L, $p = 0.097$). While the number of treatment naïve MBC patients is very small, this suggests there MIBC is a different entity than MBC without inflammatory features.

Observing the median serum levels of the other inflammatory markers, the pattern seems to hold: IBC is <u>not</u> characterized by systemic inflammation compared to LABC. In contrast, osteoprotegerin (OPG), osteocalcin (OC), eotaxin, TGF-α, IFN-α, IL-3 and IL-8 are all significantly lower in IBC than in LABC ($p < 0.05$).

Furthermore, looking at patients with metastatic disease, those with inflammatory features (MIBC) had lower levels of inflammatory cytokines than those non-IBC metastatic breast cancer including OPG, OC, eotaxin, G-CSF, FLT-3L, IFN-α, IL-17, IL-7, IL-8, and MIP-1β ($p < 0.05$), although for most of these cytokines, treatment seemed to contribute to the elevated levels as only OPG, OC and FMS-like tyrosine kinase 3 ligand (FLT3L) remained significant when looking at only treatment naïve patients.

The TH1/TH2 balance (a sum of the HD standardized z-scores for IFN-γ, IL-12p70 and IL-2 and subtracting IL-4, IL-5, IL-6, IL-10 and IL-13) seems to favor TH2 in IBC and MIBC, again primarily in the treatment naïve patients.

Figure 2. 2

Inflammatory breast cancer has lower inflammatory cytokines. The serum cytokine data is presented as median values. As in the previous figure, low values are colored blue and hi values are colored yellow. Note that the colors represent scaling, not significance. All units are in pg/mL except CRP that is in mg/L. An expanded version of the figure is on the following page. IBC = inflammatory breast cancer, LABC = locally advanced breast cancer, MBC = metastatic breast cancer (without inflammatory features), MIBC = metastatic breast cancer with inflammatory features, HD = healthy donors.

	IBC	LABC	MBC	MIBC	HD
CRP	9.0	6.9	22.0	11.9	5.6
IL-1α	12	17	27	19	22
IL-1β	2	2	2	2	2
IL-1RA	27	17	7	26	0
IL-6	4	3	6	6	0
TNF-α	7	8	9	8	11
TNF-β	8	10	14	10	20
IL-8	26	33	50	26	34
bFGF	86	94	131	91	136
VEGF	332	354	480	286	322
IFN-γ	29	32	36	24	39
IL-2	2	3	1	2	0
IL-12p40	13	20	25	19	10
IL-12p70	7	6	12	6	5
IL-13	6	5	5	7	0
IL-4	0	0	0	0	0
IL-5	1	1	1	1	1
IL-9	3	2	3	4	3
IFN-α	17	32	40	21	33
IL-17	19	24	27	13	22
TGF-β	33172	29268	28290	33517	31942
EGF	151	215	198	170	75
TGF-α	5	7	7	5	7
Flt-3L	13	56	92	30	46
G-CSF	56	65	97	58	113
GM-CSF	44	50	66	49	61
IL-15	1	2	3	3	2
IL-3	0	0	0	0	0
IL-7	7	15	25	10	26
sIL-2Rα	0	0	9	5	0
GRO	668	737	991	868	1260
IL-10	9	6	9	9	7
IP-10	379	344	514	495	416
MCP-1	973	1010	1018	944	524
MIP-1α	17	17	19	16	14
MIP-1β	47	56	68	50	62
MCP-3	10	14	15	13	4
Eotaxin	111	157	179	110	134
MDC	1690	1664	1705	1554	1713
Fractalkine	63	98	113	73	91
OC	424	683	733	441	562
OPG	212	252	316	250	254
PTH	11	11	11	12	25
RANKL	0	0	0	0	0

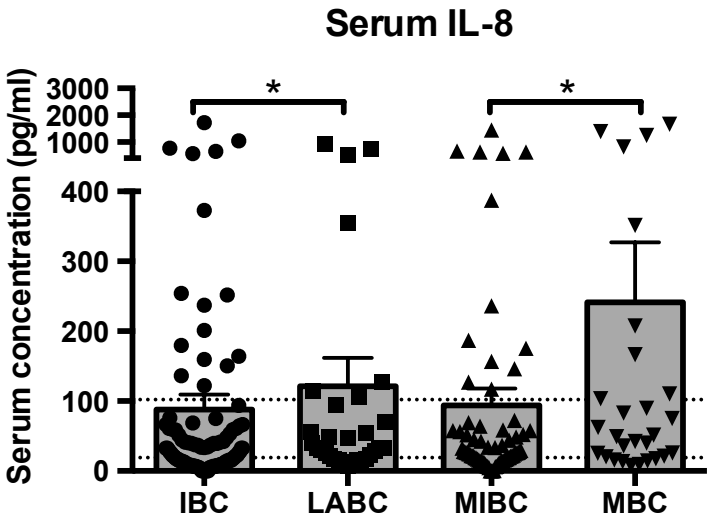
Lo Med Hi

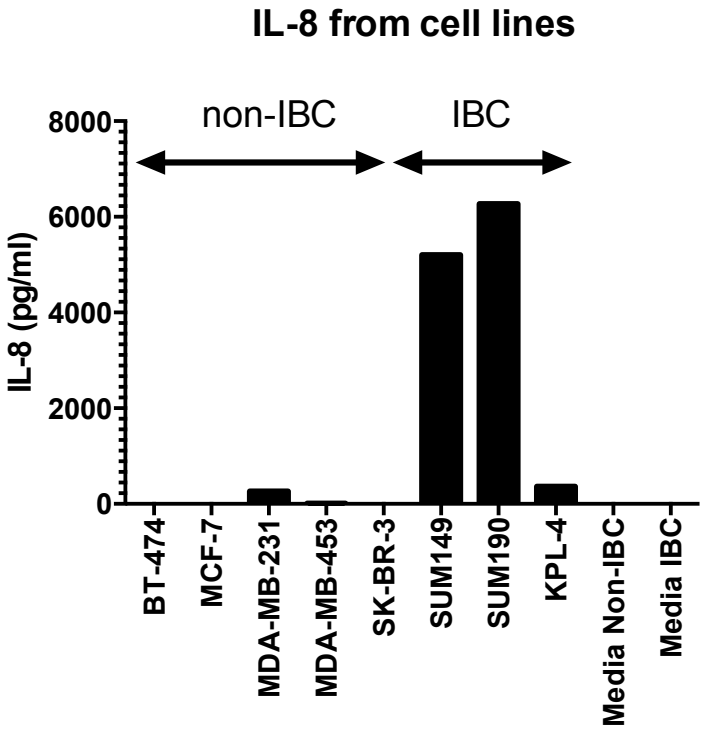
	Treatment Naive					Previously Treated				Subtype			Mets		IBC		NonIBC			IBC			Treatment	
	IBC	LABC	MBC	MIBC	HD	IBC	LABC	MBC	MIBC	IBC	MBC	MIBC	HR+	Her2+	TNBC	M0	M1	non-IBC	IBC	HR+	Her2+	TNBC	HR+	Her2+	TNBC	Naïve	Previous
CRP	9.1	6.9	22.0	11.9	5.6	8.6	6.2	6.1	15.1	15.1	23.4	9.4	11.5	8.7	12.5	8.7	12.9	12.2	10.6	7.5	17.6	24.6	14.0	8.1	11.7	8.7	15.2
IL-1a	12	17	27	19	22	15	20	54	17	8	26	26	19	14	20	13	24	25	16	25	16	26	17	13	17	17	16
IL-1β	2	2	2	2	2	2	2	2	1	2	4	3	2	2	2	2	2	2	2	2	2	3	2	2	2	2	2
IL-1RA	27	17	7	26	0	30	19	17	16	23	5	43	23	23	28	25	21	13	27	0	16	52	30	23	21	21	23
IL-6	4	3	6	6	0	5	4	10	5	1	6	11	6	4	5	4	6	4	5	4	4	5	6	4	4	4	5
TNF-α	7.01	7.52	8.85	7.83	10.68	7.28	7.91	7.41	7.47	6.58	10.11	9.86	7.03	7.9	7.6	7.03	7.99	8.07	7.34	8.83	8.16	6.86	6.62	7.76	7.68	7.91	7.76
TNF-β	7.59	9.53	13.55	10.46	19.62	7.71	9.23	3.31	9.96	6.13	17.18	12.92	10.9	8.06	9.64	7.72	11.13	13.1	9.15	13.97	9.23	13.13	10.58	7.85	9.31	10.07	10.58
IL-8	25.73	33.36	50.18	25.98	34.46	28.21	33.04	45.62	26.09	17.79	63.19	24.41	30.34	26.2	32.82	29.35	27.15	41.13	25.98	42.46	39.56	48.53	24.19	24.79	31.17	28.94	27.08
bFGF	86	94	131	91	136	88	93	91	93	79	138	87	92	92	89	88	93	100	89	97	108	100	90	89	88	95	90
VEGF	331.62	353.94	479.62	285.69	321.68	365.13	394.21	623.29	302.68	223.16	414.36	230.11	367.43	290.21	344.84	331.62	302.68	400.28	315.57	437.98	284.87	481.52	329.04	293.4	334.15	336.57	287.54
IFN-γ	29	32	36	24	39	37	33	69	35	14	34	19	34	29	25	30	27	34	26	44	26	31	26	29	18	39	24
IL-2	2	3	1	2	0	2	3	2	1	1	1	3	2	2	3	2	2	3	2	0	3	6	2	2	1	2	2
IL-12p40	13	20	25	19	10	16	18	15	13	11	30	25	16	14	19	15	20	20	15	13	18	35	17	13	15	15	18
IL-12p70	7	6	12	6	5	10	6	43	6	3	12	4	9	8	4	7	7	8	6	8	8	4	11	8	4	6	5
IL-13	6	5	5	7	0	8	7	7	5	2	4	8	9	5	4	6	6	5	6	8	3	2	9	5	5	6	5
IL-4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IL-5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
IL-9	3	2	3	4	3	3	2	3	3	1	3	5	3	3	3	3	3	2	3	2	3	4	4	3	2	3	2
IFN-α	17	32	40	21	33	17	31	121	19	14	40	29	21	19	21	20	22	33	19	31	34	35	20	18	20	21	23
IL-17	19	24	27	13	22	32	24	57	15	7	24	13	22	17	14	20	17	25	16	24	25	31	18	17	12	24	13
TGF-β	33171.94	29268.26	28289.73	33516.64	31941.8	34063.12	28914.68	22187.73	27688.67	27410.5	28622.71	29999.37	30925.76	33770.55	30025.29	32262.9	31745.99	28805.65	33424.67	28289.73	30591.48	28415.39	33501.22	34170.2	31585.19	33235.57	28622.71
EGF	151	215	198	170	75	153	199	114	148	146	205	203	167	153	199	155	180	200	155	176	192	286	167	142	181	144	201
TGF-α	5	7	7	5	7	6	8	4	7	5	7	4	6	6	5	6	6	7	5	5	8	11	6	5	4	6	5
Flt-3L	13	56	92	30	46	11	61	167	12	20	92	55	30	13	38	20	39	74	21	84	87	23	20	9	39	22	51
G-CSF	56	65	97	58	113	54	65	43	51	64	107	75	67	61	56	57	65	74	57	73	65	97	66	59	51	62	79
GM-CSF	44	50	66	49	61	44	48	37	43	45	76	67	49	47	49	45	58	60	45	55	76	58	46	44	47	47	59
IL-15	1	2	3	3	2	1	2	3	2	1	3	5	2	2	2	2	3	2	2	2	2	3	2	1	2	2	2
IL-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IL-7	7	15	25	10	26	9	15	46	8	5	22	14	11	10	8	8	12	17	8	20	16	19	9	9	8	12	10
sIL-2Rα	0	0	9	5	0	0	0	88	0	0	0	35	0	0	2	0	5	0	0	0	0	55	0	0	1	0	1
GRO	668	737	991	868	1260	672	723	436	799	661	1037	967	847	697	723	673	903	889	723	896	942	651	819	672	738	752	903
IL-10	9	6	9	9	7	9	5	2	7	7	10	13	7	8	10	8	9	7	9	3	7	11	9	8	10	7	9
IP-10	379.08	343.91	514.11	495.43	415.63	373.36	337.58	422.18	462.01	384.3	537.66	524.64	406.3	373.36	497.82	377.88	501.39	420.69	406.3	396.76	656.38	521.4	422.38	367.1	491.25	389.01	534.81
MCP-1	972.85	1010.02	1018.15	944.38	524.1	907.42	1015.42	1118.21	818.43	1150.53	1010.49	1070.4	968.6	907.42	1015.42	978.02	969.63	1014.04	948.01	1036.78	978.59	1015.42	944.38	899.65	989.88	852.42	1046.18
MIP-1α	17.07	16.95	19.11	15.73	13.5	17.53	16.88	20.63	15.56	12.33	19.11	16.29	17.13	17.22	16.76	16.97	16.78	17.13	16.78	17.13	14.07	18.8	17.22	17.25	15.9	16.85	16.78
MCP-3	10	14	15	13	4	13	13	16	14	8	15	11	14	11	11	12	14	14	12	14	12	16	13	10	10	13	11
MIP-1b	47.42	56.41	68.36	49.56	62.3	51.08	59.23	103.78	52.19	38.2	68.36	38.63	51.56	52.37	48.87	48.99	55.09	61.93	47.8	59.99	75.81	59.23	49.51	47.92	38.39	53.13	47.36
Eotaxin	111	157	179	110	134	112	126	140	110	101	188	111	114	119	125	117	128	163	111	146	165	171	96	111	120	120	128
MDC	1690	1664	1705	1554	1713	1714	1656	1363	1501	1326	1753	1592	1676	1714	1441	1678	1609	1669	1609	1711	1336	1907	1644	1754	1376	1697	1609
Fractalkine	63	98	113	73	91	68	93	101	61	43	132	86	81	70	63	69	77	108	66	110	112	93	79	62	57	73	79
OC	424	683	788	441	562	398	682	906	313	643	669	514	484	455	465	460	469	686	431	686	673	778	418	438	444	440	575
OPG	212	252	308	250	254	203	251	363	252	291	311	242	245	227	265	222	229	296	229	284	299	341	236	209	250	235	290
PTH	11	11	11	12	25	10	10	2	11	13	13	12	12	10	12	11	11	11	11	12	7	10	12	10	12	12	13
RANKL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

IL-8 was significantly lower in IBC than in either LABC ($p = 0.045$) or HD ($p = 0.03$). Additionally, IL-8 was significantly lower in MIBC than in MBC ($p = 0.027$). This is unexpected considering the important role IL-8 plays in maintaining breast cancer stem cells (65, 66) and that IBC cell lines produce high levels of IL-8 as we have previously shown (75). IL-8 has a serum half-life of about 10 minutes (76), so it seems unlikely that tumor sequestration of IL-8 could explain the low levels observed in IBC.

Figure 2. 3

IBC has low serum IL-8, but cell lines express high IL-8. IL-8 levels were measured in sera of IBC, LABC, MBC and MIBC patients. IBC median IL-8 levels are significantly lower than LABC (Mann-Whitney U $p < 0.05$). Likewise, median MIBC serum IL-8 is significantly lower than MBC. When IL-8 production was measured in the supernatants of breast cancer cell lines, the IBC cell lines expressed higher levels of IL-8 than non-IBC cell lines.



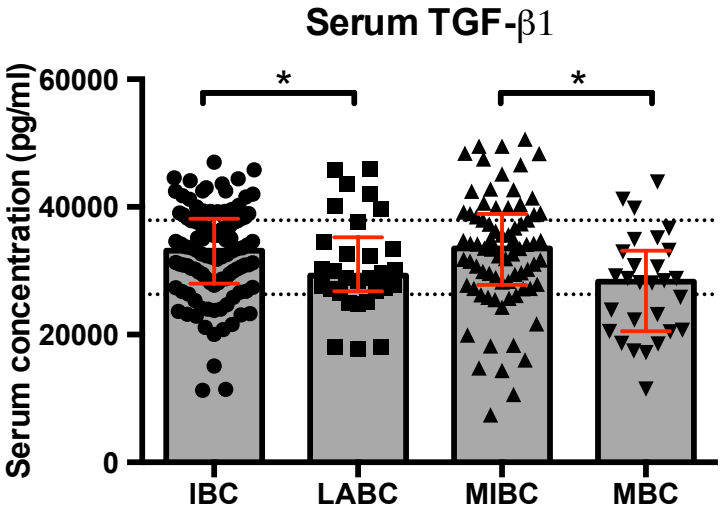


The one cytokine that was significantly elevated in IBC was TGF-β1. In all patients, there was a non significant increase in TGF-β1 in IBC compared to LABC ($p = 0.139$). However, when comparing only treatment naïve patients, IBC (median TGF-β1 34.0 mg/L) was significantly higher than LABC (median 28.9 mg/L). Comparing MIBC to MBC, MIBC was significantly higher both in all patients ($p = 0.005$) and in an analysis restricted to treatment naïve patients ($p = 0.002$). Interestingly, the IBC patients have normal levels while the MBC patients have lower than normal levels when untreated ($p = 0.039$). Likewise, MIBC has significantly higher levels of TGF-β1 than MBC ($p = 0.005$ in all patients and $p = 0.002$ in treatment naïve patients). This is particularly surprising given that recent gene expression micro-array data of laser micro-dissected tumors show that IBC tumors have attenuated TGF-β signaling (77).

Together with the CRP data and general inflammatory profile, these data suggest that IBC has more controlled systemic inflammation, possibly mediated by the anti-inflammatory properties of TGF-β1. Interestingly, in addition to its anti-inflammatory properties, TGF-β1 is the prototypical inducer of EMT, the process that allows tightly clustered epithelia cells to gain migratory and self-renewal abilities while impeding proliferation. The CRP data suggesting less inflammation contradicts the central hypothesis and anecdotal observations from the clinic therefore we are repeating the CRP analysis using a high-sensitivity clinical test from Siemens. Preliminary analysis shows median values of HD = 1.8, IBC = 3.0, LABC = 2.5 MBC = 4.3 and MIBC = 7.3 with both MBC and MIBC significantly higher than normal.

Figure 2.4

TGF-β is significantly elevated in IBC. Dotted lines show the 25th and 75th percentile range of the healthy donors. Bars show the median with interquartile range. Asterisks indicate significance by Mann-Whitney test at $p \leq 0.05$.



Chapter 3: Inflammatory Breast Cancer Hematology Profile

INTRODUCTION

The immune system is the body's defense against potentially harmful cells and organisms. Everything from viruses to bacteria to multicellular parasites to cancer must be eliminated or controlled to sustain life. The human body has protective layers starting with the skin as a protective barrier and progressing down to the single cell level where multiple pathways have evolved to protected against intracellular attack. However, the hematopoietic system comprised of red and white blood cells, lymph nodes spleen, bone marrow and thymus is unique in its ability to circulate throughout the body.

It is thought that the immune system evolved to protect the host against infection. The mammalian immune system is a highly complex network of specialized and interacting cells with a seemingly endless array of sub-types. A brief introduction to each of the major classes is provided prior to discussing them in the context of inflammatory breast cancer in the following sections. However, the immune system and the inflammation it induces have a darker side as there is increasing evidence that inflammation plays a crucial role in cancer development.

Cancer is a heterogeneous system that has a multitude of interactions with the microenvironment at multiple steps during tumorigenesis and metastasis. Interactions with the immune cells at each step of multistage metastasis can be pro-tumorigenic or anti-tumorigenic. First, as a nascent primary tumor, mutant cancer cells must evade the immune system that is capable of recognizing and attacking novel antigens that may arise with the transformation process. As it grows, the tumor requires growth factors and angiogenic factors that can be autocrine or provided by the microenvironment. Invasion of surrounding tissue requires degradation of extracellular matrix. Many tumor cells acquire this invasive phenotype, but macrophages that specialize in tissue restructuring

can also be recruited. To reach a metastatic site, tumor cells from the primary tumor transit by way of the blood stream or lymphatic vessels where they must not only survive the turbulence of transport, but must continue to evade immune surveillance. Only a very small proportion of tumor cells that enter the circulation are able to survive this process. Upon reaching the pre-metastatic niche, the tumor cell again must invade the surrounding tissue that must be conducive to tumor growth. At each stage inflammation and regulation of the immune response plays critical. In the 5th chapter of this thesis, we will provide evidence that inflammatory factors from activated immune cells are capable of inducing epithelial-mesenchymal transition (EMT) in inflammatory breast cancer cells. However, in this chapter, we will first try to define the scope of the peripheral immune system in IBC.

POPULATION STUDIED

In order to establish a hematological profile of peripheral blood cells in inflammatory breast cancer, we analyzed peripheral blood samples obtained under MD Anderson protocol Lab08-0199 "Reactivation of Epstein Barr virus in patients with breast cancer." This IRB-approved protocol had a planned enrollment of 120 patients and 30 healthy female donors and was open to patients starting a new line of therapy. The planned enrollment was for 30 patients with stage III inflammatory breast cancer and 30 patients with locally advanced breast cancer (LABC) (stage IIB or higher) for comparison. In addition, the protocol was to enroll 30 patients with metastatic breast cancer with inflammatory features (from here on referred to as metastatic IBC, or MIBC) and 30 patients with non-IBC metastatic breast cancer (MBC). We hypothesized that IBC patients would be worse than LABC patients, and that MIBC would be worse than

both IBC and MBC. Patients were recruited between October 2008 and April 2012 from the Nellie B. Connally Breast Center and the Morgan Welch Inflammatory Breast Cancer Research Program and Clinic at the University of Texas MD Anderson Cancer Center. There was an over-enrollment in the MIBC arm of the study with a dearth of IBC patients as several patients with metastases were recruited to the Stage III (non-metastatic) IBC arm of the study. These patients were enrolled prior to staging and were thought to have locally advanced disease, but due to incomplete staging at the time of enrollment and the rapid progression of IBC, were subsequently found to have progressed to MIBC at the time of enrollment. As a consequence, the protocol was modified to increase the number of allowable MIBC patients. Additionally, 5 patients with secondary IBC were excluded from the analysis. For this analysis, total enrollments included 32 patients with IBC, 26 patients with LABC, 26 with MBC and 54 with MIBC and 34 healthy female donors (HD) as controls.

Patient characteristics are presented in Table 3.1 Patient Characteristics on page 17. The healthy donor pool was significantly younger than patients (Student's t-test, $p < 0.001$). However we thought it prudent to include younger healthy donors, as IBC is known to afflict young women. Indeed, Morgan Welch, the namesake of the IBC program at MD Anderson, was 24 years old when she died of IBC. Ten of the healthy donors included in this study were between 24 and 30. Excluding these 10, the mean age of healthy donors was 46.4 years old, providing a rough approximation to the study population with no significant difference in age between the pruned healthy donor population and any of the groups (Student's *t*-test > 0.05). There was no significant difference between IBC and LABC with respect to age (Student's *t*-test $p < 0.05$) or the distribution of race, tumor grade, subtype (HR+, Her2 amplified or TNBC), (χ^2 test $p > 0.05$), or the administration of prior systemic therapy (Fisher's Exact test $p > 0.05$). The

locally advanced group included 14 patients (54%) with stage II tumors, whereas by definition, all of the IBC cases are stage III. As such, there is a slight stage mismatch between IBC and LABC. Additionally, IBC had a significantly higher proportion of samples with reported lymphatic invasion: 85% of IBC cases noting the presence or absence of lymphatic invasion were reported as positive vs. 30% of LABC (Fisher's Exact test $p = 0.0002$). It should be noted, however, that dermal lymphatic invasion is a confirmatory diagnostic criteria for IBC, therefore pathologists examining a suspected IBC case are specifically looking for the presence of lymphatic invasion. As such, there can be a bias for the positive reporting of lymphatic invasion in the IBC cases.

Comparing MBC with MIBC, there was no significant difference with respect to age (Student's *t*-test $p > 0.05$) or the distribution of patient race, tumor grade, lymphatic invasion, or prior use of systemic therapy (Fisher's Exact test $p > 0.05$). As shown in Table 3.2 Tumor Intrinsic Subtypes in Metastatic Disease, on page 48, MIBC had a significant enrichment in patients with TNBC tumors (χ^2 test, $p = 0.0413$). This is in concordance with known studies of IBC (78).

Table 3.1 Patient Characteristics for Cellular Studies

Patients in this study were recruited under MD Anderson IRB-approved protocol Lab08-0199. Patients included locally advanced breast cancer patients with and without inflammatory features (IBC and LABC, respectively) and stage IV metastatic patients with and without inflammatory features (MIBC and MBC, respectively). Tumor subtypes are derived from pathological report, TNBC tumors lacked expression of ER and PR, and Her2 amplified; tumors were classified as Her2 amplified or Her2+ if they were IHC 3+ or FISH+; tumors were classified as luminal if they were positive for ER or PR staining but not Her2 amplified. Abbreviations: HR, Hormone receptor positive IBC; LABC, Locally Advanced Breast Cancer; MBC, Metastatic Breast Cancer; MBIC, Metastatic breast cancer with inflammatory features; TNBC, triple receptor negative breast cancer

Table 3.1

		IBC	LABC	MBC	MIBC	HD
N		32	26	26	54	34
Age	Mean (range)	54.7 (32-76)	51.4 (31-67)	54.5 (35-75)	53.0 (31-76)	40.7[c] (24-60)
Race	Asian/Pacific Islander	2	0	1	0	
	African American	1	4	3	4	
	Spanish/Hispanic	2	6	5	4	
	Caucasian	26	16	17	46	
Stage	II	-	14	-	-	
	III	32	12	-	-	
	IV	-	-	26	54	
Grade	1	1	3	0	0	
	2	12	8	7	15	
	3	18	15	13	37	
Treatment Status	Previous Systemic Therapy	3	2	19	36	
	Treatment Naïve	29	24	7	18	
Subtype	Luminal (HR+)	9	13	14	15	
	Her2+	16	9	7	15	
	TNBC	6	4	5[b]	24[b]	
Lymphatic Invasion	Positive	22[a]	6[a]	8	36	
	Negative	4	14	5	3	

a) IBC vs. LABC $p < 0.005$; b) MIBC vs. MBC, $p < 0.05$; c) HD vs. all others, $p < 0.05$

Table 3.2 Tumor Intrinsic Subtypes in Metastatic Disease

Intrinsic tumor subtypes were approximated by IHC expression patterns and FISH. There is a significant enrichment of triple receptor negative tumors in MIBC compared with MBC (χ^2 test, $p = 0.0413$).

Table 3.2

	MBC	MIBC
Luminal (HR+Her2-)	54%	28%
Her2+	27%	28%
TNBC	19%	44%

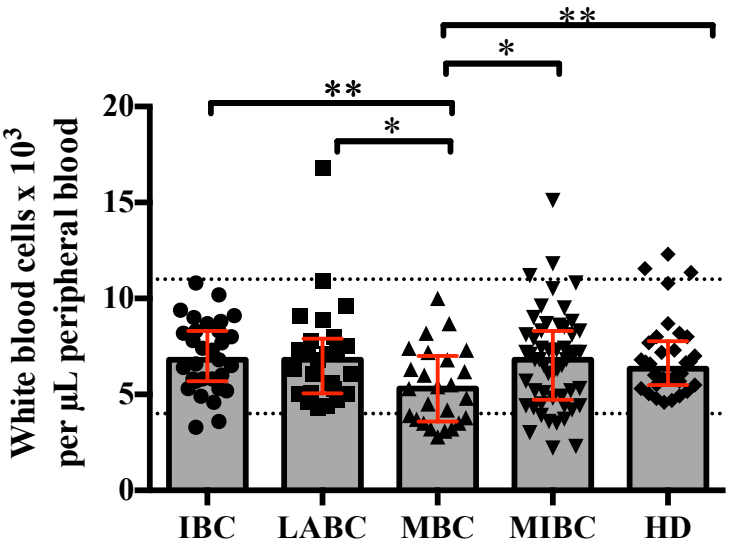
TOTAL PERIPHERAL BLOOD WHITE BLOOD CELL COUNT IS NORMAL IN IBC

White blood counts (WBC) measure the total circulating leukocytes. Normal ranges are between $4x10^3$ and $11x10^3$ cells/μL. In general, a low WBC can leave a patient susceptible to infection whereas a high WBC is indicative of an infection or hematoproliferative disorder but can also increase with smoking or corticosteroid administration. White blood cell counts also tend to decrease with age. In general, a total WBC between $3x10^3$ and $11x10^3$ cells/μL of blood is considered normal consisting on average of 50-70% neutrophils, 25%-35% lymphocytes, 4-6% monocytes, 1-3% eosinophils, 0.4%-1% basophils. To get a proper picture, both absolute WBC and relative (percent) values of individual leukocyte types should be considered. Table 3.3 White Blood Count and Leukocyte Differential – Absolute counts on page 59 and Table 3.4 White Blood Count and Leukocyte Differential – Relative Values on page 61 summarize the data from these studies. The WBC is shown graphically in Figure 3.1 White blood counts in IBC are normal on page 51. The data is presented graphically in Figure 3.2 White blood count and leukocyte differential on page 63. The table highlights significant differences between groups. However, for these tables and throughout this dissertation, differences between IBC and MBC, and LABC and MIBC are ignored as these are not meaningful comparisons.

Median WBC was (in thousands of cells/μL) 6.80 for IBC, 6.80 for LABC, 5.30 for MBC and 6.80 for MIBC. The average HD WBC was 6.35. MBC had the lowest WBC, significantly lower than that of HD, IBC, and even MIBC.

Figure 3.1 White blood counts in IBC are normal

WBC from IBC, LABC, MBC, MIBC and HD are shown as median + interquartile range. The normal range of 4–11 x 10^3 leukocytes per μL of blood is shown with dotted lines. WBC from both IBC and MIBC was not significantly different from normal. However, the median WBC in MBC was significantly lower than normal although still within the normal range. 36% of MBC patients were leukopenic with WBC below 4 x 10^3 cells per μL. Significant differences as determined by Mann-Whitney U test are denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$), or *** ($p \leq 0.001$).



	IBC	LABC	MBC	MIBC	HD
# < normal	2	0	9	7	0
% < normal	6%	0%	36%	13%	0%

WHITE BLOOD COUNT AND DIFFERENTIAL

Looking at total WBC gives only a very granular picture where IBC and MIBC both appear to be no different from that of HD. However, examining subsets of cells gives a broader picture. Throughout this chapter we will continue to subdivide peripheral blood leukocytes into smaller and smaller subsets. In the following chapter, we will look at a few specific subsets and ask whether there are any functional differences in the cells.

At a first pass, peripheral blood leukocytes can be subdivided in to granulocytes and agranulocytes. Granulocytes, which include neutrophils, eosinophils and basophils, are also called polymorphonuclear leukocytes due to the varying shape of the nucleus and staining of intracellular granules. The most common granulocyte is the neutrophil, the short-lived, armed-to-the-teeth bacteria killing machine. These cells generally live about 6 days with a circulating half-life of less than a day and are continuously regenerated by the bone marrow under physiological conditions. Neutrophils are very important as a first-line defense against infection; however, they have also been implicated in promoting metastasis. For example, high neutrophil levels, specifically the neutrophil to lymphocyte ratio, have been associate with poor clinical outcome in breast (79) and ovarian cancers (80), as well as nasopharyngeal (80), hepatocellular (81), gastric (82), colorectal (83), and renal cancers (84). They can be recruited to the tumor microenvironment by chemoattratctants or chemokines such as IL-8 (85) or Damage-associated molecular pattern molecules DAMPS such as high-mobility group box 1 (HMGB1)(86) that have been shown to be overexpressed in cancer (87). Neutrophils can have strong angiogenic activity by the release of matrix metalloprotease (MMP) 9 and VEGF(88). In vitro, neutrophils can enhance the migration of breast cancer cells in an

intercellular adhesion molecules (ICAM)-dependent manner (89). Additionally, neutrophils are capable of establishing a pro-thrombocytic state through the creation of neutrophil extracellular traps through the release of DNA (90). TGF-β can polarize neutrophils towards a pro-tumor N2 (type-2 immune response) state typified by high levels of arginase 1 and decreases tumor-directed cytotoxicity, whereas lack of TGF-β signaling can induce anti-tumorigenic N1 neutrophils that activate CD8 cells and are more cytotoxic (91). Additionally, factors in the microenvironment can extend the life of neutrophils. Perhaps most provocatively, neutrophils can prepare pre-metastatic sites prior to the arrival of tumor cells. Using a mouse model of breast cancer, Granot et al showed that "tumor-entrained neutrophils" (TENs) accumulate in the lung and decrease tumor seeding efficiency. They showed that neutrophils arrive at premetastatic site prior to detectable tumor cells; without the TGF-β suppressive effects from the tumor microenvironment, they are able to acquire an anti-tumorigenic N1 phenotype and are capable of killing tumor cells (92). In contrast, Yan et al showed that immature myeloid cells accumulate in the lung and prepare the metastatic niche by releasing MMPs and promote vascular remodeling (93). Thus, neutrophils are capable of greatly influencing the natural history of breast cancer.

Eosinophils, named for their affinity for the acidic dye eosin (94, 95) are typically associated with allergic reactions due to their release of histamine. They are typically associated with type-II immune responses (which will be discussed in Chapter 4) that help fight multicellular parasites. Eosinophils release factors such as leukotrienes (derived from arachadonic acid), major basic protein, eosinophil cationic protein (which is similar to porforin), eosinophil-derived neurotoxin, eosinophil peroxidase, CD30L (a TNF-α family member) and a number of pro-inflammatory cytokines (96). Although eosinophils have been shown to induce angiogenesis (97) and are active in breast

development (98), much research is required to understand the possible roles eosinophils may have in cancer.

Basophils, the least common class of granulocytes, are named for the affinity to blue, basophilic dyes. They tend to accumulate at site of ectoparasite infections such as ticks and allergic reactions. IgE-primed basophils degranulate releasing histamine and inflammatory factors. As they are targeted to the skin, they have potential relevance in IBC. Although they arise from different progenitors, basophils in blood are similar to mast cells in tissue, which have been shown to be immune suppressive (99). The immune suppressive effects are mediated by IL-10 (100). Mast cells are recruited to the skin via draining lymph nodes (101) by the chemokine receptor CXCR4 (102). Although the relevance of mast cells to cancer biology is starting to agglomerate, very little is known about basophils cells: in fact a Pubmed search of "basophil" and "breast cancer" only returned 11, mostly unrelated hits.

In addition to granulocytes, peripheral blood leukocytes are comprised of two primary types of agranulocytes that can be readily distinguished by morphology alone: lymphocytes and monocytes.

Monocytes are the precursors to the major phagocytic cells of the body. Sharing a common myeloid progenitor with the granulocytes, monocytes give rise to microglia in the brain, Kupffer cells in the liver, osteoclasts in the bone and the professional antigen presenting cells: monocyte-derived dendritic cells (mDC, which can be found in blood and will be discussed separately). These differentiated cells, and to a lesser extent the circulating monocytes, engulf pathogens, debris, and apoptotic cells (and bone in the case of osteoclasts), but they are more than just trash compactors. Critical to immune function, after sampling the environment, they process and then present the antigens they encounter. Antigen presentation is the critical first step in initiating an adaptive immune

response that allows the immune system to build a memory base to novel antigens. As antigen presenting cells, they are capable of modulating the immune response by providing or retracing critical co-stimulatory signals and regulating the cytokine milieu of the responding cell. These signals produced by antigen presenting cells regulate weather an immune response is initiated and what type of response is produced. Therefore, these cells could be critical in regulating the inflammatory environment.

Lymphocytes are derived from a common lymphoid progenitor during hematopoiesis, separate from the cells discussed, thus far. Generally small, round and non-descript, lymphocytes in blood are comprised primarily of T cells, B cells and Natural Killer (NK) cells. These cells will be discussed only briefly here and in greater detail later. T cells and B cells make up the adaptive arm of the immune system: T cells are responsible for the cellular response and B cells the humoral (antibody) response. After leaving the bone marrow, T cells mature in the thymus (and thus the T-cell nomenclature) where they are "educated." As discussed later, T-cells respond to antigens presented by antigen presenting cells such as monocyte and their progeny and can either elicit a cellular response that can lyse cells including tumor cells, induce an inflammatory response, or provide growth factors for a B-cell antibody response. In humans, B-cells remain in the bone marrow during maturation (however, the term B-cell refers not to bone-marrow, but instead to the bursa of Fabricius, the avain site of hematopoiesis and B-cell maturation that lacks a direct mammalian homologue). Natural killer cells are innate cytotoxic lymphocytes first described for their ability to quickly recognize and kill "non-self" cells and now recognized to have broader immune effector and modulatory activities. Therefore the number of lymphocytes in blood could play a role in tumor control and inflammation.

Thus, different cell types can contribute to the hematologic state of a patient. As noted above, MBC has a decrease in total white blood count. Table 3.3 White Blood Count and Leukocyte Differential – Absolute counts on page 59 and Figure 3.2 White blood count and leukocyte differential on page 63 show the differential. In IBC, peripheral blood counts of neutrophils, lymphocytes, monocytes, eosinophils and basophils are not significantly different from those of healthy donors. MBC, however, has significantly lower than normal counts of neutrophils, lymphocytes and basophils ($p = 0.037$, <0.001, and 0.017 respectively) than those of HD. To facilitate viewing multiple parameters of different scales, each variable in was standardized to the healthy donors as shown in the "radar plots" on the right side of Figure 3.2 White blood count and leukocyte differential.

In comparing neutrophil distributions in advanced breast cancer, we observed an increase in neutrophil counts and relative frequency in IBC, LABC and MIBC but not MBCs. Notably, MIBC did not have the drop in neutrophils. This may suggest that the neutrophils in MBC are associated with the protective effect against metastasis as suggested by Granot (92). However, this is contracted by Kaplan-Meier analysis which shows that patients with higher than average neutrophil counts (greater than the 95^{th} percentile of the HD) have decreased overall survival (median survival of 9.0 months for hi neutrophil counts vs. 34.4 months for average neutrophil counts, $p = 0.044$ with a hazard ratio of 2.5). However, the numbers are very small (7 patients with high neutrophil count and 5 deaths), and it is likely that the neutrophil count is a surrogate for infection. Nevertheless, it is noteworthy that a simple blood test can be so illuminating.

All patient groups with the exception of LABC had significantly lower than normal lymphocyte counts (Mann-Whitney U $p < 0.05$) and metastatic (M1) patients had lower than lymphocyte counts than non-metastatic (M0) patients. While there was no

significant difference between IBC and LABC, this begins to suggest that the hematology in IBC "worse" than non-IBC and is there is a fundamental difference that makes IBC a unique entity.

Unique to MIBC (seen as a purple line on the radar plot in Figure 3.2 White blood count and leukocyte differential), there is a significant increase in monocyte counts relative to HD, non-metastatic IBC and MBC ($p < 0.05$). However, while MBC has a significant drop in WBC as noted above, these patients do not exhibit decreased monocyte counts. This is borne out when looking at the relative proportions of monocytes where MBC has a non-significant trend towards elevated proportions of monocytes. As monocytes are the precursors of most antigen presenting cells (APC) (i.e. dendritic cells and macrophages), our results suggest that sufficient numbers of APC to present to the adaptive arm of the immune system. However, their functional ability to present antigen will be examined in Chapter 4.

Table 3.3 White Blood Count and Leukocyte Differential – Absolute counts

White blood cell counts and differentials were collected as part of routine clinical visits. Data is reported as numbers of cells per μL. The complete white blood count includes neutrophils, lymphocytes, monocytes, eosinophils basophils and unclassified cells. Both mean and median are presented. Test statistics are based off the Mann-Whitney U test. Significant differences are acknowledged by superscript.

Table 3.3

Cells per μL		Disease				
		IBC	LABC	MBC	MIBC	HD
WBC x 10^3	Count	32	26	26	54	34
	Mean	6.99	7.07	5.38	6.75	6.89
	Median	6.80	6.80[d]	5.30[b,d,g]	6.80[b]	6.35[g]
	Standard Deviation	1.87	2.67	1.98	2.53	2.02
Neutrophils	Count	32	26	26	54	34
	Mean	4431	4387	3373	4519	4037
	Median	4460	3800	2999[b,g]	4309[b]	3544[g]
	Standard Deviation	1434	2574	1529	2142	1422
Lymphocytes	Count	32	26	26	54	34
	Mean	1898	2060	1387	1459	2220
	Median	1881[c,e]	1932[d]	1339[d,g]	1243[c,h]	2189[e,g,h]
	Standard Deviation	608	596	635	827	710
Monocytes	Count	32	26	26	54	34
	Mean	463	461	435	575	466
	Median	431[c]	461	428[b]	516[b,c,h]	446[h]
	Standard Deviation	191	138	218	252	172
Eosinophils	Count	32	26	26	54	34
	Mean	152	121	138	147	139
	Median	100	136	120	88	124
	Standard Deviation	129	65	122	210	99
Basophils	Count	32	26	26	54	34
	Mean	28	33	39	30	45
	Median	26	29	21[g]	23[h]	33[g,h]
	Standard Deviation	15	15	76	24	45

a IBC vs. LABC $p < 0.05$
b MBC vs. MIBC $p < 0.05$
c IBC vs. MIBC $p < 0.05$
d LABC vs. MBC $p < 0.05$
e HD vs. IBC $p < 0.05$
f HD vs. LABC $p < 0.05$
g HD vs. MBC $p < 0.05$
h HD vs. MIBC $p < 0.05$

Table 3.4 White Blood Count and Leukocyte Differential – Relative Values

The relative proportions of peripheral white blood cells are displayed. The complete white blood count includes neutrophils, lymphocytes, monocytes, eosinophils, basophils and unclassified cells. Both mean and median are presented. Test statistics are based off the Mann-Whitney U test.

Table 3.4

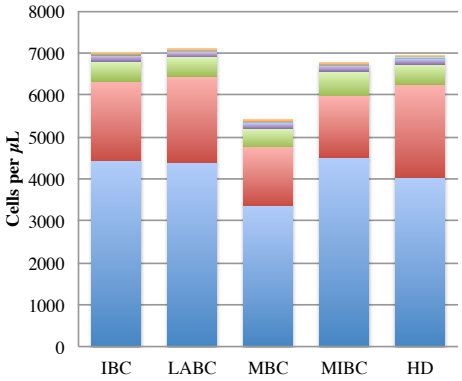
Percent Total Leukocytes		Disease				
		IBC	LABC	MBC	MIBC	HD
Neutrophils	Count	32	26	26	54	34
	Mean	62.6	59.6	62.0	65.9	57.8
	Median	63.2[e]	61.1	62.8	66.1[h]	57.8[e,h]
	Standard Deviation	8.2	10.9	9.8	10.3	7.4
Lymphocytes	Count	32	26	26	54	34
	Mean	28.0	31.1	26.4	22.3	32.6
	Median	26.4[c,e]	30.8	26.2[g]	21.7[c,h]	33.3[e,g,h]
	Standard Deviation	7.8	9.1	9.7	9.8	6.7
Monocytes	Count	32	26	26	54	34
	Mean	6.6	6.8	8.1	8.8	6.9
	Median	6.9[c]	6.5	8.0	8.7[c,h]	6.9[h]
	Standard Deviation	1.7	2.0	3.3	3.0	1.9
Eosinophils	Count	32	26	26	54	34
	Mean	2.2	1.9	2.6	2.3	2.0
	Median	1.7	2.1	2.0	1.4	1.7
	Standard Deviation	1.7	1.0	1.8	2.5	1.2
Basophils	Count	32	26	26	54	34
	Mean	.4	.5	.6	.5	.6
	Median	.4[e]	.4	.4	.4	.6[e]
	Standard Deviation	.3	.3	.8	.3	.5
Other	Count	32	26	26	54	34
	Mean	.2	.1	.1	.3	.1
	Median	.2[e]	.0	.0	.0	.0[e]
	Standard Deviation	.2	.2	.2	.7	.1

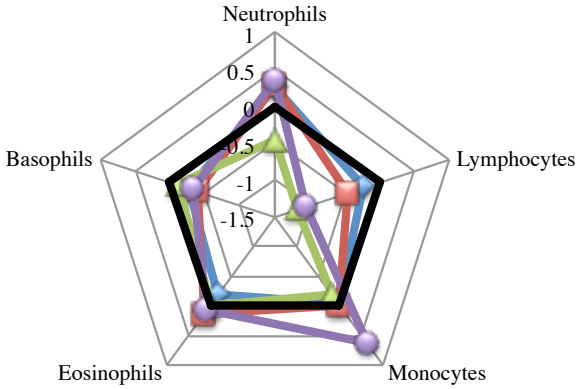
a IBC vs. LABC $p < 0.05$
b MBC vs. MIBC $p < 0.05$
c IBC vs. MIBC $p < 0.05$
d LABC vs. MBC $p < 0.05$
e HD vs. IBC $p < 0.05$
f HD vs. LABC $p < 0.05$
g HD vs. MBC $p < 0.05$
h HD vs. MIBC $p < 0.05$

Figure 3.2 White blood count and leukocyte differential

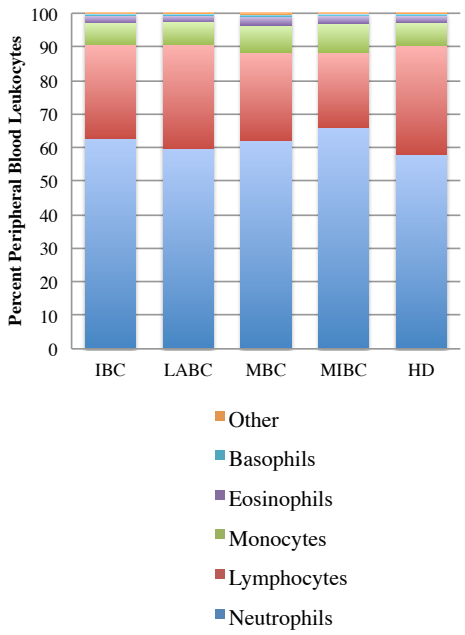
White blood counts and leukocyte differentials are depicted for each group. Average counts and percentages are shown on the left and a standardized "radar plot" is shown on the right. White blood counts are shown as healthy-donor standardized values by the equation $(\mu_{j,i} - \mu_{j,HD}) / (SD_{j,HD})$ where μ_{HD} is the mean of the healthy donors, SD_{HD} is the standard deviation of the healthy donors and μ_i is the mean of IBC (red square), LABC (blue diamond), MBC (green triangle), or MIBC (purple circle) for each j variable (neutrophil count, lymphocyte count, monocyte count, eosinophil count, and basophil count). Each parameter is presented as a radial axis emanating from the center of the plot with the Z-scaled magnitude of each variable proportional to the distance from the center of the plot. As the data are standardized to healthy donors, the HD group appears as a solid black line at 0. A scaled average that is less than healthy donors falls inside the HD circumference and a scaled average that is higher than healthy donors falls outside the HD black circumference. Note that statistics presented in the text and tables are based on non-parametric tests as most variables are not normally distributed; therefore there is no threshold value on these figures that mark a significant divergence from HD.

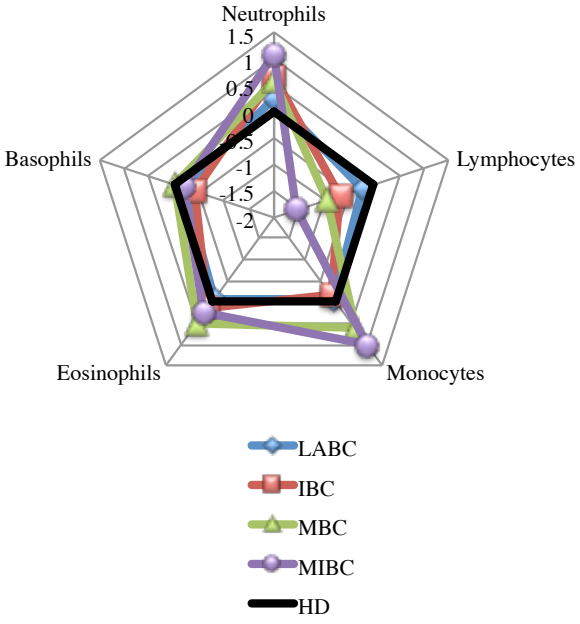
Absolute Counts





Relative Proportions





We can conclude that IBC patients have no major deficiencies in there general classes of leukocytes. This is important to ward off infections and because several therapies, traztuzumab for example, require the presence of white blood cells for full efficacy. In contrast, metastatic patients, both MBC and MIBC, exhibit marked lymphopenia, and all breast cancer patients show a slight decrease in lymphocytes. Lymphocyte subsets are discussed below.

LEUKOCYTE IMMUNOPHENOTYPES

As mentioned above neutrophils, basophils eosinophils, monocyte and lymphocytes can be quickly identified and enumerated based on their forward-scatter (size) and light-scatter (granularity) characteristics and are routinely observed in the basic laboratory White Blood Count and Leukocyte Differential. However, to differentiate lymphocyte subsets such as T, B, and NK cells, as well as dendritic cells, additional expression patterns must be interrogated. For this study we employed fluorophore-labeled antibody staining with and cell enumeration by flow cytometry to characterize leukocyte subytpes, which will be discussed following a brief introduction.

Introduction to immunophenotypes

B cells are one of the two main components of the adaptive immune system and are responsible for producing antibodies. These cells are considered adaptive because unlike innate immune cells, the receptors on the cell surface recognize antigen and elicit the immune response are not encoded in the genome and therefore are not immediately ready to fight an infection or clear mutant cells. Instead, developing B cells undergo somatic recombination of the B-cell receptor producing a diverse repertoire of mature B

cell receptors. During development, self-reactive B cells are deleted to limit auto-immunity and other cells wait silently until they are activated. Each B cell has a single, genetically encoded receptor. When a B-cell is activated by binding its cognate antigen by the B-cell receptor, the cell proliferates to produce progeny with identical receptors. B-cells undergo further somatic hypermutation once activated, a process that allows the population affinity for the antigen to be fine-tuned. Once affinity is optimized, a small population is converted to a memory B cell and the rest become antibody factory plasma cells. As such, when first exposed to antigen, adaptive immune cells require time to ramp up and provide protection, but secondary responses are much faster.

In addition to fighting pathogens, it is widely accepted that B cells play a role in tumor control as well. It is well known that many cancer patients develop auto-antibodies to p53, c-myc and HER-2/neu although they are rarely sufficient to kill malignant cells (103). This led to the development of one of the first drugs to target Her2 amplified tumors. The humanized monoclonal antibody traztuzumab, marketed as Herceptin by Genetech (now a member of the Roche Group), binds to the extracellular domain of the Her2 protein. This binding interferes with both Her2 heterodimerization (and therefore mitogenic signaling) and activates antibody-dependent cellular cytotoxicity (ADCC) (104).

Furthermore, B-cells, by the nature of their highly attuned receptors, act as very efficient and specific antigen presenting cells. In addition to producing antibodies, B-cells also secrete cytokines. As such, they are capable of shaping the immune response of a cognate T cell. For example, B_{reg} cells secrete IL-10 and have anti-inflammatory properties (105).

As there is a flip side to everything, B cells are also capable of promoting cancer. For example, Lisa Coussens has shown that antibodies to extracellular matrix can

increase tumor invasion and promote metastasis (106). Immune complexes consisting of antibodies and complement proteins act as focal points for inflammation. Circulating immune complexes deposit in the tumor microenvironment due to the leaky vasculature of the tumor (107-109). These pre-metastatic lesions can require adaptive immune cells (T-cells and B-cells) to promote innate immune infiltrates that drive full metastatic progression (109, 110). In addition, B-cell derived cytokines such as lymophotoxin-β (TNF-β), WNT16 and TNF-α can promote inflammation and tumor growth (111-113). Finally B cell cytokines such as IL-10 (114) and immunoglobulins (115) are able to inhibit the TH1 anti-tumor response from T-cells.

As mentioned in chapter 2, T cells are largely responsible for shaping the adaptive immune response. T cells express a T-cell receptor (TCR) in the cell surface. Similar to the B-cell receptor, T-cell receptor variability is the result of somatic recombination resulting in clonal T-cells each with unique antigen specificity. Once primed, T cells form antigen memory that allows for rapid secondary immune responses. There are two major classes of T cells: CD8+ cytotoxic T cells (Tc) cells and CD4+ T-helper cells (Th). CD8+ cells are the cytotoxic effector cells have evolved to kill infected cells by recognizing non-self antigens presented on the surface of infected cells by on major histocompatibility complex I (MHC-I). As cancer is often characterized by genomic instability, novel peptides are frequently produced that can be targeted by CD8+ T cell killing. In contrast, CD4+ Th cells recognize peptides expressed on MHC-class II. As MHC-II expression is restricted to professional antigen presenting cells, CD4+ cells do not target the cells they recognize (at least, not for killing). The professional antigen presenting cells (APC) such as monocytes, macrophages, dendritic cells and B-cells, take up antigen from the environment and present it to Th cells. Upon recognizing its cognate antigen, the activated T-cell responds by producing cytokines that "help" the immune

process which will be explored in the following chapter. However, in brief, type-I cytokines (TH1) promote the cytotoxic immune response while type-II cytokines (TH2) promote the humoral response and inhibit the cytotoxic response. A third type of CD4+ Th cell is the regulatory T cell (T_{Reg}). These cells inhibit the immune response by secreting TGF-β and IL-10 and through cell-cell contact through molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death-1 (PD-1). T_{Reg} require IL-2 and express very high levels of CD25, the IL-2 receptor alpha. T_{Reg} typically express the transcription factor FoxP3 but can be enumerated by cell surface markers alone as CD3+CD4+CD25^{bright}CD127- as shown in Figure 3.3 FACS Analysis of Leukocyte Subsets.

Like CD8 cells, NK cells are cytotoxic lymphocytes capable of killing non-self, virus-infected, or abnormal cells. In contrast to CD8+ cytotoxic T cells (T_c) that must first be primed, NK cells do not require priming and are capable of killing nearly immediately. Although NK cells are innate cells, incapable of the genomic rearrangements of T and B cells of the adaptive immune system, it would be inappropriate to consider them a more primitive cell type. In fact, many NK receptor families are not seen outside of mammals (116, 117) suggesting that NK cells in their modern form evolved after T cells. There are over a dozen NK activating and inhibitory receptors that recognize the major histocompatibility complex Class I (MHC I), but each NK cell expresses only a subset of receptors.

There is no single marker to adequately define NK cells. Typically for human cells, once CD3+ T cells are excluded, the remaining CD56+ lymphocytes are often considered NK cells. CD56, also known as Neural Cell Adhesion Molecule (NCAM), is involved in cell adhesion and may play a critical role in human NK cell development

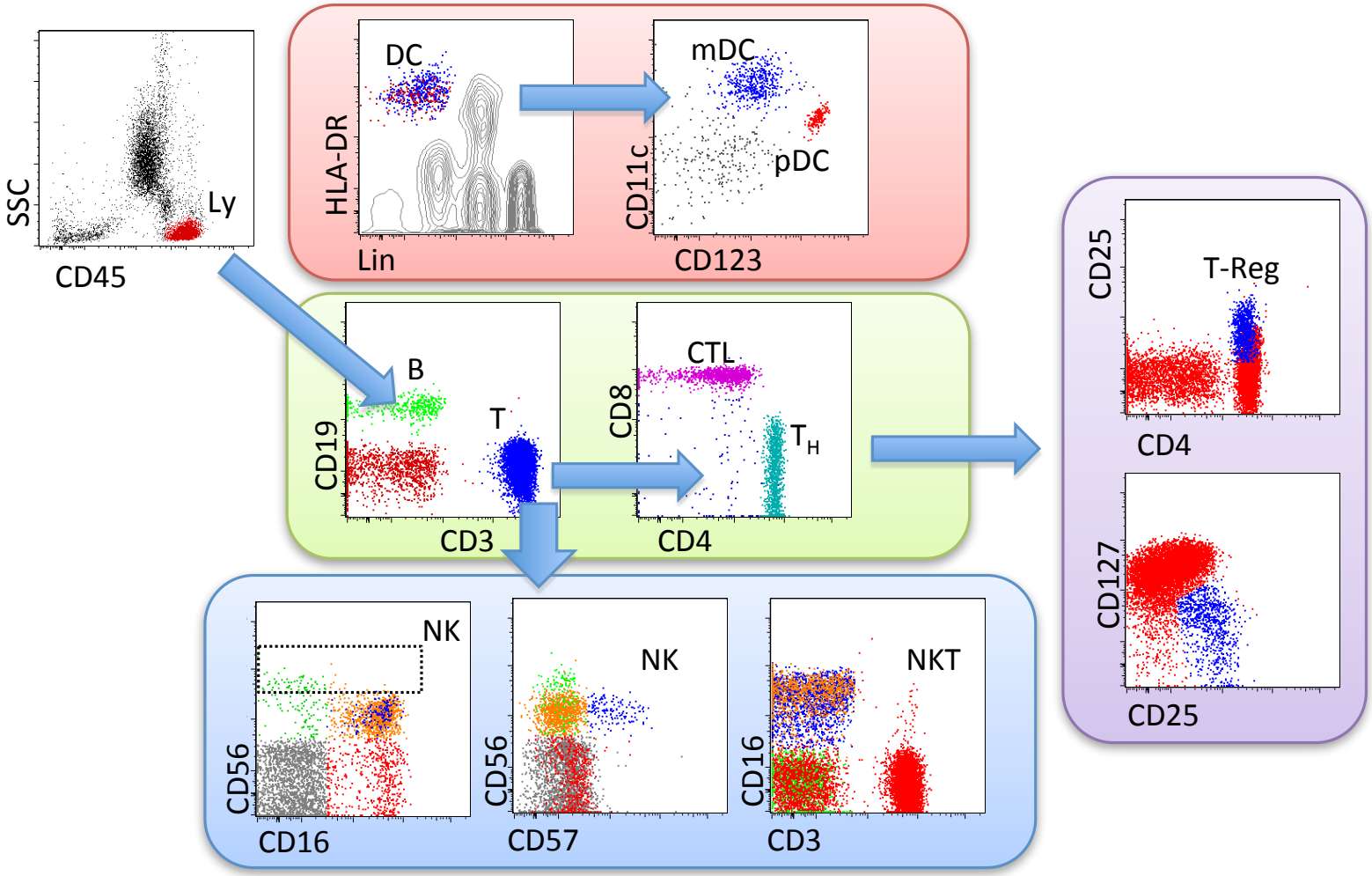
(118). However, it should be noted that CD56 is not expressed by mouse NK cells and therefore is not the definitive NK marker. For clinical tests, NKs are typically stained with a cocktail of antibody reagents consisting of anti-CD56 (NCAM) and anti-CD16 (the low affinity FcγIII receptor that facilitates binding and recognition of IgG immunoglobin). This cocktail captures both the CD56+ NK and the rare CD56- subset that has been shown to be functionally impaired (119). The two primary subsets of NK cells identified based on their expression of CD56: $CD56^{bright}$ and $CD56^{dim}$. $CD56^{bright}$ NK cells are developmentally immature and constitute about 10% of NK cells in peripheral blood but are enriched in secondary lymphoid organs. They generally lack CD16 expression (and are therefore incapable of antibody-dependent cellular cytotoxicity) and have low cytotoxicity, but produce copious amounts of cytokines such as IFN-γ, TNF-α and granulocyte colony stimulating factor (G-CSF). For their ability to secrete cytokines, $CD56^{bright}$ are occasionally referred to as regulatory NK.

As mentioned above, T cells do not respond to free antigen, rather they require peptides (or occasionally lipids) to be processed and presented on MHC class I or class II. Only professional antigen presenting cells express MHC class II with dendritic cells as the most potent. As such, they are critical in determining what antigens T cells will see and in what conditions and form the critical link between the innate and adaptive immune system. Dendritic cells scour the tissue rapidly processing antigen. Once activated through innate pattern recognition receptors that respond to "danger signals" (120), they migrate to lymph nodes where they increase their antigen presenting capabilities and interact with T cells. The most common dendritic cells are derived from monocytes. In the skin and mucosal surfaces these are referred to as Langerhans cells. In the blood they are myeloid derived dendritic cells (mDC). mDC are defined by high expression of HLA-class II (HLA-DR+) and absence of the lineage specific markers for

other agranulocytes (Lin-), specifically CD3 (T cells), CD14 (monocytes), CD16 and CD56 (NK), and CD19 and CD20 (B cells). mDC have lack expression of the IL-3 receptor (CD123) and do express the inactivated-C3b complement receptor 4 (CD11c). A second class of dendritic cell that does not derive from the myeloid line is referred to as plasmacytoid dendritic cells (pDC). These cells are also HLA-DR+ and Lin-. But in contrast to mDC, pDC are CD123+ and CD11c- (see Figure 3.3 FACS Analysis of Leukocyte Subsets). While mDC are the most potent antigen presenting cell, pDC are the professional IFN producing cells, synthesizing massive quantities of the anti-viral cytokine IFN-α which has been shown to have strong anti-tumor effects and frequently used as a anti-tumor therapy (121) but can be immune suppressive (122). As such dendritic cells are critical in control immune induced inflammation.

Figure 3.3 FACS Analysis of Leukocyte Subsets

Whole blood was stained with monoclonal antibodies and analyzed by 6-color flow cytometry. A lymphocyte gate was set based on low light scatter (SSC^{lo}) and expression of the common leukocyte antigen, CD45. Using this lymphocyte gate as an anchor population (SSC^{lo} CD45+), B cells were defined as $CD19^{+}$ cells and T cells were defined as $CD3^{+}$. Within the CD3+ gate, Tc were defined as CD3+CD8+ cells and Th cells as $CD3^{+}CD4^{+}$. T-regulatory (T_{Reg}) were defined as CD3+CD4+$CD25^{bright}$CD127-. Within the CD3-negative population, NK were defined as CD56+ or CD16+. Regulatory NK are $CD56^{bright}$ (outlined with dotted line). NK cells capable of mediating ADCC (ADCC NK) are CD16+. Finally, CD57+ NK cells are terminally differentiated NK cells that can no longer proliferate and produce minimal cytokines. Separately, within the leukocyte gate, NK cells that also express the T-cell receptor (NKT) were defined as cells that are $CD3^{+}$ and $CD16^{+}$ or $CD56^{+}$. Dendritic cells (DC) were enumerated from total white blood cells without anchoring on lymphocytes. First, cells that expressed HLA-DR^{+} (MHC II) but lacked lineage-specific receptors (Lin-) for T (CD3-), monocytes (CD14-), NK cells (CD16- and CD56-), B cells (CD19- and CD20-) were first gated to selectively quantify DC. Within this gate, myeloid-derived DC (mDC) were defined as $CD11c^{+}CD123^{-}$ and the reciprocal CD11c-CD123+ population was used to quantify plasmacytoid dendritic cells (pDC).



As seen in Table 3.5 Distribution of Leukocytes including T-cell subsets, NK cells and DC subsets on page 78 and Figure 3.4 Distribution of Leukocytes including T-cell subsets, NK cells and DC subsets on page 80, IBC has a significant increase in the percentage of CD3+ T cells of about 5 percentage points compared to LABC, MIBC, and HD ($p = 0.037$, 0.022 and 0.002, respectively). While this change is significant, it is unlikely to be of biological significance; indeed, the absolute count of T cells is not significantly different from HD ($p = 0.220$). Although this is whole blood and not tumor, it should be noted that the presence of tumor infiltrating T cells is well established as a positive predictive factor in breast cancer (123).

B cells are reduced in IBC

Instead this number is capturing the reciprocal decrease in CD19+ B cells. A shortage of B cells is one of the more prominent hematological characteristics of both IBC and other cancers (non breast: (124-128), after metastatic progression or before. Both IBC and MIBC have significantly lower percentage of B cells than HD ($p = 0.001$ and $p < 0.001$, respectively).

CD4 and CD8 T cells

CD4 relative proportions of CD4 cells are high in IBC compared to HD ($p = 0.033$) and MIBC ($p = 0.008$). As the difference is small, this is likely due to the decrease in B cells noted above.

CD4 counts in metastatic disease, both MBC and MIBC are significantly depressed compared to HD ($p < 0.001$) and non-metastatic breast cancer ($p = 0.001$) and a similar pattern is seen with CD8 cells; however, IBC and MIBC are not significantly distinguished from LABC and MBC, respectively.

Recently, low CD4+ T-cell counts have been reported to be related to poor survival in both treatment naïve and chemotherapy-treated breast cancer patients (129). In this paper, they used a cut-off of 700 CD4+ T cells per microliter as the threshold for severe lymphopenia. Interestingly, Manuel et al showed that in addition to the number of lymphocytes in blood, the diversity of the T-cell receptor repertoire is also an independent prognostic indicator factor of overall survival in breast cancer patients. In this study, they used 23 PCR primer pairs upstream and downstream of VDJ recombination sites to enumerate the T-cell receptor diversity (130). With next-generation sequencing technology, it is now possible to have a much clearer understanding of this diversity. I will be pursing this field of study as a postdoctoral fellow.

CD4 cell counts were a significant predictor of progression free survival in breast cancer patients. The cut-point of CD4+ T cells of 700 was a highly significant predictor of progression-free survival (PFS) in the whole cohort with a median survival of 10.0 months for CD4 < 700 cells/μL but 23.3 months for CD4 > 700 cell/μL with a hazard ratio of (3.1, 95% confidence interval 2.7 to 9.70). But CD4 count was not a significant predictor when looking at IBC, LABC, MBC, or MIBC separately, irrespective of metastasis, or treatment status, but was significant in both IBC and non-IBC (log rank p = 0.004 and p < 0.001). However, CD4 count was not predictive of overall survival (p = 0.1761). Furthermore, CD4 count failed to remain significant in a multivariate analysis that included stage (M1 vs. M0), presence of inflammatory features (IBC vs. non-IBC), hormone receptor (ER, PR) and Her2 status. In this model, only metastasis (HR 6.0 p < 0.001) and IBC (HR 1.7 p = 0.048) remained significant (note using CD4 count as a continuous variable in Cox multivariate analysis was a significant contributor to PFS).

T-Regulatory Cells are increased in MIBC

The recent success of T_{Reg} target therapies such as anti-CTLA-4 or anti-PD-1 confirms the relevance of T cells in tumor immunity (131-133) and suggests that restoration of the immune system could promote anti-tumor immunity. It has been suggested that tumor evoked B-regulatory cells with constitutive high Stat3 can induce T_{Regs} in breast cancer (134). T_{Reg} cells inhibit cellular cytotoxicity responses and therefore play a key role in the development of immune evasion. In these data, we see that patients with metastatic disease have higher levels of T_{Reg} than HD (MBC and MIBC vs. HD $p < 0.001$ and $p = 0.027$, respectively) and higher than patients with non-metastatic disease (MIBC vs. IBC $p = 0.079$ and MBC vs. LABC $p = 0.026$).

T_{Reg} require the T-cell growth factor IL-2 and express very high levels of the IL-2 receptor-alpha CD25, which can be shed into circulation (sIL-2Rα) upon activation without decreasing the activity of IL-2(135). Patients with increased levels of soluble CD25 (sIL-2Rα) had inferior overall survival. It is likely that these high levels of sCD25Rα are shed from activated T_{Reg}. Indeed, in HD, sIL-2Rα has a strong correlation with T_{Reg} count (Spearman $\varrho = 0.462$, $p = .0012$). Interestingly, there this correlation is lost in non-IBC patients ($\varrho = -0.133$, $p = 0.364$) and reversed in IBC ($\varrho = -0.242$, $p = 0.046$). However, with the high number of samples that had sub-quantifiable levels of sIL-2Rα (50.6%), this should be viewed skeptically.

In multivariate analysis including age, stage, IBC diagnosis, ER, PR and Her2 status CD4 count and T_{Reg} count, both CD4 and T_{reg} were significant predictors of progression-free survival and overall survival. For PFS, high T_{Reg} had a hazard ratio of 5.0 (95% CI 1.3 – 19.2, $p = .018$) whereas low CD4 counts had a hazard ration of 2.6

(95% CI 1.4 – 4.7, p = 0.002). For OS, high T_{Reg} counts had a hazard ratio of 2.6 (95% CI 1.3 – 5.3, p = 0.010) and low CD4 counts had a hazard ratio of 2.4 (95% CI from 1.3 – 4.7, p = 0.009). T_{Reg} is a significant predictor of prognosis in both IBC and non-IBC. This suggests that T_{Reg} promote tumor growth by inhibiting T-cell function.

Natural Killer (NK) cells

IBC had significantly higher percentage of $CD56^{dim}$ compared to LABC (p = 0.037) while LABC had a significantly lower percentage of $CD56^{bright}$ regulatory NK than HD (p = 0.014). Together these suggest that IBC has less cytokine producing NK and a larger number of cytotoxic capable NK cells. However, as we saw in Chapter 2, IBC also has a higher level of circulating TGF-β1 that may hinder the cytotoxic potential of these cells. This would be consistent with mouse data published by Olkhanud et al that shows that tumor metastasis in the 4T1 mouse model requires T_{Reg} (which are enhanced by TGF-β) to kill NK cells (136). In this model, the authors showed that transferring 4T1 cells to a NOD/SCID mouse that lacks T and B cells but not NK cells failed to produce lung metastasis. However, adoptively transferring splenocytes including T_{Reg}s or T_{Reg} alone allowed for the establishment of lung metastases. In such a manner, there may be higher proportions of NK in circulation, but they may be unable to control tumor seeding in the microenvironment. Furthermore, the cytotoxic potential of the circulating NK cells is unknown. We have developed NK cytotoxicity tests that can be run on fresh, whole blood so that circulating soluble factors like TGF-β cellular factors like T_{Reg} can be included in the functional testing and we have just begun to test patient samples. It will be interesting to see if higher levels of mature NK cells in IBC are fully capable of killing tumor cells.

Finally, peripheral blood dendritic cell subsets were enumerated. The percentage of mDC was significantly lower in IBC (median of 41.5% of Lin-HLA-DR+) than in LABC (median of 51.8%, $p = 0.013$). Accordingly, IBC had significantly fewer mDC than LABC ($p = 0.025$) and both IBC and MIBC had significantly fewer mDC than HD ($p = 0.001$ and 0.004, respectively) as an absolute cell count. Furthermore, IBC, MBC and MIBC not LABC had significantly fewer pDC than HD ($p = 0.014$, $p = 0.004$, $p = 0.024$ and $p = 0.421$, respectively).

Table 3.5 Distribution of Leukocytes including T-cell subsets, NK cells and DC subsets

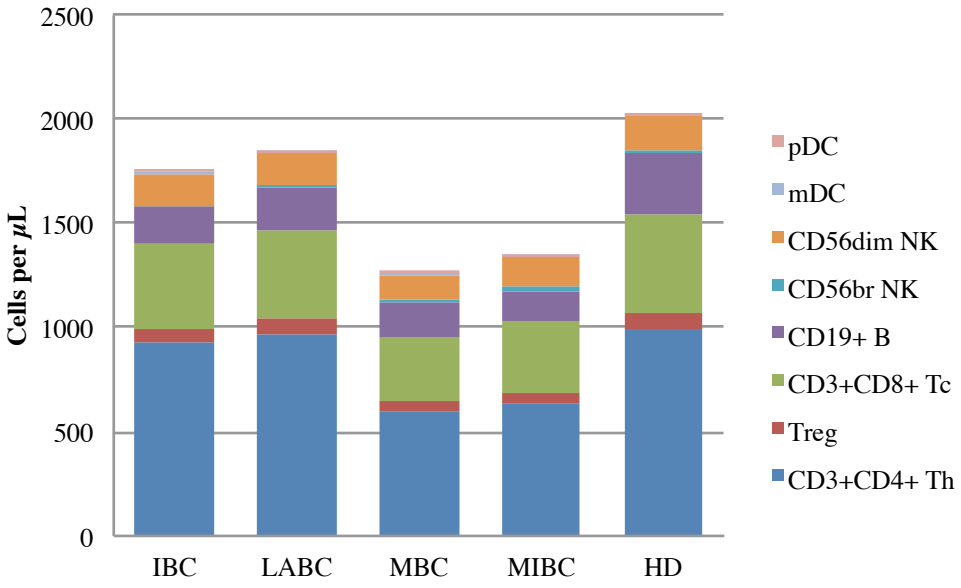
Leukocytes were enumerated by FACS as shown in Figure 3.3 FACS Analysis of Leukocyte Subsets. Each subset is shown first as a relative percentage and as an absolute count on the following line. Lymphocyte subsets (T, B, and NK) are represented as percent of lymphocytes with the exception of T_{Reg} that are presented as percentage of CD4 cells. Since they are not lymphocytes, the two dendritic cell subsets, mDC and pDC, are represented as the percentage of total $Lin^{-}HLA\text{-}DR^{+}$ cells. Significant differences were determined by Mann-Whitney U test.

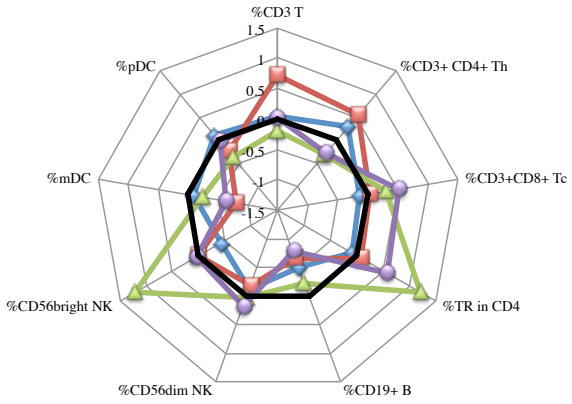
Measurement	Mean ± SEM by patient group					Significant Differences
	IBC	LABC	MBC	MIBC	HD	
No. Patients	32	26	26	51	34	
%CD3 T	79.7 ± 7.3	75.3 ± 7.7	73.9 ± 10.6	75.3 ± 8.7	75.1 ± 6.5	a,c,e
CD3+ T/µL	1517.9 ± 557.1	1557.5 ± 516.3	1023.7 ± 492.5	1100.5 ± 643.0	1681.4 ± 586.8	c,d,g,h
%CD3+ CD4+ T_h	52.0 ± 8.6	50.0 ± 7.3	45.2 ± 10.5	45.4 ± 11.4	47.6 ± 7.9	a,c,e
CD3+ CD4+ T_h/µL	993.3 ± 397.1	1041.2 ± 396.3	646.4 ± 347.9	687.3 ± 463.4	1064.8 ± 376.7	c,d,g,h
%CD3+CD8+ T_c	21.7 ± 9.7	20.7 ± 5.3	23.4 ± 8.5	24.7 ± 11.0	21.6 ± 6.0	n.s.
CD3+CD8+ T_c/µL	410.5 ± 218.2	424.9 ± 159.9	311.0 ± 162.7	345.0 ± 266.2	483.1 ± 240.2	d,g,h
%T_{REG} in CD4	7.0 ± 2.2	6.7 ± 1.9	9.0 ± 2.7	7.9 ± 2.2	6.9 ± 1.8	d,g,h
T_{REG}/µL	68.8 ± 30.9	69.1 ± 27.4	53.5 ± 27.0	52.5 ± 39.1	73.5 ± 32.1	c,g,h
%CD19+ B	9.5 ± 4.3	10.2 ± 3.9	11.4 ± 6.1	9.0 ± 5.7	12.4 ± 4.5	e,h
CD19+ B/µL	176.2 ± 89.0	211.2 ± 99.2	166.3 ± 128.3	143.1 ± 148.0	292.1 ± 170.7	e,f,g,h
%$CD56^{dim}$ NK	6.0 ± 3.7	6.3 ± 4.4	6.8 ± 5.0	7.3 ± 5.2	6.7 ± 3.6	n.s
$CD56^{dim}$ NK/µL	108.7 ± 64.3	126.9 ± 81.8	102.4 ± 88.4	101.5 ± 86.5	133.9 ± 68.1	h
%$CD56^{bright}$ NK	0.3 ± 0.3	0.2 ± 0.2	0.5 ± 0.7	1.6 ± 9.2	0.3 ± 0.2	f,d
$CD56^{bright}$ NK/µL	4.9 ± 7.4	3.6 ± 3.1	7.4 ± 10.3	18.2 ± 103.0	5.8 ± 3.6	e,f,h
%mDC in lin^{neg} $HLA\text{-}DR^{+}$	40.2 ± 12.1	48.3 ± 10.8	46.8 ± 20.0	42.3 ± 16.2	49.5 ± 11.4	a,e,h
mDC/µL	5.0 ± 3.5	8.2 ± 7.0	7.3 ± 8.8	6.0 ± 5.4	8.3 ± 5.7	a,e,h
%pDC in lin^{neg} $HLA\text{-}DR^{+}$	17.9 ± 6.7	21.5 ± 10.2	16.8 ± 8.0	20.2 ± 12.0	20.3 ± 9.9	n.s
pDC/µL	2.2 ± 1.6	3.8 ± 4.9	1.8 ± 1.3	3.8 ± 10.4	3.4 ± 2.2	e,h,g

a IBC vs. LABC $p < 0.05$
b MBC vs. MIBC $p < 0.05$
c IBC vs. MIBC $p < 0.05$
d LABC vs. MBC $p < 0.05$
e HD vs. IBC $p < 0.05$
f HD vs. LABC $p < 0.05$
g HD vs. MBC $p < 0.05$
h HD vs. MIBC $p < 0.05$
n.s. no significant difference

Figure 3.4 Distribution of Leukocytes including T-cell subsets, NK cells and DC subsets

Cell counts of peripheral blood agranulocyte (mononuclear) leukocyte subsets were evaluated in IBC, LABC, MBC and MIBC patients. The top figure shows median cell counts and the radar plots below show relative proportions on the left and cell counts on the right. As in the previous figure, the radar plots show standardized values to healthy donors (HD = 1) so that all subsets are on the same scale. Note that radar plots are calculated with mean values (to generate a Z-score) and may be affected by outliers. P-values reported in the text are from non-parametric tests to correct for this effect.





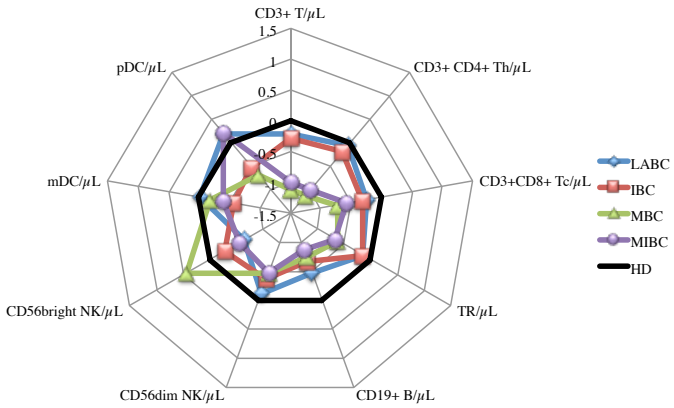
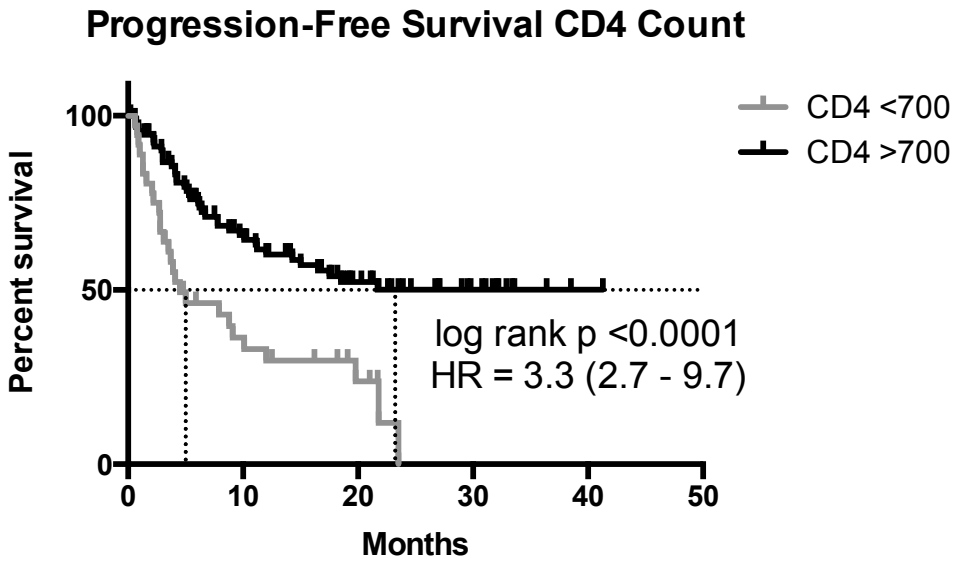


Figure 3.5 Progression free survival is decreased in lymphopenic patients



In summary, patients with IBC present a few hematopoietic differences from LABC such as decreased mDC, increased $CD56^{dim}$ NK and the percent of CD3 cells. However, for the most part, the hematology of locally advanced disease is fairly normal. Metastatic disease, however, is characterized by severe lymphopenia that is related to decreased overall survival.

Chapter 4: Inflammatory Breast Cancer T and DC Functional Studies Profile

INTRODUCTION

As mentioned in Chapter 2, T cells are responsible for shaping the adaptive immune response. Both CD4+ T-helper cells (Th) and CD8+ cytotoxic are capable of producing cytokines. However the type and quantity of the response can greatly alter the inflammatory milieu and weather the immune system promotes immune surveillance to prevent tumor growth or provides a permissive environment. In Chapter 5, we provide evidence that the inflammatory immune response can even promote cancer progression by inducing epithelial to mesenchymal transition.

Upon recognizing its cognate antigen, the activated T-cell responds by producing cytokines that "help" the immune process, which will be explored in the following chapter. Type-I cytokines (TH1) promote the cytotoxic immune response while type-II cytokines (TH2) promote the humoral response and inhibit the cytotoxic response. To a certain extent, in a fully mature effector cell, these responses are mutually exclusive and the polarization is fairly implastic. Th1 cells are stimulated by IL-12 and IFN-γ. IL-2 is generally considered to be a TH1 cytokine, although it is a growth factor for all cells including T_{Regs}. TH1 cells activate, CD8+ cytotoxic T lymphocyte, NK and macrophages and thus are generally considered to have potent anti-tumor properties. IL-4 is actively inhibited in these cells. Th2 cells, in contrast are stimulated by and produce IL-4. Other Th2 cytokines include IL-6 and IL-10. Recently, a fourth class of Th cells has been identified. TH17 cells, associated with killing extracellular pathogens and auto-immunity, produce large of amounts of IL-17. TH17 cells are induced by TGF-β, IL-6 and IL-1β while IL-23 servers as a renewal factor. TNF-a is produced by all T-cells but since it is pro-inflammatory, it will be classified as TH1 here.

It is important to note that the antigen-presenting cell that initiates the T cell response is not a passive bystander. On the contrary, in concert with the microenvironment milieu, three critical signals are provided to the naïve T-cell. First is the antigen/MHC complex. As the majority of T-cells will have very low affinity for the antigen/MHC complex, they will not be activated.

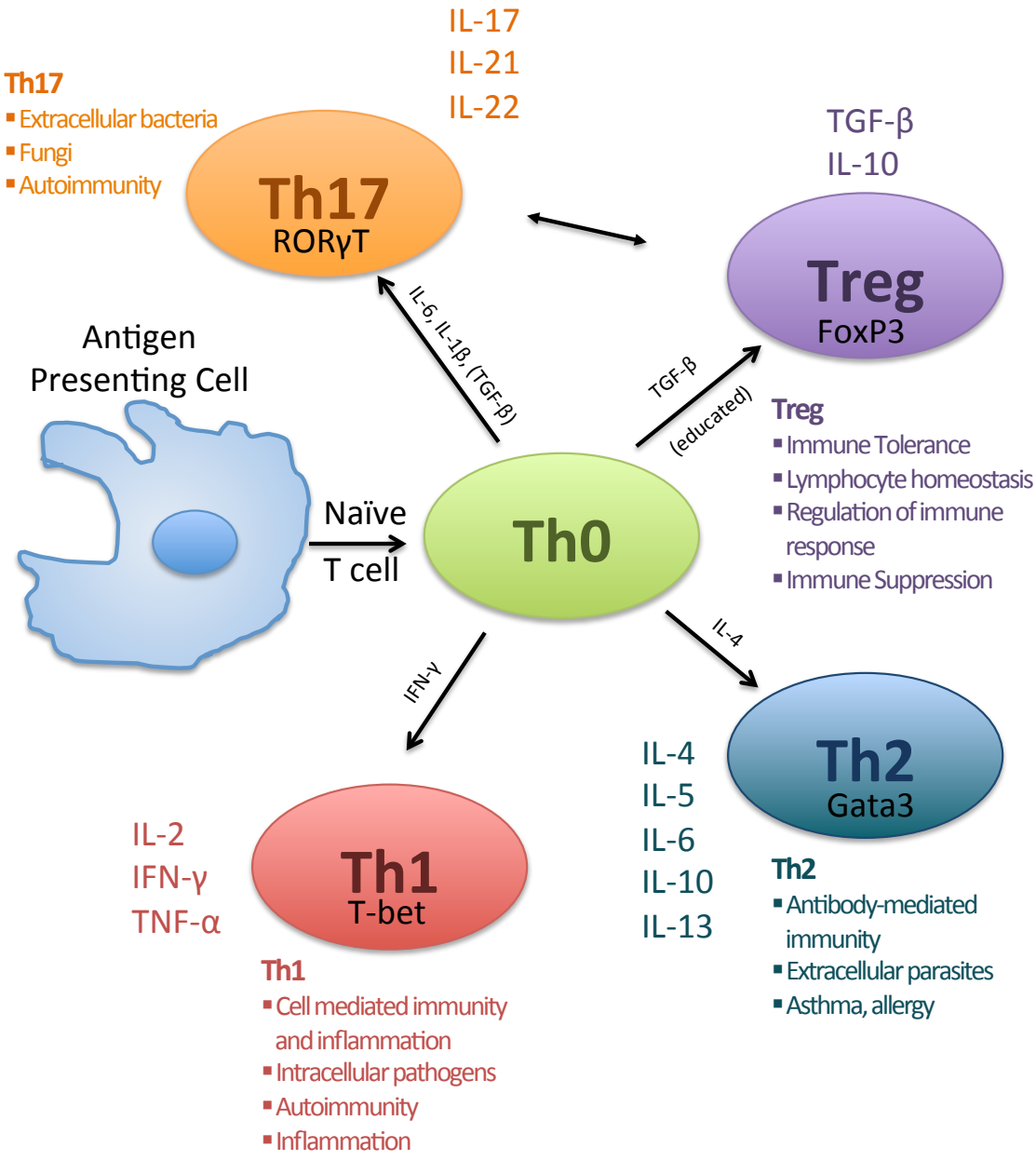
After recognizing antigen/MHC complex, T-cells must receive a co-stimulatory signal from the antigen-presenting cell to ensure that the T-cell is responding to a foreign of dangerous antigen. Antigen presenting cells express CD80 and CD86 that binds CD28 on the T-cell. Without this signal, the T-cell will become anergic and no longer respond to antigen stimulation, if in the presence of both signals.

The third signal includes inflammatory signals from the microenvironment. As the antigen presenting cells is in prolonged, close contact with the T-cell, it can produce very high local concentrations of cytokines. Exposure to IL-12 will favor a TH1 response that is associated with anti-tumor activity whereas IL-10 will oppose the TH1 response.

Therefore, to understand the possible role of the immune system in IBC, counting cells is not sufficient. Here we present functional tests that assess the propensity of T-cells to produce cytokines in IBC. This suggests the TH1, TH2, Th17 polarization. Then we will look at IBC dendritic cell ability to present antigen by the upregualtion of costimulation molecules and cytokine production.

Illustration 4.1 T-cell polarization

Antigen-presenting cells such as dendritic cells process and present antigen to T cells. The cytokine milieu of an activated naïve T cell determines the polarization the T cell that will be largely maintained on subsequent activation. TH1 cells synthesize IFN-γ and other cytokines and are involved in cell mediated immunity and tumor surveillance. TH2 cells synthesize IL-4 and are involved in the humoral response and are typically considered to promote tumor survival. TH17 cells synthesize IL-17 and other highly inflammatory factors to protect against extracellular bacteria but have been implicated in autoimmunity. T_{Reg} cells can be produced in the thymus or induced in the periphery and produce TGF-β and IL-10 as immunomodulatory factors. Adapted from *livingwellnessblog*. Th0 – Naïve CD4+ T helper cell, Th – Helper T cells, Treg – Regulatory T cells, IL- Interleukin, TNF-α– Tumor necrosis factor alpha, IFN-γ – Interferon gamma, TGF-β – transforming growth factor-beta.



POPULATION STUDIED AND METHODS OVERVIEW

As in Chapter 3, patients were recruited under protocol Lab08-019. Peripheral blood was obtained from 32 IBC patients, 26 LABC, 26 MBC 54 MIBC and 34 HD (refer back to Table 3.1 on page).

To test T-cell function and polarization, thawed peripheral blood mononuclear cells (PBMC, white blood cells consisting of monocytes, T-, B- and NK- lymphocytes and dendritic cells but depleted of granulocytes and red blood cells by density centrifugation with soluble factor from serum washed away) were stimulated overnight with plate immobilized anti-CD3 antibody that activated T-cells by cross-linking the clustering the T-cell receptor (TCR). Anti-CD28 antibody was added to provide co-stimulation so that patient antigen presentation is not a factor in the test. The percent of cells producing cytokines was measured by flow cytometry by blocking golgi transport so that cytokines accumulate in the cell following synthesis. See Chapter 7 for methods.

Dendritic cell function was measured in two assays. Both interrogate DC response following activation through the innate receptors toll-like receptor 7 and 8 (TLR7 and TLR8). TLR7 responds to single stranded RNA in endosomes and therefore is a good model for stimulation by generic microRNA. TLR8 recognizes GC-rich nucleotides (oligo-GC). In the first assay, fresh, whole blood was stimulated for 4 hours to test for cytokine synthesis. In the second assay, fresh whole blood was stimulated overnight to test for upregulation of co-stimulatory molecules and activation markers.

IBC T CELL FUNCTION IS MOSTLY NORMAL BUT ENRICHED FOR TC17

The only cytokine with significant differences in synthesis between IBC and LABC is the percentage of CD4 cells producing IL-10. IBC patients had median serum

levels of 5.3 pg/mL while LABC had 2.9 pg/mL ($p = 0.029$). As IL-10 is often anti-inflammatory, this is consistent with the higher levels of TGF-β1 observed in serum.

In fact, serum TGF-β1 correlated with the relative proportions of both CD4 and CD8 cells producing IL-10 (Pearson $\varrho = .198$, $p < 0.023$ and $\varrho = .197$, $p = 0.024$, respectively). In IBC patients, the CD4 IL-10-serum TGF-β correlation was even stronger (Pearson $\varrho = .344$, $p = 0.007$). Furthermore, in IBC patients sIL-2Rα, shed from activated Th and T_{Reg} was also strongly correlated with IL-10 production by both CD4 and CD8 cells (Pearson $\varrho = .480$, $p < 0.001$ and $\varrho = .436$, $p < 0.001$, respectively). As T_{Reg} perpetuate the anti-inflammatory signaling of IL-10 and TGF-β, this suggests that closer study of T_{Reg} in IBC are warranted even though no differences in circulating T_{Reg} were observed. However, metastatic disease presents a different picture. CD8 IL-10 producing cells in MIBC are significantly reduced in both relative proportion ($p = 0.01$ vs. HD and $p = 0.017$ vs. IBC) and number ($p < 0.001$ vs. HD and $p = 0.004$ vs. IBC) while the proportion of few other T cells are similarly affected. Only IL-4 (another cytokine that inhibits the cellular response) is reduced in MIBC ($p = 0.012$).

Compared to healthy donors, IBC cytokine production is fairly normal although there is a weak trend for IBC T cells to be more reactive. This is consistent with the data published by Mourali and Levine over 30 years ago (5) and suggests that IBC patients are not immune suppressed and are capable of producing inflammatory factors in response to stimuli.

However, upon inspection of the standardized "radar" plots in Figure 4.1 on page 98, two features are prominent. Breast cancer leukocytes are greatly enriched for CD8+ T cells producing IL-17 and to a lesser extend IFN-γ. Compared to HD, IBC had significantly enriched CD8+ T cells synthesizing IFN-γ ($p = 0.032$) and a nearly significant increase in CD8+ T cells producing IL-17 ($p = 0.058$). However, treatment

naïve IBC patients had significantly higher proportions of CD8+ T cells that produced IL-17 ($p = 0.045$). This is an intriguing finding for IBC.

IL-17 producing T-cells (TH17 and TC17) have recently been described as separate and unique from the traditionally acknowledged TH1/TH2 dichotomy. As originally described, these cells play a critical role in experimentally induced autoimmune encephalomyelitis (EAE) where they have been shown to be the critical cells responsible for autoimmune reactions (137). IL-17 knock-out mice are incapable of inducing an auto-immune response.

There has been considerable controversy over the role of IL-17 in tumor progression.

For example, IL-17 induces a number of pro-angiogenic factors such as VEGF, macrophage inflammatory protein-2, and prostaglandin E1 and E2 from tumor and tumor stromal cells (138). In vivo, mice with IL-17 overexpression have increased tumor microvessel density compared with IL-17 -/- mice (138, 139).

Furthermore, although MIBC patients are severely lymphopenic, they do not have a reduction in these cells. In psoriasis-like skin inflammation, the majority of CD8+ T cells infiltrating the skin co-express both IL-17 and IFN-γ and are able to orchestrate skin inflammation (140). As IBC is a skin disease, this can be highly relevant. Recently classified as Tc17 cells, The IL-17+ CD8 T cells are a strange subset. Although CD8+ T cells are generally strong cellular killers, TC17 cells have been shown to produce the highly inflammatory factors associated with inflammation, but lack the cytotoxic effector molecules such as granzyme B (141). As such, patients with high levels of Tc17 cells may be subjected to highly levels of inflammation, but have a deficit in the tumor controlling TH1 response.

No differences in T cell cytokines synthesis were observed between MBC and MIBC in all patients or in treatment naïve patients (all $p > 0.05$). Consistent with the reduced cellular immunity profile noted above, MIBC patients have significantly higher percentages of CD8+ T cells that produced IL-10.

Looking at absolute counts of cells (cells/μL) in treatment naïve patients, MBC consistently (but not significantly) displays fewer inflammatory cells than MIBC ("inflamocytopenia"?). Notice in Figure 4.1D the green MBC line is clearly "inside" the purple MIBC line. It should be noted that all absolute counts are essentially derived form the white blood count (discussed in Chapter 2). Any variance in this measure will propagate throughout the dataset. With that consideration (and the additional caveat that the number of untreated MBC patients in this study is very small), this suggests that prior to treatment, there are more inflammatory-cytokine producing cells in MIBC than in MBC. Furthermore, TNF-α synthesis in particular seems to be elevated in MIBC relative to MBC. While this study is performed on blood cells and not on tumor infiltrating lymphocytes (a protocol to test this has recently opened), and uses polyclonal activation rather than antigen specific stimulation, this data gives us an idea of how T-cells will respond when they encounter antigen within the tumor microenvironment: simply that there are a larger number of cells capable of producing inflammatory factors in IBC. In Chapter 5 we hypothesize that inflammatory factors derived from these cells such as TNF-α drive the metastatic progression by promoting epithelial to mesenchymal transition. A unique response to this increased source of inflammation might contribute to the distinctive features and rapid progression characteristic of IBC.

Table 4.1

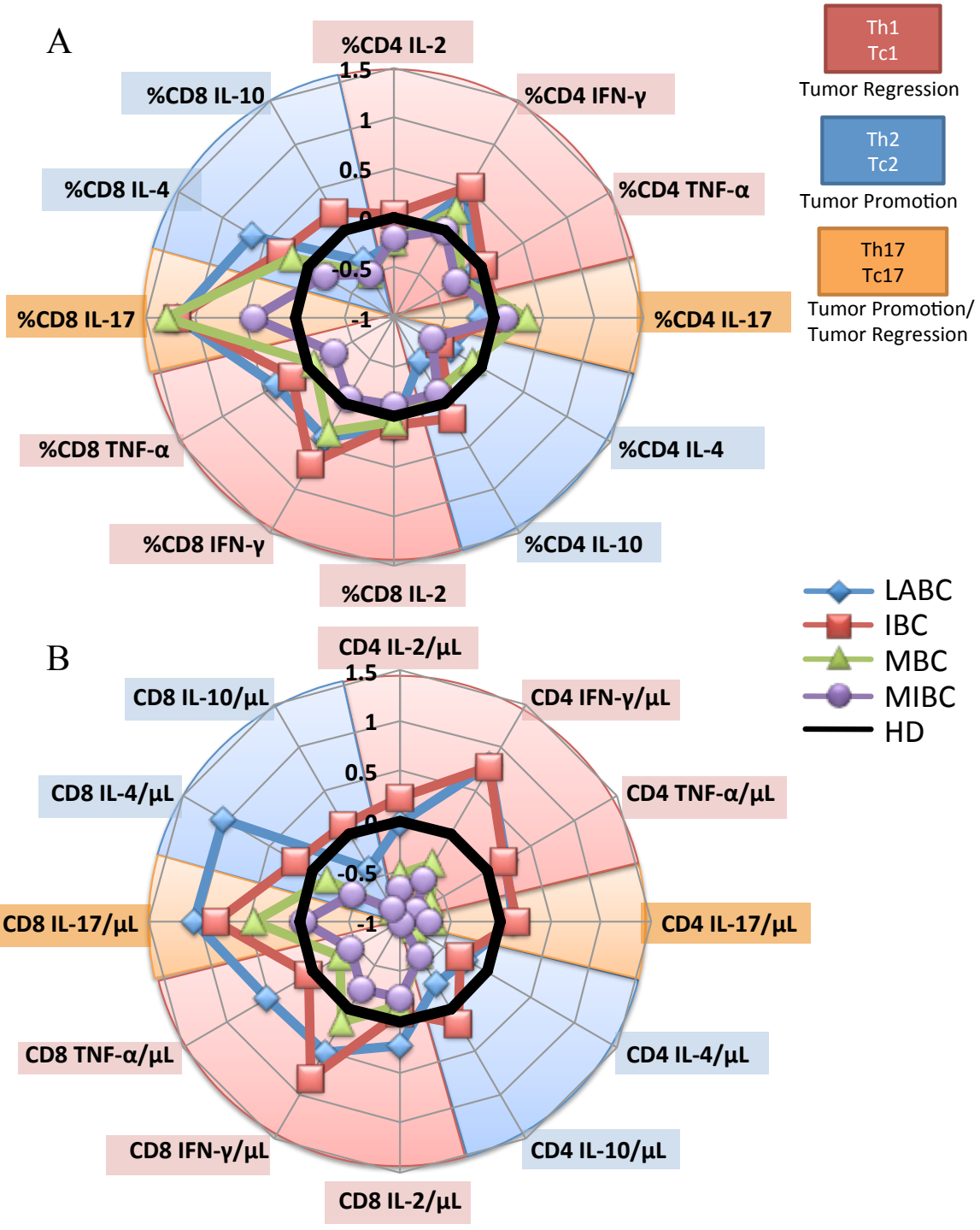
T cell cytokine production. Peripheral blood mononuclear cells were stimulated through the TCR with immobilized anti-CD3 antibody and soluble anti-CD28 antibody overnight to elicit cytokine synthesis. The percentage of cells producing cytokines was enumerated by flow cytometry. Data are shown as the mean ± s.e.m. percentage of CD4+ T or CD8+ T cells synthesizing cytokine or standardized to the number of CD4+ T cells or CD8+ T cells per μL of blood that can produce cytokine upon stimulation. Statistics are based on the non-parametric Mann-Whitney U test.

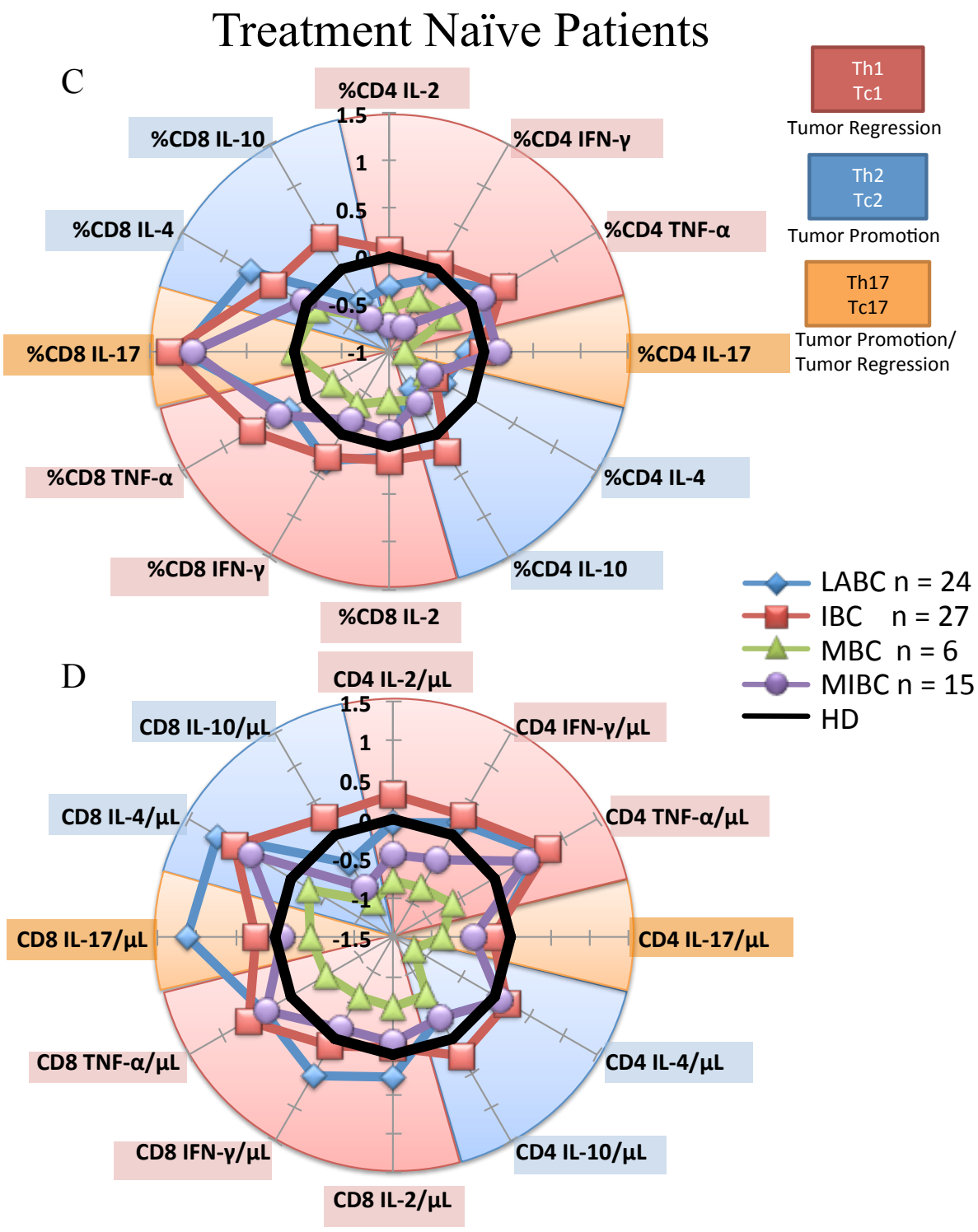
a IBC vs. LABC $p < 0.05$
b MBC vs. MIBC $p < 0.05$
c IBC vs. MIBC $p < 0.05$
d LABC vs. MBC $p < 0.05$
e HD vs. IBC $p < 0.05$
f HD vs. LABC $p < 0.05$
g HD vs. MBC $p < 0.05$
h HD vs. MIBC $p < 0.05$
n.s. no significant difference

				Mean ± S.E.M.					
				IBC	LABC	MBC	MIBC	HD	Sig. Differences
N				29	25	25	42	26	
TH1	IFN-γ	CD4	% pos.	11.6 ± 6.4	11.3 ± 6.4	10.2 ± 6.1	9.0 ± 6.1	9.0 ± 5.0	n.s.
			cell/µL	110.8 ± 65.8	111.4 ± 68.2	64.6 ± 54.9	56.8 ± 52.4	78.5 ± 41.6	c,d,f,h
		CD8	% pos.	16.3 ± 9.1	14.2 ± 7.7	13.6 ± 8.6	10.8 ± 7.2	11.2 ± 7.2	c, e
			cell/µL	69.4 ± 54.8	59.9 ± 32.3	48.6 ± 43.4	36.2 ± 41.1	43.5 ± 32.7	c,f
	IL-2	CD4	% pos.	15.4 ± 7.3	13.0 ± 7.0	13.2 ± 7.9	13.5 ± 9.9	15.1 ± 8.2	n.s
			cell/µL	158.9 ± 103.3	138.1 ± 93.9	94.0 ± 80.6	81.0 ± 81.9	139.6 ± 86.0	c,h
		CD8	% pos.	7.0 ± 5.8	7.0 ± 6.1	6.7 ± 5.0	5.8 ± 5.8	6.4 ± 6.0	n.s.
			cell/µL	22.8 ± 13.9	31.7 ± 34.7	21.2 ± 16.7	19.1 ± 29.0	25.4 ± 26.2	c
	TNF-α	CD4	% pos.	22.8 ± 9.9	21.9 ± 11.1	20.1 ± 10.9	19.3 ± 12.0	22.4 ± 10.5	n.s
			cell/µL	228.9 ± 123.6	232.7 ± 150.8	136.7 ± 107.3	119.6 ± 111.6	208.4 ± 110.5	c,d,g,h
		CD8	% pos.	14.3 ± 8.7	15.9 ± 10.5	12.2 ± 7.6	10.2 ± 8.1	12.7 ± 8.5	c
			cell/µL	51.6 ± 31.7	69.5 ± 59.3	38.0 ± 27.5	33.2 ± 38.8	49.7 ± 37.5	c,d,h
TH17	IL-17	CD4	% pos.	2.9 ± 1.4	2.6 ± 1.3	3.2 ± 2.6	2.9 ± 1.9	2.8 ± 1.4	n.s.
			cell/µL	27.6 ± 13.9	27.1 ± 16.3	17.5 ± 14.3	16.9 ± 15.9	25.5 ± 12.2	c,d,g,h
		CD8	% pos.	2.2 ± 2.3	2.2 ± 1.7	2.3 ± 3.2	1.5 ± 1.5	1.1 ± 0.9	f
			cell/µL	8.3 ± 8.0	9.1 ± 8.6	6.7 ± 9.6	4.7 ± 5.1	4.9 ± 4.1	c,d,f
TH2	IL-10	CD4	% pos.	6.3 ± 4.7	3.9 ± 2.4	5.1 ± 5.0	5.2 ± 3.9	5.7 ± 3.7	a
			cell/µL	63.0 ± 51.1	43.8 ± 34.0	29.5 ± 24.7	30.0 ± 27.9	55.9 ± 43.1	c,g,h
		CD8	% pos.	5.3 ± 4.4	3.7 ± 2.4	3.3 ± 2.7	3.3 ± 2.7	4.7 ± 2.8	n.s.
			cell/µL	21.3 ± 20.9	15.3 ± 10.9	9.6 ± 8.3	9.7 ± 7.8	20.0 ± 11.9	c,g,h
	IL-4	CD4	% pos.	2.3 ± 1.3	2.4 ± 1.6	2.8 ± 2.1	2.1 ± 1.5	2.9 ± 1.3	h
			cell/µL	1.5 ± 1.3	1.8 ± 1.4	1.4 ± 1.0	1.1 ± 0.7	1.2 ± 0.9	c,g,h
		CD8	% pos.	23.4 ± 16.1	24.4 ± 18.4	14.6 ± 9.3	12.2 ± 10.2	28.3 ± 16.4	n.s.
			cell/µL	5.6 ± 5.7	8.4 ± 9.1	4.3 ± 3.4	3.3 ± 3.0	4.9 ± 3.4	h

Figure 4.1

T cell cytokine production. Peripheral blood mononuclear cells were stimulated through the T-cell receptor (TCR) with immobilized anti-CD3 antibody and soluble anti-CD28 antibody overnight to elicit cytokine synthesis. The percentage of cells producing cytokines was enumerated by flow cytometry. Data are shown as the mean percentage of CD4+ or CD8+ T cells synthesizing cytokine (A and C, top) or the number of CD4+ or CD8+ T cells per μL of blood that can produce cytokine upon stimulation (B and D, bottom). The figures on the first page (A and B) show the data for all patients and the figures on the next page are restricted to treatment naïve patients. As in previous figures, the radar plots show standardized values with HD = 1 so that all subsets are on the same scale. Note that radar plots are calculated with mean values (to generate a Z-score) and may be affected by outliers. P-values reported in the text are from non-parametric tests to correct for this effect.





DENDRITIC CELL FUNCTION

As mentioned in Chapter 3, IBC has significantly fewer mDC than LABC. However, the ability of those cells to produce cytokines and present antigen could be critical to tumor and immune surveillance and the regulation of inflammatory processes in the tumor microenvironment.

To assess these capabilities in DC, we stimulated whole blood with toll-like receptor (TLR) 7 and 8 and assayed for cytokine synthesis and surface expression of costimulatory molecules, activation and maturation markers. In parallel, blood was left unstimulated to assess constitutive levels of activation. Both myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC) were evaluated. Cytokines included the inflammatory cytokine TNF-α, the TH1 cytokines IFN-γ and IL-12 and the anti-inflammatory/TH2 cytokine IL-10. Activation markers included CCR5, which is expressed on resting DC in the tissue, and CCR7, which helps activated DC home to the lymph node where they interact with T-cells. Costimulatory molecules CD80 and CD86 provide the crucial second signal to T-cells that licenses T-cell activation. Finally, CD40 is a TNF-receptor family member that ligates CD40L on T cells to activate both cells. As this full complement of cell types, stimulation conditions and assayed molecules resulted in a huge amount of data; only the significant differences between IBC and LABC or MIBC and MBC will be presented.

As seen in Table 4.2 and Figure 4.2 IBC has greater synthesis rates of inflammatory cytokines and poorer antigen presentation capability. Compared to LABC, on a per cell basis (as measured by mean fluorescent intensity, MFI), IBC had greater constitutive amounts of TNF-α synthesis by pDC ($p = 0.036$). Similarly, compared to

MBC, MIBC had greater constitutive and TLR-induced synthesis of IL-10 by mDC. Furthermore, IBC both and MIBC had greater induced expression of the activation marker CCR7 by both mDC and pDC than the non-inflammatory controls (LABC and MBC). As IBC is characterized lymphatic invasion, increased expression of CCR7 (which targets DC to the lymph nodes) will tend to bring these inflammatory cells in contact with tumor.

In contrast, although IBC DCs are more likely to become activated and migrate to the lymphatic vasculature, they are less likely to initiate a cytotoxic response when they arrive. As seen in the following table and figure, compared to LABC, IBC has lower numbers of mDC constitutively expressing CD40, CD80 and CD86 ($p = 0.001$, 0.011, and 0.017, respectively); lower numbers of cells capable of expressing CD80 and CD86 ($p = 0.038$ and 0.043, respectively) after exposure to TLR signaling; and lower numbers of pDC constitutively expressing CD86 ($p = 0.001$). Similarly, compared to MBC, mDC from MIBC express lower levels of CD86 following stimulation.

It should be noted that while increased expression of co-stimulatory molecules such as CD80 and CD86 show that a dendritic cell is capable of providing the required "signal 2" to T cells, measurement of these markers does not actually test the cells' ability to present antigen. An alternative approach would be to pulse DC with a recall antigen such as tetanus toxoid and measure T cell responsesresponse. Furthermore, while TLR may be activated in response to antigen in the tumor microenvironment, neither the DC activation not T cell activation studies here test antigen specific responses.

Table 4.2

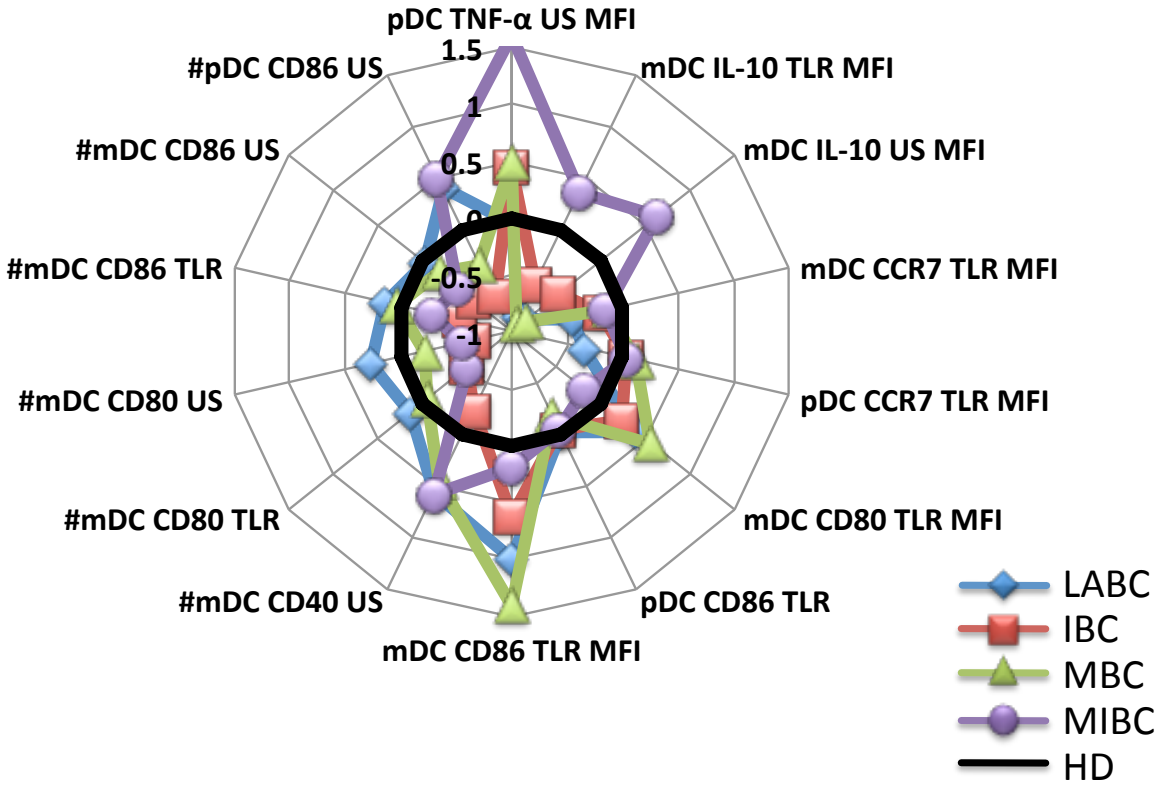
Dendritic cell function. Peripheral blood mononuclear cells were stimulated through the toll-like receptor (TLR) 7 and 8 for 4 hours to elicit cytokine synthesis or overnight to induce expression of stimulatory and activation surface proteins (TLR). In parallel, cells were left unstimulated to measure constitutive expression levels (US). Marker expression was recorded as a mean fluorescent intensity (MFI) that correlates with the average number of receptors expressed on the surface of each cell. The percentage of cells expressing activation and costimulatory molecules as measure by flow cytometry was used the enumerate cells per μL with the given expression pattern (#). Data are shown as the mean ± s.e.m. percentage of mDC or pDC expressing marker or mean ± s.e.m MFI of the specific marker on mDC or pDC. Statistics are based on the non-parametric Mann-Whitney U test.

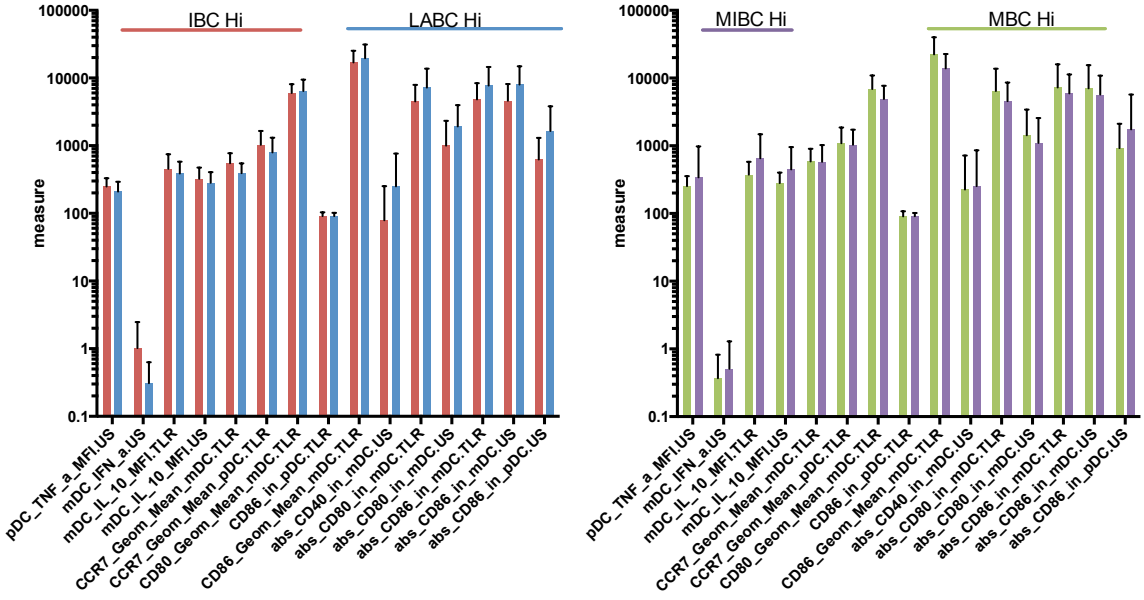
	Mean ± S.E.M.					
	IBC	LABC	MBC	MIBC	HD	Sig Differences
n	32	26	26	51	34	
pDC TNF-α US MFI	249 ± 81	210 ± 82	253 ± 103	342 ± 638	213 ± 80	a
mDC IL-10 TLR MFI	457 ± 289	388 ± 190	374 ± 206	655 ± 822	576 ± 221	b,e,f,g
mDC IL-10 US MFI	325 ± 149	282 ± 124	281± 119	449 ± 505	377 ± 113	b,f,g
mDC CCR7 TLR MFI	552 ± 220	393 ± 154	587 ± 314	580 ± 447	665 ± 592	a,d
pDC CCR7 TLR MFI	1028 ± 6234	797 ± 513	1088 ± 770	1036 ± 694	1009 ± 631	a,d
mDC CD80 TLR MFI	6015 ± 2075	6448 ± 3024	6896 ± 4017	4918 ± 2783	5375 ± 2684	b,c
mDC CD86 TLR MFI	16953 ± 8254	19593 ± 11570	22444 ± 17519	13917 ± 8705	12660 ± 6926	b,e,f,g
#mDC CD40 US	79.2 ± 173.3	250 ± 512	226 ± 491	253 ± 6023	127 ± 216	a,c,d,e
#mDC CD80 TLR	4502 ± 3417	7227 ± 6552	6423 ± 7313	4554 ± 401	6638 ± 4240	a,e,h
#mDC CD80 US	999 ± 1329	1921 ± 2045	1426 ± 1998	1106 ± 1465	1651 ± 996	a,e,g,h
#mDC CD86 TLR	4843 ± 3541	7725 ± 6839	7268 ± 8683	5929 ± 5405	7117± 4244	a,e,h
#mDC CD86 US	4609 ± 3554	7989 ± 6905	7045 ± 8503	5693 ± 5155	8126 ± 6151	a,e,h
#pDC CD86 US	624 ± 681	1648 ± 2169	921 ± 1190	1744 ± 3948	1257 ± 973	a,d,e,g

a IBC vs. LABC $p < 0.05$
b MBC vs. MIBC $p < 0.05$
c IBC vs. MIBC $p < 0.05$
d LABC vs. MBC $p < 0.05$
e HD vs. IBC $p < 0.05$
f HD vs. LABC $p < 0.05$
g HD vs. MBC $p < 0.05$
h HD vs. MIBC $p < 0.05$

Figure 4.2

IBC dendritic cell function has high inflammatory cytokines and low antigen presentation. Dendritic cells were assayed in fresh whole blood to determine cytokine production, activation markers, and antigen presentation capability. The same data are presented in the top and bottom plots. The top "radar" plot shows Z-score standardized data to so that all variables are on the same scale. The lower plots highlight differences between IBC and non-IBC. The left plot shows IBC in red and LABC in blue whereas the right plot show MIBC in purple and MBC in green. Bars are mean ± s.e.m.





In summary, there is a trend for inflammatory breast cancer patients to have greater numbers of cells, both T and DC, capable of producing inflammatory cytokines when activated. Furthermore, there is an increased prevalence of DC and T cells (possibly T_{Reg}) that produce IL-10 and CD8+ T cells that produce IL-17 (Tc17) suggesting that there may be a deficit in cell-mediated cytotoxicity. In the next Chapter, we will test weather activated cells are capable of inducing aggressive features in IBC and non-IBC cell lines.

Chapter 5: Immune cells induce epithelial to mesenchymal transition in inflammatory breast cancer cells

ABSTRACT

Inflammatory breast cancer is the most insidious form of locally advanced breast cancer, characterized by diffuse inflammation of the breast. The disease progresses rapidly with about 1/3 of patients having distant metastasis at staging. However, it is unknown if inflammation could contribute to the rapid progression associated with IBC. Here we ask whether soluble factors from activated immune cells are capable of inducing IBC cells to undergo epithelial to mesenchymal transition (EMT), a cellular program associated with increased migration and invasion that is necessary for metastasis. We found that conditioned media (CM) from activated T-cells induces IBC cells to express many factors related to the EMT program including fibronectin, vimentin, transglutaminase 2 (TG2) and ZEB1. Interestingly, although invasion and migration increased, E-cadherin, a cell-adhesion molecule typically expressed in IBC tissue, also increased following exposure to immune factors. The increased expression of E-cadherin was observed in 3 of 4 IBC cell lines but in none of the non-IBC lines examined. A combination of TNF-α, IL-6, and TGF-β was able to recapitulate the EMT induction in IBC and CM pre-absorbed with neutralizing antibodies against these factors decreased EMT. These data suggest that release of TNF-α, IL-6 and TGF-β by activated immune cells are capable of inducing EMT in IBC cells and thus, may contribute to the aggressiveness of IBC.

INTRODUCTION

Inflammatory breast cancer (IBC) is the most aggressive form of locally advanced breast cancer. It is characterized by diffuse erythyma and edema of the breast often mistaken for mastitis. Yet it is often said that IBC is not a true inflammatory condition, with inflammation arising from the characteristic tumor emboli blocking the dermal lymphatics. The tumor progresses quickly often within a few weeks or months and is often metastatic at diagnosis. The rapid onset of metastasis suggests that tumor cells are disseminating through the lymphatics or blood at an early stage of disease. However, although tumor cells are in regular contact with immune cells trafficking through the lymphatics, they are not killed by immune cells and suggest a blunted or immune suppressed response. In contrast, Mouliari and Levine postulate that since IBC patients (referred to as rapidly progressing breast cancer) are able to produce delayed-type hypersensitivity reactions to standard recall antigens and at least one breast tumor lysate, that IBC patients may in fact present with an augmented cellular immune response to tumor (5). However, the effect of this postulated immune response on the tumor cells and their metastatic potential is only beginning to be explored. Recently, Mohammed and colleagues showed that when factors secreted by the monocytic cell line U937 are added to cultures of the IBC cell line SUM149, the tumor cells develop enhanced migratory and invasive features (142) and increased expression of fibronectin (143). Activated immune cells are known to produce factors such as TNF-α, IL-6, IL-1β and TGF-β. These characteristics suggest that immune cells may induce an EMT in IBC.

EMT is a set of biological processes that occur as epithelial cells loose their sedentary characteristics and gain a motile phenotype. EMT as described by Elizabeth Hay (144) is important during physiological roles such as embryogenesis where cells migrate during gastrulation or neural crest formation (145). Kalluri and Weinburg coin such processes type I EMT (146) and express genes such as *Sox, Snail, and Slug* which encode transcription factors that control EMT. Type II EMT involved in wound repair, tissue regeneration, and fibrosis is characterized by inflammation. TGF-β signaling is frequently involved. Neoplastic cells undergoing type III EMT hijack hallmarks of both of these programs producing a metastatic pathology. EMT is characterized primarily by a loss of E-cadherin expression. Transcription factors such as Snail (147), Slug (148) and Zeb1 (149) bind directly to E-box regions in the E-cadherin promoter and repress protein expression (144). Twist (150), Mesenchyme Forkhead 1 (FoxC2) (151) and tissue transglutaminase (TG2) (152, 153) have also been shown to regulate EMT. We have recently demonstrated that detection of any of these EMT-related transcription factors (EMT-TFs) in the peripheral blood of breast cancer patients can serve as a surrogate for circulating tumor cells (CTC) in breast cancer patients (154).

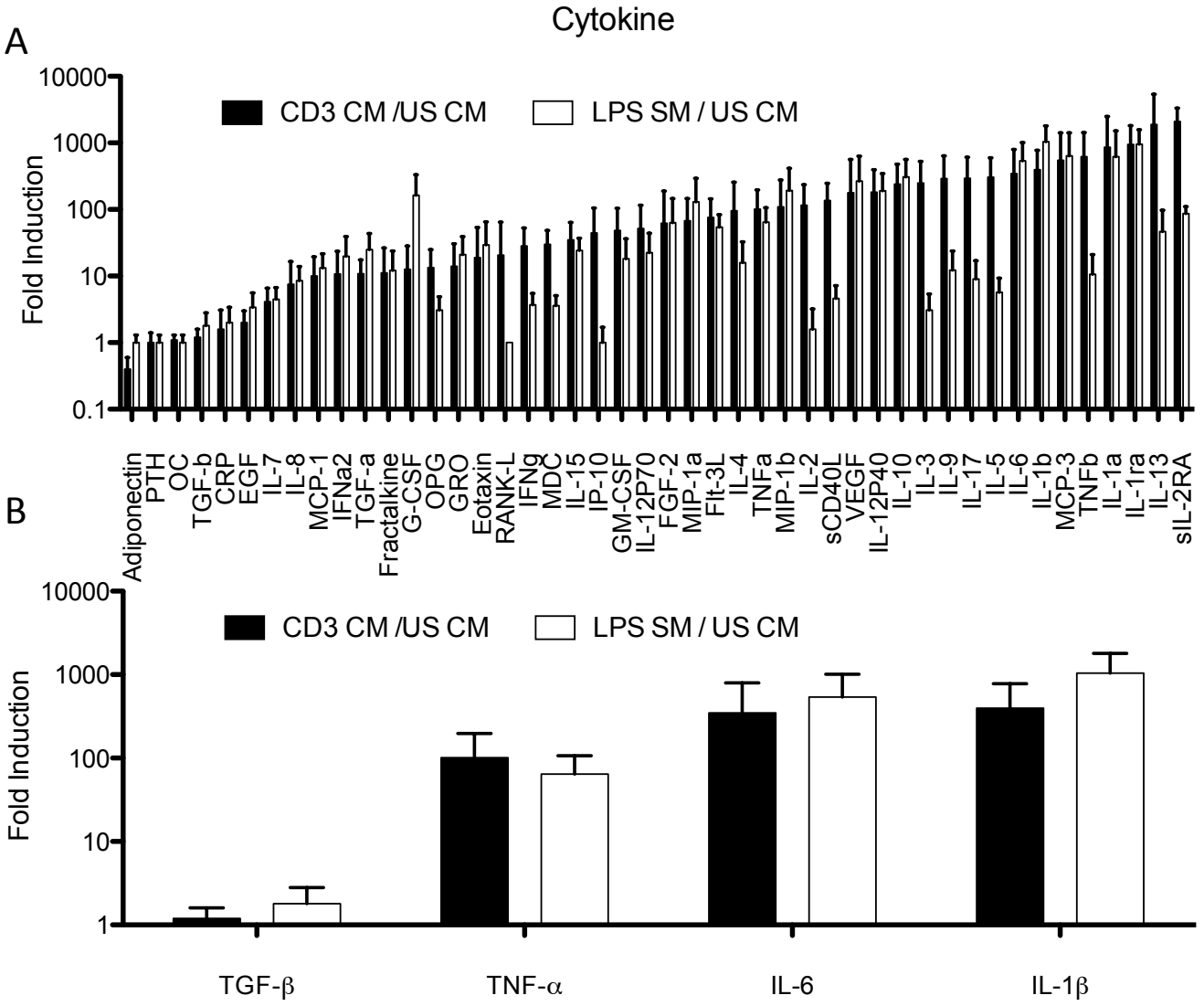
Although cytokines such as TNF-α, TGF-β1 (155), IL-6 (156) and IL-1β have been shown to induce EMT in breast cancer cells, the source of these factors has not been studied. In the current study, we activated freshly collected peripheral blood mononuclear cells (PBMC) from healthy donors (HD) to secrete soluble factors that, in turn, were added to cultures of SUM149 IBC cells. We found that secreted factors from activated immune cells are capable of inducing EMT in IBC cells.

Cytokine Profile of primary human PBMC immune cell conditioned media

To test the ability of primary human leukocytes to induce EMT in IBC cells, we cultured SUM149 cells in the presence of CM of PBMC cultures. Bacterial lipopolysaccharide (LPS), a toll-like receptor (TLR)-4 agonist was used to generate conditioned media from activated monocytes (LPS-CM). Plate-immobilized anti-CD3 antibody plus soluble anti-CD28 antibody were used to generate conditioned media from activated T-cells (αCD3-CM). PBMC were left unstimulated to generate control conditioned media (US-CM). Each condition was cultured for 48 hours prior to harvesting. Cytokine concentrations in the resultant CM samples were measured by Luminex multiplex bead assay and found to contain high levels of inflammatory cytokines including TNF-α and IL-6 (Figure 5.1 on page 114). Compared to US-CM, the αCD3-CM contained at least a 100-fold increase in the following factors: IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-13, IL-17, monocyte chemotactic protein (MCP)-3, macrophage inflammatory protein (MIP)-1β, soluble CD40 ligand (sCD40L), soluble IL-2Rα/soluble CD25α (sIL-2RA), TNF-α, TNF-β and VEGF. A 100-fold increase or greater was likewise observed in the LPS-CM in the following cytokines: G-CSF, IL-1α, IL-1β, IL-1 receptor antagonist (IL-1RA), IL-6, IL-10, IL-12p40, MCP-3, MIP-1α, MIP-1β, and VEGF. In the 5 culture supernatants of PBMC activated for 48h with anti-CD3 antibody (αCD3-CM), TGF-β had a modest 1.6-fold increase; TNF-α had an average 101-fold increase; while IL-6 had an average 347-fold increase. These data suggest that inflammatory cytokines previously shown to induce EMT (157) are secreted by activated immune cells.

Figure 5.1

Activated PBMC secreted EMT-promoting factors. Healthy donor PBMC were stimulated with LPS, plate-immobilized anti-CD3 and soluble anti-CD28 antibodies or left unstimulated for 48 hours before conditioned media (CM) were harvested. Cytokine concentrations in the CM supernatants were measured by Luminex multiplex array in 5 representative HD. (a) Relative expression of 46 cytokines, chemokines and growth factors. (b) Relative expression of TNF-α, TGF-β and IL-6 in LPS-CM, αCD3-CM relative to US-CM (solid bars).



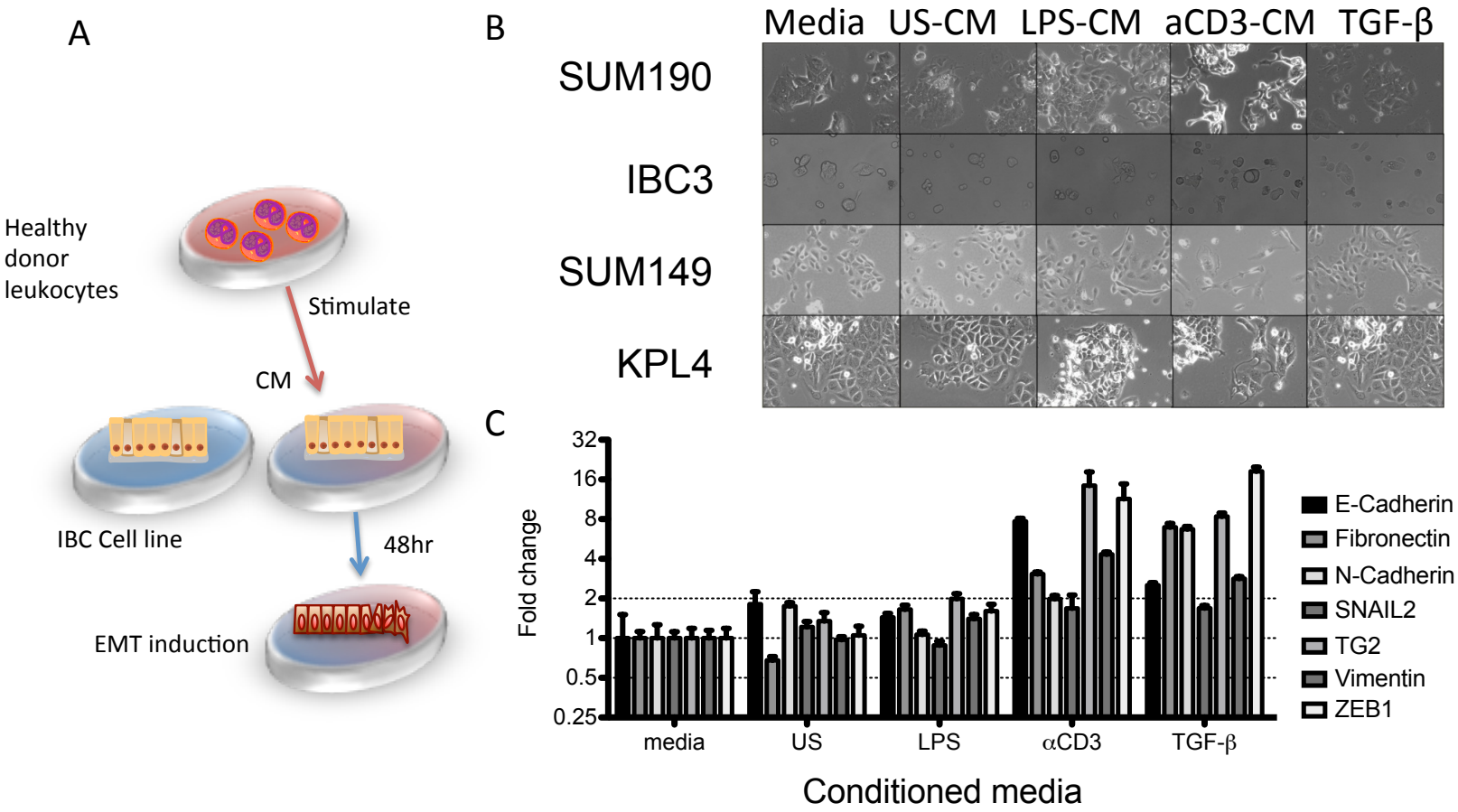
Immune conditioned media induces EMT transcription factors in SUM149 cells

Epithelial to mesenchymal transitions (EMT) have been reported in response to inflammatory cytokines typically produced by products of activated immune cells such as TNF-α and TGF-β. To test weather the full milieu of factors secreted by activated immune cells are capable of inducing EMT, SUM149 cells were incubated with αCD3-CM, LPS-CM or US-CM.

To test the effect of immune cells on breast cancer cells, immune CM was diluted 1:4 with IBC media and incubated with established 2D cultures of SUM149 cells for 48 hours prior to evaluating EMT (see Figure 5.2a on page 114). After 48-hours, SUM149 cells treated with αCD3-CM exhibited a mesenchymal or stressed morphology with elongated projections (Figure 5.2b). Assessment of the SUM149 cells exposed to αCD3-CM by qRT-PCR showed at least a 2-fold increase in transcripts of fibronectin, N-cadherin, TG2, vimentin and ZEB1 and slightly less in SNAIL1. Interestingly, although these increased EMT-related factors are typically associated with decreased E-cadherin expression, SUM149 cells showed a substantial increase E-cadherin expression in response to αCD3-CM (Figure 5.2c). However, E-cadherin expression is a hallmark of inflammatory breast cancer and is typically highly expressed even in stage IV tumors. These data suggest that soluble factors secreted by activated immune cells are able to induce EMT in epithelial cells.

Figure 5.2

Conditioned media from activated healthy donor PBMC induces expression of EMT-related transcription factors in SUM149. (a) Healthy donor peripheral blood mononuclear cells were stimulated with LPS to stimulate monocytes, plate-immobilized anti-CD3 and soluble anti-CD28 antibodies to stimulate T-cells or left unstimulated for 48 hours before conditioned media was collected. These immune CM were added to established SUM149 cultures at 25% of media volume. RPMI-1640 culture media was used as a negative control (0) and 2ng/mL TGF-β was used as a positive control. SUM149 cells were incubated with immune CM for 48 hours prior to mRNA extraction. (b) Morphological changes are consistent with stress and EMT can were observed at this time point. (c) Expression levels of EMT-related transcription factors SNAIL and ZEB1, and TG2 were quantified by Taq-Man® qRT-PCR. αCD3-CM and to a lesser extent LPS-CM, induced large increases in ZEB1 and TG2.



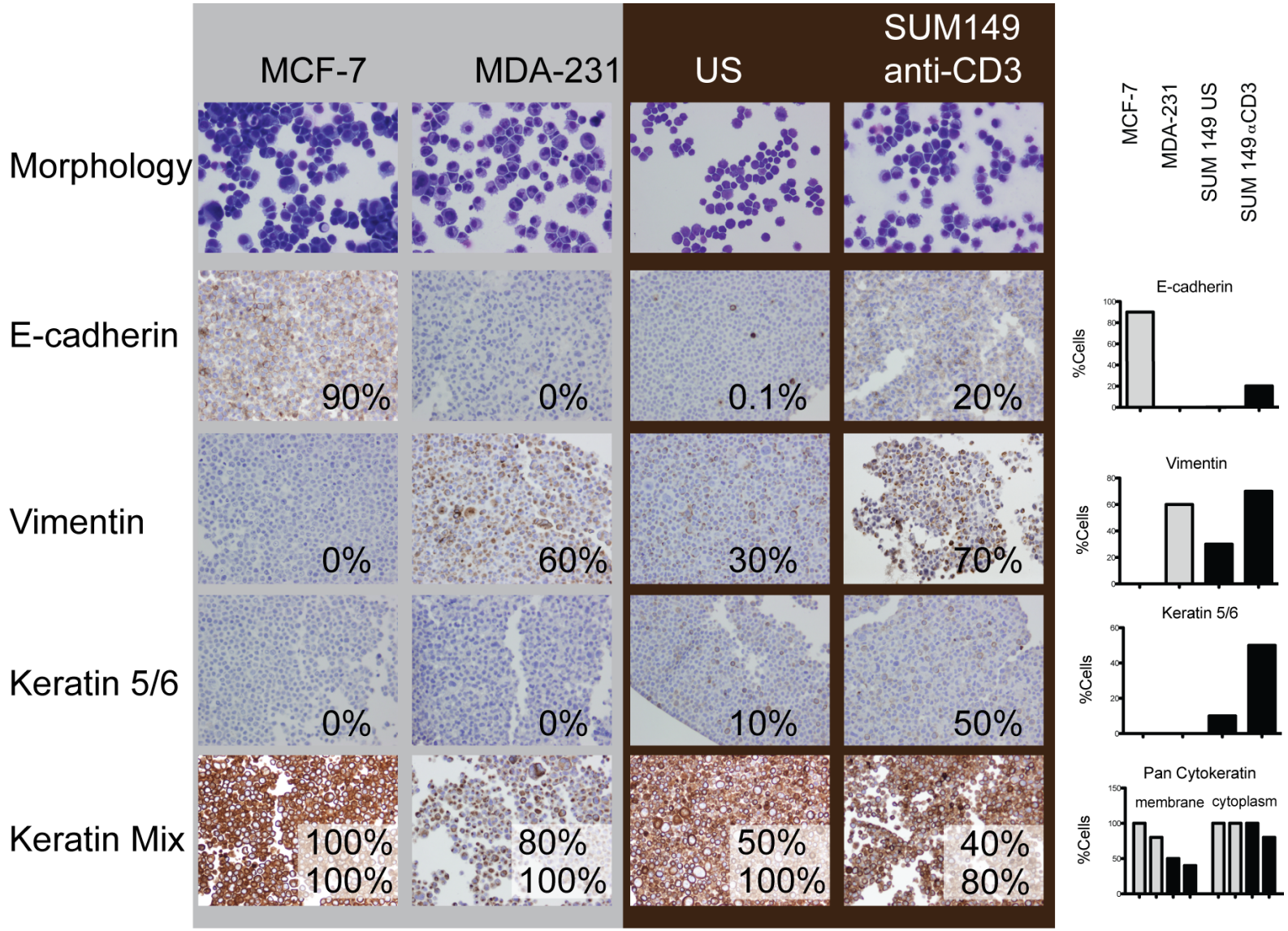
Immune conditioned media induces phenotypic changes in SUM149 cells

MCF-7 breast cells were used as a representative epithelial cell line and MDA-231 cells were used as a representative mesenchymal breast cell line. Cells were grown on plastic, trypsinized and embedded in paraffin for immunohistochemistry (IHC) evaluation, as shown in Figure 5.3 on page 120. MCF-7 cells expressed high E-cadherin and pan-cytokeratin but lacked expression of vimentin or cytokeratin (CK)5/6. In contrast, MDA-231 cells stained for vimentin in greater than 50% of cells while lacking E-cadherin expression. In these cells, cytokeratins (stained with an anti-CK antibody cocktail) are localized mostly to the cytoplasm and not at the membrane. Punctate staining is suggestive of golgi localization.

SUM149 cells treated with αCD3-CM up-regulated the expression of vimentin and E-cadherin, similar to the gene expression data. (However, LPS-CM treated cells showed a slight decrease in vimentin.) Cytokeratin 5/6 staining, typical of basal-like breast cancers but not the MDA-231 cell line, increased following treatment with αCD3-CM. Finally, pan-keratin localization to both the membrane and cytoplasm decreased following αCD3-CM treatment, consistent with decrease in epithelial structure. Together, the increases in vimentin and keratin 5/6 in conjunction with the decreased staining from the keratin cocktail suggest that activated immune cells induce breast epithelial cells to a more basal phenotype.

Figure 5.3

Immune conditioned media induces phenotypic changes in SUM149 cells. Immune conditioned media (anti-CD3 CM) was added to established SUM149 cells and cultured for 2 days (black); MCF-7 and MDA-231 grown in standard media were used as controls (grey). Paraffin-embedded cell blocks were prepared and analyzed by IHC. Morphology of the cells is visualized with Diff-Quick stain. The percentage of positive cells is listed in each image and shown in the bar graph at right. Pan cytokeratin expression is shown for both percent of cells with membrane localization (top number) and cytoplasmic localization (bottom number). MCF-7 cells show a characteristic epithelial phenotype with high E-cadherin, low vimentin, low keratin 5/6 expression and strong membrane and cytoplasmic localization of cytokeratins. MDA-231 cells are mostly mesenchymal with low E-cadherin, high vimentin and decreased cytokeratin expression. Following exposure to CD3-CM, SUM149 cells show increased expression of E-cadherin, vimentin, keratin 5/6 staining and decreased pan cytokeratin staining. (US-CM, Conditioned Media from unstimulated PBMC; LPS-CM, Conditioned media from LPS-stimulated PBMC; αCD3-CM, Conditioned media from PBMC stimulated through the T-cell receptor with immobilized ani-CD3 and soluble anti-CD28) antibodies.



Immune Cell conditioned media induces EMT profile in multiple breast cell lines

To test immune induction of EMT in additional genetic backgrounds, immune CM was added to a panel of breast cancer cell lines. Cell lines included the non-tumorigenic cell line MCF-10a, hormone receptor positive MCF-7, triple receptor negative and highly mesenchymal MDA-231, androgen receptor-positive MDA-453, and HER2-amplified SKBR3. In addition, several IBC cell lines were evaluated including: HER2-amplified KPL4, SUM190, and IBC-3 cells. As with the SUM149 cells, immune-conditioned media was diluted 1:4 with IBC media and added to established 2D cultures of above-mentioned IBC cell lines for 2 days. Cells were also treated with 2ng/ml TGF-β as a positive control for induction of EMT. All cell lines were treated with the same pool of conditioned media. Cells exposed to immune CM are generally less dense and appear spindly and stressed. To test for EMT-inducing transcription factors, RT-PCR was performed on mRNA extracted from the cell lines using the Fluidigm® Integrated Fluidic Chip Dynamic Array system to array the samples and the Fluidigm® BioMark™ HD for thermocycling and data acquisition. Results are summarized in Figure 5.4. In general, most cell lines showed increases in expression of EMT-related factors following treatment with immune CM. ZEB1 and TGM2 were the most induced factors along with the inflammatory factors prostaglandin E (PGE) and IL-8. Consistent with an induction of EMT, epithelial cell adhesion molecule (EpCAM) expression decreased with αCD3-CM treatment in every cell line except SUM149. However, as shown in Figure 5. 5, the EpCAM response is attenuated in all of IBC cell lines ($p=0.0197$). Likewise, although

the non-IBC cell lines showed no change in E-cadherin expression at this time-point, 3 of the 4 IBC cell lines (SUM149, SUM190, IBC3 but not KPL4 cells) showed a paradoxical increase in E-cadherin expression ($p = 0.0411$). Her2-amplification was also associated with an attenuated EpCAM response ($p = 0.210$ when SUM149 cells were removed from the analysis); however, it must be noted that 3 of the 4 Her2+ cell lines in this study are IBC cell lines and 3 of the 4 IBC cell lines are Her2+. Comparing intrinsic molecular subtypes, luminal cells had a higher induction of Forkhead box protein C2 (FOXC2) than the basal-like cells MCF-10a, MDA-MB-453, and SUM149 cells following treatment with αCD3-CM ($p = 0.0384$) (although MDA-453 is TNBC, it is androgen receptor positive and not basal-like) (158-160). While these data confirm that immune induction of EMT is common among a range of breast cancer subtypes, IBC cells have an abnormal response characterized by a shift towards EMT while maintaining or increasing homotypic adhesion.

Figure 5.4

EMT induction by immune conditioned media is not unique to IBC. Immune conditioned media from a single healthy donor was added to 2-day old cultures of 9 breast cancer cell lines. Thereafter, mRNA was isolated from the cultures and analyzed by qRT-PCR using TaqMan® hydrolysis probe assays on the Fluidigm® BioMark™ HD system using a single 48.48 Dynamic Array Integrated Fluidic Circuit "chip". Data are represented as $2^{-\Delta\Delta Ct}$ fold change with the unconditioned media for each cell line serving as the reference sample and GAPDH as the endogenous control. Unconditioned media appears as a solid black line at 1 in the center of each plot; points falling outside or inside the black solid circle represent increased or decreased relative expression, respectively. αCD3-CM induced EMT-related transcription factors to varying degrees in all cell lines. Zeb1 and TG2 are the most consistently increased EMT-related factors across cell lines and are highlighted with asterisks.

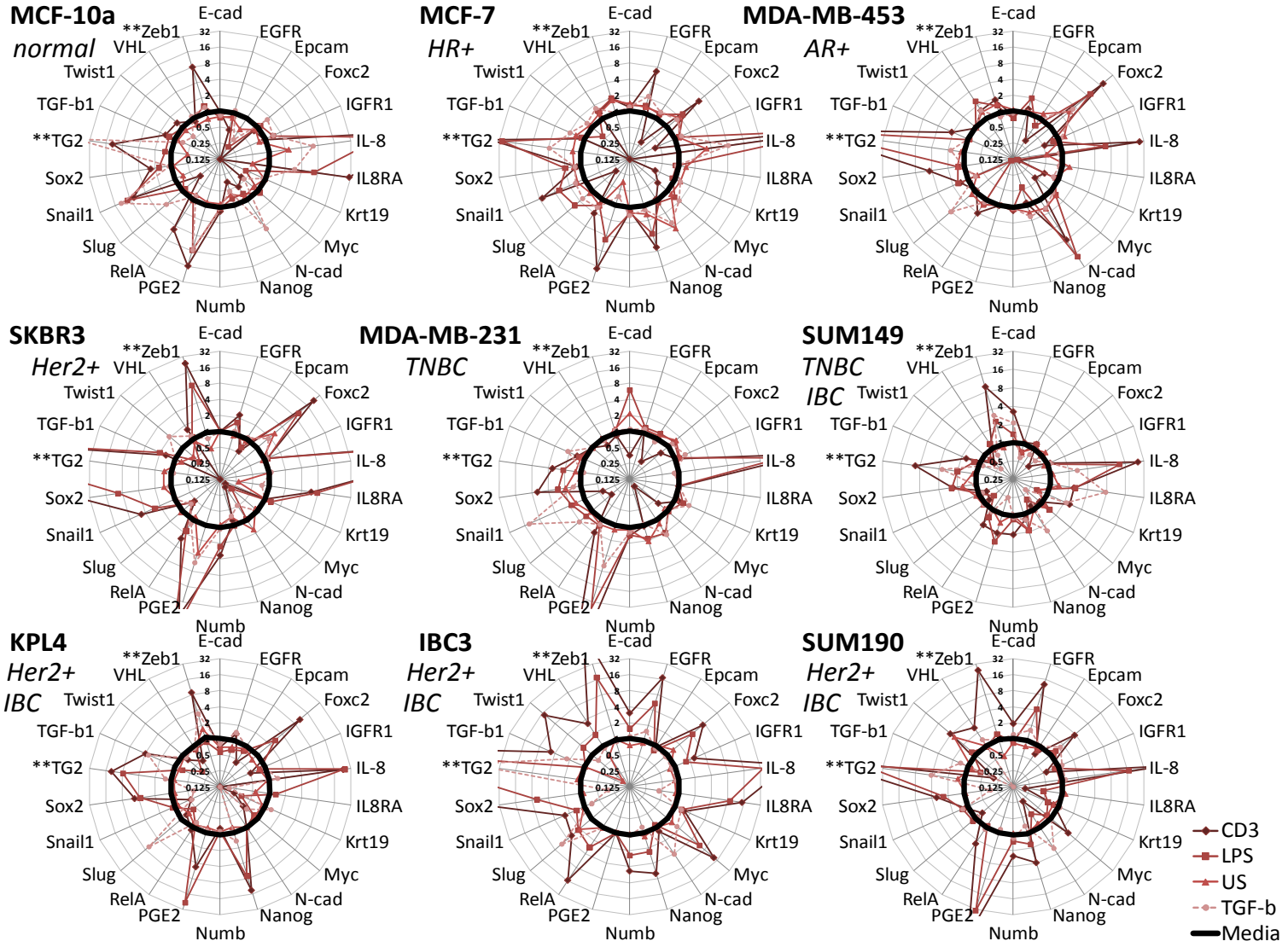
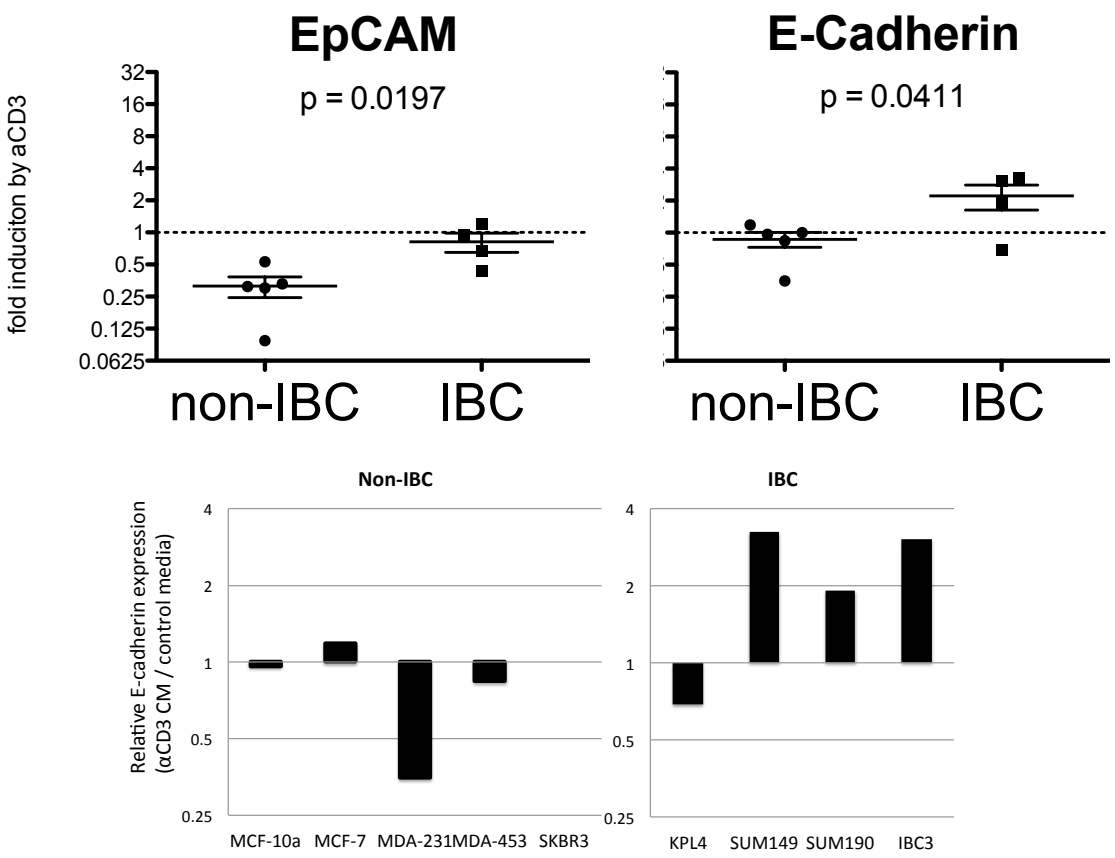


Figure 5. 5

IBC has unique response to immune conditioned media. Following treatment with αCD3-CM, IBC cell lines IBC-3, KPL4, SUM149 and SUM190 had a decrease in EpCAM mRNA compared to those of non-IBC cell lines and with the exception of KPL4, that had increased expression of E-cadherin.

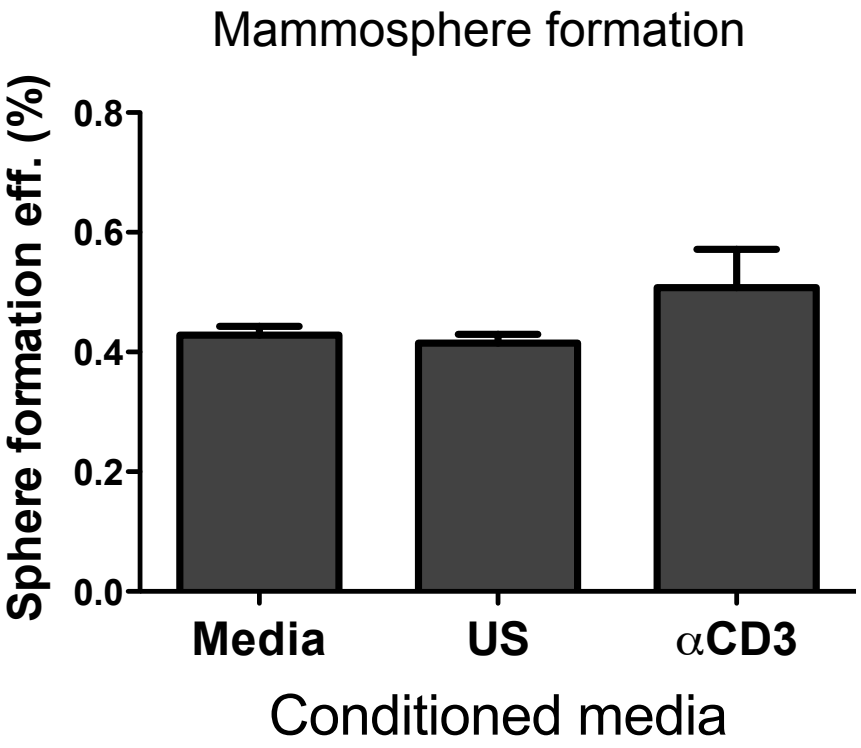


Immune CM has minimal effect on mammosphere formation

To test the functional changes associated with exposure to immune conditioned media, SUM149 cells were placed into mammosphere media following 2 day pre-incubation with CM, as above. Sphere forming efficiency in mammosphere media allows for enumeration of cells with the stem cell properties of self-renewal and differentiation potential (161, 162) that are associated with EMT (163). Cells are added as a single cell suspension and grown in 3D. After 1 week, "mammospheres" are counted. As shown in Figure 5.6 on page 129, there was a non-significant trend towards increased sphere formation following incubation with αCD3-CM (0.43% vs. 0.50%, p = 0.256). Interestingly, the spheres forming from the αCD3-CM were significantly smaller than the spheres from cells grown in unconditioned control media (mean ± sem 175μm ± 5.7 μm vs. 150μm ± 9.6, p = 0.048). This suggests that while EMT and stem cell-like properties may be induced by exposure to immune activated CM, proliferation is significantly inhibited, at least in short-term cultures.

Figure 5.6

Immune conditioned media has negligible impact on mammosphere forming ability. SUM149 cells were pre-treated with conditioned media and placed into mammosphere media. There was no significant increase in mammosphere formatting efficiency by immune conditioned media; however, there was a trend for αCD3-CM to have a higher sphere forming efficiency.



Real-time cell analysis reveals increased migration, invasion and adhesion following incubation with immune conditioned media.

Changes in transcription factors could be identified following 48 hours of incubation with immune conditioned CM. To understand the kinetics of this change, real-time analysis of cell growth was measured using the xCelligence real time cell analyzer (Acea Bioscicens, Inc, San Diego, CA). This technology measures resistance to the flow of ionic electrical current (impedance) between electrodes laid out in a grid on the bottom of cell culture well in contact with the growing cells. Measuring electrical impedance is both non-invasive and label free. Impedance increases with a number of factors, primarily cell growth and adhesion. As the surface area covered by the growing cells increases, there is less free surface area on the electrodes for the free flow of current. Similarly, as the cells adhere more closely to the substrate and to each other, impedance increases as ions cannot access the electrode beneath the cell (164). The xCelligence system measures this impedance at a defined base-line and calculates changes as a cell index (165). As such, the system can measure cell growth and adhesion, and with a modified Boyden chamber plate with the electrodes on the bottom of the membrane, migration can be measured as well as cells migrate unto the electrodes.

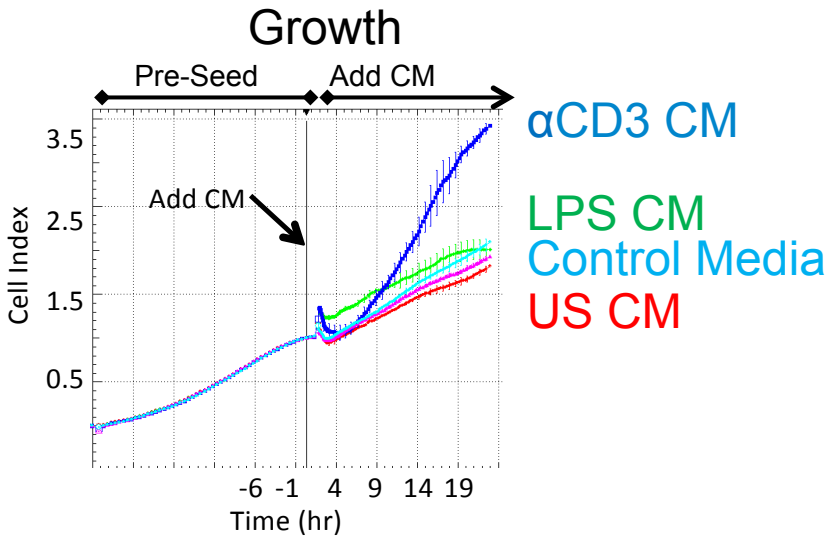
Following addition of immune CM, changes could be detected within 10 hours (Figure 5.7 on page 133). Interestingly, although αCD3-CM clearly decreased cell counts by the traditional trypan blue exclusion counting method (Figure 5.8 on page 135), the real-time analysis showed a marked increase in the Cell Index. In fact, in 2 days, $8x10^3$ αCD3-CM treated SUM149 cells achieved a cell index about 2 times larger than $20x10^3$ SUM149 cells allowed to grow in IBC media for 5 days. ($20 x10^3$ SUM149 had a

cell index of 2.54 after 22 hours of attachment and achieved a maximum cell index of 3.09 at 70 hours, post seeding. In contrast, $8x10^3$ αCD3-CM-treated SUM149 had a cell index of 1.20 after attachment that rapidly increased to 3.1 within 14 hours of treatment and 6.07 within 43 hours of treatment). As the Cell Index measured by the xCelligence is affected by cell morphology and adhesion in addition to cell growth, these data suggest that immune CM-exposed IBC cells adhere more tightly. This increased adhesion is consistent with the observed increase in E-cadherin expression by IBC cell lines.

The increase in E-cadherin and EpCAM in IBC cells concurrent with the upregulation of EMT factors is inconsistent with current models for EMT. We therefore tested the migratory ability of SUM149 cells exposed to immune conditioned media using the xCelligence system. Using a CIM (cellular migration/invasion) plate we measured SUM149 cell migration towards fetal bovine serum (FBS, a common chemoattractant) following exposure to immune conditioned media. The SUM149 cells show a very rapid increase in the cell-index of αCD3-CM-exposed cells suggesting an induced migratory capacity (Figure 5.7). Similar migration patterns were observed when the plates were pre-coated with 15% Matrigel™ and when cells were pre-treated with immune CM for 48 hours and loaded into the chambers in equal numbers (data not shown). Combined, these data show that the induced changes in IBC cells paradoxically increase both adhesion and migration.

Figure 5.7

Immune Conditioned Media induces adhesion and migration in SUM149. SUM149 were grown on an xCelligence® E-plate at an initial density of $5x10^3$/well and exposed to αCD3-CM, LPS-CM and US-CM at time 0. Cell index, a statistic that summarizes cell number, viability, and morphology, is measured at 15-minute intervals. The cell index was standardized at the vertical line, prior to adding the CM that can be seen on the graph as a transient spike in cell index. Robust changes are observed at 9 hours after treatment with cells exposed to αCD3-CM showing rapid increases in cell index. To quantify migration, $50x10^3$ SUM149 cells in immune conditioned media were seed into the top wells of xCelligence CIM plates with FBS as a chemoattractant in the bottom wells. Migration is measured as an increase in impedance as cells migrate into the lower chamber. Migration towards FBS is enhanced by immune activation factors. αCD3-CM (blue line) induces rapid migration of SUM149 cells. LPS-CM enhancement of migration (green line) is noted from 7 to ~36 hours, but is not significantly different from controls (US, Media, no FBS; red, pink and light blue) at later time points. Error bars are one standard deviation.



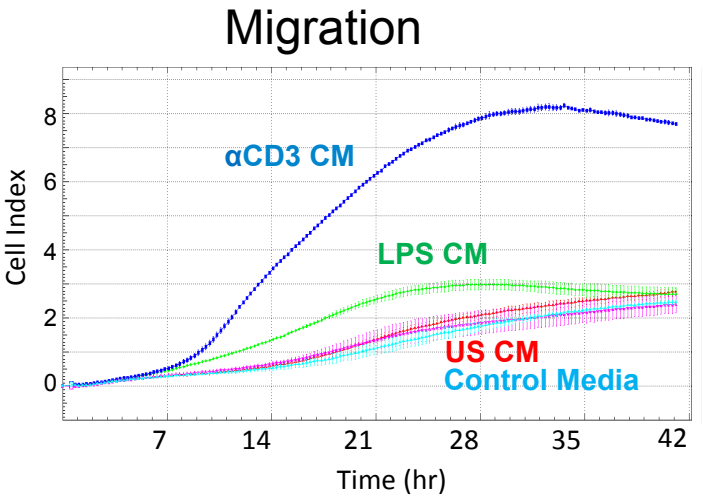
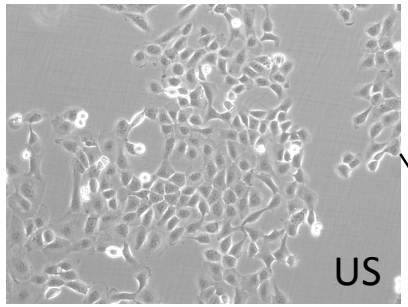
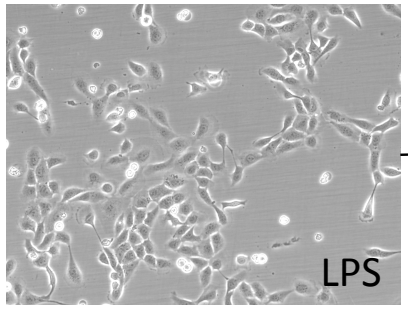
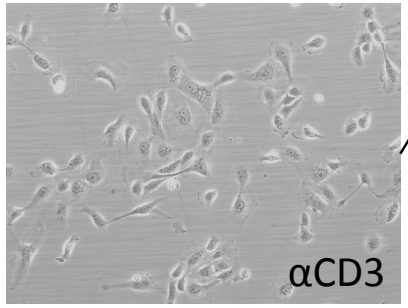


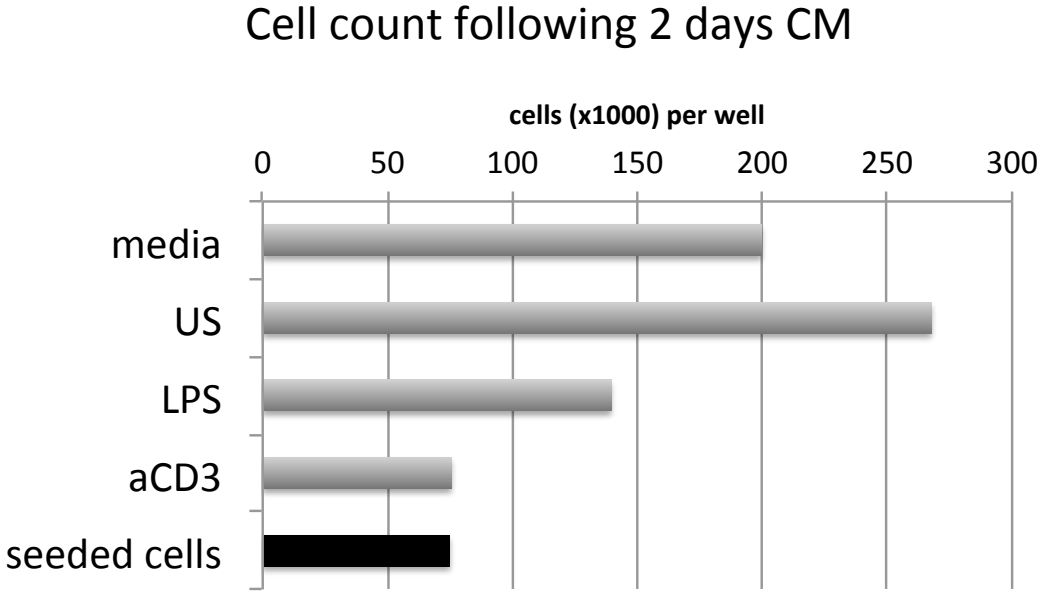
Figure 5.8

Immune Conditioned media decreases tumor cell proliferation. Cells were seeded into 6-well plates at an initial titer of $75x10^3$ cells per well. After 2 days for cell attachment, CM was added to each well. Following 2 days of culture with immune conditioned media, cell titers were determined by determining viable cell counts using the trypan blue exclusion method. Cells exposed to αCD3-CM had cell counts lower than the initially seeded cells and lower than LPS-CM, US-CM or media control. Both LPS-CM and αCD3-CM induced a fibrolblast-like cell morphology with longer projections and less of a cobble-stone appearance.









	% viabilty	count per ml ($x10^6$)
Serum free	95.9%	0.465
media	97.1%	0.675
US	99.2%	0.595
aCD3 – 2well	93.9%	0.31
LPS	94.4%	0.335

Neutralizing TNF-α, TGF-β and IL-6 reverses immune induced EMT

Factors such as TNF-α, TGF-β and IL-6 have been associated with EMT induction were found elevated in cells exposed to αCD3-CM. TNF-α induces signaling through NF-κB and IL-6 induces signaling through Stat3. Therefore, if IBC cell cells are responding to TNF-α and IL-6 in the conditioned media, we would expect to see elevated levels of phosphorylated NF-κB (p65), the upstream IκB, and Stat3 in IBC cells following addition of immune conditioned media. We measured phosphorylation levels of Stat3 (Ser727), Stat3(Tyr705), IκB (Ser32) and the p65 subunit of NF-κB (Ser536) in SUM149 cell lysates using Luminex multiplex polystyrene beads (Figure 5.9). αCD3-CM and LPS-CM induced Stat3 and NF-κB phosphorylation in SUM149 cells within 1 hour that subsided over 2 days (the time course of the co-cultures shown above). This supports the hypothesis that IL-6 and TNF-α are acting on the SUM149 cells.

As shown above, addition of TNF-α, TGF-β, and IL-6 synergistically induced EMT in SUM149 cultures. Therefore, we hypothesized that reducing these factors would mitigate the induction of EMT by immune activation. To test this hypothesis, αCD3-CM was pre-absorbed with neutralizing antibodies prior to incubation with SUM149 culture and EMT-related transcripts were quantified, as before. Compared with αCD3-CM, the pre-absorbed CM showed a reduction in E-cadherin, EpCAM, fibronectin, N-cadherin, SNAIL2, TG2, vimentin and ZEB1 (see Figure 5.10 on page 140). This suggests that TNF-α, TGF-β and IL-6 are partially responsible for the immune changes observed in IBC cells.

Figure 5.9

Immune conditioned media activates TNF-α, and IL-6 pathways in SUM149. Immune conditioned media (αCD3-CM, LPS-CM, US-CM, and media control) were added to established SUM149 cultures. Cell lysates were collected after 10 min, 30 min, 60 min and 2 days before being analyzed using Millipore Milliplex MAP polystyrene beads and quantified by mean florescent intensity (MFI) using the Luminex analyzer (the 48 hour sample was only analyzed for αCD3-CM and media control). Phosphorylation of Stat3 Ser727 and Tyr7005 were measured from the IL-6 signaling pathway and phosphorylation of IκB Ser32 and NF-κB p65 Ser536 were measured from the TNF-α pathway. The MFI of each analyte was corrected with the GAPDH loading control for each sample to account for loading variation. All four analytes show increased phosphorylation following addition of conditioned media from αCD3 and LPS activated PBMC but not unstimulted PBMC or control media.

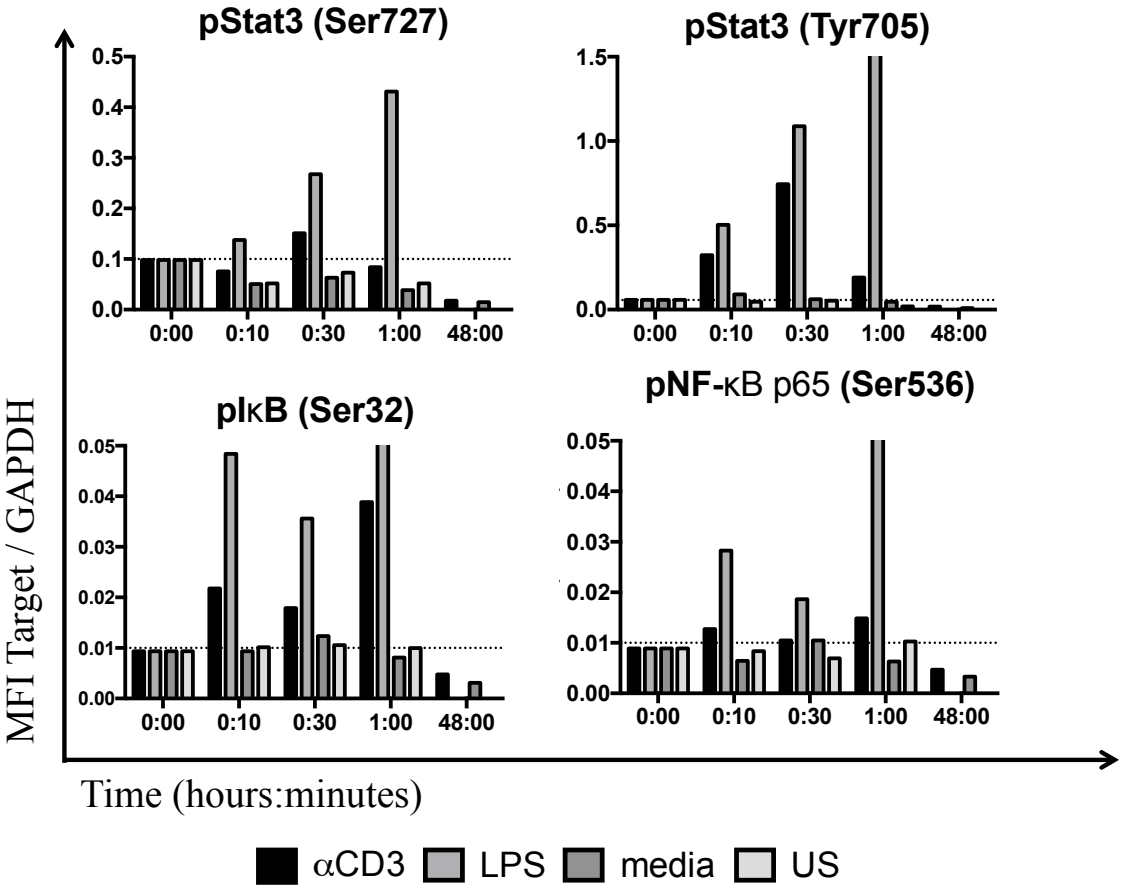
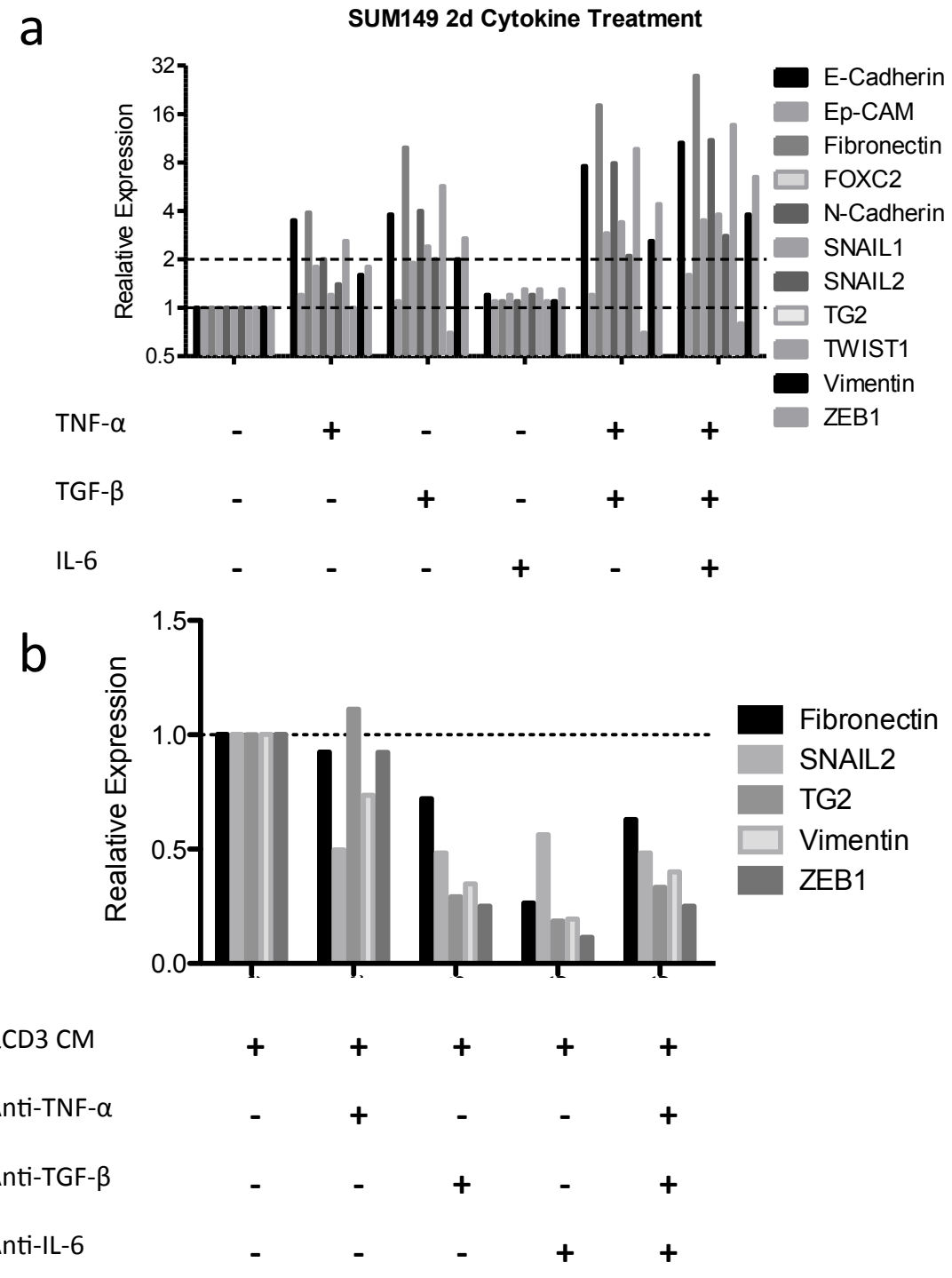


Figure 5.10

TNF-α, TGF-β and IL-6 induce EMT in SUM149. a) TNF-α, TGF-β and IL-6 were added to established SUM149 cultures and assayed for EMT-TF, as before. All three cytokines act additively to induce EMT-TF. b) Neutralizing antibodies against TNF-α, TGF-β and IL-6 were added to αCD3-CM prior to incubating with SUM149 cells. Compared to αCD3-CM without neutralization, EMT-TFs were reduced.



Patient Data

To determine whether increased cytokine production by activated immune cells is correlated with the induction of EMT in breast cancer patients, we compared the ability of peripheral blood T cells to produce TNF-α with the expression of EMT-transcription factors in blood (EMT-CTC). Fresh 5 ml whole blood samples were depleted of CD45 leukocytes and subjected to RT-PCR analysis for the measurement of EMT-related transcription factors. Matched thawed archived peripheral blood mononuclear cells were stimulated overnight with plate-bound anti-CD3 and soluble anti-CD28 antibodies for measurement of intracellular TNF-α synthesis as measured by flow cytometry (166, 167). Matched data were evaluable from 16 breast cancer patients. Six patients had detectable EMT-CTC. Of these, 5 had greater than 220 CD3+ T cells per ml of blood capable of producing TNF-α upon activation (Fisher's Exact test $p = 0.036$). Although the numbers are very small, as seen in Table 5.1, higher numbers of TNF-α producing CD3+ cells are associated with an increase in EMT-CTC in breast cancer patients. However, a CD3-TNF count greater than 225 was associated with longer progression-free survival ($p = 0.001$).

Table 5.1

T cells producing TNF-α correlates with EMT-CTC in patients. As shown in Chapter 4, patients PBMC were stimulated through the T-cell receptor overnight and interrogated for intracellular TNF-α synthesis by multiparameter flow cytometry. Additionally, 5 mL of blood was depleted of CD45+ leukocytes to enrich for circulating tumor cells and interrogated for expression of EMT-related factors. There was a significant correlation between the detection of at least 1 EMT-related factor in the CTC-enriched fraction and presence of 225 CD3+ T-cells per μL of blood capable of producing TNF-α. A cut-off of 225 cells per μL was established by receiver operator curve analysis.

		CD3 Cells producing TNF-α		
		<225	>225	Total
EMT CTC	no EMT	**9**	2	11
	any EMT	1	**4**	5
	Total	10	6	16

χ^2 Fisher Exact test p = 0.036

Discussion

Here we show that inflammatory breast cancer cells respond to inflammatory signals from innate and adaptive immune cells with a program reminiscent of EMT that renders the tumor even more aggressive. We used PBMC from healthy donors activated with LPS and immobilized anti-CD3 plus soluble anti-CD28 antibodies as a model for inducing inflammation. To mediate cell-cell killing effects from HLA-mismatch, we collected only the conditioned media from the activated cells and applied it to established cultures of IBC cells. In this model, SUM149 cells respond to immune induced inflammation with increased EMT factors as shown by PCR, stem cell properties as shown by mammosphere formation and migration as shown with the real-time cell analysis. Paradoxically, unique to IBC, these changes were also associated with an increase in E-cadherin, EpCAM, and cell adhesion, all hallmarks of IBC.

TNF-α levels in the conditioned media of anti-CD3-stimulated PBMC were higher in than LPS conditioned media. As TNF-α is known to induce EMT, this can partially explain the superior increase in EMT induction by anti-CD3-stimulated PBMC. The differences in induced TNF-α levels may be related to cell number as T cells out-number monocytes in PBMC by about 4 to 1. In fact, untreated IBC patients have significantly more monocytes than non-IBC patients.

While CD4+ and CD8+ T cells are capable of producing copious amounts of TNF-α and thus inducing EMT, our data show that T cell synthesis of TNF-α is positively correlated with survival. This study used immobilized anti-CD3 antibody to

polyclonally activate peripheral blood T cells non-specifically through the T-cell receptor in both the enumeration of TNF-α producing T-cells in IBC patients and for the production of conditioned media from healthy donors. These cells may not have the same cytokine response as tumor-infiltrating lymphocytes that conditioned the cytokine milieu in vivo (168, 169). Conversely, parallel studies on TNF-α production by TLR-activated peripheral blood dendritic cells were associated with shorter survival. Together, these observations suggest that although immune cells are capable of inducing EMT and potentially metastasis, the immune surveillance, particularly that provided by the adaptive immune system, may offer an important barrier to disease progression. Indeed, a recent Van Laere et al report showed that a gene signature associated with a TH1 immune response was associated with attainment of a pathological complete response in IBC patients (170).

E-cadherin has represented a conundrum in IBC research. In most carcinomas, loss of E-cadherin is associated with advanced disease and increased invasion and metastasis as the homotypic cell adhesions are decreased. IBC, in contrast, has been shown to have high levels of E-cadherin expression despite the rapid progression of the disease. In fact, tight clusters of tumor emboli are a hallmark of the disease.

In this study, we showed that E-cadherin expression increased in IBC cell lines after exposure to αCD3-CM. This is in contrast to the typical loss of E-cadherin expression at metastatic progression. In experimental systems, loss of E-cadherin can induce increased invasiveness and metastasis. As a cell-adhesion molecule, expression of E-cadherin on the cell surface prevents cell motility, invasion and metastasis. However, loss of E-cadherin also increases several mitogenic signaling cascades including mitogen

activated kinase (MAP) and rat sarcoma viral oncogene (Ras). Conversely, reintroduction of E-cadherin expression in poorly differentiated cell lines can restore an epithelial phenotype (for review see F.J. Rodriguez et al (171)). Furthermore, E-cadherin can increase stem cell viability through upregulation of apoptotic inhibitory gene Bcl-XL and inhibiting Caspase-3 (172).

However, inflammatory breast cancer tumors typically express high levels of E-cadherin despite the highly aggressive nature and rapid progression of the disease. Although E-cadherin expression at the protein expression is elevated, mRNA levels are lower than other E-cadherin positive breast cancers due to altered protein trafficking (173). Additionally, although IBC is typified by high levels of E-cadherin, constitutive SUM149 expression is low but can be increased with treatment with EGFR-targeted therapy as shown by Ueno and Zhang (73).

In our survey of 9 breast cancer cell lines, we found that E-cadherin expression increased in 3 of the 4 IBC cell lines following exposure to immune factors. In contrast, none of the non-IBC cell lines showed a similar increase. It would be interesting to check in the NF-κB, SMAD or Stat response elements in IBC E-cadherin promoters are different from non-IBC (this is relatively easy to check *in silico* once a full sequence is available for IBC cell lines).

The data here showed that spiking IL-6 into the media had little effect on the induction of EMT, yet depleting it form the conditioned media drastically reduced the observed EMT at the 2 day time point. The real-time cell analysis experiments were not performed with neutralizing antibodies due to the difficulty in handling the cells in small volumes, but it might this might be a good tool for exploring this observation. It is

possible that IL-6 has a minimal effect in inducing EMT but has a strong effect in maintaining the EMT-like state. For example, it has been shown that IL-6 is critical for maintaining and expanding a cancer-stem like population of trastuzumab resistant cells (63). The data here suggest that multiple pathways need to be blocked to prevent EMT induction but preventing the maintenance of this state might be a better target. Time-based experiments such as the real-time cell analysis can test this hypothesis.

NF-κB signaling has been shown to induce EMT including down-regulation of E-cadherin through ZEB1 in MCF-10a cells (174). Interestingly, the number of $CD3^+CD8^+$ cytotoxic lymphocytes producing TNF-α was correlated with the measurement of EMT in the blood of breast cancer patients, but the number of $CD3^+CD4^+$ T-helper cells was not. Despite the fact that CD8+ T cells are capable of direct tumor lysis, this observation is consistent with previous work by Santisteban et al that showed that immune-editing by CD8+ T cells can induce EMT in a mouse Her2/neu transgenic model (175).

One of the major factors upregulated in αCM-CM is IL-17. IL-17 has been associated with auto-immune reactions as might be induced by tumor-immune interactions. However, one of the major targets of IL-17 is endothelial cells. This highly inflammatory cytokine induces endothelial cells in-turn to produce inflammatory factors IL-6, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1β, TGF-β1, TNF-α, IL-8 and MCP-1, many of the same factors discussed here (176, 177). IL-8 in particular has been shown to supports breast cancer stem cells in a SUM149 model (65, 66). As the frequency of lymphocytic infiltrates in IBC tumor emboli is still uncertain, this model allows for the possibility that immune activation could produce factors such as IL-17 that, in turn, release EMT-related factors TNF-α, IL-6 and TGF-β1 into tumor emboli.

Further studies are needed to show that cytokines from activated immune cells increase metastasis in vivo. However, the patient data presented here suggest that therapeutic options should not broadly target immune cells. Rather, therapies that alter the immune response by favoring adaptive immune responses or shifting antigen specific responses towards a TH1 polarization would be more beneficial.

It can be argued that the phenomenon presented here is a selection of resistant cells rather than the induction of EMT. Inflammatory factors (such as TNF-α) induce cell death in the majority of cells and select for resistant cancer stem cells that are characterized by EMT phenotypes (163). In fact, our data confirm that αCD3-CM greatly reduces cell counts. However, the kinetic data from the xCellignce platform suggest selection of resistant cell is unlikely to account for the increase in EMT phenotype as the cell index increases within just a few hours following addition of immune-CM to the culture of SUM149 cells, far faster than stem cells would be able to repopulate a nascent niche evacuated by recently killed differentiated cells.

The combination of increased invasion and increased adhesion is observed with the xCelligence platform and suggested by the PCR data is confusing but not unprecedented. The increased cell index has also been reported in the benign prostate hyperplasia cell line BPH-1 in response to TGF-β1 (178) using this platform. The authors argue although TGF-β1 has anti-proliferative effects, the induction of EMT induces cell spreading that increase cell index.

Twist is one of the primary transcription factors responsible for driving EMT, yet we did not observe a consistent change in Twist expression in this study. However, redundancies in signaling pathways make single nodes dispensable. For example, developmental models in drosophila showed that only the complete loss of both snail and twist abrogated mesenchymal features (179) and therefore lack of induction of a

single transcription factor should not imply that the developmental program is not induced. Notably, while Twist is required to maintain the EMT program, Twist expression is dispensable in the early stages of EMT initiation. In fact, Tran et al. showed that under transient TGF-β1, Snail1 actively represses Twist1 expression, which later increases. They show that in MCF-10a cells after 2 days of TGF-β1 treatment, the time point used in the majority of experiments in this study, twist1 expression is lower than in untreated cells (180).

TGF-β can be a powerful inducer of EMT. Cell culture experiments first showed that TGF-β induced EMT in mouse mammary epithelial cells. Miettinen et al showed that TGF-β decreased epithelial markers E-cadherin, desmoplakin I and II and ZO-1 while increasing fibronectin and cytosketal rearrangements. TGF-β1, TGF-β2 and TGF-β3 are all capable of inducing EMT (181).

After binding TGF-β, TGF-β type II receptors phosphorylate the TGF-β type I receptor(182) and dominant negative forms of TGF-β type II receptor can inhibit mammary EMT *in vivo* (183). Expression of an activated form of the TGF-β type I receptors ActRIB/ALK-4 or ALK-5 can also induce EMT in mouse mammary tissue(184) while dominant negative forms of ALK-5 can inhibit EMT (185).

Binding of TGF-β induced signaling through both Smad2 and Smad3 by c-terminal phosphorylation by TGFβRI. Smad3 and Smad4 form trimmers with Smad4 before translocating to the nucleus where they regulate transcription. Smad signaling directly regulates 3 families of transcription factors related to EMT: Snail family, ZEB family and the basic helix-loop-helix (bHLH) family (e.g. Twist) (186).

Chapter 6: General Discussion

The central hypothesis of this dissertation is that **Inflammatory breast cancer is distinguished from other non-inflammatory breast cancers by unique immunological characteristics that contribute to the rapid progression of the disease.** I have presented data on inflammatory soluble factor from blood, hematology profile and functional abilities of peripheral blood T and dendritic cells from inflammatory breast cancer patients. The number of IBC patients in this study is unprecedented and could not have been accrued at any other institution. While there are a few clear differences in the average expression of certain factors, notably an increase of TGF-β in the serum and a possible decrease in serum CRP, the presence of highly inflammatory Tc17 cells and the relative increase of inflammatory cells in MIBC compared to MBC, this work has shown that there **is not a clear immune signature that distinguishes IBC from non-IBC**. However, the responses of IBC cells to immune-mediated inflammation show that inflammation can alter the aggressive tendencies of disease by invoking an epithelial to mesenchymal transition that is associated with increased invasion and migration. This induction of EMT by immune inflammatory factors is not unique to IBC as a similar response was seen in cell lines representing an array of breast cancer subtypes. Surprisingly, unique in IBC this EMT was only partial, with an unconventional upregulation of E-cadherin that is a hallmark of IBC.

This work fails to provide strong evidence in support of the central hypothesis that there is a unique IBC signature. While this work comes close to proving the hypothesis wrong, the factors studied were expertly selected and not comprehensive; therefore we cannot say that there is not a unique signature. The negative cannot be proven as there are almost always new factors that can be considered. As life as we know

it is mostly defined by genetics, whole genome sequencing would seem to capture most of the possible states as the human genome is finite, at least technically (all combinations of base pairs without considering insertions or deletions is nearly infinite at $4^{3x10\text{^}9} = 9.6 \times 10^{1806179973}$ – that's a nine followed by over 4 billion zeros). Yet, even this does not account for interactions with the environment. This merely serves to suggest that a comprehensive screen would be nearly impossible. Never the less, the data presented here do not provide a clear unique signature for inflammatory breast cancer.

The lack of an IBC-specific profile is consistent with previously described work that approached inflammatory breast cancer from tumor genetics. Gene expression and hybridization data using microdissected tumors from the Inflammatory Breast Cancer Consortium others has failed to provide a consistent gene signature within the tumor (170). Furthermore, deep sequencing suggests that common driver mutations, while present, are individually very rare, further suggesting that no single pathway has yet provided a clear target for research and therapy.

In general, the median levels of soluble inflammatory mediators were lower in IBC than in LABC. This was surprising finding as we had expected to see higher levels of inflammation in IBC. However, it is consistent with the observed high levels of the anti-inflammatory agent TGF-β1.

TGF-β1 is highly ubiquitous and pleiotropic. In terms of cancer progression, its actions can both inhibit primary tumor growth and promote metastasis by inducing EMT. Here we show that higher levels are associated with IBC. As all the patients in this study had advanced disease, it is possible that the tumor has progressed past a point where the inhibitor growth effects of TGF-β1 would be most apparent.

Furthermore, we observed lower levels of IL-8 in IBC than in non-IBC. Again, considering that IBC cell lines produce high levels of IL-8, this was not expected. Of

course, IL-8 is produced by many cell types and local production of soluble factors can be greatly diluted in circulation.

These observations of soluble factors suggest that modulation of inflammation may be related to disease progression in IBC. The idea that immune cells promote tumor growth is not new. Virchow first proposed the connection between inflammation and cancer in 1836 (review (187)). Dvorak's 1986 statement that cancer is "a wound that does not heal" (188) is almost accepted as gospel. Cancer, he argues, induces changes in the stroma similar to wound repair with changes in the extracellular matrix and increased production and secretion of growth factors.

Studying the tumor microenvironment is tricky in inflammatory breast cancer. In primary tumor samples, the amount of untreated tissue obtained from core biopsies is minuscule at best and even with ultrasound guidance often return minimal tumor. Since the majority of patients are treated with neoadjunantneoadjuvant therapy, the larger samples obtained at surgery have been heavily treated. There is currently no mouse model for inflammatory breast cancer as the molecular driver is still undefined. *In vivo* studies are limited to xenografts of human derived cell lines. Therefore, we are limited to describing the samples that are available and contriving co-culture experiments as we have done in Chapter 5. In Chapters 2-5 we have described immune paramatersparameters from blood of IBC patients. Blood represents an ideal source of minimally invasive tissue that can be samples longitudinally to observe changes over time. While the work described here is limited to baseline samples, it lays a foundation for future studies that will observe changes.

We observed normal levels of T cells in IBC patients but reduced levels in metastatic breast cancer, both with and without inflammatory features. A CD4 T-cell count of 200 or less cells/μL is severely immune deficient (this meets the definition of

acquired immune deficiency symdromesyndrome – AIDS) and often requires prophylactic treatment to prevent pneumocystis pneumonia and mycobacterium avium complex. We observed 6 patients with CD4 T-cell counts below 200 (3 MIBC and 3 MBC, all previously treated with systemic therapy) and an additional 31 patients below the "normal" limit of 500 (20 MIBC, 6 MBC, 3 IBC and 1 LABC) only 8 of whom where treatment naïve. This suggests that while treated patients maybe immune compromised, pre-treatementtreatment patients have a large number T cells that might be capable of inducing an immune response.

Therefore, we tested the ability of T cells to respond to antigen in Chapterchapter 4. As shown in Figure 4.1D on page 98, both IBC and MIBC patients have CD4+ T cells in numbers comparable to HD that are able to synthesize cytokines. This suggests a highly intriguing possibility for an immune therapy approach. It is noteworthy that the percentage of cells responding with cytotoxic, TH1 related cytokines are suppressed in MIBC (but not IBC). This suggests that an immune-based approach might be more feasible in stage-III rather than stage IV disease. In liquid tumors, it has been observed that vaccination strategies are most successful against minimal residual disease. If similar principles are involved in breast cancer, it might be best to harvest T cells from patients prior to therapy, expand them *in vitro* and return activated cells to the patient following systemic therapy and surgical de-bulking. As we also observed slight defects in dendritic cell number and ability in IBC patients (Figure 4.2 on page 105), the in vitro stimulation may be a good option of eliciting an anti-tumor immune response.

In dendritic cell responses, IBC had an increase in pDC synthesized TNF-α and mDC synthesized IL-10. pDC activity tends to promote innate immunity through recruitment and activation of macrophages with interferons. In contrast, mDC are more related to adaptive immunity, however the observed increases in IL-10 synthesis would

suggest that a cytotoxic immune response would be inhibited. Furthermore, LABC had significantly higher expression levels of costimulatory and activating cell surface proteins CD80, CD86 and CD40 than IBC. Together theses suggest that IBC has a defect in the ability of DC to polarize T-cells towards an anti-tumor TH1 response.

Immune Induction of EMT

We have shown that TNF-α, TGF-β and IL-6 can contribute to the aggressiveness of IBC by enhancing EMT. TGF-β can be hard to target clinically because of its ubiquity and pleiotropic effects (ie., this stuff is everywhere and does everything). We are proposing here that immune derived TGF-β contributes significantly to the induction of EMT within the tumor microenvironment. The Morgan Welch Inflammatory Breast Cancer Research Program and Clinic at MD Anderson has recently proposed to test BP-100.1.01 (liposomal antisense Grb-2) in IBC patients since Grb-2 is involved in the signaling cascade initiated by EGFR signaling. However, Grb-2 is also involved in T cell receptor (TCR) signaling (189). As such, administration of this drug should decrease T cell responsiveness. Hui Gao showed such an effect following treatment of CML patients with imatinib mesylate while designed to inhibit BCR-ABL tyrosine kinase signaling, also inhibits Lck signaling at the top of the T-cell receptor signaling cascade (167). Similarly, if the anti-Grb2 drug does reduce antigen specific immune responses, activated T_{Reg} cells might produce less TGF-β. It would be easy to test polyclonal T-cell responses to this drug using the methods presented in this thesis. Additionally, new TGF-β antibodies such as clone 9016 from R&D (190) not available at the initiation of this study may allow for the direct quantification of TGF-β production by T-cells. Simultaneously, EMT can be measured in circulating tumor cells (154). Following from the data presented here, we would expect that after initiation of tBP-100.1.001, there would be a blunted T-cell cytokine response including decreased TGF-β synthesis that would

correlate with decreased EMT. Inhibiting the T_{Reg} response would also have the benefit of increasing NK activity within the tumor microenvironment.

Inhibiting TGF-β production by T-cells is a dangerous proposition however. While this might decrease T_{Reg} activity, it will also decrease the TH1 response. We have shown in Chapter 4 that a strong T-cell cytokine response is related to increased survival.

The EMT data shown in Figure 5.10 on page 140 suggests that IL-6 might be a good target for preventing the maintenance of EMT. The spiking data shows that adding IL-6 alone to unconditioned media had little effect on the induction of EMT at 48 hours. However, depleting IL-6 from the conditioned media greatly reduced the EMT induction. This suggests that an anti-IL-6 therapy would not be able to reduce induction of EMT, but it might prevent EMT cells from persisting for extended periods of times. Further study would be required to determine the appropriate time frame. If on the one hand, EMT-induced cells are able to extravasate from the primary tumor, but blocking IL-6 reduced the EMT phenotype before they circulating tumor cells reach the metastatic site, an anti-IL-6 therapy might prevent metastasis by preventpreventing the tumor cellcells from invading new tissue. However, if the time period is longer, blocking IL-6 might be help the tumor cells undergo MET (mesenchymal to epithelial transition) or reverse EMT at a new metastatic site, allowing the cells to take up residence. In this case, the anti-IL-6 therapy would induce metastasis. Further experiments with the real-time cell analysis might help tease this apart, but only an in vivo model can really test this effect.

Several anti-IL-6 drugs are currently in clinical trials. It is possible to target either the soluble IL-6 ligand (such as siltuximab), the soluble IL-6 receptor required for IL-6 signaling in most non-hematopoietic cells (such as anti-IL-6 receptor antibody tocilizumab) (191), the GP130 subunit of the IL-6 receptor (192), or Jak2-Stat3 signaling

(193). Interestingly, metformin targets Stat3 in TNBC tumors, possibly offering generally well -established therapy.

Alternatively, inflammation in general can be targeted such as through the widely discredited Cox-2 enzyme, the prostaglandin (EP) receptors, or C-reactive protein. As the data we presented here suggest that a strong T-cell response is beneficial to patients, a therapeutic approach that can alter the TH1/TH2 balance in favor of TH1 would seem preferable to an approach that targets T cells non-discriminately. For example, dendritic cells exposed to prostaglandins take on a type-2 polarized effector DC polarization and induce TH2 polarization in T-cells by producing less IL-12 (194). This would suggest that a Cox-2 inhibitor, might be beneficial, if toxicities can be overcome. Long chain fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in nutraceuticals such as fish oil alter the cyclooxygenase metabolism by favoring the production of PGE_3 over PGE_2 have proven anti-inflammatory and anti-proliferative properties (195); however, they do not seem to alter the TH1/TH2 balance (196). This would suggest that while treatment with EPA (fish oil) might be beneficial in controlling tumor growth both directly and by reducing inflammatory factors, it might not be the strongest candidate for an optimal immune response. Alternatively, inflammation in general can be targeted such as through the widely discredited Cox-2 enzyme, the prostaglandin (EP) receptors, or C-reactive protein. As the data we presented here suggest that a strong T-cell response is beneficial to patients, a therapeutic approach that can alter the TH1/TH2 balance in favor of TH1 would seem preferable to an approach that targets T cells non-discriminately. For example, dendritic cells exposed to prostaglandins take on a type-2 polarized effector DC polarization and induce TH2 polarization in T-cells by producing less IL-12 (194). This would suggest that a Cox-2 inhibitor, might be beneficial, if toxicities can be overcome. Long chain fatty acids such

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Alternatively, statins (or HMG-CoA reductase inhibitors), used to control blood cholesterol levels have a strong anti-inflammatory effect as well (197). As these drugs are generally well tolerated and likely quite beneficial as IBC patients tend to be obese, statins offer a highly intriguing possible therapy.

In total, the data here suggest multiple possible therapeutic targets, however, we lack a well-validated system for testing.

Proposed Animal Model

Clearly one of the major limitations of this work is the lack of *in vivo* data. We have presented ex vivo data to show that immune cells in IBC are competent. We have performed co-culture experiments of normal donor leukocytes with IBC cell lines. But we do not know how these cells interact in the microenvironment. Presently, all IBC *in vivo* models are xenografts of human-derived cell lines in immune compromised mice. Developing a true *in vivo* model would require an understanding of the molecular basis for IBC that currently does not exist.

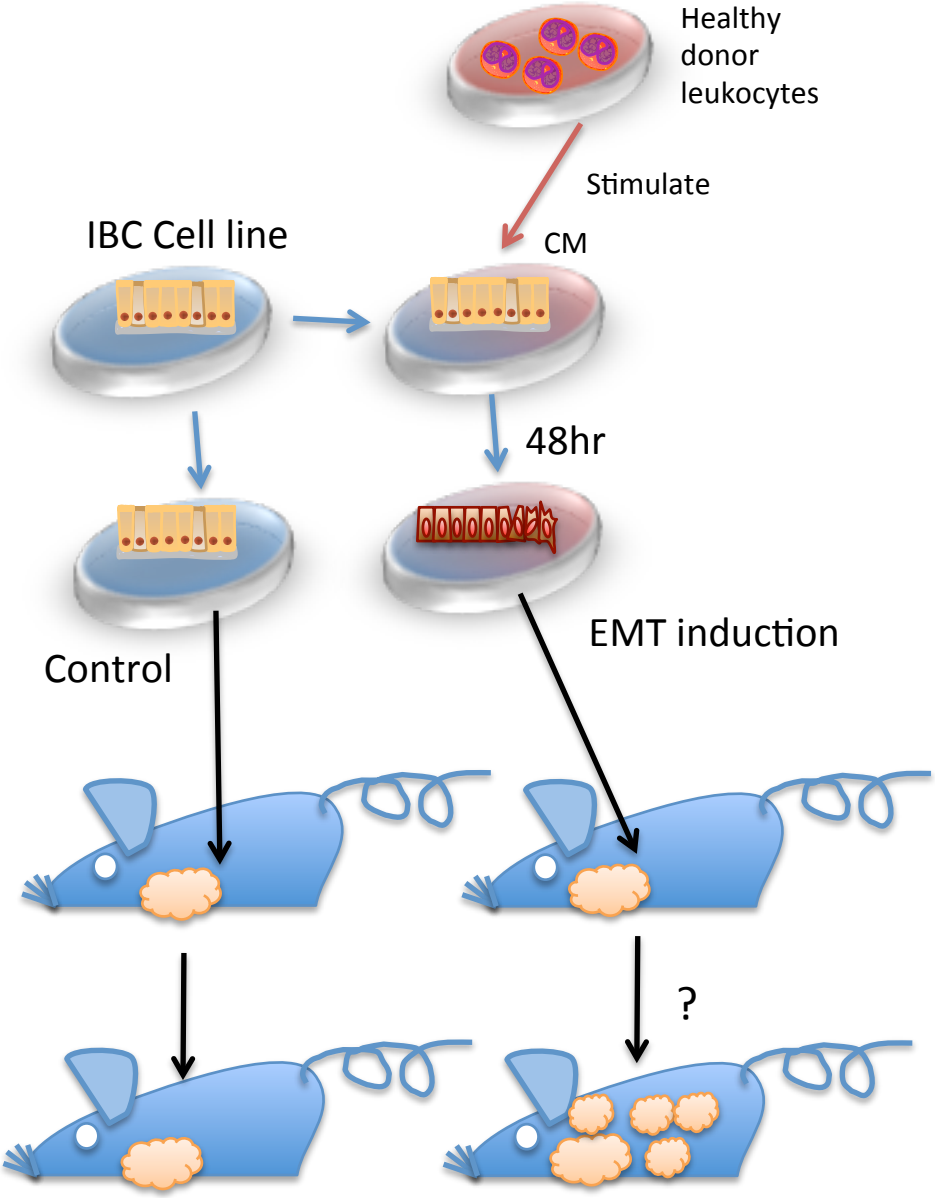
One alternative that might yield some answers would be to induce tolerance to the human cell in the mouse model. Some researchers have been able to transplant human cell lines into mice with fully intact immune systems by overexpressing CCL21 in the tumor cell. CCl21 recruits T cells to the tumor, but the majority of the cells recruited are

T_{Reg} that lead to immune tolerance. In a melanoma model, tumors with low CCL21 showed antigen specific immunity and whereas tumors expressing high levels of CCL21 induced tumor tolerance with high levels of TGF-β and even protect distant, non-CCL21 expressing tumor co-implanted at the same time, even in the non-syngeneic cell lines (198). Such a model might allow transplanting human IBC cell lines into immune competent mice. However, would this truly model IBC? Probably not! For one, it seems like the microenvironment plays a critical role, if the host is left genetically unperturbed, we might miss a critical part of the disease. The data we presented here suggest that a normal immune response is capable of inducing aggressive features in breast cells, including the non-tumorigenic cell line MCF-10a. So the question is: what dysregulation in immune activation can lead to this?

As an alternative to a genetically engineered model, combinations of *in vitro* stimulation and xenograft implantation may also get closer to providing an *in vitro* model for the interactions of immune cells and IBC tumors. For example, we have shown here that phenotypic changes are induced in IBC cells when exposed to soluble factors from immune cells. These EMT-induced cells can be transplanted into immune compromised mice once exposed to conditioning factors *in vitro* (see Illustration 6.1). As IBC cell lines are tumorigenic and metastatic, the end point would have to be the rate and number of metastases formed. These results would require a sufficiently powered experiment, as the results are unlikely to yield a clear, unambiguous answer. To help establish the timing of metastasis (particularly since the effects of the immune induced EMT are likely transitory), the primary tumor can be removed once it is established.

Illustration 6.1

Proposed in vivo model. Since no mouse model of IBC exists, tumor-immune interactions would have to be modeled in vitro and then transferred to a xenograft setting. To test weather immune-induced EMT contributes to metastasis, we propose exposing IBC cell lines to immune conditioned media in vitro prior to transplantation into the cleared mammary fat pads of immune compromised mice. The number and rate of metastases would be used as an endpoint.



Perhaps even more fundamental than a lack of an in vivo model, we currently have a very poor understanding of what leukocytes are involved within IBC tumors. This limitation is due, in part, to the very scarce pre-treatment samples available. Currently, a core biopsy is routinely taken prior to surgery. We have recently initiated a protocol in collaboration with Dr. Radvanyi to retrieve tumor-infiltrating lymphocytes from these samples to better characterize immune states as they are within the tumor. We hope this will validate the findings we saw in blood and in our *in vitro* studies.

In summary, inflammatory breast cancer is a complex and highly aggressive disease that has not yet been defined on the molecular level by examining the tumor alone. We proposed that tumor host interactions, specifically between immune cells and tumor cells could help explain some of the unique etiology of IBC. We failed to define a clear IBC signature within soluble factors from the blood or from circulating hematopoietic cells. However we noted differences between IBC and non-IBC that suggest immune regulation might play a role in the disease. We showed that like most breast cancers, IBC could be induced to a more aggressive state by soluble immune factors including IL-6, TNF-α and TGF-β as demonstrated by the induction of EMT-related factors and migration in SUM149 cells. Strikingly, we found that one of the most perplexing hallmarks of IBC, the persistence of E-cadherin expression in a highly metastatic disease, can be mediated by these immune factors. Blocking these interactions specifically with targeted therapy or by targeting inflammation in general might offer a beneficial therapeutic approach.

Chapter 7: Methods

PATIENT RECRUITMENT

All patient studies were completed at The University of Texas MD Anderson Cancer Center. For cellular immune studies, patients were recruited to lab protocol Lab08-0199 " Reactivation of Epstein - Barr virus in Patients with Breast Cancer." The serum profiling studies also included patients recruited under the Inflammatory Breast Cancer Registry, 2006-1072. All patients provided written informed consent in compliance with the World Medical Association Declaration of Helsinki. Eligibilty criteria for Lab08-0199 included women with advanced breast cancer starting a new line of therapy. Initial target enrollment called for enrollment of 30 patients from each of the following 4 diagnoses: locally advanced breast cancer (LABC), inflammatory breast cancer (IBC), metastatic breast cancer (MBC) with IBC, and metastatic breast cancer with inflammatory features (MIBC). Enrollment of patients with IBC was later extended to 200 patients. The registry protocol contains two cohorts of patients diagnosed with IBC. Cohort I is restricted to newly diagnosed patients. By design, patients should be untreated at the time of sample collection, however discrepancies in the timing of enrollment, treatment initiation and sample collection account for several samples collected after treatment. Cohort II includes patients with a diagnosis of IBC that have already initiated treatment. These are usually patients that were diagnosed in the community and referred to MD Anderson.

Clinical correlates were collected by chart review from the MD Anderson electronic medical record system ClinicStation. Pathology results and lab tests were entered into the database by the author or other lab members, but tumor staging and survival times were all evaluated and entered into the database by clinicians with a medical doctorate.

Clinical staging was based on AJCC criteria (9). The tumor markers estrogen receptor (ER) and progesterone receptor (PR) were evaluated by immunohistochemistry (IHC) of fine needle aspirates or surgical excisions of the primary tumor, as reported by a board certified pathologist. Tumors were scored as positive if 5% of the cells were positive for the marker. Her2 was evaluated by immunohistochemistry and fluorescent in-situ hybridization (FISH) when available. Tumors were considered Her2 positive if they were scored 3+ on IHC or had a Her2/CEP 17 ratio >2 by FISH. Amplification by FISH was considered the gold standard such that if there were discrepant scores by FISH and IHC, the FISH categorization was used for classification (ie., an IHC 3+, FISH- tumor was classified as Her2 negative). The proliferation marker Ki67, which is a good surrogate marker for discriminating between Luminal A and Luminal B intrinsic subtypes is rarely requested at MD Anderson and was not included in the analysis.

SAMPLE COLLECTION

All samples were collected at University of Texas MD Anderson Cancer Center and processed in the lab of James M. Reuben, PhD. Peripheral blood samples were collected by venipuncture into evacuated tubes. Samples collected under Lab08-0199 included a single 10 mL red-top tube containing no anti-coagulants for serum analyses and six 10 mL green-top tubes with heparin anti-coagulant for cellular analyses. Samples collected under 2006-1072 included one 10 mL red-top serum tube and two 10 mL purple top tubes with EDTA anti-coagulant. Lab08-0199 samples were processed within 24-hours of drawing.

FREEZE SERUM AND PLASMA

Plasma was collected from green heparinzed tubes and serum from red-top tubes without anti-coagulant. Tubes were centrifuged for 20 minutes at 1200 g. Serum was harvested from above the clot without disturbing the pellet and plasma was harvested above the buffy coat leaving at least a few millimeters of plasma to prevent collecting cells. Samples were aliquoted into three 1-mL tubes with silicon O-rings to minimize loss to evaporation. Aliquots were frozen temporarily at -20˚C until the storage box is filled and transferred to -80˚C for long-term storage.

FREEZE PBMC

Anti-coagulated blood (heparin for most studies) was diluted with at least an equal volume of phosphate buffered saline (PBS) up to a total volume of 30-35 mL. The diluted blood was slowly layered over room temperature Ficoll-Paque Plus (GE Healthcare Life Sciences, Pittsburg, PA). This solution consists of a mixture of the hydrophilic polysaccharide Ficoll PM400 and sodium diatrizoate. This produces a solution with a density of 1.078 g/mL, intermediate to the density of a red blood cell (1.096 g/mL) or polymorphonuclear leukocyte (1.06 – 1.10) and the density of a mononuclear leukocyte. After a 30-minute centrifugation at 400g with the break off, peripheral blood mononuclear cells (PBMC) were harvested from the interface between the Ficoll and the diluted plasma. The centrifuge break must remain off to prevent vibrations from disturbing the phase interfaces. PBMC consist of lymphocytes and monocytes although typically had a small erythrocytic and granulocytic contamination was present, particularly in patient samples. PBMC were washed two times by adding PBS to the cells and followed by a 10-minute centrifugation at 300g at room temperature to pellet the cells. To freeze the cells, the cell pellet was resuspended in 1-mL of freezing buffer consisting of 10% dimethyl solfoxide (DMSO) and 90% heat inactivated, certified

low endotoxin fetal bovine serum (FBS, Life Technologies, Carlsbad, CA). As endotoxin activates PBMC through the TLR-4, it is critical that only high-quality FBS is used. Cell aliquots were positioned in a "Mr. Frosty" cryogenic freezing container and placed into a -80˚C freezer overnight. The isopropanol in the freezer container maintains a 1˚C/min freezing rate which prevents cell rupture. After equilibrating to the -80˚C temperature, cell aliquots were transferred to a liquid nitrogen freezer for long-term preservation.

THAWING CELLS

To thaw cryopreserved cells, an aliquot was removed from the liquid nitrogen tank and rapidly thawed by placing the frozen tube in a 37˚C water bath with gentle agitation for approximately 1 minute. Once the cells had thawed, the mixture was transferred to a polypropylene conical tube. Complete media consisting of RPMI supplemented with10% FBS and antibiotic was added drop-wise to restore physiologic osmolality. The cells were washed twice with complete media by centrifugation at 400g for 10 minutes, counted by trypan blue exclusion assay using a hemocytometer with an improved Neubauer grid and resuspended in complete media at 1 x 10^6 cells per mL.

CELL CULTURE

All cells were cultured in tissue-culture treated plastic vessels and incubated in a 5% CO_2 atmosphere at 37˚C. PBMC were incubated in RPMI 1640 (Corning, Manassas, VA) supplemented with 10% low endotoxin certified FBS and antibiotic (penicillin/streptomycin) or antibiotic/antimycotic (penicillin/streptomycin/amphotericin B; Life Technologies, Carlsbad, CA).

IBC cell lines used in this study include: SUM149PT, SUM190PT, KPL-4, and IBC-3. SUM149PT cells, SUM190PT and the non-IBC cell line SUM159PT (abbreviated

to SUM149, SUM190, and SUM159PT respectively throughout this dissertation) were obtained courtesy of Dr. Stephen Ethier (Kramanos Institute, MI, USA) and are commercially available (Asterand, Detroit, MI). Cells were cultured in Ham's F-12 media supplemented with 10% FBS, 1 μg/mL hydrocortisone, 5 μg/mL insulin and antibiotic-antimycotic (referred to as "IBC media").

IBC-3 cells were provided courtesy of Dr. Wendy A. Woodward (36) (MD Anderson Cancer Center, Houston, TX) and cultured in Ham's F12 with 10% FBS and 5 μg/mL Insulin/L with 100 μg/L Hydrocortisone and antibiotic-antimycotic. KPL4 was kindly provided by Dr. Junichi Kurebayashi (Kawasaki Medical School, Japan) and was maintained in DMEM/F12 medium supplemented with 10% FBS and antibiotic-antimycotic.

MDA-MB 231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM/F12 50/50 culture media supplemented with 10% Gibco® certified FBS (Life Technilogies™ , Grand Island, NY).

The normal mammary epithelial cell line MCF-10a was cultured in DMEM/F12 supplemented with 5% horse serum (not FBS), 20 ng/mL EGF, 100 ng/mL cholera toxin 0.01 mg/mL insulin, and 500 ng/mL hydrocortisone. Cholera toxin increases intracellular levels of cAMP and increases growth in epithelial cells and helps the growth of normal human mammary epithelial cells but has heterogeneous effects on tumor cell growth which typically have elevated cAMP (199).

Cell line culture conditions are summarized in Table 7.1 Cell Lines.

Table 7.1 Cell Lines

Breast cancer cell line culture conditions are listed. SUM149, SUM190, KPL4 and IBC-3 are the IBC model cell lines. SUM149 is TNBC while the other IBC cell lines are Her2+. Additionally, SKBR3 was used as a Her2+ cell line. MDA-MB-231 was used as the prototypical mesenchymal breast cancer cell line. SUM159 was used as a claudin-low cell line. MCF-7 and T47D were used as luminal, HR+ cell lines. MDA-453 was used as a TNBC, androgen receptor positive cell line. MCF-10a was used as a normal, non-tumorigenic cell line.

Cell Line	Source	Culture Media	Type	Type	ER	PR	Her2	EGFR
IBC-3	Wendy Woodward (MD Anderson)	Ham's F12 5% FBS HI	IBC	Luminal B	N	N	P	N
SUM149PT	Stephen Ethier (Asterand, Detroit, MI)	Ham's F12 5% FBS HI	IBC, TNBC	Basal-like	N	N	N	P
SUM159PT	Stephen Ethier (Asterand, Detroit, MI)	Ham's F12 5% FBS HI	TNBC	Claudin-low	N	N	P	P
SUM190PT	Stephen Ethier (Asterand, Detroit, MI)	Ham's F12 5% FBS HI	IBC	Luminal B	N	N	P	P
KPL-4	Dr. Junichi Kurebayashi (Kawasaki Medical School, Japan)	DMEM/F12 10% FBS	IBC	Luminal B	N	N	P	P
MCF-10a	ATCC	DMEM/F12 , Cholera Toxin 5% Horse Serum, EHI	non-tumorigenic	Basal-like	N	N	N	P
MCF-7	ATCC	DMEM/F12 10% FBS	epithelial	Luminal A	P	P	N	N
MDA-MB-231	ATCC	DMEM/F12 10% FBS	mesenchymal	Claudin-low	N	N	P	N
MDA-MB-453	ATCC	DMEM/F12 10% FBS	androgen+	Unclass.	N	N	P	N
SK-BR-3	ATCC	DMEM/F12 10% FBS	Her2+	Her2	N	N	P	P
T47D	ATCC	RPMI 10% FBS	epithelial	Luminal A	P	P	P	P

HI – Hydrocortisone 100μg/mL, Insulin 5μg/mL
EHI – EGF 20ng/mL, Hydrocortisone 100μg/mL, Insulin 5μg/mL
FBS – Fetal Bovine Serum

CM Preparation

Immune conditioned media as described in Chapter 5 was prepared from healthy donor PBMC. Fresh peripheral blood was collected from healthy volunteers by venipuncture into heparinized Vacutainer collection tubes (BD, Frankiln Lakes, NJ). The mononuclear cell fraction (PBMC) was collected by density gradient centrifugation over Ficoll-Paque media (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). PBMC were cultured at an initial density of 1 x 10^6 cell per mL in RPMI 1640 supplemented with 10% Gibco® certified fetal bovine serum (FBS, Life Technilogies™ , Grand Island, NY) plus antibiotic-antimycotic. To activate the PBMC, cells were stimulated with plate-bound anti-CD3 (coated at 8 μg/mL) plus soluble anti-CD28 antibodies (200), 10 μg/mL lipopolysaccharide (LPS), or left unstimulated. Following 48 hours of culture, conditioned media were collected and centrifuged at 400 g to pellet cells and debris. The supernatant was clarified with a 0.22 μm pore filter and frozen in multiple aliquots at -80˚C. Prior to culturing with breast cancer cell lines, the conditioned media was diluted 1:4 with IBC media (note – migration assays used serum-free IBC media). For cell lines other than SUM149, the appropriate media was used as noted in the "Cell Culture" section above. TGF-β1 (R&D Systems, Minneapolis, MN) was used at 2 ng/mL in the appropriate media as a positive control for EMT induction.

Flow Cytometry Basics

Multi-Color Flow Cytometry

Most flow cytometry acquisitions were performed on a BD LSR II (Becton, Dickinson and Company, San Jose, CA) equipped with two lasers and 6-color, 8-

parameter capability. The red laser is a 635-nm 20 mW HeNe laser supporting 2 fluorescent channels in a trigon filter set. The blue laser is a 488 nm 20 mW Coherent Saphire air-cooled argon laser providing the side-scatter channel (SSC) and 4 fluorescent channels plus the side scatter channel arranged in an octagon format. Data was acquired in BD FACS Diva version 6.1.1. Data was analyzed in FlowJo data analysis software for flow cytometry (TreeStar, Inc, Ashland, OR) on a PowerMac computer running Mac OS X. Both the software and the computer were upgraded over the course of the studies. FlowJo versions included release 8.7.1 through 9.6.1. All data was reanalyzed and confirmed after the transition from version 8 to 9.

4-Color ICC

In addition some 4-color experiments, most notably the intracellular cytokine syntheses from the TCR-stimulated PBMC in the Lab08-0199 protocol, were acquired using a BD FACS Calibur flow cytometer equipped with a 488 nm air-cooled blue argon laser supporting 3 fluorescent channels plus forward channel light scatter (FSC) and SSC and a 635 nm red diode laser for a the single APC channel. The Calibur flow cytometer is driven by a PowerMac G4 running OS 9.1.0 and CellQuest 3.3. This 4-color data was analyzed in Cell Quest Pro version 6 on a PowerMac running Mac OS X. The host analysis computer was upgraded over the course of the studies. The current machine is configured with a 2.8 GHz quad-core Intel xenon processor with 16 GB of RAM, a 1 TB system hard drive, a 1.5 TB drive for data, and a 2 TB back-up drive that runs hourly back-ups via Apple Time Machine.

FRESH PHENOTYPES

Fresh whole blood from collected in hepainized collection tubes was processed within 24 hours of collection. Aliquots of 200μL were reacted with fluorophore-conjugated monoclonal antibodies in panels including up to 6 colors (BD Bioscience, San Jose, CA). Reactions were incubated in the dark at room temperature for 30 minutes. Subsequently, the red blood cells were lysed with BD FACS/Lyse for 10 minutes at room temperature and the washed twice by centrifugation using a Helmer UltraCW cell washer (Helmer Scientific, Noblesville, IN) in PBS. Samples were resuspended in PBS prior to acquisition. Cytometric data was acquired using the BD LSR II. Invitrogen CountBright beads were added to the dendritic cell tube to enumerate absolute counts of dendritic cells.

DC FUNCTIONAL TESTS

Fresh whole blood from collected in heparinized collection tubes was processed within 4 hours of collection, as pDC in particular can be difficult to find in older samples. Aliquots 4 aliquots of 1mL were reacted placed in polypropylene 15 mL conical tubes. Two tubes were used for overnight stimulations for upregualtion of co-stimulatory molecules and activation markers with either TLR7 and TLR8 agonists TLR075 and TLR097 (Invivogen, San Diego, CA) or left unstimulated. CL075 activates TLR8 and TLR7 to a lesser extent. CL097 is a water-soluble derivative of the imidazoquinoline compound R848 and stimulates TLR 7 and 8. The other two tubes were similarly reacted with the TLR agonists or left unstimulated for 4 hours for quantification of cytokine synthesis, however, if the samples were received after noon, the samples were left at room temperature and stimulated the following morning. Brefeldin A was added the final 3 hours to block Golgi transport and maintain de novo synthesized cytokines in the cell. Following stimulation, samples were fixed and red blood cells were lysed with 10 mL of

BD FACS/Lyse and washed 2 times with PBS. Aliquots of 200 μL were reacted with fluorophore-conjugated monoclonal antibodies in panels including up to 6 colors (BD Bioscience, San Jose, CA). Reactions were incubated in the dark at room temperature for 30 minutes. Subsequently, samples were washed twice by centrifugation in PBS. Samples were resuspended in PBS prior to acquisition. Cytometric data was acquired using the BD LSR II.

SERUM CYTOKINE ANALYSIS (LUMINEX)

Serum samples were collected as described above from MD Anderson protocols Lab08-0199 and 2006-1072. Samples were analyzed in batch using Milliplex bead kits (EMD Millipore Corporation, Billerica, MA) according to the manufacture protocol with overnight incubations and an additional point added at the low end of the standard curve and acquired using a Luminex LX100 (Luminex Corporation, Austin, TX) running BioPlex control and analysis software Version 5.0, (BioRad, Hercules, CA).

T-CELL STIMULATION THROUGH T-CELL RECEPTOR (ANTI-CD3 STIMULATION)

To interrogate T-cell function, T-cells were activated polyclonally through the T-cell receptor. Anti-CD3 antibody immobilized to plastic can cross-link T-cell receptors on T-cells establishing a localized cluster of receptors (supramolecular activation cluster or SMAC) reminiscent of an immunological synapse. This facilitates phosphorylation of immunoreceptor tyrosine-based activation motifs. Soluble anti-CD28 antibody is added as a "signal 2" to mimic co-receptor binding. In these experiments, anti-CD3 antibody (Beckman Coulter, clone UCHT1) was added to tissue-culture 6-well plates at 8 μg/mL in 625 μL per well. The tyrosine residues on the antibody react with the benzene rings in the polystyrene to facilitate binding at alkaline pH, but binding is sufficient in PBS at

physiological pH with a 6-hour incubation at 37˚C and in a 5% CO_2 humidified atmosphere. Prior to freezing, the pre-coated plates were air-dried in a laminar flow hood overnight. To stimulate T-cells, 50 μL of 150 μg/mL anti-CD28 antibody (BD340975, clone L293) was added to a thawed plate pre-coated with anti-CD3 antibody. Five (5) mL of PBMC were added to each well at 1-2x10^6 per mL in complete RPMI for a total of 5-10 x 10^6 PBMC per well.

For intracellular cytokine synthesis assays, cells were stimulated overnight at 37˚C in a 5% CO_2 humidified atmosphere. Next, cells were removed by gentle scrapping utilizing a 1-mL pipette tip. Cells were fixed for 10 minutes with BD FACS Lyse/Fix (a solution that contains <1.5% formaldehyde and <5.0% diethyene gycol at working concentrations).

For experiments utilizing conditioned media, cells were stimulated for 2 days at 1x10^6 cells/mL. After culture, media was aspirated and filtered with a 0.22 micron syringe filter to excluded cellular factors and contaminants.

mRNA

Trizol / Chloroform Extraction

Following treatments, samples were frozen in 700 μL Qialzol lysis reagent (Qiagen Germantown, MD) at -80˚C and processed in bulk. Total RNA was extracted with chloroform and processed using the Qiagen miRNeasy Mini Kit on a Qiacube automation unit according to manufacture protocol (Qiagen, Valencia, CA) to standardize the RNA isolation procedure. All RNA preparation and handling steps took place in a laminar flow hood, under RNase-free conditions. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE).

Typically 10 μg of RNA was recovered (range 0.1μg – 62μg). cDNA was synthesized by reverse transcription of 1μg of RNA and random primers using the high-capacity cDNA reverse transcription kit (Life Technologies, Foster City, CA).

2-Step RT-PCR

GENE EXPRESSION ANALYSIS BY QUANTITATIVE POLYMERASE CHAIN REACTION

Gene expression was quantified with real-time reverse transcription-polymerase chain reaction (RT-PCR) using TaqMan® hydrolysis probe gene expression assays (Life Technologies) using a 7900HT Fast Real-Time PCR System (Life Technologies), following the manufacturer's instructions. Universal thermo cycling conditions were used. Assay setup was performed on a Qiagility liquid handler (Qiagen) to standardize pipetting. The amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize gene expression levels, and the relative expression of each gene was calculated using the equation $2^{-\Delta Ct}$, where ΔCt = mean $Ct_{gene} - Ct_{GAPDH}$, or $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{gene} - Ct_{GAPDH})$ conditioned media – $(Ct_{gene} - Ct_{GAPDH})$ control media. TaqMan® assays were purchased from ABI: GAPDH: Hs02758991_g1, CDH1 (E-cadherin): Hs01023894_m1, CDH2 (N-cadherin): HS00983056_m1, EPCAM: Hs00158980_m1, FN1 (fibronectin): Hs00365052_m1, KRT19: Hs00761767_s1, SNAI1 (SNAIL1): Hs00195591_m1, SNAI2 (Slug): HS00161904_m1, TGM2 (TG2): Hs00190278_m1, TWIST1: Hs00361186_m1, VIM (Vimentin): Hs00185584_m1, and ZEB1: Hs00232783_m1. Data were analyzed with the use of the 7900 Fast System SDS software, Version 2.4 Standalone. All experiments were performed in triplicate.

Fluidigm RT-PCR

To analyze the EMT profile of multiple cell-lines, a high throughput PCR technology was employed. Multiplex qRT-PCR was performed using Fluidgm® integrated fluidic circuits. This system consists of microfluidic chips that mix each sample with each assay in a 1:1 relationship and an qRT-PCR thermocycler and data acquisition platform know as the BioMark HD. The chips utilized for these experiments were 48.48 Dynamic Array™ chips containing 48 assay wells and 48 sample wells. The microfluidics distribute each 5μL sample mixture containing cDNA and mastermix between 48 TaqMan® hydrolysis probe assays. This produces an array of 2,304 wells with a 35 nl reaction volume. This automated system increases consistency and saves both hands-on pipetting time, sample volume, and reagent volume. However, to ensure equal distribution into each of the very small 35nl wells, a pre-amplification step must be performed prior to arraying the samples. Without the pre-amplification, rare cDNA will be subjected to a binomial distribution amongst the wells, where some wells will start with a single copy of the cDNA and others will have 0 or 2 (note – typically the single cDNA will reach threshold at about cycle 30, thus any data with a CT value around 30 or higher is of little analytic value with a small number of replicate wells).

Prior to quantitative real-time PCR (qRT-PCR) analysis, 2.5 μL of cDNA was subjected to 14 cycles of multiplex sequence specific amplification using TaqMan® PreAmp Master Mix and TaqMan® Gene Expression Assays. The product was diluted 1:5 in TE. The cycling program was 95˚C for 10 minutes followed by 14 cycles of 95˚C for 15 seconds and 60˚C for 4 minutes. qRT-PCR was performed using the Fluidigm® 48.48 dynamic array integrated fluidic circuit and BioMark HD® analysis system using the GE 48x48 Standard v1 thermal protocol. Samples were run in duplicate. TaqMan® hydrolysis probe assays used for pre-amp and qRT-PCR. Data were analyzed with the

BioMark Real-Time PCR Analysis Software Version 2.0 (Fluidigm). Expression changes were calculated using the ΔCt method using beta-2-microglobin as the endogenous control. For each cell line, the media control was used as the normalizer.

CELL BOCK AND IHC

Cells from each of the cell lines were made into cell blocks for immunohistochemical (IHC) staining. Control cells (MCF-7 and MDA-MB-231) were grown in the appropriate media as noted above and SUM149 cells were exposed to CM for 48 hours. Cells were harvested following trypisnization and washed twice in PBS. $5x10^5$ cells were cytospun unto poly-lysine coated slides, air-dried for overnight and then fixed in (%?) ethanol prior to Diff-Quick staining. Additionally, $2x10^7$ cells for each cell line were fixed in 10% formalin for 16 hours, pelleted by centrifugation and embedded in paraffin, as previously described (201). Cell blocks were sectioned and used for IHC staining.

MAMMOSPHERE

SUM149 cells cultured in monolayer were exposed to CM for 48 hours as above, trypsonized, washed twice with PBS to remove serum and resuspended in serum-free media containing FGF-b, and EGF "mammosphere media", as previously described (161, 202) and cultured for 6 days prior to counting. Conditioned media was not added to the mammosphere culture.

xCELLIGENCE REAL-TIME CELL ANALYSIS

To test the changes in migratory and invasive capacity of SUM149 IBC cells, cell migration was quantified with the xCelligence Real Time Cell Analyzer Cell Invasion Migration (CIM)-plate 16 platform (Acea Biosciences, San Diego, CA) using FBS as a chemoattractant for SUM149 cells. The CIM plate is essentially a Boyden chamber that measures increasing impedance on the lower surface of the semi-permeable membrane as cells migrate into the lower chamber. To ensure that chemoattractant factors in the CM did not affect migration, 25% CM was placed in both the upper and lower chambers. The CM was diluted with serum-free IBC media in the upper chamber while the lower chamber used IBC media supplemented with 10% FBS. Serum free media in the lower chamber was used as a negative migration control. Cells were suspended in the appropriate media and added to the upper chamber as per manufacture protocol. Impedance was measured on the receiving surface in the lower chamber as a measure of cell migration and normalized to a Cell Index with RTCA software version 1.2.0.0909 (Roche Applied Science Indianapolis, IN, under license from Acea Biosciences, Inc., San Diego, CA).

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