Microfluidic Model Systems to Evaluate Endothelial Cell Phenotype in Disease

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Abstract

Macrophages are known contributors to cancer progression in the primary site, but significantly less is known about their roles in secondary tumor formation. They are likely involved in tumor cell adhesion and transmigration of endothelial cells in the secondary site since macrophages are integral conductors of immune cell recruitment during the immune response and experimental evidence indicates that macrophages recruited to the secondary site before the arrival of tumor cells. However, current model systems are limited in their ability to observe and quantify these early interactions. We have developed a tunable, microfluidic model system consisting of an endotheilalized vessel channel perfusing a 3D collagen matrix that can be seeded with macrophages to recapitulate the basic structure of the extravasation site. We will use this model to quantify human macrophage-endothelial cell interactions in the context of metastasis by measuring permeability, tumor cell adhesion, and tumor cell transmigration. By treating devices with tumor cell conditioned media before the addition of tumor cells, we will quantify how macrophages change tumor cells extravasation phenotypes. We will also use M1 and M2 polarizing molecules to determine if macrophage polarization can create tumor-inhibitory or tumor-promoting responses. Since tumor cell adhesion and extravasation may depend on leukocyte adhesion mechanisms, we also developed a microfluidic model that can measure changes in leukocyte adhesion and velocity to endothelial cells in healthy and thrombotic states. We demonstrate that this model system may be used with endothelial and blood cells from a single patient, highlighting its utility in personalized medicine. Additionally, this model can be used to evaluate tumor cell-blood interactions and how these influence initial tumor cell adhesion in extravasation. As both these systems can probe and quantify early extravasation events,

they can be used in tandem to gain a better understanding of the mechanisms driving tumor cell extravasation.

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Chapter 1: Motivation for studying macrophage-endothelial cell interactions in the context of extravasation

Metastasis is the dissemination of cancer cells from the primary tumor to a secondary site. Once cancer metastasizes, it is extraordinarily difficult to treat due to the inability to locate and control the spread of disease. For example, the 5-year survival rate for nonmetastatic, stage 0 or 1 breast cancer is 100%, decreases to 72% at stage 3, and drops dramatically to 22% once distant metastases—such as lung liver, and brain—have formed.[1] Although these tumors likely metastasize before diagnosis,[2] it is important to understand why they metastasize to prevent formation of additional metastases.

The “seed and soil hypothesis” suggests that secondary tumor formation occurs where the microenvironment is well-suited for tumor growth.[3] Breast cancer generally metastasizes to the brain, bone, liver, or lung,[3-7] and it is thought that some biological or chemical factors at these secondary sites makes them ideal “soil” for breast cancer metastases.

Organotropic metastases are associated with increased endothelial surface receptor expression in the secondary site.[5,8-13] However, this change in endothelial phenotype is not necessarily a direct result of these signals, but a secondary effect of resident cell stimulation by primary tumor signals.[4,8,9,14-16] Macrophages are phenotypically diverse cells that regulate many homeostatic functions such as regulating tissue function, clearing pathogens and debris, and regulating the immune response. Macrophages are highly sensitive to environmental cues and stimulate endothelial activation in response to these external stimuli during the immune response.[17] This interaction with endothelial cells allows macrophages to regulate the recruitment of leukocytes to sites of inflammation via expression of proteins such as P-selectin, E-selectin, vascular cell adhesion receptor

(VCAM-1), intercellular adhesion receptor (ICAM-1), and von Willebrand factor (VWF).[18,19] Since tumor cells are thought to parasitize these leukocyte adhesion and extravasation mechanisms, we hypothesize that organotropic endothelial activation by primary tumor signals is mediated by macrophages in the secondary site.

While a few macrophage-targeted therapeutics for cancer have been successful in pre-clinical trials and have entered clinical trials, they have not yet been successful.[20-25] The lack of macrophage-targeted treatments translating from pre-clinical to clinical trials indicates that there is a knowledge gap in how human macrophages interact with tumor cells in the secondary site.[26] Bridging this gap requires the development of 3D *in vitro* model systems that recapitulate the secondary tumor site with human cells.

From what is known of macrophage influence on cancer cell extravasation, it is important to incorporate and understand the interactions among macrophages, cancer cells, and endothelial cells. Since endothelial function is highly regulated by shear stresses imposed by blood flow,[27-31] it is important to develop model systems that impose shear on the endothelium. Understanding these factors has led to the development of several *in vitro* endothelialized microfluidic systems to study tumor cell extravasation.[28,32-34] One microfluidic model system found that human macrophages in the tissue alone had no effect on tumor cell extravasation;[31] however, systemic tumor signals have been implicated with aiding cancer cell extravasation to specific regions, and these factors may influence macrophage interactions with tumor cells in the metastatic site.[4,8,9,14,15,35-37] Since we hypothesize that these systemic tumor signals polarize macrophages to promote a pro-extravasation phenotype in endothelial cells, we aim to develop a microfluidic model system that accounts for these important physical characteristics of the metastatic site to investigate these mechanisms.

Chapter 2: Macrophages influence endothelial cell phenotype, altering cancer cell extravasation

This chapter contains material from an article previously published in *Trends in Cancer*, and is reproduced with permission. Doak GR, Schwertfeger KL, Wood DK. Distant Relations: Macrophage Functions in the Metastatic Niche.[8]

Introduction

While there are several cells that likely contribute to tumor cell metastasis, we hypothesize that macrophages in the secondary site contribute to generating an ideal environment for tumor cell extravasation and secondary growth. There is now a wealth of research demonstrating the importance of macrophage populations within the primary tumor to promote metastatic progression. These tumor associated macrophages (TAMs) act via multiple mechanisms to promote tumor growth, inhibit tumoricidal immune response, initiate angiogenesis, and activate matrix remodeling.[38-45] A less studied but growing area of research is to understand how macrophages at the metastatic site contribute to the later stages of metastasis including secondary site arrest, extravasation, and early colonization. Recent work suggests that metastasis associated macrophages (MAMs), which include both tissue resident macrophages and bone marrow derived macrophages (BMDMs), promote metastasis in the secondary site. Depletion of macrophages at any stage of metastatic progression decreases the metastatic potential of disseminated cancer cells, indicating that interactions with MAMs influence processes such as cancer cell adhesion to the vasculature, transmigration of the endothelium, and colonization of secondary tumors.[37,46-48] In this review, we will discuss the various functions of macrophages in establishing distant metastases including formation of the pre-

metastatic niche, extravasation of circulating cancer cells, and colonization of secondary metastases. A more thorough understanding of these MAMs and their associated mechanisms of metastatic progression may lead to novel therapeutic intervention to prevent further metastatic development and tumor re-seeding.

Macrophage phenotypes and activation influence cancer progression.

The mechanisms underlying macrophage recruitment and activation during the immune response provide insights into the potential contributions of macrophages in the tumor microenvironment. Macrophages represent a diverse population of immune cells that are generally classified as tissue resident macrophages or BMDMs (Table 1). Resident macrophages are derived from yolk sac or fetal liver progenitors to aid in embryonic development and ultimately differentiate into several subtypes to perform tissue-specific functions, phagocytose wastes and foreign bodies, and initiate the immune response.[49] BMDMs are typically recruited to tissues as myeloid-derived cells, which fully differentiate within the tissue and can be polarized in response to signals sensed within the tissue. Traditional macrophage polarization is classified as classical “M1” activation or alternative “M2” activation. Typically, M1 polarization is thought to promote pro-inflammatory, tissue destructive functions and M2 promotes anti-inflammatory, tissue repair functions. Although these definitions imply that M1 and M2 macrophages are distinct entities that perform specialized functions, this classification system is now considered an oversimplification since specific phenotypes are distributed along a continuum to allow for a smooth transition between pro-inflammatory, anti-inflammatory/recovery, and homeostatic states.[48,50-52]

However, in many diseased states the balance of polarization is skewed towards chronic inflammation (M1-like) or tissue repair (M2-like). In tumors, cancer cells generally promote the maintenance of a more M2 macrophage polarized state to aid in evading the

immune response, promoting angiogenesis, releasing pro-tumoral growth factors, and remodeling the local tissue; however, several M1-associated cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 may be pro- or anti-tumorigenic depending on the tumor type and the environment.[53,54] In fact, many macrophage subpopulations may contribute to tumor progression and metastasis. While tumor associated macrophages are thought to primarily consist of BMDMs, it is often difficult to distinguish resident macrophages from tumor associated macrophages.[44] A recent mass cytometry-based study of macrophage populations isolated from renal cell carcinomas identified up to 17 distinct macrophage populations,[55] further highlighting the need to better understand how phenotypic diversity in macrophages influences cancer progression. Since it is likely that a diverse array of macrophage populations at the metastatic site perform a range of functions to facilitate metastatic colonization, it is important to clearly characterize and not over-generalize macrophage phenotypes and functions to understand how they influence metastasis.[52]

Primary tumor-derived factors influence seeding and regulation of macrophages in the metastatic site

Recent studies of metastatic homing to specific organs highlight the diverse roles of resident macrophages and the recruitment of BMDMs in aiding the formation of distinct pre-metastatic niches (Figure 2.1). Once localized to the metastatic site, BMDMs create an inflammatory environment where tumor cells colonize, recruit additional BMDMs, and exploit BMDM pro-tumoral functions while the tumor persists (Figure 2.2). Some metastatic models have revealed that systemic soluble and exosomal signaling from the primary tumor induces resident cells in an organotropic manner to recruit BMDMs before the arrival of cancer cells (Table 2). Understanding how BMDMs are initially recruited to the pre-metastatic niche may help prevent the initiation of this malignant cycle.

Primary tumor secretome impacts bone marrow derived cell recruitment and function in the secondary site.

The ability of cancer cells to localize to specific organs is potentially dependent on the ability of the primary tumor to establish bone marrow derived cell clusters—containing progenitors of BMDMs along with other bone marrow-derived cell types—in the pre-metastatic niche.[4,9,14] For example, systemic signaling produced by intradermally injected Lewis lung carcinoma (LLC) tumors or LLC conditioned media promotes fibronectin expression in the lungs and liver, which are where intradermally injected LLC tumors generally metastasize. Bone marrow derived cells expressing the fibronectin receptor very late antigen (VLA)-4 localize to these fibronectin-rich regions where they promote neovascularization and metastasis in a vascular endothelial growth factor (VEGF)-1 dependent manner. However, in the absence of primary tumor signals bone marrow-derived cell clusters failed to form, resulting in significantly fewer metastases. Similarly, primary tumor signals from highly metastatic B16 melanomas in the same model initiate pre-metastatic bone marrow derived cluster localization to the lungs, liver, testes, spleen, and kidneys, which are consistent with metastatic burden of B16 tumors. Interestingly, LLC cells injected through the tail vein of the B16-educated mouse model seeded in all traditional B16 affected organs rather than just the lungs and liver, which are the normal sites for LLC seeding.[14] These data highlight the importance of soluble factors derived from the primary tumor in establishing the pre-metastatic niche and contributing to organotropic metastases.

Recent studies have elucidated specific factors within the primary tumor secretome that contribute to initiation of the lung pre-metastatic niche.[4,9] In LLC and B16 tumor secretomes, VEGF-A, TNF-α, and transforming growth factor (TGF)-β induce lung endothelial cell secretion of monocyte chemoattractants S100A8 and S100A9.[9] In addition

to directly recruiting bone marrow derived cells, S100A9 may also increase their retention in fibronectin-rich regions by activating β2 integrins—including CD11b—which increases leukocyte affinity to fibronectin in vitro.[56,57] Activation of the endothelium via VEGF-a, TNF-α, TGF-β, S100A8, and S100A9 is associated with increased endothelial permeability, which enhances extravasation of recruited monocytes and cancer cells *in vivo*.[58-62] In an orthotypically injected MDA-MB-231 mammary tumor model, primary tumor cell-derived lysyl oxidase (LOX) localized to fibronectin-rich tissues in the lung where it cross-linked collagen IV, resulting in increased affinity to CD11b+/VEGFR1+ monocytes. This binding promoted monocyte expression of the collagen cleaving protein, matrix metalloproteinase (MMP)-2, which aided cancer cell extravasation by reducing invasion impedance and recruiting BMDMs via production of chemotactic collagen fragments.[4] While these data highlight some mechanisms of early bone marrow derived cell recruitment and function correlated with fibronectin expression in the lungs, further investigation is required to better understand how primary tumor secretomes impact organotropic pre-metastatic niche formation.

Exosomes influence macrophage localization and function.

In addition to soluble growth factors, primary tumor-derived exosomes have also been shown to impact the pre-metastatic niche. Exosomes are vesicular carriers secreted by cells for intercellular transport, and exosome integrin expression promotes localization to specific organs.[5,15,35,36] As highly phagocytic cells, macrophages engulf exosomes and process the cargo allowing distant, primary cancer cells to alter secondary macrophage phenotypes. Tail vein injection of exosomes from organotropic breast (MDA-MB-231—lung, MDA-MB468—lung and liver) and pancreatic cells (BxPC-3 and HPAF-II—liver) into

mice revealed that exosomes with α6β4 and α6β1 integrins predominantly localize to the lungs while exosomes with αVβ5 were associated with liver localization. In both cases, these exosomes were found colocalized with macrophages in fibronectin-rich environments.[5] Exposure of macrophages to exosomes has been shown to directly promote their ability to further enhance pro-tumoral immune cell recruitment. Exosomes collected from lung cancer cells lines contain microRNA-21 or microRNA-29a which bound as ligands to toll-like receptor (TLR)-7 in mice (TLR-8 in humans) and induced expression of IL-6 and TNF-α in an NF-κB-dependent manner.[63] Because both TNF-α and IL-6 are cytokines associated with leukocyte recruitment during the immune response and tumor promotion, these studies warrant further investigation into the effects of primary tumor-derived exosomes on MAM function in the metastatic site.

Primary tumor regulation of resident macrophages in the metastatic site.

In addition to enhancing the recruitment of macrophage precursors into the metastatic site, primary tumor-derived factors can also impact tissue resident macrophages within that site. The primary tumor secretome can promote accumulation of lung resident alveolar macrophages that generate a pro-tumorigenic pre-metastatic niche for cancer cells. In the MET-1 mammary tumor model, accumulation of alveolar macrophages was found in the lung, which correlated with increased levels of the leukocyte chemoattractant, C5a. This accumulation of alveolar macrophages occurred before the arrival of cancer cells, indicating that primary tumor signals initiate lung secretion of C5a. Furthermore, depletion of alveolar macrophages significantly reduced lung metastasis of MET-1 cells, which correlated with a shift in the immune profiles from tumor-supportive Th-2 cells to tumoricidal Th-1 cells.[16] Further investigation into how the primary tumor initiates C5a production in the lungs may illuminate mechanisms to prevent this pre-metastatic niche initiation.

In addition to soluble factors, primary tumor-derived exosomes can also impact resident macrophages in distant sites. Isolation of exosomes from liver-tropic pancreatic ductal carcinoma (PAN02) cells shows that PAN02 exosomes containing migration inhibitory factor localize to the liver. Upon arrival, Kupffer cells (liver-resident macrophages) engulf the PAN02 exosomes and activate local fibroblasts to secrete fibronectin, contributing to the formation of pre-metastatic nodules where BMDMs localize.[36] These studies collectively suggest that primary tumor-derived factors can impact the function of resident macrophage in distant sites. Additional studies are needed to delineate the mechanisms of resident macrophage regulation and function in these and other distant tissue sites.

Macrophages Influence Adhesion, Extravasation, and Early Colonization of Cancer Cells

In the secondary site, MAM interactions with arriving cancer cells have been shown to promote metastatic behavior (Table 3). In many cases, the same mechanisms that macrophages use to recruit leukocytes during inflammation, such as enhancing adhesion and transmigration, are used to recruit cancer cells into the metastatic site. MAMs can further aid secondary tumor formation using the same mechanisms they utilize during tissue repair, such as inhibiting tissue-destructive immune response, promoting cellular growth, initiating angiogenesis, and remodeling the matrix. Furthermore, these MAMs can continually recruit BMDMs to maintain the pro-tumoral immune environment. Since MAMs appear to play major roles throughout metastatic progression, understanding the mechanisms through which MAMs drive tumor cell extravasation and early colonization may reveal therapeutic targets.

The presence of MAMs during extravasation promotes metastatic colonization.

Several studies have provided strong evidence that the presence of MAMs during the metastatic extravasation process is critical to successful formation of a metastatic

lesion.[37,46-48] Qian *et al*. injected breast cancer cells into the tail veins of mice following macrophage depletion with L-clodronate and found that the number of cancer cells arrested in the lung of macrophage-depleted mice dropped significantly over a 36-hour period post injection, suggesting that macrophages aid retention of disseminated cancer cells in the lung. Additionally, doubling times of tumor growth were twice as high in mice with macrophages than those without, highlighting the importance of macrophages in sustaining a pro-tumoral microenvironment. The authors also found that extravascular cancer cells in these models were in direct contact with CD11b+ BMDMs, indicating that adhesion of cancer cells to MAMs aids transmigration of the endothelium.[46]

In addition to interacting with disseminated tumor cells, MAMs also prevent tumoricidal cell infiltrate. Headley *et al*. observed shedding of 0.5-25 um portions of cytoplasm from cancer cells arrested in the vasculature.[48] These mitochondria-containing “cytoplasts” remained within the lung microvasculature and preceded three waves of monocytic cells: neutrophils (~15 minutes), conventional monocytes (15min-6hrs), and non-alveolar macrophages, patrolling monocytes, and dendritic cells (6-12hrs). Of these phagocytic cells recruited to the metastatic site, non-alveolar macrophages and conventional monocytes were mostly responsible for engulfing the cytoplasts and extravasating into the pre-metastatic niche. Engulfment of cytoplasts increased macrophage expression of adhesion and chemotactic receptors important in cancer progression including vascular cell adhesion molecule (VCAM)-1, exosomal surface protein CD63, and natural killer cell adhesion mediator CD155.[48,64-67] However, in CCR2 knockout models, immunostimulatory CD130+ dendritic cells and CD8+ T cells dominated the cytoplast-engulfing lung infiltrate, suggesting that CCR2+ BMDMs are important in promoting an immunosuppressive environment.[48] Furthermore, inhibiting recruitment of MAMs via the CCR2-CCL2 axis by

genetic or chemical means reduced metastatic burden of tail vein injected MET-1, MDA-MB-231, and melanoma cells in the lungs,[37,46-48] demonstrating the importance of this axis in metastatic progression. Taken together, these studies suggest that the presence of MAMs at the time of extravasation aids formation of metastatic lesions through direct interactions with tumor cells and indirect regulation of immune responses.

Mechanisms by which macrophages enable metastatic extravasation and colonization.

Recent studies have explored potential mechanisms through which macrophages contribute to metastatic progression. MAMs expressing CCR2 are directly implicated in metastatic progression,[37,46-48] and CCR2-mediated regulation of CCL3 has specifically been highlighted as a potential therapeutic target.[37] Stimulation of CCL2/CCR2 induces CCL3 in MAMs, which contributes to MAM retention in the metastatic site in a CCR1-dependent manner. Furthermore, the CCL3/CCR1 axis was found to be important for direct binding between macrophages and tumor cells, in part through an α4-integrin dependent mechanism.[37,46,47] α4 integrins bind to VCAM-1, which is expressed by leukocytes and endothelial cells,[68] but can also be overexpressed in cancer cells.[69] Chen *et al.* showed that mammary fat pad injections of the lung tropic MDA231-LM2-4175 breast cancer cell line in which VCAM-1 levels were knocked down grew at the same rate, but created significantly fewer lung metastases due to decreased binding affinity to macrophages. When modeling extravasation via tail vein injection of MDA231-LM2-4175 cells, reduced VCAM-1 expression did not affect accumulation of cancer cells in the lungs during the week after injection, but significantly decreased formation of metastases. Binding of macrophage α4 integrins to VCAM-1 in breast cancer cells activates downstream PI3K-AKT pro-migratory, pro-survival pathway in the cancer cells, which reduces cancer cell apoptosis and promotes secondary tumor formation.[64,65]

Additional macrophage-derived factors, such as VEGF and endothelin-1 (ET-1), have also been implicated in promoting metastatic colonization. In mammary tumor models, monocyte/macrophage VEGF has been shown to contribute to retention of MET-1 mammary tumor cells in the lung. Furthermore, in vitro studies have shown that VEGF promotes endothelial permeability and transendothelial migration of MET-1 cells. Macrophage-derived VEGF has also been linked to cancer cell retention in the lung.[47] Studies using LLC lung cancer and B16 melanoma models suggest that VEGF also acts on VEGFR1-expressing macrophages and endothelial cells to induce production of MMP9 and monocyte chemoattractant, CXCL12.[70] MMP-9 contributes to tumor progression by increasing vascular permeability,[70] degrading the ECM, cleaving growth factors that ultimately aid in tumor proliferation,[71] and angiogenesis to nourish the highly metabolic tumor.[42] Furthermore, MAM-induced VEGF-VEGFR1 signaling maintains gradients of the CXCL12 chemokine, which is a strong chemoattractant for monocytes and CXCR4+ tumor cells, contributing to the cycle of BMDM recruitment.[14,47,72] Macrophage-derived ET-1, a soluble vasoconstrictor, has been shown to contribute to metastatic colonization. Tail-vein injection of muscle-invasive bladder cancer cells that secrete soluble vasoconstrictor, endothelin-1 (ET-1), leads to improved recruitment of BMDMs and ultimately increased metastatic burden compared to ET-1 knock-down controls. Inhibition of macrophage ET-1 receptor ET_{aR} significantly reduced metastatic burden in mice by preventing downstream production of pro-tumoral cytokines such as IL-6, CCL2, COX-2, and MMPs, which are all associated with BMDM recruitment, activating angiogenesis, and secondary tumor formation.[73,74]

Macrophages recruited to secondary locations not only influence extravasation through direct interactions with cancer cells, but can also interact with other cells within

the pre-metastatic niche to promote metastasis. As alluded to in previous sections, macrophage-endothelial cell interactions can enhance metastatic potential of cancer cells by increasing endothelial affinity and permeability which aids circulating cancer cell adhesion and transmigration into the tissue.[47,58-62,70,75] Additionally, cancer associated fibroblasts (CAFs) polarize TAMs to perform pro-tumoral functions in the primary site.[76] In the secondary site, CAFs and MAMs collaborate to increase metastatic potential of arriving tumor cells. In both lung and liver metastases, the primary tumor promotes fibroblast secretion of fibronectin, which recruits BMDMs to the secondary site.[4,5,14,36] BMDMs recruited to the liver promote pancreatic ductal carcinoma (PDAC) metastasis by secreting granulin, generating a fibrotic environment by activating hepatic stellate cells into myofibroblasts. These myofibroblasts ultimately increase growth of secondary tumors by secreting periostin that activates Wnt and αVβ3-Akt/PKB signaling pathways in PDAC cells. Depletion of granulin prevented MAM-dependent development of the fibrotic environment and subsequent PDAC metastatic growth while inhibition of periostin significantly reduced metastatic outgrowth of PDAC cells by blocking the CAF/PDAC paracrine loop.[77] Understanding how macrophages promote pro-tumoral functions of other cell types within the pre-metastatic niche—including endothelial cells and CAFs—may illuminate several therapeutic targets that can serve as combination therapy to alleviate metastatic progression.

While these studies highlight the importance of MAM-derived factors in tumor cell extravasation and growth in the metastatic site, given the diversity of mechanisms through which TAMs in the primary tumor site contribute to tumor growth and progression, it is likely that there are additional MAM-related factors that contribute to metastasis. For example, it is widely accepted that TAMs promote intravasation via the CSF-1/EGF

paracrine loop.[38-45,75] Specifically, secretion of CSF-1 by cancer cells recruit BMDMs to the primary tumor where they secrete EGF, which contributes to vascular permeability and cancer cell transmigration.[75] Additionally, cancer stem cells have been identified in circulation and may be better suited to infiltrate and colonize the secondary site than other tumor cell populations since they can quickly adapt to new environments.[78] Macrophage crosstalk with cancer stem cells in the primary tumor promotes development, maintenance, self-renewal, and the epithelial to mesenchymal transition of cancer stem cells via several mechanisms which could also promote early tumor development in the secondary site.[78,79] Whether these mechanisms or other known pathways through which primary TAMs function are replicated in the metastatic site remains to be investigated. Understanding the similarities and differences in TAM and MAM function is critical for developing the most effective macrophage-targeted therapies that will target macrophage function in both sites.

Macrophages are a potential therapeutic target in the secondary tumor microenvironment

Numerous strategies are being pursued to target macrophages in the primary tumor, including ablation of TAMs residing in the tumor, impeding further recruitment of BMDMs, and polarizing TAMs toward a tumoricidal phenotype.[20,21] Several of these treatment strategies targeting the CSF1-CSF1R,[22] CCL2-CCR2,[23] and VEGF-VEGFR1[14,75] axis effectively reduce primary tumor burden and metastasis in murine models.[14,37] However, clinical trials targeting TAMs have thus far been less successful.[21] Inhibition of the CSF1/CSF1R pathway decreases metastatic burden in murine models and is generally well tolerated in patients, but is ineffective in reducing recruitment of BMDMs to the primary tumor in clinical trials.[21,22] Recruitment of BMDMs to the primary tumor may be maintained when the CSF1 pathway is inhibited via stromal cell secretion of other BMDM recruitment

cytokines. Kumar et al. demonstrated that CSF1 downregulates CAF secretion of CXCL1, which is a granulocyte/monocyte recruiting cytokine. Inhibition of CSF1 alone promotes upregulation of CXCL1 and, therefore, increases recruitment of granulocytes and BMDMs. However, inhibition of CSF1R and CXCL1 receptor, CXCR2, significantly reduces BMDM recruitment and primary tumor growth in mice.[80] These data show the importance of understanding the various mechanisms that contribute to BMDM recruitment and finding the optimal combination of targets to prevent BMDM recruitment to the pre-metastatic niche.

Similarly to CSF1R inhibition, blocking BMDM recruitment via CCL2 has shown promise in pre-clinical studies. In murine models, ablation of CCR2+ MAMs reduces metastatic burden, possibly due to tipping the immune cell infiltrate from pro-tumoral CCR2+ MAMs towards anti-tumoral CD130+ dendritic cells and CD8+ T cells.[37,48] However, because ablating CCR2+ macrophages directly is not currently a clinical option due to necessary homeostatic functions of macrophages, clinical trials have focused on reducing CCL2 levels with anti-CCL2 antibodies. Stage 1 trials with anti-CCL2 monoclonal antibody carlumab demonstrated that it is well tolerated by patients, but carlumab on its own does not consistently suppress free CCL2 levels and ultimately increased CCL2 levels over time.[24] The complications of carlumab treatments are likely due to short-term affinity of carlumab, overcompensation of cells expressing CCL2, and increased sensitivity of macrophages to CCL2 through upregulation of CCR2.[24,25,81] For example, some murine models of mammary cancer associate cessation of anti-CCL2 agents with accelerating metastatic progression by increasing influx of monocytes to the primary and secondary tumors. In these models, monocytes/macrophages in the lungs increased metastatic growth of cancer cells in an IL-6/STAT3 dependent manner via VEGF-A driven

neovascularization. When both CCL2 and IL-6 are inhibited, there is reduced metastatic burden.[25] While there is much to be understood about how these therapeutics influence macrophage recruitment and function in the secondary site, these data emphasize the importance of not only inhibiting BMDM recruitment, but concurrently targeting MAM functions to effectively target metastasis.

Concluding Remarks

Therapeutically targeting macrophages in cancer is complex due to the various roles of macrophages in physiology; however, macrophages influence metastatic progression and may be an integral component in combination therapy. Further research is required to fully understand the mechanistic contributions of macrophages to organotropic metastases, early cancer cell-macrophage interactions in the secondary site, influence of cancer cell and macrophage heterogeneity on metastatic potential, and how these interactions influence the immune response. Understanding these key interactions that drive tumor cell-macrophage interactions throughout the metastatic cascade will provide insights that we can leverage with better drugs and delivery vehicles to skew MAMs towards anti-tumoral phenotypes and break the deadly cycle of metastatic progression.

Tables

Table 1: Macrophage subsets, ontogeny, and function

Table 1: Macrophage Subsets, Ontogeny, and Function				
Macrophage	**Ontogney**	**Function**	**Identifying Markers**	**Ref**
BMDM	Monocyte-derived	Patrol vasculature for pathogens Recruited as part of the immune response Phagocytose pathogens and clear debris Promote tissue reconstruction	Patrolling: CX_3CR1-high CCR2-negative LY6C-low Inflammatory: CD11b-high F4/80 CCR2 LY6C-high	56
Alveolar Macrophages	Yolk sac and fetal liver progenitors	Patrol and eliminate inhaled particles Maintain lung homeostasis	F4/80-low CD11b-low CD11c-high CD68 Siglec F MARCO CD206 Dectin-1 Galectin-3	49
Kupffer Cells	Yolk sac progenitors	Clear pathogens and toxins	F4/80 CD11b-low CD169 CD68 Galecin-3 CD80-low	49
Osteoclasts	Monocyte-derived	Bone resorption Create pre-metastatic niches for disseminated tumor cells	Calcitonin receptor	49
TAMs	Monocyte-derived Resident macrophages?	Promote tumor growth Inhibit tumoricidal immune response Initiate angiogenesis Activate matrix remodeling	<u>Murine:</u> LY6C MHC II molecules CX_3 CR1 CCR2 L-selectin TIE2	44

		Aid invasion and intravasation	Human: CD14 CD312 CD115 CD16	
MAMs	Monocyte-derived Resident macrophages?	Aid metastatic functions including pre-metastatic niche formation, extravasation, angiogenesis, and immune evasion	Monocyte derived: F4/80 CD11c-low CD11b VEGFR1-high CCR2-high	37,46-48

Table 2: Macrophage interactions in promoting pre-metastatic niche formation

Table 2: Macrophage Interaction in Promoting Pre-Metastatic Niche Formation				
Primary Tumor	**Secondary Site**	**Primary Tumor Signal**	**Secondary Site Response**	**Refs**
Lewis lung carcinoma (LLC) B16 melanoma	Lungs, liver Lungs, liver, spleen, testes, and kidney	Conditioned media from LLC/B16 cells or intradermal injection of LLC/B16 cells	Fibronectin expression pre-determines VLA4+ bone-marrow derived cell cluster localization to organs where each cell type generally metastasizes	[14]
Lewis Lung Carcinoma (intradermal injection)	Lungs	TNF-α, TGF-β, VEGF-a	Stimulates endothelial secretion of S100A8/9 which recruits BMDMs	[9,14]
Hypoxic ER- breast cancer	Lungs	LOX	Localizes to fibronectin rich areas, cross-links collagen IV,	[4]

			promotes BMDM recruitment	
Pancreatic (PAN02)	Liver	TGF-β exosomes	Activate Kupffer cells which initiates fibronectin secretion in liver	[36,70]
Lung-tropic breast cancer (MDA-MB-231, MDA-MB-468), osteosarcoma, Rhabdomyosarcoma, Wilms' tumor, and melanoma exosomes	Lung	Aplha6β4 α6β1	Cancer exosomes expressing α 6 integrins localize in lung with macrophages before cancer cells arrive	[5]
Liver-tropic breast cancer 9MDA-MB-468), uveal melanoma, colorectal cancer, pancreatic cancer(BxPC-3, HPAF-II), and gastric cancer exosomes	Liver	αVβ5	Cancer exosomes expressing β5 integrins localize in the liver with macrophages before cancer cells arrive	[5]

A-549 and SK-MES lung cancer lines	RAW 264.7 murine macrophages Lungs of WT and TLR7-/- mouse models	miR-21 and miR29a in exosomes	miR-21 and miR-29a containing exoosmes are phagocytoced by macrophages and activate TLR-7 in macrophage endosomes which induces a pro-metastatic immune response by activating NF-κB transcription of TNF- α and IL-6	[63]
Hypoxic ER- breast cancer	Bone	LOX	Activates transcription factors that upregulate osteoclastogenesis	[7]
Breast Renal NSCLC	Bone	TGF-β	Activate osteoclastogenesis and MMP secretion	[82]

Table 3: Macrophage influence on cancer cell arrest, extravasation, and colonization

Table 3: Macrophage Influence on Cancer Cell Arrest, Extravasation, and Colonization

Primary Tumor	Secondary Site	Macrophage population	Macrophage function	Cancer Interaction	Refs
PyMT breast cancer cells	Lungs	CD11b/Ly6C/ VEGFR1/CC R2/CSFR1+ macrophages recruited to secondary site via CCL2 axis	Secretion of VEGFa increases permeability of endothelium	This subset of macrophages is in direct contact with extravasacular breast cancer cells Increased vascular permeability increases metastasis	[46,47]
Polyoma-middle-T MET-1 cancer cells	Lungs	Recruited monocytes	VEGF secretion by recruited monocytes	VEGF expressing monocytes significantly increase retention of MET-1 cells in the lung	[47]

MDA-MB-231:4175	Lungs	CD11b+ MAMs	Recruited via CCL2 axis which induces MAM CCL3 secretion and MAM retention via CCL3/CCR1 axis	The CCL3/CCR1 axis leads to direct binding of tumor cells to MAMs via α4 integrin binding to VCAM-1 in breast cancer cells	[37]
B16 Melanoma	Lungs	CCR2+ BMDM	Engulf cytoplasts and upregulate adhesion receptors VCAM-1, CD-38, CD-63, CD-88, CMKLR-1, and CD-155	Melanoma cells secrete shear-induced “cytoplasts” that initiate pro-tumoral immune cell recruitment Lack of macrophages results in recruitment of anti-tumoral immune cells	[48]

MMTV-PyMT indced mammary tumor cells	Lungs	TAMs	BMDMs are recruited via CSF-1 gradients and secrete EGF to aid tumor cell invasion MAMs are recruited to the lung in a CSF-1 dependent manner	Secrete CSF-1 to recruit macrophages. Migrate along EGF gradients produced by macrophages. Intravasate due to this mechanism	46,58,75
Lung-tropic breast cancer cells (MDA231-LM2-4175)	Lungs	CD11b+/ VLA4+ BMDMs	Macrophages bind to VCAM-1 with α4 integrins	Binding of breast cancer VCAM-1 to α4 activates pro-migratory and survival PI3K-AKT	65,83
--	--	Macrophage activation	Promote endothelial expression of VCAM-1	Aids TAM localization and upregulates α4 integrins in macrophages which tethers breast cancer cells	65

MB49 murine bladder cancer	Lungs	BMDM	Arrive to extravasation site before cancer cells and produce CCL2, COX-2, MMPs and IL-6 Depletion of ET-1 from cancer cells decreased macrophage influx to the lung	Secondary tumor growth, not primary tumor, is affected by ET-1 expression ET-1 recruits macrophages to secondary site to aid immune response and angiogenesis	[73,74]
Pancreatic Ductal Carcinoma (PDAC)	Liver	BMDM	Recruited to liver and secrete granulin which promotes myofibroblast differentiation	Myofibroblasts secrete periostin which promotes PDAC metastatic growth via αVβ3-AKT/PKB signaling	[77]

Figures

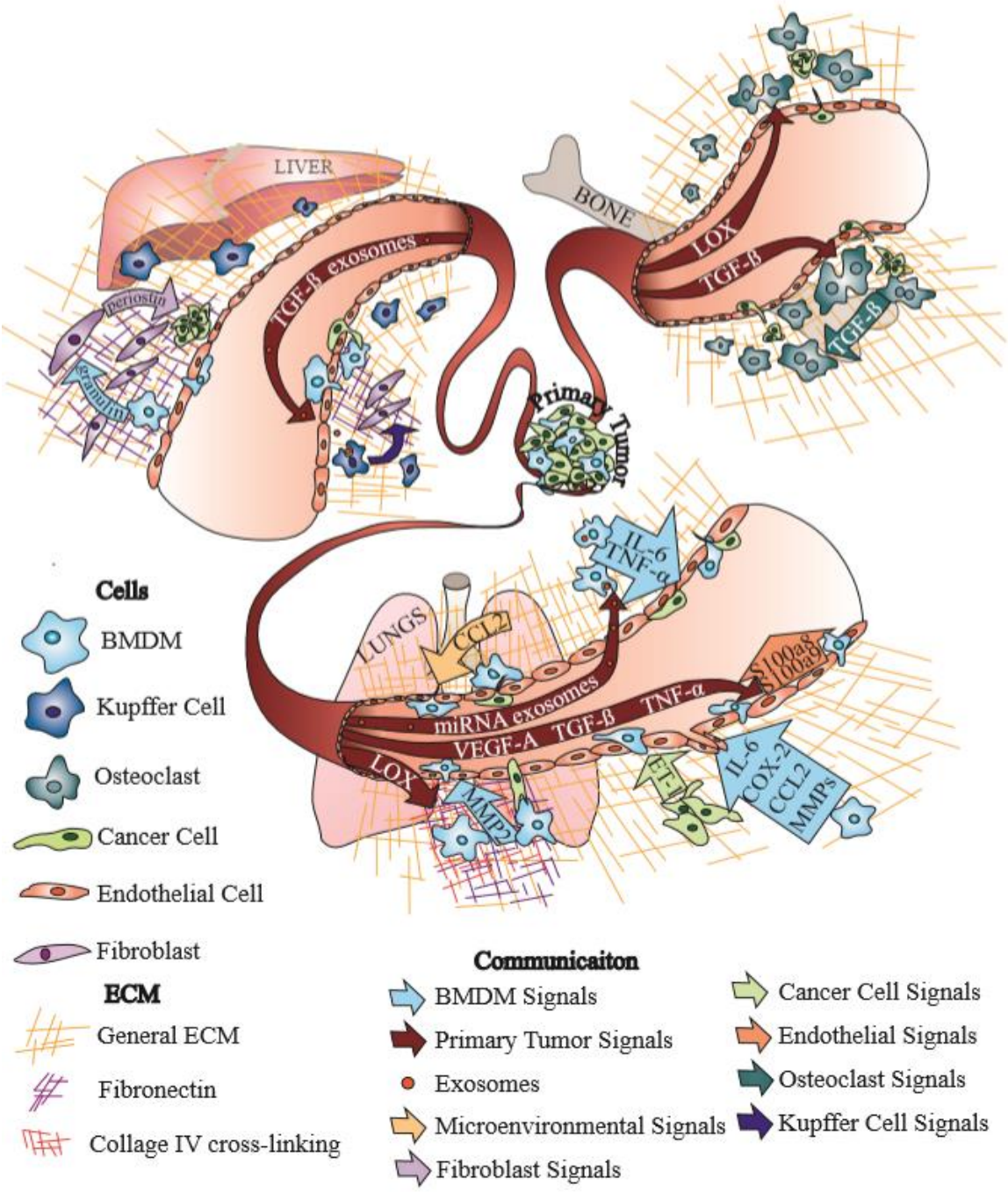


Figure 2.1: Macropahge Roles in Organotropic Extravasation

In bone, primary, hypoxic ER- breast tumors initiate metastasis to the bone microenvironment though secretion of LOX which promotes osteoclastogenesis and cross-linking of collagen 4 which aids BMDM adhesion and extravasation.[7] TGF-β secretion by primary tumors also promotes metastasis to the bone by activating

osteoclastogenesis and secretion of MMP9 which helps form pre-metastatic lesions.[82] In the liver, Kupffer cells engulf TGF-exosomes from the primary tumor which activates fibroblast-secretion of fibronectin that aids adhesion and extravasation of BMDMs.[36,70] Once in the liver, BMDMs secrete granulin which promotes fibroblast secretion of periostin. Periostin secretion generates a fibrotic environment that supports tumor cell colonization in the liver.[77] LOX secreted by hypoxic ER- mammary cancer cells also localizes to fibronectin-rich regions in the lung, cross-links collagen IV, and promotes BMDM recruitment.[4] BMDMs recruited by CCL2 aid extravasation of cancer cells.[46-48] Primary tumor secreted VEGF-a, TGF-β, and TNF-α activate S100A8&9 in endothelial cells which recruits BMDMs that predetermine lung metastases.[9,14] Extravasated BMDMs engulf exosomes containing miR-21 or miR-29a and secrete IL-6 or TNF-α to promote endothelial expression of surface receptors VCAM-1 and E-selectin, which increases BMDM and CTC affinity to the endothelium.[63]

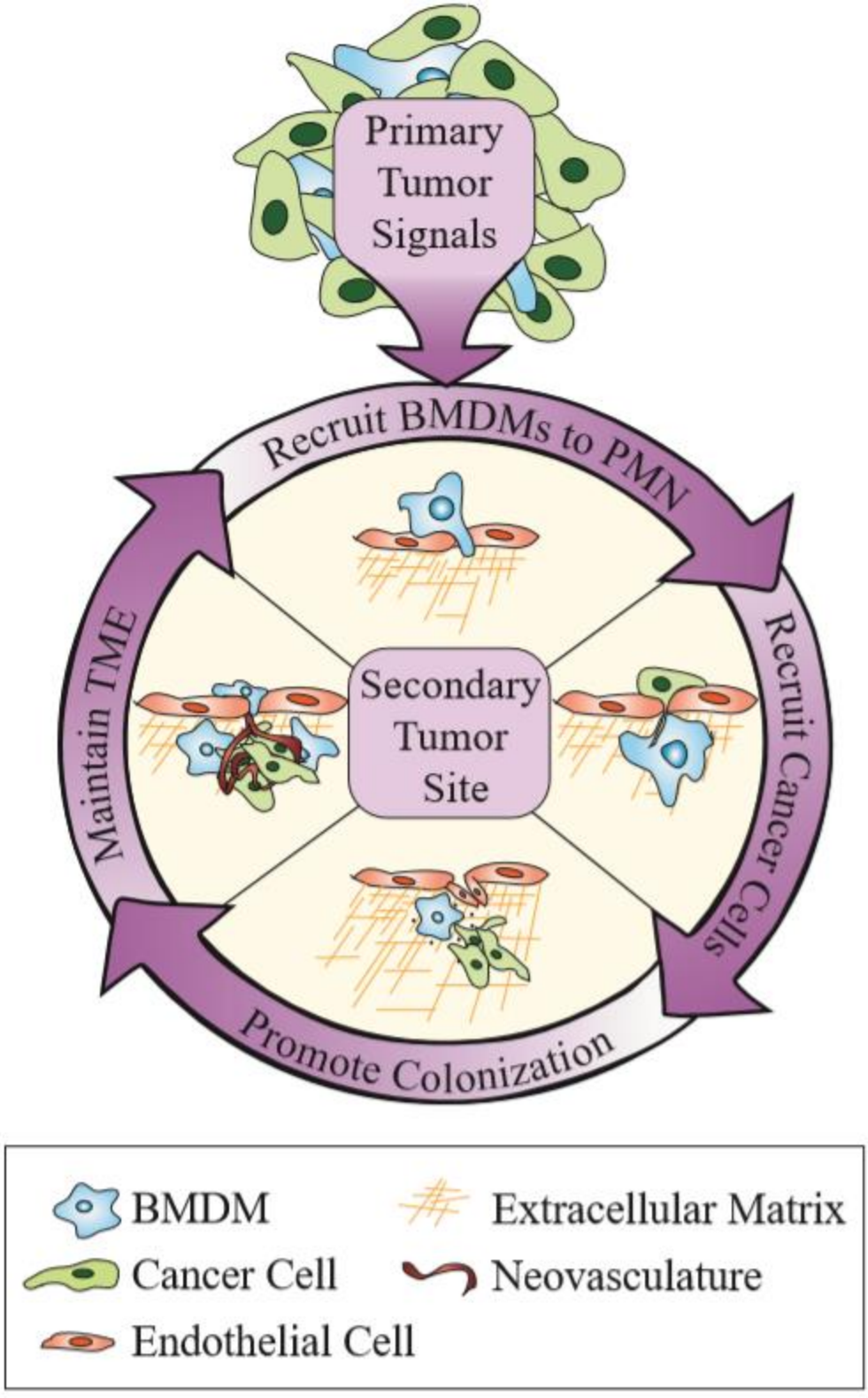

Primary Tumor Signals
Recruit BMDMs to PMN
Recruit Cancer Cells
Promote Colonization
Maintain TME
Secondary Tumor Site
BMDM
Cancer Cell
Endothelial Cell
Extracellular Matrix
Neovasculature

Figure 2.2: Cycle of BMDM Recruitment Maintains Secondary Tumor Microenvironment.

There is a "vicious cycle" of osteolytic tumor development in the bone microenvironment [36], but evidence suggests there is also a cycle of BMDM recruitment in the lung and liver microenvironments that aids metastatic colonization. The primary tumor can promote formation of the pre-metastatic niche (PMN) by activating resident macrophages via soluble and exosomal signaling. Bone marrow derived macrophages (BMDMs) are recruited to these regions of increased endothelial activation and increased fibronectin expression.[4,5,9,14,36,63,70] At the secondary site, BMDMs promote cancer cell retention in the secondary site through direct contact, signaling, and interactions with the endothelium. Tumor cell-MAM interactions further promote tumor growth through secretion of growth factors, promotion of angiogenesis, and maintenance of the immunosuppressive environment.[46-48,58,65,73-75,83] As the tumor grows, BMDMs and cancer cells are likely continually recruited to this inflammatory, tumor microenvironment (TME) through increased endothelial permeability, affinity to the endothelium, and maintenance of chemokine gradients.

Chapter 3: Model system to study macrophage influence on endothelial cell phenotype

Introduction

Cancer metastasis leads to over 90% of cancer-related deaths, but the mechanisms driving metastasis are not well understood. Once cancer cells enter the bloodstream they must extravasate into the tissue to complete the metastatic process. Since extravasation is thought to be a major bottleneck in cancer metastasis, understanding the mechanisms that lead to successful extravasation may lead to the development of metastasis-suppressing therapeutics. This process requires endothelial adhesion, transmigration, and eventual colonization of the pre-metastatic niche.

Current models to study cancer metastasis mostly consist of murine models and traditional 2D cell culture assays, which are—and will always be—important in the fight against cancer. However, these model systems do have translational limitations including differences in cell behavior in 2D vs 3D, a lack of physiologically relevant mechanical stimuli, biological complexity, and basic differences in murine and human biology.[26,84,85] To fill this gap, we aimed to build a simple, tunable extravasation microenvironment consisting of an endothelialized vessel perfusing a 3D ECM channel. We also incorporated flow over the endothelial cells since endothelial function is highly regulated by shear stress. To visualize how macrophages influence tumor cells adhere and transmigrate under shear, we believe that microfluidic models are ideal candidates to fulfill these experimental criteria.

Several existing microfluidic models that consist of manufactured or spontaneously formed vascular channels perfusing 3D tissues.[28,32-34,86-88] While these model systems have their own advantages such as self-assembled microvessels of physiologic size,[31,33,87] we chose to adapt an existing microfluidic model system developed by Bischel, et. al. 2012

to study factors influencing extravasation events.[86] This model allows us to create an endothelialized “vessel” channel perfusing a 3D ECM that can contain resident cells. Although this method generates a larger vessel diameter than what would be found physiologically, it can be generated consistently. To add flow through the vessel channel, we designed a detachable resistor to control gravity-driven fluid flow. We can also flow tumor cells through the channel and quantify their behavior in response to exogenous signals and other cell types over time. Before using this model system to study how macrophages influence tumor cell extravasation, we first wanted to validate that this system can be used consistently, and that we can measure changes in endothelial and tumor cell phenotypes.

Materials and Methods

Device Fabrication

A passive pumping microfluidic device fabrication protocol was optimized from procedures published by Bischel et al 2012 (Figure 3.1).[86] The devices, a simple, straight channel later filled with a collagen hydrogel, were fabricated using standard soft photolithography techniques using PDMS (10:1 base: curing agent; Dow Sylgard 184 Elastomer Kit). The channels were designed to be 500 um tall and the optimal length was determined to be 5 mm for optimal vessel stability. One side of the channel was punched with an 11-gauge punch (inlet) and the other with a 1 mm biopsy punch (outlet). To aid in connecting flow to the fragile collagen tube interface, PDMS spacer cubes punched with 11-guage needle were bonded to the top of the microfluidic inlet.

Collagen solution and microfluidic devices were prepared in parallel. First, collagen I (Corning) was neutralized using 10x phosphate buffer solution (PBS) and nucleated for 90 minutes on ice before loading into devices. To prepare devices, patterned PDMS and

glass slides were plasma etched and bonded to create a complete microfluidic device. Immediately after bonding, 1 mg/mL Poly-D-Lysine was added to devices to ensure the channels remained hydrophilic and aid in collagen adhesion to the channel walls. After 30 minutes incubation, the PDL was aspirated and the channels were rinsed with cell culture grade water.

To form a collagen channel, water was aspirated and collagen was perfused through the microfluidic channel. Using the mismatch in viscosity between the collagen solution and cell culture media, we used passive pumping to flow media through the center of the collagen solution within the device and tunnel a space for a "vessel" in the collagen gel (Figure 3.1a).[86] Specifically, this was achieved by dispensing 1uL drops of cell culture media on the outlet of the device. Because collagen is thermally polymerized, we can preserve this configuration by increasing the temperature of the device until a collagen hydrogel is formed. We complete the setup by slowly filling the inlet tube with media. Devices were maintained in a humidified and CO2 controlled incubator at 37C for at least 30 minutes to ensure complete polymerization of the collagen gel. This process was repeated for each device (Figure 3.1). This procedure ultimately results in a "vessel" channel tunneling though a 3D collagen I hydrogel that will serve as a basis of our extravasation model.

Endothelialization

Human lung Microvascular Endothelial cells (HMVEC-Ls, Lonza) were cultured in Endothelial Growth Medium-2-MV (Lonza) and used between passages 3-5 according to supplier protocols. Cells were rinsed with HBSS and passaged with 0.05% trypsin, counted, centrifuged, and resuspended at a concentration of $5x10^6$ cells/mL. Between 30-

60 minutes after collagen channel formation, two 1 uL drops of cell suspension were added to each device outlet and seeded via passive pumping. Devices were inverted to ensure HMVEC-Ls adhered around the entire collagen channel and incubated for 30 minutes at 37˚C. Devices were then turned right-side-up and one drop of cell suspension was added to the outlet before overnight incubation. Devices were stored in a large petri dish with water for this overnight incubation to prevent channel dehydration. At this stage, the “vessel” channel is endothelialized and culture under shear will promote a confluent, viable endothelium in this model system (Figure 3.1a).

Perfusing devices to generate shear on the endothelium

To control flow rate, resistors with channel dimensions of 75,310 x 150 x 150 um were also fabricated using standard soft photolithography techniques, both ends punched with a 13-gauge hole punch (McMaster Carr), and bonded to glass slides (GLOBE). Tubing was cut into 6cm lengths and plugged directly into the resistor outlet to connect the resistor to the device (Tygon, Lot# 24924745). A p1000 pipet tip with 1mL of media was added to the inlet and pushed through the resistor until a drop of media came out of the end of the tubing. To connect tubing to the soft, collagen channel-interface, a p200 pipet tip was cut in two places—between 10 and 50 uL lines—and gently inserted into the cube inlet on the passive pumping device. A gel-loading tip was used to load media into the inlet cube and pipet tip, taking care to not to touch the delicate collagen gel in the inlet. The tubing from the resistor was added to the pipet tip in the microfluidic device, ensuring bubbles are not introduced to the system. Fresh medium was added daily by adding 1mL EGM to a 1 mL pipet tip in the inlet of the flow resistor (Figure 3.1).

Immunofluorescence Staining of Endothelial Cells in Devices

After incubation under flow for 48 hours, resistors were removed from the endothelialized devices and PBS was added to the inlet pipet tip of the channels to rinse media from the channel. Then formalin (INFO) was added to the device for 30 minutes at room temperature to fix the cells. After another PBS wash, blocking solution (1xPBS with 10% fetal bovine serum (FBS)) was added. Conjugated CD31 antibody (BioRAD APC) was diluted 1:10 in blocking solution with 1:1000 dilution of Hoechst (ThermoFisher) for at least 1 hour at room temperature to stain for endothelial tight junctions and nuclei. Devices were rinsed 3 times with PBS and left overnight to remove excess fluorophore from the collagen. Optical sectioning of the endothelial layers was completed with a Zeiss Axio-Observer with an Apotome attachment.

Measuring Permeability

After flow was maintained for 48 hours, permeability was quantified to see if changes in endothelial barrier function could be measured. Normal culture media, 10 ug/mL TNFα in media, or 5 mM EDTA was added to the inlet channel of respective devices and incubated for 4 hours prior to imaging. Permeability was measured by adding 70 kDa TRITC-dextran to the inlet pipet tip and imaging the center slice of the collagen channel on the Zeiss Axio-Observer with the Apotome attachment at one minute intervals for 20 minutes (Figure 3.3a). We calculated the endothelial permeability to 70 kDa TRITC dextran using a formula derived by Bischel et al:

$$P = \frac{1}{\Delta I}\left(\frac{dI}{dt}\right)\frac{r}{2}$$

where ΔI is the step change in the fluorescent intensity immediately after adding fluorophore and $\frac{dI}{dt}$ is the rate of change in intensity across the endothelium over the first

5 minutes after fluorophore has entered the channel.[86] Since there is some effect of background intensity in these measurements, we subtracted the average intensity of the PDMS space from the vessel and collagen intensity measurements before calculating the permeability.

To calculate the permeability of the endothelium separately from that of the ECM, we added EDTA for at least 30 minutes to some devices to release tight junctions between the endothelial cells. This allows for measurement of the permeability of the endothelial cell-altered ECM alone, which can be subtracted from the total permeability of the ECM and ECs using the formula:

$$\frac{1}{P_{EC}} = \frac{1}{P_{tot}} - \frac{1}{P_{ECM}}$$

Introducing Cancer Cells to Devices

Cancer cells were introduced at least 48 hours after flow was introduced to the endothelialized devices. Cancer cells used in these experiments include highly invasive, triple-negative (MDA-MB-231) and less invasive, estrogen receptor positive (MCF7) breast cancer cell lines. Cancer cells were passaged according to recommended supplier protocols, counted, centrifuged, and resuspended at 1.25×10^6 cells/mL. The resistor tubing was removed and 20 uL of cancer cell suspension was added to each channel so that a total of 10,000 cancer cells were perfused through each device. Each resistor was re-filled with media and the tubing was added back to each device so cancer cells would flow through the channel. Devices were incubated for at least two hours before adhesion was measured. After 2 hours, devices were imaged on an inverted Zeiss Axio Observer with an Apotome attachment to obtain optically-sectioned images of adhered cancer cells. A line between the collagen-endothelial cell interface was drawn by one researcher using

only the bead color channel before overlaying the green, cancer cell channel. A separate, blinded researcher counted the number of cells adhered to the endothelium and the number of cells in the collagen space based on the line drawn along the interface (Figure 3.4a). Adhesion and invasion were quantified and compared between MDA-MB-231 and MCF7 cells.

Statistics

All data sets were tested for normality using the Shapiro-Wilk test. Statistical significance of normally distributed data was done with either an unpaired t-test (when comparing 2 sets of data) or one-way ANOVA corrected for multiple corrections with Dunn's method. If the data were not normally distributed, then Mann-Whitney U test (for two comparisons) or Krustal Wallis test (for multiple comparisons) corrected with Dunn's multiple corrections method was used instead. Statistical significance indicated by: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results

Endothelialization of microchannels—validation with staining

We evaluated endothelial confluency within our model system by staining for endothelial tight junction marker CD31. Optical sectioning and 3D reconstruction of CD31 staining indicates a confluent endothelial layer lining the entirety of the collagen channel (Figure 3.2 b.). By comparing the brightfield images of devices with CD31-validated confluent monolayers, we can ensure that cancer cell adhesion in the devices is due to adhesion interactions with endothelial cells rather than bare spots in the device.

Addition of flow—quantifying flow rates

Flow-induced shear stresses on endothelial cells are important for basic endothelial cell functions such as maintaining physiological permeability, tight junctions, and modulating adhesion molecules.[27-29,89,90] Successful addition of flow is necessary to control and maintain a physiologically-relevant phenotype. We designed detachable resistors to add flow to collagen microchannels, which allows us to set-up a greater number of devices than could be done with pumps. Flow rates and channel diameters were measured to calculate shear stresses on the endothelial cells. We determined that the average channel diameter was 370 +/- 55 um and the shear stress fell between 0.001-0.04 dynes/cm^2. Although this flow rate is low, any shear on the endothelium improves endothelial function and could be increased if alternate resistors or pumps are used.

Sprouting endothelial vessels lead to increased extravasation potential of breast cancer cells

We observed that adding flow to devices too soon after endothelial cell seeding led to formation of endothelial sprouts into collagen. Although studying sprouting in extravasation was not our primary objective, we observed that tumor cell arrest and extravasation were qualitatively increased in devices with sprouts than those that did not sprout (Figure 2.2 c.). This increase in arrest may be due to the direct flow of cells into the cavities where they are contained and have endothelial tracks to migrate along into the tissue. These sprouts also likely have increased vascular permeability, which would aid transmigration. We hypothesize that angiogenic tissues may be prime “soil” for metastatic seeding due to the structure and immune profile in these tissue-regenerating regions.

Inflammatory Cytokine, TNFα Increases Endothelial Permeability

Endothelial cells alter their permeability in response to various microenvironmental factors, including immune cell secretion of cytokines.[40,58-62,70,75,91] An increase in permeability leads to increased solute exchange across the endothelium and may aid immune cell transmigration. It is hypothesized that circulating tumor cells can also take advantage of increased permeability to help them through the transmigration stage of extravasation. One well-known modulator that increases endothelial permeability is Tumor Necrosis Factor (TNFα).[88] We demonstrated that we can measure differences between TNFα treated devices ($1.5x10^{-5}$ dynes/cm^2) and untreated controls ($5x10^{-6}$ dynes/cm^2) in this model system (Figure 3.3b). Although these permeability values are higher than what would be found *in vivo*,[88,92] since we can measure significant changes in endothelial permeability with this model system, we can use this as an additional metric for measuring environmental influence on endothelial barrier function.

Highly Invasive MDA-MB-231 cells s show increased extravasation potential compared to MCF7 cells

To evaluate the ability to measure differences in extravasation potential between cell types, we compared highly invasive MDA-MB-231 cancer cell adhesion and invasion to less invasive MCF7 breast cancer cells. Optical sections of the microfluidic device shows that green MDA-MB-231 cells and MCF7 cells in the endothelialized channel (Figure 3.4a). However, it appears as though more MDA-MB-231 cells extravasate and invade into the collagen as denoted by the green cells invading the orange bead space. When quantified, we observed a small difference in initial adhesion of cancer cells to the endothelium between MDA-MB-231 cells and MCF7 cells, indicating that both cell types have similar endothelial adhesion properties. However, MDA-MB-231 cells are much more invasive

than MCF7 cells in our system (Figure 3.4b), suggesting that if either of these cell lines enter the vasculature, MDA-MB-231 cells have increased extravasation potential than MCF7 cells due to their highly invasive nature. We found that this model system may be used to answer further questions about factors contributing to extravasation phenotype.

Conclusions

We have developed a model to study how microenvironmental factors influence extravasation of cancer cells. Building upon existing model systems, we have optimized a microfluidic device that consists of a vascularized microchannel perfusing a 3D collagen matrix. The endothelium is consistently confluent as demonstrated by immunofluorescent staining of endothelial tight junctions. This allows for any cancer cell that adheres to the vasculature to extravasate into a 3D tissue. We distinguished differences in extravasation potential between two breast cancer cell lines—highly invasive MDA-MB-231 and less invasive MCF7 cells. While approximately the same number of MDA-MB-231 and MCF7 cells initially adhered in the device, a much higher percentage of highly invasive MDA-MB-231 cells transmigrated the endothelium than less invasive MCF7 cells. While these differences are likely due to intrinsic differences in tumor cell migratory phenotype,[93] the fact that there was such a large difference in cell invasiveness implies that this model may be used to distinguish cancer cell extravasation potential.

We also demonstrated that we can exogenously alter and measure changes in endothelial permeability. Endothelial cells activated by TNFα were more permeable to 70 kDa TRITC-dextran than untreated endothelial cells. Since we demonstrated that we can measure changes in endothelial barrier function, we may be able to relate these changes to extravasation phenotypes. This model system was used by Bischel et al. to study cytokine-induced endothelial sprouting,[86] and we observed pressure-induced endothelial

sprouting. When cancer cells were added to these devices, it appeared as though tumor cells used endothelial sprouts as tracks to exit the vasculature, indicating that the presence of sprouting alone may aid tumor cell extravasation. Macrophages may also contribute to endothelial sprouting, which may be a mechanism by which macrophages aid tumor cell extravasation.

Utilizing this microfluidic system we can quantify both permeability and tumor cell extravasation, and can begin to elucidate what factors are most important in tumor cell extravasation. By determining the most impactful mechanisms by which tumor cells extravasate, we can test treatments in this model system and focus on successful treatments to test in other more expensive and time-consuming model systems.

Figures

Figure 3.1: Procedure for microfluidic, extravasation model

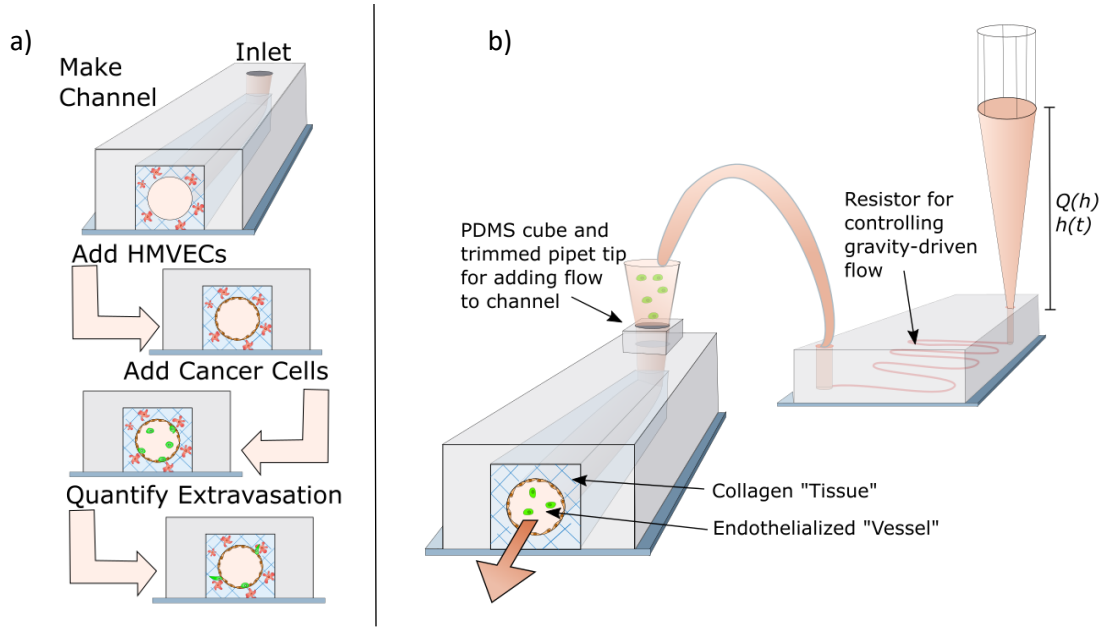


Figure 2.1: Device set-up. a) A channel is made through collagen via passive pumping before hydrogel polymerization. HMVEC-Ls are added via passive pumping to form a confluent endothelial layer. Cancer cells are added and their behavior is quantified. b) Gravity-driven flow is added to channel via an external resistor. A PDMS cube and pipet tip are necessary for adding flow without disturbing the collagen-PDMS interface. Flow rate Q(h) is determined by the height of media in the inlet and the geometry of the resistor. The height of media changes with time h(t), so media needs to be replenished at least once every 24 hours.

Figure 3.2 Validation of endothelial viability in extravasation model

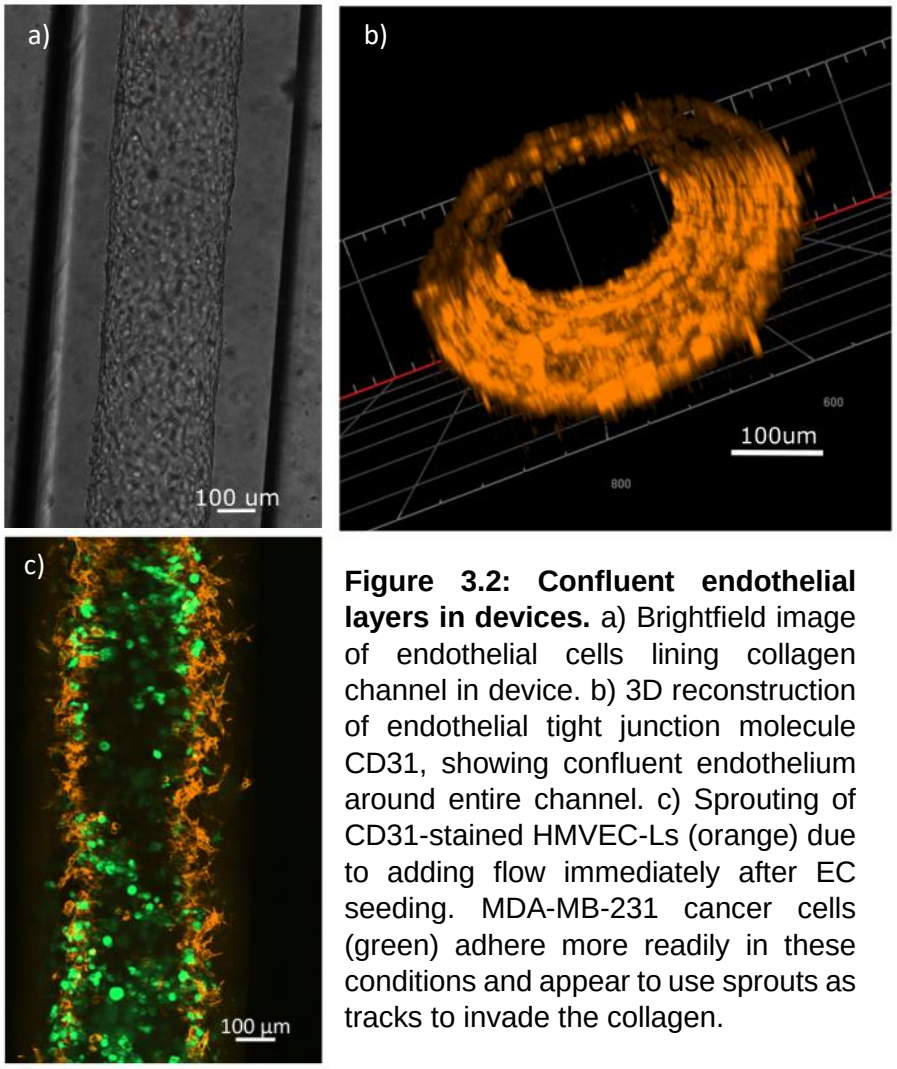


Figure 3.2: Confluent endothelial layers in devices. a) Brightfield image of endothelial cells lining collagen channel in device. b) 3D reconstruction of endothelial tight junction molecule CD31, showing confluent endothelium around entire channel. c) Sprouting of CD31-stained HMVEC-Ls (orange) due to adding flow immediately after EC seeding. MDA-MB-231 cancer cells (green) adhere more readily in these conditions and appear to use sprouts as tracks to invade the collagen.

Figure 3.3 Quantification of endothelial permeability in extravasation model

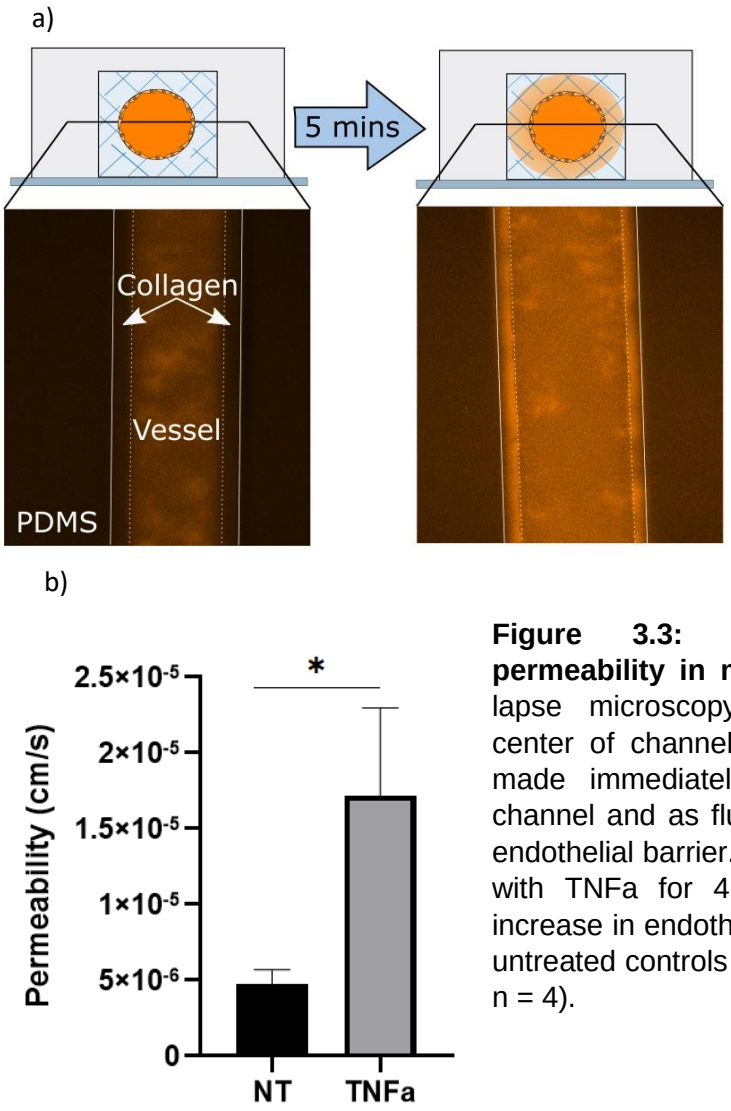
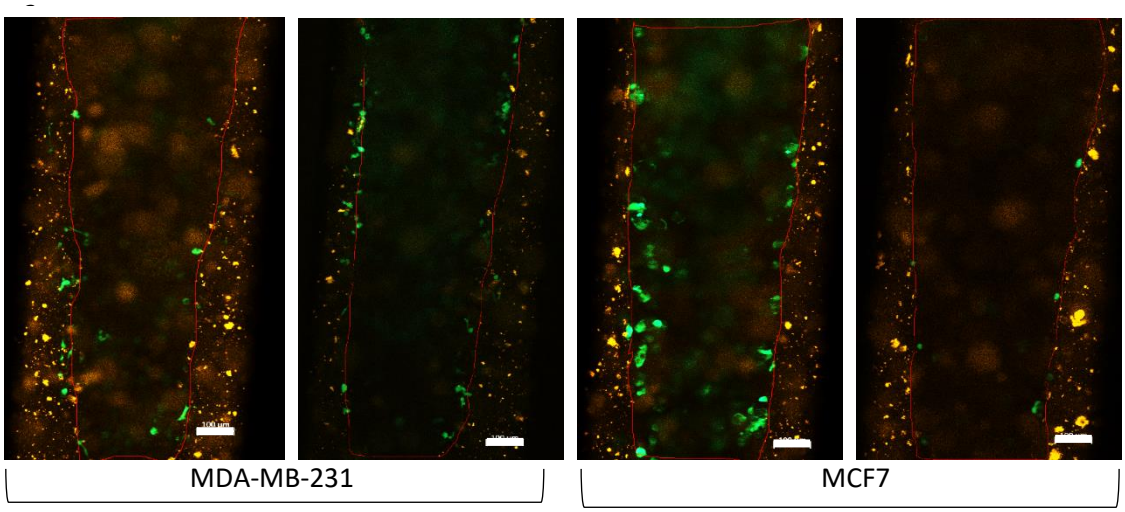


Figure 3.3: Measuring changes in permeability in microfluidic model. a) Time-lapse microscopy captured images through center of channel so measurements could be made immediately after fluorophore entered channel and as fluorophore diffused across the endothelial barrier. b) Stimulation of endothelium with TNFa for 4 hours caused a significant increase in endothelial permeability compared to untreated controls ($p = 0.0198$; NT $n = 12$, TNFa $n = 4$).

Figure 3.4: Quantification of extravasation phenotype of two breast cancer cell lines



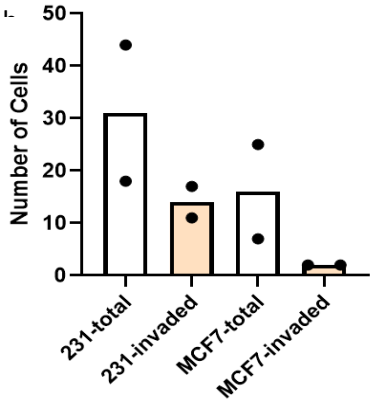


Figure 3.4: Quantification of extravasation in our model system. a) Optically sectioned images of 231 vs MCF7 invasion. Beads in collagen (orange) denote collagen space. Red lines were drawn using orange channel only and the cell channel (green) was overlaid so adhesion and invasion could be quantified by a blinded individual. “Adhered” cells we defined as those on the inner side of the red line and “invaded” cells were those mostly on the bead side of the red line. Scale bars 100 um. b) Quantification of MDA-MB-231 vs MCF7 extravasation from optically sectioned images. The total number of cancer cells (white bars) are similar between cell types, but the number of cells invaded were much higher for 231s (n = 2).

Chapter 4: Murine macrophages alter endothelial cell-cancer cell interactions

Introduction

To develop treatments that inhibit extravasation, we must first understand what makes the extravasation site suitable for tumor cells; several factors likely influence the "soil" tumor cells that eventually "seed."[3] Due to their ability to alter endothelial phenotype to recruit immune cells, direct tissue-repair functions, and their primary tumor-promoting properties, macrophages may be an important contributor to priming the pre-metastatic niche.[8] Additionally, macrophages promote pro-tumoral immune response, but can also polarize tumoricidal immune cells, which highlights macrophage immunotherapy potential.[8,22,41,94-96] However, macrophage roles in extravasation are not well understood. Literature indicates that primary tumor signals cause macrophage localization to secondary sites before the arrival of tumor cells, indicating that primary tumor signals and macrophages work in tandem to generate a tumorigenic pre-metastatic niche.[4,5,8,9,14-16,36] While these *in vivo* models are important for identifying these correlations, elucidating specific mechanisms is difficult due to the intrinsic complexity and difficulty visualizing early interactions at the extravasation site. Therefore, we have adapted a tunable, microfluidic model to study extravasation and we have incorporated macrophages in this model system to visualize how macrophages influence endothelial-tumor cell interactions in the secondary site.

Materials and Methods

Cell culture

Human lung microvascular endothelial cells (HMVEC-L) and MDA-MB-231 breast cancer cells were cultured as previously described.

RAW 264.7 Macrophages

RAW 264.7 macrophages (RAW macrophages) were generously gifted from Dr. Tanya Freedman's lab and cultured according to their protocols. Cells were grown in DMEM + 10% FBS and cultured to ~70% confluency before being passaged. RAW macrophages were passaged with 5mM EDTA after being rinsed with HBSS and split 1:10 into T75s. When lifted for experiments, RAW macrophages were counted, centrifuged, and resuspended at $1x10^6$ cells/mL for resuspension into collagen that was prepared as previously described.

Conditioned media collection

MDA-MB-231 cancer cells generously provided by Dr. Paolo Provenzano's lab were grown to 70% confluency in T-150 flasks in DMEM + 10%FBS. Media was removed and replaced with fresh DMEM+10%FBS and incubated at 5% CO_2 and 37˚C for 24 hours before being collected. Fresh cell culture media was added after collection and then collected after 48 hours. The conditioned cell culture media (CM) was pooled and filtered with a 0.02 um media filter (Corning) and aliquoted before freezing in working aliquots at -80˚C. For any CM experiment, 40% CM was combined with 60% EGM-2 MV (Lonza). Control DMEM was incubated in T150 flasks for 24 hours, collected, frozen, and diluted to 40% to serve as a control for CM treatment.

Microfluidic model set-up

As described previously, devices were prepared for loading collagen (Figure 3.1). After mixing and nucleating the collagen, the volume was split in half before adding the proper amount of DMEM (Blank) or $1x10^6$ cells/mL RAW macrophages in DMEM (RAW macrophage) into the collagen for a final concentration of 6 mg/mL collagen. Collagen and endothelial cells were added to all collagen channels as previously described.

After overnight incubation, half the blank and RAW macrophage devices were perfused with control media while the other half had CM flow introduced. Control and CM media was replaced every 24 hours for the duration of the experiment. After 48hrs, 10,000 MDA-MB-231 cells were added to each channel as previously described (Figure 3.1). Images were taken every 24 hours for 2 days and images were processed for cancer cell adhesion and transmigration.

Data Analysis for Adhesion and Extravasation

Z-stacks from each device were exported and processed using a custom macro in ImageJ (NIH). Using similar principles as described for processing MCF-7 vs MDA-MB-231 adhesion and transmigration described above, we wrote a custom macro to automatically quantify adhesion and transmigration. One macro was written to threshold and count the green cells to obtain a “total” cell count. A separate macro was written to threshold and determine the collagen area by “connecting the dots” made by beads in the collagen. Collagen segmented images were overlaid the cell images to subtract out cells that were outside the collagen to obtain an “invaded” cell count. For each device, the number of cells “Invaded” was divided by the “total” number in each device to obtain the invasion fraction.

Statistics

All data sets were tested for normality using the Shapiro-Wilk test. Statistical significance of normally distributed data was done with either an unpaired t-test (when comparing 2 sets of data) or one-way ANOVA corrected for multiple corrections with Dunn’s method. If the data were not normally distributed, then Mann-Whitney U test (for two comparisons) or Krustal Wallis test corrected with Dunn’s multiple corrections method

was used instead. Statistical significance indicated by: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results

To study how macrophages influence tumor cells extravasation in the secondary site, we used RAW macrophages in the collagen of our microfluidic model (Figure 4.1a). Using this model system, we will measure the effects of primary tumor signals on macrophage-endothelial interactions by measuring permeability, tumor cell adhesion, and invasion fraction of adhered tumor cells.

RAW Macrophage Viability in model system

To confirm that macrophages are alive in our system, we stained macrophages for viability in 3D collagen gels. Measuring viability with a Calcein AM live and Hoechst nuclear stain, we found that 89% of macrophages were viable within the collagen channel of our model system for at least 7 days.

We also wanted to ensure that the macrophages maintained their differentiation status. We stained RAW macrophages with murine macrophage marker F4/80 and found that all cells within the tissue stained positive for F4/80. When devices were fixed and imaged 48 hours after the addition of MDA-MB-231s, it appears that some tumor cells interact with the F4/80 positive macrophages within the tissue (Figure 4.1a,b). These data taken together indicate that RAW macrophages are viable in this model and retain macrophage phenotype throughout the duration of these experiments.

Permeability is not influenced by macrophages or CM

Macrophages are known influencers of endothelial permeability in the immune response, and evidence indicates that macrophages also influence permeability in cancer.

The “seed and soil” hypothesis is that cancer cells are disseminated throughout the body but will form secondary tumors where the “soil” is ideal for the “seed” to grow.[3] We hypothesize that macrophages in the secondary site partially influence the “soil” by increasing endothelial permeability to arriving tumor cells. Since primary tumors secrete cytokines into the bloodstream, we also hypothesized that these systemic tumor signals would affect macrophages in the secondary site. To test these hypotheses, we added macrophages to the collagen and generated conditioned medium from tumor cells to act as systemic tumor signals. In this model system, we hypothesized that macrophages or conditioned media alone would have minimal effect on endothelial permeability, while the combination of macrophages and conditioned media would significantly increase permeability.

Permeability significantly decreases in devices with untreated ECs, CM-treated ECs, ECs exposed to macrophages, and ECs exposed to CM-treated macrophages when compared to EDTA-removed endothelial controls (Figure 4.2). TNFα treated devices do not significantly alter permeability when compared to EDTA, indicating that these molecules increase endothelial permeability. However, our permeability measurements show no significant change in permeability by adding macrophage, conditioned media, or the combination of the two compared to untreated controls (Figure 4.2). While this is a surprising result, we hypothesize that macrophages and tumor signals have little impact on endothelial permeability in this model system and probably do not influence extravasation potential of arriving tumor cells. Therefore, any change in tumor cell extravasation potential is due to alternate EC surface expression, interactions directly with macrophages via adhesion (Figure 4.1), or from cytokine signaling among macrophages, ECs, and tumor cells.

Macrophages treated with CM influence cancer cell adhesion

Since bone marrow-derived macrophages have been associated with aiding intravasation of tumor cells[8,40] and secondary tumor development is often associated with macrophage-rich sites,[5,9,14,36] we hypothesized that tumor cells would transmigrate more efficiently across endothelial cells if macrophages resided within the tissue. Primary tumor signals are present in the bloodstream of patients with metastatic cancer,[97] so we also hypothesized that primary tumor signals—modeled with CM—would further increase extravasation potential by activating macrophages to promote a pro-metastatic phenotype in endothelial cells. To investigate these hypotheses, we analyzed adhesion and transmigration potential with CM, macrophages, or a combination of CM and macrophages.

Our data corroborates other recent data that there is no increase in 231 adhesion to ECs with RAW macrophages alone (Figure 4.3a).[31] With conditioned media alone, there is a slight, but statistically insignificant, increase in initial adhesion of tumor cells to the endothelium (Figure 4.3a) which indicates CM may upregulate surface receptors on HMVEC-Ls as it is known to do with HUVECs.[10] However, when macrophages are stimulated by CM for 48 hours, the number of MDA-MB-231 cells adhered to the endothelium is significantly decreased compared to CM alone (Figure 4.3a). We hypothesize that while CM may upregulate MDA-MB-231 adhesion molecules on ECs, some interaction between the CM and macrophages decreases the expression adhesion molecules, resulting in a decrease in MDA-MB-231 adhesion. Since adhesion is the first step to extravasation, understanding these mechanisms may provide insight into treatment options that may alleviate the spread of metastatic cancer. In understanding these interactions, we may also gain some insight into which tumor cells are more likely

to adhere than others. Are the tumor cells that adhere in the presence of macrophages intrinsically different than those that adhere in the absence of macrophages? Are those that adhere in the presence of macrophages more likely to extravasate than those that do not? To answer how macrophages and primary tumor signals influence extravasation potential, we analyzed how many of the total number of cells in each device had transmigrated into the tissue space.

Macrophages treated with CM affect extravasation

To quantify the effect of macrophages on extravasation potential of adhered 231s, we divided the number of extravasated cells by the total number of cells in the device. We hypothesized that cells adhered in the devices with macrophages would extravasate more readily into the collagen space than those that were not exposed to macrophages due to macrophage interactions with the endothelium and directly with cancer cells.

The invasion fraction does not increase between 24 and 48 hours with macrophages or CM alone but does significantly increase with the combination of CM and macrophages (Figure 4.3b). This indicates that the macrophages exposed to the CM may not initially promote adhesion, but over time these macrophages may be better suited to aid tumor cell extravasation. When compared with the initial adhesion data (4.3a), although fewer tumor cells adhere to the endothelium when it has been exposed to CM-primed macrophages, these tumor cells may be better suited for transmigration.

Investigation into CM-mediated Reduced Cancer Cell Adhesion

The inhibition of initial tumor cell adhesion in the presence of CM-treated macrophages was surprising, so we decided to investigate how macrophages influence adhesion under other conditions. Is this effect MDA-MB-231 specific? Does stimulation of the endothelium

with cytokines that are known to increase endothelial expression of adhesion receptors also increase tumor cell adhesion? To answer these questions, we used this model to quantify adhesion with two distinct triple-negative breast cancer cell lines, HS578T cells and MDA-MB-231 cells, with TNFα stimulated endothelial cells.

TNFα does not impact endothelial adhesion of MDA-MB-231 cells

Some studies show that MDA-MB-231 CM can promote tumor cell adhesion to the endothelium by upregulating EC adhesion receptors such as E-selectin, P-selectin, and ICAM-1.[10] We hypothesize that tumor cell CM may upregulate these adhesion receptors which increases tumor cell adhesion, but CM-treated macrophages counteract this effect by direct signaling with ECs. Since TNFα is a known promoter of endothelial expression of E-selectin, P-selectin, and ICAM-1 expression in HMVEC-Ls,[88,98] we used TNFα as a positive control. We have previously shown that TNFα stimulation of HMVEC-Ls in this model system significantly increases endothelial permeability (Figure 4.4), but here we will probe its effect on tumor cell adhesion.

We first tested if TNFα increased HMVEC-L expression of ICAM-1 in a 2D monolayer using IF. We found that 4hr TNFα stimulation greatly increased HMVEC-L expression of ICAM-1, and that CM-stimulation of ECs may have slightly decreased ICAM-1 expression (Figure 4.4 a). Because of this strong increase in ICAM-1 expression, we hypothesize that MDA-MB-231s will adhere more readily to TNFα stimulated ECs than untreated or CM-stimulated ECs in our model system. However, endothelial cells treated with TNFα in this model system exhibited baseline adhesion (Figure 3.5c), indicating that tumor cell CM activates endothelial cells differently than TNFα. Tumor cell adhesion is dependent on these adhesion receptors, so understanding what adhesion receptors are responsible is important. We hypothesize that TNFα with macrophages would also increase adhesion of

231s compared to macrophages alone or with CM since TNFα is considered an M1-stimulating cytokine.[53,54] Interestingly, it seems that TNFα induces the same adhesive phenotype as TNFα-activated endothelial cells alone (Figure 4.4c). Although endothelial permeability with TNFα was increased, this does not necessarily translate to adhesive phenotype.

Although there does not seem to be increased adhesion with TNFα treatment, we fixed and stained these devices for ICAM-1 expression to see if there are changes in expression or tumor cell localization. ICAM-1 expression seems to be decreased in the CM-treated macrophage condition compared to the other experimental conditions which is consistent with the initial adhesion results (Figure 4.4a). It appears that TNFα-stimulation upregulates ICAM-1 expression similarly to the 2D screen (Figure 4.4a) which implies that ICAM-1 may not be the primary EC receptor utilized by tumor cells in this model. However, some of the adhered tumor cells are co-localized with ICAM-1, which indicates that some tumor cells utilize ICAM-1 or that there may be other receptors—such as P- or E- selectin—that are necessary for endothelial adhesion. It is also possible that the sites with increased ICAM-expression in this model also have other endothelial receptors upregulated in the non-TNFα conditions, so tumor cell adhesion is dependent of the local combination on endothelial adhesion receptors.

RAW Macs alone seem to inhibit 578T adhesion

To evaluate if CM-stimulated macrophages also altered the adhesion of other tumor cell lines, CM was made from HS-578T breast cancer cells. Endothelial cells alone did not seem to be influenced by HS-578T CM (Figure 4.5). We observed that macrophages alone seemed to decrease initial cancer cell adhesion as much as CM-stimulated macrophages compared to their respective macrophage-free controls, although these changes in

adhesion were insignificant (Figure 4.5). This data implies that macrophages alone do influence the endothelium, but HS-578Ts are more sensitive to these changes than MDA-MB-231 cells. It also seems that HS-578T CM has less of an impact on endothelial and macrophage function since CM exhibits little effect with or without macrophages. Since not all triple-negative breast cancer cell lines behave similarly and should be studied independently.

Conclusions

Macrophages are known contributors to primary tumor progression and intravasation of tumor cells, but their roles in extravasation a not as well understood.[8] To study the potential roles of macrophages in tumor cell extravasation, we used a microfluidic model that consists of an endothelialized vessel channel boring through a 3D collagen hydrogel seeded with RAW macrophages. Some *in vivo* studies have found that primary tumor signals and exosomes can alter macrophage function in the secondary site to aid tumor metastasis.[5,9,14,15] We modeled these primary tumor signals using tumor cell CM to evaluate how the cytokines from primary tumor may impact distant metastases.

Our permeability measurements did not indicate any significant changes in endothelial permeability with the addition of macrophages, CM, or the combination of the two. While this result was surprising, there may be some changes in endothelial permeability that this model is not sensitive enough to detect. While large changes in endothelial permeability likely allow tumor cells to more easily transmigrate, we concluded that the impact of global endothelial permeability was negligible in this model system, but there may be some impact of local changes in permeability.

Upon the addition of tumor cells under flow, we found that macrophages alone do not alter 231 adhesion to ECs. While primary tumor signals may slightly increase endothelial affinity to tumor cells, CM-treated macrophages significantly decreased tumor cell adhesion to the endothelium. Since there was a slight increase in adhesion with CM compared to untreated, blank controls, we hypothesize that some factors in the CM increase adhesion molecules on the surface of HMVEC-Ls. Some evidence has indicated that MDA-MB-231-CM upregulates STAT-3 signaling in HUVEC monolayers. This upregulation of the STAT-3 pathway results in a significant upregulation of E-selectin, P-selectin, and ICAM-1 which are all associated with increased tumor cell adhesion.[10] However, CM seemed to reduce ICAM-1 expression in 2D and 3D but exhibited increased tumor cell adhesion while the addition of TNFα upregulated endothelial expression of ICAM-1, but tumor cell adhesion was unaffected. Upon staining microfluidic devices for ICAM-1, we found that while some tumor cells were adhered in high ICAM-1 regions, others were not. While this could be due to tumor cells blocking the ICAM-1 molecules from ICAM-1 antibodies, the lack of adhesion to TNFα treated devices implies that other adhesion receptors are more important for endothelial adhesion. Therefore, since CM seems to upregulate these important receptors, we hypothesize that RAW macrophages exposed to CM secrete signals that counteract the upregulation of these surface adhesion molecules, leading to decreased MDA-MB-231 adhesion.

Even though initial adhesion decreased, the invasion fraction between 24 and 48 hours significantly increased when CM-treated macrophages were present, but the invasion fraction was insignificant between 24 and 48 hours in all other conditions. If macrophages exposed to CM reduce adhesion molecules that aid tumor cell adhesion to the EC, but adhered tumor cells in these devices also exhibit increased invasion efficiency,

the lack of adhesion molecule interactions may allow tumor cells to more easily transmigrate. This would suggest that there is an optimal number of adhesion receptors that allows for tumor cells to stick to ECs, but also not too many that they are unable to unclasp those bonds.

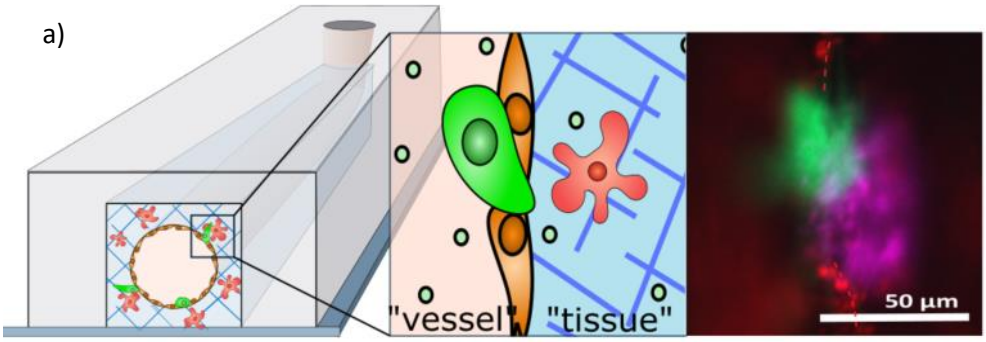
Tumor cells—even from established cell lines—are intrinsically heterogenous.[99] An alternative hypothesis is that there is something unique about tumor cells that adheres to ECs in the presence of CM-stimulated macrophages that allows for them to more easily transmigrate, such as alternate adhesion receptors that activate unique pathways from those that adhere in the other conditions, or there is some paracrine loop between tumor cells and CM-treated macrophages such as the EGF-CSF-1 paracrine loop that is hypothesized to aid intravasation.[75]

Direct contact with macrophages is thought to promote extravasation.[65] Since tumor cells appear to interact with macrophages in this model system (Figure 4.1), direct contact with macrophages on the basal side of the endothelium may aid tumor cell extravasation (Figure 4.1). These interactions may be catalyzed by systemic signals, such as those in CM. Alternatively, macrophages pre-conditioned with CM may be prepared to eradicate tumor cells on arrival while non-stimulated macrophages do not recognize the threat. These pre-conditioned macrophages may eradicate some tumor cells as they arrive, but the tumor cells that are left are better suited to extravasate. Some macrophages may be activated to eradicate the arriving tumor cells, but these macrophages self-destruct after consuming the tumor cells. This may alter endothelial permeability to tumor cells so they can more easily transmigrate, leaving the remaining macrophages primed to aid tumor cell extravasation.

While this model does not account for the intrinsic complexities of the secondary tumor microenvironment, it does provide some surprising insight into the potential role of macrophages in the pre-metastatic niche. When studying macrophage influence on tumor cell extravasation, it is important to account for systemic signals from the primary tumor. Several factors from the primary tumor secretome are associated with increased metastatic burden,[97] so understanding how these signals affect the pre-metastatic niche may illuminate some metastasis-inhibiting therapeutics. We aim to use this model to probe some of these developing hypotheses and utilize human macrophages to further study these macrophage-endothelial cell-tumor cell interactions.

Figures

Figure 4.1: Macrophages interact with tumor cells during extravasation



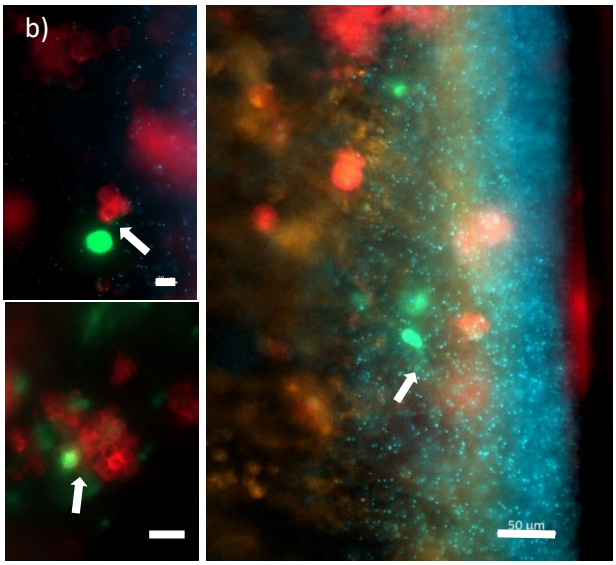


Figure 4.1: macrophages interact with extravasating tumor cells in this model system. a) Schematic of microfluidic model to study how primary tumor signals alter macrophage interactions with tumor cells during extravasation. Fluorescent image of MDA-MB-231 cell (green Extravasating endothelium (red) while in direct contact with macrophage (Purple). b) F4/80 positive macrophages (red) are present in devices and may interact with MDA-MB-231tumor cells (green) in collagen space denoted by blue beads. White arrow indicates extravascular tumor cell making protrusion towards cluster of F4/80+ macrophages. Images are overexposed in green channel to show cytoplasmic protrusions since GFP is nuclear. Scale bars: top left = 20 um, bottom left and right = 50 um

Figure 4.2 Permeability of the endothelium is unaffected by macrophages and CM

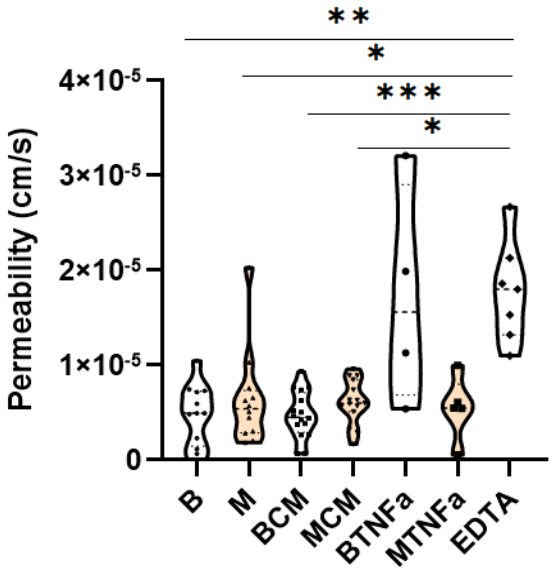


Figure 4.2: Permeability of endothelium does not significantly change in the presence of CM and/or RAW macrophages. Compared to EDTA-treated devices, untreated controls (B; p = 0.0014), RAW macrophage-only (M; p = 0.0100), CM-only (BCM; p = 0.0005), and CM-treated RAW macrophage (MCM; p = 0.0378) had significantly lower permeabilities. Neither TNFα-treated condition was significantly different than EDTA-treated controls, indicating permeability was higher in the TNFα-treated conditions. Since there is little influence on permeability with macrophages and/or CM, we assume any changes in extravasation are not due to altered endothelial permeability.

Figure 4.3: CM-treated macrophages inhibit MDA-MB-231 adhesion but promote transmigration

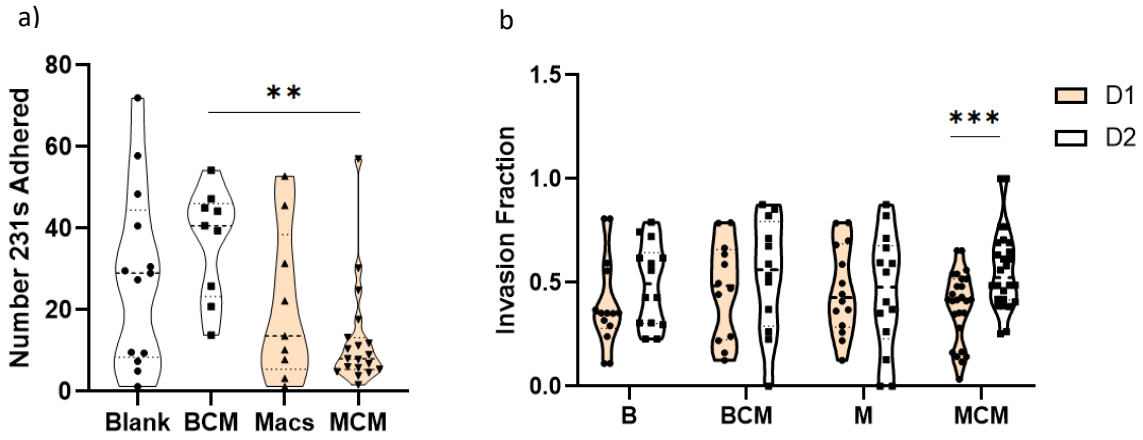


Figure 4.3: RAW macrophages exposed to CM alter tumor cell extravasation. a) RAW macrophages (M) alone do not seem to influence initial tumor cell adhesion compared to untreated controls (B), but there is a significant decrease in adhesion between CM-treated macrophages (MCM) and CM-only controls (BCM; p = 0.0039). b) Invasion fraction (# 231s in collagen/# 231s in device) is significantly increased in devices with CM-treated macrophages between 24 and 48 hours, but not in any other condition (p = 0.0009). Macrophages alone do not promote tumor cell extravasation, but extrinsic signals from the primary tumor may alter these interactions.

Figure 4.4: Endothelial activation with TNFa does not promote MDA-MB-231 adhesion

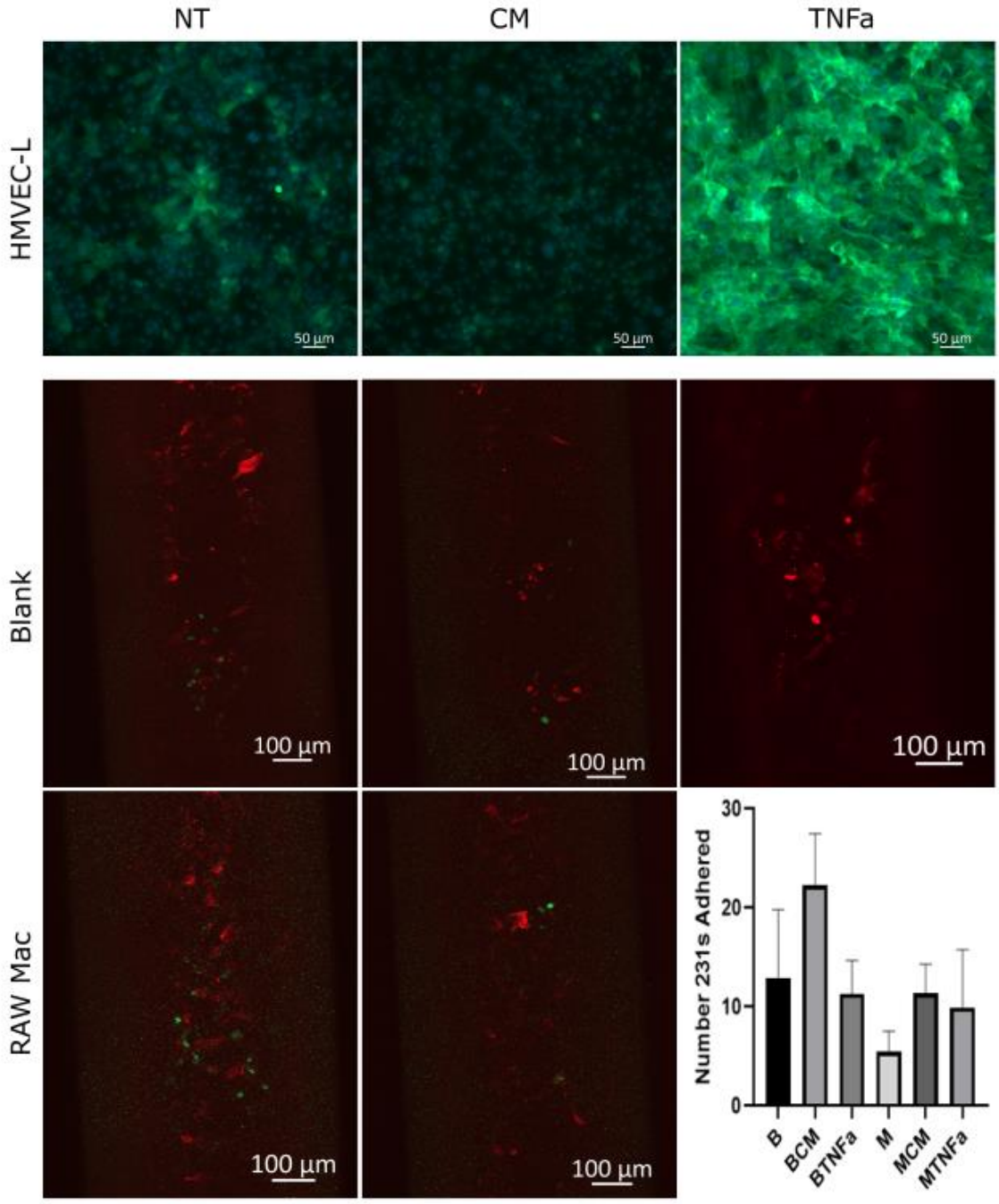


Figure 3.5: TNFα alters endothelial expression of cell adhesion-proteins but does not significantly image tumor cell adhesion. a) ICAM-1 expression (green) is greatly increased when activated with TNFα, and may inhibited by 231 CM. b) ICAM-1 (red) expression seems increased when HMCEC-Ls are treated with TNFα but is qualitatively decreased when macrophages are pre-treated with CM. MDA-MB-231s (green) are sometimes co-localized with ICAM-1 high regions but are not exclusively adhered in these areas. c) There is no significant change in 231 adhesion in the presence of TNFα, but there are similar trends in the CM-treated devices that we saw previously. CM seems to promote adhesion, but reduce expression of ICAM-1 which is unexpected

Figure 4.4: Hs578T breast cancer cell adhesion seems to be inhibited by macrophages

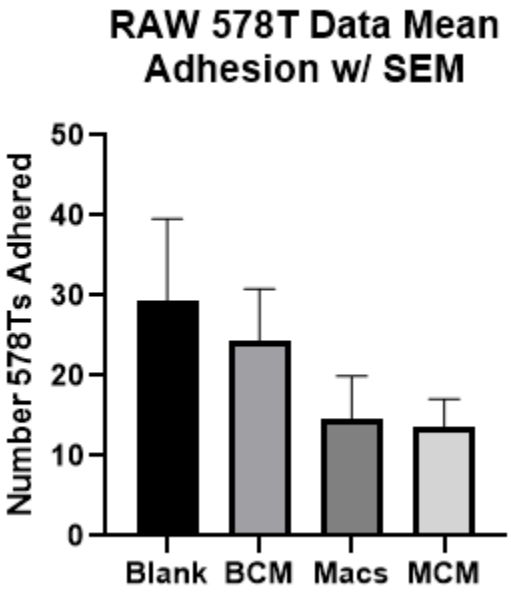


Figure 4.4: HS578T Adhesion to the endothelium may be affected by Macrophages. Although insignificant, trends indicate that HS578T adhesion is reduced in the presence of RAW macrophages, but CM has little impact.

Chapter 5: Human Macrophages primed with CM alter Endothelial Cell-Cancer Cell Interactions on a Donor-Specific Basis

Introduction

Macrophages are known contributors to cancer progression in the primary site, but their roles in distant metastasis formation are not as well understood. Recent *in vivo* studies have highlighted potential roles of macrophage in secondary tumor seeding and progression, but due to intrinsic biological complexity and difference in human and murine macrophages understanding distinct mechanisms is difficult.[26] Several studies have found that organotropic metastases are associated with recruitment of bone marrow-derived macrophages to the pre-metastatic niche.[4,8,9,14,46-48] While some of these studies examine how these murine macrophages aid tumor extravasation, less is known about how human bone-marrow derived macrophages alter extravasation. One microfluidic model examining roles of blood-derived human monocytes and macrophages in extravasation demonstrate that macrophages alone do not alter extravasation potential of tumor cells.[31] While our microfluidic model system also found that murine macrophages in collagen do not affect tumor cell extravasation, pre-treatment of these cells with tumor cell conditioned media indicate that primary tumor signals may alter macrophage function in the secondary site (previous section). We aim to use our microfluidic model to create an all-human *in vitro* system to study how human macrophages may contribute to metastatic progression and if these interactions are altered in response to systemic stimuli.

Materials and Methods

Cell culture

HMVEC-Ls and MDA-MB-231 were cultured as described previously.

Human PBMC-derived macrophages

Human peripheral blood mononuclear cells (PBMCs) were isolated from donor blood obtained from Memorial Blood Center (St. Paul) using protocols and help from Dr. Schwertfeger's lab. Due to donor variability, 5 donors were used in these experiments. Approximately 10mL of blood from Trima cones were drained into conical tubes, diluted with 30mL 1x PBS (Corning). We added 15mL Isolymph (CTL Scientific) to two separate conical tubes and 20mL of blood solution was carefully layered on top of the Isolymph. To isolate the buffy coat, we centrifuged the tubes at 800xg for 20 minutes with the brake off. After centrifugation, we collected the buffy coat and transferred it to a new 50mL conical tube. After washing the buffy coat with 30mL of 1x PBS, we centrifuged again at 400 x g for 10 minutes and resuspended the pellet in 10mL of 1xdPBS before counting. We then either froze the cells in RPMI + 50%FBS + 10% DMSO (Sigma) at $2.5x10^7$ cells/mL or completed the monocyte enrichment step.

We positively selected CD14+ monocytes from the PBMCs using magnetic activated cell sorting (MACS). PBMCs were thawed, resuspended in complete RPMI, and centrifuged at 300x g for 10 minutes. The cell pellet was resuspended in 80 uL/10^7 PBMCs in sterile FACS buffer (10 mM EDTA + 0.5% FBS + PBS) and 20uL CD14+ magnetic microbeads (Miltenyi Biotec). This solution was mixed well and incubated at 4˚C for 15 minutes before being washed with 1 mL of FACS buffer. After centrifugation at 300 x g for 30 min, up to 10^8 cells were resuspended in 500 uL FACS buffer. A MACS column (Miltenyi Biotech) was placed in a MACS magnet (Miltenyi Biotech) and rinsed with 3 mL buffer before adding the PBMC + CD14+ magnetic bead solution. The column was washed 3 times with 3 mL FACS solution until all the solution washed though the column. The column is removed from the magnet and placed into a collection tube where 5 mL FACS

solution was flushed though the column with the provided plunger. Cells were either plated or frozen at 3 x 10^6 cells/mL in the PBMC freezing media.

To differentiate CD14+ monocytes to macrophages, we plated 1 x 10^6 CD14+ monocytes in each well of a low-retention 6 well plate (Corning) in RPMI + 10% FBS + 1% pen/strep + 60 ng/mL M-CSF (Biolegend) and incubated at 37˚C for 5 days. After 5 days, we added additional media supplemented with M-CSF to create a final concentration of 60 ng/mL and incubated for an additional 2 days before use.

We removed the media and non-adhered cells before adding 5 mL of RPMI to the well. Using a 1 mL pipette, we gently washed the bottom of the plate to lift the cells from the bottom of the well and added them to a 15 mL conical tube. Cells were counted, centrifuged, and resuspended at 5x10^5 cells/mL.

Macrophage staining and polarization

For polarization staining experiments, macrophages were plated in 96-well plates at 1x10^4 cells/well and incubated overnight. Media was changed and replaced with control media (40% DMEM + 60% EGM-2 MV), conditioned media (40% MDA-MB-231 conditioned DMEM + 60% EGM-2 MV), control media with M1 cytokines 20 ng/mL IFN-gamma (PeproTech) + 100 ng/mL LPS (Sigma Aldrich), or control media with M2 cytokines 20 ng/mL IL-4 (PeproTech) + 20 ng/mL IL-13 (PeproTech). After incubation for 24 hours, cells were rinsed twice with 1x PBS, fixed with formalin for 1 hour at room temperature, washed 3 times with 1x PBS, and blocked and permeabilized with 10% FBS and 0.1% tween20 for 30 minutes. Cells were stained for human macrophage marker CD68 (RnDSystems), M1 marker iNOS (NovusBio), or M2 marker CD206 (RnDSystems) and counterstained with 1 ug/mL hoescht to visualize nuclei.

Microfluidic Devices with Human Macrophages

Microfluidic devices were set up as described previously except human macrophages were added to half the unpolymerized collagen at a final concentration of $5x10^5$ cells/mL before being loaded into devices (Figure 3.1). After the overnight incubation following channel formation and endothelialization, devices received either control media or conditioned media for 24 hours as explained previously. If necessary, TNFα, M1, or M2 treatments were added 24 hours before permeability was measured or cancer cells were added. Permeability and cancer cell adhesion and transmigration were measured as previously described (Figure 3.3).

Data Analysis for Adhesion and Extravasation

Z-stacks from each device were exported and processed using a custom macro in ImageJ (NIH). Using similar principles as described for processing MCF-7 vs MDA-MB-231 adhesion and transmigration described above, we wrote a custom macro to automatically quantify adhesion and transmigration. One macro was written to threshold and count the green cells to obtain a “total” cell count. A separate macro was written to threshold and determine the collagen area by “connecting the dots” made by beads in the collagen. Collagen segmented images were overlaid the cell images to subtract out cells that were outside the collagen to obtain an “invaded” cell count. For each device, the number of cells “Invaded” was divided by the “total” number in each device to obtain the invasion fraction.

Statistics

All data sets were tested for normality using the Shapiro-Wilk test. Statistical significance of normally distributed data was done with either an unpaired t-test (when comparing 2 sets of data) or one-way ANOVA corrected for multiple corrections with

Dunn's method. If the data were not normally distributed, then Mann-Whitney U test (for two comparisons) or Krustal Wallis test corrected with Dunn's multiple corrections method was used instead. Statistical significance indicated by: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results

We evaluated how systemic signals influence interactions between human macrophages and endothelial cells in the context of extravasation. While there has been a wealth of information about macrophages in cancer done with murine models, it is important to also study macrophage-tumor cell interactions with human cells.[26] By seeding human macrophages into our extravasation mode, we have visualized interesting interactions and ultimately uncover mechanisms that can be used to prevent further metastatic spread in cancer patients.

Macrophage Viability and Phenotype

To ensure that we successfully differentiated CD14+ monocytes to macrophages, we stained macrophages with human macrophage marker CD68. IF staining shows that these cells are macrophages (Figure 5.1a), indicating that we successfully differentiated monocytes and can use them as macrophages in experiments.

We stained human macrophages in 3D collagen gels with calcein to indicate viability and counterstained the nuclei with DRAQ5 to get a live and total count (Figure 5.1 b,c) We found that macrophages in collagen were 98% viable after 5 days in culture, showing that macrophages can survive in collagen for the duration of our experiments. We also stained macrophages for viability in our devices to ensure nothing about the loading process or co-culture with endothelial cells altered macrophage viability. Macrophages in

the collagen (Figure 5.1b,c) and endothelial cells (Figure 5.1c) are highly viable in this system after 5 days. Since macrophages remain alive in our model system, we assume that macrophages can alter phenotypes in this model system.

Permeability is affected by macrophages in a donor-dependent manner

Endothelial permeability measurements were done to analyze if human macrophages, primary tumor signals, or macrophages exposed to primary tumor signals altered endothelial function. We hypothesize that macrophages and tumor signals alone would slightly increase endothelial permeability—which would result in increased transmigration—and the combination of these factors would greatly increase permeability. Since TNFα is a known permeability-increasing cytokine, we also treated devices with TNFα as a positive control.

These experiments were conducted with macrophages isolated from two blood donors. When permeability data is combined, there is a significant increase in endothelial permeability when TNFα is added to macrophage-free devices compared to untreated controls (Figure 5.2c), which is consistent with previous mouse macrophages data from this model system (Figure 4.4). However, macrophages treated with TNFα have little impact on permeability compared to TNFα only controls, indicating that these changes in permeability may be purely from TNFα-endothelial interactions. The combined data also suggest that macrophages alone tend to increase endothelial permeability, but when separated by donors there is a significant increase with donor 1, but not with donor 2 (Figure 5.2 a,b). Additionally, CM-treated macrophages from donor 1 seem to slightly increase permeability while the same treatment from donor 2 seems to have little effect. Macrophages from donor 1 may be in a basal, immune-recruiting state while those from donor 2 are more immune suppressive. Although the influence on endothelial permeability

differ depending on donor, it appears as though macrophage phenotypes can alter permeability in this model system which may impact tumor cell extravasation.

Adhesion of MDA-MB-231a is affected by macrophages

Cancer cell adhesion to the endothelium is an important step in metastasis. Some studies have shown that for a cancer cell to transmigrate, it must first have direct interactions with surface receptors on endothelial cells.[100,101] When we evaluated the effects of primary tumor signals and macrophages on tumor cell adhesion with murine RAW macrophages (Figure 4.3a), we found that CM alone slightly increased MDA-MB-231 adhesion to ECs but macrophages treated with CM seemed to negate this effect by significantly decreasing initial tumor cell adhesion to the endothelium. This result was surprising since the current literature suggests that primary tumor signals in the secondary site activate macrophages, which then activate endothelial cells.[8,102] We were also surprised to find that TNFα does not increase MDA-MB-231 adhesion to the endothelium since TNFα upregulates endothelial adhesion receptors that are implicated in tumor cell adhesion in response.[10,88] To determine if these surprising results translate to an all-human system, we replaced the murine macrophages with PBMC-derived human macrophages.

Adhesion of MDA-MB-231 Cancer Cells in Response to CM and/ or Human Macrophages

In experiments with macrophages from donor 1, we found that CM treated devices exhibited an insignificant increase in initial MDA-MB-231 adhesion, macrophages alone had little effect on MDA-MB-231 adhesion, but CM-treated macrophages had a significant decrease in initial MDA-MB-231 adhesion compared to CM-treated devices (Figure 5.3a). Permeability measurements indicated that these macrophages were more influential on endothelial permeability, so it was surprising that this effect did not translate to any change

in adhesion. Similarly to murine RAW macrophages (Chapter 3), human macrophages that are exposed to primary tumor signals may reduce tumor cell adhesion in the secondary site, contrary to what is shown in the literature.

We repeated these experiments with macrophages from donor 2 and found a slight, insignificant decrease in MDA-MB-231 adhesion between CM-treated devices and CM-treated macrophage devices. While there was also less of an increase in adhesion with CM-stimulated ECs, there is a slight—but insignificant—decrease in adhesion when macrophages are present in the devices (Figure 5.3b). While macrophages from donor 2 may not be as sensitive to CM as donor 1 macrophages, they may be intrinsically anti-inflammatory since there is some loss of adhesion in both macrophage conditions.

In both sets of experiments, we observed that TNFα treatment alone slightly decreased initial tumor cell adhesion to the endothelium, which was surprising but reflects our data from previous studies. However, we were surprised to see that TNFα treated macrophages from both donors significantly increased initial adhesion to the endothelium unlike what we observed with RAW macrophages (Figure 5.3c). Although not significant, this trend is apparent when donors are evaluated separately as well (Figure 5.3 a,b). This indicates that these human macrophages are more sensitive to TNFα stimulation that may be polarizing them to an “immune-recruiting” phenotype which alters the endothelium to aid tumor cell adhesion. One potential hypothesis is that patients with increased systemic TNFα may have increased tumor cell adhesion due to macrophage polarization and not necessarily from direct effects of TNFα on the endothelium. Overall, macrophage-dependent mechanisms that can promote tumor cell adhesion during extravasation are regulated by exogenous signals, highlighting the need to understand primary tumor

secretome influences on macrophages as a potential target in limiting circulating tumor cell adhesion.

Invasion Fraction of 231s is Not Impacted by CM-treated Macrophages

Although we observed some changes in initial tumor cell adhesion, we were curious how macrophages and primary tumor signals impact the ability of tumor cells to transmigrate the endothelium. We hypothesized that extravasation efficiency is correlated with endothelial permeability, since an increase in permeability implies that there are more holes in tight junctions that tumor cells could extravasate through. We found that with RAW macrophages, the invasion fraction between 24 and 48 hours was significantly increased when macrophages were pre-treated with CM compared to all other treatment groups (Figure 4.3b). We hypothesize that human macrophages will have a similar influence on tumor cell extravasation.

We found that invasion fractions between 2 and 24 hours increased significantly in untreated, CM-only, macrophage-only, and CM-treated macrophage conditions (Figure 5.4). Additionally, there was a significant increase in invasion fraction in all conditions except TNFα treated macrophages. We hypothesize that increases between 2 and 24 hours are significant since studies suggest that transmigration occurs over a scale of hours.[103] For this measurement, tumor cells only have 2 hours to adhere and transmigrate before the first timepoint, so not all transmigrating cells would complete the migration by then. However, we were surprised to see that when treated with TNFα, there is no significant change in tumor cell extravasation between the 2 and 24 hour timepoints due to the relatively higher endothelial permeability and intrinsic invasiveness of MDA-MB-231s. similarly, it is more surprising that there is no significant increase in transmigration between 2 and 48 hours in the TNFα treated macrophage condition. The discrepancy in

macrophages treated with TNFα increasing adhesion but decreasing invasion fraction indicates that macrophage phenotypes that promote adhesion do not necessarily promote extravasation. One hypothesis for this phenomenon is that upregulation of adhesion molecules may help tumor cells adhere to the endothelium but impede transmigration due to the increased number of bonds that would need to be released to migrate. Alternatively, adhesion molecules upregulated by TNFα-stimulated macrophages may be adhesive to both invasive and less invasive tumor cells, while the other conditions may upregulate adhesion molecules that adhere to basally more invasive cells.

M1 and M2 Polarization in Extravasation Potential of MDA-MB-231s

Since we have observed that CM-treated macrophages seem to decrease and TNFα-treated macrophages seem to increase endothelial adhesion of tumor cells, we hypothesize that macrophage polarization may play a role in extravasation potential of tumor cells. Although TNFα treated macrophages upregulate adhesion, there is a smaller change in the fraction of adhered tumor cells that ultimately transmigrate the endothelium compared to other treatment groups. We hypothesize that TNFα stimulation of macrophages results in a more M1-like phenotype which would promote expression of endothelial surface receptors and increased permeability. Since the M1-like phenotype leads to increased immune adhesion and infiltration, we hypothesize that this would also lead to increased tumor cell extravasation unlike what we observed with TNFα treated macrophages. We further hypothesize that CM generates a M2-like polarization in macrophages, which would result in lowered permeability and reduced surface receptor expression. While this would not aid tumor cell adhesion and extravasation, we hypothesize that M2 polarized macrophages may aid secondary tumor colonization since M2 polarization is synonymous with tissue repair.[50-52] To test this hypothesis, we polarized

macrophages with wither M1(IFN-gamma + LPS) or M2 (IL-4 + IL-13) cytokines for 16 hours before measuring permeability or adding tumor cells.

Macrophage polarization with M1 and M2 Cytokines

Before investigating the roles of macrophage polarization in our microfluidic model system, we wanted to vizualize how stimulation with M1 or M2-like cytokines altered human endothelial cell and macrophage phenotype. To see how these cytokines alter endohtelial cell phenotype, we first treated HMVEC-Ls with CM, TNFα, M1 (LPS + IFN-gamma), or M2 (IL-4 + IL-13) cytokines to observe changes in ICAM-1 expression. As shown in literature TNFα greatly increases and CM slightly increases ICAM-1 expression compared to untreated controls.[10,98] It also appears as though M1 cytokines induce a TNFα-like upregulation of ICAM-1 while M2 cytokines alone appear more similar to CM-stimulated HMVEC-Ls (Figure 5.5b).

We also treated macrophages from donors 8 and 9 to see how their macrophages respond to M1/M2 stimulation. From IF staining of M1 marker iNOS and M2 marker CD206, we found that M1 and M2 stimulation seem to decrease both iNOS and CD206 compared to untreated and CM-treated macrophages (Figure 5.5a). This is surprising since stimulation with these cytokines should induce M1 or M2-like polarization. However, donor 9 exhibited an increase in CD206 expression with M1 stimulation and a decrease in CD206 exression with M2 stimulation, which is the opposite of what we would expect from this experiment. Additionally it appears as though untreated macrophages from donor 9 tend to have more CD206-positive cells than CM-treated macrophages—although these gene expresion changes are not what we expected—CM and M2-stimulated macrophages exhibited a more similar phenotype than M1-stimulated macrophages. Overall, macrophages from donor 9 seem to have an increased responsiveness to cytokine

stimulation, so we hypothesize this will translate to more dramatic alterations in extravasation phenotypes than experiments done with donor 8.

Permeability is influenced by macrophage polarization

During the immune response, polarization of macrophages is an important factor in altering endothelial permeability to solutes and arriving immune cells.[104] We hypothesized that CM would generate a M2-like phenotype in macrophages, which is correlated with a more anti-inflammatory phenotype. If true, M2-stimulation would result in a smaller increase in permeability and reduced tumor cell adhesion to the endothelium. Alternatively, we hypothesized that M1-cytokines would induce a similar response to TNFα-treated macrophages—increasing permeability and tumor cell adhesion. As controls, we treated macrophage-free devices with M1 and M2 cytokines to elucidate macrophage influence on the endothelium from direct cytokine influence on the endothelium.

These experiments were conducted with macrophages from 3 separate donors (donors 7, 8, and 9). While it appears that CM and macrophages alone increase permeability in these donors, CM-treated macrophages seem to reduce permeability back to basal levels similarly to permeability data from Donor 2. This indicates that in 4/5 donors, CM-treated macrophages may inhibit the permeability-increasing effects of CM by some form of anti-inflammatory activation. Surprisingly, M1 (IFN-gamma + LPS) and M2 (IL-4 + IL-13) cytokines had no influence on endothelial permeability on the endothelium alone (Figure 5.6). However, in devices with macrophages there seems to be an increase in endothelial permeability when macrophages were stimulated with M1 and M2 cytokines compared to macrophage-free controls (Figure 5.6). When compared to macrophage-only controls, M1 stimulation cause a slight increase in permeability—almost to the level of

TNFα treated macrophage—while M2 stimulated devices seem to have a similar distribution as macrophage-only controls (Figure 5.6). Although the changes are statistically insignificant, these trends indicate that macrophage polarization does alter endothelial permeability in this model system, so polarization may play other roles in tumor cell adhesion and extravasation.

Initial Adhesion with M1/M2 stimulation

We found that polarizing macrophages towards an M1 or M2 phenotype may alter endothelial permeability, so we hypothesize that tumor cell adhesion and transmigration may also be affected by macrophage polarization. Initially, we wanted to observe how macrophage-polarizing molecules impact endothelial expression of adhesion molecules such as ICAM-1. We found that M1 cytokines (IFN-gamma + LPS) qualitatively upregulated ICAM-1 expression almost to the level of TNFα, while M2 cytokines (IL-4 + IL-13) may slightly increase expression to the level of CM stimulation.

When data initial adhesion data from all donors is combined, there are no statistically significant changes in adhesion (Figure 5.7d). In macrophage-free controls, M2 cytokines seem to downregulate endothelial adhesion of 231s. It appears there is a slight, but insignificant, decrease in tumor cell adhesion when macrophages are present except for M2 and TNFα-treated macrophage conditions. M2-stimulated macrophages exhibit a noticeably larger increase in adhesion compared to the macrophage-free M2 control, which indicates that M2-polarized macrophages may upregulate endothelial adhesion of circulating tumor cells.

Since we showed that different donors exhibit different tumor cell adhesion properties to the same stimulation (Figure 5.7a,b,c), we wanted to analyze data based on

macrophage donor. All three donors seem to exhibit a decrease in 231 adhesion when macrophages are pre-treated with CM, but only donor 9 showed a significant decrease (Figure 5.7c). Since there appears to be no change in adhesion between untreated and CM-treated devices in these experiments, we hypothesize that CM-treated macrophages reduce tumor cell adhesion.

Changes in tumor cell adhesion under M1/M2 stimulation seem to have a more variable response across donors. There is a significant upregulation of 231 adhesion when donor 7 macrophages are treated with M2-stimuli compared to untreated macrophage controls. Since macrophages from donor 7 also exhibit an increase in adhesion compared to macrophage-free devices treated with M2 cytokines, it seems that some interaction between M2-polarized macrophages and endothelial cells that leads to increased tumor cell adhesion. M1 stimulation only exhibited a small, insignificant increase in adhesion compared to macrophage-only controls but were approximately the same as endothelial cells treated with M1 cytokines, so M1 stimulation does not alter adhesion (Figure 5.7a). This is surprising since M1-stimulation is associated with upregulation of endothelial adhesion molecules that aid tumor metastasis.

We expected macrophages from donor 9 to cause an increase in 231 adhesion since these macrophages had a greater baseline M1/M2 marker expression and were more responsive to M1/M2 stimulation than macrophages from donor 8 (Figure 5.7b,c). Overall, macrophages from donor 8 seem to promote adhesion more than macrophages from Donor 9 which indicates that in some cases, baseline polarization may lead to reduced endothelial activation. However, donor 8 may also have slightly increased 231 adhesion with M1 and M2 stimulation, while donor 9 appears to have a slight decrease in adhesion

after both M1 and M2 treatment. Taken together, macrophages can alter tumor cell adhesion to the endothelium, but these effects may be patient-specific.

Extravasation is influenced by macrophage polarization

To test the hypothesis that tumor cell extravasation is altered by macrophage phenotype in the secondary site, we compared what fraction of tumor cells were in the collagen. Two hours after tumor cells were added to devices, there was a significant increase in the fraction of transmigrated cells in M2-treated macrophages compared to macrophages-only controls (Figure 5.8a). Although not significant, it appears as though tumor cells also transmigrate more easily when macrophages are treated with M2 cytokines compared to macrophage only (M), macrophage-free M1 (BM1), and macrophage-free M2 (BM2) controls (Figure 4.8a). Macrophages stimulated with cytokines associated with immune recruitment allow for increased transmigration rate, which is synonymous with increasing endothelial permeability to immune cells. There is no change in invasion fraction between any treatments after 24 hours, but after 48 hours there is a significant increase in tumor cell transmigration fraction in M2-treated macrophages compared to M2 cytokines alone (MM2 vs BM2; Figure 5.8 c). Since it appears that BM2 invasion fraction decreased dramatically between 24 and 48 hours, there may be some experimental discrepancy caused by tumor cells flowing in from the inlet after 24 hours.

As described previously, MDA-MB-231 invasion fractions in untreated, CM-treated, macrophage, and CM-treated macrophage devices increased significantly between 2-24 hours and 24-48 hours. Although this trend does not hold in M1 or M2 treated macrophage devices, it appears as though tumor cells in these devices invade more quickly, so almost all the MDA-MB-231 cells transmigrate soon after adhesion (Figure 5.8d). This indicates

that macrophages polarized towards M1 or M2 may promote more efficient extravasation behavior, either by altering endothelial function or through some form of direct interaction with tumor cells.

Conclusion

Macrophage roles in extravasation are not well-defined, but it is likely that their microenvironment is influential on early interactions between macrophages and tumor cells in the metastatic site. Since macrophages can alter endothelial phenotype, we hypothesized that macrophages may influence early extravasation events by altering endothelial cell phenotype, which is dependent on macrophage response to systemic signals. We tested this hypothesis by measuring endothelial permeability to solutes, endothelial adhesion of tumor cells, and the transmigration efficiency of adhered cells.

In response to modeled primary tumor signals (CM) we found that there was a slight, but insignificant, trend in endothelial permeability. While macrophages and tumor CM alone seem to increase permeability, macrophages treated with CM seem to decrease the effects of CM alone. We found this result surprising since literature suggests that primary tumor signals activate macrophages to promote immune-recruiting behavior, which is thought to be a mechanism in preparing the pre-metastatic niche to recruit tumor cells.[5,9,14,15] However, this decrease in permeability suggests that tumor pre-conditioned macrophages suppress endothelial activation, which may restrict extravasation of tumor cells. This hypothesis is supported by data showing that macrophages treated with CM seem to also inhibit tumor cell adhesion to the endothelium, since endothelial activation is synonymous with upregulation of adhesion receptors that are thought to increase endothelial adhesion. In experiments with RAW macrophages, although CM stimulated macrophages decreased tumor cell adhesion, they seemed to promote transmigration.

However, these human experiments indicate that extravasation occurs at a similar rate across all treatment groups until almost all tumor cells extravasate.

TNFα is a known stimulant of endothelial activation and is known to increase endothelial permeability and upregulation of adhesion receptors on endothelial cells (Figure 4.4b, 5.4b),[54,88,98] so was used as a positive control in these experiments. We did see a significant increase in endothelial permeability with the addition of TNFα and the addition of macrophages did not further alter this effect. Interestingly, endothelial adhesion of tumor cells is decreased with the addition of TNFα while TNFα treated macrophages seem to upregulate endothelial adhesion. Even though the endothelium is more permeable, it appears endothelial transmigration does not occur as quickly in TNFα treated devices as untreated or CM treated conditions between 2 and 24 hours. This indicates that endothelial permeability does not directly translate to invasion potential of tumor cells, and that endothelial adhesion molecules may decrease mobility of tumor cells. Overall, TNFα activation of endothelial cells is different than TNFα activation in the presence of macrophages. Since endothelial permeability is approximately the same, we assume that there is activation in both cases, but the endothelial cells are activated to recruit different cells from the blood under each condition—which changes tumor cell "recruitment."

Macrophages are known to recruit different immune cells based on their polarization,[104] so polarization may also change tumor cell extravasation potential. TNFα is considered an inflammatory cytokine that is associated with a "M1-like" immune response, when macrophages are thought to recruit tumor-suppressing immune cells that are programmed to destroy tissue. When macrophages are polarized to a more "M2-like" phenotype, they recruit tumor-supportive immune cells that rebuild tissue. Since

macrophages are a highly regulated cell, over stimulation with inflammatory cytokines in a tissue-destructive environment can program them to recruit immune suppressive immune cells to balance the system and return to homeostasis.[17] Since TNFα is associated with highly inflammatory environments, we hypothesize that endothelial cells exposed to TNFα alone are activated to promote infiltration of pro-inflammatory immune cells while macrophages in the tissue are overstimulated by TNFα and secrete cytokines to recruit immune-suppressive immune cells. Based on our data and this hypothesis, tumor cells have a greater affinity to endothelial cells subjected to M2-like macrophages and have a lower affinity in M1-like environments.

When we added M1 (IFN-gamma + LPS) or M2(IL-4 + IL-13) cytokines to devices, there seemed to be no change in permeability without macrophages, but there is an apparent increase with macrophages. This supports the hypothesis that both M1 and M2 macrophage polarization activates endothelial cells to increase permeability. Since M1 polarization is associated with pro-inflammatory immune recruitment, we hypothesize that tumor cell adhesion to these endothelial cells will reflect TNFα controls. We also hypothesized that M2 polarization will reflect endothelial adhesion characteristics of TNFα-stimulated macrophages. It appears as though these hypotheses were correct: adhesion in macrophage-free TNFα devices is approximately the same as in M1-stimulated macrophages while adhesion in TNFα-treated macrophage devices is approximately the same as M2-stimulated macrophage devices. However, there is variability in M1/M2 stimulated macrophage devices between donors which indicates that macrophage-tumor cell interactions may vary by patient.

While overall adhesion profiles of M1 and M2-treated devices reflected TNFα and TNFα treated macrophage devices respectively, the invasion fraction profiles seem quite

different. The violin-plot distribution in M1 and M2 treated macrophage conditions after 2 hours indicates that almost all the cells that adhered in these conditions transmigrated within the first 2 hours, while these distributions were not observed in the other experimental conditions until the 24 hour timepoint. At 2 hours, this change was significant between M1-treated macrophages and the macrophage-free control. Since the M1 and M2 macrophage-free controls took 24 hours to resemble the M1 and M2 macrophage invasion fraction distribution, we hypothesize that M1/M2 polarized macrophages have some interaction with tumor cells that increases their rate of extravasation. Several mechanisms could be responsible for this change including increase in endothelial permeability, change in EC surface adhesions, direct tumor cell-macrophage signaling or physical contact.

We have used this model system to demonstrate that macrophages can influence extravasation of tumor cells. Macrophages likely interact with tumor cells in several ways, but we investigated mostly endothelial cell-mediated influences. Since macrophages were influential in altering endothelial permeability, adhesion, and transmigration, understanding and targeting these macrophage-induced mechanisms may lead to metastasis-inhibiting therapeutics.

Figures

Figure 5.1: Macrophages are viable in our microfluidic model

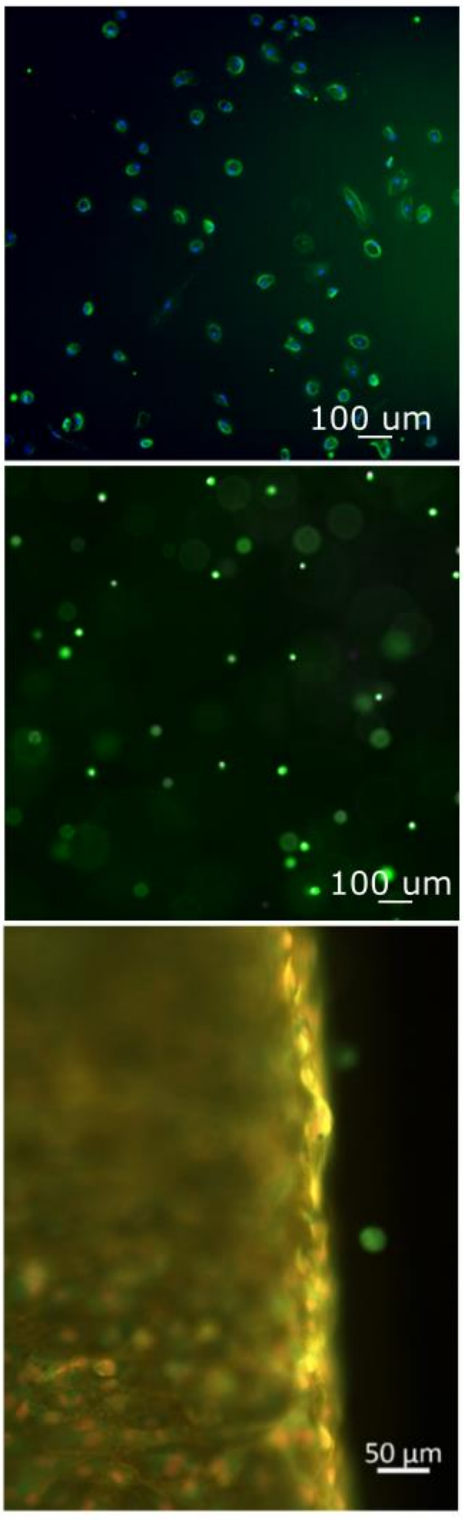


Figure 5.1: Human-derived macrophages are differentiated and viable in experiments. a) CD68+ macrophages (green) show that macrophages are differentiated before use in microfluidic experiments. b) Human macrophages are highly viable in 3D collagen gels (green = live, pink = nucleus). c, d) Macrophages are viable in our model system for the entirety of our experiments (5 days from seeding). Endothelial cells are also highly viable (green = live, orange = CD31, red = nuclear).

Figure 5.2: Permeability of endothelial cells influenced by human macrophages

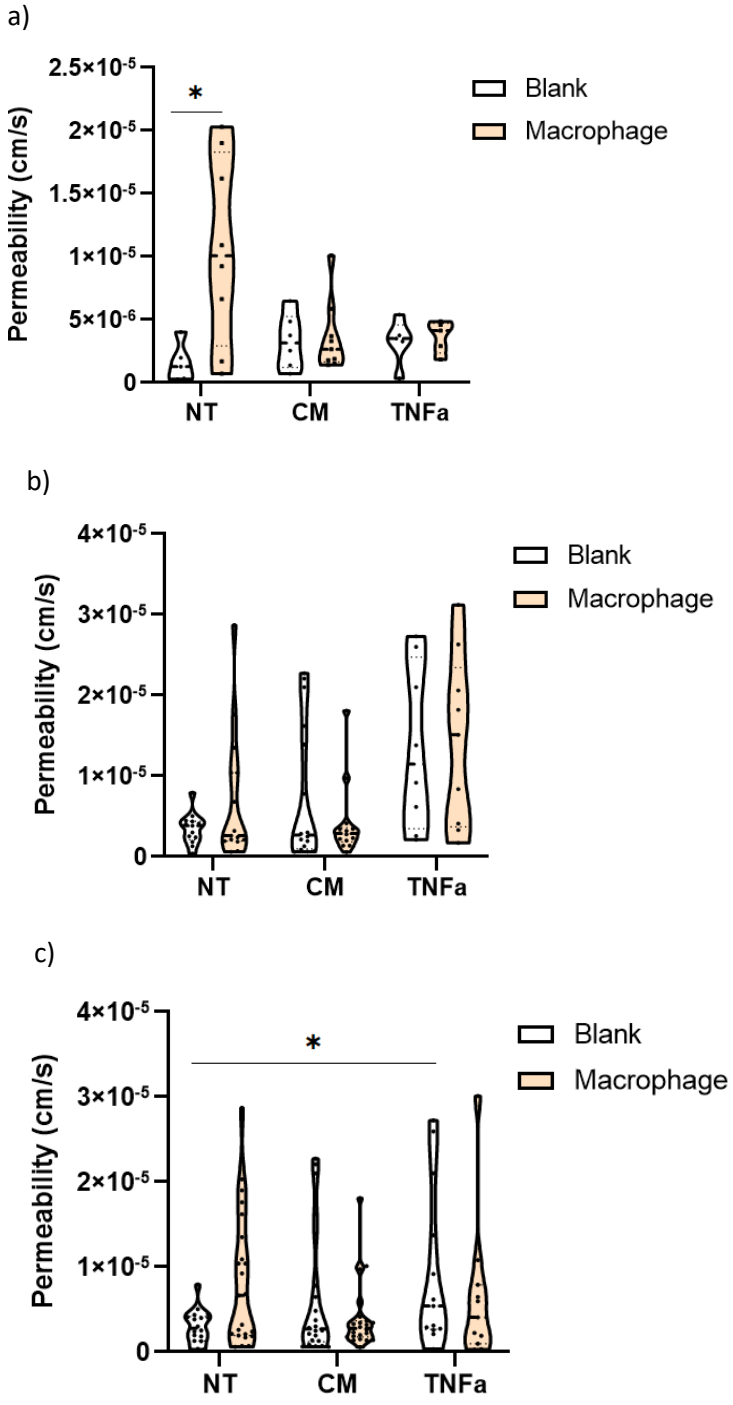


Figure 5.2: Permeability is not significantly altered with human macrophages and or CM or TNFα.

a) Donor 1:permeability of endothelial cells seems to increase with addition of donor 1 macrophages (Kruskal Wallis with Dunn's test--B vs M p = 0.02) (B n=5; BCM n=6; M n=4; MCM n=9; TNFα n=5; MTNFα n=5).

b) Donor 2 macrophages do not seem to affect endothelial permeability. TNFa stimulation does seem to stimulate increased endothelial permeability (Krustkal Wallis with Dunn's test—B n=13; BCM n=16; M n=15; MCM n=14; TNFα n=8; MTNFα n=9).

c) Data compiled from these experiments indicates that there is a significant change in untreated and TNFα treated devices (2way ANOVA with Tukey's test p = 0.0264) while macrophages seem to slightly increase permeability—likely due to the large increase with donor 1(B n=18; M n=23; BCM n=22; MCM n=23; BTNFα n=13 MTNFα n=11)

Figure 5.3: Human macrophages alter adhesion and transmigration in response to external stimuli

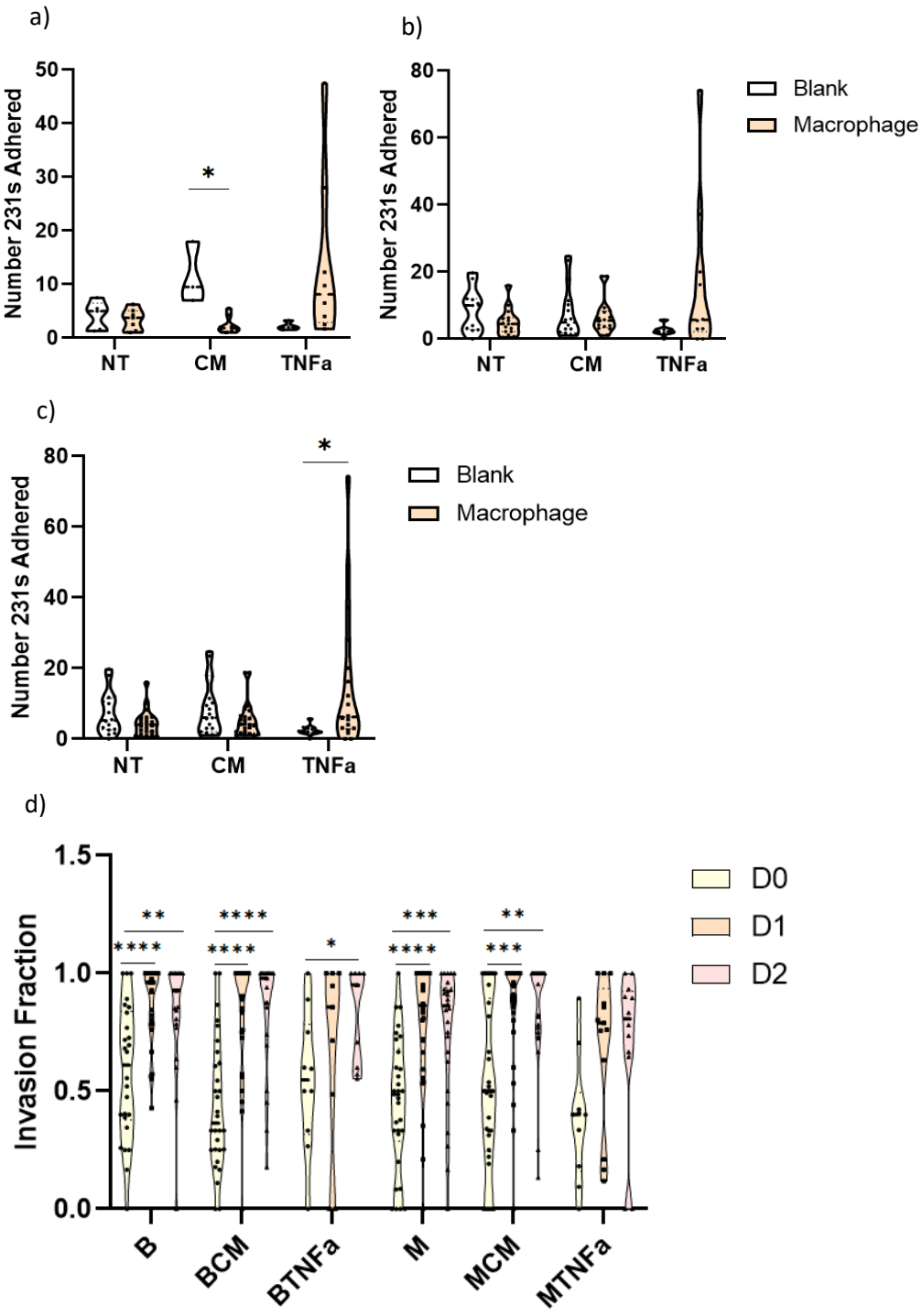


Figure 5.3 Macrophage influence on tumor cell adhesion and transmigration.

a) Adhesion of tumor cells is significantly inhibited by donor 1 macrophages treated with CM while macrophages treated with TNFα seem to increase adhesion.

b) Donor 2 macrophages seem to only affect tumor cell adhesion when exposed to TNFα.

c) When these data sets are compiled, there is a significant increase in tumor cell adhesion when macrophages are treated with TNFα.

d) The invasion fraction of tumor cells significantly increases in untreated (B), CM-treated (CM), macrophage only (M) and CM-treated macrophage (MCM) conditions between 2-24hrs (D0-D1). TNFα treatment seems to inhibit tumor cell extravasation rate since it takes more time for these distributions to resemble those of the not TNFα treated conditions. (* p<=0.5; ** p<=0.01; *** p<= 0.001; **** p<=0.0001)

Figure 5.4 M1/M2 polarization in macrophages and endothelial cells

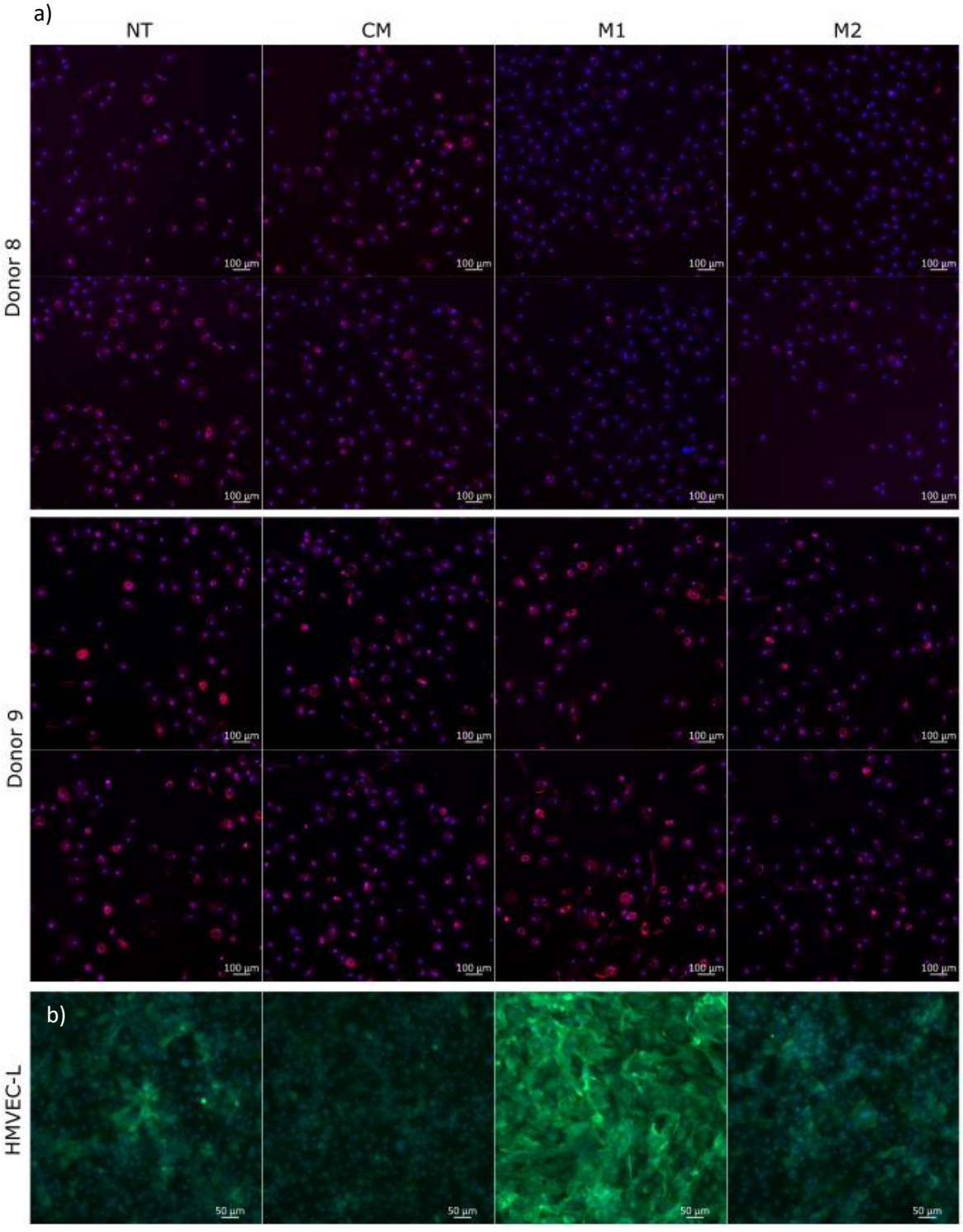


Figure 5.4: M1/M2 cytokines alter HMVEC-L and Human macrophage expression for some donors. a) M1 marker iNOS (pink) and M2 marker CD206 (red) were qualitatively compared when human macrophages were stimulated by M1 or M2 cytokines. M1/M2 stimulation alters macrophages increases expression of these markers for donor 9, but decreases them in Donor 8. Surprisingly, CD206 expression appears to be increased in M1 stimulation of Donor 9 macrophages, which is the opposite of what is expected. b) HMVEC-Ls treated with TNFα or M1 cytokines (IFN-gamma + LPS) upregulate ICAM-1 expression while M2 cytokines (IL-4 + IL-13) do not affect expression. CM may slightly decrease ICAM-1 expression compared to untreated controls.

Figure 5.5: M1 and M2 macrophage may alter endothelial permeability

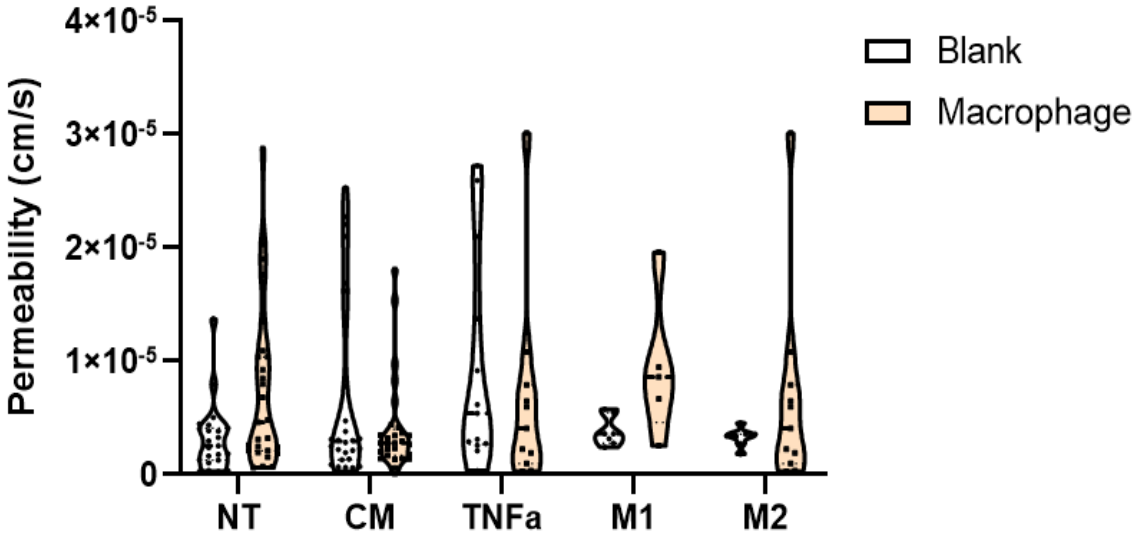


Figure 5.5: M1 and M2 polarized macrophages increase endothelial permeability. Exposed to M1 and M2 cytokines, endothelial cells do not exhibit a large change in permeability; however macrophages exposed to M1 and M2 cytokines seem to have a more dramatic—but insignificant—effect on permeability. These distributions resemble TNFα treated devices and devices with macrophages alone.

Figure 5.6: M1 and M2 cytokines alter endothelial adhesion in a donor-dependent manner

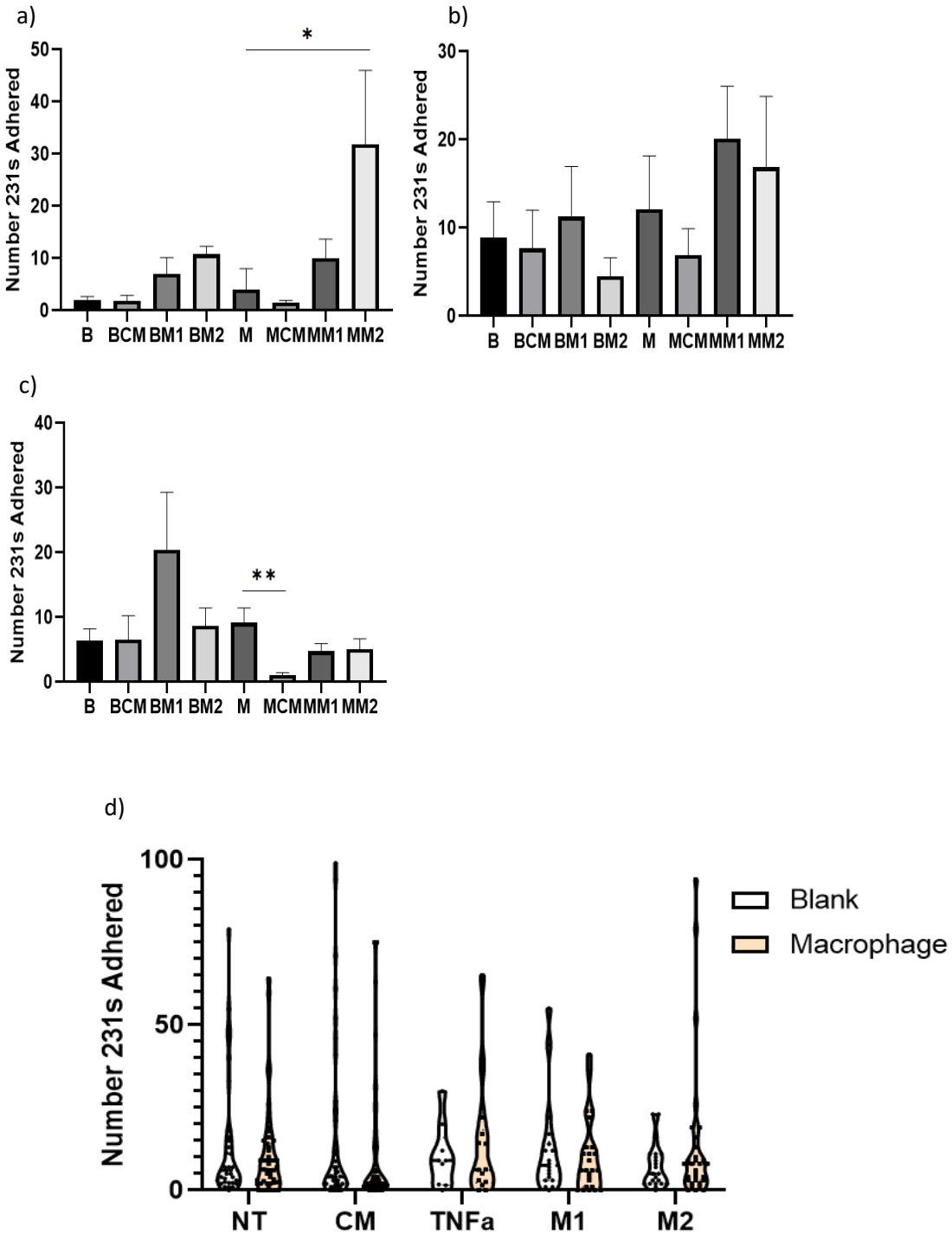


Figure 5.6: Macrophages from different donors have differential effects to M1 and M2 stimulation. a,b,c) Although baseline adhesion differs slightly, untreated and CM-treated devices have approximately the same adhesivity and CM-stimulated macrophages seem to reduce adhesion compared to untreated macrophage controls while there is some variability in M1/M2-stimulated endothelial cells. a) Donor 7 macrophages treated with M2 cytokines significantly increase tumor cell adhesion compared to macrophages alone. b) Donor 8 macropahges do not significantly alter tumor cell adhesion to endohteial cells c) Donor 9 macropahges significantly decrease adhesion when exposed to CM compared to macrophage-only controls. d) Overall, there is slight decrease in 231 adhesion between macrophage-only and CM-treated macrophage devices, indicating that CM has a consistent adhesion-inhibiting effect on the endothelium. (* p<=0.5; ** p<=0.01; *** p<= 0.001; **** p<=0.0001)

Figure 5.7: Invasion fraction of adhered tumor cells is influenced by macrophage polarization

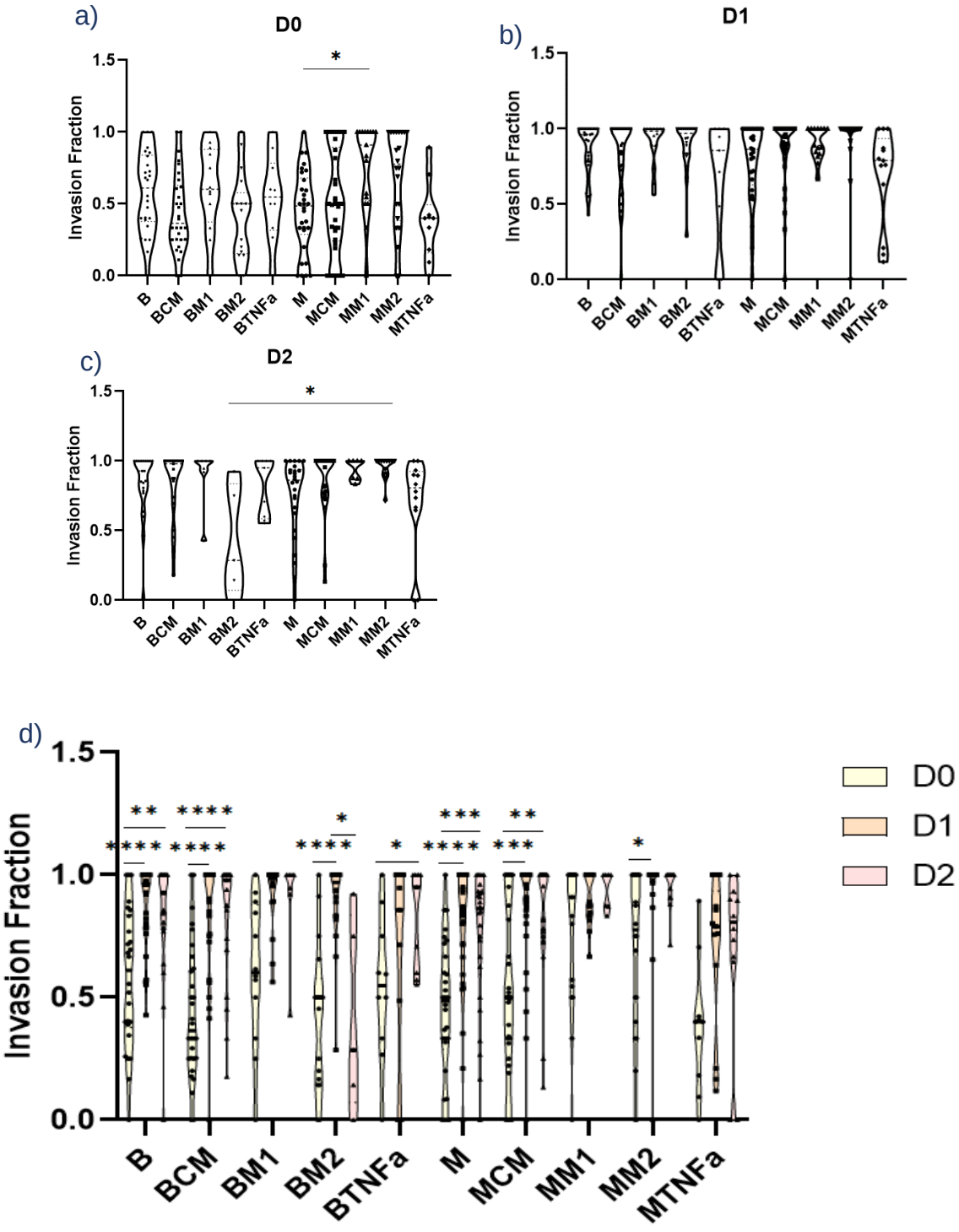


Figure 5.7: 231s may extravasate more quickly when M1/M2 macrophages are present. a) After 2 hours, a higher proportion of tumor cells transmigrated when exposed to M1 macrophages than untreated macrophages. While not significant, there is a similar trend between macrophages-only and M2-polarized macrophages. b) There are no significant changes at 24 hours, c) but there is a significant different in invasion fraction between macrophage-free M2 controls and M2-polarized macrophages at 48 hours. d) comparing the overall invasion fractions over time reveals that in most conditions there is a significant increase between 2-24 hours. While this is not true of M1-polarized macrophages, it appears this is due to higher fractions of 231s invading before 2 hours than there being any inhibition between 2-24 hours. It also appears as though a lower fraction of 231s extravasate when TNFα is in the system.

(* p<=0.5; ** p<=0.01; *** p<= 0.001; **** p<=0.0001)

Chapter 6: Leukocyte Adhesion to Endothelial Cells is altered by Endothelial Activation

Introduction

The endothelium is a highly regulated transport barrier that controls the flux of nutrients, wastes, and immune cells so that our tissues can remain in homeostasis. However, endothelial phenotype is likely an important part of organotropic tumor cell metastasis.[8] Endothelial cells are highly regulated by extrinsic immune cells to promote adhesion and transmigration of immune cells to sites of injury.[19] This adhesion also upregulates proteins important in transendothelial migration.[19] We have shown that macrophages residing in the tissue may differentially alter endothelial-tumor cell adhesion when stimulated by CM, M1 or M2 cytokines, or TNFα. We hypothesize that these tumor cells parasitize leukocyte-EC adhesion mechanisms to aid in adhesion and extravasation. Before investigating whether tumor cells parasitize leukocyte endothelial adhesion mechanisms, we first aim to understand leukocyte adhesion mechanisms in a simplified model system.

After endothelial activation by cytokines such as IL-1 or TNFα, the endothelium initially retracts tight junctions to increase vascular permeability, upregulates P-selectin expression to aid rolling of leukocytes, and releases pro-thrombotic von Willebrand factor (VWF) to aid platelet accumulation. Once adequate time has passed for gene expression and protein synthesis, leukocyte adhesion molecules such as VCAM-1 and ICAM-1, pro-thrombotic tissue factor, and immune-recruiting cytokines.[18] Together, these mechanisms promote rolling, adhesion, and accumulation of leukocytes and platelets that perform tissue repair functions which help the tissue return to homeostasis.

However, dysregulation of these endothelial-blood interactions can lead to several conditions such as myeloproliferative neoplasms (MPNs) which are a form of blood cancer. Up to 40% of MPN-related deaths are attributed to thrombotic events which highlights the need to understand clotting mechanisms in these patients.[105,106] Patients with MPNs are diagnosed with overproduction of red blood cells (polycythemia vera (PV)), platelets (essential thrombocythemia (ET)), or decreased blood cell production due to bone marrow fibrosis (myelofibrosis). Since 95% of PV and almost 50% of ET patients have a Janus kinase-signal transductor and activator of transcription (JAK-STAT)-2 mutation (*JAK2-V617F)*,[106-110] we aim to better understand how this mutation alters thrombosis.

Current treatments for PV and ET focus on reducing activity or concentration of excess blood cells with aspirin, hydroyurea, and phlebotomy to reduce the risk of an inflammatory immune response; however, these patients remain at an increased risk of thrombotic events, indicating that increased concentration of blood cells alone is not fully responsible for thrombosis.[105] Investigations into *JAK-V617F* mutation in vascular endothelial cells have revealed other potential mechanisms of increased thrombosis.[106] Some studies have shown that endothelial cells with the *JAK2-V617F* mutation express more P-selectin and VWF, which indicates a basal level of endothelial activation. Additionally, leukocytes adhered more readily to *JAK2-V617F+* endothelial cells under static and flow conditions,[106] implicating this mutation in ECs may promote a pro-thrombotic phenotype which is exacerbated by a high concentration of blood cells.

We aim to further investigate the mechanisms that drive thrombosis in MPNs. Since murine models with *JAK2-V617F+* ECs exhibit a bleeding phenotype[111] and EC function is directly correlated to shear stress,[27-30] we will develop a microfluidic model to visualize

and quantify blood cell-EC interactions. While there are existing microfluidic models that focus on evaluating hematological interactions with endothelial cells,[88,106,112] we will develop a custom microfluidic model made of PDMS for sufficient gas exchange for long-term culture, with enough surface area so ECs can be treated and evaluated via RNA-seq, and that can maintain physiologically-relevant shear stress.

Materials and Methods

Shear Device Fabrication

This device was designed with channel dimensions of 0.4 m x 1 mm to generate enough surface area for endothelialization. Master molds of devices were made using standard soft photolithography methods[113] to a height of 50 um, which is tall enough for relatively simple, consistent endothelial seeding but short enough to maintain physiologically relevant shear with a reasonable amount of media. Devices were cut out with a scalpel, inlet and outlet holes were punched with a 1mm biopsy punch, and devices were bonded to glass slides using plasma (Figure 6.1).

Endothelialization of Devices

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords and cultured on gelatin-coated cell culture flasks in EGM-2 (Lonza). Before adding cells to channels, devices were coated with gelatin for 10 minutes and rinsed with HBSS. HUVEC or HMVEC-L (P2-5) were rinsed with HBSS and trypsinized (0.05% Trypsin) for 2 minutes, or until cells detached from the plate. Cells were counted and centrifuged at 1200 RPM for 3 min and resuspended at a concentration of $10x10^6$ cells/mL. Using a 200 uL pipetter, 50 uL of cells were added to each device and pushed through until cells filled the entire channel. Devices were incubated at 37 ˚C for 30 minutes before pushing through the cell suspension again. After an additional 30 minutes incubation, flow was added to

devices using syringe pumps loaded with 3mL syringes filled with EGM-2 media at a rate of 50 uL/hr for 18 hours or until confluent endothelial monolayers form. Syringes were then replaced with 60mL syringes and flowrate was changed to 2100 uL/hr to expose endothelial monolayers to physiologically relevant venous shear (15 dyne/cm^2) for 48 hours before flowing blood or cancer cells. This flow rate was determined for this target shear stress using the following equation:

$$\tau_w = \frac{6\mu Q}{wh^2}$$

Where τ_w = wall shear stress (dyne/cm^2), μ= viscosity of cell culture media (dyne*s/cm^2), Q = Volumetric flow rate (mL/s), w is the width of the channel, and h is the height of the channel.

ImmunoFluorescent Staining of Endothelial Phenotype

Immunofluorescent (IF) staining was done on devices that did not receive blood flow to evaluate endothelial phenotype. Syringes were used to rinse devices with 1x dPBS before fixing with formalin for 15 minutes. Devices were then blocked with 10% FBS in 1xPBS for at least 30 minutes at RT or stored at 4˚C until stained. Primary antibodies included CD31 (1:100, RPE, BioRAD), VWF (RnDSystems), or P-selectin (ThermoFisher). Secondary antibodies were (ThermoFisher). All devices were counterstained with hoescht. Images were acquired on the Zeiss Axio Observer.

Blood Flow Over Devices

Four hours prior to blood flow, some devices were treated with 10 ng/mL TNFα, 4 μM Rux, 10 ng/mL TNFα+ 4 μM Rux, or DMSO (vehicle control) to stimulate endothelial adhesion molecule expression and see if we can inhibit the effects of TNFα stimulation. Blood was collected according to IRB standards and 1 mL was treated with calcein for 30

minutes to stain white blood cells and platelets green. Devices were imaged on a Zeiss Axio Observer inverted fluorescent microscope to ensure the confluencey of endothelial cells prior to flowing blood. Blood flow was added to the device via a syringe pump loaded with a 1 mL syringe at 150 uL/hr. This flow rate was used to ensure the visualization of rolling, sticking, and flow given the fluorophore and camera streaming within this set-up. Blood was flown over the endothelial cells for 15 minutes and about twenty 10-second videos were taken at a frame rate of 6.4 videos/second to analyze changes in leukocyte velocity and adhesion.

Data Analysis

Each video was processed with ImageJ (NIH) for adhesion, and the ImageJ Trackmate plugin for velocity.[114] To prepare videos for velocity measurements and to extract adhesion data, median fluorescence of each pixel was determined and subtracted from the video using an ImageJ macro.[115] The resulting “median” image consists of all the cells that adhered to the device, while the resulting video had these removed so cells with zero velocity did not attribute to the measurement. At least 10 videos were processed for each device.

Results

To study how endothelial activation affects thrombosis in healthy and MPN leukocytes, we aimed to develop a microfluidic model that could sustain endothelial cell cultures under physiologic shear for at least 5 days.

CD31

We first wanted to confirm that we could consistently seed endothelial cells in this model system and that they form a confluence monolayer along the channel. When

stained for endothelial tight junction marker CD31, we see that HUVEC form a confluent monolayer along the channel and are seemingly aligned with the direction of flow (Figure 6.2a).

TNFα induces a change in HUVEC phenotype

Since we can generate confluent endothelial layers consistently in this device, we wanted to study how activated endothelium influences endothelial cell phenotype. Since TNFα is a known stimulator of endothelial activation, we treated devices with TNFα for 4hrs before making measurements. HUVEC treated with TNFα in our microfluidic model exhibited increased VWF and P-selectin expression (Figure 6.2a,b). We hypothesize that this upregulation of VWF and endothelial adhesion receptors—such as P-selectin—will cause rolling and adhesion of leukocytes that will result in a global decrease in blood velocity.

Blood flow/data processing/TNFα treatment

To determine if we can measure differences in blood flow in response to endothelial activation with TNFα, we analyzed healthy, calcein-stained blood flow in our microfluidic channels. Median fluorescence of each pixel throughout the video was subtracted from the video, leaving an image showing adhered cells (Figure 6.2 a, b) and a video with only flowing cells (Figure 6.2 c, d). Videos acquired at approximately the same location and time after initiating blood flow in the device qualitatively show increased adhesion of leukocytes with TNFα stimulation (Figure 6.2 a, b) and decreased velocity (Figure 6.2 c,d). Additionally, the TrackMate flow tracks of leukocytes in the untreated device (Figure 6.2c) appear more linear than the flow tracks in the TNFα treated device (Figure 6.2d), indicating that there is increased rolling on TNFα-stimulated endothelium. Quantitatively one donor had a less dramatic change in leukocyte adhesion than the other donors, but there is a

TNFα-dependent increase in leukocyte adhesion compared to untreated controls (NT) across all donors (Figure 6.3a NT vs TNFα).

When endothelial cells are activated by TNFα, the leukocyte adhesion receptors that promote adhesion first cause leukocytes to roll along the endothelium. We hypothesize that TNFα activated HUVEC will slow the overall flow of blood since more leykocytes will roll along the endothelium than in untreated controls. However, we saw a small, but less dramatic, decrease in average leukocyte velocity than we were expecting in response to TNFα (Figure 6.3b NT vs TNFα). The decrease in velocity of controls may be caused by experimental variability or intrinsic donor to donor variability. While more experiments should be done to determine the cause of this change in velocity, we can stimulate endothelial activation with TNFα and measure resulting changes in leukocyte adhesion, but changes in velocity after TNFα treatment is more subtle.

Ruxolitinib experiments

Since we observed changes in leukocyte adhesion and velocity over TNFα stimulated HUVEC, we aimed to therapeutically inhibit the effects of TNFα on endothelial cells in this model system. Since TNFα is thought to promote JAK/STAT-induced endothelial activation,[106] we used JAK/STAT inhibitor, ruxolitinib (Rux), to ameliorate endothelial activation by TNFα. We hypothesize that Rux treatment alone would result in similar adhesion and velocities as untreated and vehicle (DMSO) controls, while Rux-treated TNFα devices (TNFα+Rux) would exhibit slightly increased adhesion and decreased velocity, but not to the level of TNFα only devices.

This experiment was completed using two separate healthy donors, with one donor tested on two separate occasions. All three experiments show that Rux and DMSO do not

alter leukocyte adhesion to the endothelium compared to untreated controls (Figure 6.4), indicating that the drug or vehicle alone do not alter endothelial adhesion. When devices were treated with TNFα+Rux, two donors exhibited decreased adhesion compared to TNFα-treated devices as expected, but one donor had greatly increased adhesion compared to TNFα alone (Figure 6.4). Although this spike is likely due to experimental error and this experiment should be repeated to verify these changes, we concluded that we can measure changes in leukocyte adhesion in response to therapeutics.

We also measured changes in velocity of leukocytes in response to TNFα, Rux, DMSO, and TNFα+Rux. Although the untreated control exhibited a larger change in velocity than expected, it appears that DMSO and Rux alone do not alter the velocity of leukocytes in this model, which was expected. However, TNFα+Rux treated devices appear to have a decreased velocity compared to Rux-only but is indistinguishable from TNFα-only devices (Figure 6.3b). However, this Rux concentration is on the low-end of therapeutically relevant, so a higher dose may be necessary to discern differences.

Patient Data +/-TNFα

Since we can measure changes in adhesion and velocity of healthy donor leukocytes in response to endothelial activation, we aimed to see if we could observe these changes in MPN patient blood. We hypothesize that there will be a similar increase in MPN adhesion to TNFα-activated endothelium compared to untreated endothelium as there was with healthy blood, but there will be a basal increase of MPN endothelial adhesion compared to healthy donors.

Blood from two MPN patients was analyzed in two separate experiments. Similarly to healthy controls, there is an increase in MPN leukocyte adhesion to HUVEC activated with

TNFα compared to untreated controls (Figure 6.5a). When compared to previously collected healthy donor data, we also see a basal increase in MPN-leukocyte adhesion to the endothelium in both untreated and TNFα conditions (Figure 6.5a). We concluded that MPN leukocytes and platelets are basally more adhesive than healthy leukocytes. When comparing velocities of healthy donors and *MPN* patients on healthy endothelial cells, we observe a slight increase in velocity (Figure 5.4b). While this increase may be within the noise of the measurement, it is surprising that the velocity is not decreased given the apparent increase in adhesion. *MPN* blood does decrease velocity over TNFα activated endothelial cells compared to donor leukocytes. The change in *MPN* leukocyte adhesion and velocity over TNFα activated endothelial cells indicates that this mutation promotes an elevated thrombotic phenotype—particularly when endothelial cells are activated. Since *JAKV617F* endothelial cells exhibit baseline activation,[106] the *JAK2V617F* in both endothelial cells and blood cells likely promotes an exaggerated phenotype. Therefore, understanding how MPN leukocytes and platelets interact with endothelial cells from MPN patients will provide a greater understanding of these mechanisms and an improved platform to testing therapeutics.

Blood Outgrowth Endothelial Cells may provide an opportunity to study MPN EC-Blood Interactions

Treatment of MPN patients is difficult because we do not fully understand how MPN blood cells interact with MPN endothelial cells. While *JAK2-V617F*+ endothelial cells can be made using genetic tools,[106,111] using a specific patient's endothelial cells with their blood provides an opportunity to personalize their treatment to their unique biology. Blood outgrowth endothelial cells (BOEC) can be isolated from human peripheral blood, providing an opportunity to study endothelial biology on an individual level.[116,117] We aim

to retrieve BOEC from MPN patients and test anti-thrombotic treatments with patient-matched blood to predict how individual patients will respond to treatment and develop new treatment strategies.

Using healthy donor BOECs, we first wanted to see if we could exogenously activate BOECs with TNFα and measure changes in adhesion and velocity. When comparing adhesion all the videos taken for each device, there was a significant increase in healthy leukocyte adhesion to endothelial cells when activated with TNFα, except for donor 2 (Figure 6.6a). Since there is some distribution of adhesion depending on where the video is taken in the device, we compared the total number of adhered leukocytes in each device between untreated and TNFα treated devices for each donor. Although there is no significant change between these groups, there is an increase in total adhesion for all donors except donor 2 (Figure 6.6b).

We also observed a decrease in velocity of leukocytes on TNFα-activated BOEC (Figure 6.6c). When compiling all the videos from both blood donors that were tested with one BOEC donor, we found that there was a significant decrease in velocity when BOEC were stimulated with TNFα (Figure 6.6d). We hypothesize BOEC may be a viable option for studying mechanisms of MPNs and illuminate treatment options that could reduce the frequency of thrombosis in these patients.

Conclusions

Endothelial activation is an important process during the immune response but can be detrimental to heath is dysregulated. We have developed a model system capable of supporting endothelial cell growth under physiologically relevant shear stresses for at least 5 days. This model system can be used for visualizing changes in endothelial activation

with IF or through quantifying direct interactions with blood cells. We also showed that we can alter EC-blood interactions using Rux, demonstrating the utility of this platform for testing therapeutics. Additionally, we can use donor-derived BOEC to coat microfluidic channels which demonstrates the capability of this model to pair MPN patient-derived endothelial cells with their blood for personalized medicine. Since we observed increased adhesion of MPN blood donors compared to healthy donors and literature suggests that MPN ECs are basally activated,[106] we hypothesize that the pro-thrombotic behavior of MPN blood cells will be exacerbated in the presence of MPN endothelial cells. Using this model system of dynamic endothelial-blood cell interactions of MPN patients along with traditional model systems will provide a clearer picture of how thrombotic events occur and what the best strategies are to prevent them.

In addition to better understanding blood cancers, we can utilize this microfluidic model to more fully explore the roles of endothelial activation on circulating tumor cell adhesion. It is hypothesized that circulating tumor cells parasitize intrinsic immune-recruiting receptors on ECs to adhere and transmigrate the endothelium. However, in our extravasation microfluidic system we found that TNFα alone apparently reduces MDA-MB-231 tumor cell adhesion compared to untreated controls, but TNFα treated macrophages seem to upregulate endothelial adhesion of tumor cells (Figure 5.3c). Since some of our data support a clear upregulation of leukocyte adhesion to TNFα stimulated endothelial cells, we hypothesize that tumor cell-EC interactions may be more complex than simply adhering to leukocyte adhesion receptors. Some evidence has shown that tumor cell adhesion to ECs alone is not enough for transmigration, but interactions with circulating immune cells may play an important role. This model system is also poised to quantify tumor cell circulation with whole blood, which may highlight some of these

interactions. For example, it is hypothesized that tumor cells cluster with platelets which allows for better adhesion with endothelial cells. Not only does this increase the probability of adhesion, but interactions with platelets also activates pro-migratory pathways in tumor cells that helps them invade.[118,119]

Ultimately, we have developed a tunable model to study how flow impacts endothelial interactions with circulating cells. While this model has many potential uses outside the scope of what was discussed here, we hope to continue to use this model to uncover novel mechanisms and treatments to prevent thrombotic events in MPN patients.

Figures

Figure 6.1-microfluidic model system to study endothelial-leukocyte interactions

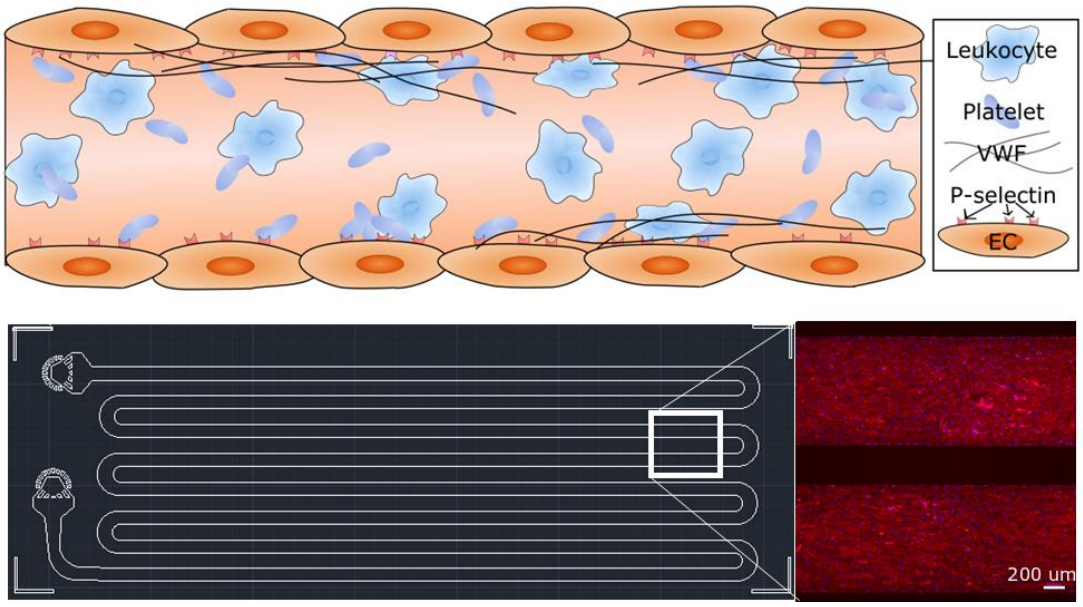


Figure 6.1: Microfluidic model to study endothelial adhesion of leukocytes under shear. a) Endothelial cells (ECs) are activated to upregulate thrombotic factors such as von Willebrand factor (VWF) and P-selectin. This results in increased adhesion, and ultimately thrombosis. b) Microfluidic model consists of a 0.4 m x 1mm x 50um channel that can be seeded with endothelial cells to quantify thrombotic behaviors of blood cells. c) HUVEC seeded in microfluidic model after 48hrs under 15 dyne/cm^2 shear stress stain positive for CD31 (red). scale bar 200um.

Figure 6.2 Endothelial activation with TNFα alters protein expression and leukocyte behavior

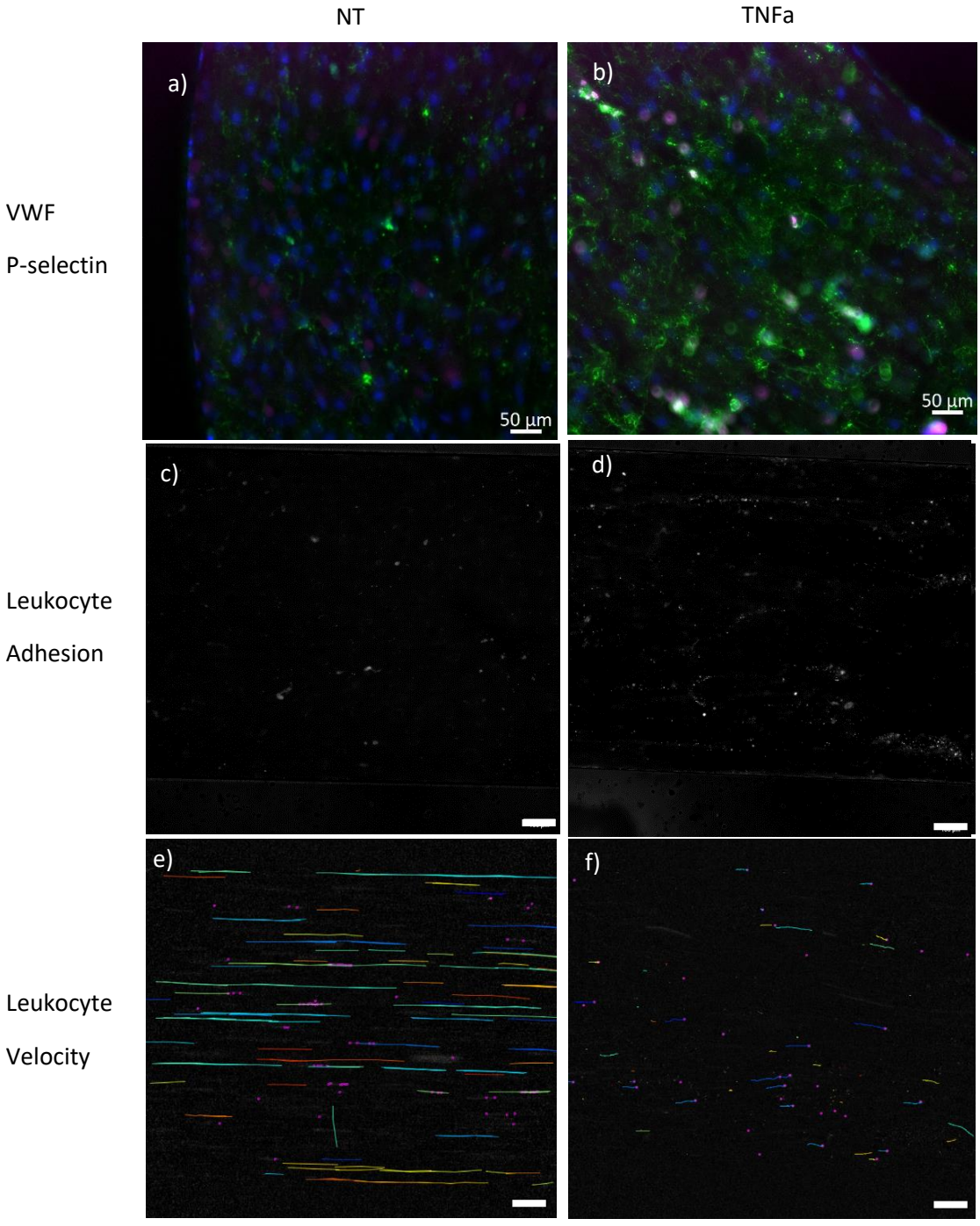


Figure 6.2: Endothelial cell phenotype in microfluidic model changes with TNFα treatment. a,b) HUVEC treated with TNFα (b) upregulate von Willebrand factor (green, VWF) and P-selectin (pink) compared to untreated HUVEC (a) Scale bars 50 um. c,e) Median image and corresponding video of calcein-labeled leukocytes adhered (c) and flowing (e) over untreated HUVEC. d,f) Median and corresponding video of calcein-labeled leukocytes adhered (d) and flowing over (f) TNFα treated HUVEC. c) Untreated HUVEC exhibit less adhesion at the same time after blood flow and location in device as (d) TNFα treated HUVEC. e) Lines tracking leukocyte velocities in untreated HUVEC device are longer and more linear than those tracking leukocytes flowing over (f) TNFα treated device. This data indicates that TNFα treatment activates endothelial cells to promote a rolling and adhesive phenotype compared to untreated controls. Scale bars 100 um.

Figure 6.3--- TNFα upregulation of leukocyte adhesion is mostly inhibited by JAK/STAT inhibition

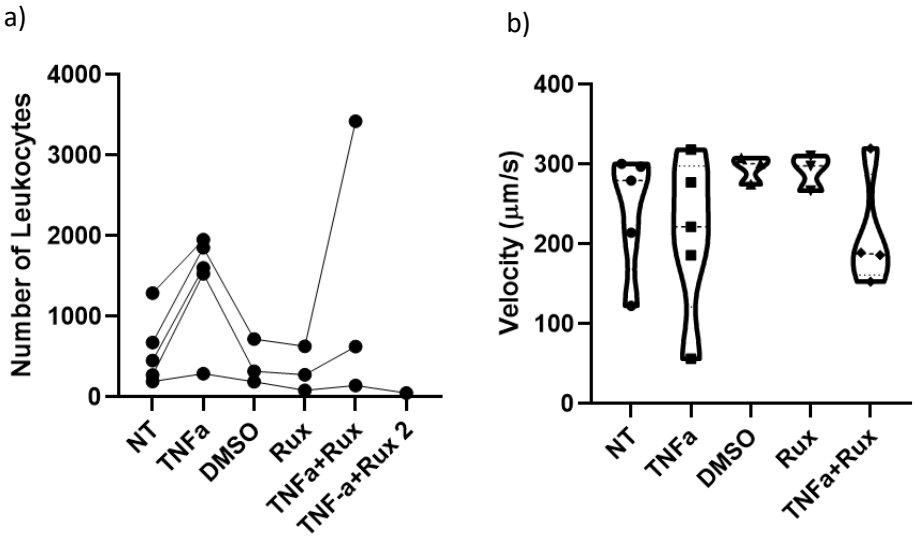


Figure 6.3: Ruxolitinib may alleviate the pro-thrombotic effects of TNFα treated HUVECs on leukocyte adhesion and aelocity. a) Across 5 different experiments, TNFα activation of HUVECs seems to upregulate endothelial adhesion of leukocytes (NT vs TNFα). Ruxolitinib alone and DMSO vehicle controls had no effect on leukocyte adhesion compared to NT. Ruxolitinib reduced the pro-adhesive effects of TNFα in all but one experiment (Rux + TNFα vs TNFα). b) Velocity of leukocytes is not affected by DMSO or Rux alone, but seems to be more variable in untreated, TNFα , and TNFα +Rux treated HUVEC, indicating that more rolling phenotypes are observed in these conditions.

Figure 6.4—MPN *leukocytes have greater baseline adhesion than healthy leukocytes*

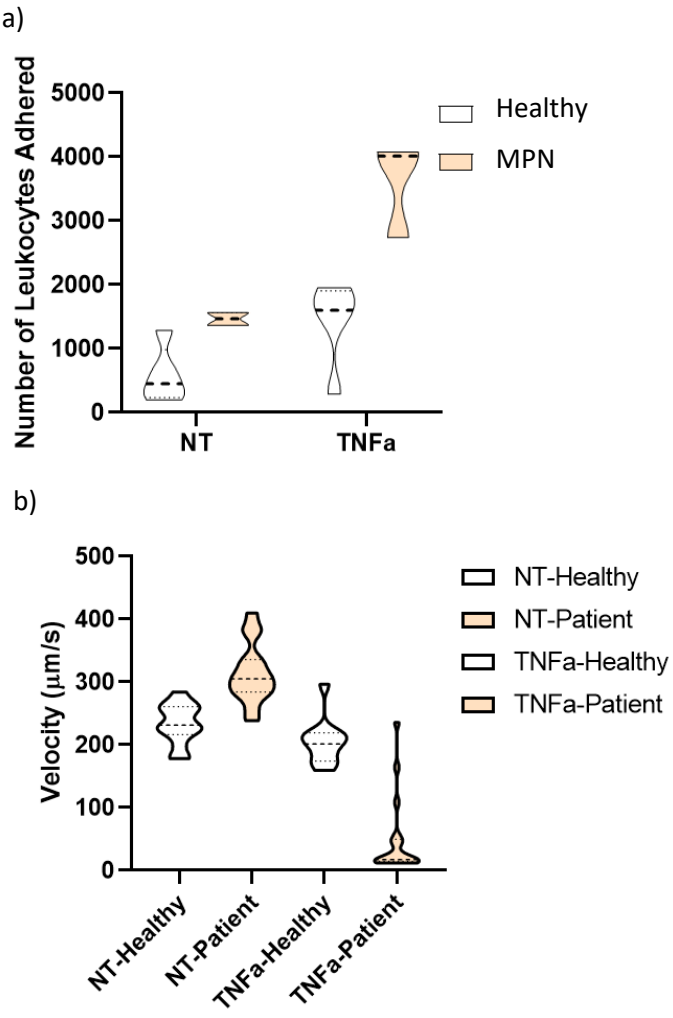


Figure 6.4: *MPN* patient blood exhibits a more thrombotic phenotype than healthy donor blood. a) *MPN* blood cells appear to be more adhesive than blood cells from healthy donors. This adhesive phenotype is exacerbated by endothelial activation with TNFα. b) Velocity of leukocytes from *MPN* patients seems to increase over untreated endothelial cells, but decrease over TNFα activated HUVECs.

Figure 6.5—Leukocyte adhesion and velocity with BOEC

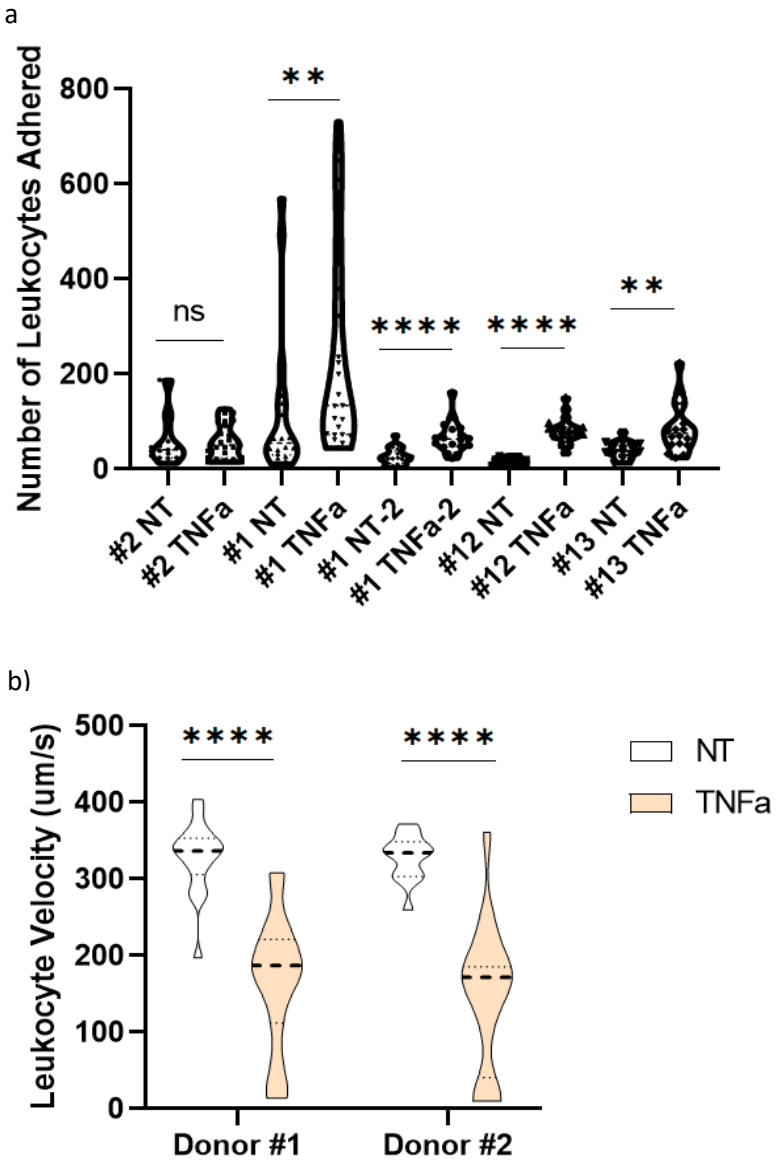


Figure 6.5: Blood outgrowth endothelial cells exhibit alterations in leukocyte adhesion and velocity upon TNFα stimulation. a) Leukocyte and platelet adhesion is generally increased in TNFα treated BOEC devices compared to untreated controls, except for donor 2 (t-test: **0.01> p >=0.001; **** p<0.0001). b) There is a significant decrease in velocity of leukocytes from both donor 1 and 2 when exposed to TNFα treated BOEC (Mann Whitney U test : ****p=<0.0001).

Chapter 7: Overall Conclusions / Potential Impact / Meaning of Demonstrated Applications

We hypothesized that macrophages in the secondary site influence endothelial cell interactions with arriving tumor cells, so we developed a microfluidic model to probe and quantify these mechanisms. We demonstrated that we can generate endothelialized microchannels tunneling thorough collagen gels via passive pumping as described by Bischel et al.[86] We adapted this model system to support gravity-driven flow to generate shear on the endothelium and quantify changes in tumor cell adhesion under flow. To measure endothelial function, we measured changes in permeability to TNFα—a known modulator of endothelial permeability. Upon addition of tumor cells, we were able to measure differences in invasive potential of highly invasive MDA-MB-231 and less invasive MCF7 breast cancer cells. We also demonstrated the ability to culture RAW 264.7 macrophages in this model system and measure phenotypic differences in tumor cell adhesion and extravasation—likely driven by tumor signal-macrophage-endothelial cell interactions. Ultimately, we seeded human blood-derived macrophages in the ECM and found that exogenous signaling alters endothelial cell-macrophage interactions in the context of metastasis, but these results vary by donor. Overall, these findings indicate that macrophage influences on endothelial adhesion and transmigration of tumor cells may illuminate potential extravasation-inhibiting mechanisms.

Recent literature suggests that primary tumor signals influence macrophage-endothelial cell interactions in the secondary site, which ultimately increases the probability of oragnotropic metastases.[8] Much of what is known of these interactions comes from *in vivo* models that measure changes in ultimate metastatic burden, but it is difficult to elucidate how these mechanisms influence initial tumor cell adhesion and

extravasation. To investigate how primary tumor signals and macrophages influence endothelial-tumor cell interactions, we perfused devices with CM—to model primary tumor signals—with or without RAW 264.7 macrophages. While macrophages alone did not alter initial tumor cell adhesion compared to macrophage-free controls, we found that CM-treated macrophages surprisingly reduced the adhesion of tumor cells in the secondary site compared to CM-treated endothelial cells (Figure 4.3a). However, the tumor cells that adhere to endothelial cells exposed to CM-treated macrophages seem to be more invasive than tumor cells in the other conditions. We hypothesized that increased extravasation fraction would correlate with increased permeability, but we saw no change in endothelial permeability due to macrophages, CM, or CM-treated macrophages.

Although these results were surprising, we found that these trends held when murine, RAW macrophages were replaced with human-blood-derived macrophages in this model system. When data from all donors are combined, it appears that there is a slight decrease in initial tumor cell adhesion when macrophages are stimulated with CM compared to macrophages alone (Figure 5.6a). When evaluated by donor, MDA-MB-231 adhesion in CM-treated macrophage devices is decreased compared to CM-treated controls in 2/5 donors, and decreased adhesion compared to macrophage-only controls in 4/5 donors. Although not all these changes are significant, these trends indicate that CM-educated macrophages can counteract upregulation of endothelial affinity to tumor cells caused by either CM or macrophages alone.

With the RAW macrophage experiments, we found a significant change in extravasation fraction of tumor cells in devices with CM-treated macrophages between 24 and 48 hours with no changes in any other condition; however, there were no clear differences in invasion fraction in response to human macrophages or CM-treated human

macrophages (Figure 5.7c). When looking at the shape of the distributions of untreated, CM, macrophage, and CM-treated macrophage invasion fraction at 2 hours (D0) (Figure 5.7a), we see that more CM-treated macrophage devices had all adhered cells transmigrate within 2 hours than CM and macrophage devices alone. Although insignificant, this trend also implies that tumor cells that adhere in devices with CM-treated macrophages transmigrate more easily than when exposed to CM or macrophages alone. Similarly to the RAW macrophage data, these subtle changes in extravasation fraction are not associated with changes in endothelial permeability.

From these data, we hypothesize that primary tumor signals—modeled by CM—do influence extravasation potential of MDA-MB-231 breast cancer cells, but not in the manner we expected. The apparent decrease in initial adhesion of MDA-MB-231 cells in devices with CM-treated macrophages indicates that there is some mechanism that causes macrophages to inhibit endothelial expression of surface receptors that aid tumor cell arrest. However, it appears that although fewer tumor cells adhere initially, these cells have an increased ability to extravasate than cells adhered in the other conditions. We hypothesize that endothelial expression of surface receptors is proportional to tumor cell arrest but inversely proportional to tumor cell extravasation; therefore, if CM-treated macrophages reduce endothelial surface adhesion expression, then tumor cells that are able to adhere will have a smaller energy barrier to breaking these bonds and extravasating.

It is also possible that macrophages are polarized by tumor signals to promote different surface receptors expression, such as E-selectin, P-selectin, and VCAM-1, on endothelial cells than what is observed with untreated, CM, or macrophage-only conditions. While these receptors may have a lower affinity to MDA-MB-231 cells, these surface receptors

may activate more migratory phenotypes from the tumor cells leading to faster transmigration. Additionally, changes in endothelial cell expression can lead to recruitment of different subtypes of tumor cells, so in this case there are fewer adhered cells,[5] but these cells are intrinsically more migratory. There are several components of tumor CM that may promote this behavior, and analysis of these factors and their potential effects on macrophage polarization may provide insights to adhesion-inhibiting therapeutics.

Macrophage polarization is thought to play a major role in tumorigenisis: M1-like polarization is considered anti-tumoral while M2-like polarization is considered pro-tumoral. We stimulated macrophages in this model system with M1 (IFN-gamma + LPS) or M2 (IL-4 + IL-13) to evaluate how endothelial cells responded to these stimuli with and without macrophages. Generally, M1 cytokines resulted in increased endothelial affinity of tumor cells to the endothelium in the absence of macrophages; however, there was a donor-dependent change in adhesion when macrophages were present (Figure 5.6 a-c). Human lung microvascular cells upregulate ICAM-1 in response to M1 stimuli, which may aid in tumor cell adhesion to the endothelium, but the variation in adhesion in the presence of macrophages may be due to variability in macrophage polarization (Figure 5.5). For example, macrophages from donor 8 had lower baseline activation than macrophages from donor 9. Untreated macrophages from both donors seem to have approximately the same adhesion profile, but adhesion in response to M1-stimualted macrophages trend in opposite directions. Macrophages from donor 8 seem to downregulate M1 (iNOS) and M2 (CD206) markers in response to M1 stimulation, but tumor cell adhesion is greater. Alternatively, macrophages from donor 9 have an apparent increase in CD206 expression in response to M1 stimulation, but adhesion is downregulated. Although opposite of what is expected from M1 macrophages, upregulation of M2-like phenotype may induce an

endothelial phenotype with a lower affinity to tumor cells. In contrast, M2-stimulation of macrophages seems to increase tumor cell adhesion to the endothelium for donors 7 and 8, but slightly decreases adhesion for donor 9. We hypothesized that M2 polarization would increase adhesion since M2-stimulated macrophages still recruit immune cells via endothelial surface expression, but these adhesion receptors potentially differ from those found with M2 stimulation.

While adhesion was variable among donors, M1 and M2 stimulated macrophages appear to promote extravasation more quickly than their macrophage-free M1 and M2 counterparts (Figure 5.7d). Although there was no statistical increase between D0 and D1 as seen in the untreated, CM, macrophage, or CM-treated macrophage conditions, there may be more MDA-MB-231 cells extravasating before the 2 hour timepoint than in other conditions, as indicated by the significant increase in extravasation fraction between macrophage-only and M1-macrophage conditions (Figure 5.7a). Although not significant, M2-macrophages exhibited a similar distribution as M1-macrophages. These data indicate that polarization is influential in tumor cell extravasation. Since both M1 and M2-like macrophages are associated with promoting immune recruitment, these macrophages exposed to these cytokines likely induce an endothelial phenotype that aids extravasation, which is what we see in our model system. There is some variable change in permeability in response to M1 and M2 cytokines which may impact extravasation efficiency, but these mechanisms are not yet clear. We can use this model system to further probe these interactions in response to differential macrophage polarization to identify mechanism for altering endothelial-tumor cell interactions via macrophages.

To better understand the roles of permeability and adhesion of tumor cells in the secondary site, we studied the effects of TNFα on endothelial function and MDA-MB-231

extravasation potential. We showed that TNFα in this model system increases endothelial permeability, as predicted by literature. Since TNFα is associated with increasing permeability, endothelial cell adhesion molecules, and adhesion and transmigration of leukocytes and tumor cells,[53,54,97,120,121] we hypothesized that this would translate to greater MDA-MB-231 adhesion and transmigration. We also expected this behavior in TNFα stimulated macrophages. However, TNFα seemed to decrease initial tumor cell adhesion compared to untreated controls while TNFα significantly reduced MDA-MB-231 adhesion compared to TNFα-treated macrophages (Figure 5.3a-c). This is particularly surprising since we show that TNFα stimulated endothelial expression of ICAM-1—a endothelial adhesion molecule that promotes leukocyte adhesion and is associated with promoting tumor cell metastasis—does not correlate to increased MDA-MB-231 adhesion. We conclude that changes in endothelial cell surface expression in response to TNFα do not necessarily correlate with increased adhesion of circulating tumor cells, but TNFα stimulated macrophages seem to upregulate endothelial adhesion receptors that have a higher affinity to MDA-MB-231 cells. Extracting and comparing the genomic sequencing of these populations of endothelial cells may provide insights to what surface receptors are associated with MDA-MB-231 metastases. Efficacy of therapeutics blocking this receptor could be carried out in this model system as well before carrying out experiments with murine models. Although tumor cells have diverse adhesion receptor expression, developing a strategy to identify, target, and analyze these changes may aid in reducing the probability of extravasation.

Although TNFα-treated macrophages seem to promote adhesion of tumor cells and increase endothelial permeability, MDA-MB-231s in TNFα treated devices seem to extravasate more slowly than those in devices with no treatment, CM, macrophages, and

CM-treated macrophages based on the shape of the extravasation fraction distribution (Figure 5.3d). These surprising trends may be the result of increased endothelial adhesion molecule expression, as mentioned previously. TNFα may also be acting directly on MDA-MB-231 cells to make them less transmigratory. However, some research suggests that TNFα makes MDA-MB-231 cells more transmigratory in transwell systems[120], which indicates that transmigration efficiency is different in 2D than 3D and highlights the importance of studying these effects in several model systems.

TNFα has been implicated as both an inhibitor and promoter of tumor progression, partially due to its ability to promote an immune-recruiting immune phenotype.[54] However, since high levels of systemic TNFα are correlated with clinical breast cancer stage,[97] we hypothesize that this may be due to TNFα interactions with the microenvironment of the secondary site—such as macrophages—that counteract direct TNFα influences on endothelial cells which ultimately changes interactions with tumor cells. Dysregulated TNFα signaling in macrophages is associated with several chronic immune diseases, and TNFα inhibitors have been used clinically to treat cancer and several autoimmune disorders; however, toxicity of TNFα inhibitors cause detrimental side-effects.[54,122,123] Therefore, this model system can be used to probe these mechanisms behind TNFα-macrophage interactions in extravasation may provide improved therapeutic targets.

We have demonstrated that macrophages have differential effects on MDA-MB-231 extravasation depending on circulating signals. Although we only used this model to probe macrophage polarization by specific cytokines or CM, this model could also be used to study therapeutics or other exogenous factors on macrophage-endothelial interactions in the context of metastasis. It may also be possible to extend the timescale of these experiments to observe how macrophages prune early colonization events. Additionally,

there are several cell types—including resident macrophages—in the secondary site that likely impact metastatic potential of circulating tumor cells. This tunable model system could also be used to further investigate effects of other resident or circulating cells on tumor cell extravasation. There is also potential to introduce tumor cells to the ECM space to analyze how resident cells—and recruitment of other tumor cells or immune cells from the circulation—influence early tumor growth.

Within this model system, endothelial adhesion of tumor cells seems to be impacted by systemic cytokine activation of macrophages. While there are subtle changes in extravasation fraction, by 48 hours most adhered tumor cells transmigrate. While extravasation is considered the rate-limiting-step of metastasis, adhesion may be the rate-limiting step of extravasation. Therefore, understanding the mechanisms that change tumor cell adhesion to the endothelium may illuminate therapeutic targets to restrict metastatic burden in patients.

We developed a separate microfluidic model system to elucidate how blood flow over endothelial cells alters thrombotic events in healthy and diseased states (Chapter 6). We show that we can stimulate endothelial activation of HUVEC and BOEC with TNFα, and that this activation alters leukocyte adhesion and velocity profiles. Additionally, we can model leukocyte adhesion mechanisms in this device and probe these mechanisms with therapeutics such as ruxolitinib.

We also demonstrated that we could measure changes in adhesion and velocity of leukocytes from patients with myeloproliferative neoplasms (MPNs), indicating that we can distinguish phenotypes from healthy and diseased states. Additionally, we cultured healthy blood donor-derived endothelial cells (BOECs) and were able to measure changes

in velocity and adhesion when endothelial cells were activated by TNFα. Since patients with MPN likely have increased basal activation of endothelial cells in addition to more thrombotic blood cells,[106] it is important to understand how these cells interact to determine optimal treatments. Since we can culture endothelial cells from blood donors, we may be able to extract BOEC from MPN patients so that we can evaluate patient rheology over their own endothelial cells and help determine the best treatment on an individual level.

This model system can also be used to evaluate direct tumor cell adhesion to endothelial cells. Although we found that leukocytes adhered more readily to TNFα activated endothelial cells in this shear microfluidic model, we did not see increased adhesion of MDA-MB-231 cells in our 3D microfluidic system unless macrophages were present. We conclude that tumor cells do not necessarily parasitize intrinsic leukocyte adhesion receptors in flow; however, tumor cells circulating with blood cells may increase tumor cell adhesion. By incorporating whole blood or individual blood components, we can visualize circulatory and endothelial interactions in real-time to help us understand how blood cells in circulation alter initial tumor cell adhesion. We can also visualize how blood cells influence extravasation potential alone or in response to resident cells in our 3D model. For example, we found that MDA-MB-231 tumor cells may extravasate more quickly depending on macrophage phenotype. Exiting the vasculature more quickly may be an advantage since circulation is thought to be a dangerous place for tumor cells due to increased interactions with circulating immune cells such as natural killer cells. Ultimately, both tunable model systems are poised quantify tumor cell interactions within the pre-metastatic microenvironment and may provide insight to the mechanisms that drive extravasation.

Overall, we demonstrated that when studying immune interactions, it is important to not only pool results and look at trends across a population, but there are insights to gain from analyzing data on personalized scale. Microfluidic model systems such as these open the door to studying patient samples on a personalized level to gain insights to their disease and provide treatments based on these results. They also provide flexibility in utilizing different cell types, exogenous stimuli, and different forms of ECM to quantify extravasation potential in more specific extravasation microenvironments. Although not demonstrated here, there is opportunity to use more traditional assays in combination with this model system such as sequencing and comparing endothelial cell phenotypes across treatments and collecting media outputs to perform ELISAs on proteins of interest. Utilizing *in vitro* model systems such as these in combination with traditional *in vitro* and *in vivo* assays may lead to an improved understanding of metastatic events in a broad range of cancers.

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