DIETARY MODULATION OF MYELOID DERIVED SUPPRESSOR CELL BIOLOGY IN PATHOPHYSIOLOGY AND PHYSIOLOGY

by

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To my Wife, Jennifer and my Children, Samuel and Maggie, for all their love and support. Also, to my parents, Terry and Cyndi Calvert for raising me to follow my dreams.

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$1,25(OH)_2D$	1, 25 dihydroxyvitamin D_3
APC	antigen presenting cells
ARG1	arginase 1
BRB	black raspberries
BM	bone marrow
CCL2	CC ligand 2
CXCR2	CXC receptor 2
FC	flow cytometry
FSC	forward scatter
GM-CSF	granulocyte macrophage colony-stimulating factor
ivgMDSC	in vitro generated myeloid derived suppressor cell
iMDSC	immature myeloid derived suppressor cell
pMDSC	peripheral myeloid derived suppressor cell
tMDSC	tumor derived myeloid derived suppressor cell
G-MDSC	granulocyte myeloid derived suppressor cell
G-CSF	granulocyte-colony stimulating factor
HIF-1α	hypoxia-induced factor-1 alpha
IPTM	intraperitoneal tumor cells
IFN-γ	interferon gamma
IL	interleukin
LOOCV	Leave One Out Cross Validation
MEC	Mixed Edge Cover
M-MDSC	monocyte-like-MDSC
MDSC	myeloid derived suppressor cell
MPO	myeloperoxidase
NVBM	naïve bone marrow
NOS2	nitric oxide synthase 2
NF-κB	nuclear factor kappa B

PBMC	peripheral blood mononuclear cells
PGE2	prostaglandin E2
RNS	reactive nitrogen species
ROS	reactive oxygen species
SSC	side scatter
SP	spleen
SVM	support vector machine
TCR	T-cell receptor
Treg	T regulatory
TLR	toll-like receptor
TGF	transforming growth factor
TU	tumor
TAM	tumor -associated macrophage
TUSP	tumor bearing spleen
TNF-α	tumor necrosis factor alpha
TME	tumor microenvironment
VDR	Vitamin D Receptor

ABSTRACT

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T-cells are present in the immune system to fight against invaders. Once their job is done, suppressing their activity is an important step in maintaining a proper immune response. Myeloid derived suppressor cells (MDSCs) are immune cells that suppress T-cell activity. Currently, MDSCs are defined as a heterogeneous population of immature cells that are derived in the bone marrow and travel to the site of inflammation or cancer. Two major subtypes of MDSCs have been identified in mice and humans, monocyte-like MDSCs (M-MDSC) and granulocyte MDSCs (G-MDSC). G-MDSCs typically make up the majority of the total population of MDSCs but are less T-cell suppressive than M-MDSCs. One of the major problems in the study of MDSCs is that the current marker system for subtypes does not differentiate between precursor MDSCs (lacking suppressive ability) and functional MDSCs (those with suppressive ability). Therefore, using cancer models in mice, we investigated the development and potential to classify precursor MDSCs from functional MDSCs. While MDSCs have been highlighted as a target cell to inhibit in cancer, in other conditions, such as pregnancy, MDSCs have been shown to be beneficial in maintaining a normal pregnancy. Therefore, targeting the increase of MDSCs in abnormal pregnancy conditions like pre-eclampsia may act as a prevention or therapeutic strategy. Finally, it is known that many dietary components can act as modulators of immune cells. Specifically, the polyphenol like phytochemical, curcumin has been shown to act as an anti-inflammatory agent with the potential to modulate multiple immune cells. Therefore, we propose two different studies to investigate the potential of curcumin as either an inhibitor and/or promotor of MDSCs in a disease-specific context. Together the role of phytochemicals as immunomodulators of MDSCs is still very young, in part due to the complexity of phytochemicals themselves, but the studies cited here provide evidence that the field is ripe for additional questions to be asked.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

In the United States, cancer is the second leading cause of death and accounts for about 25% of all deaths [1]. Sporadic cancers can result from the accumulation of genetic mutations in somatic cells. If these abnormal cells are not cleared by the immune system a tumor may form. Cancer treatments include conventional strategies like surgical removal of the tumor, radiation therapy, and chemotherapy. Recently immunotherapies are showing great promise in the treatment of cancer [2]. These can be used alone or in combination with conventional therapeutic regimens.

Immunotherapies use immunomodulators to boost or restore the host immune system and improve its ability to clear tumor cells [3]. During tumor formation, cytotoxic T-cells (CD8+) can recognize antigens on mutated cells as foreign and target them for killing. However, as the tumor develops it adapts to evade T-cell surveillance. For example, the tumor may acquire new gene mutations that decrease cell surface antigen presentation [4]. This decreases tumor cell recognition by cytotoxic T-cells. Additionally, the tumor will release chemokines to recruit immune cells into the tumor microenvironment (TME). Careful phenotypic analyses have indicated that a large proportion of cells within the TME have suppressive features. These include phenotypic subsets such as the regulatory T-cell (Treg) and the myeloid derived suppressor cells (MDSC) that directly inhibit host T-cell function through a variety of mechanisms [5]. Although certain tumors may present histologically as being “T-cell rich,” further analysis indicates these T-cells are poorly functional at eliciting antitumor immune responses [6]. In a recent review, Makkouk and Weiner discussed several immunomodulatory therapies where specific immune cells are targeted to increase the host immune function, break immune tolerance, and aid tumor cell clearance [7]. Consistent with this approach, several studies have shown that depletion of MDSCs in mice increased the ability of host immune cells to clear tumor cells and reduce tumor growth [8-11]. Because of this, MDSCs are considered a critical target for enhancing the efficacy of immunotherapy.

Evidence exists to suggest that nutrition could play a significant role in the patient response to immunotherapy, including alteration of MDSC function. In 2004, Kaminogawa et al. summarized studies on the impact of foods and nutrition on immune function. Their analysis

suggested that nutrients and dietary bioactives can modulate both the innate and adaptive immune cells in healthy individuals, and in cancer patients [12]. For example, in cancer patients, diets supplemented with L-arginine, RNA, omega-3 fatty acids or their combination, increase macrophage phagocytosis, T-cell activation and proliferation, and antigen-specific antibody production by B cells [13-15]. Similarly, vitamin A increases interleukin (IL) -2 production, T-cell proliferation and cytotoxicity, and dendritic cell migration to lymph node resulting in a more active adaptive immune response [16, 17]. Curcumin has been shown to modulate both innate and adaptive immune cells and reduces the production of pro-inflammatory signals such as IL-6 and IL-1 [18]. Finally, Vitamin D and polyunsaturated fatty acids, on the other hand, increase the human monocyte response to bacteria increasing innate immune responses [19, 20]. The field of MDSC biology is continually growing and specific studies on the impact of diet or isolated nutrients on MDSC function are now appearing in the literature. These studies suggest that dietary factors may act as MDSC immunomodulators that could complement cancer treatment [21]. In this introduction, background will be provided to understand where how these cells develop, their role in cancer, methods used to isolate and study them, current immunotherapies, and how my research questions move the field of MDSC biology forward.

1.2 Immunology and Cancer

1.2.1 Basics of Immunology and Cancer

An overview of cancer biology: Cancer develops through multiple means but can be described as the unregulated growth of mutated cells. The hypothesis that cancer develops via multiple genetic mutations (multi-hit model) was first formally formulated by Knudson [22] in 1971. In general, the multi-hit model suggests that a single gene mutation is not enough to promote a normal cell to become neoplastic. This hypothesis has gained great acceptance within the field of cancer biology and has led to the discovery of specific gene mutations involved with cancer development [23, 24]. These multiple gene mutations each add a new feature to the forming neoplastic cell. For example, in colon cancer the hypothesized order of gene mutations is: loss of APC, Activation of K-ras, loss of tumor suppressor gene DCC, and loss of p53 [23, 25]. As the cell replicates, a polyp forms (due to loss of APC) and will first progress to a benign adenoma (activation of K-ras and loss of DCC) and finally progress to a malignant carcinoma (loss of p53).

Once a malignant carcinoma is formed the next stage of cancer development is to metastasize and spread to other organs in the body. Less is known about the processes involved in metastasis, but several hypotheses exist, and it is an active area in cancer research. While the multi-hit model has provided great insight into the molecular mechanisms involved in tumor development, it is lacking the impact of the local microenvironment as an effector of cell neoplasia development. The local microenvironment includes multiple cytokines, growth factors, and immune cells, all of which will alter local cell development.

An overview of the immune system: The immune system is divided into two major response types, innate and adaptive, where the innate is responsible for activating the adaptive (reviewed in [26]). The division of the immune system into two parts is often attributed to two features of the cells in each. First, they are separated by the rate at which they respond to infection or tissue damage where the innate response is the immediate response and the adaptive requires several days to become activated. Second, they are divided by their functional abilities where innate cells use a non-specific method of pathogen clearing, aid in wound healing, and activate the adaptive response, while the adaptive response involves infection clearing is very specific. Each response will be discussed separately to identify the functions of the major cell classes involved in each response type.

The innate immune response is made up of a large group of immune cells (reviewed in [27]). These cells can be represented by their major class types being monocytic (monocytes, macrophages, and dendritic cells), granulocytes (neutrophils, basophils, eosinophils and mast cells), and, innate lymphocytes (natural killer cells (NK) and γδT-cells). While the cells within each class have very specific purposes, the general purpose and response to infection or pathogen will be discussed by the classes.

Granulocytic cells are polymorphonuclear and represent the largest number of innate cells found in the body. These cells are regarded as the first responders to infections or tissue damage. Specifically, during the initial stages of an infection or tissue damage, tissue resident macrophages will release cytokines that initiate an increase in neutrophil production at the bone marrow. Neutrophil then travel via circulation to the specific site where they enter the tissue and begin the inflammatory response. This is achieved by proinflammatory mediators, chemoattractants, chemokines and the binding of specific adhesion molecules presented in the vasculature as a result of downstream signaling from cells within the infection or damage site. This method of homing to

the infection or damage site is used by all immune cells. The other granulocytic cells are responsible for fighting parasitic infections, mediating immune tolerance, wound healing, and are the main cells involved in allergic responses.

In contrast, monocytic type cells are mononucleated cells that differentiate from monocytes. Monocytes are generated in the bone marrow and are found in bone marrow and circulating in the blood. In circulation, upon contact with a specific adhesion molecule or chemokine, they enter the specific site of infection and differentiate into macrophages based on signals at the site. Macrophages are responsible for the phagocytosis of pathogens and the release of cytokines. There are two know major types of macrophages M1 and M2. M1 are the primary phagocytic cells and play a major role in inflammation while M2 are responsible for aiding in the termination of inflammation and cell repair. Phagocytosis is often mediated by antibodies that attach to targeted cells. This process is called opsonization. M2 cells are actively involved in the regulation of the inflammatory response via cytokine release. However, not all monocytes become macrophages, some monocytes will undergo differentiation at the bone marrow and become dendritic cells and enter circulation. However, not all dendritic cells are monocytic derived and come from the bone marrow, tissue resident dendritic cells also exist and act as first responder dendritic cells. These cells also slightly differ in their transcription and cytokine profile compare to monocytic dendritic cells. However, all dendritic cells are considered the true antigen presenting cells (APC) that activate the adaptive immune response via cell-to-cell contact. This is achieved by phagocytosis of opsonized cells and the creation of antigens that are presented to B and T-cells. Finally, within the innate response exists a non-cellular response known as the complement system. This system can be activated by three mechanisms but all result in a number of proteins binding to the cell which creates either the membrane attack complex or the soluble terminal complement complex, both of which create pores in the membrane killing the cell.

The adaptive response is made up of only two major types of cells, T and B cells. T-cells are further divided into two classes based on function and can be identified by their T-cell receptor, either CD8 or CD4. CD8+ T-cells are referred to as cytotoxic T-cells and are responsible for the targeted killing of other cells. Meanwhile, CD4+ cells are referred to as T helper cells (Th) cells and are responsible for aiding in the activation of cytotoxic T-cells, B cells and facilitate tissue repair. B cells are responsible for the production of antibodies. Both T and B cells also contain a subset of cells classified as memory cells that are long lived and are responsible for increased

infection clearance during a secondary infection. Finally, both T and B cells contain a subset of cells known as regulatory cells (Treg and Breg, respectively) that aid in the shutdown of inflammatory responses. These cells are important in the suppression of autoimmune diseases and the termination of the inflammatory response.

1.2.2 Immune Response to Tumor Development

Elimination, equilibrium, evasion are the three stages of the immune response to tumor development. A seminal paper in cancer biology is Hanahan and Weinberg's "The Hallmarks of Cancer" [5]. In this paper, they describe specific functional aspects (hallmarks) of cancer that can be used to define cancer as a whole. Emerging hallmarks reflect the complex interactions between the developing tumor cells and the immune system, i.e. "avoiding immune destruction". The interaction between the tumor and the immune system can be described by two major phases; immunosurveillance and immunoediting. Immunosurveillance is the normal physiologic role of a healthy immune system and in the context of cancer, it will prevent abnormal cells from surviving and establishing a tumor. During immunosurveillance, monocytes, neutrophils, and dendritic cells travel through circulation in response to specific chemokine signals. This leads to their extravasation into sites where pathogens, infected cells, or abnormal cells reside (e.g. an inflammatory site). During this process, innate cells may also activate the adaptive response to aid in clearance i.e. promote the recruitment and activation of T and B cells. In contrast, immunoediting is the process by which immune cells interact with cells from a growing tumor and inadvertently influence the expansion of the tumor. There are three stages of immunoediting: elimination, equilibrium, and escape [28]. The elimination stage of immunoediting is essentially immunosurveillance in the context of cancer cells. However, in addition to the signals and processes that occur during immunosurveillance, activated dendritic cells will activate the adaptive immune system. Meanwhile, additional signals from the tumor cells also activate an innate immune response. If the mutant cells are not cleared during elimination, an equilibrium between tumor cells and immune cells may be established. This stage may continue indefinitely. However, the complex tumor microenvironment presents a selective pressure that can lead to immune escape. During this stage, tumor cells begin to replicate at a faster rate than they are cleared by the immune system. In addition, the tumors cells develop two escape mechanisms. First, they decrease their expression of MHC I molecules which will make them less visible to cytotoxic T-cells. Second,

tumor cells will increase production of chemokines that recruit immunosuppressive cells like MDSCs and Tregs to the tumor site. During a normal inflammatory response, these immunosuppressive cells are recruited to prevent cytotoxic T-cell induced autoimmune disorders, aid in cell repair and ending the inflammatory response. However, at the tumor site, these cells protect the tumor from cytotoxic T-cell clearance.

Immune response to tumor development: The response of the immune system to developing and established tumors is very complex and is still an active area of research. Therefore, the following section will be a brief overview of the innate and adaptive responses to tumors. This is not meant to be a thorough review of each cellular response but, is meant to provide a brief background into the role of each response.

Adaptive response: The adaptive immune response is initiated by cell to cell contact from both innate APC's as well as signals from non-immune cells near the tumor. The response of B cells to tumor development was recently reviewed by Yuen et al. [29] covering both pro and anti-tumor responses. As with T-cell, there is a subtype of B cells known as B regulatory cells that exhibit an immunosuppressive function at the tumor site. However, it is still unclear whether these cells play an important role in all cancers. In contrast, anti-tumor B cells seems to be present in a majority of solid tumors. These cells are primarily responsible for the release of antibodies that aid in opsonization of targeted cells and activate the complement system. Within the tumor B cells release IgG which targets the tumor cells for phagocytosis and antigen presentation by dendritic cells. Finally, B cells have been shown to aid in the activation of T-cells by acting as APCs as well as through antigen independent mechanisms (CD27-CD70 interaction). However, cytotoxic T-cells have been the major area of study in tumor cell clearance by the adaptive response due to their direct targeting killing of cancer cells. In a review by Topfer et al. [30] the response of T-cells to developing and established tumors was thoroughly discussed. As discussed earlier, within the general population of T-cells exist T regulatory cells which have been associated with pro-tumor activity. These cells are hijacked by the tumor to aid in immune escape, specifically to shut down cytotoxic T-cells. However, the major response by T-cells is anti-tumor, from both Th and cytotoxic T-cells. Th cells respond by activating cytotoxic T-cells via release of IL-2. Additionally, these cells release IFN-γ which aids in T-cell recognition of target cells by increasing the MHC-I molecules and enhances the abilities of macrophages and NK cells. Cytotoxic T-cells, however, tend to take center stage in the anti-tumor response as they specifically target tumor cells for

killing. Additionally, they release IL-12 which promotes further clonal expansion. However, the major pitfall in the adaptive response is that they may not reach the tumor cells before coming in contact with immunosuppressive cells, specifically those of the innate response.

Innate response: Innate immune cells are the first responders to mutated and damaged cells. In a review focused on the translational potential of cancer immunotherapies, Lie and Zeng [31] provide a thorough review of the innate cellular responses to tumors. Therefore, the following is a brief overview of their findings. The first cells to arrive at the developing tumor are neutrophils and mast cells which begin clearing dead cells and releasing cytokines and chemokines that aid in the recruitment and activation of macrophages and dendritic cells. While granulocytic cells are most known for their pro-tumor response, supporting tumorigenesis and metastasis, they play an important role in initiating the innate response. Next, both Macrophages and dendritic cells arrive at the site and begin phagocyting apoptotic cells and start the process of recruiting the adaptive response. Macrophages have a somewhat convoluted relationship with tumor cells. During the early stages of tumor development these cells aid in tumor cell clearance while in later stages they are hijacked by the tumor to become tumor associated macrophages (TAM). TAMs are thought to be a major component in tumor immune escape by inhibiting cytotoxic T-cells and production of anti-inflammatory cytokines. In contrast, dendritic cells maintain their role as the primary APC and activate tumor cell specific B cells and T-cells. Lastly, NK cells are the primary killing cell of the innate response and upon arrival to the tumor site begin killing tumor cells. This is accomplished via expression of receptors for specific molecules expressed on the dying, damaged, infected, or tumor cells. As the tumor cells evolve these molecules are often down regulated. As a final note, both monocytic and granulocytic cells are known to be hijacked by the tumor and become MDSCs and represent the major immunosuppressive facilitator in immune escape by the tumor.

Immunosuppressive cells at the tumor site: Multiple immunosuppressive cells exist at the tumor site from both innate and adaptive responses. It is important to know that under physiologic condition these cells play important roles in wound healing by decreasing the inflammatory response, reduce chronic inflammation, reduce graph-verses host disease, and are mediators of the maternal-fetal tolerance during pregnancy [32]. However, during tumor development these cells are hijacked via cytokines released by the tumor and aid in tumorigenesis. In the adaptive response B and T regulatory cells target their cytotoxic counterparts and promote cell death. Within the

innate response the most prominent immunosuppressor cells are the TAMs and MDSCs. It has been hypothesized that a majority of TAMS are differentiated from Monocytic MDSCs [33] and therefore MDSCs are a major component of tumor immune escape.

1.2.3 MDSCs

MDSCs have been studied for several years under a variety of conditions but their defining feature is their ability to suppress T-cell function [34]. Originally MDSCs were identified as cells causing immune cell tolerance and called natural suppressor cells [35]. Further research has shown that "natural suppressor cells" play a vital role in keeping the immune system in check, preventing autoimmunity and, are involved in maternal-fetal tolerance [32]. It is this function that becomes problematic in the context of cancer and is used by tumors as a means of immune escape.

Early studies identified MDSCs, in mouse tumors, as cells expressing extracellular markers for both myeloid (CD11b+) and granulocytes (Gr1+). In 2008, the population of MDSCs was officially defined as two subsets; a polymorphonuclear cell-like MDSC defined as CD11b+Ly6GhiLy6Cmed (G-MDSC) and a monocyte-like MDSC defined as CD11b+Ly6GlowLy6Chi (M-MDSC) [36]. These markers have facilitated relatively efficient isolation of MDSCs from mice and allowed for a better understanding of their function. Functional analysis showed that M-MDSCs have more T-cell suppressive ability than G-MDSCs but that G-MDSCs are more abundant in most murine tumor models [8]. The two MDSC subtypes are also thought to exist in humans. However, because several different combinations of cell surface markers have been used to identify MDSC subtypes in humans [37], it can be difficult to compare results across studies. However, recently it suggested that the minimal set of markers for Human MDSC subtypes should be; M-MDSC: CD11b+HLA-DRlow/-CD14+CD15- and G-MDSC: CD11b+CD14-CD15+ [38].

Gabrilovich et al. [39] summarized the T-cell suppressive mechanisms that have been proposed for MDSC. First, MDSCs can promote CD4+ T-cells to differentiate into immunosuppressive Treg cells. While the exact pathways leading to Treg differentiation by MDSCs have not been clearly defined, a combination of events such as the release of IL-10, tumor growth factor beta (TGF-β and, cell-to-cell interactions between CD40-CD40 ligand have been proposed. Second, MDSCs generate oxidative stress through the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Increased oxidative stress inhibits activation

of CD8+ effector T-cells by multiple mechanisms involving the T-cell receptor (TCR). RNS is produced by MDSC through the expression of high levels of nitric oxide synthase 2 (NOS2 or iNOS). RNS has been shown to bind to the TCR and other proteins via nitration of tyrosine residues that attenuate the capacity for downstream signal transduction events related to antitumor immune responses [40]. Oxidative stress from ROS may also interfere with IL-2 signaling, induce the loss of the TCR ζ chain and ultimately leave the T-cell in a form of anergy (a state in which T-cells are unable to completely activate and do not become cytotoxic). Third, RNS from MDSC interferes with cytotoxic T-cell homing and viability by binding to chemokine (C-C motif) ligand 2 (CCL2). Finally, certain MDSC subsets express high levels of arginase 1 (ARG1) and have increased L-arginine uptake that may deplete arginine from the local environment. This deprives cytotoxic T-cells of an essential nutrient, causes the loss of the TCR ζ chain, and induces growth arrest and anergy. Because of their proposed roles in MDSC-mediated T-cell suppression, the upregulation of ARG1 and NOS2 have been used as functional markers of these cells [41]. Using the extracellular markers CD11b, GR1, Ly6C, and Ly6G combined with ARG1 and NOS2 expression levels, the stages and functional development of a MDSC can be partially followed from its origin in the bone marrow to its final functional stage in the tumor.

1.2.4 The Life History of MDSCs in Cancer: From Bone Marrow to Tumor

Tumor MDSCs have a complex lifespan: Expansion and commitment of precursors in peripheral sites precedes differentiation and activation in the tumor: MDSCs found in the tumor have a complex development beginning in the bone marrow where they reside as non-functional precursors. In response to the appropriate inflammatory signals, these precursors can enter circulation and travel to the secondary lymphoid tissues where they become partially activated. These cells are subsequently recruited to the tumor where they gain additional suppressive function via their interactions with several potential factors derived from the tumor microenvironment. One potentially confusing aspect of MDSC biology is that markers used to define functionally suppressive MDSC in the tumor (e.g. in mice: CD11b, Ly6C, Ly6G) are also expressed on MDSC precursor cells found in the bone marrow and spleen, i.e. the stage of MDSC development currently cannot be accurately determined based on cell surface markers alone. However, advances in machine learning have shown that immune cell phenotypes can be determined using basic antibody panels [42]. This technique could be used to identify different stages of MDSC development based

on the current marker set and will be discussed further in Chapter 3. As a result, the current use of the term "MDSC" for both precursor cells and functional tumor-derived cells can be confusing. Recently, Bronte et al. [38] proposed the term "eMDSC" to define the immature "early stage MDSC" that reside outside the tumor environment in humans. However, this nomenclature does not resolve the inherent confusion about the stage of development as they do not apply this to any MDSC found in mice. Therefore, the following nomenclature for MDSCs will be used in this chapter: (a) immature MDSC (iMDSC) for cells from the bone marrow; (b) peripheral MDSC (pMDSC) for cells isolated from peripheral lymphoid tissues like spleen and lymph nodes; (c) in vitro generated MDSC (ivgMDSC) that result from stimulation of cultured iMDSCs or pMDSCs; and (d) tumor MDSC (tMDSC) that are the functional, mature cells isolated directly from the tumor. We believe that this nomenclature better reflects the etiology and function of MDSCs and that this clarity will aid in the interpretation of M-MDSC research as well as the development of anti-M-MDSC therapies. This is summarized in Figure 1.

From iMDSCs to pMDSCs; Signals from the tumor invoke change: The change from iMDSC to pMDSC begins with the expansion of iMDSCs in the bone marrow and is followed by an activation step at the spleen. Multiple cytokines and environmental signals influence iMDSC expansion, Dolcetti et al. [10] summarized data on iMDSC expansion in mice from multiple tumor models and concluded that tumor-derived granulocyte macrophage colony-stimulating factor (GM-CSF), is one of the main cytokines responsible for the expansion of iMDSCs at the bone marrow and spleen of tumor-bearing mice. In addition, Casbon et al. [43] found that tumor-derived granulocyte-colony stimulating factor (G-CSF) induced differentiation of bone marrow iMDSCs toward tMDSCs in a murine breast cancer tumor model. Finally, Marigo [44] found that tumor-derived GM-CSF, G-CSF or a combination of the two were sufficient to induce expansion of murine bone marrow iMDSCs in culture. They also showed that the addition of IL-6 with GM-CSF could induce development of the T-cell suppressive function in cultured bone marrow derived iMDSCs [44]. Similarly, GM-CSF and IL-6 have been shown to induce human peripheral blood mononuclear cells (PBMC) into suppressive MDSCs in culture [45]. GM-CSF, G-CSF, and IL-6 influence iMDSC expansion through receptor tyrosine kinases that activate STAT3 or C/EBPβ to induce transcription of genes encoding cell cycle regulators [46].

After expansion of iMDSCs in the bone marrow, these cells are recruited to, and accumulate in, the secondary lymphoid tissue where they are further influenced by tumor-derived cytokines to become pMDSCs. Spleen pMDSC accumulation is associated with increased tumor burden [8, 47]. Interestingly, in a study where mice were injected subcutaneously with one of several different tumor cell lines, there were differences in number and ratio of G- to M-pMDSCs depending on the cancer cell line. This suggests that there are specific but as yet unidentified factors from different tumors that could drive pMDSC expansion and differentiation toward M- or G- pMDSCs.

In the spleen, pMDSCs gain production of IL-10, ROS, myeloperoxidase (MPO) and peroxynitrite as well as expression of NOS2 and ARG1 mRNA [46]. Additionally, many have shown that spleen pMDSCs gain suppressive function however, these studies all have used assays exceeding 48 h co-culture with T-cells. When spleen pMDSCs are cultured in a shorter assay, 12-18 h, they are not suppressive [48, 49]. This suggests that signals from the T-cells are inducing their suppressive function during the longer suppression assays. Additionally, these data suggest that immediate suppressive potential of spleen pMDSCs is not being tested. Therefore, throughout this dissertation, the length of time for suppression studies will be noted and provides evidence of the need for a higher level of clarity when discussing MDSCs. Signals that specifically induce development of M- subtype phenotype are Interferon gamma (IFN-γ), prostaglandin E2 (PGE2) and, tumor necrosis factor alpha (TNF-α) (reviewed in [46]). IFN- γ is released by T-cells; it binds the IFN- γ receptor to activate STAT-1 which regulates NOS2 and ARG1 expression. PGE2 production results from increased COX-2 production by tumor cells. It binds to the EP2/4 receptor which leads to increased expression of ARG1. Finally, TNF-α from tumor cells acts via its receptor to activate the nuclear factor kappa B (NF-κB) signaling pathway, leading to cell survival. Several factors have been found that activate both subtypes. IL-1βand toll-like receptor (TLR) ligands signal through the NF-κB pathway to increase RNS production, while IL-4 and IL-13 activate the STAT-6 signaling to increase ARG1 activity. Finally, IL-6 selectively induces development of the G-subtype via a STAT-3 pathway to increase T-cell suppressive activity and transcription of the NOS2 gene to increase RNS production.

<u>pMDSCs migrate to the tumor site to become fully functional MDSCs:</u> MDSCs, like other immune cells, find their way to the tumor via homing signals and chemotaxis. This was shown elegantly

by Katoh et al. [9] who used chemokine (C-X-C motif) receptor 2 (CXCR2) KO mice to demonstrate that recruitment of G-pMDSCs into the inflamed colonic mucosa of mice treated with AOM and DSS was essential for the development of colon tumors. In the absence of CXCR2, they showed that pMDSCs remained in circulation and did not accumulate in the DSS-damaged colon. This finding was confirmed in breast cancer models by Sharma et al. [11] who showed that mice lacking CXCR2 had decreased accumulation of CD11b+Gr1+ MDSCs at the tumor. A recent study by Chun et al. [50] suggests that Chemokine (C-C Motif) Ligand 2 (CCL2) is also critical for pMDSC migration to colon tumors. This study found that knock-down of CCL2 reduced MDSC accumulation at implanted CT26 mouse colon cell tumors. Additionally, intratumoral injection of CCL2 into CCL2 knockdown tumors increased the accumulation of MDSCs into the tumor [50]. Other factors released by the tumor (e.g. CCL4, CCL5 [51]) may also aid in the recruitment of MDSCs. However, further investigation is needed to fully understand the migration of pMDSCs to the tumor site.

The development of tMDSCs in the tumor microenvironment is influenced by both pro and anti-inflammatory signals generated by the complex interaction between tumor and immune cells. Along with the cytokines and immune cells in the tumor microenvironment, the tumor microenvironment is known to be hypoxic. As early as 2009, hypoxia was identified as a potential regulator of tMDSC T-cell suppressive function [52]. Later, Corzo et al. [53] adoptively transferred pMDSCs into tumor-bearing mice and found that the transferred pMDSCs rapidly gained T-cell suppressive function. Further, they cultured pMDSCs with GM-CSF under normoxic and hypoxic conditions and showed that hypoxia appeared to mimic the tumor microenvironment by increasing the ability of cultured pMDSCs to develop T-cell suppressive function. Finally, they showed that myeloid cell-specific deletion of hypoxia-induced factor-1 alpha (HIF-1α) prevented the development of T-cell suppressive function development in tMDSCs from mouse tumors [53]. Collectively these data show that hypoxia-induced gene regulation mediated by HIF-1α is an important step in the final activation of the tMDSC function.

Together, the life of an MDSC has multiple potential immunotherapy target sites, from iMDSC expansion to decreasing the function of tMDSCs. Several of the studies listed above have suggested potential target pathways to decrease MDSC development and reduce tumor burden. Additionally, as discussed earlier, nutrients and dietary agents can act as immunomodulators and

may act upon some of these pathways. We will summarize the existing evidence that links the use of nutrients and dietary agents with modulation of MDSC development and function in Chapter 4.

Immunotherapies Strengths and weaknesses: Several immunotherapies have already been approved and are currently being used to treat various cancers. According to the National Cancer Institute the major types of immunotherapy either directly promote the immune system to attack cancer cells (checkpoint inhibitors, adoptive cell transfer, monoclonal antibodies, and treatment vaccines) or generally support the immune system (cytokines, and Bacillus Calmette-Guérin (BCG, specific to bladder cancer)) [54].

Checkpoint molecules on T-cells promote cell cycle arrest or anergy and act as a break system in normal physiology but, some tumor and immunosuppressive cells, have been shown to contain the ligands for these molecules. Therefore, checkpoint inhibitors block the programed death 1 receptor (PD-1) or T lymphocyte associated protein 4 (CTLA-4) on T-cells, resulting in a larger number of T-cells able to clear tumor cells [3]. However, a major drawback to this therapy is that its high efficacy is only found in low percentage of patients due to both tumor cell and immune cell susceptibility to the treatment [3, 55].

Another therapy to enhance T-cells is adoptive transfer. Adoptive transfer therapy currently consists of two different types of transfers including ex vivo expanded (by IL-2) host tumor infiltrating T-cells or genetically engineered T-cells expressing chimeric antigen receptors (CAR). CAR therapy is a modification of tumor infiltrating T-cell therapy where T-cells are removed from the blood, modified and expanded ex vivo to directly recognize the patient's tumor, then reinjected back into the patient, where they can seek out the specific tumor cells [56]. The downsides to adoptive transfer therapies are, first the increase in T-cells is not always enough to overpower immunosuppressive cells at the tumor, it may not be possible to get tumor infiltrating T-cells from all tumors, and these treatments are very expensive.

Additional immunotherapies work by acting on cancer cells to enhance the immune response such as monoclonal antibodies. These therapies utilize antibodies that target specific proteins on cancer cells making them more susceptible to immune responses and in some cases these antibodies will also aid in reducing tumor cell replication, as in the HER2 antibody trastuzumab (Herceptin®). These antibodies have shown much promise for aiding in targeting the tumor for clearance but can come with several side effects. While less than traditional

chemotherapy, the side effects can be serious depending on the antibody. Finally, vaccines can be used to initiate an immune response against tumor cells. With this treatment vaccines are made from pieces of lysed tumor cells ex vivo or from known proteins expressed by the tumor ie. Sipuleucel-T (provenge®) which is uses prostatic acid phosphatase protein for treating prostate cancer. The major weakness to this type of therapy is that it takes several weeks to develop the vaccines and they are specific to each patient, aside from treatments like Sipuleucel-T (provenge®).

Treatments that generally support the immune system are often used as adjuvants to other treatments and can cause serious side effects. For example, early studies with IL-2, which promotes cytotoxic T-cell activation, resulted in capillary leakage allowing fluid to enter organs causing damage or failure [57]. Other cytokines are also used but the lessons learned during the IL-2 trials have reduced their use as a primary treatment. The other type of generally supportive treatment is the use of a weakened form of the bacteria Bacillus Calmette-Guérin. While BCG treatment has shown success, it is only available for bladder cancer. However, others are studying the possibility of using the same technique with different bacteria strains for other cancers [58, 59].

Overall the current milieu of immunotherapies has not been able to break past the problem of immunosuppression at the tumor site. Primarily due to the T-cells and targeting/tagging the tumor cells. However, some immunotherapies like checkpoint inhibitor have been shown to decrease the T-cell suppressive ability of MDSCs [60].

1.3 Flow Cytometry: Current and Upcoming Techniques

1.3.1 General Use for Studying Cells: Strengths and Weaknesses

One of the critical tools used to study the cells of the immune system is flow cytometry. As discussed by McCoy [61], flow cytometry utilizes the concept of photoemission from fluorochrome molecules and light scatter to measure and analyze the physical characteristics of cells. Primary or cell lines are prepared by staining the cells with fluorochromes prior to flow cytometry analysis. Many fluorochromes are covalently bonded to antibodies for a specific cell marker, both intra- and extracellular. However, some fluorochromes are not attached to antibodies but are simply dyes that bind proteins or DNA, ie. propidium iodide binds DNA and is used for live dead discrimination. Generally, multiple lasers are used to excite the fluorochromes to a

specific emission where photodetectors can then analyze each signal. When the cells are placed into a flow cytometer, each cell is analyzed individually at a high rate (as high as 10k cells/sec depending on cell size). Therefore, a single flow cytometry sample file may consist of data for millions of cells depending on the proportion of the target cell within the sample. Based on these signals a user can use a method called gating which utilizes bi-plots of the markers. During sorting, gating is used to determine the percentage of specific cells within the heterogeneous mix of cells and select the specific populations to be isolated. However, for analysis, gating can be done during the initial analysis or after using gating software i.e.. FlowJo (Tree Star).

Traditional flow cytometry has been limited to around 16-18 color combinations due to the limitations of lasers and photodetectors. However, new advances in the field of flow cytometry are increasing this number with the potential to reach upward of 100 markers. While this technology is still relatively new, many studies are already seeing its benefits for cell lineage and multiple phenotype characterization. Another advancement in the field is the concept of ad hoc analysis of flow cytometry data via multidimensional analysis. This method utilizes multiple markers identified on a cell but instead of being limited to a bi-plot analysis, identifies cells based on expression levels of multiple markers at the same time in a multidimensional space.

There are two basic types of flow cytometry, but both utilize the same principles spelled out above, cell sorting-also known as Fluorescence-Activated Cell Sorting (FACS) or pure analysis. In FACS, cells are labeled and kept alive throughout the process in order to be used as a pure population post sort. This process involves a specific type of cytometer that allows the user to collect cells with specific markers. When done correctly this generally yields a cell purity (based on markers) of >90%. In contrast, cells used in pure analysis flow cytometry can be either live or fixed (ie. dead). This allows the investigator several options that are not available during FACS. For example, post fixation of cells, specific intracellular markers may be used that would otherwise alter the function of the cell. As previously stated, one weakness of traditional flow cytometry includes a limited number of markers. This weakness is magnified by the fact that specific cell types may not have pure marker sets and therefore may be pure by marker but not by function. For example, neutrophils in mice are known to express CD11b and ly6G or Gr-1, yet these are the same markers used for isolating the G-MDSC subtype. Another weakness is that, while minimum standard information about how flow cytometry data is collected has been suggested [62], these standards are still not practiced in many publications. Therefore, there is a need for standard flow

cytometry method reporting, marker panels with higher precision for specific cell types, and methods for analyzing flow data based on known cellular functions.

1.3.2 High Throughput Methods Utilizing Large Marker Panels

New advances in flow cytometry have opened the possibility of lineage tracing using high-throughput methods with large cell marker panels. The most notable method for high throughput flow cytometry is mass cytometry, also known as Cytometry Time-of-Flight (CyTOF) [63]. This method, as reviewed in [64] requires special cytometers that include mass spectrometry. Here, antibodies are no longer bound to fluorochromes but have heavy metal ions attached to them. Current panels are around 30-40 markers allowing for more exploratory approaches to understanding cell lineage. As this technology continues, it will aid in our understanding of not only lineage but, will also help identify specific markers that may be used in traditional FACS. Like all methods, CyTOF has weaknesses when compared to traditional flow. First, cells are fixed prior to analysis and therefore, cell sorting is not possible with CyTOF. Second, the number of premade panels is still very limited and may not include all markers of interest. Finally, CyTOF requires special machines and marker panels that are very costly and make their current availability limited. Therefore, until more advances are made in this method, other approaches may have great merit in the understanding of cell phenotypes.

1.3.3 Multidimensional Flow Analysis: Utilizing Existing Data in *ad hoc* Analysis

Post hoc multidimensional analysis of flow cytometry (MDF) data has emerged as another successful tool in understanding cell phenotypes. There are multiple software packages that do this type of analysis [65, 66] two examples that represent the major types of packages include; Spanning-tree Progression Analysis of Density-normalized Events (SPADE [67]) and FlowMatch (package for Bioconductor in R [68]). SPADE represents software packages that are designed primarily for the use of understanding hierarchal clustering and lineage mapping. FlowMatch represents a group of software packages that are designed for the purpose of comparing data sets of samples and discovery of unique groups of cells.

Common to all multidimensional software packages each cell within the collected sample is viewed in multidimensional space to create groups or clusters of cells with similar markers. The

major differences are in the algorithms used for processing the data, sample comparisons, and the final output.

SPADE creates a node tree of different groups of cell types based on marker expression. These branched cluster trees can then be adjusted, similar to a heat map, to show the intensity of specific markers. One of the greatest advantages to these packages is the connections within the given tree, providing information about the node's relationships such as lineage [65]. However, as stated above, packages such as SPADE were not specifically designed to compare across multiple sample types but within samples.

In contrast, FlowMatch samples are used to create a representative template of the clusters within a specific set of samples such as bone marrow. At this level, FlowMatch is very similar to SPADE; however, the output plots are quite different. FlowMatch produces hierarchical trees and 2D plots of the templates that represent specific sample sets. The novelty of FlowMatch is that it will compare samples based on cell populations from different sample sets such as comparing bone marrow to spleen. These template plots can provide information about unique clusters of cells within a sample. For example, FlowMatch was recently used to identify a unique set of cells found in Acute Myeloid Leukemia (AML) patients [42]. While these tools are slowly gaining popularity and use [66, 69] more studies are needed to understand their future use in immunology and, potentially, clinical settings.

1.4 Nutrition and the Immune System

Several studies have looked at diet and dietary bioactives as effectors of the immune system in physiological and pathophysiological conditions. One of the challenges in this field is that individual bioactive components have different potentials based on the environment in which they are present. These environments vary based on physiological and pathophysiological conditions making it challenging to apply knowledge generated from one system to another. Therefore, this section includes an overview of several reviews on nutrition, vitamins, and phytochemicals as immunomodulators in a variety of physiological and pathophysiological conditions. The two major responses from the immune system can be categorized as pro- and anti-inflammatory (immunostimulatory or immunosuppressive, respectively) as these two responses involve signaling to inhibit one another. Thus, dietary bioactives will be discussed in these two settings,

identifying the specific modulation of the innate and adaptive responses in an effort to understand how they may act in different physiological and pathophysiological conditions.

1.4.1 Effects of Overall Diet and Nutrition

The role of the diet as an immunomodifier is an important aspect of diet that has been studied alongside the advances in the field of immunology and referred to as nutritional immunology or immunonutrition. Understanding the roles of various nutrients within foods as immunomodulators is critical to understand the maintenance of a healthy immune system. It is important at this point to define a significant problem in the investigation of diet on immunomodulation, systems biology vs a reductionist approach. In systems biology, often where dietary studies exist, the role of a single molecule and its pathway is not as critical as the overall effect of the diet on the entire system(s). In contrast, the reductionist approach tries to identify the ways in which a single molecule will affect specific pathways within a cell(s). This means that when discussing the role of diet (a combination of multiple molecules and types) a reductionist approach is not appropriate. Therefore, the following will focus on the systematic effect of diet on the immune system and less focused on individual cells, as will be done with vitamins and phytochemicals.

The role of diet as a modulator of the immune system has been reviewed in various physiologic and pathophysiological conditions [12, 70, 71]. In a review by Kaminogawa et. al, the immunomodulatory properties of vitamins and minerals, carbohydrates, proteins, and lipids were categorized and discussed by physiological states; healthy individuals, those with hypersensitivity, and immunocompromised individuals [12]. Therefore, this categorization will be used in the following summary of reviews diet as immnuomodulators.

In healthy individuals, several compounds have been identified to maintain the homeostasis of the immune system. For example, in the innate response the primary phagocytes (macrophages, dendritic cells and neutrophils) and cytotoxic cells (NK cells) are influenced by dietary content of vitamins and minerals along with fatty acids and lactic acid. These nutrients can improve delayed-type hypersensitivity, increase bacteria phagocytosis by macrophages, dendritic cells and neutrophils, and increase NK cell activity [12]. These actions support a role for dietary vitamins and minerals, fatty acids and lactic acid in the retention of the innate system homeostasis and inhibition of pathological states. Additionally, dietary nutrient content effects the adaptive

response, including cytotoxic T-cells and mature antibody producing B cells. For example, dietary content of vitamins and minerals along with fatty acids and oligosaccharides increase the T-cell response and antibody production by B cells. Together this provides a brief summary of a few dietary nutrients in their aid of a pro-inflammatory response and maintenance of a healthy immune system. However, a differential response to nutrients exists in those with pre-existing inflammatory conditions.

In patients with hypersensitivity (i.e. autoimmune and allergy responses) and chronic inflammation, specific nutrient-rich diets can aid in the reduction or completion of the inflammatory response. While Kaminogawa et. al discuss several different pathophysiological states, Magrone et al have reviewed evidence for functional foods as nutraceuticals to treat diet-related diseases [12, 71]. During hypersensitivity and chronic inflammation, dietary content of vitamins and minerals, fatty acids and caloric intake have all been shown to alter both the innate and adaptive responses to be anti-inflammatory by reducing inflammatory cytokine production, oxidative stress, and altering the Th1/Th2 balance toward an anti-inflammatory response [12, 71][1]. Similarly, Lopez et al. have reviewed evidence that dietary alterations (increased ω-3 fatty acids; eicosatetraenoic acid and docosahexaenoic acid) can also reduce other pro-inflammatory states such as rheumatoid arthritis [72]. Lopez et. al also suggest that there is evidence that vitamin deficiency, such as vitamin D and folate, is associated with the worsening of this condition. Taken together, these reviews show that nutrients can play an important role in the inhibition of chronic inflammation seen in hypersensitivity and rheumatoid arthritis.

Finally, specific nutrient-rich diets have been associated with increasing the host immune responses in immunocompromised individuals [12, 72]. In immunocompromised individuals, such as those with HIV/AIDs, multiple secondary diseases are a major concern. According to Lopez et al. these secondary pathophysiological states can cause a decrease in dietary intake leading to weight loss, maldigestion and malabsorption contributing to malnutrition [72]. Therefore, they suggest that increased protein and polyunsaturated fatty acids intake, as well as overall healthy food consumption, will lead to reduced secondary effects and promote the adaptive immune response, specifically CD4 T-cells, the major target of HIV/AIDs. Together these data provide a basic look at the complexity that exists in dietary modulation of the immune system and the

[1] Magrone et. al table 1 shows multiple studies with treatment types, immune markers, and responses. Treatments in this table are diet based and include several well-known diets such as the Mediterranean diet.

importance of understanding the pathophysiologic state of the host as well as the role of the entire immune system response.

1.4.2 Effects of Vitamins

The role of vitamins in maintaining a healthy balance between anti- and pro-inflammatory settings from the immune system has been well documented in multiple model systems [73-77]. However, the focus of many of these reviews is on the anti-inflammatory immunomodulatory capabilities of vitamins which often only accounts for vitamin action during inflammatory pathophysiological conditions. However, the role of vitamins in cancer immunology is becoming a major field of interest where vitamins aid both adaptive and innate immune cells in the clearing of cancer cells. In this setting, vitamins are no longer anti-inflammatory but, act as promoters of a pro-inflammatory response. Two examples of vitamins that exhibit this dual role are vitamin A (and its metabolite all-trans retinoic acid: ATRA) and vitamin D (specifically its hormonal form 1,25-dihydoxyVitaminD3: 1,25D).

The effects of Vitamin A and D as transcription regulators of the immune system under various conditions have been previously reviewed in detail [74, 78-80]. Both vitamin A and D are ligands to specific receptors (retinoic acid receptor (RAR) and vitamin D receptor (VDR), respectfully) which once bound become transcription factors that regulate transcription. This section will highlight specific effects and mechanism of action of Vitamin A and D on specific immune cell types and their functions. The cell targets of vitamin A and D include neutrophils, monocyte/macrophages, dendritic cells, and T and B cells. The major functions of these cells were covered in Section 1.2 therefore only a brief description of their general functions will be stated here.

In the innate response, neutrophils are the most abundant immune cell found in circulation and are considered the first responders to pathogens and are modulated toward anti- and pro-inflammatory conditions by vitamins A and D. These cells are involved in phagocytosis and the release of cytokine signaling to promote inflammation as well as recruit other immune cells. Under inflammatory conditions vitamins, A and D act as anti-inflammatory agents against neutrophils by reducing the number of neutrophils in circulation and migration to the inflammatory site [81, 82]. However, at the inflammatory site, they act more as pro-inflammatory agents and stimulate

neutrophil maturation, phagocytic ability, and production of extracellular traps [79, 83]. This pro-inflammatory response is also seen in complex environments such as cancer.

Monocytes are a mature cell with multiple fate options and are responsible for macrophage cell infiltration into pathological sites and are modulated by vitamins A and D by promoting class switching between M1 and M2 subtypes [78, 81]. Macrophages play a dual role in the immune system and are divided into two subtypes M1, which promote inflammatory responses via cytokines and phagocytosis, and M2 which aid in tissue repair and termination of the inflammatory response via cytokines. In monocytes, vitamin A and D promote the differentiation of monocytes toward the M2 macrophage phenotype and potentiate the production of the anti-inflammatory cytokine IL-10 [78, 80, 84]. While in a pathogen inflammatory response these vitamins appear to be anti-inflammatory, under different pathophysiologic conditions they are pro-inflammatory. Vitamin A and D have both been shown to increase chemotaxis of monocytes to the pathogenic site, phagocytosis, and release of the pro-inflammatory cytokine IL-1β [74].

Dendritic cells can also be divided into two main categories as pro-inflammatory, those that activate the T and B cell responses, and tolerogenic, which aid in the termination of inflammation and these responses are modulated by vitamins A and D. Under pathogen-induced inflammation vitamin A and D reduce dendritic cells differentiation from monocytes, promote tolerogenic dendritic cells and the release of anti-inflammatory cytokines [74]. In contrast, both vitamins aid in the maturation of differentiated dendritic cells by promoting MHC expression which is critical for the activation of the adaptive immune response and increasing the inflammatory response [17, 74] .

In the adaptive immune response, T-cells and B cells are both active during inflammation and their response is modulated by vitamins A and D. There are two major types of T-cells; CD4 T helper cells, and CD8 T-cells. CD4 T helper cells are modulators of the adaptive immune response and mediators of inflammation termination while CD8 cytotoxic T-cells are the primary killing cell of the adaptive response and also include the memory T-cells. The other major cell type of the adaptive response are the B cells. There are two major types of B cells; B regulatory cells which aid in inflammation termination and B cells which aid in pathogen clearance via antibody production and also include memory B cells. Both vitamins A and D reduce the inflammatory response of adaptive cells by promoting the production of regulatory T and B cells, reducing the number of cytotoxic T and B cells, and increasing the number of memory T and B cells [74, 78].

Interestingly, both vitamins have been shown to be critical for the proliferation and longevity of T and B cells [74, 79]. However, in the context of cancer, both vitamins have been shown to reduce tumor growth by increasing cytotoxic T-cells and B cells [74, 79]. Therefore, the contradictory functions of vitamin A and D illustrate the dual role of vitamins and exemplify the role of the environment in the immunomodulatory role of vitamins.

1.4.3 Effects of Phytochemicals

The term phytochemical implies a large group of compounds and is defined as non-nutritive plant chemicals expressed as secondary metabolites which may have protective or disease-preventing properties in humans and animals [85]. According to Liu et al, as of 2004, over 900 compounds have been identified from plant sources [86]. In general, these compounds can be divided into the following major classes of compounds based on physical properties such as structure and functional groups present: phenolics (also known as polyphenols), carotenoids, alkaloids, Nitrogen-containing compounds, and organosulfur compounds [86]. The effects of many phytochemicals on the immune system under various pathophysiologic states have been previously reviewed in detail [87-92]. Epicatechin gallate, resveratrol, and curcumin are three examples of polyphenols that have been studied as immunomodulatory and will be further discussed here.

Primarily seen as anti-inflammatory agents, epigallocatechin gallate (a flavonoid) and resveratrol both act on the innate and adaptive immune responses. In the innate response, these polyphenols target monocyte/macrophages, neutrophils and NK cells. In macrophages, epigallocatechin gallate (a flavonoid) and resveratrol have been shown to reduce pro-inflammatory interferons and interleukins [93] respectively. Additionally, epigallocatechin gallate is involved in the reduction of adhesion molecules found on monocytes, macrophages, and neutrophils reducing their ability to enter the inflammatory site. Meanwhile, resveratrol has been shown to reduce the expression of CD28 and CD80 receptors on macrophages and dendritic cells involved in the activation of the adaptive response. In the adaptive response, both polyphenols have been shown to decrease cytotoxic CD8 T-cell and B cell activation while increasing Tregs. Together these demonstrate a small portion of the many potential actions by which polyphenols can act as anti-inflammatory molecules.

As with diet and vitamins, epigallocatechin gallate and resveratrol also act as pro-inflammatory immunomodulators in specific pathophysiologic conditions such as cancer. For example, in the innate response, low dose administration of resveratrol has been shown to increase macrophage function and enhance macrophage and dendritic cell induced IL-12 and INF-γ production [94], both important for the activation of T-cells. Additionally, resveratrol enhances NK cell activation and tumor cell killing [95]. Additionally, epigallocatechin gallate has been shown to enhance the induction of IL-12, a CD8 activating cytokine, in UVB-immunosuppressed mice. However, the cell responsible for the release was not determined. Within the adaptive response, in the pathophysiologic condition of existing cancer, epigallocatechin gallate and resveratrol increase CD8 T-cell activation by promoting dendritic cells antigen presentation and Th1 cells, respectively [94].

Whereas epigallocatechin gallate and resveratrol have a limited number of studies and reviews on their effects on the immune system; curcumin has been one of the most studied immunomodulatory phytochemicals in multiple pathophysiologic conditions [96-99]. The effects and molecular actions of curcumin on the major cells of the immune system have been reviewed by Jagetia and Aggarwal [100]and will be summarized here. In the innate system, curcumin is an immunomodulator of all the major cell types; neutrophils, macrophages, dendritic cells, and NK cells. As an anti-inflammatory immunomodulator of the innate cells, curcumin inhibits the activation of neutrophils, reduced the production of ROS and NO production by macrophages, reduces the expression of CD80 and CD86 (important for dendritic cell activation of T-cell) and pro-inflammatory cytokines by dendritic cells, and reduced cytotoxicity in NK cells. Within the adaptive response, curcumin acts on both T and B cells to reduce inflammation. In T-cells (both CD4 and CD8) curcumin inhibits T-cell proliferation, IL-2 expression, and it has been shown to increase Tregs in mice with colitis [101]. As a modulator of B cells, curcumin reduces the proliferation of immature B cells and inhibits B cell proliferation and production of memory B cells in the presence of Epstein–Barr virus (known to be responsible for mononucleosis). Together these cellular responses provide evidence that curcumin acts as an anti-inflammatory immunomodulator under specific pathophysiologic conditions, primarily those that are inflammatory in nature.

The effects of curcumin on the immune system are not limited to anti-inflammatory responses but in conditions like cancer can be pro-inflammatory. The innate response to curcumin,

in pathophysiological conditions like cancer, increases total white blood cells, increases phagocytosis of macrophages, enhances antigen endocytosis in dendritic cells (albeit overall curcumin suppresses dendritic cells function), and increase NO and cytokine production as well as Th1 activation mediated by NK cells. While the major adaptive cell response to curcumin is anti-inflammatory some pro-inflammatory responses have been shown. In T-cells, curcumin has been shown to increase cell proliferation of CD4 T-cells in the spleen, increased proliferation, cytotoxicity, and INF-γ production by CD8 T-cells [94]. Similarly, in B cells curcumin increases B cell proliferation at the intestinal mucosa of mice. Finally, curcumin has been found to boost the adaptive response to tumors by reducing the suppressive action of MDSC's [102]. Overall curcumin, like other phytochemicals, has a differential response to the immune system depending on the pathophysiological condition of the host. Therefore, these compounds are an important area of study for immunomodulation within different pathophysiologic states to determine their beneficial or detrimental role on the immune system.

Dietary nutrients composition, vitamins, and phytochemicals are powerful immunomodulators that rely heavily on the health status of the host. This discussion provides evidence that the pathophysiologic condition of the host plays a major role in the overall impact of dietary nutrients composition, vitamins, and phytochemicals on the whole immune system response. This information supports the need for more studies to understand how dietary nutrients composition, vitamins, and phytochemicals may be used in multiple disease states to enhance or suppress the immune system.

1.5 Research Questions

Based on the information discussed here, it is clear that several questions regarding MDSC and the use of dietary bioactives as immunomodulators remain. However, before looking at the potential of bioactives it is important to understand more about the basic biology of MDSC. The major question addressed in Chapter two is, do MDSC isolated from different tissue represent different stages of MDSC development? This question is based on the lack of studies comparing MDSC isolated from peripheral tissues and the tumor site. The results will lead us to a better understanding of MDSC biology and how to develop more specific strategies for targeting these cells. Following this line of thinking, chapter three addresses the question, can we classify the difference between BM, SP, and TU MDSCs based on common flow cytometry markers? The

results from this study not only aid in the understanding of MDSC biology but also help to lay a foundation for the use of MDSCs as a potential biomarker for cancer stage. While cancer has been used as a model to study MDSC biology, these cells play an important role in maintaining a normal inflammatory response. In cancer, MDSCs and regulatory cells are hijacked by the tumor and therefore aid in immune escape but, in other pathophysiologic conditions MDSCs help in reducing the negative effects. Dietary agents are a key component to any disease state and altering a patient's intake during disease progression may be a useful way to target MDSCs and aid in the treatment of several diseases, for example, cancer, and preeclampsia.

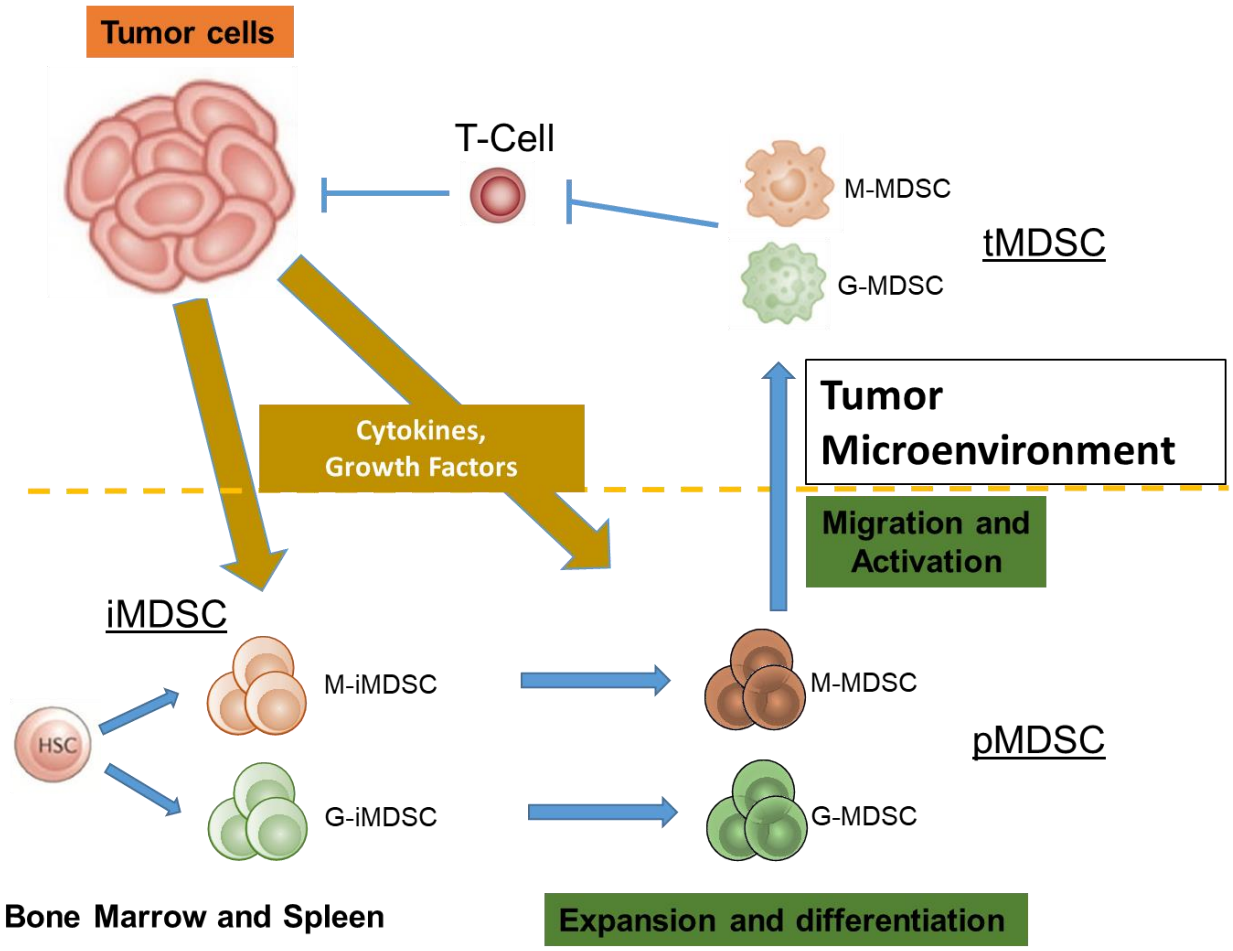

Figure 1.1 Alternate Differentiation of Myeloid Cells by Tumor-Derived Factors.

In the context of cancer, T-cells will enter the tumor site and begin clearing tumor cells by recognition of tumor antigens. As this occurs tumor cells secrete cytokines and growth factors into circulation that change the physiologic myelopoiesis. Cytokines and growth factors released from the tumor influence hematopoietic stem cell (HSC) increasing production of M- and G-iMDSC. Continued exposure to tumor-derived factors causes M- and G-iMDSCs to migrate to the spleen and undergo partial differentiation into peripheral MDSC (p-MDSC). From the spleen, p-MDSC enter the circulation and migrate to the tumor site where they are activated by factors found in the tumor microenvironment. Once fully activated t-MDSC are able to suppress T-cell cells. Modified from [39]

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CHAPTER 2. MONOCYTIC MYELOID DERIVED SUPPRESSOR CELLS FROM TUMOR HAVE LIMITED DIFFERENTIATION POTENTIAL

Portions of this work were published and presented at the 2018 American Association for Cancer Research conference. Additionally, a version of this work is currently being submitted for publication.

Calvert RD, Fleet JC, Chen Y, Pothen A, Rajwa B, Fournier PG, Juarez P, Guise TA, Ratliff TL, Elzey BD. Abstract 4741: Monocytic myeloid derived suppressor cells (M-MDSC) from spleen are multipotent while tumor M-MDSC have limited plasticity2018;78(13 Supplement):4741-. doi: 10.1158/1538-7445.AM2018-4741 J Cancer Research.

2.1 Abstract

The tumor microenvironment (TME) contains monocytic myeloid-derived suppressor cells (M-MDSC, $CD11b^{+}Ly6C^{hi}Ly6G^{lo}$) that suppress cytotoxic T-Cell-mediated immune surveillance and allow tumors to grow. M-MDSC-like cells are also found in the bone marrow (BM) and spleen (SP) of tumor-bearing mice and are often used as surrogates for tumor (TU) M-MDSCs. We conducted a series of experiments to test if these three cell types are functionally similar. Our group has previously demonstrated that although TU and SP MDSCs suppress T-cell proliferation in the standard long term (72h) suppression assays, TU MDSCs, but not SP MDSCs, suppress T-cell proliferation in short-term suppression assays (12-16h) [8, 9]. We verified these findings that in a short-term T-cell suppression assay (16 h) only TU M-MDSCs suppressed T-cell proliferation. To further investigate this difference, we looked at the transcriptome of SP and TU MDSCs and gained insight into pathways that may be associated with activation or differentiation. Additionally, the transcriptome gave us insight into the magnitude of transcript and pathway differences between SP and TU MDSCs. This suggests SP M-MDSCs are reprogramming through differentiation as they become TU M-MDSCs. Others have shown that SP M-MDSCs can differentiate toward other end-stage myeloid cells, but it is not clear whether the ability to differentiate extends to TU M-MDSCs. Therefore, we used *in vitro* differentiation models to determine if BM, SP, and TU M-MDSCs differentiate to different cell fates. Upon treatment, SP M-MDSCs acquired the markers of osteoclasts (Ocs) (TRAP), G-MDSC (loss of Ly6C, gain of Ly6G), dendritic cells (DCs)

(CD11c), and macrophages (F4/80). In contrast, TU M-MDSCs could only differentiate into macrophages. Because these assays do not fully represent the environment that M-MDSCs are found in tumor bearing mice, we also cultured the BM, SP and TU M-MDSCs with differentiation cytokines and tumor extract supernatant (TES) to mimic the tumor microenvironment (TME). These assays demonstrated that BM, SP and TU M-MDSCs do not possess the same abilities to differentiate into G-MDSCs, DCs, and Ocs. Culturing SP M-MDSC with EL-4 cell TES, caused them to become resistant to signals for conversion to G-MDSC or DCs and more responsive to the macrophage-inducing treatment. Our data suggest that SP M-MDSC are immature, multipotent cells and the addition of TES causes them to mature and behave more like a less plastic TU M-MDSC. The major finding of these experiments is that TU M-MDSCs are a more mature phenotype that are morphologically and functionally distinct from the M-MDSC precursors found in BM and SP.

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2.2 Introduction

During the development of tumors, myeloid derived suppressor cells (MDSCs) play a critical role in the inhibition of host T-cell-mediated tumor clearance. Therefore, MDSCs create a

challenge for developing immunotherapies for cancer focused on increasing T-cell mediated tumor clearance. Thus, understanding MDSC development and function is an important step toward developing successful immunotherapies for cancer that will suppress MDSCs and increase T-cell responses to clear tumors. MDSCs are a heterogeneous population of cells consisting of two major populations, monocyte-like (M-MDSC) and granulocytic (G-MDSC) cells. In mice, these are defined by three markers where M-MDSC are CD11b+Ly6ChiLy6Glow and G-MDSC are CD11b+Ly6intLy6Ghi [1]. These two populations exhibit differential abilities to suppress T-cells in vitro and in vivo where M-MDSCs have been shown to have a much higher suppressive ability over G-MDSCs [2]. Additionally, M-MDSCs possess a high level of plasticity. This plasticity, or potential to differentiate into other cell types, is a mechanism to control the T-cell suppressive ability of M-MDSCs and therefore understanding this differentiation potential is important for controlling tumor immune evasion. One example of MDSC differentiation potential is that total MDSCs (CD11b+GR-1+) from the bone marrow (BM) differentiate into mature osteoclasts (Ocs) which do not have T-cell suppressive activity [3]. A second example of MDSC differentiation potential is that the M-MDSC subtype from the spleen (SP) differentiates into macrophages, dendritic cells (DCs), and G-MDSCs also lacking T-cell suppressive activity, while G-MDSCs do not [4].

The current paradigm for MDSC development of T-cell suppressive activity suggests a two-step model consisting of expansion and activation steps [5]. Tumor-derived cytokines and growth factors initiate an expansion of MDSCs in the BM and SP followed by their activation to have T-cell suppressive activity. However, it is unclear where the activation of MDSCs takes place. Many investigators suggest that activation takes place at both the SP and tumor (TU) [6] and often use SP MDSCs as a surrogate for TU MDSCs. This is helpful for researchers as more MDSCs can be isolated from the SP than the TU but may not be accurate to MDSC biology. For example, SP MDSCs were recently shown to utilize a different set of T-cell suppressive mechanisms than TU MDSCs and found to be less suppressive of T-cells than TU MDSCs [7]. In addition, our group demonstrated that although TU and SP MDSCs suppress T-cell proliferation in long term suppression assays, TU MDSCs, but not SP MDSCs, suppress T-cell proliferation in short-term suppression assays [8, 9]. Therefore, we asked if these unique activities of TU M-MDSCs are due to their activation or differentiation from BM and SP MDSCs using a variety of methods including T-cell suppression assays, transcriptome analysis, and in vitro and in vivo differentiation assays.

2.3 Materials and Methods

2.3.1 Reagents

Routine laboratory chemicals, DNAse I, Trypsin-EDTA 0.25%, and the Acid Phosphatase Leukocyte (TRAP) Kit were purchased from Sigma Chemical (St. Louis, MO). RPMI, Alpha MEM without ribonucleosides and deoxyribonucleosides, and DMEM with 2 mM L-Glutamine were purchased from Sigma (St. Louis, MO) or Corning (Manassas, VA). Fetal bovine serum (FBS) was purchased from Corning (Manassas, VA). 1 M Hepes and 100 mM sodium pyruvate were purchased from Media Technology (Manassas, VA). Penicillin/Streptomycin (10,000 units/mL) was purchased from HyClone (South Logan UT). Liberase TM was purchased from Roche (Basel, Switzerland). Liberase TM was diluted to 5 mg/ml and stored at -20oC until use. The EdU Click-iT kit was purchased from Life Technologies (Carlsbad, CA). RNAeasy kit was purchased from Qiagen (Hilden, Germany). Tri-reagent (Zymo Research) and Direct-zol RNA MiniPrep Plus kits were purchased from Zymo Research (Irvine, CA). Tri-reagent was stored at 4oC. All antibodies, TruStain FcX and Zombie Violet were purchased from Biolegend (San Diego, CA) (Table 1). All recombinant murine cytokines were purchased through PeproTech (Rocky Hill, NJ) (Table 2). Anti-Rat IgG compensation beads were purchased from BD (San Jose, CA). M-MLV reverse transcriptase, 5X first strand buffer, BSA, deoxynucleotide triphosphate, Rnasin, random hexamers, and oligo-dT primers were purchased from Invitrogen (Carlsbad, CA). Primer/probe sets were purchased from Integrated DNA Technologies (IDT, Skokie, Il).

2.3.2 Mouse Lines and Husbandry

Female C57Bl/6J and BALB/cJ were purchased from The Jackson Laboratory (Barr Harbor, ME) and C57Bl/6N purchased from the Purdue Transgenic Mouse Core Facility (West Lafayette, IN). Prostate OVA-expressing mice-3 (POET-3) mice were generated and maintained as previously described [10]. $Rag^{-/-}$ OT-I mice were generated and maintained as previously described [11]. Mice were housed in specific pathogen-free conditions with a 12 hr light/dark cycle. Mice were fed a standard chow diet (Teklad 2018) and water *ad libitum*. All of the mouse use described in this paper was approved by Purdue University or the IUPUI Animal Care and Use Committees.

2.3.3 Cell Culture

The following cell lines were used in our studies: RM-1 cells (a generous gift from Dr. Tim Thompson, Baylor University), EL-4 (a generous gift from Dr. Dmitry Gabrilovich, The Wistar Institute), and 4T-1, murine breast cancer cells.

All cells were grown in humidified incubators with 5% CO2 at 37 oC. EL-4, 4T-1 and, OT-I T-cells (from Rag-/- OT-I mice spleens) were cultured in complete RPMI (RPMI containing 10% FBS, 1 mM HEPES, 1 mM Na pyruvate, 100 U/mL Pen/Strep; RPMI-C). EL-4 culture also included 55 μM β-mercaptoethanol. RM-1 cells were cultured in complete DMEM (DMEM containing 10% FBS, 1 mM Na pyruvate, 1 % Pen/Strep; DMEM-C). 4T-1 and RM-1 cells were passaged 1:10, at 70% confluency. Non-adherent EL-4 cells were maintained between 1 x 105 and 1 x 106 cells/ml.

During some M-MDSC differentiation experiments, TES was added to RPMI-C. The protocol for creating TES provided by Dr. Dmitry Grabrilovich. Briefly, TES was derived from subcutaneous tumors that developed in C57BL/6J mice 14 days after injection of 5 x 106 EL-4 cells. Tumors were harvested, minced, and ~500 mm3 of tissue was cultured with RPMI (with 10% FBS, 1 % Hepes, and 1% Pen/Strep) for 18 h. At 18 h the supernatant was removed and purified by filtering through a 70 μm cell strainer followed by centrifugation at 200 xg for 5 min at 4 oC. The supernatant was then transferred to a new tube and centrifuged at 13300 xg for 20 min at 4 oC. Finally, the supernatant was sterile filtered (0.22 μm), aliquoted, and stored at -80 oC until use.

2.3.4 Tumor Formation and Inflammation

Tumor cells were suspended in PBS for all injections. Concentrated cell suspensions (5x 107 cells/ mL, EL-4; 107 cells/ mL RM-1, 3 x 107 cells/ mL, 4T-1) in PBS were kept on ice prior to injection of 100 μL of cell suspension.

To generate TU MDSC for the microarray analysis, RM-1 TU cells were harvested for injection during their exponential growth phase (~70% confluent) and 1 x 106 cells were injected into the peritoneal cavity of C57Bl/6J mice. 7 d later, TU MDSCs were harvested from ascites by serial lavage with sterile PBS. MDSCs were isolated as described below (section 2.3.5). To generate activated peripheral MDSCs from the SP, POET-3 mice were injected with 5 x 106

activated OT-I cells to induce prostate inflammation as we have previously described [12]. 6 days later the mice were killed, spleens harvested, and MDSCs were isolated as described below (section 2.3.5).

To generate MDSCs for in vitro differentiation experiments, 6-10 female 6-8-week-old C57Bl/6 mice were injected subcutaneously (s.c.) with 5 x 106 EL-4, 106 RM-1, or 3 x 106 4T-1 (BALB/cJ mice) cells. Tumors were allowed to grow for 14 d and did not exceed 2 cm in diameter prior to harvest. Tissue and tumors were pooled to isolate enough MDSCs for each experiment. MDSCs were isolated as described in section 2.3.5.

Finally, to generate MDSCs from osteolytic BM for Oc differentiation experiments, intracardiac inoculation of tumor cells was performed as previously described [13]. Briefly, 8 to 12-week-old female BALB/cJ mice were anesthetized and 1 x 10^5 4T1 cells in 100µl PBS were inoculated into the left ventricle over 30 seconds. Tumors were allowed to grow for 7-10 d prior to harvest. The development of osteolytic lesions in hind limbs were identified prior to BM harvest from osteolytic and non-osteolytic bones using a Faxitron MX-20 X-ray machine (Faxitron X-ray Corporation, Tucson, AZ). Lesion area was confirmed with histology and quantified using MetaMorph analysis system software (Universal Imaging Corporation, Bedford Hills, NY).

2.3.5 MDSC Isolation

MDSCs were isolation from BM, SP, solid tumors, or i.p.-induced tumors. Mice were euthanized then BM was removed from femurs and tibias by flushing the marrow cavity with RPMI-C. Spleens were physically disrupted between two frosted microscope slides in RPMI-C. Solid tumors were removed, minced and digested with 5 mg/mL Liberase TM and 1 mg/mL DNAse I in PBS at 37 oC with shaking. MDSC associated with tumors from the peritoneal cavity were harvested via peritoneal lavage with sterile PBS.

Following dissociation, digestion, or collection of cells, all samples were treated with Ammonium-Chloride-Potassium lysis buffer (ACK), to remove red blood cells, for 2 minutes at RT. Samples were then filtered through a 70 µm cell strainer and spun at 300 x g, for 5 min to pellet cells. Cells were then stained following blocking of Fc receptors with TruStain FcX (1:50) or 50% mouse serum in the dark at RT for 10 minutes. In some experiments, Zombie Violet (1:100) was added to the TruStain FcX master mix to identify and remove dead cells. Specific antibodies against CD11b, Ly6C, and Ly6G were added and cells were incubated in the dark for 20-30

minutes on ice. For the cell differentiation studies, SP and TU samples were stained first with CD11b-FITC and prepared for magnetic-activated cell sorting (MACs) to enrich the CD11b population of cells as per the manufacturer instructions (Miltenyi Biotec; Auburn, CA). Following MACs enrichment, cells were incubated with antibodies for Ly6C and Ly6G. CD11bhi Ly6Chi Ly6Glow (M-MDSC) and CD11bhi Ly6Cmed Ly6Ghi (G-MDSC) were isolated using a BD FACS Aria III with cell purities >90% (Figure 2.1A). (See section 2.3.7)

2.3.6 Experimental Designs

Tissue M-MDSC T-cell suppression activity: M-MDSCs were isolated from BM, SP, and solid TU of 4T1 tumor-bearing mice, the cells from each replicate (3 mice per replicate) were pooled to ensure enough cells. Single cell suspensions of tumors were prepared and M-MDSCs (CD11bhi Ly6Chi Ly6Glow) were isolated by fluorescence-activated cell sorting (FACS) as described in Section 2.3.7. M-MDSCs from each tissue were then resuspended at 106 cells/ml. One-hundred thousand MDSCs were plated together with either pre-activated CD8+T-cells in the presence of 5 μg/ml anti-CD3 antibody and 2.5μg/ml anti-CD28 antibody in flat bottom 95 well plates for the 16 h assay or with inactivated CD8+T-cells and with the anti-CD3 or CD28 antibodies for the 72 h assay. Positive control wells contained purified CD8+ T-cells placed on a feeder layer of naive whole SP cells. Negative controls wells contained either purified CD8+ T-cells placed on a feeder layer of naïve spleens cells but without added anti-CD3 or CD28 antibodies or purified CD8+ T-cells placed on a feeder layer of naïve spleens cells without added EdU. Pre-activation of CD8+ T-cells for the 16 h assay was done using single cell suspension of splenocytes from normal BALB/cJ mice. Red blood cells were lysed with ACK lysis buffer and splenocytes from 2 spleens were placed in upright T25 tissue culture flasks with 10 mL RPMI-C containing 5μg/ml anti-CD3 and 2.5μg/ml anti CD28 for 24 hrs. Miltenyi positive T-cell selection magnetic bead kits were used to obtain purified activated CD8+ T-cells (>95% pure as verified by flow cytometry). One and one-half h prior to the end of the 16 h or 72 h suppression assay, 10μM EdU was added to the appropriate wells. Cells were then harvested from the plates and prepared for FACS analysis (see section 2.3.7).

Microarray analysis of SP and TU MDSC subtypes: Cells were collected from the peritoneal cavity of mice with intraperitoneal RM-1 tumors and from the spleens of POET-3 mice with OT-1 cell induced prostatic inflammation were collected. Cells from 3-5 mice were pooled

to make each replicate sample and four replicate samples were processed for each tissue. MDSC subtypes were isolated by FACS as described in Section 2.3.7. RNA was isolated from cell pellets of each tissue/MDSC subtype (n=4 per group) using the RNAeasy kit (Qiagen) and following manufacturer instructions. Microarray analysis was conducted, and the data were analyzed as described in section 2.3.9.

Induced differentiation of M-MDSC from BM, SP, and TU: A series of experiments were conducted to determine whether M-MDSCs from BM, SP, and solid TUs from the same mice could respond to signals for differentiation toward various myeloid cell fates. Three to seven replicate experiments were conducted for each study. Each replicate was comprised of a cell pool from 6-10 mice. BM, SP, and solid TUs M-MDSCs were cultured in RPMI-C media plus specific cytokine treatments for 3 days; the studies were also conducted in the presence or absence of 20% TES to assess whether signals from the tumor microenvironment (TME) changed the fate potential of the tissue M-MDSCs. For all experiments, 105 cells were seeded into wells of a 96 well plate; technical replicates were then pooled to represent a single biological replicate that was used for statistical analysis. Following the 3 d treatment period, media and non-adherent cells were collected. Adherent cells were harvested with 0.25% Trypsin-EDTA for 10-15 minutes or until cell layer had dispersed. Adherent and non-adherent cells were pooled and prepared for flow cytometry analysis (see section 2.3.7).

Here we examined the conversion of M-MDSCs to G-MDSCs using two treatment designs. First, we followed the differentiation protocol reported previously by Youn et al. [4]. For this approach, SP and TU M-MDSCs were cultured in RPMI-C with 10 ng/mL GM-CSF in the presence or absence of 20% TES. Second, we repeated these experiments without GM-CSF to determine whether TES alone was a sufficient stimulus for this conversion. Following, we examined the potential of M-MDSCs to differentiate into DCs (CD11c+). For this, BM, SP, and TU M-MDSCs were cultured in RPMI-C with 10 ng/mL GM-CSF and 2 ng/mL IL-4 in the presence or absence of 20% TES. Additionally, we examined the potential of BM, SP and TU M-MDSCs to differentiate into macrophages. M-MDSCs were cultured in RPMI-C supplemented with 25 ng/mL M-CSF in the presence or absence of 20% TES.

Finally, we examined the potential to differentiate BM, SP, and solid TUs M-MDSCs into Ocs using two different models. In the first model, BM, SP and TU M-MDSC from mice with solid 4T1 tumors were plated at 5 x 105 cells/cm2 in αMEM-C (10 % FBS, and 2 mM L-

Glutamine) supplemented with 50 ng/mL M-CSF and 100 ng/mL RANKL. Half of the media was renewed every 2 d, and at 8 days of culture the cells were fixed in formaldehyde-acetone-citrate from the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma, St. Louis, MO). Fixed cells were stained for Tartrate Resistant Acid Phosphatase (TRAP) activity according to the manufacturer's instructions. The number of TRAP-positive Ocs (i.e. cells with at least 3 nuclei) was counted in each well. In the second model, mice were given intracardial injections of 4T1 cells as described above in section 2.3.4. M-MDSCs were isolated from SP of tumor-bearing mice and from the marrow of bones containing osteolytic lesions (OL+) and paired bones that did not have osteolytic lesions (OL-). These SP, OL+, and OL- M-MDSC were cultured for their ability to undergo Oc differentiation as described above.

2.3.7 Flow Cytometry and Analysis

T-cell suppression assay: Following co-culture and EdU treatment of M-MDSCs and OT-1 cells, total cells were harvested from the plates and stained with antibodies against the cytotoxic T-cell antigen CD8 and the myeloid antigen CD11b. They were then fixed and analyzed for EdU levels using the EdU Click-iT staining kit, following manufacturer instructions (Life Science Technologies, Waltham MA). In brief, following fixation cells were resuspended in permeabilization buffer for 10 min then incubated with Click-iT reaction master mix for 30 min at RT. Samples were washed and resuspended in 150 ul permeabilization buffer then data was collected for CD8, CD11b, and EdU on a BD Fortessa cell analyzer (San Jose, CA). Data were analyzed using Flow Jo v.10 (Tree Star; Ashland, OR), i.e. selection of non-debris events, gating for single cells, biplots for cytotoxic T-cells (CD11b- CD8+ cells) and for proliferating cells (SSC-A x EdU+). OT-I cells incubated without EdU were used to create the positive EdU gate.

Differentiation studies: As described above, cells were subjected to Fc blocking and, in some cases, live dead dye (Zombie Violet, Biolegend). An antibody cocktail consisting of CD11b-FITC, Ly6C-PE-Cy7, Ly6G-APC, F4/80-PE, and CD11c-PerCP was added to the cells followed by incubation for 20 min at 4 oC in the dark. Prior to staining a small aliquot representing each tissue was removed for controls. Aliquots were used as Fluorescence Minus Two (FMT) isotype controls via staining with IgG isotypes: CD11b-FITC, Ly6C-PE-Cy7, Ly6G-APC, IgG-PE, and IgG-PerCP. After staining, cells were fixed in the dark with 10% neutral buffered formalin (NBF) for 15 min at 4 oC. Data for fixed pre-culture controls (Figure 2.1B) and post 3 d culture samples,

as well as single-color compensation samples containing compensation beads (BD, San Jose, CA), were collected on a BD Fortessa cell analyzer then analyzed using Flow Jo v.10. Prior to setting final gates, samples were roughly gated to see if the compensation matrix from the initial analysis displayed a good separation of populations. When the compensation matrix resulted in spectral overlap, a new matrix was made within FlowJo using single color controls. Following compensation verification, live-singlet-CD11b+ cells were gated based on the location of positive populations (Figure 2.1B). For final M and G gates, M-MDSC were gated as Ly6ChiLy6G- and G-MDSC Ly6CintLy6Ghi based on pre-culture controls. In addition to the M-MDSC and G-MDSC gates, a histogram was used to quantify the total percentage of Ly6Ghi cells. Finally, to identify macrophages and DCs, we used isotype controls to locate the F4/80 and CD11c negative population using a quadrant gate (marker list of cell types can be seen in Table 2.3). All gates were confirmed across samples to ensure true population separation.

2.3.8 RNA Isolation and qPCR

SP and TU M-MDSC were cultured as above in 96 well plates then harvested into Tri-reagent (Zymo Research, Irvine, CA). Samples were then flash frozen in liquid nitrogen and store at -80 oC until all replicates were collected. RNA was isolated using Direct-zol RNA MiniPrep Plus kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. cDNA was created using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) in a 10 µL reaction with the following reagents: 5X first strand buffer, 0.05 mg/mL BSA, 0.05 mM of each deoxynucleotide triphosphate, 0.6 U/µL Rnasin, 0.005µg/µL random hexamers, 0.005µg/µL oligo-dT primers and0.25µg sample RNA in 4.85 µL. The reverse transcriptase PCR reaction was incubated for 1.5 h at 37oC followed by inactivation of enzymes at 95oC for 5 min. The final cDNA sample was diluted to 100 µL and stored at -20 oC until use. qPCR was used to analyze cDNA for message for specific targets. Premade PrimeTime® Assays were purchased from Integrated DNA Technologies (IDT, Skokie, Il) for: Nos2 (IDT Assay: Mm.PT.56a.43705194), Arg1 (Mm.PT.58.8651372), VDR (Mm.PT.58.7050931), Ccr1 (Mm.PT.58.32053786), Slc7a11 (Mm.PT.58.29117975), Stfa2l1 (Mm.PT.58.41576651), Mpo (Mm.PT.58.5251395), Spp1 (Mm.PT.58.29117975) and, Nr1d1 (Mm.PT.58.17472803) gene expression and, r18s (Hs.PT.39a.22214856.g) was used as a housekeeping gene. qPCR was conducted using IQ Supermix (Bio-Rad) and 10 pmol of each primer/probe set with the following cycling conditions:

95 oC (3 min.) followed by 50 cycles of 95 oC for 15 sec., 60 oC for 30 sec. and, 72 oC for 30 sec on a Bio-Rad CFX96 thermocycler using Bio-Rad CFX Manager software. Relative expression levels were determined using the delta-delta Ct method [14].

2.3.9 Microarray Analysis and Bioinformatics

Transcript levels in each sample were determined using the Affymetrix Mouse Gene 1.0 ST V1 GeneChip (ThermoFisher, Waltham MA; 27,543 probe sets). RNA labeling, chip hybridization, and chip scanning were carried out at the Purdue Genomics Facility using standard Affymetrix protocols. Chips were scanned, and raw data was saved into CEL files. Microarray data and CEL files can be accessed at the NCBI Gene Expression Omnibus (GSE116596).

The sample array files were examined for quality and RMA normalized using RMAExpress (rmaexpress.bmbolstad.com) (quartile normalization, gene level analysis, NUSE, and RLE plots). Arrays for all of the samples met the quality control criterion and were used in downstream analysis. Probesets were annotated to genes using BRB-Array Tools V 4.6.0 Beta_1 (https://brb.nci.nih.gov/BRB-ArrayTools/index.html). Prior to statistical analysis, the genes in the bottom 25% of expression were removed leaving 22,390 probesets. Differential gene expression was conducted using Significance Analysis for Microarrays [15] within BRB-ArrayTools (500 permutations, 5% FDR, 70% false negative detection rate). Statistical analysis was conducted on four pairwise comparisons: SP G- vs M-MDSC; TU G- vs M-MDSC; SP G-MDSC vs TU G-MDSC; SP M-MDSC vs TU M-MDSC. between the four sample groups. Bioinformatic analysis was conducted using MetaCore (Clarivate Analytics, Philadelphia, PA). For each relevant two-way comparison, analysis was conducted for the combined up and down-regulated genes as well as separately for up or down-regulated genes only. The core MetaCore analysis includes enrichment analysis for curated pathways, Gene Ontology, process networks, and diseases. In addition, de novo network construction can be conducted.

2.3.10 Statistics

Statistical analyses were conducted using SAS Enterprise Guide v 5.1 (SAS Institute Inc. Cary, NC). Evaluation of histograms and the Shapiro-Wilk test of normality ($p<0.05$) were used to assess whether the data were normally distributed. When data from flow cytometry was not

normally distributed it was transformed using cube-root, natural log or, (2Arcsin√x/100) [16] transformation. After confirming the distribution, data was assessed for outliers using Cook′s D statistic and studentized residuals by leverage plot, and outliers were removed. While statistical tests were conducted on transformed data, data are reported as the mean + SEM of non-transformed data. One-way ANOVA was used to determine significance for T-cell suppression assays and the comparison of TRAP+ cells among sites. The examination of tissue M-MDSC for their response to differentiating agents in the presence or absence of TES was conducted using a split-plot design to account for the interdependence of responses within each replicate. For all analyses, comparisons among multiple treatment groups were conducted using Tukey's HSD. A p-value < 0.05 was considered statistically significant.

2.4 Results

2.4.1 M-MDSCs from TU, but not BM and SP, Suppress T-cells in a Short-Term Assay.

To confirm our previous finding that BM, SP and TU M-MDSCs possess different T-cell suppressive activities we conducted long (72 h) and short (16 h) term T-cell suppression assays. Consistent with previous observations [17], we found that BM, SP, and TU M-MDSCs from tumor-bearing mice all have T-cell suppressive activity in a 72 h T-cell suppression assay (Figure 2.2A). However, we found that BM and SP M-MDSCs have little to no T-cell suppressive activity in a 16 h T-cell suppression assay (Figure 2.2B). In contrast, TU M-MDSCs had high T-cell suppressive activity in a 16 h T-cell suppression assay (Figure 2.2B). These results confirm our previously published observations on MDSCs in the inflamed prostate or in prostate tumors [12] and suggest that M-MDSCs from peripheral sites must undergo an activation or program of differentiation in response to T-cell and/or tumor-derived factors to acquire T-cell suppressive activity.

2.4.2 The Transcriptome of TU M-MDSCs is Dramatically Different from SP M-MDSCs.

To explore the molecular changes that define the difference between SP and TU MDSCs, we conducted a microarray analysis on SP and TU MDSCs from RM-1 tumor bearing mice. Of the 22,392 probe sets analyzed, 7,602 probe sets were differentially expressed in one of the four

pairwise comparisons we conducted (TU vs SP M-MDSCs, TU vs SP G-MDSCs, G vs M-MDSCs in TU, and G vs M-MDSCs in SP).

Specifically, in TU compared to SP M-MDSCs and G-MDSCs, we found a large number of differentially expressed genes (4513 and 4638 genes, respectively) (Figure 2.3A). Seventy-five percent of the genes that are differentially expressed between SP and TU MDSCs were common to both subtypes; these include the classic MDSC markers of T-cell suppressive function Arg1 and Nos2. The top 5 up and down-regulated genes in this group are shown in Figure 2.3A. However, additional interesting genes in this category include the vitamin D receptor (VDR, 26-28-fold increased), folate receptor 2 (Folr2, 5-12 fold increased), the cationic amino acid transporter Slc7a2 (17-26 fold increased), chemokines like Ccl2 (16 fold increased), and Ccl17 (16-25 fold increased). In addition, there are transcripts that are unique to SP or TU for both M-MDSC (n=1162) and G-MDSC (n=1287) (Figure 2.3A). Similarly, there are a number of genes that are differentially expressed between G-MDSC and M-MDSC in the TU (n=872, Figure 2.3B). These transcripts are distinct from those that define the difference between the subtypes in the SP (n=908 of which only 191 overlap with the TU subset comparison).

To gain insight into the functional differences between SP and TU MDSCs, as well as the difference in G- and M-MDSC subtypes in the TU environment, we conducted bioinformatics analysis for the comparisons reflected in Figure 2.3A. A summary of the top 10 enriched pathways or process networks for the analysis of the combined-, up-, and down-regulated genes are provided in Appendix A and B and in an abbreviated version in Table 2.4. The most prominent pathway enrichment common to both subtypes was for HIF-1 signaling; this included upregulation of HIF-1 mRNA by 3 (G-MDSC) to 3.9-fold (M-MDSC). Similarly, up-regulation of an expression network centered around CREB1 and NF-κB were prominent in the genes common to both subtypes in SP vs. TU MDSCs. Signatures more specific to TU M-MDSCs include downregulation of cell cycle, up-regulation of chemotaxis/Ccl2 signaling, and activation of pathways for myeloid and macrophage differentiation. Signatures more specific to TU G-MDSCs include up-regulation of pathways for cell-matrix interactions or extracellular matrix remodeling, up-regulation of ATP/ITP metabolism, and up-regulation of a network centered around p53. Therefore, our microarray data suggest that SP and TU MDSCs represent two distinct cell types possibly due to a differentiation process and not only activation.

2.4.3 Different Transcriptomes and Transcriptome Responses to Tumor Environment in SP and TU M-MDSCs.

Based on our microarray data (shared in section 2.4.2) we chose nine genes to analyze by qPCR to validate the microarray results: Arg1, Nos2, Mpo, Spp1, Nr1d1, Stfa2l1, Slc7a11, Ccr1, and VDR and test the impact of TES on SP M-MDSC transcript levels. Freshly isolated SP and TU M-MDSC were compared to SP M-MDSC cultured 3d in RPMI +/- 20% TES (Figure 2.12). We chose these conditions to determine first, that freshly isolated SP and TU have differential expression of the selected targets mRNA levels and second, the ability of TES to make SP M-MDSCs more similar to TU M-MDSCs in terms of gene expression. To do this, pre-cultured cells (freshly isolated) were used as controls for *in vivo* mRNA levels and RPMI alone cultured cells were used as a control for the TES treatment in our *in vitro* assay. Within the nine targets, eight genes were confirmed to be present at higher levels in TU M-MDSCs compared to SP-M-MDSCs as was seen in the microarray. The culture of SP M-MDSCs in RPMI alone resulted in varying results within our targets where Arg1, Nos2, Ccr1, and VDR mRNA levels did not change while Spp1, Stfa2l1 and Slc7a11 mRNA levels were increased, and Mpo mRNA levels were decreased. We then asked if TES altered SP M-MDSC transcript levels to more similar transcript levels found in TU M-MDSCs? Addition of TES resulted in a differential response suggesting SP M-MDSC transcript levels had not become like TU M-MDSC levels. For example, TES increased expression of Arg1, and Ccr1 decreased expression of Stfa2l1 and Nr1d1, and no effect on Nos2, Mpo, Spp1, Nr1d1, Slc7a11, and VDR mRNA levels. These data confirm our findings in our microarray and suggest that signals from the TME are not enough to fully modulate the transcripts levels of SP M-MDSC to be similar to the levels found in TU M-MDSC.

2.4.4 Differential Ability of M-MDSCs from BM, SP, and TU to Differentiate to New Cell Types.

Based on our microarray data (shared in section 2.4.2) we chose nine genes to analyze by qPCR to validate the microarray results: Arg1, Nos2, Mpo, Spp1, Nr1d1, Stfa2l1, Slc7a11, Ccr1, and VDR and test the impact of TES on SP M-MDSC transcript levels. Freshly isolated SP and TU M-MDSC were compared to SP M-MDSC cultured 3d in RPMI +/- 20% TES (Figure 2.12). We chose these conditions to determine first, that freshly isolated SP and TU have differential expression of the selected targets mRNA levels and second, the ability of TES to make SP M-

MDSCs more similar to TU M-MDSCs in terms of gene expression. To do this, pre-cultured cells (freshly isolated) were used as controls for *in vivo* mRNA levels and RPMI alone cultured cells were used as a control for the TES treatment in our *in vitro* assay. Within the nine targets, eight genes were confirmed to be present at higher levels in TU M-MDSCs compared to SP-M-MDSCs as was seen in the microarray. The culture of SP M-MDSCs in RPMI alone resulted in varying results within our targets where Arg1, Nos2, Ccr1, and VDR mRNA levels did not change while Spp1, Stfa2l1 and Slc7a11 mRNA levels were increased, and Mpo mRNA levels were decreased. We then asked if TES altered SP M-MDSC transcript levels to more similar transcript levels found in TU M-MDSCs? Addition of TES resulted in a differential response suggesting SP M-MDSC transcript levels had not become like TU M-MDSC levels. For example, TES increased expression of Arg1, and Ccr1 decreased expression of Stfa2l1 and Nr1d1, and no effect on Nos2, Mpo, Spp1, Nr1d1, Slc7a11, and VDR mRNA levels. These data confirm our findings in our microarray and suggest that signals from the TME are not enough to fully modulate the transcripts levels of SP M-MDSC to be similar to the levels found in TU M-MDSC.

2.4.4.1 Unlike BM and SP M-MDSCs, TU M-MDSCs Do Not Differentiate into G-MDSCs and SP M-MDSC Differentially Respond to the TME.

To determine if SP and TU M-MDSC are able to differentiate to the G-MDSC subtype, SP and TU M-MDSCs from EL-4 Tu bearing mice, were cultured for 3 days in the presence of GM-CSF (standard protocol for G-MDSC differentiation) and +/- 20% TES (to determine the effect of the TME), then harvested for FACS analysis (Figure 2.4C). We found that in the presence of GM-CSF alone SP M-MDSCs had a significantly greater ability to differentiate to G-MDSCs compared to TU M-MDSCs (15% SP and 1.5% TU, $p<0.05$) (Figure 2.4D *left*). Additionally, to determine if signals from the TME would hijack the standard differentiation, we found that the addition of TES did not significantly alter the ability of either SP or TU M-MDSC to differentiate to G-MDSC like cells (SP: 15% to 10%, TU: 1.5% to 1.3%) (Figure 2.4D *right*).

Interestingly, M-MDSCs are able to differentiate to G-MDSCs in the absence of GM-CSF. To determine if there are differences in the ability of BM, SP and TU M-MDSCs to differentiate to G-MDSCs in the absence of GM-CSF we did the same analysis in the absences of GM-CSF (Figure 2.4 A and B). We found, in the EL-4 tumor model, that in the absence of GM-CSF, BM and SP M-MDSCs strongly shifted toward G-MDSCs (58% and 50% differentiation, respectively).

In contrast, TU M-MDSCs did not shift towards G-MDSCs (<1% differentiation) ($p < 0.05$). These data were also confirmed in the, 4T1 and RM-1 tumor models (Figure 2.5).

Additionally, we asked if signals from the TME would hijack the differentiation in the absence of GM-CSF and would make BM and SP M-MDSCs more like TU M-MDSCs (gating is shown in Figure 2.4A). In the presence of TES, BM and TU M-MDSCs had similar responses as in the absence of TES. BM M-MDSCs retained a high percentage of shift to G-MDSCs (58% differentiation) while TU M-MDSCs retained a low percentage of shift to G-MDSCs (1.5% differentiation). However, in the presence of TES, SP M-MDSCs had significantly less ability to shift to G-MDSCs (50% to 21.6% differentiation) (Figure 2.4B *right*). Interestingly, the SP response supports the idea that the TME will convert peripheral M-MDSCs toward a TU M-MDSC phenotype, while the BM response did not.

2.4.4.2 Unlike BM and SP, TU M-MDSCs Do Not Differentiate to DCs and the TME Reduces the Ability of BM and SP M-MDSCs to Differentiate to DCs.

Another potential non-suppressive cell type M-MDSCs have been shown to differentiate into are DC's. Multiple studies have suggested that MDSC development is in part due to tumor cytokines hijacking the normal differentiation process of monocytes into DCs [19, 20]. Therefore, the ability to shift M-MDSCs into DC's, is important as a means to reduce MDSC T-cell suppressive activity and to restore normal DC differentiation. To address the potential difference between BM, SP and TU M-MDSCs to differentiate toward DC's, we asked if BM, SP, and TU M-MDSCs could differentiate into DCs under standard conditions (GM-CSF + IL-4) (Figure 2.6). We found that when cultured in the presence of cytokines alone BM and SP M-MDSCs showed a significantly higher gain of CD11c expression, the marker of DCs, (20% and 33%, respectively $p<0.05$)) compared with TU (1.5%, $p<0.05$) (Figure 2.6B *left*). These same effects were seen in the 4T1 and RM-1 tumor models as shown in Figure 2.7.

More recently, Tcyganov et al. [21] suggested that M-MDSCs differentiate into DCs at the tumor site. Therefore, to determine if signals from the TME would hijack the standard DC differentiation, we also asked if the presence of TES would alter the ability of BM and SP M-MDSCs to become DCs (Figure 2.6). We found that in the presence of TES, BM and SP M-MDSCs had a significant decrease in ability to differentiate to DCs (gain CD11c) compared to cytokine alone (Figure 2.6B *right*, 7%, and 6%, respectively, $p<0.05$) suggesting that signals from the TME

will cause BM and SP M-MDSCs to behave more like TU M-MDSCs in terms of differentiation potential to DCs. TU M-MDSCs were not affected by the presence of TES and remained unable to differentiate into DCs (gain the CD11c marker) (<1%).

2.4.4.3 M-MDSCs from BM, SP, and TU Differentiate into Macrophages.

Another major innate cell type suggested to be an alternative endpoint of M-MDSC differentiation is the macrophage. At the TU, tumor-associated macrophages have been identified as F4/80^{+} cells, while M-MDSCs at the tumor are F4/80^{-} (Figure 2.1A). Recently, it was suggested that M-MDSCs enter the tumor site and differentiate to tumor-associated macrophages [2, 21]. Therefore, to determine if BM, SP and TU M-MDSC have the same ability to differentiate toward macrophages, we asked if BM, SP and TU M-MDSCs would equally differentiate toward macrophages (gain in F4/80) in the presence of M-CSF (standard macrophage differentiation conditions). We found that BM, SP and TU M-MDSCs were all able to differentiate toward macrophages (gain F4/80) in the presence of M-CSF alone (Figure 2.8A). TU and SP M-MDSCs showed the highest ability to differentiate toward macrophages (54.6% and 62.7%, respectively), compared to BM (36.9%) although not statistically different (Figure 2.8B *left*). Consistent to these findings, no difference in the ability of BM, SP, or TU M-MDSCs to differentiate toward macrophages was seen in the RM-1 tumor model (Figure 2.9). These data suggest that BM, SP and TU M-MDSCs are not different from each other in their ability to differentiate to macrophages.

To determine if the TME would hijack the standard macrophage differentiation of BM and SP M-MDSC toward TU M-MDSCs, we added TES to the differentiation procedure above. We found that the addition of TES resulted in a significant increase in differentiation toward macrophages (gain of F4/80) of SP M-MDSCs (7.27% increase, $p<0.05$) (Figure 2.8B *right*). Interestingly, BM and TU M-MDSC were not significantly affected by the presence of TES and therefore these data do not support a difference between BM, SP, and TU M-MDSC in their ability to differentiate to macrophages.

2.4.4.4 Unlike BM and SP, TU M-MDSC Resist Differentiation to Ocs *in vitro.*

Previous studies have shown that BM and SP total MDSCs differentiate into mature Ocs resulting in loss of bone mass. Specifically, it has been shown that BM and SP total MDSCs

cultured with M-CSF and RANKL will result in a large number of TRAP+ cells, a marker of Ocs [22]. Therefore, to determine if BM, SP and TU M-MDSC have the same ability to differentiate toward Ocs, we repeated this assay utilizing the same culture conditions but using M-MDSC from EL-4, 4T1 and RM-1 tumor models. In the EL-4 model, we found that BM M-MDSCs had the highest number of Ocs cells, followed by SP (7-fold less compared to BM), and finally TU M-MDSCs (4-fold less compared to SP (Figure 2.10A, Figure 2.11: similar results from 4T1 and RM-1 models)).

2.4.4.5 M-MDSCs Resist Differentiation to Ocs in an *in vivo* Mouse Model with a Tumor.

To determine if signals from the tumor would hijack standard Oc differentiation and push the BM and SP M-MDSCs to behave more like TU M-MDSCs *in vivo*, we isolated M-MDSCs from the 4T1 mouse metastatic cancer model. More specifically, 4T1 mouse tumor cells were injected into mice by intracardiac injection to induce metastasis to the bone. M-MDSCs were isolated from SP and BM from osteolytic (containing tumors) and non-osteolytic (no tumors) bones. Osteolytic sites were confirmed by X-ray following tumor challenge (Figure 2.10B). M-MDSCs from the SP and BM from osteolytic bones (OL+) and non-osteolytic bones (OL-) were cultured in M-CSF and RANKL for 8d and stained for TRAP and counted. Figure 2.10C shows representative images of post 8d culture phenotypes. By observation, we found large Ocs formed in both the SP and OL- BM samples while OL+ BM samples show very few large Ocs. Therefore, we counted Ocs (TRAP+ cells) and counts confirmed what was seen by microscopy, both SP and OL- BM M-MDSC were able to differentiate toward Ocs while OL+ BM M-MDSCs showed significantly lower ability (Figure 2.10D, $p<0.001$) which suggest that signals from the TME induce a change in BM M-MDSCs ability to differentiate in to Oc similar to TU like M-MDSCs.

2.5 Discussion

To determine if TU M-MDSCs represent an activated or differentiated cell from BM and SP MDSCs, we conducted multiple experiments. The difference between activation and differentiation can be difficult to tease out as many assay's give results that could be interpreted in either direction. Therefore, we began by confirming our previous observations that BM and SP M-MDSC are different from TU M-MDSC in T-cell suppression activity. To further investigate

this difference, we looked as the transcriptome of SP and TU MDSC subsets and gained insight into pathways that may be associated with activation or differentiation. Additionally, the transcriptome gave us insight into the magnitude of transcript and pathway differences between SP and TU MDSC. We confirmed our transcriptome analysis by qPCR of specific targets while also investigating the impact of the tumor environment on SP M-MDSCs to make them more similar to TU M-MDSCs. To distinguish between activation and differentiation we defined differentiation as a developmental step toward a final phenotype with limited differentiation potentials. Therefore, we used *in vitro* differentiation models to determine if BM, SP, and TU M-MDSC cells differentiate to different cell fates. Because these assays do not fully represent the environment that M-MDSCs are found in tumor bearing mice, we also cultured the BM, SP and TU M-MDSCs with differentiation cytokines and TES to mimic the TME. These assays demonstrated that BM, SP and TU M-MDSCs do not possess the same abilities to differentiate into G-MDSCs, DCs, and Ocs. Taken together, these analyses provide insight into the development of M-MDSCs and determine the potential role of differentiation focused immunotherapies on MDSCs found in different tissues.

MDSCs characteristic functions are their ability to suppress T-cell proliferation and IFN-γ production [1]. MDSC function is commonly assessed using a three-day co-culture assay that combines MDSCs and T-cells [24-26]. However, our group has previously found that the total ($CD11b^+Gr1^+$) BM and SP MDSC population from naïve and TU bearing mice have T-cell suppressive function when studied in a 3-day assay, but not when they are tested in a 12 h [9] or 16 h [8] assay. Here, we extended this observation by showing that when BM, SP, and TU M-MDSCs from the same mice are isolated, only TU M-MDSC suppressed T-cell proliferation in both a 16 and 72 h assay. BM and SP M-MDSCs were not T-cell suppressive in a 16 h assay but they acquired T-cell suppressive function when they were studied in a 72 h assay. This is likely due, in part, to the influence of INF-γ released by the activated T-cells on BM and SP M-MDSCs but does not clarify if this step is activation or differentiation [27].

Our microarray analysis reveals large differences in the transcriptome of SP and TU MDSCs which is consistent with previously published work and provides hints at whether the activities found in TU M-MDSC are from activation or differentiation [28, 29]. In a study comparing the transcript profile of G-MDSCs ($CD11b^+Ly6G^+$) from SP and TU of BALB/cJ mice with AB12 tumors, Fridlender et al. [28] detected a total of 10,172 significant genes in their

samples with 5344 differentially expressed genes (FDR 2%, fold change >1.7). Of the 5344 differentially expressed genes, many are consistent with results described in this study such as increased levels of Arg1, Dab2, Spp1, and Ear11. In another study that compared the transcriptome of M-MDSC ($CD11b^{+}Ly6C^{hi}Ly6G^{-}$) from Influenza A-infected lung or SP of infected mice [29], a total of 11,034 significant genes were identified in their samples, 2,346 of those genes were differentially expressed (fold change >2), including genes from ones identified in this study such as; Ccl2, Il6, Tgfb3, and Nos2. Based on the number of genes and transcription factors differentially expressed, our data suggest that TU MDSCs are not simply SP MDSCs that have entered the tumor (or site of inflammation) but that they are cells that have undergone a vast molecular reprogramming consistent with differentiation.

The current two-stage model of MDSC development proposed by Condamine et al. [5] suggests that transcription regulators such as STAT 1, 3, and 6; IRF8, C/EBP alpha, NF-κB, and Notch are involved in the activation/expansion and development of MDSC T-cell suppressive function. Transcription factor network analysis of our data support a role in the change from SP to TU M-MDSC for these transcriptions factors, as well as HIF1α [7]. Our data also suggest that transcription factors like CREB1 and ESR1 as well as pioneer factors like PU.1 and Sp1 may contribute to the differentiation of SP to TU MDSC. Additionally, Condamine et al. suggest that other non-transcription factor pathways are involved in the expansion/activation and development of MDSC T-cell suppressive activity such as COX-2, ER stress, retinoblastoma protein Rb1, and adenosine receptors A2b. Similar to our transcription factor findings, our data support these pathways to be important in the SP to TU transition (Appendix A and B). However, future studies will be necessary to validate and fully understand their roles in the differentiation of MDSC subtypes.

The main aspect of MDSC biology that can determine if the nature of the change, activation or differentiation, from BM or SP to TU M-MDSC, is due to differentiation potentials is a developmental step toward a final phenotype with limited differentiation potentials. The differentiation of BM and SP M-MDSC toward other phenotypes has been demonstrated in several *in vitro* studies [4, 18, 30, 31]. However, it is difficult to compare these studies, and therefore assess the similarity of cells from different sites, because there is a lack of consistency in the conditions used to induce differentiation. For example, in studies that examined the ability of SP M-MDSC to become G-MDSC, macrophages, or DCs, some groups culture the cells in media

alone [31, 32] while others culture cells in the presence of cytokines and/or tumor cell conditioned media [4, 18, 33]. In addition, none of the studies on MDSC differentiation directly examine TU M-MDSC. As such, our study is novel because it is the first to directly compare BM, SP and TU M-MDSC under identical conditions and assess their ability to differentiate into other myeloid cell fates. Our data clearly shows that although TU M-MDSCs can become macrophages, they have limited ability to become DCs, Ocs, or G-MDSCs compared with BM and SP M-MDSCs. Therefore, our data conflict with the model presented in a recent review [21] which suggests that M-MDSCs are the same regardless of the site of isolation. Our data provide evidence that BM, SP and TU M-MDSCs represent different stages of M-MDSC development. In fact, our data suggest that only TU M-MDSCs are the true, functional M-MDSCs and that they are a final differentiated M-MDSC phenotype with limited alternative cell fates. In addition, our data suggest that BM and SP M-MDSCs represent earlier, non-functional stages of the TU M-MDSC akin to the early-stage MDSCs (eMDSCs). identified in human blood [1].

Because the standard differentiation assays do not fully represent the conditions M-MDSCs will experience in tumor bearing mice, we asked if the presence of TES, to mimic the TME, with the differentiation cytokines would alter the BM and SP M-MDSCs to be like TU M-MDSCs with less ability to differentiate into other cell types. We found that BM M-MDSCs lose their ability to differentiate into Ocs when they are in close proximity to experimentally induced metastatic bone tumors. In other words, the bone TME converts them into TU M-MDSCs. We also tested whether this conversion could occur *in vitro*. Within the field of MDSC biology, multiple models have been utilized to mimic the tumor environment *in vitro,* including the use of tumor cell conditioned media [34], TES [4], or purified cytokines [27] in the presence or absence of hypoxia [7]. However, researchers have not compared how M-MDSCs from different tissues respond to these conditions nor have they directly compared the differentiation potential of *in vitro* differentiated cells to TU MDSCs. This makes it difficult to know whether the *in vitro* methods accurately mimic the TME and if the *in vitro* derived M-MDSCs from the BM or SP truly reflect the biology of the TU M-MDSC. Our data are the first to directly compare the impact of TES on BM, SP and TU M-MDSCs and our data demonstrate that signals from the TME reduce the differentiation ability of BM and SP M-MDSCs. However, while TES treated BM and SP M-MDSCs behave similar to TU M-MDSCs and respond strongly to macrophage-differentiating signals, they are not identical to TU M-MDSCs. TES did not reduce the GM-CSF mediated conversion of SP M-MDSCs to G-MDSCs

to the level seen with TU M-MDSCs. Similarly, treatment of SP M-MDSCs with TES induced only a subset of genes associated with freshly isolated TU M-MDSCs. Thus, our data show that *in vitro* TES differentiated SP M-MDSCs are not equivalent to TU M-MDSCs and we suggest caution when using these cells as a surrogate for the TU M-MDSC. Future studies should examine what additional stimuli are needed to fully differentiate BM or SP M-MDSC into TU M-MDSC, e.g. modeling interactions between M-MDSCs and TU cells [35] or the hypoxic tumor environment [7].

The problem of understanding if BM and SP M-MDSC undergo activation or differentiation into TU M-MDSC has been a problem within the field for example, MDSCs are described as a heterogeneous population of immature cells having phenotypic, morphological, and functional heterogeneity [1]. However, this diversity has led to confusion regarding the use of the term MDSC. Recently a group of scientists proposed characterization standards to define MDSCs and to serve as a guide and framework for consistent communication in MDSC research [1]. As part of this effort, a less mature, non-suppressive precursor for MDSCs was recognized to be present in human blood that were defined as eMDSCs. Although it has been argued that mice do not have eMDSCs, MDSCs isolated from both peripheral tissues (BM, SP) do not possess immediate T-cell suppressive function and are more plastic (immature ie early-stage) compared to those isolated from the tumor site. The current paradigm put forward by Condamine et al [5] defines MDSC development by a two-stage model that begins with their expansion from immature myeloid cells in the BM, followed by migration of these cells through the SP and/or into tumors where the MDSC is "activated" to a phenotype that is T-cell suppressive. In this model, the cells from SP and TU are considered nearly equivalent [17]. However, we have demonstrated that MDSCs found at the TU represent a more differentiated cell than those found in the peripheral sites. As such, in the context of cancer, we propose that only TU MDSCs should be called MDSCs.

Taken together, our data suggest that BM and SP M-MDSCs are precursors for TU M-MDSCs that undergo a differentiation step at the tumor site. Their T-cell suppressive ability transcript profile, and differentiation ability are all distinct from the TU M-MDSC. Therefore, we propose two changes to the field of MDSC biology. First, short-term T-cell suppression assays should be used as the new standard for testing immediate suppressive ability of MDSCs. Second, new nomenclature for M-MDSCs that reflects these differences: immature M-MDSC (M-iMDSC) for cells from the BM, peripheral M-MDSC (M-pMDSC) for cells isolated from peripheral

lymphoid tissues like SP and lymph nodes, in vitro M-MDSC (M-ivMDSC) that result from stimulation of cultured M-iMDSC or M-pMDSC, and TU MDSC (M-tMDSC) that are the functional, mature cells isolated directly from the TU (Figure 2.13, does not show ivgMDSC). We believe that this nomenclature better reflects the etiology of the M-MDSC and that this clarity will aid in the interpretation of M-MDSC research and development of anti-M-MDSC therapies.

2.6 Tables and Figures

Table 2.1 Antibodies Used for Flow Cytometry and Cell Sorting.

Antibody	Clone	Manufacturer
CD8	53-6.7	Biolegend
CD11b	M1/70	Biolegend
Ly6C	HK1.4	Biolegend
Ly6G	1A8	Biolegend
CD11c	N418	Biolegend
F4/80	BM8	Biolegend
Rat IgG2a,k	RTK2758	Biolegend
Armenian Hamster IgG	HTK888	Biolegend

Table 2.2 Cytokines and Growth Factors Used for Cell Treatments

Cytokine	Final concentration	Manufacturer
GM-CSF	10 ng/mL	PeproTech
M-CSF	25 ng/mL, 50 ng/mL	PeproTech
IL-4	2 ng/mL	PeproTech
RANKL	100 ng/mL	PeproTech

Table 2.3 Identification Parameters for Cell Types Using Flow Cytometry

Antibody (Flow Cytometry or IHC)	**G-MDSC**	**Macrophage**	**Dendritic Cell**	**Osteoclast**
CD11b (general myeloid cell marker)	+	+	+	+
Ly6C	Int	-	-	-
Ly6G	High	-	-	-
F4/80	-	High	-	-
CD11c	-	-	High	-
TRAP (IHC)	-	-	-	+

Table 2.4 Summary of Functional Enrichment Analysis of MDSC Array Data

Group	Pathways	Network Hubs	Process Networks
Common to Sp to Tu Transition across M- and G- subtypes			
UP	Transcription_HIF-1 targets	Sp1	Apoptosis_apoptotic mitochondria
	Oncostatin M signaling via Jak-Stat	CREB1	Chemotaxis
	PGE2 pathways in Cancer	c-Myc	Proteolysis_Ubiquitin proteasomal proteolysis
	Transport Clatherin_coated vesicle cycle	Jak/Stat5	Protein Folding in normal condition/ER and cytoplasm
	IL-1 signaling pathway		
Down	IFN-alpha/beta signaling via JAK/STAT	NF-κB	Cell cycle G2-M
	Antigen presentation by MHC class I	Stat3	Antigen presentation
			TCR signaling
Specific to M-MDSC, Sp to Tu Transition			
UP	ETV3 affect on CSF-1 promoted macrophage differentiation	CREB1	Chemotaxis
	PGE2 pathways in Cancer		GO terms for localization and migration
	M-CSF-receptor signaling pathway		
Down	DNA damage ATM/ATR regulation of G2/M checkpoint	NF-κB	Cell cycle Core/G2-M/S phase/Mitosis
	Cell cycle_Initiation of mitosis	Ubiquitin	
		CDK1	
Specific to G-MDSC, Sp to Tu Transition			
UP	Transcription_HIF-1 targets	CREB1	Integrin-mediated cell-matrix interactions
	Cell adhesion_ECM remodeling		Proteolysis_Ubiquitin proteosomal proteolysis
	ATP/ITP metabolism		Regulation of EMT
	IL-1 signaling pathway		
	Clathrin-Mediated Cell adhesion		
Down	T-cell co-signaling receptors	NF-κB	Phagocytosis
	IFN-alpha/beta signaling via JAK/STAT	Jak/Stat	Phagosome in antigen presentation
		c-Myc	TCR signaling
Tu G-MDSC vs Tu M-MDSC			
G>M	Cell adhesion_ECM remodeling	p53	Cell cycle_ G2-M
	Regulation of EMT	Tgfbr2	Proteolysis_ECM remodeling
	Cell cycle_Initiation of mitosis		Connective Tissue Degradation
	Transcription regulation of granulocyte development		
M>G	G-CSF-induced myeloid differentiation	c-Myc	Innate inflammatory response
	CCL2 signaling/CCL2-induced chemotaxis	c-Src	Leukocyte chemotaxis

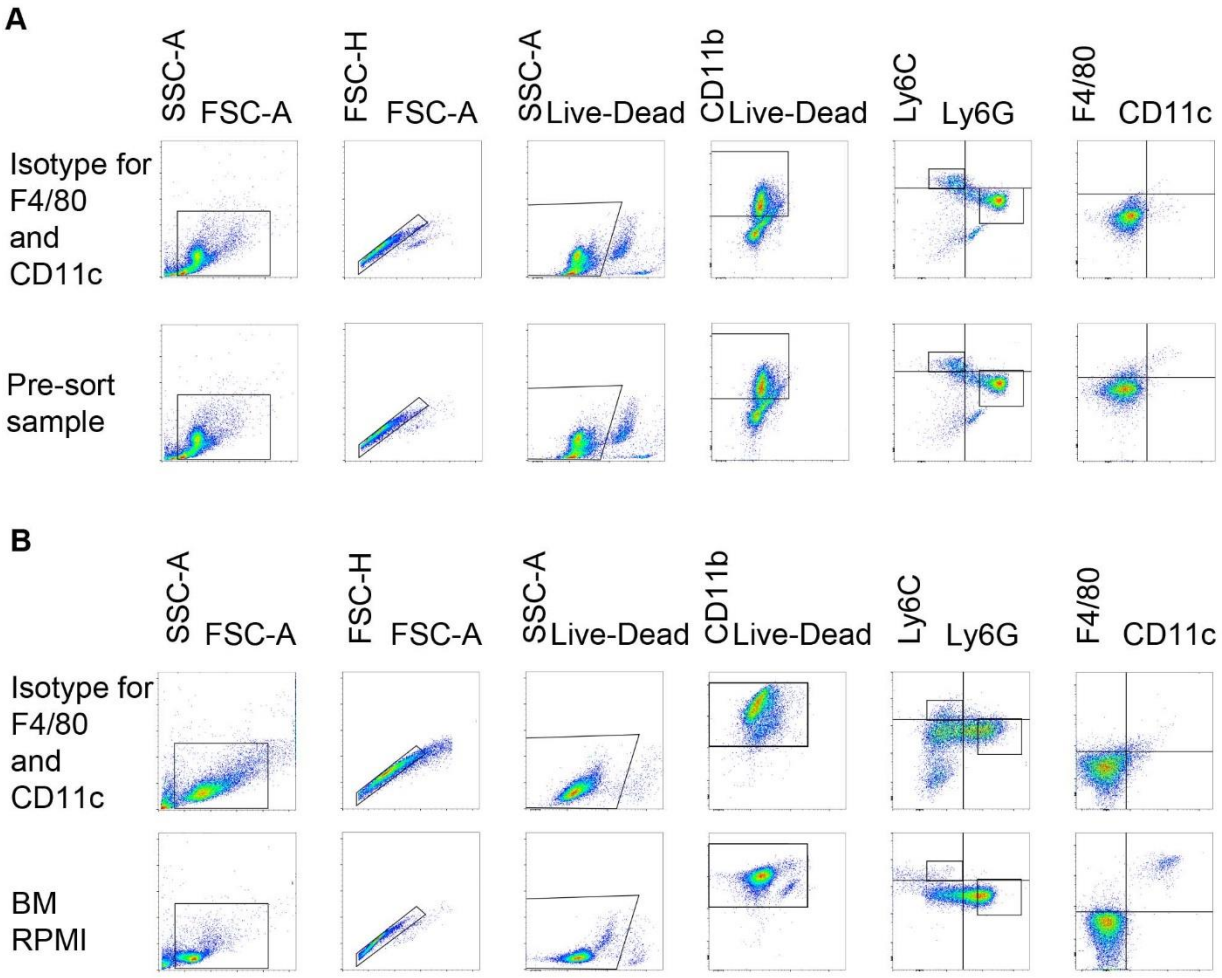

Figure 2.1 Gating Strategies and Sort Purity for Pre- and Post-culture.

Single cell suspension from bone marrow (BM), spleen (SP), and tumor (TU) of EL4 tumor-bearing mice were stained with antibodies for CD11b, Ly6C, Ly6G, F4/80 and CD11c. **(A)** Representative plots of the gating strategy of pre-culture samples. Double isotype control staining, fluorescence minus two, methods were used to determine the F4/80 and CD11c expression prior to culture. **(B)** Representative plots of post-culture gating strategy using the same method as pre-culture with isotypes to determine the $F4/80^{-}$, $CD11c^{-}$ gate.

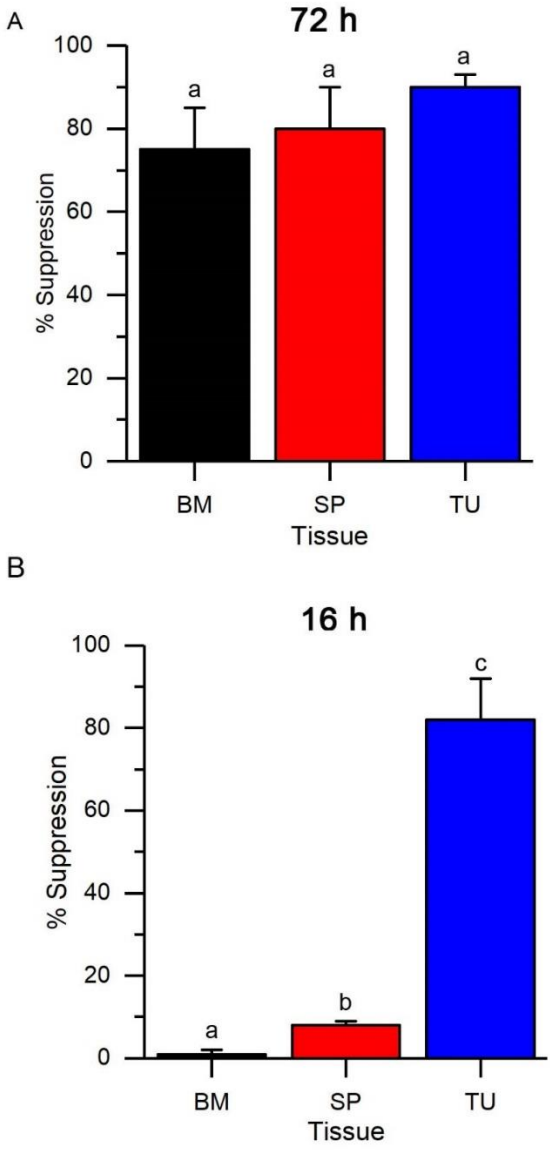

Figure 2.2 Only Tumor M-MDSCs Suppress T-cell Proliferation in Short-Term Assay.

M-MDSCs ($CD11b^{+}$, $Ly6C^{hi}$, $Ly6G^{low}$) were isolated from bone marrow (BM), spleen (SP), and solid tumors (TU) of 4T-1 tumor-bearing mice then co-cultured with purified $CD8^{+}$ T-cells at a ratio of 1:1 for **(A)** 72 hr or (**B**) 16 h. Bars represent the mean + SEM for n=3 observations per group. Bars with different letter superscripts are significantly different from one another (Tukey's HSD, $p<0.05$)

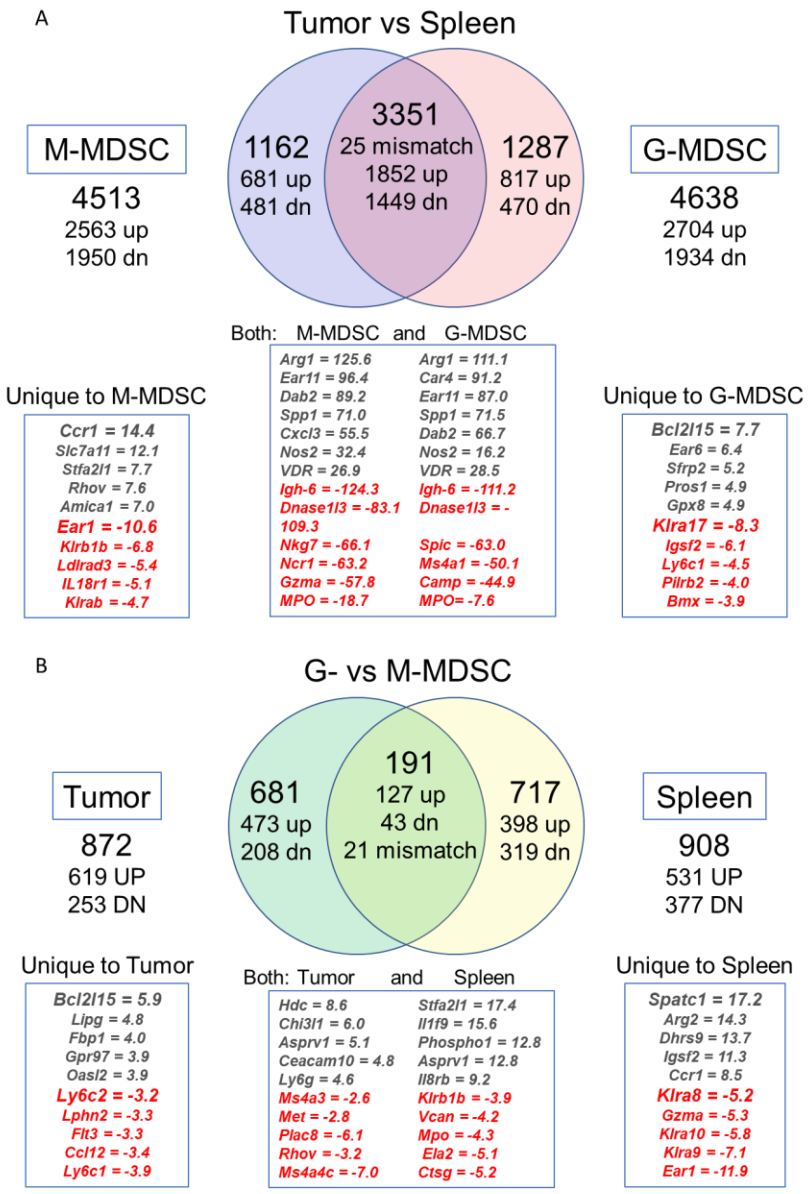

Figure 2.3 Figure 2.3: Spleen and Tumor MDSC Subtypes have Different Gene Expression Patterns.

M- and G-MDSCs were isolated from the peritoneal ascites of mice with intraperitoneal RM-1 tumors (TU) or from the spleens (SP) of POET-3 mice with OT-1 cell induced prostatic inflammation. RNA was isolated and the transcript profile of each SP or TU MDSC subtype was assessed by Affymetrix microarrays. **(A)** Venn diagram of the differentially expressed genes (DEG, 1.5 FC, 5% FDR) identified as M- *(left)* or G-MDSC *(right)* transitioned from SP to TU MDSC (n=4 biological replicates per group). A list of the top 5 up- or down-regulated DEG for SP-to-TU transition for both M- and G-MDSC *(center)* or for each subtype is shown. (**B**) Venn diagram of the differentially expressed genes (DEG, 1.5 FC, 5% FDR) identified between M- and G-MDSC within the SP *(right)* or TU *(left)* (n=4 biological replicates per group). A list of the top 5 up- or down-regulated DEG for the M- to G-MDSC comparison *(center)* or for each tissue type is shown.

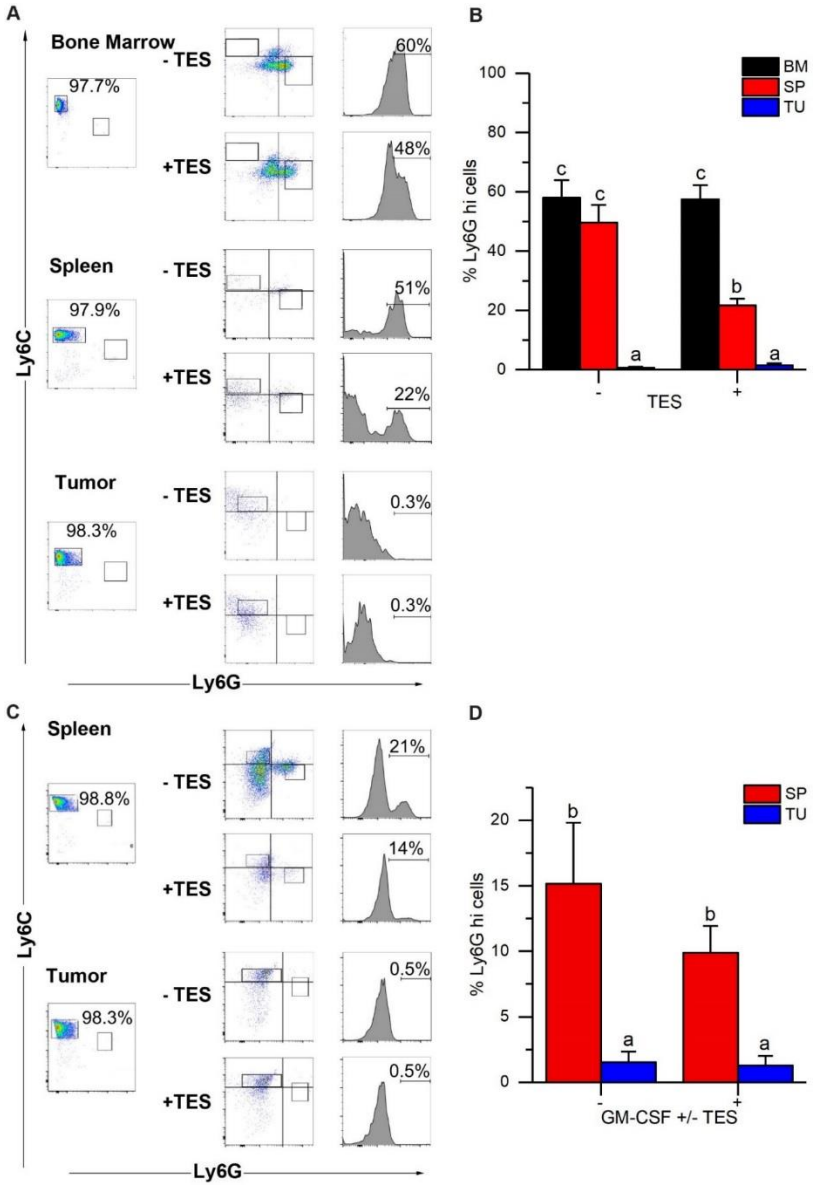

Figure 2.4 Unlike BM and SP M-MDSCs, TU M-MDSCs Do Not Differentiate into G-MDSCs.in the EL-4 Tumor Model

Bone marrow (BM), spleen (SP) and solid tumor (TU)M-MDSC were isolated from of EL-4 tumor-bearing mice. Cells were cultured for 3 days with (**A, B**) basal medium (RPMI-C) or tumor extract supernatant (TES), or **(C, D)** GM-CSF or GM-CSF + TES then harvested from plates and stained for FACS analysis. **(A, C)** ***(left)*** Representative bi-plots of post-isolation purity of M-MDSC from BM, SP, and Tu, ***(center)*** Post-culture Ly6G x Ly6C bi-plots and, ***(right)*** histogram of the Ly6G signal distribution. **(B, D)** Summary graphs showing the percent of M-MDSC that gained Ly6G expression in the experiment. Bars represent the mean + SEM for (**B**) n=8 or **(D)** n=5 biological replicates for each tissue. Statistical analysis was done on transformed data (natural log) using Mixed Design ANOVA. Values with different letter superscripts are significantly different from one another ($p<0.05$, Tukey's HSD).

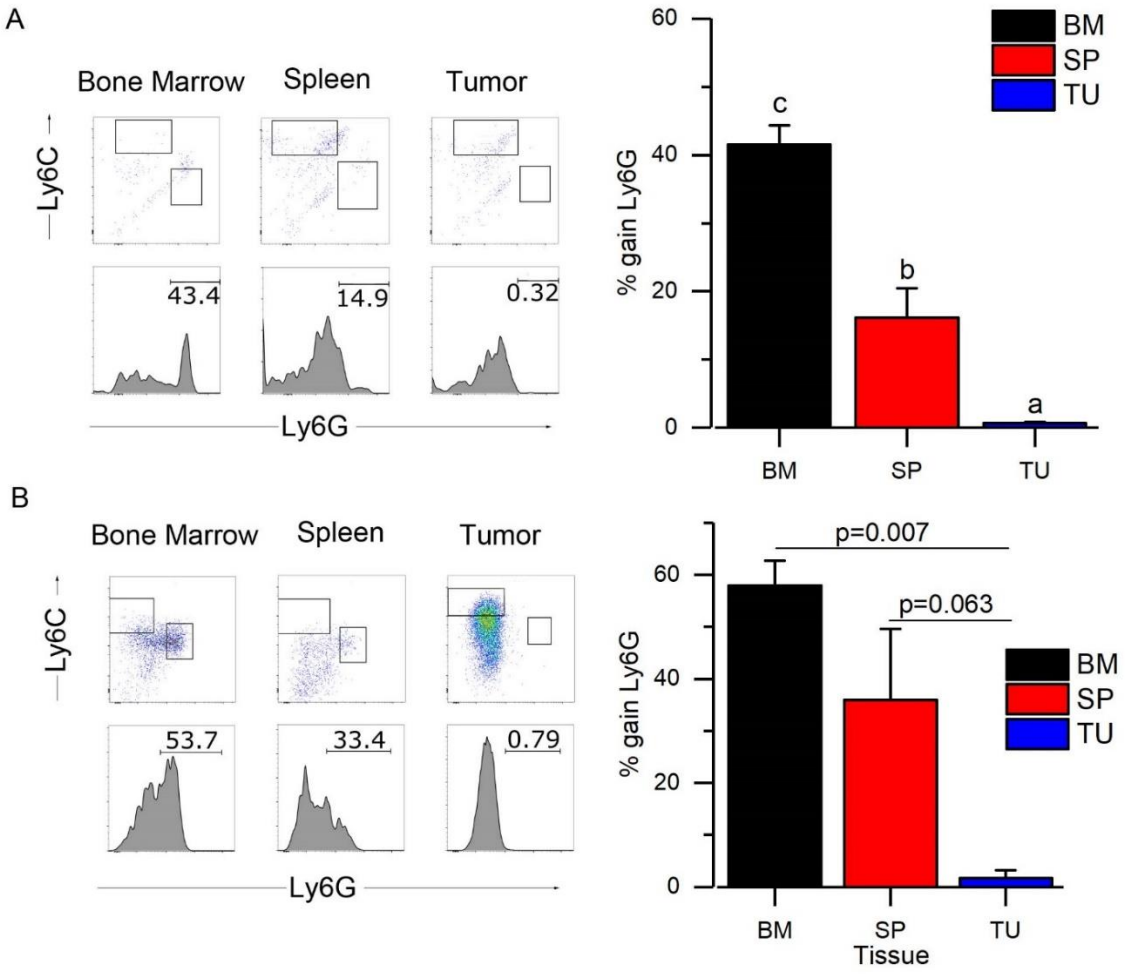

Figure 2.5 Unlike BM and SP M-MDSCs, TU M-MDSCs Do Not Differentiate into G-MDSCs in the RM-1 and 4T1 Tumor Models.

4T1 and RM1 tumors were allowed to grow for 14 d after sc injection then bone marrow (BM), spleen (SP) and solid tumor (TU) M-MDSCs were isolated and cultured for 3 days in RPMI-C. After 3 d cells were harvested from plates and stained for FACS analysis. Results from M-MDSC from **(A)** 4T1- or **(B)** RM1-tumor-bearing mice. Shown are representative plots of the post-culture of Ly6C x Ly6G gate. Histograms were used to calculate the percent of $CD11b^{+}$ cells expressing Ly6G. Bars represent the mean+ SEM for RM-1 data n=3, 4T1 n=4-5. In **(A)**, bars with different letter superscripts are significantly different from one another ($p < 0.05$). In **(B)** the p-values for the comparisons are shown.

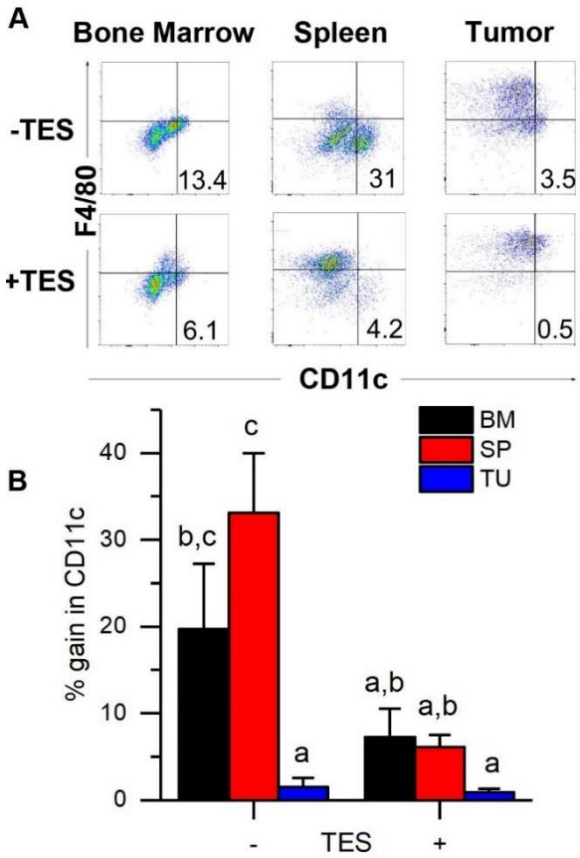

Figure 2.6 Figure Unlike BM and SP M-MDSCs, TU M-MDSCs Do Not Differentiate to DCs in EL-4 mouse Tumor Model

Bone marrow (BM), spleen (SP) and solid tumor (TU) M-MDSC were isolated from of EL-4 tumor-bearing mice and cultured for 3 days in the presence of GM-CSF and IL-4, +/- TES, and then analyzed by Flow cytometry for markers of macrophages (F4/80) and DCs (CD11c). **(A)** Representative flow cytometry plots of post-culture of M-MDSC. **(B)** Summary plot of $CD11c^{+}$ cells. Bars represent the mean + SEM for n=3 biological replicates for each tissue. Statistical analysis was done on transformed data (natural log) using Mixed Design ANOVA. Values with different letter superscripts are significantly different from one another ($p<0.05$, Tukey's HSD).

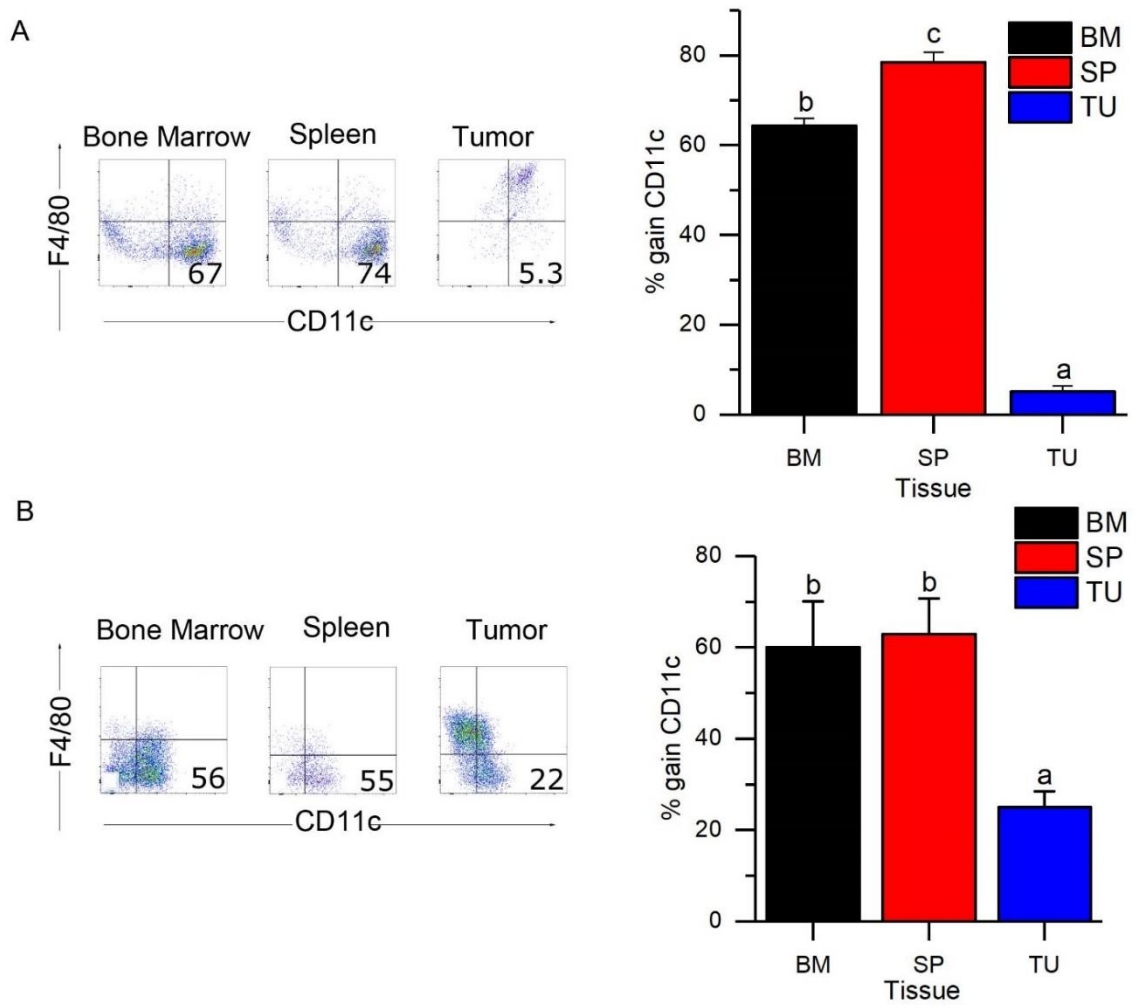

Figure 2.7 Unlike BM and SP M-MDSCs, TU M-MDSCs Do Not Differentiate to DCs in RM-1 and 4T1 Mouse Tumor.

4T-1 and RM-1 s.c. tumors were allowed to grow for 14d then bone marrow (BM), spleen (SP) and solid tumor (TU) M-MDSCs were isolated and cultured for 3 days in RPMI-C with GM-CSF (10 ng/mL) and IL-4 (2 ng/mL). After 3d cells were harvested from plates and stained for FACS analysis. Results from M-MDSC from 4T-1**(A)** and RM-1 **(B)** tumor-bearing mice **(A-B, left)** Shown are representative plots of post-culture of CD11c x F4/80 gate BM, SP and TU. **(A, *right*)** Bar graph showing M-MDSC from BM and SP are significantly more able to gain CD11c compared to TU M-MDSC ($p<0.05$). **(B, *right*)** Bar graph showing M-MDSC from BM are significantly more able to gain CD11c compared to TU M-MDSC ($p<0.05$). Statistics were done using ANOVA on raw percentage data post Shapiro-Wilks normality assessment and Cook's D outlier test.

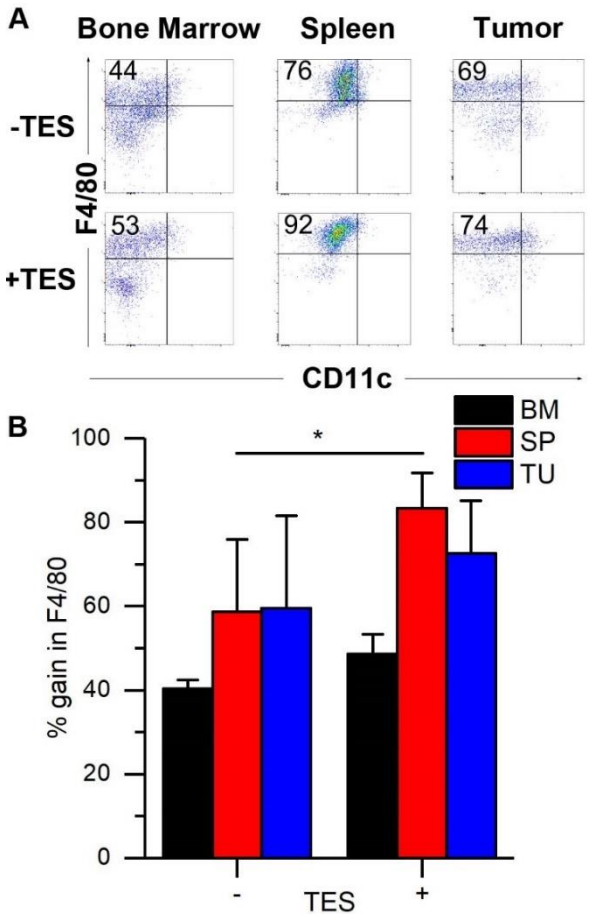

Figure 2.8 BM, SP M-MDSCs, and TU M-MDSCs Differentiate to Macrophages

Bone marrow (BM), spleen (SP) and tumor (TU)M-MDSCs were isolated from EL-4 tumor-bearing mice and cultured for 3 days in the presence of M-CSF +/- TES, and then analyzed by Flow cytometry for markers of macrophages (F4/80) and DCs (CD11c). **(A)** Representative flow cytometry plots of post-culture of M-MDSC **(B)** Summary plot of $F4/80^{+}$ cells. Bars represent the mean + SEM n=3 biological replicates for each tissue. Statistical analysis was done on transformed data (natural log) using Mixed Design ANOVA. Values with different letter superscripts are significantly different from one another ($p<0.05$, Tukey's HSD).

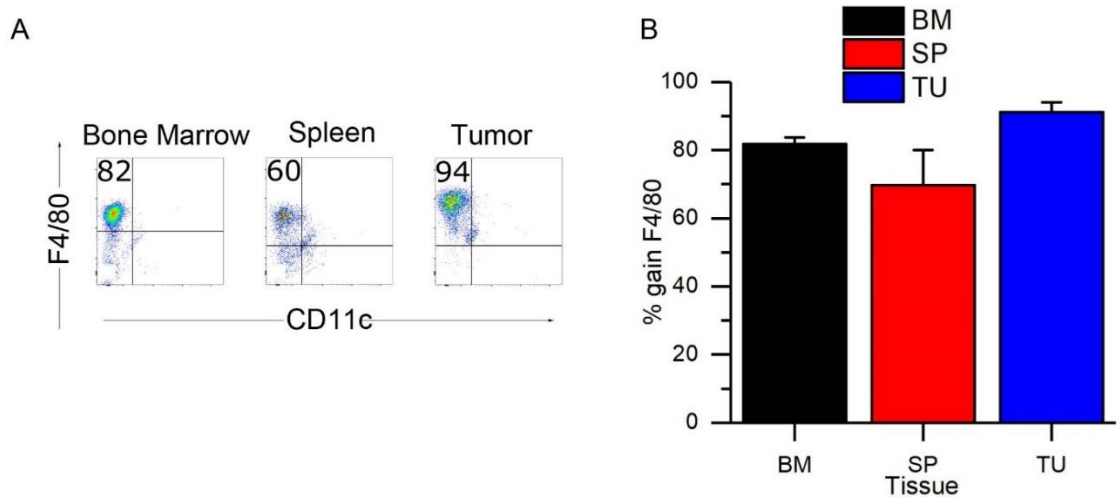

Figure 2.9 BM, SP M-MDSCs, and TU M-MDSCs from RM-1 and 4T1 Tumor Models Confirm Findings that Cells From All Tissue are able to Become Macrophages

RM-1 s.c. tumors were allowed to grow for 14d then bone marrow (BM), spleen (SP) and solid tumor (TU) M-MDSCs were isolated and cultured for 3 days in RPMI-C with M-CSF (25 ng/mL). After 3d cells were harvested from plates and stained for FACS analysis. (A) Representative plots of post-culture BM, SP, and TU M-MDSCs on a CD11c x F4/80 gate (B) Bar graph of percent differentation data. BM, SP and, TU M-MDSC are not significantly different in their ability to gain F4/80 ($p<0.05$). Statistics were done using ANOVA on raw percentage data post Shapiro-Wilks normality assessment.

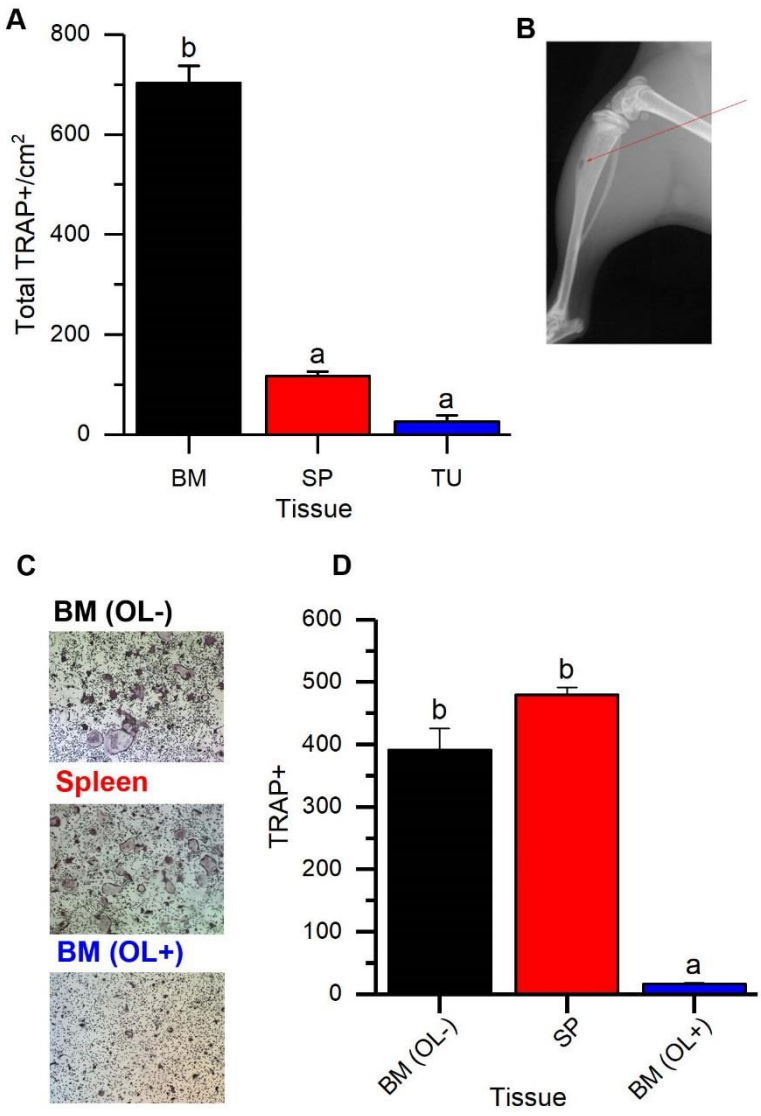

Figure 2.10 Unlike BM and SP M-MDSCs, TU M-MDSCs do not Respond to Oc Differentiating Signals

(A) Bone marrow (BM), spleen (SP) and solid tumor (TU) M-MDSC were isolated from EL-4 tumor-bearing mice and cultured 8 d with M-CSF and RANKL. TRAP+ cells were counted post culture. Bars represent the mean + SEM (n=3). Statistical analysis done on raw counts using one-way ANOVA. Values with different letter superscripts are significantly different ($p<0.05$, Tukey's multiple comparisons). **(B-D)** Mice received an intracardiac injection of 4T-1 cells and bone tumor metastases were allowed to grow 10 d. M-MDSC were then collected from bone marrow regions showing osteolysis (OL+), contralateral bones with not osteolysis (OL-), or the SP of the tumor-bearing mice. M-MDSC were cultured for 8 d with M-CSF and RANKL and TRAP+ cells were counted. **(B)** Representative X-ray of Osteolytic site on the tibia. **(C)** Representative microscopy images, showing large, TRAP+ Ocs in M-MDSC cultures from OL- bone marrow, OL+ bone marrow, and SP. **(D)** Graph of TRAP+ Ocs cells following the treatment period. Bars represent the mean+ SEM (n=3). Values with different letter superscripts are significantly different ($p<0.05$, Tukey's HSD).

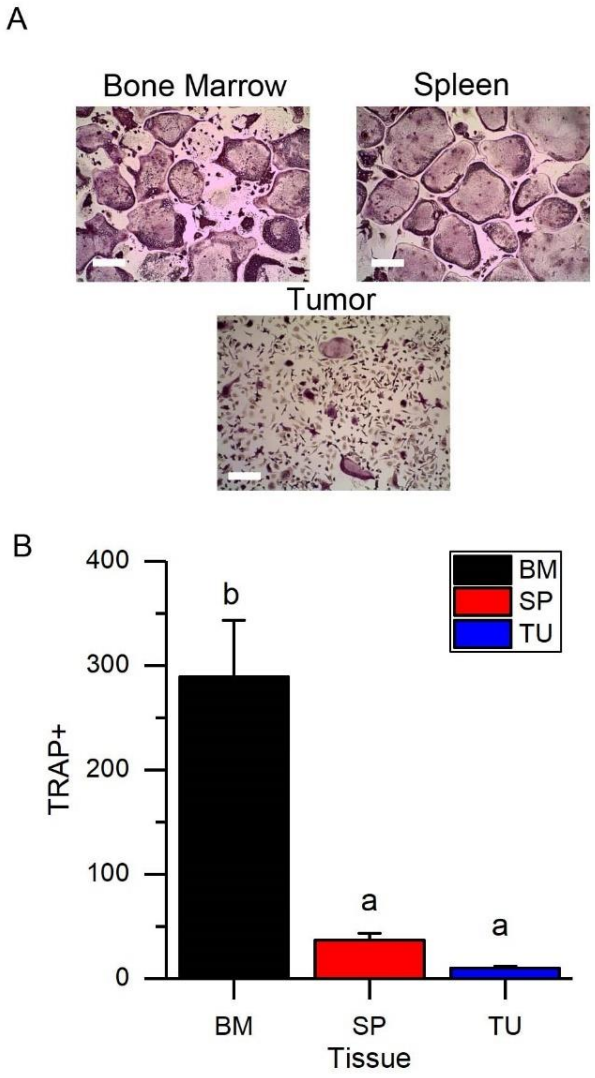

Figure 2.11 Unlike BM and SP M-MDSCs, TU M-MDSCs do not Respond to Oc Differentiating Signals RM-1 and 4T1 Tumor Models and Confirm EL-4 Tumor Model Findings

RM-1 and 4T1 s.c. tumors were allowed to growth for 14d then bone marrow (BM), spleen (SP) and solid tumor (TU) M-MDSCs were isolated and cultured in M-CSF (50 ng/mL) and RANKL (100 ng/mL) for 8d and were stained for TRAP+ cells and counted. **(A)** Representative micrographs of TRAP staining of M-MDSC from 4T1 s.c. tumor-bearing mice post culture (original magnification 40X). Bar represents 200 μm. **(B)** $TRAP^+$ cells from RM-1 derived tissue M-MDSC. Graphs show the number of TRAP+ cells/cm^2 counted from each tissue. Letters designate statistical significance ($p<0.05$) determined by ANOVA with Bonferroni's Multiple Comparison Test.

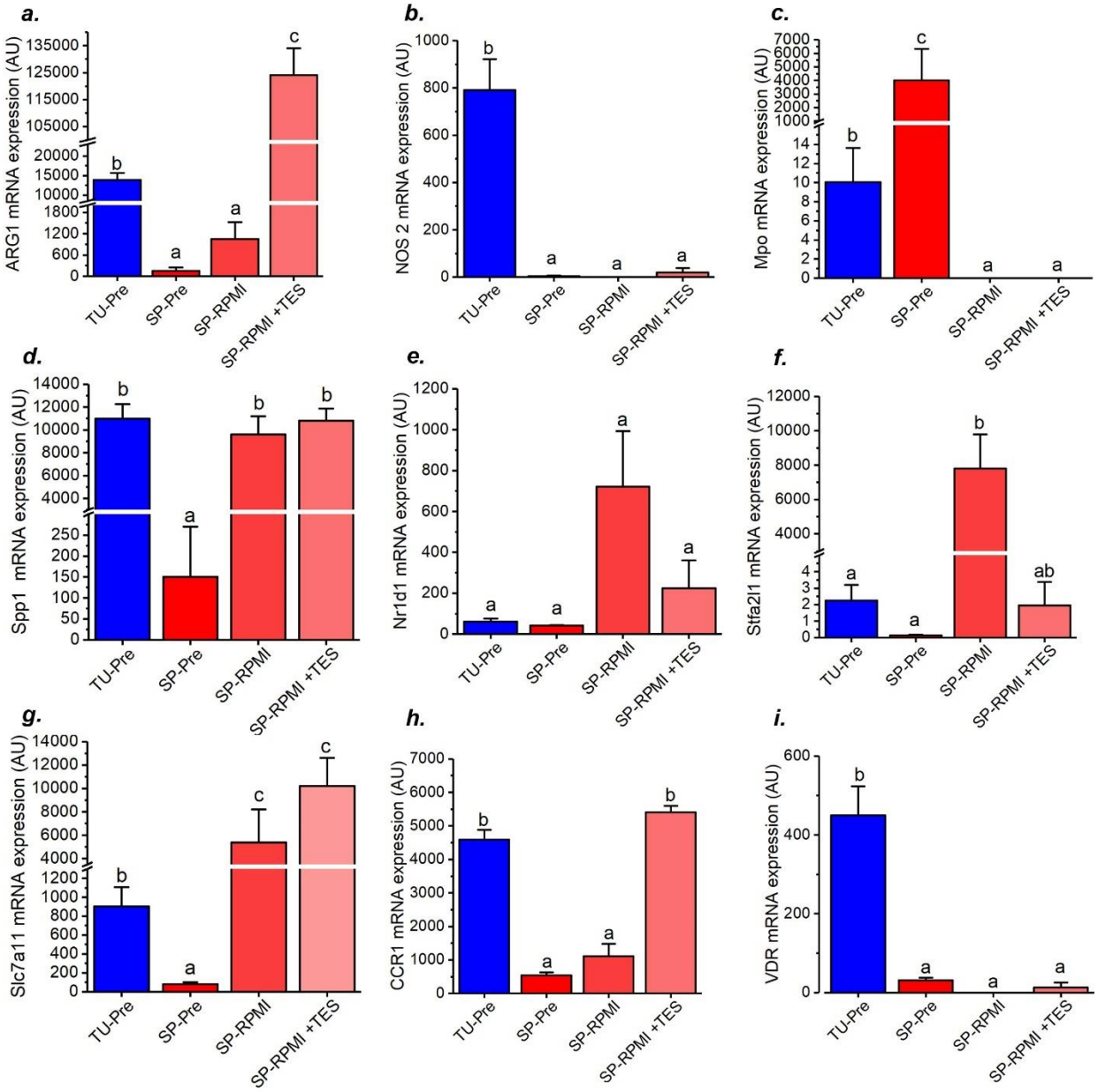

Figure 2.12 Different Transcriptomes and Transcriptome Responses to Tumor Environment in SP and TU M-MDSCs.

Tumor (TU) and spleen (SP) Pre-culture samples were prepped for RNA isolation directly following sorting. Some SP samples were cultured in RPMI or RPMI +TES (20%) for 3d then harvested for RNA isolation and qPCR. RNA was analyzed for mRNAs found to be differentially expressed between SP and TU M-MDSC in our microarray experiment. Bar graphs show mean + SEM (n=3-5 biological replicates per target). Within each panel, bars with different letter superscripts were significantly different from one another ($p<0.05$, Fisher's protected LSD).

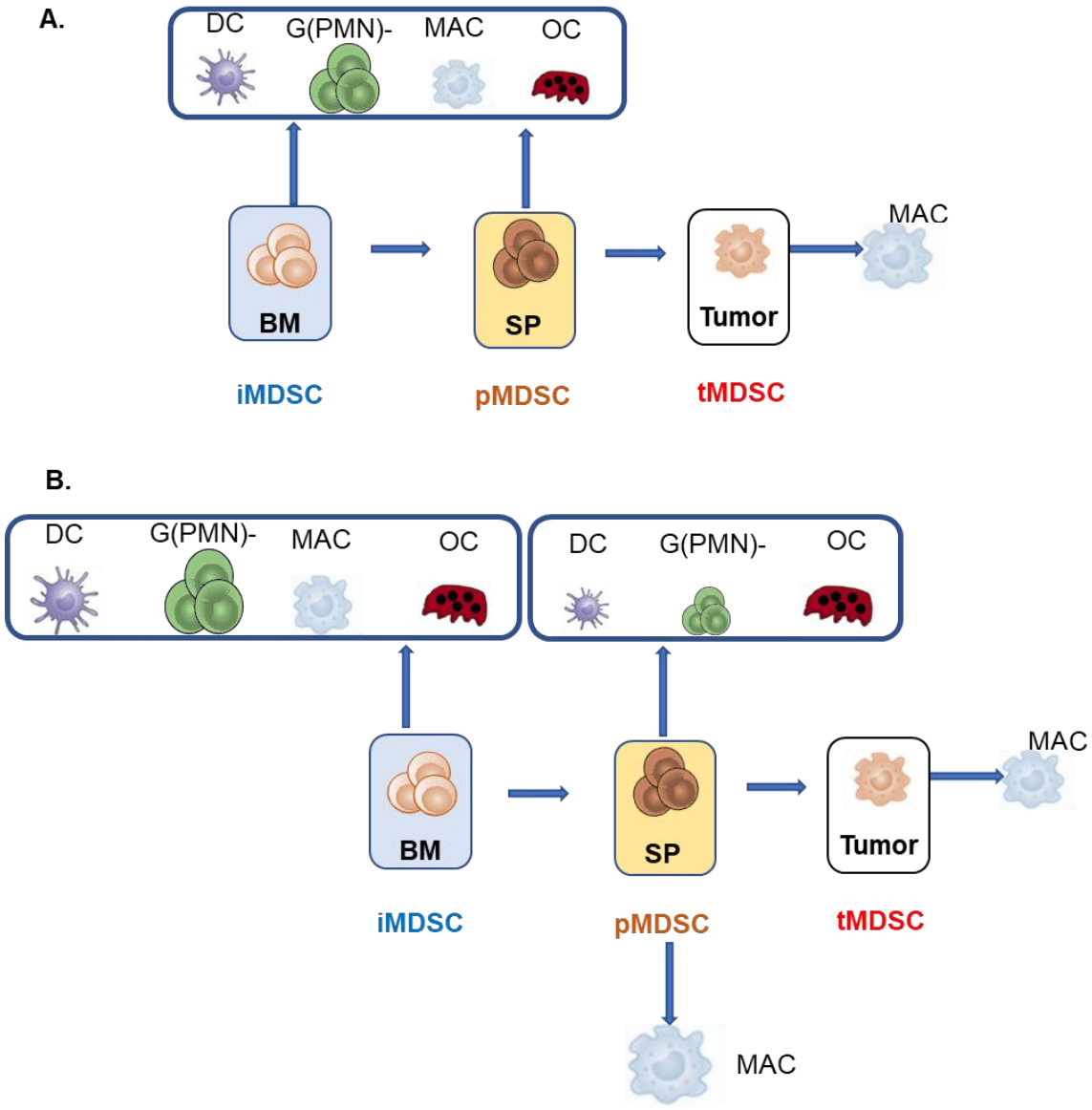

Figure 2.13 Model of Differentiation Potential Related to Tissue of Isolation and New Proposed Nomenclature

Models of potential differentiation from bone marrow (BM), spleen (SP) and tumor isolated M-MDSC. Each model also shows suggested new nomenclature for each M-MDSC based on the location of isolation. **(A)** Model of the potential of M-MDSC isolated from different tissues to differentiate toward alternative cell types by cytokine treatment *in vitro*. **(B)** Model of the potential of M-MDSC isolated from different tissues to differentiate toward alternative cell types by cytokine treatment +TES *in vitro*.

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2.8 Chapter 2 Appendix

Appendix A: Pathways Enriched in subsets

Pathways enriched in subsets of comparisons from analysis of M-MDSC and G-MDSC from spleen and tumor of mice. Refer to the Venn diagram in Figure 3 of the manuscript and the legend below the table for a definition of the subgroups analyzed.

	Common MDSC			G-MDSC Only			G-MDSC All			M-MDSC Only			M-MDSC All		
Maps	Both UP and DN	UP Only	DN Only	Both UP and DN	UP Only	DN Only	Both UP and DN	UP Only	DN Only	Both UP and DN	UP Only	DN Only	Both UP and DN	UP Only	DN Only
Action of GSK3 beta in bipolar disorder										X	X				
ATP/ITP metabolism					X			X							
B CELL Immune response_B cell antigen receptor (BCR) pathway						X			X						
B CELL Role of B cells in SLE			X			X			X				X		X
B cell signaling in hematological malignancies						X			X						X
Cell adhesion_Cadherin-mediated cell adhesion					X			X						X	
Cell adhesion_ECM remodeling					X			X							
Cell cycle_Chromosome condensation in prometaphase										X		X			
Cell cycle_Initiation of mitosis										X		X			
Cell cycle_Role of APC in cell cycle regulation										X		X			
Cell cycle_Role of Nek in cell cycle regulation					X			X		X		X			
Cell cycle_Spindle assembly and chromosome separation										X		X			

Appendix A cont.

Cell cycle_The metaphase checkpoint										X		X			
Cell cycle_Transition and termination of DNA replication												X			
Cytoskeleton remodeling_Regulation of actin cytoskeleton nucleation and polymerization by Rho GTPases											N				
Cytoskeleton remodeling_Regulation of actin cytoskeleton organization by the kinase effectors of Rho GTPases					X			X							
Development_PIP3 signaling in cardiac myocytes		X												X	
Development_Regulation of epithelial-to-mesenchymal transition (EMT)					X			X							
DNA damage_ATM / ATR regulation of G2 / M checkpoint										X		X			
Glomerular injury in Lupus Nephritis				X			X						X		
HYPOXIA Transcription_HIF-1 targets	X	X		X	X		X	X					X	X	
HYPOXIA Transcription_Hypoxia- and receptor-mediated HIF-1 activation					X			X							
Hypoxia-induced EMT in cancer and fibrosis					X			X							
Immune response_IFN-alpha/beta signaling via JAK/STAT	X		X	X		X	X		X				X		X
Immune response_IL-1 signaling pathway	X	X		X			X				N		X	X	
Immune response_IL-10 signaling pathway				X			X								
Immune response_IL-3 signaling via JAK/STAT, p38, JNK and NF-κB		X												X	
Immune response_IL-4-induced regulators of cell growth, survival, differentiation and metabolism					X			X							

Appendix A cont.

Immune response_IL-4-responsive genes in type 2 immunity				X			X								
Immune response_Inhibitory PD-1 signaling in T-cells	X		X										X		X
Immune response_OX40L/ OX40 signaling pathway			X												X
Immune response_Role of DPP4 (CD26) in immune regulation	X												X		
Multiple myeloma (general schema)											Y				
MYELOID Immune response_Antigen presentation by MHC class I: cross-presentation		X												X	
MYELOID Immune response_ETV3 affect on CSF1-promoted macrophage differentiation											X				
MYELOID Immune response_M-CSF-receptor signaling pathway											N				
MYELOID Immune response_Oncostatin M signaling via JAK-Stat		X												X	
MYELOID Immune response_Oncostatin M signaling via MAPK		X													
MYELOID Macrophage and dendritic cell phenotype shift in cancer				X			X								
MYELOID PGE2 pathways in cancer		X									Y			X	
NETosis in SLE												X			
Neurogenesis_NGF/ TrkA MAPK-mediated signaling		X													
Ovarian cancer (main signaling cascades)											Y				
Reproduction_Gonadotropin-releasing hormone (GnRH) signaling											Y				
Rheumatoid arthritis (general schema)				X		X	X		X		N				
Role of IL-23/ T17 pathogenic axis in psoriasis				X		X	X		X						

Appendix A cont.

Signal transduction_AKT signaling														X	
T-CELL Breakdown of CD4+ T cell peripheral tolerance in type 1 diabetes mellitus	X		X			X			X						X
T-CELL Immune response_Differentiation and clonal expansion of CD8+ T cells	X		X			X			X				X		X
T-CELL Immune response_T-cell co-signaling receptors, schema	X		X	X		X	X		X	X			X		X
T-CELL Immune response_T-cell subsets: cell surface markers	X		X									X			
T-CELL Immune response_TCR alpha/beta signaling pathway															X
T-CELL Immune response_Th1 and Th2 cell differentiation													X		
T-CELL SLE genetic marker-specific pathways in T-cells			X												
T follicular helper cell dysfunction in SLE	X		X			X			X						X
Transcription_Epigenetic regulation of gene expression										X					
Transport_Clathrin-coated vesicle cycle		X												X	

X = FDR < 0.05; Y = FDR < 0.1; N = FDR > -0.1 but map was listed as top 10

Common to MDSC = genes that are differentially regulated from spleen to tumor in both G- and M- subsets

G-MDSC Only = genes that are differentially regulated from spleen to tumor in only the G-MDSC subset

M-MDSC Only = genes that are differentially regulated from spleen to tumor in only the M-MDSC subset

G-MDSC All = genes that are differentially regulated from spleen to tumor using the G-MDSC subset data

M-MDSC All = genes that are differentially regulated from spleen to tumor using the M-MDSC subset data

Both = analysis conducted using both significantly differentially up and down regulated genes

UP = analysis conducted using only significantly differentially up regulated genes

DN = analysis conducted using only significantly differentially down regulated genes

Appendix B: Networks enriched in subsets

Networks enriched in subsets of comparisons from analysis of M-MDSC and G-MDSC from spleen and tumor of mice. Refer to the Venn diagram in Figure 3 of the manuscript and the legend below the table for a definition of the subgroups analyzed.

	Common MDSC			G-MDSC Only			G-MDSC All			M-MDSC Only			M-MDSC All		
Networks	Both UP and DN	UP Only	DN Only	Both UP and DN	UP Only	DN Only	Both UP and DN	UP Only	DN Only	Both UP and DN	UP Only	DN Only	Both UP and DN	UP Only	DN Only
Apoptosis_Apoptotic mitochondria		X												X	
Cell adhesion_Amyloid proteins		X			X			X						X	
Cell adhesion_Cell-matrix interactions					X			X							
Cell adhesion_Integrin-mediated cell-matrix adhesion					X			X							
Cell adhesion_Leucocyte chemotaxis	X		X	X		X	X		X				X		X
Cell adhesion_Platelet aggregation											N				
Cell adhesion_Platelet-endothelium-leucocyte interactions	X			X	X		X	X							
Cell cycle_Core										X		X			X
Cell cycle_G2-M	X		X							X		X			
Cell cycle_Meiosis										X		X			
Cell cycle_Mitosis										X		X			
Cell cycle_S phase										X		X			
CELL CYCLE Proliferation_Lymphocyte proliferation	X		X	X		X	X		X				X		X

Appendix B cont.

CELL CYCLE Proliferation_Positive regulation cell proliferation													X	
Chemotaxis	X	X									X	N	X		
Cytoskeleton_Spindle microtubules										X		X			
Development_Blood vessel morphogenesis		X			X			X						X	
Development_EMT_Regulation of epithelial-to-mesenchymal transition		X			X			X						X	
Development_Neurogenesis_Synaptogenesis														X	
Development_Regulation of angiogenesis		X		X	X		X	X					X	X	
Development_Skeletal muscle development											Y				
DNA damage_Checkpoint										Y					
DNA damage_DBS repair										X		Y			
Immune response_Antigen presentation	X		X	X		X	X		X				X		X
Immune response_BCR pathway			X			X			X						X
Immune response_Phagocytosis				X		X	X		X						
Immune response_Phagosome in antigen presentation				X		X	X		X				X		
Immune response_T helper cell differentiation	X		X										X		X
Immune response_TCR signaling	X		X	X		X	X		X				X		X
Immune response_Th17-derived cytokines											N				
Inflammation_Histamine signaling											N				
Inflammation_IgE signaling											N				
Inflammation_IL-10 anti-inflammatory response						X			X		N				

Appendix B cont.

Inflammation_IL-12,15,18 signaling			X											X
Inflammation_Interferon signaling	X		X	X		X	X		X				X		X
Inflammation_MIF signaling											N				
Inflammation_Neutrophil activation											X				
Inflammation_NK cell cytotoxicity	X		X	X		X	X		X				X		X
Protein folding_ER and cytoplasm		X			X			X						X	
Protein folding_Folding in normal condition		X												X	
Proteolysis_Ubiquitin-proteasomal proteolysis		X			X			X							
Reproduction_Feeding and Neurohormone signaling		X			X			X						X	
Reproduction_Male sex differentiation										Y					
Reproduction_Progesterone signaling												N			
Signal Transduction_Cholecystokinin signaling											N				
Transcription_Chromatin modification										X		X			

X = FDR < 0.05; Y = FDR < 0.1; N = FDR > -0.1 but map was listed as top 10

Common to MDSC = genes that are differentially regulated from SP to TU in both G- and M-subsets

G-MDSC Only = genes that are differentially regulated from SP to TU in only the G-MDSC subset

M-MDSC Only = genes that are differentially regulated from SP to TU in only the M-MDSC subset

G-MDSC All = genes that are differentially regulated from SP to TU using the G-MDSC subset data

M-MDSC All = genes that are differentially regulated from SP to TU using the M-MDSC subset data

Both = analysis conducted using both significantly differentially up and down regulated genes

UP = analysis conducted using only significantly differentially up regulated genes

DN = analysis conducted using only significantly differentially down regulated genes

CHAPTER 3. PHENOTYPING IMMUNE CELLS IN TUMOR AND HEALTHY TISSUE USING FLOW CYTOMETRY DATA

A version of this work has been published in the conference proceedings of ACM-BCB'18: 9th ACM International Conference on Bioinformatics, Computational Biology and Health Informatics

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3.1 Abstract

It has now been demonstrated that myeloid-derived suppressor cells (MDSC) are functionally distinct depending on the tissue of isolation. However, to date no one has attempted to distinguish these differences based on common markers used in flow cytometry. We present an automated pipeline capable of distinguishing the phenotypes of MDSCs in healthy and tumor-bearing tissues in mice using flow cytometry data. This pipeline includes data transformation, automated gating and clustering, cluster matching and template formation, and finally, sample classification and phenotype discovery. In contrast to earlier work where samples are analyzed individually, we analyze all samples from each tissue collectively using a representative template.

We demonstrate with 43 flow cytometry samples collected from three tissues that a set of templates serves as a better classifier than popular machine learning approaches including support vector machines and simple neural networks. Our "interpretable machine learning" approach goes beyond classification and identifies distinctive phenotypes associated with each tissue, information that is clinically useful. Hence the pipeline presented here leads to better understanding of the maturation and differentiation of MDSCs using high-throughput data.

Y. Chen and R. D. Calvert each contributed significantly to the work. Y. Chen did the programming for the project and R.D. Calvert collected biological samples, conducted gating comparison experiment, aided in programing decisions, and, added biological relevance to project.

Acknowledgements

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3.2 Introduction

Inflammation and cancer affect the formation of cells in the blood and bone marrow by altering myelopoiesis toward immunosuppressive cells and reducing maturation of innate immune cells. The heterogeneous collection of immature myeloid cells that can potentially suppress the T-cell response at the tumor are called myeloid derived suppressor cells (MDSC) [1]. In mice, monocytic MDSCs (M-MDSC; $CD11b^{+}Ly6C^{hi}Ly6G^{-/lo}$) and granulocytic MDSCs (GMDSC; $CD11b^{+}Ly6C^{int}Ly6G^{hi}$), are the best two recognized MDSC subtypes and can be separated using the markers above via Flow Cytometry (FC) [1]. In chapter 2, it was demonstrated that MDSCs derived from different tissues of tumor bearing mice represent different stages of MDSC development; MDSCs found in the bone marrow (BM) and spleen (SP) are immature precursors to the fully suppressive MDSCs found at the tumor (TM). This means that there are at least six different MDSC populations within a tumor bearing animal: both M- and G- subtypes found in bone marrow (immature MDSC, iMDSC), spleen (peripheral MDSC, pMDSC), and the tumor site (tumor MDSC, tMDSC) (Figure 3.1). Naïve MDSC subtypes from bone marrow or spleen of mice without tumors are often considered similar to those found in the bone marrow of tumor bearing animals due to their ability to gain suppressive function *in-vitro* and are referred to as iMDSC.

Since MDSCs are associated with tumor progression and increased metastasis [2], these cells are candidate targets for immune therapy. It has been shown that the number of MDSCs in human blood are associated with the tumor lymph node metastasis stages [3] in colorectal cancer patients [4]. However, it is still unclear whether more specific identification of the MDSC subtypes would better predict cancer stage. Therefore, our long-term goal is to develop a method to identify specific subtypes of MDSCs found in blood. In addition to serving as a biomarker for cancer stage, this information may also be helpful in determining the type of immunotherapies that would be most successful for a cancer patient. For example, if pMDSCs are the only MDSC present in the

blood then a therapy targeted at inhibiting chemotaxis of pMDSCs may be the better than one focused on decreasing the T cell suppressive ability of tMDSCs.

Here we ask if we can determine a set of rules, that can be followed (algorithms) by machine learning software in a flow cytometry analysis to distinguish specific subpopulations of MDSC cells. We define machine learning as a specific program that is designed to learn and adjust itself based on the sample data provided to it, without the need to manually reprogram the software. Unlike standard FC analysis, multidimensional FC analysis accounts for multiple markers at the same time to identify cell populations (ie. CD11b, Ly6G and Ly6C). This allows the investigator to assess the differential expression of multiple markers on a cell. For example, we know that M-MDSC are defined as $CD11b^{+}Ly6C^{hi}Ly6G^{low/-}$ but we do not know the how the expression of CD11b relates to Ly6C especially during MDSC development. This method will help to move the field of MDSC biology forward by 1) confirming that MDSCs found at the tumor site possess differentially expressed extracellular markers compared to precursors found in the BM and SP and 2) demonstrating that, with minimal extracellular markers, the developmental stage of MDSC can be determined. This information will aid in providing a more clear definition of MDSC development which will lead to the development of more precise immunotherapies targeting MDSCs. Based on data presented in chapter 2, we chose to analyze samples of MDSCs from naïve BM (NVBM), to represent iMDSC due to their lack of exposure to signals from a tumor, from the SPs of tumor bearing mice (TBSP, pMDSC) as a moderate maturation stage of MDSC subpopulations due to their ability to respond to differentiation signals and lack of immediate T cell suppressive ability, and from the tumor (IPTM, tMDSC) which are more mature MDSC subpopulation because MDSCs found at the tumor possess immediate T-cell suppressive ability [5, 6].

Our machine learning multidimensional FC analysis method utilizes the concept of building a template for each sample class ie NVBM, TBSP and IPTM. A template is a compact representation of the cell populations found within a collection of samples after eliminating sample-specific variations and noise. In our study, templates are made for a specific collection of MDSCs from the same type of tissue/developmental stage. A template is expected to capture the underlying distribution of cell populations within different samples based on the cell markers provided to the machine learning software. To do this each sample is analyzed and groups of cells with similar marker expression patterns are classified as a cluster of cells. In our work, we capture

the core pattern of multiple “similar” clusters across different sample classes by a meta-cluster. A meta-cluster is the representation of a specific cluster of cells found across multiple samples. Therefore, a template can be defined as is a collection of meta-clusters present in a class of samples. We illustrate this concept in Figure 3.1. Our goals are to show that the use of multidimensional FC analysis can correctly classify mixed populations of cells from MDSC samples by tissue/developmental stage and to lay the foundational work for future studies in understanding which MDSC populations exist in human blood samples, which could aid in assessing the TNM stage of patients.

3.3 Methods

3.3.1 Description of Dataset

The data were drawn from an archive of FC data-sets of ~60 individual experiments conducted over a period of 2 years and representing 94 biological samples. Below is a description of the FC methodology. Subsequently, we describe the method used to select individual samples that met specific criteria for similarity, consistency, and quality.

3.3.2 Reagents:

RPMI was purchased from Sigma (St. Louis, MO). DMEM (containing 2 mM L-Glutamine) and fetal bovine serum (FBS) were purchased from Corning (Manassas, VA). 1 M Hepes and 100 mM sodium pyruvate were purchased from Media Technology (Manassas, VA). Penicillin/Streptomycin (Pen/Strep, 10,000 units/mL) was purchased from HyClone (South Logan UT). TruStain FcX (clone 93, product #101320) was purchased from Biolegend (San Diego, CA). Fluorescent tagged antibodies for CD11b-PeCy7(clone M1/70, product #101216), Ly6C-APC (clone HK1.4, product #128016) and Ly6G-PE (clone 1A8, product #127608) were purchased from Biolegend (San Diego, CA), and BD pharma (Franklin Lakes, NJ).

3.3.3 Animals:

Male and female C57Bl/6N were obtained from the Purdue Transgenic Mouse Core Facility (West Lafayette, IN). Mice were housed in specific pathogen-free conditions with a 12 hr light/dark cycle. Mice were fed a standard chow diet (Teklad 2018) and water ad libitum. All of

the mouse use described in this paper was approved by Purdue University Animal Care and Use Committees.

3.3.4 Tumor Cell Culture:

RM-1 murine prostate cancer cells [7]were cultured in DMEM supplemented with 10% FBS, 1 mM Na pyruvate, 1% Pen/Strep at 5% CO_2 at 37ºC. Cells were passaged 1:10, at 70% confluency.

3.3.5 MDSC Generation and Isolation:

10^6 RM-1 cells were injected intraperitoneally (i.p.) into 9-15 wk old male or female C57Bl/6 mice and allowed to grow for 7 d. After 7 d, mice were euthanized according to approved AVMA methods and cells from spleen (TBSP) and/or i.p. exudate (IPTM) were harvested. An additional group of C57Bl/6 mice that did not receive tumor cells (i.e. naive) was used to harvest cells from BM (NVBM). Samples from naive and tumor-bearing mice were comprised of pools of cells from 2-5 mice. Bone marrow was removed from femurs and tibias by flushing with RPMI supplemented with 10% FBS, 1 mM HEPES, 1 mM Na pyruvate, 1% Pen/Strep (RPMI-C). SP were physically disrupted between two frosted microscope slides and then suspended in RPMI-C. Cells from the i.p. exudate of tumor cell injected mice were harvest via peritoneal lavage with PBS, individual mouse samples were pooled and resuspended in RPMI-C.

Following collection of cells, all tissue samples were pelleted then treated with Ammonium-Chloride-Potassium lysing buffer (0.15 M NH4Cl, 10 mM KHCO3, 1 mM EDTA, pH=8.0, in sterile H2O) (2 min., RT) to remove red blood cells. Samples were then diluted with PBS (1:1) and filtered through a 70 μm cell strainer. Cells were pelleted and then resuspended in PBS (100 μl/mouse for BM and SP (200-500 μl total), and 100 μl /pool of i.p. TU exudate cells) and incubated with TruStain FcX Fc receptor inhibitor (1:100, 15 min., RT) then labelled with antibodies for CD11b (PeCy7), Ly6C (APC), and Ly6G (PE) for MDSC subtype identification (1:200, 20 min., 4o C). Immediately after staining, cells were taken to the Purdue Flow Cytometry and Cell Separation Facility and analyzed using an Aria III cell sorter (BD, San Jose, CA).

3.3.6 Inclusion Criterion for Samples in the Final Flow Cytometry File Dataset:

Each data file was inspected using FlowJo v.10 software (FlowJo, LLC: Ashland, OR). Only samples that met three characteristics were included; a minimum of 10,000 events were recorded, the dataset included FSC-A, SSC-A, FSC-H, FSC-W, SSCH, SSC-W, CD11b, Ly6C and Ly6G data, and a common fluorescent compensation protocol was used. Only samples where a comparison of intensity for each fluorochrome pair (APC, PE, and PE-Cy7) showed complete separation of the fluorescent signals were included in the final dataset. The quality of each sample was examined with traditional biplots used for isolating mouse MDSC: i.e. (a) FSC-A x SSC-A, (b) CD11b-PE-Cy7 x SSC-A, (c) Ly6G-PE x Ly6C-APC [1]. Only samples where positive and negative populations of cells were clearly defined on a specific axis were included in the final dataset (i.e. FSC and SSC (neg: <30, pos. >40 on linear scale) and each fluorochrome (neg: $0\text{-}10^2$, pos. $10^3\text{-}10^4$ on log scale)). After the full review of the available flow cytometry files, we had data for 10 NVBM, 13 TBSP, and 20 IPTM (Table 1) in our final data set.

3.3.7 Data Transformation

Data transformation is a necessary step for automated, high-throughput FC data analysis, visualization, and the target cell selection. This is required due to the variance of the fluorescence signals is dependent on the signal mean, based on the stochastic nature of photoemission and the photodetection processes. Several transformation methods have been proposed in the literature for variance stabilization including biexponential (logicle), hyperbolic arcsine, and Box-Cox transformations [7-11]. Generally, the fluorescence channels are transformed using these approaches, while forward and side scatter channels remain linear and not transformed at all. Therefore, in our study we used biexponential/logicle transformation for the fluorescence channels [11] from the R library flowCore and estimated the parameters of the transformation using the *estimateLogicle* function. Figure 3.2 shows density plots of three fluorescence signals Ly6G, Ly6C, and CD11b after they are transformed. Different expression levels in each of these cellular markers are clearly visible after the transformation.

3.3.8 Automated Gating and Clustering

3.3.8.1 Automated Gating.

We employ an automated gating algorithm that works on one channel at a time (one column in the data matrix of each sample). Considering *z* to be a channel subject to gating, our general automated approach uses the following steps:

(1) The algorithm estimates the density of *z* by a kernel density estimation method (we used the density function with Gaussian kernel in the stats package in R).

(2) The peaks in the density of *z* are identified as regions of high local density and significant curvature (also called landmarks in [12]). We identify high-density regions in *z* by the "curv1Filter" function of the flowCore package [13] in Bioconductor.

(3) The boundaries of density peaks are identified by detecting minima between two adjacent density peaks. Here, a density peak represents a 1-D cluster of cells.

Further details of our automated approach and design are described in the results section.

3.3.8.2 Automated Clustering.

After we gate cells using a phenotypic feature (ie. SSC-A, FSC-A), that feature is not used in subsequent analysis. Hence, after a series of gating steps, we reduce the dimensionality of the sample (from 9-D to 3-D in this experiment). We cluster each of these gated samples to identify subtypes of cells, using the k-means clustering algorithm in R. To identify the optimum number of K-mean clusters (k-opt) , we used the S_Dbw index [14]. To accomplish this, we run k-means for different values of k, compute the S_Dbw index for each k, and select k-opt where S_Dbw index is the minimum. In our experience [8], the S_Dbw index works better with high-dimensional samples than specialized implementations of k-means, such as flowMeans [15]. However, the S_Dbw index tends to pick a higher number of clusters when used with preselected MDSCs. Therefore, we used a combination of three cluster validation criteria: Average Silhouette Width, Calinski-Harabasz index and Dunn index to validate our choice of k-opt.

In our study, we assume that cells in a cluster are normally distributed, and hence can be statistically summarized by its high dimensional mean and the covariance matrix. More specifically, a *p*-dimensional cluster from the k-means algorithm is represented by two distribution parameters μ, the *p*-dimensional mean vector, and Σ, the $p \times p$ covariance matrix.

3.3.9 Registering Cell Clusters Across Samples

After clustering each FC sample independently, we match phenotypically similar clusters across samples, a process commonly known as cluster registration. Figure 3.3 shows a model of the cluster registration process, where we illustrate that cell clusters may split, merge or remain absent in a sample due to biological or experimental perturbations, or due to artificial errors in clustering.

To match cell clusters, we used a robust variant of a graph matching algorithm called the Mixed Edge Cover (MEC) algorithm that allows a cluster in one sample to be matched with zero, one, or more clusters in the second sample [16]. The algorithm to compute an optimal MEC was developed in our prior work [16]. Here, we briefly summarize this method for completeness.

The MEC algorithm initially creates a bipartite graph from a pair of samples, where vertices in each part represent clusters from a sample. A pair of vertices (clusters) is connected by an edge whose weight is computed by the Mahalanobis distance[2] between a normally distributed pair of clusters. Next, modified minimum weight bipartite matching is computed on the bipartite graph (This concept can be seen in Figure 3.3). The resultant solution matches clusters across samples, while possibly leaving a small number of clusters unmatched (the X in Figure 3.3). For each unmatched cluster, we assign a penalty λ, which ensures that the number of such clusters remains small. The cost of a MEC is the sum of weights of all matched edges and the penalties due to the unmatched clusters. An optimal MEC can be computed by $O(k^3 \log k)$ time where O is the set of outliers and k is the maximum number of clusters in a sample [16].

The matching of clusters returned by the MEC algorithm serves two purposes. First, it registers cell clusters between a pair of samples. This allows us to track changes in different cell types (including the absence of certain types of cells) across samples. Second, we can define the overall dissimilarity between a pair of samples as the sum of the dissimilarities of the matched clusters and the penalties of the unmatched clusters. The latter is used as a building block when templates are created from a class of samples and will be discussed in the next subsection.

[2] Mahalanobis distance is a multidimensional generalization of the number of standard deviations a distribution mean (D) is from a given point (P).

3.3.10 Algorithms to Create Templates

In our prior work, we designed an algorithm to create a template from a group of similar samples [8]. To create a template, we register cell clusters in samples from a class using the MEC algorithm and merge the matched clusters into meta-clusters. Here a meta-cluster is a group of phenotypically similar clusters and have smaller Mahalanobis distances among themselves relative to their distance to other clusters. In Figure 3.4, the matched clusters are joined by dashed lines and then merged to form meta-clusters in the rightmost subfigure. Similar to the statistical representation of a cluster, we model a meta-cluster by a Gaussian distribution along with its mean and covariance matrix. Finally, a collection of meta-clusters defines a template of a class of samples as shown in Figure 3.4. The same statistical representation of a sample and a template with a Gaussian mixture model enables seamless matching of clusters and meta-clusters via the MEC algorithm.

To simplify the registration of clusters across many samples, the algorithm iteratively merges the most similar pair of samples. The similarity is computed by the cost of the optimum mixed edge cover. The pair of samples considered in the current iteration is merged to create an intermediate template. The algorithm for creating templates organizes samples in a binary tree and merges a pair of samples and intermediate templates that are the most similar in the current round (Figure 3.5).

3.3.11 Template-driven classification and Immunophenotype discovery

Given a group of FC samples belonging to m classes, we build m templates, T_1, T_2, ..., T_m, one for each class. A new sample S is compared with each of the templates via the MEC algorithm and is predicted to belong to the class whose template it is most similar (least dissimilar). This approach is a template-driven nearest neighbor classification, which is more robust than sample-driven nearest neighbor classification. The template-based classification requires m MEC computations, one with each template, making it significantly faster than nearest-neighbor classification that requires a new sample to be compared with all existing samples.

3.3.11.1 Fully Automated Classification Pipeline

In our experiment, we set up a pipeline that automatically makes templates from labeled compensated FC samples and classifies unknown compensated FC samples to existing templates. The general steps in the pipeline are described in Figure 3.6.

The key to automation lies in automating the parameter optimization steps, since the parameters depend entirely on the data distribution of input FC samples. Two parameters that significantly influence the classification result have been found: the number of clusters (k) in k-means, and the unmatch penalty (λ) in the MEC algorithm. k defines how many distinct cell populations we believe are reasonable in a given sample, and λ generally establishes the dissimilarity boundary when determining whether two clusters should be matched or left unmatched in a pair of samples. We described our strategy to select k in the previous section. Two approaches are recommended for selection of λ. First, tune λ and find the knee point when the number of meta-clusters in the template drops dramatically as previously described [16]. Second, use a multiplier (>2) of the maximal standard deviation (estimated by the square root of the trace of the covariance matrix) of clusters in the samples. The idea behind this approach is to unmatch the clusters if they hardly intersect with each other in the multi-dimensional feature space. This means that when the distance between the two clusters is greater than the sum of their estimated radii, we consider them as two distinct cell populations.

3.3.11.2 Template Evaluation and Classification Score

Due to the small dataset (43 samples) in our experiment, separating the dataset into training and testing sets is not feasible. Instead, we use the Leave One Out Cross Validation (LOOCV) technique to evaluate the prediction accuracy of the templates. In turn, we make each sample the test sample, create templates using all other samples, and then classify the sample left out to the nearest template. This method was compared with the 1-nearest neighbor method for validation of our optimization.

When comparing template data across our three template sets (myeloid, and M- and G-MDSC subsets), we only consider meta-clusters which entirely reside within the subtype range of M- or G-MDSC. Meta-clusters that are only partially involved in the subtype range are separated into the ’others’ range. Because of the large number of meta-clusters identified in each template,

only significant meta-clusters are reported. A meta-cluster is significant if it exits in more than one sample or has > 1% of myeloid cells.

To evaluate the classification confidence of the samples, we define a classification score by the following formula: classification score = $1 - d_t / \min(d_a, d_b)$. Where d_t is the dissimilarity of the test sample and the true template of the tissue it belongs to, and d_a and d_b refer to the dissimilarity of the test sample and the other two templates, respectively. (Recall that since we are doing LOOCV, we know the true template for each sample). Hence a positive score indicates a correct prediction, while a negative score identifies a misclassification. The higher the classification score is, the more confidence we have in classifying the sample.

3.4 Results

3.4.1 Selecting Myeloid Cell Subsets via Automated Gating and Clustering.

In most FC studies, a subset of relevant cells is selected for investigation by choosing a range of values in 1-D histograms or 2-D scatter plots. This process of cell selection in cytometry is called gating. For example, to study MDSCs, the MDSC subtypes must be separated from other types of cells using a sequence of gates. Traditionally, gating is performed manually by visual inspection, which is not feasible for high-dimensional dataset analysis. Alternatively, cell populations can be identified by automated clustering algorithms [17]. Here we automate both the initial gating and the clustering steps. Compared with the manual processing, automated gating, and clustering via a reproducible procedure dependent on distributions of cellular markers reduces bias and lowers variability across samples in the final data set (data not shown).

3.4.1.1 Automated Gating

Traditional gating begins with selecting viable, single cells before moving onto specific cell markers. The first row of Figure 3.7 shows the initial gates in our gating strategy which are labelled as the non-debris gate, side scatter (SSC) gate and forward scatter (FSC) gate. These gates take different aspects of forward scatter and side scatter as input to select viable, single cells, and remove all debris, dead cells, and doublets. The non-debris labeled gate uses the areas of FSC (FSC-A) and SSC signals (SSC-A) to remove debris and dead cells. While the SSC and FSC gates are common gates to select single viable cells and remove cell doublets or clumps. Following this step, myeloid cell selection and MDSC subtype selections are done based on fluorochrome

intensity (Myeloid: $CD11b^{+}$; M-MDSC: $Ly6C^{hi}Ly6G^{low}$; G-MDSC: $Ly6C^{int}Ly6G^{hi}$). Fluorochrome intensity for both M-MDSC and G-MDSC subtype selection is based on the known location of these cell types within the Ly6C and Ly6G bi-plot [18].

Our primary interest is to investigate if there are physical differences between MDSC subtypes isolated from different tissues i.e. M-MDSC from spleen vs. those from tumor. To identify different myeloid cell subtypes, we apply a sequence of gates one after another (Figure 3.7). The basic concept for calculating the boundary of each gate is described in the Methods section 2.3 and was compared to manual gating patterns across samples to confirm that our method selected commonly accepted ranges for each gate. The following describes both the mathematical approach and the results for automating our gating strategy.

To automate our gating, we determined mathematical approaches to each gate found in traditional gating. To create the non-debris gate, we select the boundary of FSC-A from half the sum of first local minimum and the first local maximum of the density curve to the 99.5% of cells, and the boundary of SSC-A from 0 to 99.75% of cells. This resulted in an average selection of 98% of total events which was higher than the 86% seen in manual gating. The SSC gate, used to define single cells, is defined based on the corresponding height (SSC-H) and width (SSC-W) markers, with the boundary of SSC-H selected from 0 to the maximal value times a regularization term, SSC-W from the minimal value to 95% of cells. Similarly, the FSC gate takes FSC-H (height) and FSC-W (width) as input, with FSC-H ranges from the minimal value to the maximal value times a regularization term, and FSC-W from the minimal value to the 97.5% of cells. These resulted in an average selection of 91% (percent of total events) of single cells which was different than the 80% seen in manual gating. Myeloid cells were selected by taking the half the sum of the minimum and maximum peaks within CD11b density plot and using the same parameters for SSC as used in the non-debris gate. This resulted in an average selection of 57% myeloid cell (of total events collected) selection which was similar to the 50% seen in manual gating. While there was a 7% difference between our automated and manual gating strategies at the final myeloid gate, this difference was not significant ($p=0.3$, T-Test of 2*Arcsin(sqrt(%)) transformed data) (data not shown).

With the key marker expression pattern of M-MDSC and G-MDSC well established, we based our gates on the known location of each subtype. Therefore, we locate M-MDSCs ($Ly6C^{hi}$,

Ly6G^{low}) by the last density peak of Ly6C and the first density peak of Ly6G, and G-MDSCs (Ly6C^{int} , Ly6G^{hi}) by the penultimate peak of Ly6C and the last peak of Ly6G. Selected myeloid cells (from the myeloid cell gate), M-MDSCs and G-MDSCs are each represented by a *n* x *m* matrix, where *n* is the number of cells, and *m* is the number of markers. Three markers are chosen to define a unique cell event: CD11b, Ly6C, and Ly6G. The vertical lines in Figure 3.2 show how this automated grating strategy successfully identified density peaks in the transformed marker space. We used these gates to select cells of interest (refer to Figure 3.7 for details). Using these calculations, we found that our automated gating selects a slightly higher number of events in each gate but with lower variation overall (data not shown).

3.4.1.2 Automated Clustering

When we consider all myeloid cells, we cluster cells using the k-means algorithm after the myeloid gate, but before M-MDSC and G-MDSC gates (Figure 3.7). In contrast, to investigate M-MDSCs and G-MDSCs in isolation, we gate on Ly6C and Ly6G to identify these subtypes and then apply k-means clustering. In both cases, we cluster cells in three-dimensional marker space (CD11b, Ly6C and Ly6G). These three-dimensional clusters are used to create templates, forming the backbone of our classification scheme. For Myeloid cells the average k-means clusters for each tissue were 9-10. While the subtype averages were 2-2.5 clusters.

3.4.2 Naïve and Tumor Templates

After we gate and cluster every sample individually, we group all samples collected from a tissue and create a template for that tissue type. A small number of templates can concisely represent a large cohort of samples by emphasizing their common characteristics while hiding small inter-sample variations due to innate biological variability among individuals, or various form of noise from the FC instrumentation [19, 20]. Therefore, unknown samples can be classified by comparing them with the cleaner and fewer class templates rather than a large number of noisy samples themselves as depicted in Figure 3.4. Therefore, we created three templates for NVBM, TBSP, and IPTM. Furthermore, to investigate different cell types in each tissue, we create templates using three different sets of cells: all myeloid cells (myeloid templates), M-MDSCs only (M-MDSC templates), and G-MDSCs only (G-MDSC templates). Because our primary focus is on the subtypes, we used the myeloid template as a verification or test templates for our

optimization and automation. Hence, we come up with nine templates as shown in Figure 3.8, where we described templates with their meta-clusters. To compare the templates, we summarize the meta-clusters within each template from the following four aspects: a number of meta-clusters, fraction of cells in all myeloid cells, average expression level of marker Ly6G and Ly6C, and an average standard deviation of the meta-clusters (Table 2). The average expression level of markers measures the approximate position of meta-clusters in the 2-D template plot, and the average standard deviation estimates the spatial extent of each meta-cluster, based on the fact that each meta-cluster is represented by a normal distribution with a mean vector and a covariance matrix.

3.4.2.1 Myeloid Cells Templates

From the myeloid cells template, for the three tissues we observed similar meta-cluster distribution patterns that contain the M-MDSC range at top left, the G-MDSC range at the middle right, and "other cell" populations at left bottom (Figure 3.8c). However, this was not surprising as there are known differences in myeloid cell populations within each tissue. As such, significant differences exist across templates. As our primary interest is in MDSCs, we analyze the meta-clusters based on the two subtypes and the "other cell" areas. In general, NVBM and TBSP have fewer meta-clusters than IPTM, especially within the "other cell" area (6, 6 and 9 respectfully, as shown in Table 3.2). Numbers and patterns of meta-clusters within the M- and G-MDSC range appear similar across tissues, but a slight shift of meta-clusters can be observed: for example, meta-clusters in M-MDSC shift upward (Ly6C increases) from NVBM to IPTM, while TBSP tends to have slightly lower Ly6C and Ly6G expression in G-MDSC. Additionally, we observe an increase in the percentage of M-MDSCs from NVBM to TBSP (19.23% to 19.87%), then followed by a drop of M-MDSCs in IPTM (15.77%). This is in contrast to a steady decrease in percent of G-MDSC population from NVBM to TBSP to IPTM (49.27%, 25.41%, 17.24% respectively). These results confirm that our method was able to classify samples to the correct tissue based on the total myeloid cell populations.

3.4.2.2 M-MDSC Templates

By contrasting the M-MDSCs templates we can identify meta-clusters across tissues only in the M-MDSC range. To avoid losing information with few meta-clusters present in each template, all meta-clusters (including insignificant ones) are used Table 3.2. We observe that IPTM

is the most distinctive template with the highest number and the broadest distribution of meta-clusters (Figure 3.8a). This is confirmed in Table 3.2 where we observe a large increase in the number of meta-clusters in the IPTM M-MDSC template compared to NVBM and TBSP (increase of 7 MC). Additionally, the average expression level of both Ly6C and Ly6G increased from NVBM to IPTM (NVBM (1.25, 3.49), TBSP (1.35, 3.51), IPTM (1.41, 3.57); Ly6C, Ly6G), which indicates an up and rightward shift of meta-clusters in the template plots. Although there is not an all-tissues common meta-cluster identified, two significant meta-clusters in NVBM template are each found in TBSP and IPTM template, respectively, hence no significant unique meta-cluster is in the NVBM template. This is contrasted by the unique cluster found in TBSP and the multiple clusters found in IPTM. These results suggest that it is possible for precursor cells from the BM and SP to exist in tissue further along the MDSC differentiation path consistent with what is known about immune cell development.

3.4.2.3 G-MDSC Templates

The G-MDSC templates are built only using cells from G-MDSC gate. These templates reveal greater divergent patterns than seen in the M-MDSC templates (Figure 3.8b). First, the location of meta-clusters alters across tissues within the G-MDSC gated area: NVBM meta-clusters aggregate at the middle left, TBSP meta-clusters are centered at bottom right, while in IPTM, they are distributed along a diagonal line, which can be confirmed from the average expression level of markers (Table 3.2). Secondly, similar to the myeloid and M-MDSC templates, the number of meta-clusters increases in the IPTM template compared to NVBM and TBSP (7 compared to 3 and 4 respectfully). Also similar to the myeloid templates, the percentage of G-MDSCs decreases from NVBM to TBSP to IPTM (62.12%, 25.28%, and 19.14% respectfully). Finally, all meta-clusters are unique in the corresponding tissue, indicating that G-MDSC templates are more distinctive than M-MDSC templates.

3.4.3 Classifying Samples Using Templates

In order to quantify how well each template performed, we calculated a classification score of each sample when using different sets of templates (Figure 3.9). With each of the three template sets: myeloid cells templates, M-MDSC templates, and G-MDSC templates, we observe that all samples are correctly classified to the true tissue (100% prediction accuracy in all template sets).

The myeloid template performed the best for TBSP and IPTM with higher classification scores compared to M- and G-MDSC templates (Figure 3.9c). As we expected, myeloid templates generally have the characteristic distribution of both G-MDSCs and M-MDSCs, and also other myeloid cells. Surprisingly, the myeloid template did not perform nearly as high for NVBM. NVBM was best classified by the G-MDSC template, while classification scores for TBSP and IPTM were the lowest with this template (Figure 3.9b). M-MDSC templates show higher classification confidence when classifying TBSP and IPTM compared with G-MDSC templates. Observation of the distribution of meta-clusters show that TBSP samples are more distinct in G-MDSCs than in M-MDCSs. One possible reason could be the higher number of IPTM meta-clusters in M-MDSC templates, making it easier to distinguish IPTM from the TBSP. These results suggest that within each template we can be confident in the classification results and comment on the biology of myeloid and MDSC cells at each tissue.

3.4.4 Classifying Samples with Other Classification Approaches

We compare the template-based classification method to five other widely used non-template-based classification approaches using the R package Caret (https://CRAN.R-project.org/package=caret).

3.4.4.1 Features Construction

We cluster each sample by k-means and construct features from the clusters. For each cluster, we select the size (cell count), the mean vector, and the covariance matrix as features, yielding a total of $1 + m + m^2$ features for one cluster, where m is the number of markers. With k clusters, an FC sample is finally represented by a feature vector of length $k * (1 + m + m^2)$. To ensure that the feature vectors have consistent meanings across samples, we label clusters in the ascending order of their mean vector. Additionally, we fix the number of clusters for this comparison so that each sample has equal number of features without missing values. Using a majority voting rule, we set $k = 10$ in myeloid cells and $k = 2$ in M-MDSCs or G-MDSCs. With all samples having three markers, this yields 130 features for a myeloid cells sample and 26 features for an M-MDSCs or G-MDSCs sample.

3.4.4.2 Classification Results

The training function implemented in Caret automatically selects the best parameter set yielding the highest accuracy. We considered five classification methods: K-nearest neighbor, support vector machine (SVM) with radial kernel, SVM with linear kernel, naive Bayes, and a neural network. For a fair comparison with FlowMatch, we again use LOOCV to evaluate each classifier and classify samples using myeloid cells, G-MDSCs only and M-MDSCs only. For each classifier and each set of cells, we report prediction accuracy, precision, recall, specificity and the F-score in Table 3. SVM-Radial and Naive Bayes generally have the highest prediction accuracies and F-Scores but had at least one sample misclassified. The simple neural network performed the worst in all cases (~ 50% less in accuracy, precision, recall, and F-score). When classifying samples by non-template based methods, G-MDSCs appear more distinguishing compared to all myeloid cells and M-MDSCs.

3.5 Discussion

In this study, we show that the use of multidimensional FC analysis can correctly classify mixed populations of cells from MDSC samples by tissue/developmental stage and to lay the foundational work for future studies. Additionally, our method can provide important biological insights into MDSC phenotyping. Overall, we make the following contributions to cell phenotyping, MDSC biology, and machine learning:

(1) **Fully automated analysis:** We present a fully automated pipeline to classify FC samples and identify phenotypes of MDSCs in three different tissues in mice. This pipeline includes data transformation, automated gating and clustering, cluster matching and template formation, and finally, sample classification and phenotype discovery. To the best of our knowledge, this is the first fully automated analysis of MDSCs, paving the way toward better understanding of the maturation and differentiation of MDSCs in high-throughput settings.

(2) **Algorithmic novelty:** By creating and manipulating templates of different tissue types, we provide an interpretable machine learning approach to the classification of MDSCs where we identify phenotypes of cell populations in each tissue in terms of metaclusters. These meta-clusters are matched to identify changes in different cell types. The template-based classification is more accurate than popular machine learning approaches such as SVM and

simple neural networks and provides more in-depth details about the specific cell populations that help us to classify the samples.

(3) New insight into MDSC phenotyping: Our primary contribution is to provide new insights into MDSC phenotyping via automated algorithms. We classify samples in three different tissue types using M-MDSCs only, G-MDSCs only, or all myeloid cells, and identify the impact of cell types on tissue classification and phenotyping. We provide these insights using a new in-house dataset, which has not been presented before.

Our study presents a new perspective in classifying and phenotyping MDSCs by using templates of NVBM, TBSP and IPTM tissues. In addition to providing a simple, yet effective, classification scheme, templates offer a cleaner way to track prototypical changes in terms of their meta-clusters. For example, we can match meta-clusters between the NVBM and IPTM templates and precisely identify changes in different cell types. In all three sets of templates, we observe different patterns of amount and distribution of meta-clusters across tissues. For example, common meta-clusters appear in all tissue templates, but plenty of unique meta-clusters exist in only one tissue, which reveals the underlying biological differences between the tissues. IPTM templates tend to have more meta-clusters than the other two tissues, and NVBM templates are generally similar to TBSP templates except in the case of G-MDSC. Increasing number of unique meta-clusters along with decreasing amount of common meta-clusters indicate a higher level of distinction between samples, which suggest G-MDSC templates are more distinguishing than M-MDSC templates when classifying NVBM, while M-MDSC templates will perform better when classifying IPTM. Consistent with other research, it was found that in all cases, there are more MDSCs in NVBM samples compared to the IPTM samples. This is most likely due to NVBM enrichment of myeloid precursors cells that express the selected markers, and IPTM consists of a broader mix of immune cell types found in the tumor microenvironment.

Identifying biologically interpretable features behind a classification decision is a powerful aspect of our approach, which is missing from popular black-box machine learning approaches such as neural networks. Non-template-based classifiers perform well when classifying samples using G-MDSCs and all myeloid cells, but none of them achieves 100% prediction accuracy as attained by the template-based method. Furthermore, traditional classification methods only generate a prediction result, with no insight into the underlying biological. By contrast, our template-based classifier clearly shows the characteristic cell populations and their distribution,

uncovering the underlying biological differences between tissue MDSC samples. Consistent with our findings in Chapter 2, we show that physical characteristics on MDSC change as the cells progress toward their more mature phenotype found at the tumor. This concept challenges the current paradigm of the development of MDSC [21, 22].

Identifying specific cell phenotypes associated with disease conditions is of utmost importance for cancer research and drug discovery. For example, in our data, we show that physical differences can be seen between MDSC found at the tumor, spleen and bone marrow which may aid in the development of drugs designed to target MDSC at a specific tissue or developmental state. Our data suggest that cells from specific tissue sites may require different treatments that pursue different outcomes for each MDSC development stage. For example, targeting MDSC found at the spleen or bone marrow may involve developing drugs that alter the differentiation of MDSC away from the suppressive phenotype ie. toward DC, neutrophils or M1 macrophages. Meanwhile, targeting MDSC found at the tumor is limited to pursuing cell death, or inhibiting the T cell suppressive mechanisms of MDSC. This is not to say that there is no merit in targeting all MDSC but, that a better understanding of their developmental biology could bring about more precise targeting. Our group has currently conducted a transcriptome analysis to identify differentially expressed transcripts in these tissues [NCBI Gene Expression Omnibus (GSE116596)]. These data confirm the findings of the current study and provide new gene targets for separately targeting spleen and tumor isolated MDSC.

In this study, we show that multidimensional flow cytometry can distinguish the physical phenotypes of M-MDSC and G-MDSC cells from three tissues representing different stages of MDSC development. Together, these studies will enable us to provide a better model of MDSC maturation and differentiation in a tumor environment that will advance MDSC biology and treatment.

3.6 Tables and Figures

Table 3.1 List of Three Tissues with Number of Samples and Number of Features Measured

Tissue	Samples	Features
Naive Bone Marrow	10	9
Tumor Bearing Spleen	13	9
Intraperitoneal Tumor	20	9

Table 3.2 Comparison of Meta-Clusters in Three Sets of Templates

Tissue M-MDSC G-MDSC Myeloid cells templates	M-MDSC Template	G-MDSC Template	Myeloid Cells Templates		
			M-MDSC range	G-MDSC range	Others range
NVBM Fraction in Myeloid Cells	18.55%	62.12%	19.23%	49.27%	28.54%
Avg. Expression Level (Ly6G, Ly6C)	(1.25, 3.49)	(3.19, 2.56)	(1.48, 3.51)	(3.44, 2.57)	(2.01, 2.02)
Avg. Standard Deviation	0.16	0.11	0.15	0.07	0.29
Number of MC	3	4	3	3	6
TBSP Fraction in Myeloid Cells	19.14%	25.28%	19.87%	25.41%	54.36%
Avg. Expression Level (Ly6G, Ly6C)	(1.35, 3.51)	(3.31, 2.50)	(1.31, 3.52)	(3.27, 2.47)	(1.47, 1.51)
Avg. Standard Deviation	0.14	0.09	0.19	0.14	0.25
Number of MC	3	3	2	2	6
IPTM Fraction in Myeloid Cells	14.38%	19.14%	15.77%	17.24%	56.78%
Avg. Expression Level (Ly6G, Ly6C)	(1.41, 3.57)	(3.27, 2.56)	(1.40, 3.53)	(3.43, 2.66)	(1.96, 1.65)
Avg. Standard Deviation	0.16	0.09	0.24	0.15	0.18
Number of MC	10	7	3	2	9

In G- and M-MDSC templates, all metaclusters, including insignificant meta-clusters, are counted in the table. Fraction in Myeloid Cells indicates the percentage of M-MDSCs or G-MDSCs in the myeloid cells selected by Myeloid cells gate. For myeloid cells templates, we show meta-clusters identified as G-MDSC, M-MDSC, and others. We only consider meta-clusters entirely reside within the approximate subtype range as G- or M-MDSC, the rests go to the others range. Due to large number of meta-clusters within the myeloid cells templates, only significant meta-clusters are counted in the table. The average expression level of markers and the average standard deviation of meta-clusters in each template are also shown.

Table 3.3 Five Statistical Measures for the Performance of Classifiers

(a) Classification using M-MDSCs

Classifier	Accuracy	Precision	Recall	Specificity	F-score
KNN (k=5)	0.744	0.751	0.782	0.874	0.752
SVM-Radial (C=0.5, Sigma=0.031**)**	0.837	0.831	0.831	0.914	0.828
SVM-Linear (cost=0.5, Loss=L1)	0.488	0.473	0.478	0.737	0.472
Naive Bayes (laplace=0, usekernel=True, adjust=1)	0.791	0.809	0.764	0.882	0.779
Neural Network (size=5, decay=0.1)	0.442	0.394	0.418	0.716	0.397
FlowMatch	1	1	1	1	1

(b) Classification using G-MDSCs

Classifier	Accuracy	Precision	Recall	Specificity	F-score
KNN (k=5)	0.93	0.916	0.908	0.952	0.909
SVM-Radial (C=1, Sigma=0.032)	0.922	0.97	0.941	0.971	0.953
SVM-Linear (cost=0.25, Loss=L2)	0.837	0.849	0.874	0.92	0.851
Naive Bayes (laplace=0, usekernel=True, adjust=1)	0.93	0.957	0.923	0.957	0.933
Neural Network (size=5, decay=0.1)	0.814	0.841	0.814	0.894	0.826
FlowMatch	1	1	1	1	1

(c) Classification using all myeloid cells

Classifier	Accuracy	Precision	Recall	Specificity	F-score
KNN (k=9)	0.721	0.787	0.8	0.87	0.728
SVM-Radial (C=0.25, Sigma=0.00392)	0.93	0.935	0.924	0.961	0.929
SVM-Linear (cost=0.25, Loss=L2)	0.837	0.84	0.856	0.913	0.847
Naive Bayes (laplace=0, usekernel=True, adjust=1)	0.977	0.861	0.831	0.986	0.978
Neural Network (size=5, decay=0.1)	0.791	0.814	0.765	0.881	0.781
FlowMatch	1	1	1	1	1

Table 3.3 shows methods with kernels or regularization terms specified, using all myeloid cells, only M-MDSCs, or only G-MDSCs. Each classifier was trained using LOOCV and the final models were selected based on the highest accuracy. All statistical measures except Accuracy are average based due to multi-class classification.

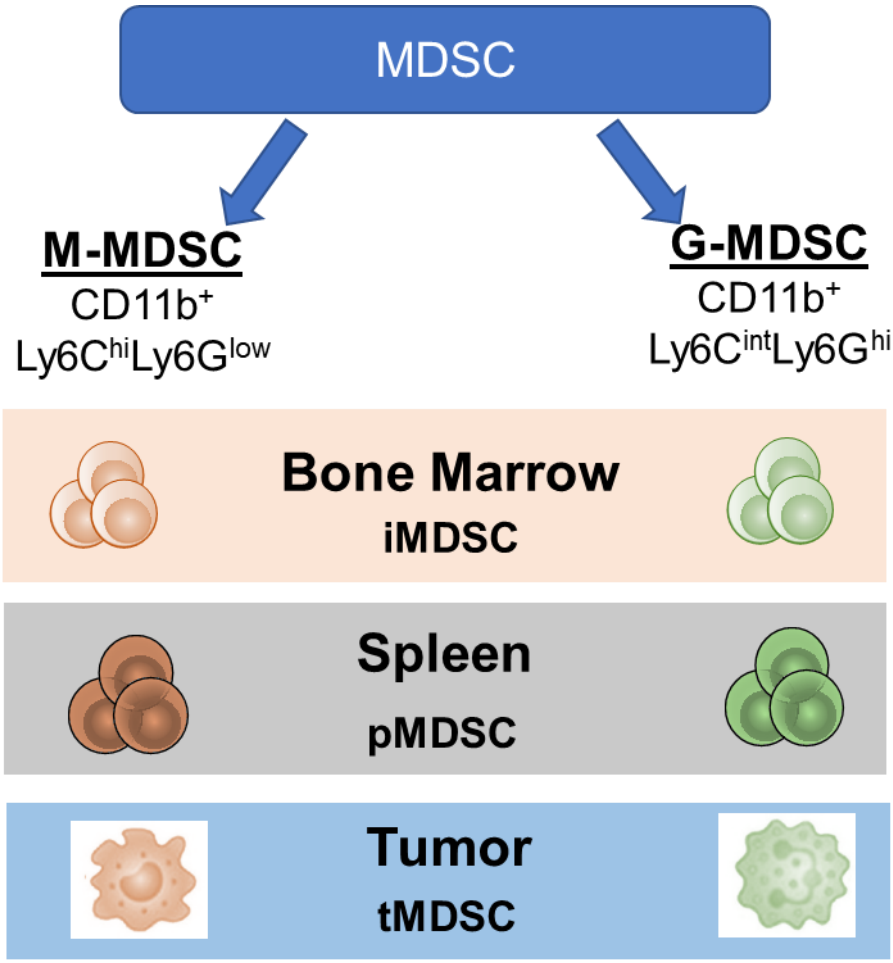

Figure 3.1 Model Showing Six Different Types of MDSCs from Tumor Bearing Mice

Additionally, naïve bone marrow and spleen cells would be considered iMDSC as they represent immature cells with the potential to gain suppressive function *in-vitro*.

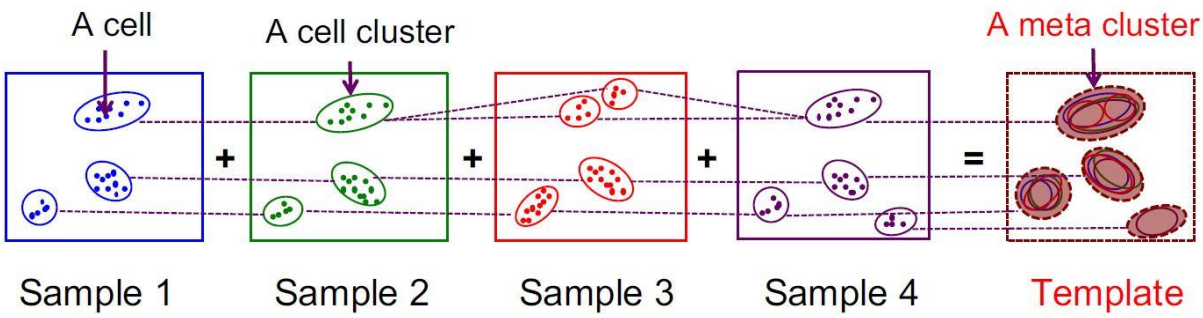

Figure 3.2 Creating a Template from Four FC Samples.

Each cluster is denoted by an ellipse. The union of registered clusters forms a meta-cluster, and a collection of meta-clusters defines a template.

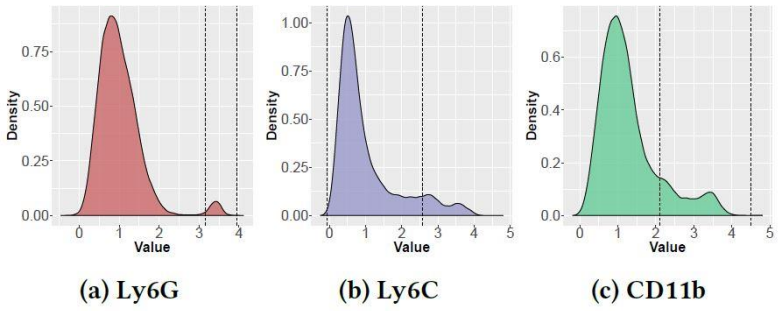

Figure 3.3 Density Plots of Ly6G, Ly6C and CD11b After They are Transformed by Biexponential/Logicle Transformation.

Before the transformation, values are concentrated around zero. Vertical lines separate cells based on their marker expression levels. A pair of vertical line serves as a gate and is identified by the automated gating approach described in Section 2.3.

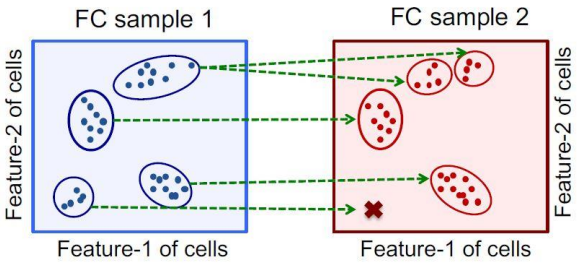

Figure 3.4 Registering Cell Clusters Across a Pair of FC Samples to Find “Similar" Types of Cells.

Here, dots and ellipses represent cells and clusters, respectively. The arrows show the matching clusters from sample 1 to sample 2. The registration algorithm should be robust enough to handle split, merge and missing clusters as shown in the figure.

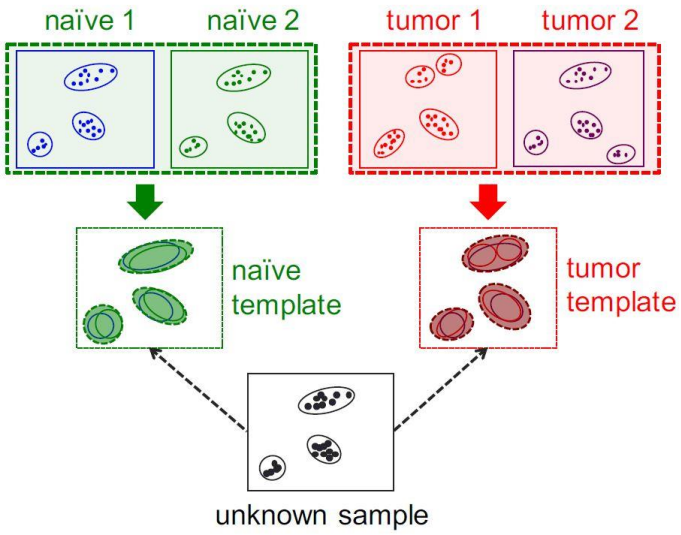

Figure 3.5 A Schematic View of a Template-Based Classification

Here a template is created from each class of samples where corresponding clusters from different samples are collapsed into a meta-cluster (shown in the middle row). Our task is to classify a new sample (shown in the bottom row) based on the naive and tumor templates. In this scheme, the new sample is classified to the class of its most "similar" template.

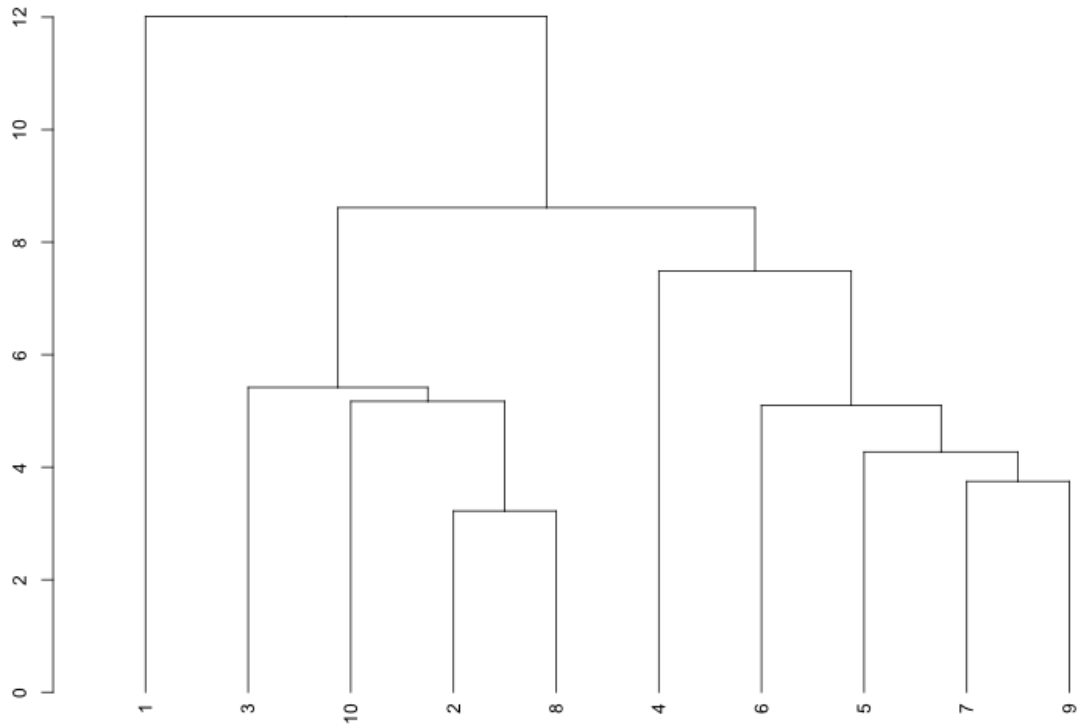

Figure 3.6 Example of Hierarchal Tree Classification.

Naïve bone marrow (NVBM) sample files are clustered by FlowMatch based on sample similarity. The resulting hierarchal tree shows all 10 samples (x-axis) grouped into 2 basic sets of samples with high similarity and one sample that was less similar. Samples 2-10 were all generated by a single user that was different from sample 1.

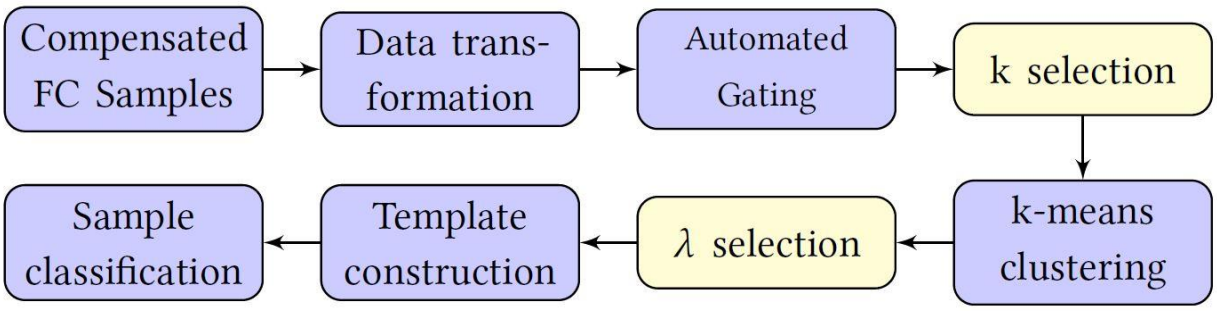

Figure 3.7 Pipeline of Automated Template-Based Classification

Purple blocks denote core steps required in the pipeline, and yellow blocks indicate parameter optimization steps.

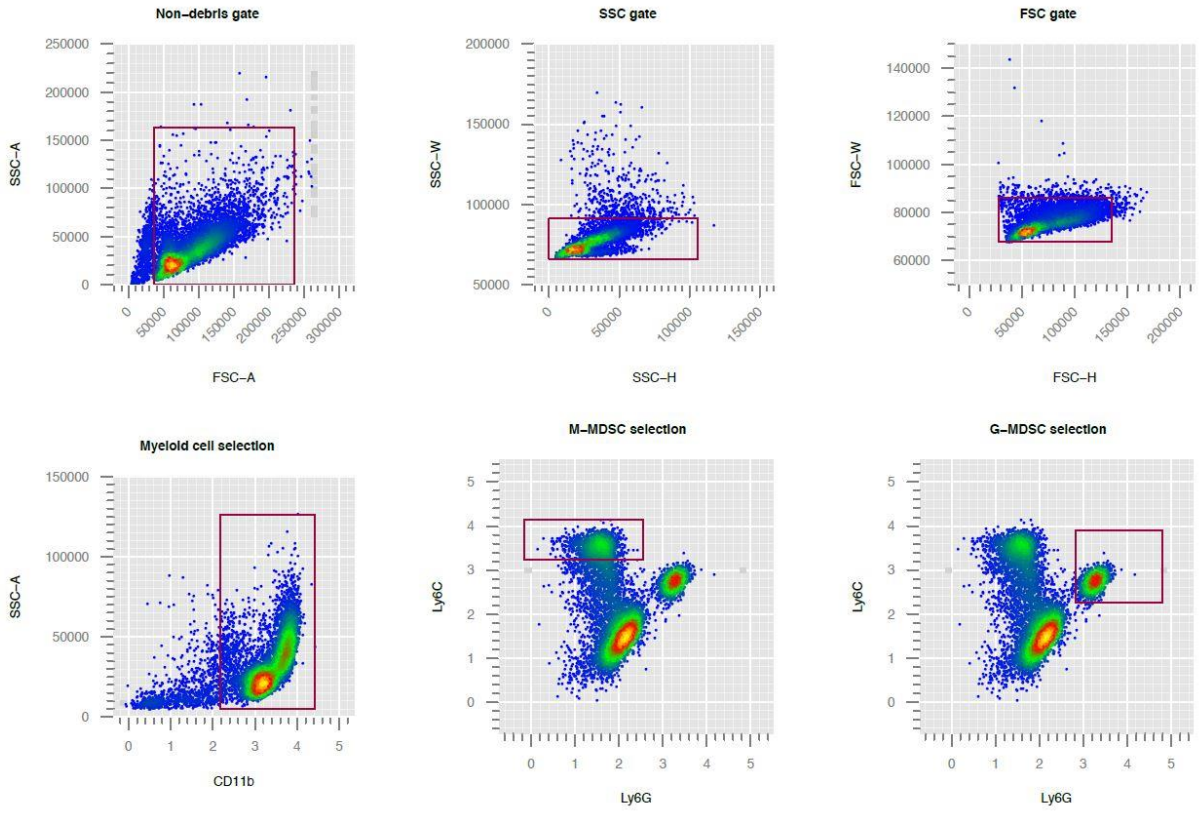

Figure 3.8 The Sequence of Gates Applied to FC Data to Select MDSC Subtype Myeloid Cells

The red rectangle in each plot shows the boundaries for the gates, which are obtained from the density distribution or the spread of data. Non-debris gate, side scatter (SSC) gate, forward scatter (FSC) gate and myeloid cell gate, are applied in sequence to select myeloid cells, followed by individual gating of M- and G-MDSC subtypes.

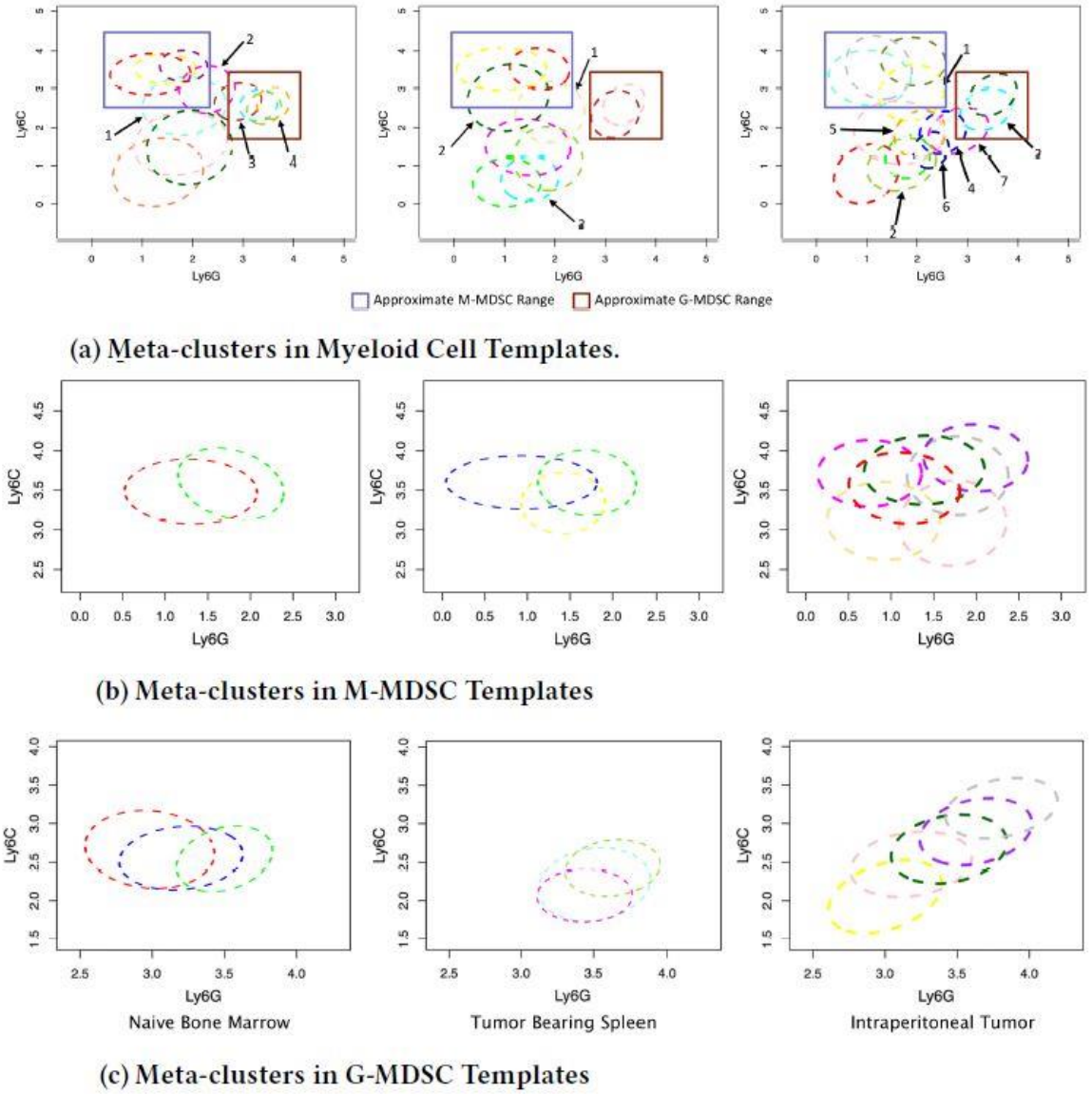

Figure 3.9 Templates for NVBM, TBSP, and IPTM Illustrated by the Meta-Clusters Present in Each Tissue.

Each meta-cluster is represented by a contour curve that includes 95% of the cells within the meta-cluster. Templates are built with a) G-MDSCs only, b) M-MDSCs only, and c) all myeloid cells. These are 2-D plots from a collection of 3-D bivariate contour plots. a) and b). Distinct meta-clusters are represented by different colors, with matched meta-clusters represented by the same color across templates. Meta-clusters that are present in only one sample are omitted for simplicity. c). Purple and red rectangles indicate the approximate areas of M-MDSC and G-MDSC, respectively. Unique meta-clusters in each template are indicated by arrows with indices specified.

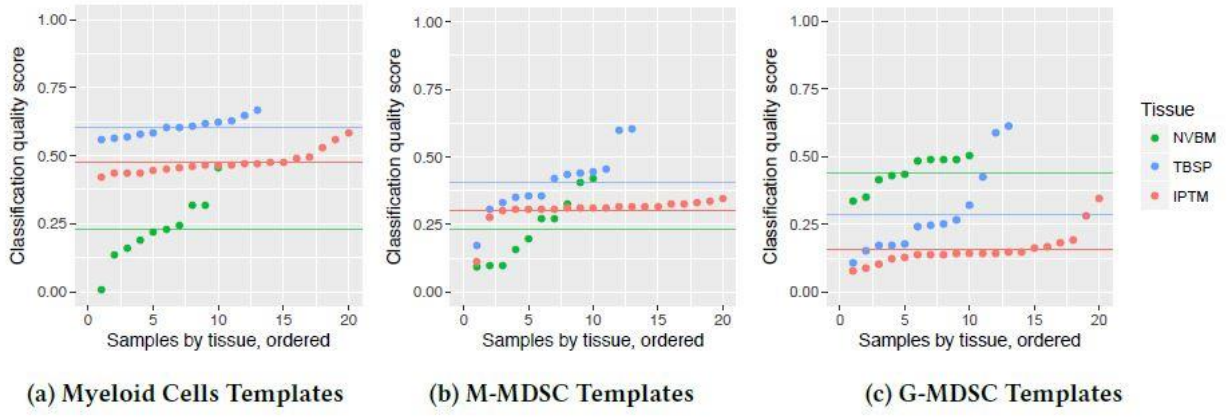

Figure 3.10 The Classification Score of Samples Classified by Nearest Templates Using LOOCV Using All Myeloid Cells, M-MDSCs only, or G-MDSCs only.

Correct prediction corresponds to classification score greater than zero. Horizontal lines indicate the average classification score of each template, respectively. Higher score indicates higher confidence in the prediction. Overall, M-MDSC templates have higher prediction confidence with TBSP and IPTM, while G-MDSC templates have higher prediction confidence for NVBM. Compared with single MDSC templates, myeloid cells templates have better classification performance when predicting IPTM and TBSP, but do not improve the prediction of NVBM.

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CHAPTER 4. NUTRITIONAL MODULATIONS OF MDSC IN DISEASE AND FUTURE DIRECTIONS

4.1 Abstract

T-cells are present in the immune system to fight against invaders. Once their job is done, suppressing their activity controls the immune response. Myeloid derived suppressor cells (MDSCs) are immune cells that suppress T-cell activity. In cancer, suppressing T-cell activity allows tumors to grow. Therefore, MDSCs have been highlighted as a target to inhibit so T-cells can do their job fighting cancer. In pregnancy, suppressing T-cell activity allows the fetus to grow. Therefore. MDSCs have been highlighted as important for promoting a normal pregnancy to progress. Dietary bioactive components may be effective modulators of MDSCs and therefore may ultimately play a role in cancer and pregnancy. Here we discuss studies that investigate nutritional modulators of MDSCs in the context of cancer and pre-eclampsia. Specifically, the polyphenol like phytochemical, curcumin will be reviewed and highlighted for future study. We propose two different studies to investigate the potential of curcumin as either an inhibitor and/or promotor of MDSCs in a disease specific context. Currently, investigation of curcumin as a modifier of MDSCs in cancer and pre-eclampsia are both needed. Together the role of phytochemicals as immunomodulators of MDSCs is still very young, in part due to the complexity of phytochemicals themselves, but the studies cited here provide evidence that the field is ripe for additional questions to be asked.

4.2 Introduction

There exists an interesting dichotomy within the role of dietary agents (vitamins and phytochemicals) and the immune system, one of supporting the inflammatory response reducing infection, and a second which supports the suppression of the inflammatory response. Many reviews have covered the connection between dietary agents and their ability to aid in the treatment of several pathophysiologic conditions such as cancer, autoimmune disease [1], graft versus host disease [2], and pregnancy-related conditions [3-6]. Specific types of cancer studied include: lung [7], prostate [8], breast [9] and colon cancer [10]. In the past, the focus of nutrition and cancer research has concentrated on the effect of the dietary agent on the tumor cells. However, nutrients

and dietary bioactive agents also have a significant role in the function of the immune system and the response to infection [11, 12]. As a result, there is great potential for nutrition and dietary agents to be modulators of cancer immunology, including myeloid derived suppressor cells (MDSCs). However, very little has been done to show the relationship between dietary agents and their potential to support MDSCs in other pathophysiological conditions. Therefore, it is useful to also investigate the role of dietary agents on the immune system, specifically MDSCs, in other pathophysiologic conditions.

Phytochemicals such as polyphenols have been shown to be immunomodulators of MDSCs. The family of molecules called polyphenols is very large and includes the three isoforms of curcumin, called curcuminoids. Curcumin, in general, is one of the most studied polyphenols in relation to the immune system and multiple pathophysiologic conditions. Therefore, curcumin will be used as an example of the potential that polyphenols and phytochemicals, may have in the effort to modulate MDSCs in two conditions that are similar yet represent opposing roles for MDSC, cancer, and pre-eclampsia.

4.3 Phytochemicals and Bioactives as MDSC Modulators in Cancer

4.3.1 Effects of Essential Nutrients on MDSC Function

A number of traditional, required nutrients have been demonstrated to influence immune function. However, only a few of the nutrients have been studied for their role in MDSC biology. The following section will review the evidence for three essential nutrients, vitamins D, A, and E, in regulation of MDSC biology.

4.3.1.1 Vitamin E:

Vitamin E is a fat-soluble vitamin with eight different isomers all possessing antioxidant properties [13]. The many isoforms of vitamin E have been studied for their ability to block the development of cancer through their direct actions on tumor cells [13]. Recently Kang et al. [14] examined the role of vitamin E on total SP MDSCs from mice injected subcutaneously with TC-1 tumor cells (a mouse B cell myeloma cell line). *In vitro* treatment with 10 μM D-α-tocopherol succinate reduced the T-cell suppressive ability of total SP MDSCs (72 hr co-culture with CD3/CD28 activated CD8 T-cells) and significantly reduced NO production in total SP MDSCs

(18 hr culture). This suggests that vitamin E reduces SP MDSC suppressive function by either inhibiting SP MDSC NO production or by acting as an antioxidant against NO. In addition, Kang et al. found that vitamin E mice treatment (+/- 2 mg/kg i.p. injection of D-α-tocopherol succinate/kg/mouse every two days for 3 treatments) reduced the number of tumor (TU) MDSCs and increased the number of CD8+ T-cells in the tumor within 3 days. Finally, using an adoptive transfer model, they found that vitamin E treatment improved the ability of tumor-specific CD8+ T-cells to reduce TC-1 cell tumor growth. This suggests that vitamin E's actions against tumor growth are due in part to reducing the accumulation and function of SP MDSCs and TU MDSCs through a NO-dependent mechanism.

4.3.1.2 Vitamin A:

Vitamin A is an essential vitamin for the body and is metabolized into the active form all-trans retinoic acid (ATRA) prior to use by cells. This form has already seen some success as an adjunct therapy for acute promyelocytic leukemia. ATRA is arguably the most studied nutrient as an immune modulator, especially for MDSC. Draghiciu et al. have reviewed the mechanistic aspects of ATRA's effect on MDSCs as well as some of the studies confirming its use in human cancers as an adjunct [15]. More recent studies have reported similar results for the use of ATRA as an adjunct for cancer therapies [16-19]. Therefore, continued study of the anti-MDSC effects by ATRA should be conducted to promote further clinical trials.

4.3.1.3 Vitamin D

Vitamin D is a conditionally required nutrient that can be produced in our skin in response to UVB radiation. The active form of vitamin D, 1, 25 dihydroxyvitamin D_3 ($1,25(OH)_2D$), is a steroid hormone that activates gene transcription through a nuclear receptor, the Vitamin D Receptor (VDR). While the best-known function of vitamin D is the regulation of Ca metabolism, multiple studies have shown that vitamin D has a role in immune function [20].

Several studies have examined the impact of vitamin D on $CD34^+$ hematopoietic progenitor cells [21-24]. Although CD34 is not a specific marker of MDSCs, these studies have been used as evidence that vitamin D modulates MDSC function [25]. CD34 is now considered a marker of stem cells but, was used as a marker of the "natural suppressor cell" identified as causing T-cell suppression in the tumor microenvironment [26]. However, Wu et al. showed a relationship

between $CD34^+$ cells and MDSCs [27], by demonstrating that $CD34^+$ cells cultured with GM-CSF and IL-6 develop the MDSC phenotype. Therefore, the use of these studies is justified as setting the foundation of vitamin D as a modulator of MDSCs. For example, cultured $CD34^+$ cells from tumor-bearing mice had reduced ability to differentiate into dendritic cells, while $1,25(OH)_2D$ treatment (10 nM, 2 wks) restored dendritic cell differentiation and improved antigen presentation [21]. Similarly, the spleen (SP) and lymph nodes of LLC-LN7-tumor-bearing treated with $1,25(OH)_2D$ (5 μmoles/kg via intraperitoneal injection 3x/week, for 2 wks) had fewer of $CD34^+$ cells and more dendritic cells [24]. These two studies suggest that vitamin D acts as an immunomodulator by increasing the differentiation of $CD34^+$ natural suppressor cells toward a dendritic cell lineage. In addition, giving LLC-LN7-tumor-bearing mice large oral doses of vitamin D_3 (240X normal dietary levels,10 d) reduced the percentage of $CD34^+$ cells and increased $CD8^+$ cells in tumors – indicating a shift to improved T-cell surveillance [22]. Additionally, a pilot study of 6 head and neck cancer patients showed that 6 wks of treatment with the vitamin D pre-hormone, 25 hydroxyvitamin D (25OHD) reduced the circulating number of $CD34^+$ cells [23]. Combined, these data suggest that vitamin D may act as a modulator of MDSC differentiation.

Recently, Chen et al [28] examined the connection between $1,25(OH)_2D$ treatment and IL-6 production by tumor cells in 4-nitroquinoline 1-oxide-induced esophageal squamous cell carcinoma. In mice treated with $1,25(OH)_2D$ for 4 weeks (0.5 μg/kg per mouse) the total number of SP MDSCs was significantly reduced. Similarly, high levels of $1,25(OH)_2D$ (1 or 10 μmol/L) reduced IL-6 stimulated STAT3 signaling and ROS production in cultured SP MDSC. Finally, $1,25(OH)_2D$ treatment reduced the IL-6 induced T-cell suppressive function of SP MDSC (72 hr co-culture with CD3/CD28 activated $CD8^+$ T-cells). These data suggest that $1,25(OH)_2D$ can inhibit SP MDSC function in part by inhibiting IL-6 activation via STAT-3 signaling. Overall, high dose vitamin D or $1,25(OH)_2D$ treatment holds promise as an inhibitor of SP MDSC function and development. However, further studies are needed to test additional questions related to the role of nutritional vitamin D status on TU MDSC development and function as well as to assess whether vitamin D status or $1,25(OH)_2D$ targets one or both of the MDSC subtypes.

Together these data suggest a potential role for vitamins in immunotherapies. While vitamin E has been the least studied, all three vitamins have been shown to modulate MDSCs in the context of cancer. Therefore, additional studies are warranted. It is unclear if these vitamins act on a specific MDSC subtype and where in the development of MDSCs they act. The data

suggests that vitamins may play a critical role in reducing the development of the T-cell suppressive phenotype which may be due to actions at the TU or could be during the development of MDSCs.

4.3.2 Effects of Dietary Phytochemicals on MDSC Biology in Cancer

Phytochemicals are chemicals produced in plants that are not essential nutrients, but which can have bioactive effects in humans and other mammals. A large body of research suggests that many different phytochemicals have anti-cancer properties [29]. However, only a few studies have examined the effect of specific phytochemicals on MDSC biology. Several studies that have examined the impact of phytochemical mixtures or specific, individual phytochemicals on MDSC biology will be reviewed here.

4.3.2.1 Korean red ginseng:

Korean red ginseng is a processed and purified extract of Panax ginseng that has been proposed to prevent tumor growth through specific metabolites called ginsenosides which are comprised of steroid glycosides and triterpene saponin phytochemicals [30]. To explore the role of Korean red ginseng on MDSC, Jeon et al. [31] injected mice intraperitoneally (ip) with Korean red ginseng (100 mg/kg/mouse, 1x daily) for two weeks prior to receiving a flank subcutaneous (sc) injection of EL-4 thymoma cells. After four weeks of daily treatments and tumor growth, SP MDSC were isolated. Although Korean red ginseng did not reduce total or subtype SP MDSC accumulation, total SP MDSC (CD11b+GR1+) from Korean red ginseng treated mice had a decreased ability to suppress proliferation of anti-CD3/28 activated CD4+ T-cells (4-day co-culture, proliferation assessed by formazan formation). This change in function was associated with a reduction in NOS2 mRNA expression and NO production by total SP MDSC from Korean red ginseng treated mice that were stimulated in culture with LPS for 24 h. Together these data suggest that Korean red ginseng reduces total SP MDSC T-cell suppressive function by inhibiting NO production. While this gives insight into how Korean red ginseng is acting on SP MDSC, no studies were conducted on TU MDSC or how Korean red ginseng effected the function of each subtype.

4.3.2.2 Black-Jack:

Black-Jack (*Bindens pilosa*) is considered a medicinal herb in Chinese medicine and has shown anti-inflammatory and immunomodulatory properties, attributed to the polyacetylenic glycoside content [32, 33]. To study the effect of *Bindens pilosa* on MDSCs, Wei et al [33] injected 4T1 breast cancer cells into the mammary fat pad of mice followed by oral gavage of *Bindens pilosa* extract (100 mg/kg BW/day). By day 42 post-implantation, treatment with *Bindens pilosa* extract significantly reduced the number of SP G-MDSCs. To better understand what phytochemicals were responsible for the immunomodulatory response they further fractionated the extract using high-performance liquid chromatography and found fraction 1 contained the most active compounds. They used this fraction to investigate the role of *Bindens pilosa* extract on the ability of MDSCs to promote tumor metastasis and recurrence. At 21 days post-implantation the tumors were carefully removed, and mice were injected with *Bindens pilosa* extract fraction 1. *Bindens pilosa* extract fraction 1 treated mice had significantly reduced tumor metastasis and recurrence. To further examine this, they used a co-injection model where SP G-MDSCs were isolated and injected with tumor cells +/- *Bindens pilosa* extract fraction 1. SP G-MDSCs were able to increase tumor growth and metastasis compared to control (4T1 only) while *Bindens pilosa* extract fraction 1 significantly reduced metastasis compared to control and SP G-MDSC groups. Together this suggests that *Bindens pilosa* extract fraction 1 inhibits the ability of SP G-MDSCs to promote metastasis but, it is unclear the mechanism by which this occurs.

4.3.2.3 Ashwagandha:

Extract from the *Withania somnifera* (Ashwagandha) contains the compound Withaferin A, a steroidal lactone triterpenoid, which has been shown to inhibit multiple pathways in tumor cells [34]. Sinha and Ostrand-Rosenberg fed tumor challenged mice (4T1 breast cancer, s.c. into mammary pad) Withaferin A three days a week post palpable tumor growth (1,2,4,8 mg/kg BW) [35]. In all four doses, Withaferin A significantly reduced the number of G-tMDSC at day 11 and 24 of tumor growth but did not affect M-tMDSC. Further, total blood MDSCs were less suppressive from mice fed with Withaferin A (1,2,4 mg/kg BW). To understand how Withaferin A was affecting MDSCs, total blood MDSCs were isolated (38 days post challenge) and cultured +/- Withaferin A (1ug/ml). When total blood MDSCs were cultured in the presence of Withaferin A (1 ug/ml) and stimulating agents (LPS and IFN-γ), Withaferin A significantly reduced IL-10

production. Finally, total blood MDSCs were cultured with PMA and Withaferin A (1 ug/ml) resulting in significantly reduced ROS production via inhibition of the STAT3 pathway. Together this data suggests Withaferin A inhibits blood MDSCs function inhibiting STAT3 signaling resulting in loss of migration to and function at the tumor site.

4.3.2.4 Red Seaweeds:

Red algae (*Porphyra dentata*) is plant used in Asian folk medicine and crude extracts contain several phytochemicals such as catechol, rutin, and hesperidin which have been identified and have anti-tumor activity [36, 37]. Okai et al. injected tumor-bearing mice (4T1, injected into mammary fat pad) ip with the sterol fraction of *Porphyra dentate* (5, 10, or 25 mg/kg/day). They found that treatment (all doses) did not change the percentage of total SP MDSCs. However, ROS production, both *in vitro* (5 and 10 mg/kg/day) and *in vitro* (5 and 10 ug/ml), was reduced by treatment with the sterol fraction. Finally, ARG activity was measured via urea content from total SP MDSCs of tumor-bearing mice and found that treatment significantly reduced Urea concentration. Together these data suggest that the sterol fraction of *Porphyra dentate* contains phytochemicals that reduce ROS production via reduction of ARG activity.

4.3.2.5 Corosolic acid:

Corosolic acid has been identified as a pentacyclic triterpene acid phytochemical from apple pumis capable of inhibiting NF-κB and Stat3 signaling in macrophages [38]. Based on these findings Horland et al. used osteosarcoma challenged mice (LM85 injected s.c.) treated orally with Corosolic acid (17.5 mg/kg, 10 days prior to tumor challenge and days 3,7,10,14,17, and 20)[39]. The number of BM MDSC and SP MDSC did not change with treatment however, SP MDSCs had significantly reduced T-cell suppressive function (1:4 T-cell:MDSC, 5d culture). Additionally, SP MDSCs had significantly reduced COX2, and CCL2 mRNA expression and pSTAT3 protein. This data suggests that Corosolic acid reduces pSTAT3 resulting in reduced mRNA expression of COX2 and CCL2 and, reduced T-cells suppression function.

Black Raspberry: Studies on human cancer cell lines suggest that phytochemicals from black raspberries (BRB), rich in flavanol polyphenols, possess anti-cancer abilities [40, 41]. To test whether BRB phytochemicals also influence tumor immunology, Mace et al. [42] used an ethanol

extract from lyophilized BRB powder that contained a large number of phytochemicals including anthocyanins and quercetins. Human peripheral blood mononuclear cells (PBMC) from healthy donors were cultured with GM-CSF and IL-6 (10 ng/ml, 7d) to induce the formation of MDSCs and the MDSCs were then isolated by flow cytometry (HLA-DR ^{low}CD11b^{+}CD33^{+}). PBMC treated with BRB (100 μg/ml) were less likely to differentiate toward MDSC in response to the cytokine treatments. Additionally, MDSCs generated in the presence of BRB were less able to suppress the proliferation of CD3/CD28-activated CD4^{+} or CD28-activated CD8^{+} T-cells (72 hr suppression assay, proliferation assessed by CFSE). MDSC differentiation and function were also examined in the presence of Quercetin-3-Rutinoside or Cynaidin-3-Rutinoside (200 μM, 7 days), two abundant metabolites of BRB. Like BRB extract, these compounds reduced IL-6 mediated STAT3 signaling, however, they were less potent suppressors of MDSC T-cell suppressive ability as compared to BRB. This suggests that additional BRB phytochemicals also contribute to the biological effects of BRB on MDSCs.

4.3.2.6 Soy compounds:

Soy is known to contain multiple isoflavones that have been shown to have anticancer activity in several different cancer models [43]. Lesinski et al. [44] examined the impact of dietary soy in men with elevated levels of prostate-specific antigen (PSA). The men were given two slices of a soy-enriched bread or control bread for 56 days each in a cross-over design. Plasma from patients fed the soy bread had reduced serum levels of pro-inflammatory cytokines (IL-1β, TGF-β, TNF-α, and IFN-γ) and MDSC-associated cytokines (G-CSF, GM-CSF, M-CSF, IL-10, IL-13, and VEGF). Additionally, there was a reduction in the number of phenotypically defined blood M-MDSC (HLA-DR^{low}CD11b^{+}CD33^{+}CD14^{+}) isolated via flow cytometry after 56 days on the soy bread. These data suggest that phytochemicals from soy may inhibit blood M-MDSC formation in humans. However, neither the mechanisms by which soy phytochemicals may act on blood M-MDSCs nor the identity of the specific bioactive phytochemicals in soy were investigated in these clinical trial samples.

The research summarized in this section shows that several phytochemicals have promise as inhibitors of MDSC in the context of cancer. However, more studies are needed to understand the mechanisms by which these different phytochemicals, and families of molecules, act on MDSCs. In addition, there are many different phytochemicals that could alter MDSC biology

including polyphenols, terpenoids, organosulfur compounds, and phytosterol [29], Upadhyay et al. [29] recently discussed the major modes of action of these classes of phytochemicals that are used in specific settings, e.g. inflammation and metabolism. Their work may serve as a foundation to select additional phytochemicals with the potential to alter MDSC function in various pathophysiological conditions.

While several phytochemicals have been studied, curcumin stands out as one example of polyphenols as immunomodulators. Multiple studies have been done to show that curcumin acts on multiple cells of both the innate and adaptive response. Additionally, the structure of curcumin has been used as a backbone to create potential therapeutics. The role of curcumin as a modulator of MDSC biology in the context of cancer and future work that would aid in expanding the field of polyphenolic phytochemicals as adjuncts to cancer treatments will be discussed. This will be presented in a grant type of format.

4.3.3 Specific Aims: Curcumin as an Example of Polyphenol Immunomodulation of MDSCs in Cancer

Curcumin, a type of polyphenol, is widely known for its anti-inflammatory properties via inhibition of several pathways known to be pro-inflammatory (i.e. NF-κB, STAT3, IL-6, TNF-a, IL-1b, HIF-1a, iNOS) [45]. Additionally, several of these pathways have also been shown to aid in the inhibition of tumorigenesis [46]. Specifically, in animal models focusing on the effect of curcumin on tumor cells, curcumin has been shown to reduce tumor growth in the colon [47, 48], skin [49, 50], breast [48, 51] and liver [52]. More recently studies have shown the role of curcumin as an immunomodulator in cancer [53-55]. Within the complex tumor environment, myeloid derived suppressor cells (MDSC) aid in immune escape from T-cell immune surveillance [56-59]. Several studies have been reviewed to show the primary pathways involved in MDSC development and gain of suppressive function (i.e. NF-κB, STAT3, IL-6, TNF-a, IL-1b, HIF-1a, iNOS) are targets of curcumin in other cells similar to MDSC such as monocytes, macrophages, and neutrophils. [45, 60, 61]. However, to date, only one group [62] has looked at the role of curcumin specifically in MDSCs. Tu et al. [62] were able to show that curcumin acted on MDSC subtypes and other immune cells and that these actions were attributed to down-regulation of NF-κB and STAT3 in MDSCs; however, a few important points remain unclear. For example, Tu et al. did not directly test the T-cell suppressive function or differentiation potential of MDSCs post-treatment with curcumin. Additionally, their pathway analysis was limited to NF-κB and STAT3,

while it is likely that other pathways such as TNF-a, IL-1b, HIF-1a, and iNOS may be affected. Therefore, it is important to continue this work by looking at the pathways involved in the effects seen by curcumin on MDSCs, specifically, T-cell suppression activity of individual MDSC subtypes and the potential of MDSCs to differentiate to cell types that do not suppress T-cell activity at the tumor.

Our hypothesis is that curcumin will reduce MDSC T-cell suppressive activity in part by promoting differentiation of M-MDSCs to cell types that do not suppress T-cell activity at the tumor via regulation of one or multiple pathways including NF-κB, STAT3, IL-6, TNF-a, IL-1b, HIF-1a, and iNOS. Because there are multiple MDSC subtypes that may respond differentially to curcumin, we will address the effects of curcumin on specific MDSC subtypes (M-MDSCs and G-MDSCs) which are known to have different activities in other systems. The rationale for the proposed research is that specific action of curcumin on MDSCs in a subtype-specific manner, as well as the mechanism of action, is unknown. Curcumin has the potential to be an adjuvant in traditional chemotherapies and immunotherapies by reducing the number and T-cell suppressive activity of MDSC's at the tumor site allowing a more robust T-cell mediated clearance of the tumor cells. Therefore, understanding the pathways affected by curcumin in MDSCs may shed light on how curcumin may alter MDSCs within the milieu of the tumor microenvironment. These hypotheses will be tested with two specific aims:

Aim 1: Identify the cellular pathways that curcumin inhibits within specific MDSC subtypes that reduces their T-cell suppressive function and improves cancer outcomes. We *hypothesize* that specific MDSC subtypes found at the tumor site of mice fed a curcumin-rich diet will have reduced T-cell suppressive activity and promotes differentiation. Additionally, we *hypothesize* that curcumin acts on multiple transcription factors (beyond NF-κB and STAT3) within MDSCs to reduce their T-cell suppressive activity and promote differentiation. We will test these hypotheses in MDSCs isolated from C57Bl/6J mice fed +/- curcumin. We will use t-cell suppression assays, in-vitro differentiation assays, multiple transcription factor binding site analysis, and transcriptome analysis.

Aim 2: Evaluate the role of curcumin to redirect the development of MDSCs toward non-T-cell suppressive cell types. We *hypothesize* that dietary curcumin will result in the differentiation of BM and SP M-MDSCs toward non-T-cell suppressive cell types such as

M1 macrophages and DC's. Additionally, we *hypothesize* that dietary curcumin will irreversibly inhibit the T-cell suppressive activity of TU MDSCs subsets. We will test these hypotheses in MDSCs isolated from C57Bl/6J mice fed +/- curcumin. We will use the adoptive transfer of MDSCs from C57Bl/6J CD45.1 and CD45.2 followed by flow cytometry analysis and T-cell suppression assays.

4.3.4 Background and Significance

4.3.4.1 Introduction to Curcumin:

Curcumin is the main active phytochemical found in turmeric and comes from the Curcuma longa Linn plant. Curcumin is known as a polyphenol but has also been classified in its own category of phytochemical called, "curcuminoids." Curcumin is known to have three major isoforms: curcumin, desmethoxycurcumin, and bisdemethoxycurcumin. Curcumin represents the most abundant and studied isoform. Specifically, curcumin has been studied for its positive effects on multiple diseases. Aggarwal et al. summarized multiple positive effects of curcumin in a review covering neurodegenerative, cardiovascular, metabolic, autoimmune diseases, and cancer [45]. Recently, Vallianou et al. reviewed the mechanism by which curcumin acts as an anti-cancer agent against cancer cells [55]. Finally, the effects of curcumin have been shown to have a positive impact on immune function under healthy and diseased states [53]. However, the effects of curcumin on immune cells during cancer development is less studied.

4.3.4.2 Curcumin's Mechanisms of Action on Cancer cells:

Curcumin has been studied for several years as an anti-cancer agent and has been found to reduce cancer progression in several types of cancer. Curcumin effects major pathways involved in multiple cancers in animal models for example, in breast cancer curcumin reduces TNF-α signaling resulting in reduced tumor formation [63], in liver hepatocellular carcinoma curcumin reduced protein levels of p21ras (a proto-oncogene) and inhibited cell cycle signaling [52] and, in oral cancer curcumin reduced cellular proliferation [64]. Equally important is understanding the multiple mechanisms by which curcumin may act on cancer cells. Due to the large number of pathways affected by curcumin (transcription factor regulation, protein kinases, adhesion molecule inhibition, enzyme inhibition), only nuclear factor kappa B (NF-κB) and signal transducer and

activator of transcription (STAT) 3 will be discussed briefly as they directly relate to MDSC biology.

NF-κB and STAT3 are master regulators of transcription and important for multiple cellular processes. NF-κB can be activated by multiple inflammatory cytokines (IL-6, IL-1, TNF-α) and once activated a subunit (p65) translocates to the nucleus regulating more than 200 genes [45, 65]. In cancer, NF-κB has been shown to upregulate genes important in the inhibition of apoptosis (bcl-2 and bcl-XL) as well as genes that promote angiogenesis and metastasis (VEGF and MMP) [65]. STAT3 is also activated by several inflammatory cytokines and growth factors (EGF, PDGF, IL-6) which activate receptor tyrosine kinases resulting in phosphorylation of STAT3. Once activated STAT3 translocates to the nucleus where it regulates multiple genes involved in cell cycle, apoptosis, and immune evasion. Inhibition of these two master regulators by curcumin has been extensively studied and summarized in multiple reviews [45, 55, 65].

4.3.4.3 Curcumin as an Immunomodulator of MDSCs in Cancer:

Curcumin has a long history as a complementary agent to cancer therapies [55]. There are several studies that focus on the impact that curcumin, curcuminoids, or curcumin analogs on MDSC in the context of cancer. Tu et al. [62] examined the role of curcumin in the CT26 cell allograph model of colon cancer. In a primary prevention model, mice were fed a high curcumin diet (2%, AIN93M diet) for four weeks beginning on the same day as CT26 cells were injected s.c. In a treatment model, mice received ip injections of curcumin (50 mg/kg body weight) for 3 weeks after CT26 tumors had developed to >1 cm diameter. Total SP MDSCs and TU MDSCs were isolated from mice at the multiple time points. In both models, curcumin reduced the accumulation of total SP and TU MDSCs. Curcumin treatment was also found to reduce the number of TU G-MDSC but not M-MDSC in MKN-45 human gastric cancer cell xenografts, suggesting the effects of curcumin may be specific to G-MDSC.

Tu et al. [62] also examined the effects of curcumin on SP MDSC plasticity *in vitro*. MDSCs have been reported to differentiate into tumor-associated (M2) macrophages [66]. When total SP MDSCs cultures were treated with curcumin (12.5 μmol/L, 48 h), curcumin induced SP MDSCs toward cells with phenotypic properties consistent with anti-tumor, M1 macrophages and reduced the number of cells harboring M2 macrophage phenotypes. Curcumin also reduced the cell-to-cell contact between SP MDSCs and gastric tumor cells in co-culture in a dose-dependent

manner, leading to reduced colony formation and reduced IL-6 production by the tumor cells. These results suggest that in addition to protecting tumor cells by suppressing cytotoxic T-cells, MDSCs can signal through cell-to-cell contact to increase tumor cell expansion. Bill et al. [67] later used curcumin analogs designed to specifically inhibit STAT3 phosphorylation and showed that they could reduce the *in vitro* differentiation of human myeloid precursors into MDSCs.

To understand the mechanism by which curcumin may suppress MDSC function, Tu et al. [62] investigated the impact of curcumin treatment on cell signaling and IL-6 production. When SP MDSCs were treated with cancer cell conditioned medium, IL-6 production was increased by >100% and was accompanied by increased signaling through STAT3 and NF-κB However, all of these effects were blocked by curcumin treatment (12.5 μmol/L). These findings are consistent with the hypothesis that curcumin inhibits an autocrine response whereby tumor-derived factors stimulate MDSC production of IL-6, which then further stimulates MDSC development. This signaling may also regulate SP MDSC differentiation into macrophages.

Singh et al. [68] subsequently tested the hypothesis that a curcumin-mediated reduction of IL-6 could improve the efficacy of vaccination against triple negative breast cancer. This group used a Listeria^{at}-based vaccine expressing the tumor-associated antigen Mage-b to induce an immune response against the triple negative breast cancer 4T1 model in mice. While immune suppression in the tumor microenvironment normally limits the effectiveness of this immunotherapy, co-treatment of mice with curcumin, enhanced vaccine-mediated suppression of 4T1 tumor growth. This was associated with a robust suppression of IL-6 production by blood M- or G-MDSCs and by TU M-MDSCs as well as increased blood MDSC production of IL-12, a cytokine that is important in activating CD4+ and CD8+ cells [69]. Consistent with the effects of curcumin on IL-6 and IL-12 production, curcumin treatment increased both the number of CD4+ and CD8+ T-cells in the circulation of vaccinated mice, as well as their activation state (measured by increases in IFN-γ levels). Indeed, curcumin has also been shown to limit iNOS production in other models and enhance the efficacy of immunotherapy with the cytokine interleukin-2 [70]. This suggests that curcumin acts at multiple levels in the tumor microenvironment and that it has potential as an adjunct treatment to immunotherapy. However, further studies focused on TU MDSCs and using additional immunotherapies are necessary to fully evaluate the translational potential of curcumin.

4.3.4.4 Aim 1. Proposed Research Plan

Identify the cellular pathways that curcumin inhibits within specific MDSC subtypes that reduces their T-cell suppressive function and improves cancer outcomes. Two hypotheses will be tested in this aim. Our ***first hypothesis*** is that specific TU MDSC subtypes of mice fed a curcumin-rich diet will have reduced T-cell suppressive activity. TU MDSC subtypes have been shown to have different degrees of suppression of T-cell proliferation. The M-MDSCs have been shown to be more suppressive over G-MDSCs however, in many tumor lines G-MDSCs appear at the tumor site in greater numbers. Our goal is to understand how curcumin affects these subtypes and their ability to suppress T-cell proliferation. We will use EL-4 subcutaneous solid tumors in C57Bl/6J mice (14 d growth) in mice fed a control diet or a curcumin-rich diet. MDSCs will be isolated using flow cytometry and used in *in vitro* 18 h T-cell suppression assay's immediately following isolation. An additional *in vitro* model will also be used where TU MDSC will be cultured with T-cells +/- curcumin (18 h). The final analysis will be done using FlowJo software and SAS for statistical analysis (ANOVA with Tukey HSD and student T-tests).

The ***innovation*** of testing this hypothesis is that the role of curcumin to inhibit the suppressive ability on MDSC have yet to be directly tested. By looking at the role of curcumin on the subtypes of TU MDSC we will be able to understand how curcumin is altering TU MDSC in the tumor microenvironment. *in vitro* suppression assay's will allow us to separate the systemic effects of curcumin and look at its effect on a single cell type. By understanding the response to the different subtypes, we can begin to look at specific mechanisms by which curcumin is affecting each cell. We expect that tumor MDSC from curcumin-fed mice will have reduced suppressive function in both MDSC subtypes but have a more profound effect on M-MDSC.

While the methods we propose to use have been well established, it is important to understand their ***limits and alternatives***. For example, the harvest of solid tumor MDSC can result in low yields of MDSC subtypes. An alternative approach would be to use an i.p. tumor model to generate MDSCs as ascites [71]. Another issue that may arise is only one subset of MDSCs may be suppressive in our model. In this case, we will focus on that subtype alone for the remaining assays. Another issue is the use of specific vs non-specific activation of T-cells. We will be using T-cells activated by a specific peptide (SIINFEKL), this approach may not be a realistic model of T-cell activation as it produces a single clonal T-cell population. Alternatively, non-specific

activation of WT T-cells (by CD3/CD38 antibody) which produces a more physiologic heterogeneous population of activated T-cells.

Our *second hypothesis* is that curcumin acts on multiple pathways (beyond NF-κB and STAT3) within MDSCs to reduce their T-cell suppressive activity and promote differentiation. While Tu et al. were able to show that curcumin altered the NF-κB and STAT3 pathways, curcumin is known to alter multiple other pathways [46, 53, 55].

Additionally, BM and SP M-MDSC but not G-MDSC were shown to differentiate into G-MDSCs, DC's and Macrophages in Chapter 2 as well as in [72] however, the pathways involved in differentiation are unknown. Therefore, for pathway analysis we will isolate BM, SP and TU MDSCs from tumor-bearing mice fed a standard or curcumin-rich diet as well as collect cells post differentiation assay, for RNAseq and ATACseq analysis. Combined these methods will identify the transcriptome changes as well as changes in transcription factor binding sites induced by curcumin in BM, SP, and TU MDSCs. These data will be followed by pathway analysis to assess if other known targeted pathways of curcumin are altered in MDSCs. Finally, based on these results we will conduct protein analysis using western blots and/or ELISA and flow cytometry.

The *innovation* of testing this hypothesis is that the full spectrum of pathways altered by curcumin in MDSCs is unknown in cancer. Of high importance, we will gain an understanding of how curcumin modulates specific pathways involved with MDSCs T-cell suppressive function. Additionally, it is unclear what pathways curcumin may alter in BM and SP MDSCs that play a critical role in altering MDSC differentiation away from the T-cell suppressive phenotype. We expect that curcumin will decrease the T-cell suppressive ability of TU MDSCs compared to controls. Additionally, we expect that curcumin will alter the transcriptome and the open chromatin regions of the genome and illuminate multiple pathways altered by curcumin in MDSCs associated with MDSC differentiation and T-cell suppression ability.

RNAseq and the *in vitro* assays will require a large number of MDSCs from each tissue site, highlighting one of the *limits* of the proposed methods. However, ATACseq requires substantially fewer MDSCs in comparison. Therefore, it may be beneficial to begin the analysis of the effects of curcumin on MDSCs with the ATACseq analysis. This would allow us to know if the treatment is causing a significant difference at the level of transcription factor binding sites on each subtype, which would relate to the transcriptome analysis. Based on the ATACseq data we

could focus our cell collection and analysis on only those with significant changes in transcription factor binding sites.

4.3.4.5 Aim 2 Research Plan

Evaluate the role of curcumin to redirect the development of MDSCs toward non-T-cell suppressive cell types. Our *first hypothesis* is that dietary curcumin will cause MDSCs to differentiate away from the suppressive phenotype and toward non-T-cell suppressive cells such as M1 macrophages and DC's. The potential to differentiate away from the T-cell suppressive phenotype is an important potential therapeutic target of immunotherapies. Therefore, we will look at the ability of BM and SP to differentiate toward G-MDSCs, DC's and M1/M2 macrophages as described in Chapter 2. We will additionally test the suppressive ability of the M- derived G-MDSCs. This will aid in determining if the resulting G-MDSCs post culture are truly G-MDSCs or have become neutrophils. Additionally, we will employ an adoptive transfer model, using C57Bl/6J CD45.1 and CD45.2 mice, where MDSCs from AIN93G +/- curcumin diet fed mice are transferred into tumor-bearing mice fed an AIN93G +/- curcumin diet to assess the ability of curcumin to initiate differentiation in MDSCs that have never been exposed to curcumin. Additionally, we will investigate the longevity of the curcumin effect on BM and SP MDSCs when transferred into AIN93G without curcumin fed tumor-bearing mice.

Our *second hypothesis* is that the inhibitory effect of dietary curcumin on TU MDSC T-cell suppressive ability is irreversible. Based on the results in chapter 2, that TU M-MDSCs do not possess the same level of differentiation ability as seen in BM and SP, we do not think that TU MDSCs will be able to undo the effects of curcumin before dying. Therefore, utilizing the same adoptive transfer model as mentioned above, we will adoptively transfer TU MDSCs and re-isolate them 3 d post transfer and subject them to a T-cell suppression assay.

As part of the *innovation* of our study, we will be thoroughly investigating the role of curcumin on the differentiation potential of BM and SP MDSCs. The investigation into the ability of curcumin to promote the differentiation of BM and SP MDSCs toward non-T-cell suppressive phenotypes will provide more details about curcumin's ability to inhibit tumor growth. These data could lead to improved combination therapies with curcumin as an adjunct. Based on Tu et al.'s [62] results we expect that curcumin will promote M-MDSCs to differentiate toward M1 macrophages at the cost of M2 and G-MDSCs. Tu et al used models to suggest potential uses for

curcumin in both primary and secondary prevention, however, they were unable to separate the systemic effects from specific effects on MDSC. Our *in vitro* adoptive transfer models will separate these effects and set the stage for clinical studies for using curcumin to target MDSC. Finally, the longevity of curcumin's effects on MDSCs is unknown but is an important factor when thinking of therapeutic use. To our knowledge, there are no studies that test the longevity of the effects of curcumin on immune cells, especially MDSCs. We expect that the effects of curcumin will last throughout the adoptive transfer process and result in TU MDSC that retain a reduced ability to suppress T-cells.

While the *in vitro* methods of differentiation are well established and tested, a major ***limit*** to this study is that adoptive transfer studies have high failure rates. As mentioned in Aim 1, the number of MDSCs needed from each tissue may be problematic. Therefore, as an ***alternative*** approach, the use of the i.p. model may be used as BM and SP MDSCs are the primary focus of these studies. Additionally, we can limit the number of *in vitro* differentiation assays to only the subtype that shows differentiation post-treatment. While the current data suggests only the M-MDSC subtype has differentiation potential, we can conduct a pilot study where both subtypes are isolated and tested, the results will determine if only one or both subtypes should be tested. There are multiple pitfalls when using the adoptive transfer model. For example, the currently published methods are not consistent with each other. Additionally, the number of cells needed is extremely high. Finally, because we want to investigate the longevity of the effects of curcumin, we will need to have highly coordinated breeding, injection and dietary schedules to produce the needed mice post isolation of TU MDSCs from +/- curcumin diet mice. We will be testing the T-cell suppressive ability of the same cells that will be adoptively transferred reducing the overall number. As an alternative to adoptively transfer MDSCs could be cultured *in vitro* +/- curcumin with T-cells and tumor cells. While possibly not as translational of data the question of longevity could still be answered.

4.3.4.6 Conclusions for Curcumin as a Modulator of MDSCs in Cancer

The use of curcumin as a modifier of MDSCs under cancerous conditions is in a great state for more investigations. New studies will help us understand the complex nature of curcumin's interactions with cells of the immune system. Additionally, if we view curcumin as an example of the potential of polyphenols to modify immune cells, new studies will also give us insight into

how other polyphenols may act on MDSCs. The aims and studies presented here will move the fields of phytochemical immunomodulation, immune cancer interaction, MDSC biology, and immune modulation of MDSC by diet forward.

4.4 MDSCs: From Bad to Good

In the context of cancer, MDSCs have been highlighted as a target cell to inhibit thereby allowing T-cells to do their job killing tumor cells; MDSCs are bad for the host. However, in other conditions, such as pregnancy, MDSCs aid in the protection of increased inflammation and protection of the fetus; MDSCs are good for the host. Therefore, increasing MDSCs may be a prevention or therapeutic strategy to reduce pregnancy complication such as pre-eclampsia. Understanding that MDSCs are hijacked by cancer has helped to understand how MDSCs play an important part in the physiologic immune system. Therefore, to better understand how these cells act in a positive manner to reduce inflammation and T-cell mediated adverse conditions, we will investigate the role of MDSCs in pre-eclampsia (PE).

4.5 Dietary Bioactives as MDSC Modulators in Pre-eclampsia

4.5.1 Immunology of Pregnancy

During pregnancy, the immune system exists in a unique state where cells are actively attempting to attack the fetus, while other cells are protecting it. This interaction is referred to as fetal-maternal tolerance. Fetal-maternal tolerance has been well studied and the role of several immune cells has been reviewed focusing on several aspects of the response [73-76]. In a review by Brozychowski et al. the authors state that pregnancy can be viewed as a mild state of systemic inflammation [75]. Multiple cytokines are released during pregnancy which leads to the activation of both the innate and adaptive immune response in an inflammatory manner. The major cells involved in this inflammatory state appear to be M1 macrophages, DC’s, and CD8 T-cells. However, at the uterus and placenta anti-inflammatory cytokines are released that recruit several regulatory cells such as T- and Bregs and, promote a shift in macrophages from M1 to M2 [73, 74]. Finally, new studies have shown that MDSCs are also present in higher percentages during normal pregnancy than during PE and other pathophysiologic pregnancy conditions [76]. Therefore, prior to discussing the potential studies for the role of curcumin as an MDSC modulator

during PE, we will review the role of immunity in pregnancy, the etiology of PE and, curcumins effects on pregnancy and PE.

To achieve fetal-maternal tolerance both the innate and adaptive systems are working against each other. In a review of the innate immune cells in reproduction, Negishi et al. discuss how pro- and anti-inflammatory cells are needed to maintain the mother's immune system while also initiating fetal-maternal tolerance. To summarize some of these cell types and events, macrophages, DC's, NK cells, and MDSCs will be discussed. Macrophages and DC's are the major antigen presenting cells which bridge the innate and adaptive immune responses. During pregnancy both cell types have multiple roles, in early pregnancy they release pro-inflammatory cytokines at the uterus which aid in the implantation of the fertilized egg but, later these cells act as important regulators of inflammation by promoting the differentiation of Tregs (DC's) or by phenotype switching from M1 to M2[74]. Therefore, the timing of the DC and macrophage response during pregnancy plays an important role in the development and maintenance of fetal-maternal tolerance.

Another major innate cell type involved in a successful pregnancy is the natural killer cell (NK). These cells are also divided into multiple subtypes based on location and function; peripheral NK (pNK) and uterine NK (uNK). During pregnancy pNK cells are needed systemically to aid in anti-viral immunity, while uNK cells and dNK cells are needed to support the fetal-maternal tolerance. While pNK cells possess the traditional markers and functions of NK cells (cytotoxicity and inflammatory cytokine release), uNK and dNK cells express specific receptors that when activated by ligands present on extravillous trophoblasts, which are involved in the maintenance of open spiral arteries that empty into the maternal lacunae[77], inhibit the cytotoxicity function and promote the release of several MMP, which aid in the trophoblast invasion of the extracellular matrix, as well as the release of angiogenic growth factors: vascular endothelial growth factors -A and -C, angiogenin, angiopoietin -1 and 2, placental growth factor, keratinocyte growth factor, and fibroblast growth factor.

MDSCs represent the one innate cell type that does not seem to have an opposing pro-inflammatory subtype. MDSCs are known in cancer to aid in tumor evasion by suppressing the cytotoxic T-cell response to tumors. In pregnancy, these cells are also involved in the suppression of T-cells as well as aiding in several other functions that aid in fetal-maternal tolerance and successful pregnancy. In a review by Ghaebi et al. [76], the role of MDSCs in pregnancy is

described as an important part of establishing and maintaining fetal-maternal tolerance. This role is attributed to their ability to downregulate the expression of the homing molecule L-selectin on immune cells, induce the differentiation and proliferation of Tregs, and inhibit cytotoxic CD8 T-cells. However, in a study by Zhao et al [78] depletion of MDSCs in mice resulted in an increase in DC and T-cell uterus infiltration. These data suggest that MDSCs play an important role in maintaining innate cell fetal-maternal tolerance as well. Together, these examples help to illustrate the complex pro- and anti-inflammatory responses that occur during pregnancy in the innate immune response.

Within the adaptive immune response, both T and B cells play important roles in successful pregnancy though pro- and anti-inflammatory responses. The important interactions between Tregs, B cells, Bregs, and MDSCs during pregnancy was recently reviewed by Ghaebi et al. [76]. Additionally, Lissauer et al have recently reviewed the role of T-cells during pregnancy [79]. Therefore, to briefly summarize the importance of T and B cells in successful pregnancy we will limit the focus to the most studies cell types: cytotoxic CD8 T-cells, Tregs, and Bregs based on these reviews.

According to Lissaurer et al., the number of both CD4 T helper cells and cytotoxic T-cell found in the maternal blood and in the decidua compartments slowly increases during pregnancy. However, the blood T-cells are less mature and play an important role in maintaining the maternal immune system, while those in the decidua are more mature and are responsible for ensuring fetal cells do not escape into the maternal blood stream. The T-cells found at the decidua release a higher percentage of IFN-γ, express TCR specific for fetal cells, and are more sensitive to elimination by regulatory cells via increased expression of the checkpoint inhibitor PD-1. Therefore, T-cells are important in pregnancy by maintaining maternal immune surveillance and inhibiting the release of fetal cells into the maternal blood stream. Similar to T-cells, B cells are increased during pregnancy in the maternal blood but, primarily work to increase antibody production to aid in pathogen resistance of the mother [76]. However, the T-cells at the decidua are also capable of infiltrating the uterus and attacking the fetus directly. Therefore, Tregs, Bregs, and MDSCs are present to reduce this interaction. All three of these regulatory cells will suppress cytotoxic T-cells via various mechanisms but share one important role, the production of IL-10.

According to Ghaebi et al., IL-10 is produced by all three regulatory cells and is a major cytokine involved in Treg expansion and maintaining DC’s in an immature state reducing the

activation of CD4 T helper cells (involved in the activation of cytotoxic T-cells and increased inflammation) which ensures the fetal-maternal tolerance.

Tregs and MDSCs have also been shown to release TGF-β which is known as an anti-inflammatory growth factor which aids in the production of additional T and B cell suppressive molecules such as indoleamine 2,3-dioxygenase and galectin-1 [76]. The last regulator of T-cells is the MDSC. During pregnancy, MDSCs are increased in the maternal blood and in the decidua. Along with their production of IL-10 and TGF-β, MDSCs found at the fetal-maternal interface possess the same characteristics as those found at a tumor. However, a direct comparison of decidua and tumor MDSCs has not been done.

Together the cells of the adaptive immune response aid in maintaining a strong maternal immune system, reduce the risk of fetal cell release into the maternal bloodstream while simultaneously inhibiting an immune response to the fetus. While MDSCs are part of the innate system their role in fetal-maternal tolerance and similarity in function to T and Bregs, it seems appropriate to place additional information about them with the regulatory cells of the adaptive response in pregnancy. When the feto-maternal tolerance is disrupted or when there is a lack of regulation of the inflammatory response, reproductive defects can occur such as miscarriage and pre-eclampsia.

4.5.2 Biology and Immunology of Pre-eclampsia

Pre-eclampsia (PE) is a condition that occurs in 3-8% of all pregnancies but is the main cause of maternal Eclampsia mortality in the world [80] and as of 2016, there was an increase in PE occurrence in first world countries [81]. Currently, PE is defined by multiple symptoms including increased blood pressure, proteinuria, maternal organ dysfunction, uteroplacental dysfunction, and restricted fetal growth [80]. However, PE can be divided into two types with different diagnosis parameters and etiologies; early- and late-onset, where early-onset is associated with increased risk of maternal and fetal complications [81]. While these subtypes are distinct in many ways, they have significant symptomatic overlap resulting in difficulty in using these as diagnostic parameters. Additionally, when looking at risk factors the subtype distinction is not used [81]. In recent years, increased studies are providing improved models of the biology of PE. Therefore, in this section, we will cover the overall risk factors, current prevention and treatment strategies, and the role of the immune system in PE.

Multiple risk factors have been identified for PE but prediction of PE has yet to be established. However, it is possible to identify women as low or high risk from PE. Mol et al. conducted a review of multiple aspects of PE including risk factors, signs and symptoms, treatment strategies, and prevention methods, which will be summarized here [80]. Women at high risk from PE will have multiple of the following comorbidities: chronic kidney disease, hypertension, diabetes, and autoimmune disorders. High-risk women may also have moderate risk factors such as first pregnancy at > 40 years, high BMI (>35), polycystic ovarian syndrome, and prior PE diagnosed pregnancies. Finally, there are clinical and lifestyle predictors but several of these need further evaluation or only show modest predictive value. These include lifestyle factors such as maternal birth weight, and clinical factors such as mean arterial pressure at 15 wk gestation and vaginal bleeding >5d during current pregnancy [80]. Women with a low risk of PE will have some of these risk factors but the combination of them will be lower, ie a woman with only vaginal bleeding >5d during current pregnancy. Based on the current status of risk factor assessment, more research is needed to better understand the role of risk factors for PE.

Despite the lack of clear etiology of PE, prevention and treatment plans have been established. As of 2019, two reviews have discussed the recommended prevention/treatment plans for women at risk of PE [82, 83]. Both groups have recommended low-dose intake of aspirin (50-150 mg/d) while only one [82] suggested that Ca supplementation (500 mg/d) was also beneficial for women who are Ca deficient. However, in a previous review of PE by Mol et al. [80] discuss the potential of other preventative interventions such as low molecular weight heparin, Vitamin C and E, Magnesium, L-arginine with anti-oxidants, Vitamin D and Ca, and diet and lifestyle changes in random controlled trials. While not all of these interventions have been studied as much as Aspirin, they show promise for use in specific demographics of women at risk of PE. Once diagnosed the suggested treatments often focus on treating single symptoms/risk factors such as hypertension and oxidative stress [80]. It is, therefore, necessary to further understand the etiology of PE to create prevention strategies and treatments.

Several factors, such as inflammation, have been associated with the progression and establishment of PE and the cells of the immune system have emerged as another component of PE [75]. Both the innate and adaptive immune responses have been shown to play vital roles in a successful pregnancy and fetal development [73]. Therefore, it is not surprising that when these

cells are dysfunctional that abnormal pregnancies can occur. To understand how immune cells are involved in PE, they will be divided as in the previous section (4.4.1).

Current data suggests that several cells of the adaptive immune response may play important roles in the development of PE. Redman et al. and Ribeiro et al. have reviewed the data on the immunology of PE with a focus on T-cells [84, 85]. Specifically, both suggest that an imbalance between Tregs and Th17 pro-inflammatory cells at the decidua promote autoimmunity reducing the placenta size and disrupting the fetal-maternal tolerance. Additionally, both suggest that the imbalance of these T-cells may be due in part to the reduced release of IDO and TGF-β by innate cells. Additionally, the cytotoxic CD8 T-cells may also play a role in the development of PE but current data only provides evidence for a subset of women with PE and more studies are needed [79]. However, it could be speculated that the dysregulation of regulatory cells (T- and B-regs, and MDSCs) may result in increased activation of cytotoxic T-cells resulting in an autoimmune and fetal-specific response [76]. While there is limited data on the role of B-cells in PE, Sarween et al. demonstrate that B-cells and the humoral immune response (B-cell and antibody response) are reduced in the serum in the context of PE, specifically, levels of subtypes of IgG1 and IgG3[86]. However, they report an increase of IgG1 placental transfer which appears to increase the activation of the complement system at the Uterus-placental interface. While Sarween et al. focused specifically on PE, other studies have shown that Bregs have an important role in reducing immunological abortions [76, 86]. More studies are needed to support the role of the B-cells and humoral response in PE. Therefore, there is limited evidence for the direct relationship with cytotoxic T- and B-cells with PE, but there is evidence for a role in the dysregulation of Tregs, Th17 cells and Bregs in PE.

Multiple cells of the innate response have been associated with PE but the most studied cells are the DC's and NK cells but, there is becoming a new emphasis on MDSCs as modulators in PE. DC's have been shown to play an important secondary role in PE by releasing IL-12 and other pro-inflammatory cytokines at the decidua switching the T-cell differentiation from Tregs toward inflammatory Th17 cells and cytotoxic T-cells [74]. NK cells have been studied and when unregulated appear to be a major contributor to the development and symptoms of PE [74, 76]. One of the main causes of uNK function is the lack of proper HLA signals from the trophoblast which do not initiate the fetal-maternal tolerance [84]. Additionally, the alternatively activated uNK cells will release cytokines such as INF-γ and IL-12 which will promote other immune cells

to become inflammatory [76, 84]. Together these data demonstrate a small amount of the information known about the role of the innate cells in PE. More studies are needed to illuminate the roles of other innate cells, specific subtypes, and most importantly, how they are dysregulated to promote PE.

4.5.3 MDSC as Helpful Cells in Pre-eclampsia

The final cells of the immune system that has recently been suggested as being important in PE are MDSCs. The study of MDSCs has mostly been in cancer, as this was the pathophysiologic state in which they were discovered [26]. However, recent studies have shown they are essential for maintaining fetal-maternal tolerance, protection against arginine auxotrophic pathogens, and reduction of spontaneous abortions [78, 87-89]. While the evidence for the importance of MDSCs is increasing, only one study has attempted to directly link MDSCs and PE and therefore will be discussed in greater detail than others. Wang et al. investigated the differences in T-cell and MDSC populations comparing normal and PE women [90]. Women included in the study were all diagnosed with severe pre-eclampsia at 20 wks of pregnancy and were age-matched to normal pregnancy and a non-pregnancy group was used as a control. The major results included significantly reduced G-MDSCs and Arg1 in PE patient peripheral blood and reduced G-MDSCs in cord blood when compared to normal pregnancy. Interestingly, there were no differences seen in the T-cell or M-MDSC subtype populations between normal and PE peripheral blood or cord blood samples. The authors conclude by suggesting that the restoration of G-MDSCs may be a possible treatment for PE [90]. However, one of the potential limits of this study is that MDSCs were collected from peripheral blood and not from placental tissue (knowing this may be impossible). As discussed in Chapter 2, the cells found in the spleen and/or blood may not be representative of the cells found at the site of inflammation ie the decidua. Finally, the study lacked any functional assays to evaluate the MDSCs were truly suppressive. Therefore, this study provides a good first look at the potential role for MDSCs as essential cells for inhibiting PE and opens the possibility for future follow up studies.

The previous sections have provided a small but important glance at the importance of the immune system in pregnancy and PE. Specifically, the study of MDSCs as a critical moderator of the development of PE is very young and has the potential to further understand the etiology of PE. Through new studies and further knowledge of PE development better treatments and

prevention strategies can be developed. As MDSCs have been shown to be altered by dietary bioactive, it is possible that treatment and prevention of PE may involve the use of dietary alterations to modify the MDSC profile in PE patients.

4.5.4 Promoting MDSC Biology with Phytochemicals and Dietary Bioactives

Unfortunately, the promotion of MDSCs by phytochemicals, or any agent, has not yet become a well-established area of research due to the majority of work being done in the context of cancer. However, *in vitro* work with MDSCs has demonstrated specific cytokines that can promote BM MDSCs into suppressive TU-like MDSCs (See Chapter 1). Aside from the generation of MDSCs for cancer research, only a few studies have shown increases in MDSCs by dietary agents or phytochemicals. While these studies are not focused on PE they may provide some initial information regarding the promotion of MDSCs by phytochemicals in the prevention or treatment of PE.

Resveratrol is a well-known phytochemical to have anti-tumor capabilities [91]. In a study by Hongbing et al., resveratrol was used in combination with high dose IL-2 treatment (HDIL2). HDIL2 treatment is for metastatic renal cell carcinoma and melanoma but, is associated with adverse symptoms such as vascular leak syndrome (VLS). VLS is brought on by an autoimmune response to endothelial cells by lymphokine-activated killer cells (LAK), a type of cytotoxic T-cell activated by IL-2. Hongbing et al. [92] hypothesized that resveratrol could help reduce VLS during HDIL2 treatment. They used mice injected subcutaneously with B16F10 melanoma cells and resveratrol administered once daily by oral gavage (100 mg/kg body weight) and isolated total SP MDSC (CD11b+GR-1+). Resveratrol treatment increased the main receptor for resveratrol, AhR, on total SP MDSC. This resulted in MDSC mediated reduction of the autoimmune response of VLS in peripheral tissues by suppressing LAK cells. Resveratrol increased the suppressive function of total SP MDSCs against LAK cells isolated from tumor-bearing mice. Similar increases in total liver MDSCs were seen in a study of resveratrol and *Staphylococcus aureus*-induced liver injury [93]. Finally, one other study has investigated the role of the taurine conjugated bile acid taurodeoxycholate (TDCA) as a modulator of LPS-induced sepsis in mice [94]. Chang et al. found that TDCA increased the number of SP G-MDSC compared to non-treated. Additionally, in an adoptive transfer model, TDCA G-MDSCs provided increased protection from sepsis compared to non-treated. Finally, they suggested that these effects were due to TDCA

modifying the protein production of anti-inflammatory molecules such as oncostatin (a cytokine in the IL-6 family), lactoferrin (non-heme iron-binding glycoprotein) and CD244 (NK cell receptor). Together, TDCA appears to be a potent inducer of MDSCs in this model.

While only two dietary agents were discussed here, they highlight the limited number of studies that exist on the potential to increase MDSCs as a form of treatment. More research is needed in this area as MDSCs have been shown to be beneficial in many pathophysiologic conditions (see Chapter 1). Taken together these studies support the hypothesis that MDSCs may be increased by dietary agents and used as anti-inflammatory cells in certain pathophysiologic conditions such as PE.

4.5.5 Specific Aims: Curcumin as an Example of Polyphenol Immunomodulation of MDSCs in the Development of Pre-eclampsia

During pregnancy, the immune system of the mother undergoes many changes including the development of fetal-maternal tolerance (the response by the maternal immune system to suppress itself from attacking the fetus). When this tolerance is not established, increased inflammation can occur resulting in pre-eclampsia (PE). PE affects 3-8% of all pregnancies and is an equal opportunity disease [80]. Recent studies have shown an important link between myeloid derived suppressor cells (MDSCs), specifically the G-MDSC subtype, and successful fetal-maternal tolerance [78, 95]. Additionally, a lack of G-MDSCs in peripheral organs and cord blood has been shown to be the major immunological difference between normal and PE pregnancy [90]. This has been confirmed in animal models that mimic aspects PE [89, 95]. Curcumin has been shown to reduce infection-induced inflammation at the placenta and fetal membranes [96] and reduce the IL-6 signaling pathway in uterine decidual cells [97]. During pregnancy, curcumin reduces placental inflammation via upregulation of AKT in mice [6] and reduced the LPS-induced PE phenotype in mice through the inhibition of TLR4 pathways [98]. While a direct link between curcumin and reduced PE occurrence has not been shown, curcumin has been shown in animal models to aid in the anti-inflammatory responses within the fetal-maternal tolerance [6, 98] and therefore may have a beneficial effect on MDSCs such as increase T-cell suppressive ability and ability to induce differentiation of Tregs which will result in reducing the damage from T-cells during PE. Therefore, it is unclear what role curcumin may have on MDSC subtypes during the progression of PE.

Our hypothesis is that curcumin reduces inflammation via increasing the number of MDSCs at the uterus and placenta resulting in restored normal fetal-maternal tolerance and improved birth outcomes during PE. Because there are multiple MDSC subtypes that may respond differentially to curcumin, we will address the effects of curcumin on specific MDSC subtypes (M-MDSCs and G-MDSCs) which are known to have different activities in other systems. The rationale for the proposed research is that specific action of curcumin on MDSCs, in a subtype-specific manner, during PE is unknown. Therefore, understanding the role of curcumin on MDSC subtypes during PE will shed light on the possibility of using curcumin as an aid to boost the anti-inflammatory effects and/or localization of MDSCs at the uterus and placenta. Additionally, following up on G-MDSCs being the primary difference between normal and PE pregnancy, G-MDSCs may be a possible biomarker for PE which may be modifiable by curcumin. These hypotheses will be tested by the following aims:

Aim 1: Identify the role of curcumin on MDSCs during pre-eclampsia at the uterus and placenta in regard to promoting fetal-material tolerance. We *hypothesize* that dietary curcumin will increase the number of MDSCs that arrive at the uterus and placenta resulting in reduced numbers of cytotoxic T- and NK cells and M1 macrophages. Additionally, we *hypothesize* that dietary curcumin will aid in the G-MDSC induced differentiation of T-regulatory cells via increased production of TGF-β. These hypotheses will be tested using a borderline hypertensive mouse strain (BPH/5) which emulates many of the symptoms of PE [99]. We will measure levels of TGF-β production by placenta/uterus G-MDSC *in vitro* as well as *in-vitro* assays with naïve T-cells (non-activated and non-differentiated cells with high differentiation potential) to measure G-MDSC induced differentiation of naïve T-cells into Tregs, flow cytometry to measure changes in MDSCs, T-cells and M1 macrophages.

Aim 2: Investigate the effect of curcumin on MDSCs as a biomarker of PE risk. We *hypothesize* that there is a level of G-MDSCs found in the placenta, and blood (during 2nd and 3rd trimester) which will correlate with women who are at risk for pre-eclampsia. Additionally, we *hypothesize* that women who are at risk for PE and consume curcumin will have increased levels of G-MDSCs compared to non-consumers resulting in reduced risk pre-eclampsia. These hypotheses will be tested using blood samples from pregnant women with standard markers for pre-eclampsia (hypertension, increased urinary protein, and low

platelet count). Women will be matched by multiple classifications categories and curcumin intake will be categorized as consumers or non-consumers based on dietary survey and, G-MDSC levels will be assessed using flow cytometry.

4.5.6 Background and Significance

4.5.6.1 Curcumin as an Anti-Inflammatory Immunomodulator

Curcumin is an effective anti-inflammatory agent in multiple pathophysiologic conditions. As described in section 4.3.4.1, the effects of curcumin have been studied under multiple pathophysiologic conditions [45]. One of the ways curcumin acts as an anti-inflammatory is by modulating immune cells. Part of the ***significance*** of this work will be to evaluate the potential of curcumin to act as an immunomodulator in the context of PE. Recently, Momtazi-Borojeni et al. reviewed the effects of curcumin on macrophages [100]. In their review, they conclude that curcumin can inhibit the formation of foam cells in atherosclerosis by modifying the expression of lipid transporters. Additionally, curcumin can act as a modulator of M1 to M2 macrophages by acting on the toll-like receptor 4 pathways. In DC's, Curcumin has been shown to reduce maturation via interactions with STAT3 and NF-κB [101]. Finally, in a study of curcumins effect on colitis in mice, curcumin shifted the ratio of Th17: Tregs resulting in increased Tregs and reduced colitis [102]. While only a brief covering the spectrum of immunomodulatory potentials of curcumin, these studies demonstrate the versatile nature of curcumin on immune cells and support the exploration of curcumin as a modulator in PE.

4.5.6.2 Curcumin Effects on Pregnancy

Limited studies have been conducted on the impact of curcumin during pregnancy and have resulted in differential results. Due to the multiple factors involved in pregnancy, the current data suggests curcumin can have both a positive or negative effect depending on the physiologic or pathophysiologic state. Additionally, these studies have been conducted in mice and cell cultures and may not truly represent the full biology in humans. Therefore, it is important to view each outcome individually to understand the translational relevance.

The ability of curcumin to aid in successful pregnancies has been shown in only three studies. Zhou et al. conducted studies using LPS-induced adverse pregnancy outcomes in mice to investigate the role of curcumin in inhibiting placental inflammation [6]. In this model curcumin

(intragastric infusion 100 lg/kg/d from GD 0.5) was found to reduce placental inflammatory cytokines (TNF-α, IL-1β, and IL-6), M1 macrophages, and increase p-AKT. These effects resulted in increased numbers of live pups, fetal weight, and placental weight. Similarly, Devi et al. utilized a rodent non-transformed decidual cell line to further understand the anti-inflammatory pathways involved in curcumin treatment. Their data showed that curcumin inhibits IL-6 production by inhibiting the STAT3 pathways. Additionally, they confirmed these results in human HuF primary fibroblasts obtained from the decidua paretalis. While no direct pregnancy outcomes were tested in this study, they demonstrated the potential of curcumin to alter inflammatory signaling by decidual cells, which could act as a mechanism for immunomodulation. Finally, Lim et al. investigated the ability of curcumin to inhibit LPS-induced inflammation in human placenta, fetal membranes and myometrium cells [96]. The culture of primary cells with curcumin (60 μM) resulted in reduced mRNA expression and protein production of IL-6 and PGE_2. These effects were due, in part, by a reduction of nuclear NF-κB. Together these studies suggest that curcumin alters the uterus microenvironment by reducing induced inflammatory signals. However, it is unclear if these same effects would be seen during normal physiologic conditions in pregnancy.

The negative responses from curcumin during pregnancy have been found in two studies using mouse models. Huang et al. utilized mouse early stage embryos and blastocytes to study the adverse effects of curcumin on fetal development [103]. However, they state that their use of *in vitro* culture was to create an environment independent of maternal influence. Therefore, their findings should be viewed with caution as their culture conditions have removed the possible beneficial effects of curcumin on the maternal status. In this study they found that culture with curcumin (45 μM) resulted in reduced development of early stage and post-implantation stage blastocyte by altering the germ layer and neurula development. Similarly, they found that low levels of curcumin in culture (6 and 12 μM) was able to reduce proliferation of the blastocyst. Together these results suggest that direct contact of curcumin on developing blastocytes will cause a negative result in fetal development. However, it is important to restate that in the absence of the maternal response, these data have very limited translational potential. The second study by Chen et al. utilized both *in vitro* and *in vitro* treatment with curcumin to investigate the potential hazardous effects during oocyte development[104]. Similar to the first study, the *in vitro* studies were done in the absence of any maternal response and should be taken with caution. They found that when *in vitro* fertilized oocytes were cultured with curcumin (0-20 μM) but only at 20 μM

found a significant increase in oocyte apoptosis and decreased proliferation. Again, demonstrating that direct contact of curcumin to oocytes has detrimental effect. They further took *in vitro* fertilized oocytes treated with or without curcumin and implanted them in female mice. The curcumin-treated mice had significantly higher rejection of implanted oocyte compared to control. Finally, in their *in vitro* study, they gave female mice curcumin enriched water (0-40 μM) and normal *in vitro* fertilized oocytes were implanted. Only the mice drinking the 40 μM curcumin water had a significant decrease in implantation take. Together, the data from Chen et al. demonstrate that curcumin may cause oocyte development issues when in direct contact with the oocyte or during implantation. However, as neither of these studies examined the role of curcumin on normal pregnancy it is still unclear what effects may occur.

While there is conflicting data on the role of curcumin during pregnancy, the studies where positive results were seen have a higher degree of translatability to humans. The positive studies used inflammatory models which provide some similarities to PE and therefore provide preliminary support of curcumin as a positive inhibitor of PE. Therefore, it is reasonable to further examine the potential of curcumin in the inhibition of PE.

4.5.6.3 Curcumin as an Immunomodulator of MDSCs in PE

Despite the large amount of research that demonstrates curcumin as an anti-inflammatory, few studies have investigated its potential in PE. However, many of the pathophysiologic conditions in which curcumin has been tested share portions of the etiology of PE. Specifically, curcumin has been demonstrated as a potent agent against autoimmune diseases [45] and graft versus host disease [2]. In both of these conditions, regulatory cells play key roles in reducing the negative effects of cytotoxic NK and T-cells. Therefore, an investigation of the use of curcumin as a potential therapeutic in PE is warranted.

4.5.6.4 Aim 1 Proposed Research Plan

Identify the role of curcumin on MDSCs during pre-eclampsia at the uterus and placenta in regard to promoting fetal-material tolerance. Two hypotheses will be tested in this aim. Our *first hypothesis* is that dietary curcumin will increase the number of MDSCs that arrive at the uterus and placenta resulting in reduced numbers of T- and NK cells and M1 macrophages. While there is currently no data on the potential of curcumin to promote MDSCs, we predict that

the MDSCs found at the uterus will benefit from the anti-inflammatory effects of curcumin and increase their functional abilities. Cytotoxic T- and NK cells at the uterus have been shown to play important roles in the promotion of PE and MDSCs are known to suppress these cells. Therefore, a curcumin-induced increase of MDSCs at the uterus will increase the suppression of these cells as well as aid in the promotion of tolerogenic Tregs and dNK cells via IL-10 production by MDSCs. Similarly, increased M1 macrophages at the uterus and placenta have been demonstrated to increase inflammation and the promotion of PE. M-MDSCs have been shown to be phenotypically similar to M2 macrophages and will aid in promoting M1 to M2 differentiation. Curcumin has been shown to aid in the M1 to M2 phenotype shifting of macrophages and combined with increased MDSCs will result in an increase in fetal-maternal tolerance. We will use a mouse model of PE (BPH/5 strain) +/- dietary curcumin and will compare to control C57Bl/6J. Uterus and placentas will be isolated and single cell suspensions will be analyzed by flow cytometry for markers M1, M2, dNK, NK, MDSCs and T-cells. Additionally, we will conduct *in vitro* analysis of MDSC subtypes isolated from the uterus and placenta for IL-10 cytokine production by ELISA, and T- and NK cell suppression (18 h co-culture method). The final analysis will be done using FlowJo software and SAS for statistical analysis (ANOVA with Tukey HSD and student T-tests).

The ***innovations*** of testing this hypothesis are that the role of curcumin as a treatment for PE and to increase MDSCs has never been tested. By investigating the role of curcumin on PE we will be able to determine if polyphenols like curcumin may be used as potentials dietary aids in preventing/treating PE. Additionally, we will be able to understand the role of curcumin on MDSCs in a new pathophysiologic condition in which little is known about the function of MDSCs. We expect that curcumin treatment will increase the number of MDSCs, M2 macrophages, Tregs, and dNK cells while decreasing the number of M1 macrophages and cytotoxic T- and NK cells.

Within our approach, there are many ***limits and alternatives*** to consider. For example, it has never been shown that curcumin can promote MDSCs. As an alternative, resveratrol may be used instead as it has been shown to increase MDSCs in other inflammatory pathophysiologic conditions. Another limit to this study is the limited number of good flow cytometry markers for the immune cells found at the uterus and placenta. As an alternative to large panel analysis, samples could be aliquoted for analysis of specific cell populations. This method would decrease the level

of difficulty during flow cytometry analysis but increase the difficulty in normalization and population comparisons. Additionally, as stated above, curcumin may have negative effects on pregnancy. Therefore, as an alternative the curcumin diet could be started after conception potentially preventing these effects.

Our ***second hypothesis*** is that dietary curcumin will aid in the G-MDSC induced differentiation of T-regulatory cells via increased production of TGF-β. For this study, we will use the same model as in our first hypothesis but focus on the G-MDSC subtype. G-MDSCs have been the main subtype reported to be affected by curcumin and found at decreased levels during PE. Therefore, we will isolate G-MDSCs from the SP (as a control), uterus, and placenta from BPH/5 strain +/- curcumin and will be used in *in vitro* assays. We will co-culture naïve T-cells with G-MDSCs followed by flow cytometry analysis to detect Tregs. Additionally, the supernatants of this assay will be assessed using WB or ELISA for TGF-β. Finally, we will use an inhibitor of TGF-β to confirm Treg it is the major pathway involved in G-MDSC induced Treg differentiation. The final analysis will be done using FlowJo software and SAS for statistical analysis (ANOVA with Tukey HSD and student T-tests).

The ***innovation*** of testing this hypothesis is the ability of curcumin to promote TGF-β production from G-MDSCs, which has never been done. Investigating this role will aid in understanding the role of TGF-β in the etiology of PE. If curcumin is shown to promote TGF-β from G-MDSCs which promotes Tregs, this will provide further preliminary data to support the use of curcumin as a promoter of MDSCs in the prevention and treatment of PE. We expect that uterus and placenta G-MDSCs will have increased production of TGF-β and the ability to differentiate naïve T-cells into Tregs compared to G-MDSCs from non-curcumin fed controls.

The method proposed has minimal ***limits and alternatives*** due to well-established protocols for these studies. However, one major limitation may be that G-MDSCs share the same markers as neutrophils and currently it is not possible to distinguish them without microscopy and/or functional tests. Therefore, an alternative would be to add a functional suppression assay along with the T-cell differentiation assay. By conducting both assays we would be able to determine the suppressive ability of the G-MDSCs isolated and confirm they are G-MDSCs and not neutrophils.

4.5.6.5 Aim 2 Proposed Research Plan

Investigate the effect of curcumin on MDSCs as a biomarker of PE risk. Two *hypotheses* will be tested in this aim. Our *first hypothesis* is that there is a level of G-MDSCs found in the placenta, and blood (during 2nd and 3rd trimester) which will correlate with women who are at risk for pre-eclampsia. Current studies have shown an association between the presence of G-MDSCs and PE. However, it is unclear if both subtypes are involved during the early stages or if only the number of G-MDSCs change in PE. Additionally, it is not known if MDSCs can be used as a marker for the risk of PE. While this hypothesis does not include the consumption of curcumin, it is necessary to first understand the levels of MDSCs found in normal and PE women. To conduct this study, we will use a cohort of women which have been shown to have an increased risk of PE. To ensure that enough women are included in the study we will enlist the aid of a statistician and an epidemiologist. For this study we will focus on early-onset PE and PE diagnosis will be done during the first 20 wks of pregnancy based on the current recommendations for PE diagnosis [80]. Blood samples will be isolated from patients at 15, and 20 wks pre-diagnosis, during the second trimester, and placenta and blood samples will be collected at birth. These samples will be blindly analyzed by flow cytometry for MDSC subtypes. All data will be recorded in excel and statistical analysis will be done in SAS (logistic regression modalities and multivariate regression).

The *innovation* of testing this hypothesis is that the use of immune cells as a marker for PE has not been done. Additionally, current prediction models include that include multiple biomarkers are still only able to predict 80% of PE cases [80]. Therefore, the addition of MDSCs as another marker will aid in increased predictability in diagnosing PE. We expect that MDSCs will appear early (at gestation age 12 weeks) in normal pregnancy while women with PE will have significantly less or none at the same time points. Additionally, we expect that by birth we will be able to generate a time course estimation of the rates at which MDSCs increase in normal and PE pregnancies to use as a predictor of PE.

Within the proposed approach there are many *limits* that exist. For example, it may not be possible to recruit enough women that can be age and parity-matched to gain the needed power to achieve true significant differences. Therefore, an alternative would be to select women with a history of PE. Finally, 15 wks may not be early enough to detect a clear difference between the levels of MDSCs in normal and PE patients. As an alternative approach if not enough women are

able to be recruited for this study, we will collaborate with other groups across the country to achieve a larger population.

Our *second hypothesis* is that women who are at risk for PE and consume curcumin will have increased levels of MDSCs compared to those who do not. While only G-MDSCs have been associated with PE, the samples used were only from birth and may not represent the MDSC subtype response in early PE. Therefore, we will look at both MDSC subtypes. To test this hypothesis, we will collaborate with several hospitals across the United States to recruit enough subjects. As with our first hypothesis, we will utilize the help of a statistician to ensure the correct sample numbers needed. We will focus on recruiting low to middle-income women who are at high risk for PE. Subjects will be divided by those who consume curcumin regularly (at least 1/ wk) and those who do not. Throughout the pregnancy food questionaries' will assess whether the consumers maintain a minimal amount of curcumin intake. At birth, we will collect data on fetal weight and other standard outcomes of PE. Additionally, maternal blood, placental, and uterus samples will be collected and analyzed for MDSCs. These samples will be blindly analyzed by flow cytometry for MDSC subtypes. All data will be recorded in excel and statistical analysis will be done in SAS (logistic regression modalities and multivariate regression).

The *innovation* of testing this hypothesis is that no one has investigated curcumin consumption as a modulator of PE risk and its relationship to MDSC populations found at birth. It has been shown that PE patients have reduced G-MDSCs at birth compared to normal birth, but it is unknown if curcumin will alter the only G-MDSCs or both in the context of PE. Finally, if the consumption of curcumin is associated with an increase in MDSCs and reduction in PE-related birth effects, this information will be beneficial in recommending diets to women at risk of PE.

The *limits and alternatives* for this hypothesis are the same as those found in our first hypothesis of this Aim. However, due to the added dietary component of curcumin, we need to start with as large of the subject base as possible to increase our chances of recruiting enough subjects.

4.5.7 Conclusion for the use of Curcumin as an Immunomodulator in PE

The use of curcumin as an inducer of MDSCs during PE is an area devoid of research. Current studies have isolated several of the aspects we wish to combine in our Aims, such as dietary intervention, MDSCs biology, and PE. However, to date, no studies have attempted these

types of experiments. Specifically, the concept that MDSCs can act as a positive cell in different pathophysiologic conditions is extremely understudied. Additionally, if we view curcumin as an example of the potential of polyphenols, and to a larger extent phytochemicals, to modify immune cells, in combination with non-cancerous pathophysiological conditions, new studies will give us further insight into how phytochemicals may increase or decrease the functions of MDSCs.

4.6 Conclusion

In conclusion, more research is needed to understand the versatility of dietary agents on MDSCs in multiple pathophysiologic conditions. As was demonstrated, MDSCs are not always a negative cell for the host but should be viewed as any other cell of the immune system. When MDSCs are viewed as an active part of the immune system studies will increase to identify their role in multiple pathophysiologic conditions instead of the overwhelming focus on their role in cancer. The field of MDSC biology has come a long way in a short time but as new roles for MDSCs are discovered, the field should embrace and encourage research to better understand their roles outside of cancer. Finally, the role of phytochemicals as immunomodulators of MDSCs is still very young, in part due to the complexity of phytochemicals themselves, but the studies cited here provide evidence that the field is ripe for additional questions to be asked. We know that not all phytochemicals act in the same manner or target the same receptors, therefore another area to be developed is the role of phytochemical combinations on MDSCs in physiology and pathophysiologic conditions.

4.7 References

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CHAPTER 5. SUMMARY AND IMPLICATIONS

5.1 The Yin and Yang of MDSCs

In the presented chapters I have addressed basic biology of MDSCs as well as potential future studies to better understand the dichotomous role of MDSCs in pathophysiologic and physiologic conditions. MDSCs fit well into the concept/model of the Yin-Yang, as MDSCs serve both a positive and negative role depending on the specific condition in which they are found. The concept of using the Yin-Yang as a model for immune cells is not unique and has been used to describe M1/M2 macrophages as well as tumor-associated macrophages [1, 2]. Other immune cells could also be placed into the Yin-Yang model beyond macrophages and MDSCs. For example, the T helper 1/2 cells secrete cytokines to reduce the production of each other depending on the environmental signals resulting in pro- or anti-inflammatory responses. This model aids in defining the roles of immune cells as positive or negative to the condition. In physiologic conditions, MDSCs act as an important regulator of immune responses however, the role in which they are studied is when they are hijacked by cancer cells and promote immune escape. Therefore, placing MDSCs into the Yin-Yang model will aid in understanding their overall functional role in pathophysiologic and physiologic conditions.

The most studied role of MDSCs is in the context of cancer where they are considered a negative/target cell and therefore the Yin. In chapters two and three, MDSCs were discussed in this context to better understand specific aspects of their basic biology. While these chapters provide data, which will aid in the development of a better nomenclature and marking system, new question emerge about the biology of MDSCs as a negative/target cell. For example, at what stage of development should the field focus on to decrease the effects of MDSCs on the host immune systems ability to clear tumor cells?

The lesser studied side of MDSCs is the Yang or MDSCs as positive cells for the pathophysiologic or physiologic condition. However, while less studied it has been known for several years that MDSCs can aid in the reduction of autoimmune disorders, graft versus host disease and chronic inflammation [3, 4]. More recently, studies have shown that MDSCs are important for a successful pregnancy [5]. Together these conditions suggest that MDSCs do in fact have a positive role in physiology [6]. Therefore, new research should include looking for therapies

that will enhance and promote the development of MDSCs to aid in the treatment of specific conditions. However, a larger question comes to the surface as we place MDSCs in the Yin-Yang model, how do we move the field forward knowing MDSCs can play a dual role?

Moving forward, the field of MDSC biology needs to take a more systems approach to understand MDSC biology. This will include the addition of lifestyle and environmental variables to experimental designs. Adding these aspects will allow the field to better understand the interaction between diet, therapies, and successful treatments of various conditions. A major reason for this approach is that, as discussed in chapter one, dietary agents are known to influence immune cells as well as have large impacts on the overall physiology of an organism. One of the known alterations to immune cells is that dietary agents can change cell differentiation programming. Therefore, viewing MDSCs in the Yin-Yang model will allow us to look for treatments and dietary agents that will push MDSCs between Yin and Yang. Albeit, this can include altering the fate of MDSCs toward non-suppressive cell phenotypes, ie. no longer MDSCs. However, it can also mean promoting MDSCs via diet to enhance the functions and production of MDSCs as a form of treatment.

Placing MDSCs in the Yin-Yang model will have large implications on the overall field of MDSC biology and potential immunotherapies. First, the Yin-Yang model will allow for an increased number of published studies to be presented. Currently, in the context of cancer, if treatment fails to reduce tumor size or enhances MDSC function, it may be viewed as a negative result and not published. However, these “failed” treatments may be useful in the Yang of MDSCs. For example, resveratrol has been shown to increase the number of MDSCs during high does IL-2 treatment of cancer[7]. However, in this study, MDSCs aided in the reduction of T-cell induced autoimmune response at the lung due to high does IL-2. This study suggested a positive role for resveratrol for cancer treatment and posited that the IL-2 treatment superseded the resveratrol effect on MDSCs at the tumor site and it should be used with caution in the context of cancer. This, however, leaves open the concept that resveratrol may be beneficial in other conditions where MDSCs are good cells, ie graft versus host, chronic inflammation, and autoimmune diseases. Finally, while much research has been done to understand the negative effects of MDSCs, we know very little about their physiologic role. When MDSCs are seen as a normal regulatory cell of the immune system, many new opportunities arise to understand their role. For example, it has been hypothesized that MDSCs are part of the normal anti-inflammatory response, or part of the

completion of inflammation [6]. However, limited work to understand this has been conducted. Understanding the basic biological role of MDSCs may open up more possibilities to treat other pathophysiologic conditions as well as maintain a well-balanced physiologic immune system.

5.2 Identification of MDSCs and Functions: Defining Developmental stages of MDSCs

The identification of MDSCs has been convoluted since they were first discovered but refining this identity has not appeared to be a focus in the field. In the last fifteen years, only two papers have directly focused on establishing standard nomenclature and markers for MDSCs, the latest published in 2016 [8, 9]. The work done in chapter two and chapter three adds to the work of understanding the basic biology of MDSCs. Based on our data we conclude that the MDSCs found at the tumor site are functionally and phenotypically different than those found in the bone marrow or spleen. This has large implications for the field in basic MDSC biology and therapeutic development.

In studying the basic biology of MDSC development and function our data has opened up many new questions. For example, what is the full developmental path of MDSCs toward the tumor? Can MDSCs migrate directly from the bone marrow to the tumor site, or do they need to migrate to the spleen first? These questions can aid in understanding the development of MDSCs and present specific tissues in which to target MDSCs. Additionally, based on our data, it is not known if bone marrow and spleen MDSCs that reach the tumor site develop into the same cell type. These unknowns impact the current *in vitro* models used to create tumor-like MDSCs. Currently, *in vitro* methods to push bone marrow and spleen MDSCs toward a tumor-like MDSC phenotype are a major problem in MDSC studies as it is unknown if the multiple methods used produce the same cell. However, accepting the new model presented in chapters two and three would push the field toward rectifying this dilemma. To achieve this, the field should establish protocols that require the comparison of all *in vitro* derived MDSCs to those found at the tumor (or inflamed site) via multiple assays prior to their use as surrogates, ie. short term suppression and differentiation potential assays. By instituting these kinds of large-scale measures in the field we will gain better insight into the true nature of MDSCs from each tissue.

Through understanding the basic biology of MDSC function and markers, improved therapeutics can be developed to target specific stages of MDSCs. Two major types of therapeutic strategic models will arise from this; targeting precursor MDSCs and targeting tumor MDSCs

directly. Each of these strategies may involve different methods. For example, a therapy to target the cells at the bone marrow and spleen could involve altering their differentiation toward non-suppressive phenotypes or when MDSCs are a positive cell, increasing their development. Targeting at the tumor, or site of suppressive action may involve therapies to increase or decrease their suppressive ability or longevity. These methods will increase the chances of therapeutic success by acknowledging the true target cells.

Another major outcome from the work presented here is the possibility of using MDSCs as a marker of disease stage. However, current markers for MDSCs also identify other immune cells, ie. neutrophils, and monocytes. This is especially problematic for human studies as blood is the primary source used to isolate and count cells. To overcome this problem, we have proposed the use of multidimensional flow cytometry (MDF) analysis to detect differences between blood MDSCs and other immune cells. However, this will need to be tested and if successful, MDF analysis of MDSCs could be used as a diagnostic tool for disease stage. Taken together, the identification of MDSC developmental stages will improve our understanding of MDSC biology, improve therapeutic development, and provide the possibility of using MDSCs as a disease stage marker.

5.3 Phytochemical Regulation of MDSCs Function and Development

Understanding the role of dietary agents and phytochemicals to alter the immune system in pathophysiologic and physiologic conditions has made several leaps forward but is still in its infancy. While we have large bodies of work pertaining to some specific nutrients or phytochemicals, as discussed and summarized in chapter one and four (ie. vitamins D, A, E, folate, curcumin, and resveratrol), we have much less information on how these may be used as examples of phytochemical types i.e. polyphenols or phenolic acids. In chapter four, the focus was on curcumin as an example of using polyphenols to alter MDSC biology. However, two critical issues arise from that data. First, polyphenols represent a very large diverse group of molecules with very different chemical properties, and second, there are very few studies that directly test the impact of polyphenols on MDSCs. Therefore, more work is needed to better understand which molecules possess the potential to generally alter immune cells and then focus in on MDSCs. The impact of such work would aid in the development of dietary interventions as well as provide new molecules that serve as a starting point for drug development.

The proposed studies in chapter four provide a small example of the different types of studies needed to move phytochemical-immune interaction research forward. Most notably, translational models are needed to ensure that immune modification by phytochemical interventions will have beneficial effects in humans. Phytochemicals represent a unique challenge as they are metabolized creating additional molecules that may also have the potential to alter immune cell functions. Therefore, attempting to isolate each individual phytochemical and its metabolites would be unrealistic. However, finding phytochemicals that represent a body of molecules would reduce this problem and is suggested. Additionally, as stated previously, a systems approach will also be necessary and is suggested.

The potential of phytochemicals as adjuncts to treatments which target MDSCs represents has widespread implications. First, because phytochemicals are naturally occurring molecules, they have the potential to be much cheaper than designed drugs. Second, phytochemical use may reduce the use of drugs with severe side effects resulting in improved patient health during treatment. Third, further study of phytochemicals may support the use of phytochemicals in disease prevention. Together these implications justify the further study of phytochemicals as immunomodulators.

5.4 Final Conclusions

Taken together the work presented in this document provide data and future directions that will advance the multi-discipline field of MDSC biology. Increasing the ability to accurately define and isolate MDSCs will aid in defining and isolating other immune cells. Many of the same methods used to uncover the developmental stages of MDSCs can be applied to other cell types. Included in these methods is the concept of viewing immune cells in a Yin-Yang model to better understand how the cells can have seemingly opposing roles depending on the environment. Similarly, the use of MDF analysis is not limited to immune cells and may be useful in multiple other application where limited cell markers are available. Finally, the use of curcumin as a model of polyphenols as immunomodulators will increase the work on other other phytochemical classes. Increasing our knowledge of how different phytochemicals alter the immune system has the potential to alter drug design and therapies for multiple pathophysiologic conditions. Therefore, more work to understand the role of dietary agents as modulators of MDSCs in pathophysiologic and physiologic conditions needs done.

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Whitman College, Walla Walla, WA — May 2014

BA in Biochemistry, Molecular Biology, and Biophysics

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Teaching experience

Fundamentals of Nutrition (NUTR 31500),

Instructor, *Purdue University* — Summer 2019

- Responsibilities:
  - Communicated with Students via Online Forum
  - Updating course design for distance course
  - Grading

Physiology and Nutrition Through the Life Cycle (NUTR 36500),

Teaching Assistant, *Purdue University* — Spring 2019

- Responsibilities: 71 students
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  - Assisted students with course content during office hours
  - Lectured on adolescent growth cycle and nutritional requirements
  - Developed student project and rubric

Food Chemistry (NUTR 45300),

Teaching Assistant, *Purdue University* — Fall 2017

- Responsibilities:
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Nutritional Assessment (NUTR 43600),

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  - Assisted students outside of class

Food Chemistry (NUTR 45300),

Teaching Assistant, *Purdue University* Fall 2016

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  - Graded all Lab Assignments
  - Assisted students outside of class

General Chemistry (Chem 125-6),

Undergraduate Teaching Assistant, Whitman College Fall 2012-2014

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Chemistry of Art (Chem 102),

Undergraduate Teaching Assistant, Whitman College Fall 2012-2014

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The Guitar Spot, Guitar/Music Instructor 2006-2014

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Mentoring

New Graduate Teaching Assistants for NUTR 36500 Spring 2019

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Incoming Graduate Students in Research Laboratory (4 students) 2015-2018

Fellow Graduate Students in Teaching Certificate Opportunities at Purdue	2016-2019
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Honors and awards

Teaching Academy Graduate Teaching Award	April 2019
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Research Experience

Phytochemical and Dietary Modulation of Myeloid Derived Suppressor Cells in Pathophysiologic and Physiologic Conditions	2018-2019
Vitamin D as a Modulator of Myeloid Derived Suppressor Cells in Cancer	2014-2018
Development of Food Chemistry Laboratories for the Home Kitchen	2016-2017
Nutritional Education Survey of Walla Walla Physicians	2013-2014

Publications

R. Calvert, J. Fleet, P. Fournier, P. Juarez, G. Burcham, J. Haverkamp, T. Guise, T. Ratliff, and B. Elzey, *Monocytic Myeloid Derived Suppressor Cells from Tumor are a Differentiated Cell with Limited Fate Plasticity*, Journal of Leukocyte Biology, in review.

Calvert RD, Fleet JC, Chen Y, Pothen A, Rajwa B, Fournier PG, Juarez P, Guise TA, Ratliff TL, Elzey BD. Abstract 4741: *Monocytic myeloid derived suppressor cells (M-MDSC) from spleen are multipotent while tumor M-MDSC have limited plasticity*. 2018;78(13 Supplement) J Cancer Research. *poster*

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Posters and Presentations

Presentations

R.Calvert and M. Allsopp, *The Early Bird Gets the Feedback: Lessons Learned from First-Time Instructors Following Formative Student Evaluations*, Lilly Conference-Designing Effective Teaching. 2019, Bethesda, MD.

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Calvert RD, Burcham GN, Ratliff TL, Fleet JC. *Myeloid Derived Suppressor Cells (MDSC) Are Vitamin D Targets and 1α, 25 Dihydroxyvitamin D (1,25(OH)2D) Inhibits their Ability to Suppress T Cell Function.* Experimental Biology: American Societry of Nutrition 2017, Graduate Student Research Award Oral Competition

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Posters

R. Calvert, C. Andoline, and C. Running. *Thinking outside the bench: Teaching chemistry of foods in kitchens.* Institute of Food Technology conference 2019

D. Cladis, C. Andoline, **R. Calvert**, and C. Running. Demonstrating protein functionality in foods: an innovative teaching lab for students in any setting. Institute of Food Technology conference 2019

C. Andoline, **R. Calvert**, and C. Running. *A teaching laboratory on the physical and chemical properties of sweeteners.* Institute of Food Technology conference 2019

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