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Abstract

Developing Therapies to Overcome Immunosuppressive Myeloid Cells in the Tumor Microenvironment

Justine Ceradoy, Master of Science, 2018

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Myeloid cells in the tumor microenvironment represent significant barriers to the development of successful cancer immunotherapies. A multi-kinase inhibitor, Regorafenib (Reg), and a DNA-PK inhibitor, NU7441 (NU) were shown in a previous study to reduce expression of immunoinhibitory proteins in adaptive immune cells while increasing stimulatory MHC-I on cancer cells. In this study, we explored whether these drugs could reverse the suppressive activity of myeloid-derived suppressor cells (MDSCs) and alternatively activated macrophages. To test this idea, we used splenocytes from tumor-bearing mice and a human monocytic cell line differentiated into suppressive macrophages and assessed Arginase activity, their ability to suppress effector T cells, and mRNA expression of immunosuppressive and activating markers. We showed that Reg/NU decrease arginase activity and increase immunoactivating markers. These data demonstrate that treatment of suppressive myeloid cells with Reg/NU confers a less suppressive phenotype and leads to the generation of a more activating phenotype.

Developing Therapies to Overcome Immunosuppressive Myeloid Cells in the Tumor Microenvironment

by
Justine Ceradoy

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List of Abbreviations

CAR – Chimeric Antigen Receptor

CTLA4 – Cytotoxic T Lymphocyte Associated Antigen 4

DAMP – Damage Associated Molecular Pattern

DC – Dendritic Cell

DC-SIGN – Dendritic Cell Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin

DNA-PK – DNA Dependent Protein Kinase

EGF – Epidermal Growth Factor

IDO – Indoleamine 2,3 Dioxygenase

IFNγ – Interferon Gamma

MAPK – Mitogen Activated Protein Kinase

MDSC – Myeloid Derived Suppressor Cell

MHC – Major Histocompatibility Complex

MHC I – Major Histocompatibility Complex Class I

M-MDSC – Monocytic Myeloid Derived Suppressor Cell

MMR – Macrophage Mannose Receptor

NK – Natural Killer

NO – Nitric Oxide

NU – NU7441

PBMC – Peripheral Blood Mononuclear Cell

PD1 – Programmed Cell Death Protein 1

PDGF – Platelet Derived Growth Factor

PDL1 – Programmed Death Ligand 1

PGE_2 – Prostaglandin E2

PI3K – Phosphatidylinositide 3-Kinase

PMA – Phorbol 12-Myristate 13-Acetate

PMN-MDSC – Polymorphonuclear Myeloid Derived Suppressor Cell

Reg – Regorafenib

ROS – Reactive Oxygen Species

TAM – Tumor Associated Macrophage

TCM – T Cell Media

TCR – T Cell Receptor

TGFβ - Transforming Growth Factor Beta

TNFα – Tumor Necrosis Factor Alpha

T_{regs} – Regulatory T Cells

VEGF – Vascular Endothelial Growth Factor

α-MSH – Alpha-Melanocyte-Stimulating Hormone

Chapter I: Background and Introduction

Tumor Immunology

One of the "enabling characteristics" of cancer, as defined in the Hallmarks of Cancer, is the development of genomic instability in tumor cells. This instability leads to the generation of random mutations throughout their genome (Hanahan and Weinberg, 2011). Considering that one of the core functions of the immune system is to recognize foreign entities in the body, it is reasonable to imagine that as tumor cells become more unrecognizable as 'self', they also become prime targets for the immune system.

As these tumor cells mutate, they express antigens derived from various sources, including proteins that become over-expressed, viral-derived proteins, as well as proteins that arise from mutations in cancer known as neoantigens. These antigens from dying tumor cells or cells being killed by antigen presenting cells are released into the tumor microenvironment and processed by dendritic cells (DCs). DCs engulf antigens, process antigens into peptides which are then presented on their cell surface via major histocompatibility complex (MHC) molecules. These small antigenic peptide-MHC complexes are referred to as epitopes. When the DCs travel through the lymphatic system, they encounter T cells in lymphoid organs. If these T cells have T cell receptors (TCRs) that recognize epitopes on DCs, they become activated. The T cells are now able to traffic to the tumor, where tumor cells will present antigens on MHC on their cell surface that can be recognized by the newly-activated T cell's TCR. This will ultimately result in the killing of the cancer cell and the release of more antigens (Chen and Mellman, 2013). Additionally, NK cells can eliminate tumor cells without needing the neoantigen in the

context of MHC (Ventola, 2017). The idea of the immune system scanning the body for malignant cells and eliminating them is called immunosurveillance (**Figure 1**).

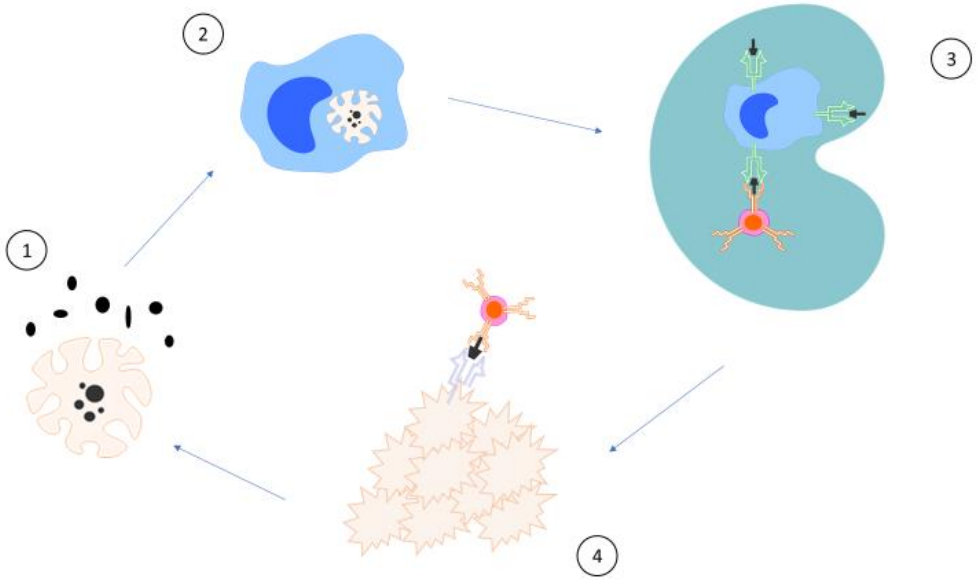

Figure 1: Tumor Immunity. A schematic of how tumor cells are recognized and eliminated by the immune system. **1)** Dying tumor cells are engulfed by APCs. **2)** Phagocytic APCs clean up the necrotic cells and process the antigens. **3)** APCs encounter T cells in lymphoid organs, present antigen, and activate them. **4)** Activated T cells travel through the blood vessels to the tumor to recognize and kill tumor cells, thus releasing more neoantigens.

The major goal in cancer immunotherapy is to induce a potent and long-lasting anti-tumor response, primarily through the activation of tumor-specific T cells. One approach to inducing such a response is through cancer vaccines. These function much like regular vaccines to induce an immune response, but rather than contain viral matter, they contain whole cancer cells, parts of cancer cells, or purified antigens (Karlitepe et al., 2015). Adoptive T cell therapy (ACT) represents another method by which to treat cancer patients. In ACT, tumor-reactive T cells are collected from the patient's tumor or blood and then stimulated *ex vivo,* so they can grow and expand. These cells are reintroduced into the patient where they have been shown to mediate a potent anti-tumor response (Perica et al., 2015). The idea of using tumor-specific T cells has developed greatly with the idea of using

genetic engineering to produce an ideal anti-cancer T cell. Chimeric antigen receptor (CAR) T cells are T cells whose TCRs have been modified. The extracellular domain resembles the variable region of an antibody that can recognize an antigen directly with no need for an MHC to present it, and the intracellular domain contains signaling motifs required for T cell activation (Rosenberg and Restifo, 2015). However, no matter what mechanism is used to activate the immune system against the cancer, it will be all for naught if the tumor is able to dampen the response.

Soluble factors in the tumor microenvironment are also able to dampen the anti-tumor response. Cancer cells, as well as other cells in the tumor microenvironment, express indoleamine 2,3 dioxygenase (IDO), an enzyme that catabolizes tryptophan (Munn and Bronte, 2016). This reaction depletes tryptophan necessary for T cell function and proliferation (Frumento et al., 2002) and creates a metabolite, kynurenine, that can promote the generation of T_{regs} (Mezrich et al., 2010). Immunosuppressive cytokines, such as IL-10 and Transforming Growth Factor Beta (TGFβ), also play a role in preventing an antitumor response. IL-10 can inhibit antigen presentation on dendritic cells and macrophages, thus indirectly preventing activation of T cells (Landskron et al., 2014). TGFβ has been found to prevent the accumulation and function of tumor-specific CD8 T cells (Chou et al., 2012). Reactive oxygen and nitrogen species can also prevent T cells from infiltrating the tumor and are toxic at certain levels (Anderson et al., 2017).

There exist multiple mechanisms of suppression in the tumor microenvironment. These mechanisms pose as a great barrier to the development of robust and sustained cancer immunotherapies. One such mechanism, which is a highly sought-after target in immunotherapy today, is the immune checkpoint axis. These are immunoinhibitory

pathways that normally prevent an overactive immune response. These mechanisms can be hijacked by tumors to avoid detection by tumor-specific T cells (Pardoll, 2012). The two most clinically relevant targets of the immune checkpoint pathways are cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1). Normally, CD28 on T cells binds to CD80/86 on APCs for costimulation, but CTLA4 on T cells can intrinsically compete with CD28 for binding to CD80/86 at the immunological synapse due to its higher affinity to both ligands (Rudd et al., 2009). Additionally, regulatory T cells (T_{regs}) express CTLA4, which can not only compete with T cells that may target the tumor for binding to CD80/86, but can also remove the ligand from APCs altogether through transendocytosis (Walker and Sansom, 2015). Whereas the inhibitory pathway of CTLA4 is involved in the initial activation of T cells, PD1 expression on T cells is induced upon activation and functions in the periphery to prevent their function in the peripheral tissue during inflammatory responses (Pardoll, 2012). This directly correlates to T cell function in the tumor microenvironment. The pro-inflammatory cytokine, interferon gamma (IFNγ), upregulates the expression of programmed death ligand 1 (PDL1) on the cell surface of tumor cells (Dong et al., 2002) and when PD1 engages its ligand, T cell proliferation and cytokine secretion is inhibited (Freeman et al., 2000). Antibodies against these inhibitory pathways have shown some level of clinical success. One anti-CTLA4 antibody, ipilimumab, was able to show an improved overall survival in patients with metastatic melanoma (Hodi et al., 2010). Another study observed that treatment with an anti-PD1 antibody, nivolumab, showed better progression-free survival than ipilimumab in melanoma patients and that a combination treatment with both

antibodies showed even higher rates of progression-free survival than each of the monotherapies (Larkin et al., 2015).

In the tumor microenvironment, there is a milieu of immunosuppressive cells that all work to prevent anti-tumor T cell effector function. T_{regs} function normally by suppressing immune responses to prevent autoimmunity and maintain self-tolerance (Sakaguchi et al., 2008). In cancer, their immunosuppressive function dampens anti-tumor responses to promote tumor progression (Chaudhary and Elkord, 2016). This subset of T cells uses many of the mechanisms of suppression that were described above, such as secretion of suppressive cytokines, IDO activity, and immune checkpoints, in order to suppress T cell activation and proliferation, but they can also do so through consumption of IL-2 (Chaudhary and Elkord, 2016). T_{regs} also have the ability to crosstalk with other populations in the tumor microenvironment, such as the immunosuppressive cells of the myeloid lineage, to sustain this pro-tumorigenic microenvironment (Lindau et al., 2013). This thesis will focus on these suppressive myeloid cells and will describe them in further detail in the next section.

Myeloid Suppressors

Among the cells of the myeloid lineage that function to suppress the immune response are Myeloid Derived Suppressor Cells (MDSCs). This population of cells is not a distinct lineage, but rather a classification of a heterogeneous group of immature myeloid cells that suppress both innate and adaptive immune responses (Parker et al., 2015). Chronic inflammation and the presence of pro-inflammatory cytokines such as IL-1β, IL-6 and Prostaglandin E2 (PGE_2) lead to MDSC formation and suppressive function, as well as other cytokines, transcription factors, and damage-associated molecular patterns

(DAMPs) (Parker et al., 2015). The heterogeneity of MDSCs can be attributed to the wide range of stimuli that can affect their development.

Despite their heterogeneity, MDSCs have been characterized as Gr-1^{+}CD11b^{+} in mice (Peranzoni et al., 2010). In humans, CD33 is a myeloid marker often used to identify MDSCs, but CD33 expression is not exclusive to MDSCs (Bronte et al., 2016). MDSCs can be further classified into two distinct subsets: polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). As their names suggest, PMN-MDSCs resemble neutrophils while M-MDSCs have a similar morphology to monocytes (Movahedi et al., 2008). These subtypes even have different cell surface markers to discern them: PMN-MDSCs are CD11b^{+}Ly6C^{lo}Ly6G^{+} in mice and CD14^{-}CD11b^{+}CD15^{+} in humans, and M-MDSCs are CD11b^{+}Ly6C^{hi}Ly6G^{-} in mice and CD14^{+}CD11b^{+}CD15^{-}HLA-DR^{lo} (Bronte et al., 2016).

MDSCs prevent T cell activation and effector function primarily through the depletion of arginine, an essential amino acid for T cells, by arginase, and the creation of reactive oxygen species (ROS) and nitric oxide (NO) through NOX and iNOS, respectively (Ostrand-Rosenberg and Fenselau, 2018; Parker et al., 2015). Reactive oxygen species can not only kill T cells through oxidative stress, but can also decrease their cytokine production and expression of CD3ζ, rendering them less functional as effector cells (Parker et al., 2015). NO can interfere with T cell IL-2 signaling through the inhibition of various signaling molecules in the JAK/STAT pathway (Mazzoni et al., 2002). Reactive oxygen species and NO together can form peroxynitrite, which can nitrate the TCR or the MHC:peptide complex, thus disrupting the immunological synapse as well as T cell function (Lindau et al., 2013). MDSCs can also suppress T cells by depleting other amino

acids necessary for function such as tryptophan–via the enzymatic function of IDO–and cysteine, the latter of which they cannot produce *de novo* (Parker et al., 2015). MDSCs are also able to use some of the other suppression mechanisms that have previously been described, such as expression of PD-L1, production of anti-inflammatory cytokines like IL-10 and TGFβ, and recruitment, induction, and activation of T_{regs} (Huang et al., 2006; Noman et al., 2014). These suppressive cells can even inhibit T cell migration: ADAM17 expression on the cell surface of MDSCs cleaves L-selectin on naïve T cells, preventing their migration to lymph nodes, and NO production decreases E-selectin expression, obstructing the infiltration of T cells to the tumor site (Parker et al., 2015). Due to their many suppressive mechanisms, MDSCs pose as a great threat to anti-tumor immunity.

Macrophages in the tumor microenvironment can also promote tumor progression and immunosuppression. Much like MDSCs, macrophages are phagocytic myeloid-derived cells that are involved in innate immunity. These cells are derived from monocytes circulating in the blood and mature into macrophages in the tissues where they will reside (Davies et al., 2013). Also, like their MDSC counterparts, their development is highly dependent on the signals received from the tissue milieu. In fact, macrophages can be polarized into two different subtypes: M1, or classically activated macrophages, and M2, or alternatively activated macrophages (Mantovani et al., 2004). As their name suggests, M1 macrophages are involved in type I immune responses while M2 macrophages are involved in type II immune responses (Italiani and Boraschi, 2014). In fact, because macrophages take up and present antigen to T cells, polarization of macrophages into M1/M2 can help determine whether a Th1 or Th2 response occurs (Mills et al., 2000). Therefore, macrophages are a major key in bridging innate and adaptive immunity and

macrophage polarization is necessary to direct these responses. It is important to note, however, this dichotomy is not perfect and there are some complexities in macrophages phenotypes (Chávez-Galán et al., 2015).

Classically activated macrophages form in the presence of IFNγ. LPS, and/or Tumor Necrosis Factor Alpha (TNFα) and produce cytokines such as IL-6, IL-23, and IL-12 to promote a proinflammatory immune response against pathogens (Mantovani et al., 2004; Tariq et al., 2017). This phenotype is also associated with higher antigen presentation, production of NO and reactive oxygen species tissue damage, and activation of the transcription factor IRF5 (Davies et al., 2013; Krausgruber et al., 2011; Nielsen and Schmid, 2017). Alternatively activated macrophages, on the other hand, form in the presence of IL-4, IL-10, IL-13, glucocorticoids, and/or immunoglobulin complexes. Macrophages that have been polarized into this subtype are involved in anti-inflammatory responses, tissue remodeling, angiogenesis, and wound healing (Mantovani et al., 2004; Tariq et al., 2017). Additionally, M2 macrophages demonstrate some of the immunosuppressive functions that MDSCs have, such as increased arginase activity and IL-10 production (Lu et al., 2015).

The differential roles M1 and M2 macrophages have in normal immune responses come into play during tumor development and antitumor immune responses M1 macrophages, for example, are usually regarded as tumoricidal. Their high expression of MHC allows for presentation of tumor antigens and can lead to the activation of an antitumor response (Chanmee et al., 2014). Additionally, tumor associated macrophages (TAMs) that resemble M1 macrophages interact with natural killer (NK) cells that produce IFNγ and target tumor cells (Zheng et al., 2017). However, chronic inflammation and the

subsequent production of substances such as NO and ROS can lead to the formation of a tumor microenvironment that promotes tumorigenesis, tumor growth, and tumor escape (Bui and Schreiber, 2007; Hanahan and Weinberg, 2011).

M2 macrophages, due to their immunosuppressive nature, are protumorigenic and are widely regarded as the TAMs in the tumor microenvironment (Tariq et al., 2017). M2-like TAMs recruit and stimulate the activation of T_{regs} as well as Th2 cells and MDSCs, effectively suppressing effector T cell function against tumors (Tariq et al., 2017; Zheng et al., 2017). Like their M1-like TAM counterparts, M2-like TAMs interact with NK cells, but rather than activate NK cells, they induce an exhausted phenotype through production of TGFβ (Zheng et al., 2017). These macrophages can also affect tumor growth more directly through the secretion of growth factors such as Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), and Platelet Derived Growth Factor (PDGF), and the promotion of angiogenesis (Tariq et al., 2017; Zheng et al., 2017). Additionally, TAMs help facilitate metastasis by releasing chemokines and cathepsins that promote adhesion to the extracellular matrix, producing VEGF to progress extravasation, and engaging tumor cells at the site of metastasis to enhance prosurvival signaling (Nielsen and Schmid, 2017). Though they differ, both suppressive macrophages and MDSCs alike contribute to the struggles clinicians and researchers have in finding promising results from immunotherapy treatment.

Clinical Relevance: Melanoma

Melanoma is a form of cancer that arises from the transformation of melanocytes, or pigmented cells rich with melanin, into malignant cells (Gray-Schopfer et al., 2007). Melanoma only accounts for 1% of skin cancers and has relatively high survival rates when diagnosed at early stages, but is one of the deadliest forms of cancer when detected later

(Domingues et al., 2018). In the United States, an estimated 9,320 people will die from melanoma in 2018, approximately one death every hour (American Cancer Society, 2018). Unlike other cancers, the number of new melanoma cases in the U. S. has increased each year with a 53% increase in the past decade (American Cancer Society, 2008, 2018).

One of the most contributing risk factors for melanoma is race and ethnicity. Skin cancer is predominantly diagnosed in non-Hispanic whites with an incidence rate of 26 per 100,000, whereas only 4 in 100,000 are diagnosed in Hispanics and 1 in 100,000 for blacks (American Cancer Society, 2018). However, survival rates for black patients are far lower than those of white patients with a 26% difference in five-year relative survival rates at all stages of melanoma, which is, in part, due to the fact that black patients are more likely to be diagnosed at advanced stages than white patients (Siegel et al., 2018). Great disparities in incidence rates are also prevalent when looking at age and gender: women have higher incidence rates before age 50, but men's incidence rates are double and triple that of women's by age 65 and 80, respectively (American Cancer Society, 2018). Other risk factors include family history, presence of atypical moles, and exposure to ultraviolet radiation from both indoor and outdoor tanning (American Cancer Society, 2018; Tolleson, 2005).

Before transforming into melanomas, melanocytes mature from differentiated neural crest cells during embryonic development and reside primarily in the skin, eye, inner ear, and leptomeninges, the covering of the brain and spinal cord (Domingues et al., 2018; Tolleson, 2005). Keratinocytes regulate the homeostasis and melanin production of melanocytes by secreting factors such as alpha-melanocyte-stimulating hormone (α-MSH) in response to ultraviolet radiation (Slominski et al., 2004). By producing melanin,

melanocytes help provide protection to the skin from ultraviolet radiation-induced damage; this explains why skin color and ability to tan are the most reliable predictors of melanoma risk (Lin and Fisher, 2007).

Mutations in regulatory genes in melanocytes can allow them to escape control by keratinocytes and lead to their proliferation, spread, and transformation. Earlier stages of transformation lead to the formation of benign nevi, also known as moles, which can then turn into dysplastic nevi that are more atypical in growth and appearance (Miller and Mihm, 2006). In the radial growth phase, also known as the primary malignant phase, melanomas progress horizontally in the epidermis (Gray-Schopfer et al., 2007; Miller and Mihm, 2006). Once melanomas reach the vertical growth phase, they begin to invade the dermis reticular dermis and at and eventually become metastatic, able to successfully spread and proliferate into other tissues and organs (Miller and Mihm, 2006).

Though, like other cancers, melanoma is a genetically complex disease, studies have given light to the signaling pathways crucial for its development and survival, giving way to possible targets for therapies. Two of the major pathways involved in the progression of melanoma are the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositide 3-kinase (PI3K) pathway. Both pathways are associated with survival and proliferation, and the PI3K pathway is also involved in metastasis, survival, and angiogenesis (Gray-Schopfer et al., 2007). As a result, when any protein involved in this pathway is mutated such that the pathway is constitutively active, the melanoma can thrive. The most common mutations in these pathways are in the *BRAF* gene, with 50-70% of melanomas having a BRAF mutation, the most common of which is the V600E mutation – a substitution of a glutamate for valine at the 600^{th} position that causes the

serine/threonine kinase BRAF, and therefore the MAPK/ERK pathway, to be constitutively active (Gray-Schopfer et al., 2007). Other commonly mutated genes in these pathways include *NRAS*, *AKT*, and *PTEN*. Unlike the other aforementioned genes, PTEN functions normally as an negative regulator, rather than an activating kinase or effector, and therefore loss of PTEN function, as it is in 5-20% of late-stage melanomas, leads to overactivity of the PI3K pathway (Gray-Schopfer et al., 2007).

The discovery of these essential pathways and common mutations in melanoma has naturally led to the development of targeted therapies to dampen the signaling and prevent the progression of the melanoma. As of now, there are no approved therapies that target the PI3K pathway for melanoma, but many are in clinical trials. Currently there are 4 approved therapies for melanoma that target the MAPK pathway: vemurafenib, dabrafenib, trametinib, and cobimetinib (Domingues et al., 2018). Vemurafenib, a selective BRAF-mutant inhibitor, has shown some promise. The drug has shown tumor regression in 90% of patients with the V600E *BRAF* mutation as well as improved response rates, progression-free survival, and overall survival (Domingues et al., 2018). Dabrafenib, the other approved selective BRAF-mutant inhibitor, has also had some success, causing regression of brain metastases in patients with the V600E mutation (Long et al., 2012). Despite the success of BRAF inhibitors, half of the patients treated with dabrafenib and vemurafenib showed disease progression 6-7 months after treatment due to the development of resistance to the drugs and approximately 20% of patients with BRAF mutations show intrinsic resistance to BRAF inhibitors, leading to unresponsiveness to treatment and relapse (Arozarena and Wellbrock, 2017; Long et al., 2012). To combat resistance to these BRAF inhibitors, MEK inhibitors such as trametinib and cobimetinib

became potential solutions due to their activity downstream of BRAF. These drugs have been approved to be used in combination with BRAF inhibitors and have improved overall survival and progression-free survival in BRAF-inhibitor naïve patients (Arozarena and Wellbrock, 2017; Domingues et al., 2018).

Despite some levels of clinical success, targeted therapies are not effective alone to combat melanoma, due to the heterogeneity of the disease and resistance mechanisms. Additionally, many patients show adverse effects in response to treatment. To maximize the efficacy of these targeted therapies while reducing adverse effects, researchers and clinicians alike have shown interest in using them in combination with other therapies. Combinations with immunotherapies have been of particular interest due to the immunomodulatory potential of BRAF and MEK inhibitors. Both BRAF and MEK inhibitors have shown upregulation of tumor antigen presentation (Hu-Lieskovan et al., 2015). Additionally, BRAF inhibitors alone have improved T cell effector function and homing to the tumor (Hu-Lieskovan et al., 2015). Therefore, not only can BRAF and MEK inhibitors directly affect the growth and spread of tumors, they also improve anti-tumor activity by the patient's immune system (Hu-Lieskovan et al., 2015). One study found that a combination of dabrafenib, trametinib, and adoptive cell transfer showed complete tumor regression and improved T cell infiltration and cytotoxicity. The same study also tested a combination of dabrafenib, trametinib, and anti-PD1 therapy and found that the combination improved antitumor activity compared to individual therapies and the BRAF/MEK combination alone (Hu-Lieskovan et al., 2015). Trials with combinations of targeted BRAF and MEK inhibitors and immunotherapies are still ongoing, but these early successes show a potential cure for those with advanced *BRAF* mutant melanomas.

Introduction to Project

A previous study in our lab used high throughput flow cytometry to identify compounds that could potentially be used to enhance the efficacy of immunotherapy (Tsai et al., 2017). Two lead therapies, Regorafenib (Reg) and NU7441 (NU) were selected based on their immunomodulatory potential. Reg is a multi-kinase inhibitor that can target various tyrosine kinases while NU is a DNA-dependent protein kinase (DNA-PK) inhibitor. Currently, Reg is FDA-approved for metastatic colorectal cancer, gastrointestinal stromal tumors, and hepatocellular carcinomas.

In the initial screening, both Reg and NU were able to decrease expression of PD-L1, a mechanism of immune suppression that has had clinical success as an immunotherapy target, while increasing expression of major histocompatibility complex class I (MHC I), an essential component of anti-tumor immunity, in a melanoma cell line. These drugs were able to reduce expression levels of other CD8 T cell immunoinhibitory proteins such as CD55, CD155, CD73, and CD271. In mice bearing B16 melanomas, treatment with Reg alone or in combination with immune adjuvants that activate T cells, reduced tumor growth kinetics in a CD8 T cell-dependent mechanism (Tsai et al., 2017).

Based on the immunomodulatory potential of Reg and NU, we hypothesize that treating tumor-derived myeloid cells with Reg or NU will differentiate them into cells that lose their T cell suppressive function and acquire an ability to activate T cells. This study aims to determine the functional, phenotypic, and genotypic changes that Reg and NU have on suppressive myeloid cells from melanoma mice models as well as macrophages derived from a human monocytic cell line.

Chapter II: Validating Differentiated THP-1 Monocytes as a Model

One of the models that we used during our studies was the THP-1 cell line. This is human monocytic cell line derived from acute monocytic leukemia (Tsuchiya et al., 1980). To differentiate these monocytes into macrophages, cells were treated with phorbol 12-myristate 13-acetate (PMA) for 48 hours. After PMA treatment, they were treated with either IFNγ for 48 hours to differentiate them into M1 macrophages or with IL-4 and/or IL-13 to differentiate them into suppressive M2 macrophages (Chanput et al., 2014). Before beginning our assays with Reg and NU, it was imperative to validate these monocytes differentiated with IL-4/IL-13 as a proper model for suppressive macrophages. This chapter will focus on the genotypic and phenotypic characterization of macrophages derived from the THP-1 cell line.

First, we investigated whether these monocytes resembled macrophages upon differentiation. Before differentiation, the monocytes are spherical and able to stay in a single cell suspension (**Figure 2A**). After 48 hours of PMA treatment, the cells become adherent and can stick to the bottom of the well of the cell culture plate, resistant to washes with media (**Figure 2B**). Once the cells are given cytokine, either IFNγ or IL-4, for 48 hours after PMA treatment, they become flatter and more elongated (**Figure 2C-D**). Therefore, there are some macrophage-like phenotypic changes after treatment with PMA alone as well as with cytokine treatment after PMA.

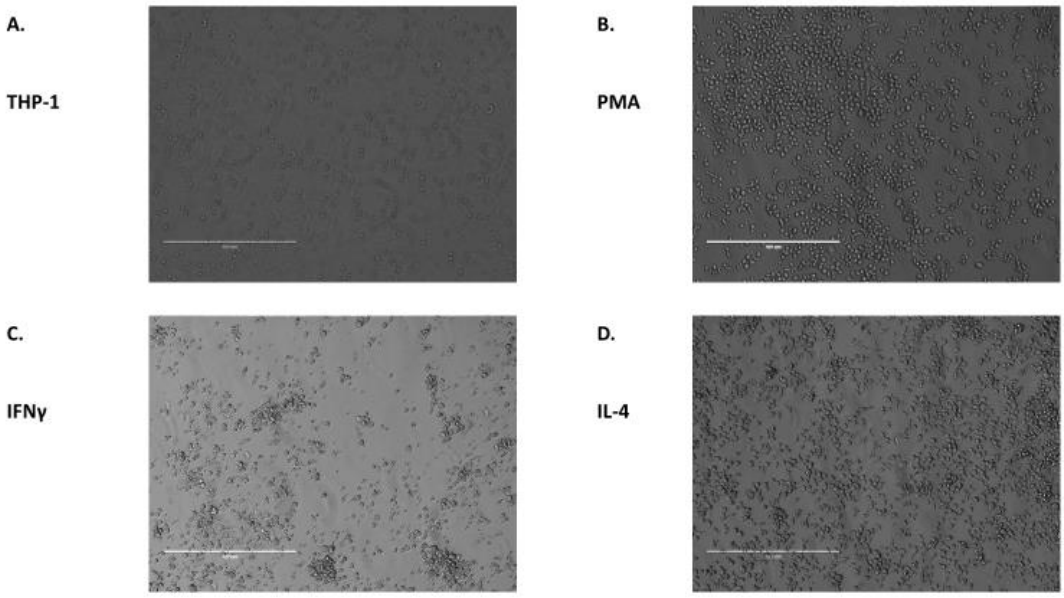

Figure 2: THP-1 Cell Morphologies. Microscopy images of THP-1 cells before PMA treatment (**A**), after 48 hours of PMA treatment (**B**), after 48 hours of PMA treatment and then 48 hours of IFNγ (**C**), and after 48 hours of PMA treatment and then 48 hours of IL-4 (**D**)

To determine whether the THP-1 monocytes that were differentiated using IL-4 could be called M2-like macrophages, we first assessed mRNA expression of markers associated with the M2 phenotype. CD206, also called the macrophage mannose receptor (MMR), is a marker for M2 macrophages (Rőszer, 2015). CD206 expression was not significantly different from baseline in THP-1 cells differentiated with PMA but was five times higher in THP-1 cells treated with IL-4 post-PMA treatment and six times higher in THP-1 cells that received both IL-4 and IL-13 (**Figure 3**).

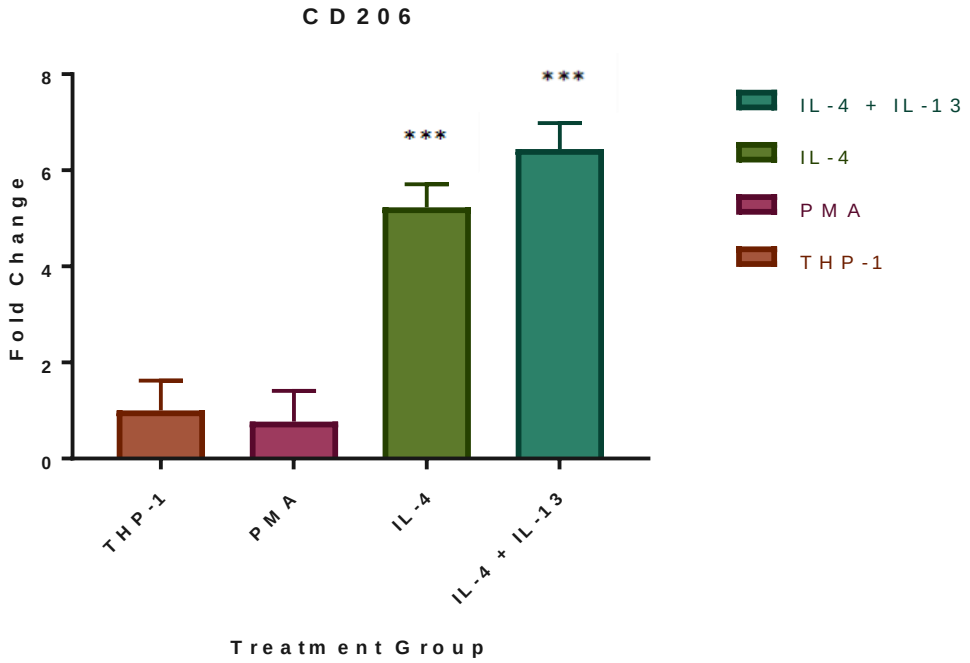

Figure 3: CD206 mRNA Expression in Differentiated THP-1 Cells. Expression of CD206 mRNA from RT-qPCR. Data is normalized to the average of triplicate reactions measuring GAPDH. Error bars represent standard deviation. * = $p<0.05$; **=$p<0.01$; ***=$p<0.001$ compared to control THP-1 group based on multiple t tests using Holm-Sidak for multiple comparisons.

CD209, also known as dendritic cell-specific Intercellular adhesion molecule-3-Grabbing nonintegrin (DC-SIGN), is a marker of dendritic cells, but is also present on certain tissue macrophages (Rőszer, 2015). Inflammatory signals that are associated with the M1 phenotype, such as IFNγ, decrease CD209 expression, while IL-4 increases CD209 expression (Relloso et al., 2002). PMA treatment increased CD209 expression six-fold compared to baseline, while IL-4 treatment increased expression more than twenty-fold and treatment with both IL-4 and IL-13 increased expression thirty-fold (**Figure 4**).

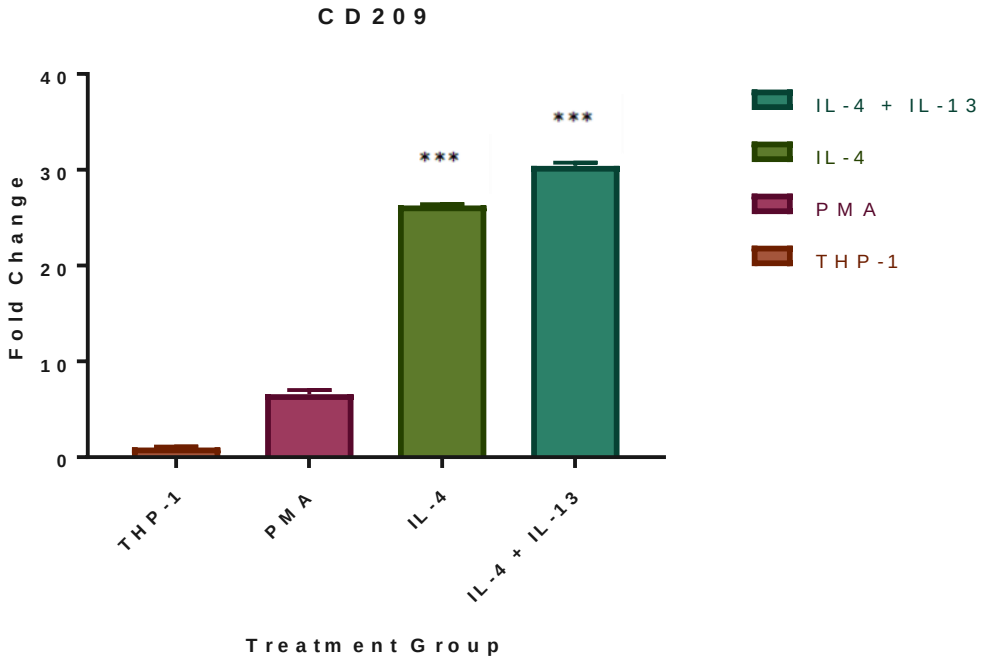

Figure 4: CD209 mRNA Expression in Differentiated THP-1 Cells. Expression of CD209 mRNA from RT-qPCR. Data is normalized to the average of triplicate reactions measuring GAPDH. Error bars represent standard deviation. * = $p<0.05$; **=$p<0.01$; ***=$p<0.001$ compared to control THP-1 group based on multiple t tests using Holm-Sidak for multiple comparisons.

Due to their anti-inflammatory nature, M2 macrophages secrete IL-10 (Mantovani et al., 2004). PMA increased IL-10 expression nearly eight-fold. IL-10 expression in IL-4 treated THP-1 cells increased seventeen-fold compared to baseline while THP-1 cells treated with IL-4 and IL-13 increased IL-10 expression ten-fold (**Figure 5**).

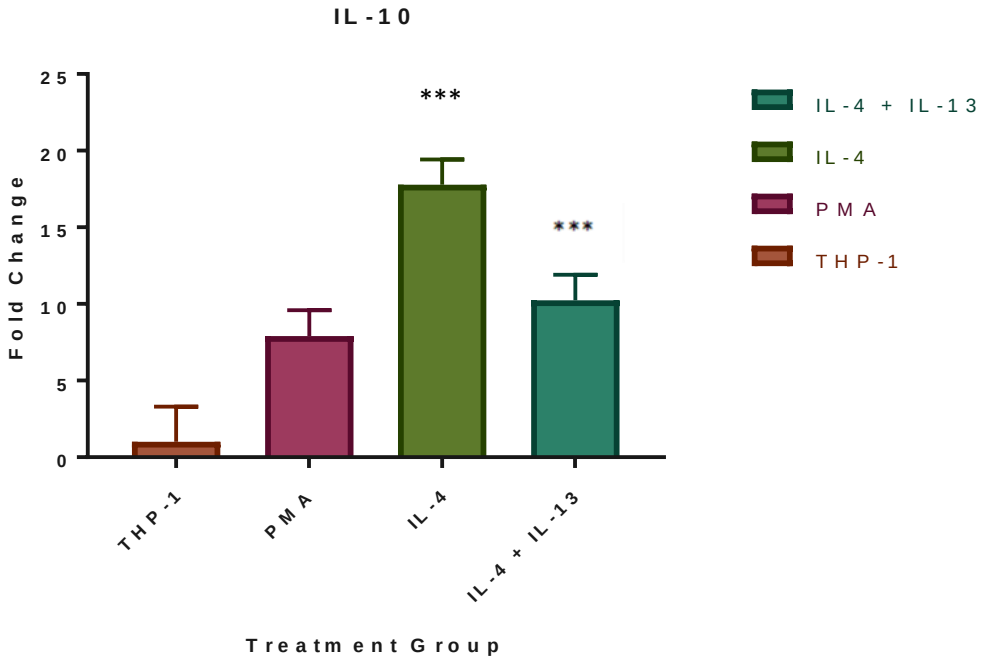

Figure 5: IL-10 mRNA Expression in Differentiated THP-1 Cells. Expression of IL-10 mRNA from RT-qPCR. Data is normalized to the average of triplicate reactions measuring GAPDH. Error bars represent standard deviation. * = $p<0.05$; **=$p<0.01$; ***=$p<0.001$ compared to control THP-1 group based on multiple t tests using Holm-Sidak for multiple comparisons.

To further investigate whether these treatment groups result in suppressive macrophages, we looked at surface expression of both CD206 and CD163 using flow cytometry. Some M2 macrophages that express CD206 also express CD163, a scavenger receptor (Rőszer, 2015). PMA treatment (**Figure 6B**) increased the number of single positive cells CD206 cell to 1.83% and the number of CD206+CD163+ to 0.50%, compared to 0.27% and 0.031% in untreated THP-1 cells, respectively (**Figure 6A**). Additionally, PMA treatment increased single positive CD163 expression from 0.014 in untreated THP-1 cells to 6.65%. IL-4 treatment increased both single positive CD206 expression, single positive CD163 expression, and double positive CD206+CD163+ expression to 3.49%, 4.11%, and 0.83%, respectively (**Figure 6C**). Combination IL-4 and IL-13 treatment showed similar results to the IL-4 treatment group with 3.90% of the cells

expressing CD206 alone, 3.04% cells expressing CD163 alone, and 0.65% expressing both CD206 and CD163 (**Figure 6D**).

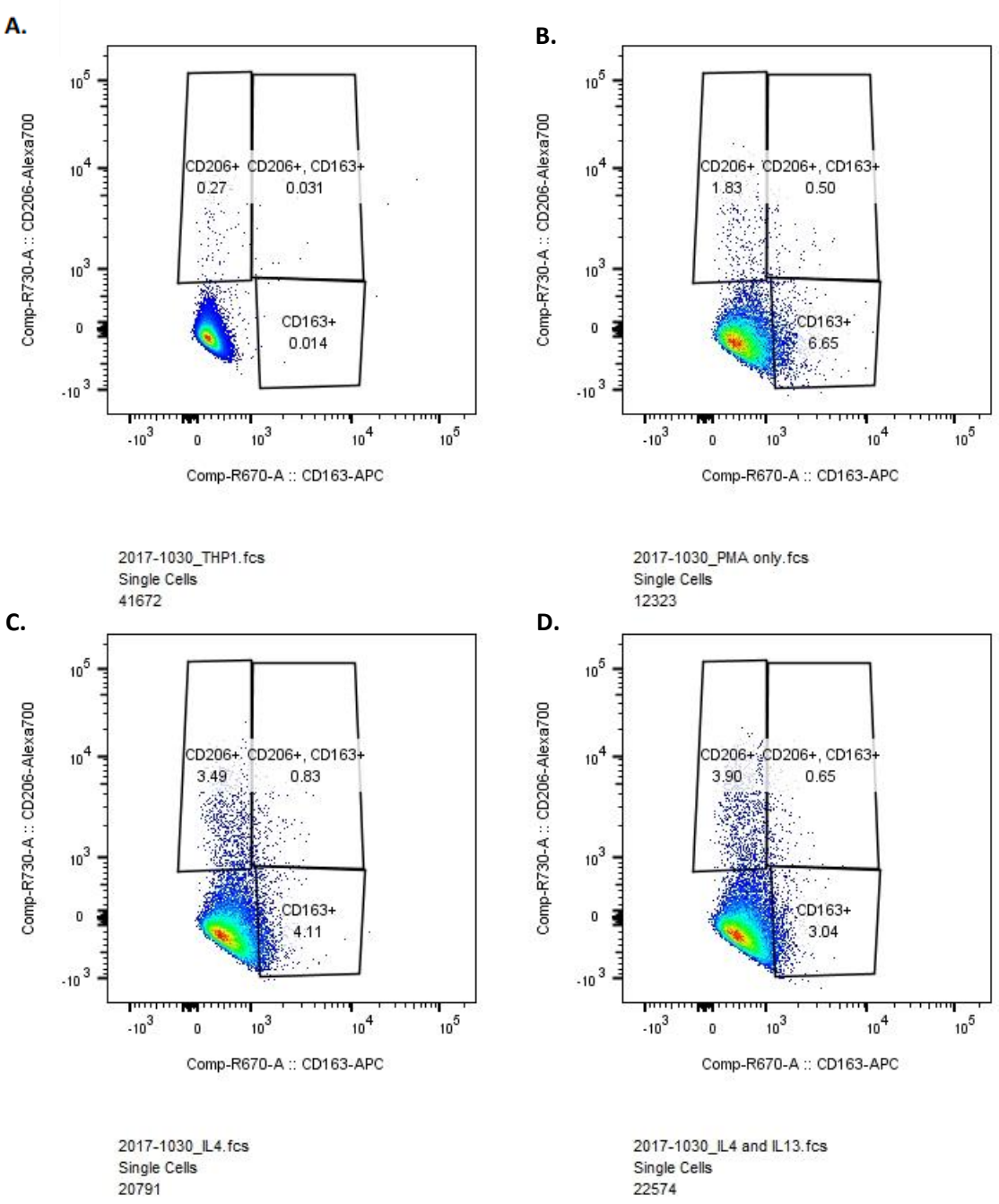

Figure 6: Surface Expression of CD206 and CD163 in Differentiated THP-1 Cells. Expression of CD206 and CD163 in THP-1 cells before PMA treatment (**A**), after 48 hours of PMA treatment (**B**), after 48 hours of PMA treatment and then 48 hours of IL-4 (**C**), and after 48 hours of PMA treatment and then 48 hours of IL-4 and IL-13 (**D**).

Based on these data, we concluded that the IL-4 and IL-4 + IL-13-treated THP-1 cells can serve as a model for suppressive macrophages for future experiments with our drugs. We also determined from these results that treatment with IL-4 was sufficient to produce a suppressive phenotype and proceeded to work only with IL-4.

Chapter III: Effects of Reg/NU Treatment on Myeloid Cells

This chapter will focus on the functional, phenotypic, and genotypic characterization of suppressive myeloid cells that have been treated with Reg or NU. The two models used are the IL-4-treated M2-like macrophages as described in the previous chapter as well as MDSCs derived from a B16 GM-CSF melanoma mouse (**Figure 7**).

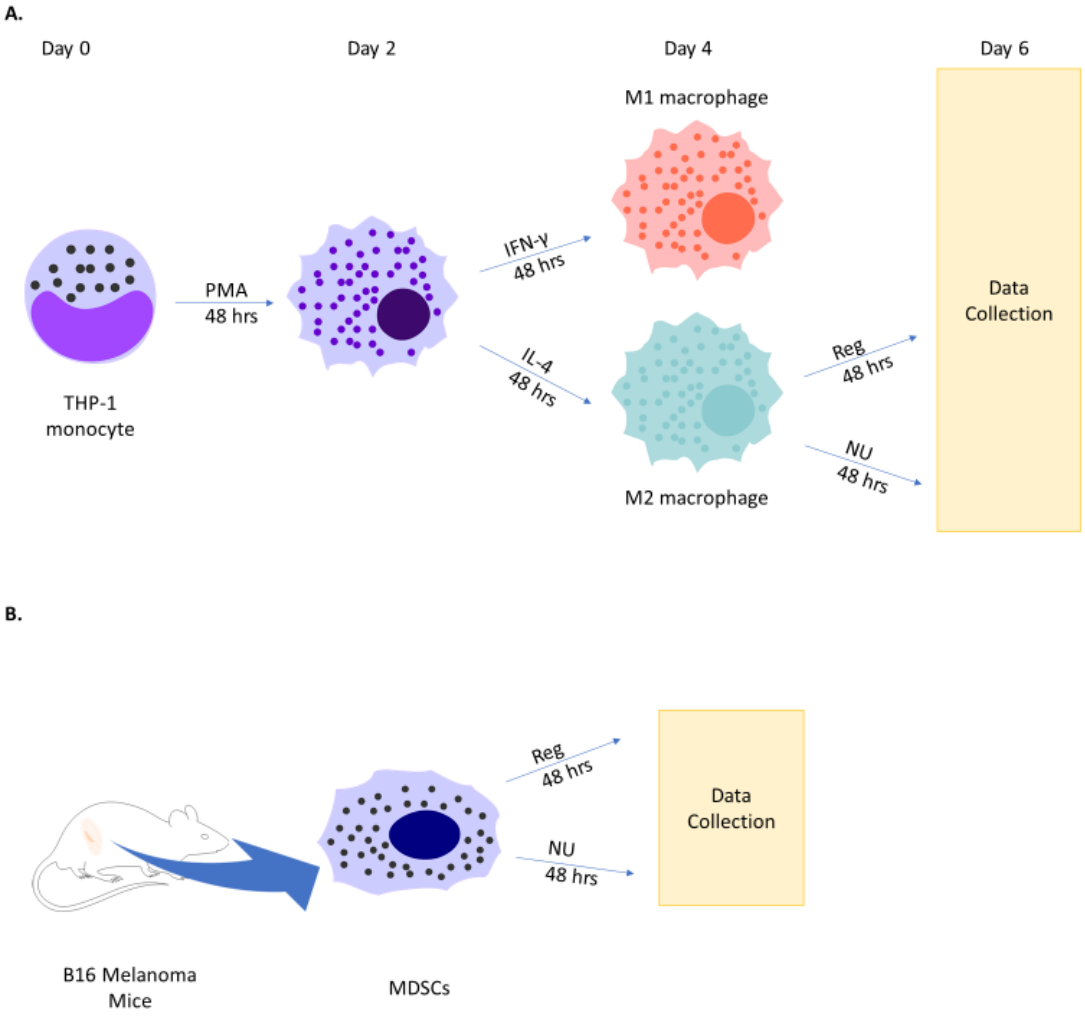

Figure 7: Experimental Design. A graphical representation of the human cell line (A) and melanoma tumor-bearing mouse (B) models used in this thesis. (A) THP-1 monocytes were differentiated into M1 and M2 macrophages as described in **Chapter II**, then the M2 macrophages were treated with Reg or NU for 48 hours. MDSCs were isolated from the spleen of mice with melanoma tumors and then treated with Reg, NU, or DMSO for 48 hours.

T cell suppression models

We wanted to explore the effect that Reg and NU have on effector T cell activation. For the mouse model, drug-treated splenocytes, of which 44.5% of cells were Gr-1+ (**Figure 19** in index), were co-cultured with murine CD8+ T cells labeled with eFluor450, a proliferation dye that gets diluted as cells proliferate, at various T cell to splenocyte ratios for 5 days. The T cells we used are TCR transgenic CD8+ T cells reactive towards an epitope expressed in the gp10025-33 protein expressed on melanoma. Alternatively, M2-like THP-1 cells were co-cultured with human T cells derived from the blood of a healthy donor. Mouse pmel T cells were activated by providing splenocytes gp100 as well as IL-2. For the human model, T cells from PBMCs were activated using anti-CD3 and IL-2. We were unable to optimize both models of suppression. The THP-1 model was too suppressive, showing high levels of eFluor450 staining even with the IFNγ-treated M1-like group (**Figure 8**). As for the mouse model, it was too activating, even at higher ratios of MDSCs (**Figure 9**).

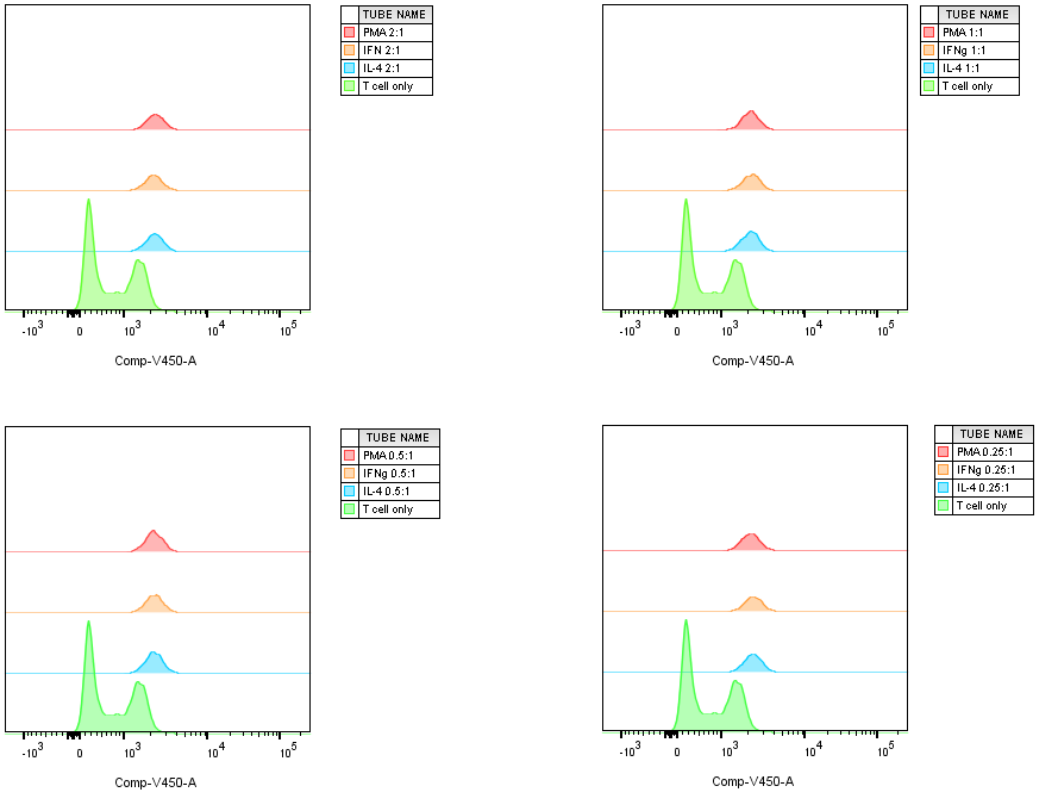

Figure 8: T Cell Suppression Assay – THP-1. Histograms of eFluor450 expression of CD8+ T cells from healthy human blood samples that were coincubated with differentiated THP-1 cells at various ratios, with a constant number of 500,000 T cells per well.

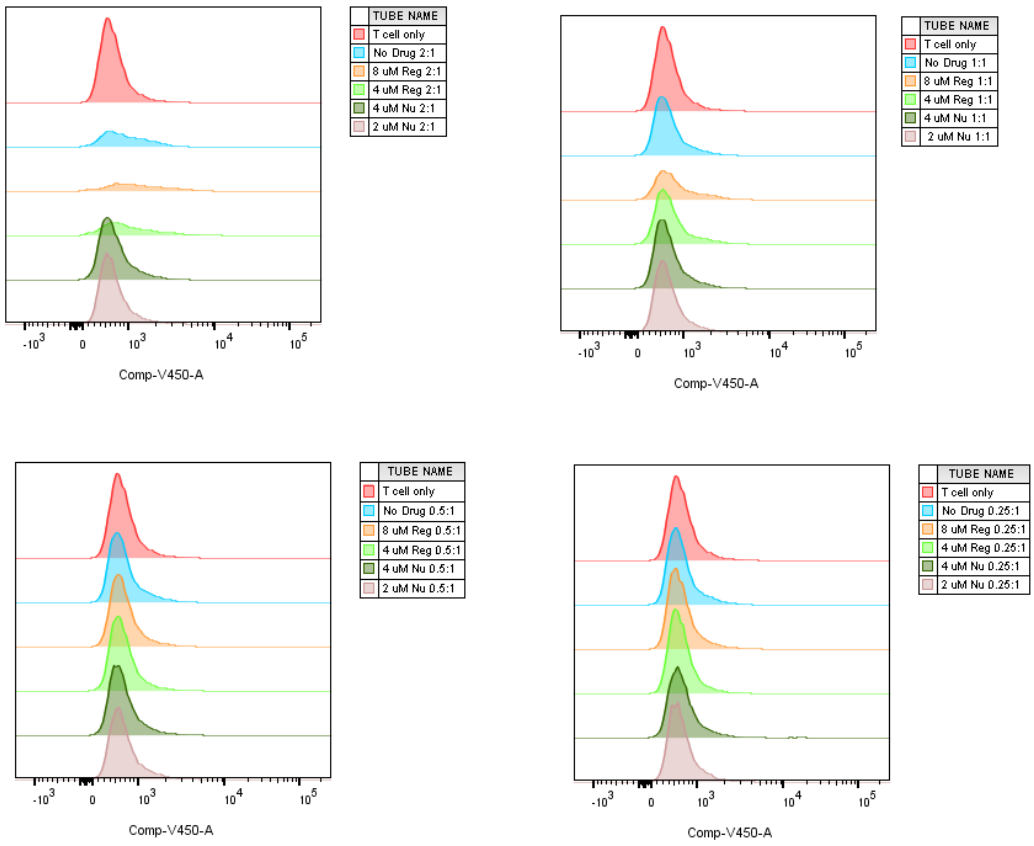

Figure 9: T Cell Suppression Assay – MDSCs. Histograms of eFluor450 expression of CD8+ pmel T cells that were coincubated with splenocytes derived from a B16 GM-CSF mouse at various ratios, with a constant number of 500,000 T cells per well.

Arginase activity in M2-like macrophages decreases after treatment with Reg/NU

Considering arginase activity is a suppressive mechanism shared by both MDSCs and M2 macrophages, we investigated the impact that treatment with Reg or NU had on arginase activity. Lysates were incubated with arginine substrate for both one hour and two hours. For all arginine assays in this thesis, there are no error bars because the assays were not performed in triplicate. This is for several reasons, one of which is the protocol by which the manufacturer provides. The protocol calls for 100 µL of lysate with 40 µL for the sample well and 40 µL for the sample blank well, both wells are used in the calculation of arginase activity. To deviate from the protocol would require some level of optimization, such as higher volume of lysate which may make it difficult to see differences between

treatment groups. Another reason for the lack of triplicates is the sensitivity of the assay, especially since it is a colorimetric assay. A number of things could affect the levels of activity that are calculated, including incubation time, insoluble precipitates, as well as whether the lysates are fresh or from -80ºC. In regards to incubation time, as seen in this section, there are nuances between the one-hour and two-hour incubation periods in terms of the activity levels, but overall the trends remain the same. Additionally, if a single well or row of wells received arginine slightly earlier than the other groups, that could make a difference. Ideally, high throughput pipetting would be used because it allows for the same exact incubation time for every single well. A replicate of this experiment utilized triplicates for the assay, however the lysates were frozen in -80ºC rather than freshly collected, which led to low levels of arginase activity across the board. Nonetheless, there is every intention to repeat these assays.

For the one-hour incubation period (**Figure 10**), undifferentiated THP-1 monocytes and PMA-treated THP-1 cells have relatively high baseline levels of arginase activity, with 21.89 units/L and 32 units/L, respectively. Differentiation with IFNγ decreased arginase activity to 10.26 units/L whereas differentiation with IL-4 increased arginase activity to 28.96 units/L. M2-like macrophages treated with 2 μM NU showed a decrease in activity relative to IL-4 treatment alone to 24.97 units/L and those treated with 4 μM Reg had an activity of 3.03 units/L.

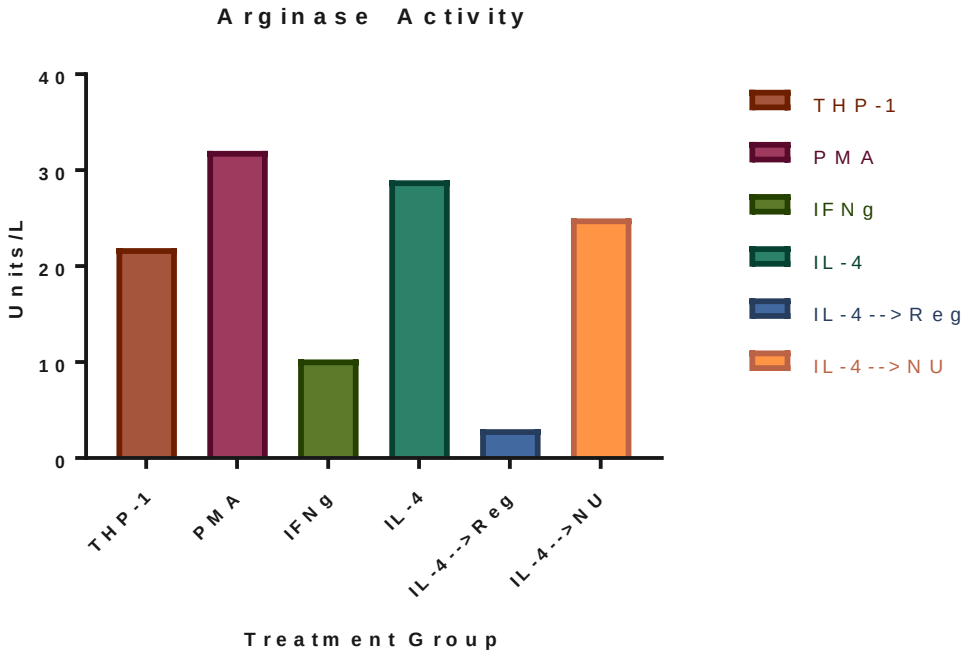

Figure 10: THP-1 Arginase Activity – 1 Hour Incubation. Arginase activity of differentiated THP-1 macrophages before and after treatment with 4 µM Reg or 2 µM NU.

For the two-hour incubation period (**Figure 11**), undifferentiated THP-1 monocytes and PMA-treated THP-1 cells still have high baseline levels of activity, with 12.87 units/L and 12.28 units/L of activity, respectively. Differentiation with IFNγ decreased arginase activity to 6.96 units/L whereas differentiation with IL-4 increased arginase activity to 16.58 units/L. NU-treated M2s showed a decrease in activity relative to IL-4 treatment alone to 11.55 units/L while those treated with 4 µM Reg had an activity of less than 1 unit/L.

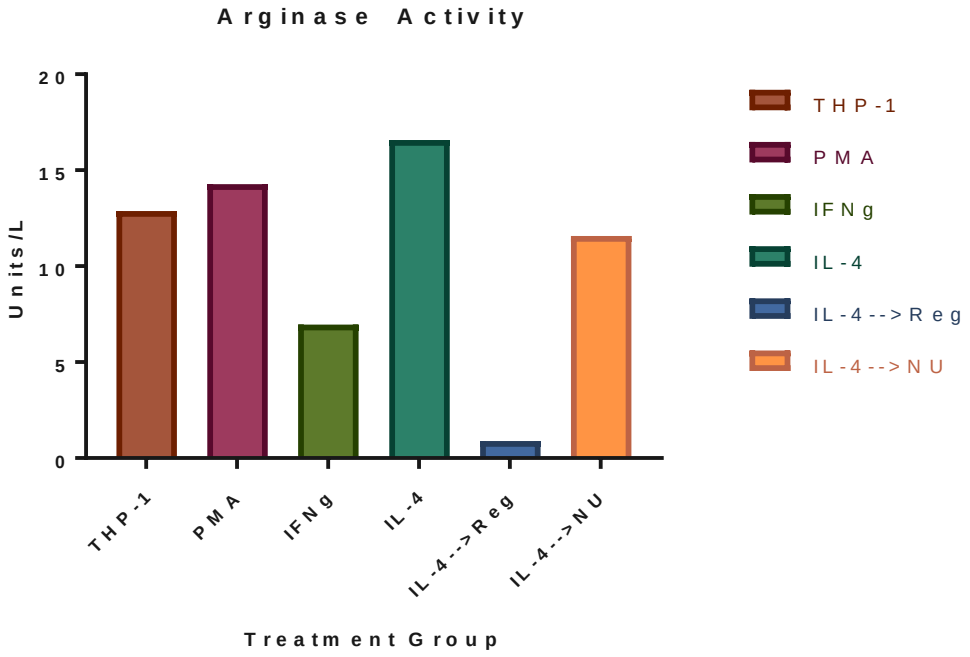

Figure 11: THP-1 Arginase Activity – 2 Hour Incubation. Arginase activity of differentiated THP-1 macrophages before and after treatment with 4 µM Reg or 2 µM NU.

mRNA expression of inflammatory markers increases in M2-like THP-1 cells after Reg/NU

Considering that treating M2-like THP-1 cells with Reg or NU decreases suppressive arginase activity, we sought to determine how drug treatment impacted the expression of inflammatory markers associated with the M1 phenotype. Whereas the suppressive activity of M2 macrophages are in part dependent on arginase activity, M1 macrophages' activity to activate T cells are more associated with iNOS activity (MacMicking et al., 1997). Treating M2-like macrophages with Reg increased iNOS expression six-fold and NU treatment increased iNOS expression three-fold, compared to untreated M2s (**Figure 12)**. Both drug groups had higher levels of iNOS mRNA expression than M1-like macrophages.

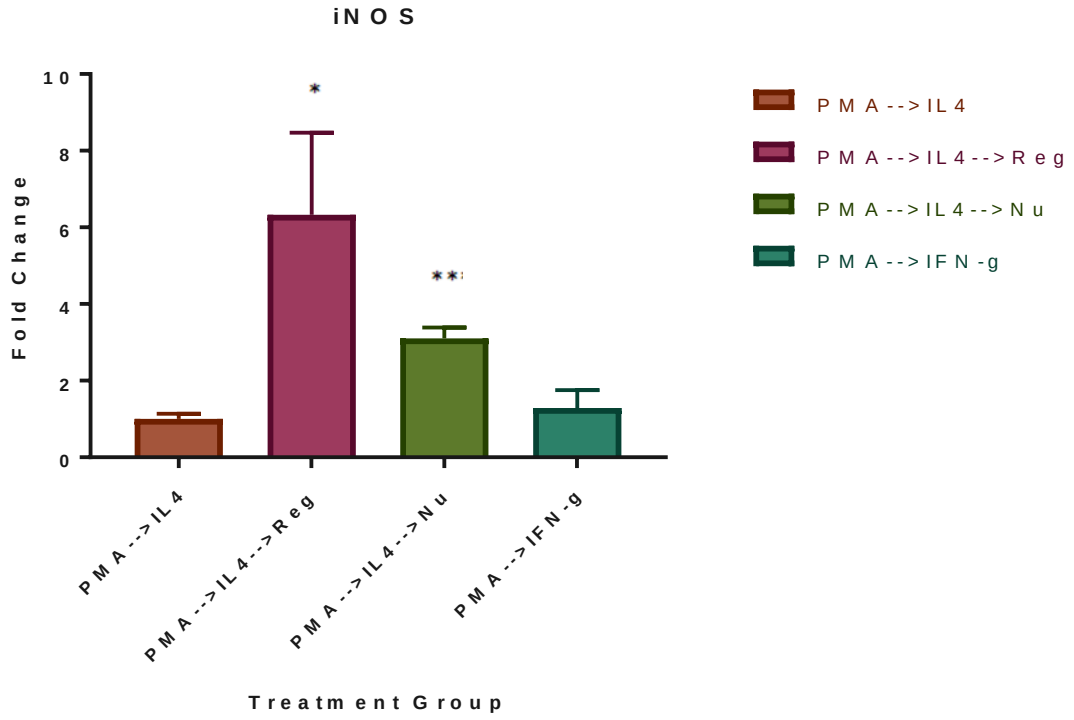

Figure 12: iNOS mRNA Expression in Drug-Treated THP-1 Cells. Expression of iNOS mRNA from RT-qPCR. Data is normalized to the average of triplicate reactions measuring GAPDH. Error bars represent standard deviation. * = $p<0.05$; **=$p<0.01$; ***=$p<0.001$ compared to control IL-4 group based on multiple t tests using Holm-Sidak for multiple comparisons.

CCR7 is a chemokine receptor for CCL19 and CCL21 whose expression increases after M1 polarization (Raggi et al., 2017; Yuan et al., 2015). CCR7 expression was 2.2 times higher in Reg-treated M2s and 3.62 times higher in NU-treated M2s, compared to M2s that did not receive drug treatment (**Figure 13**). These levels were similar to M1-like macrophages, which expressed CCR7 2.65 times higher than the M2-like macrophages.

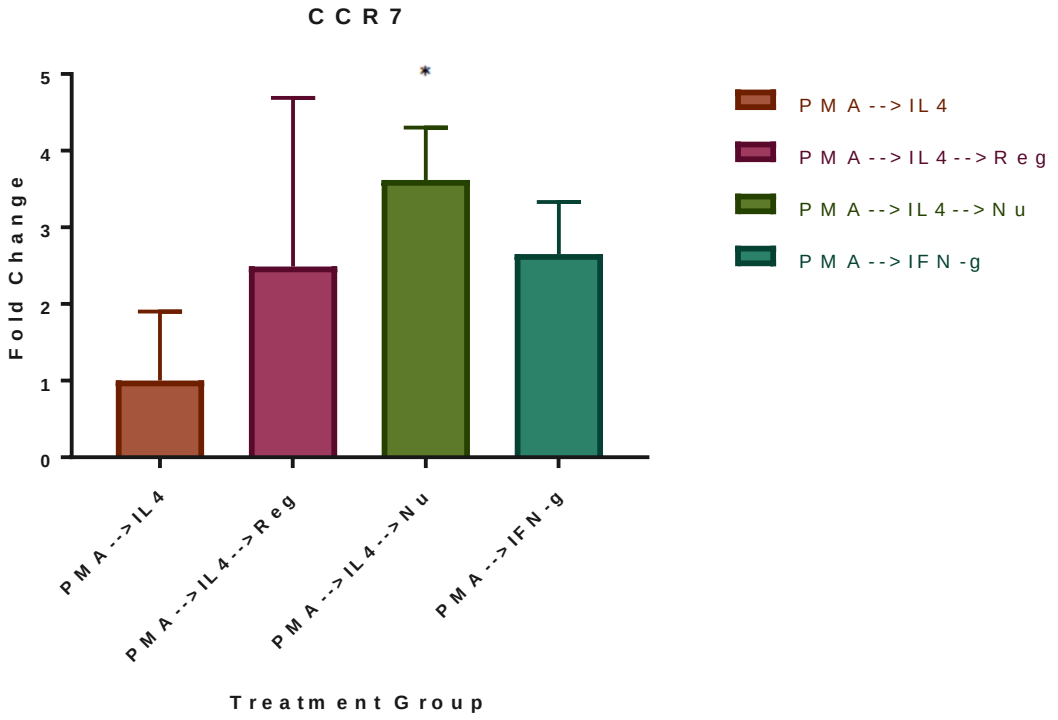

Figure 13: CCR7 mRNA Expression in Drug-Treated THP-1 Cells. Expression of CCR7 mRNA from RT-qPCR. Data is normalized to the average of triplicate reactions measuring GAPDH. Error bars represent standard deviation. * = $p<0.05$; **=$p<0.01$; ***=$p<0.001$ compared to control IL-4 group based on multiple t tests using Holm-Sidak for multiple comparisons.

NF-κB is a key transcription factor in M1 polarization and regulates a number of inflammatory genes, including COX2. Reg and NU treatment greatly increased COX2 mRNA expression to 27 and 28 times higher than that of untreated M2s, respectively. This is similar to M1s, whose COX2 expression was 26 times higher than that of M2s. (**Figure 14**).

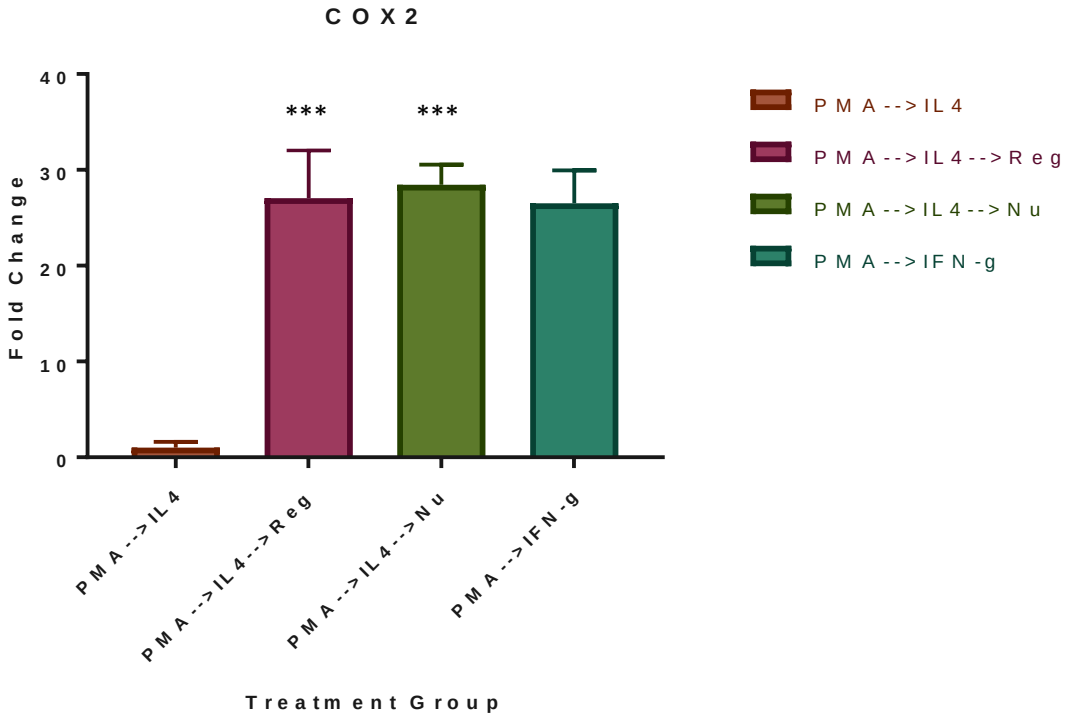

Figure 14: COX2 mRNA Expression in Drug-Treated THP-1 Cells. Expression of COX2 mRNA from RT-qPCR. Data is normalized to the average of triplicate reactions measuring GAPDH. Error bars represent standard deviation. * = $p<0.05$; **=$p<0.01$; ***=$p<0.001$ compared to control IL-4 group based on multiple t tests using Holm-Sidak for multiple comparisons.

The production of IFN-γ by macrophages has been a great debate within immunology (Frucht et al., 2001). This mechanism, if it does exist, would allow for autocrine regulation of M1 polarization. Treatment with Reg increased IFN-γ expression seven-fold compared to untreated M2s (**Figure 15**). Similarly, M1-like macrophages increased IFN-γ expression five-fold comparted to untreated M2s. However, NU greatly decreased IFN-γ expression in M2s to 14%.

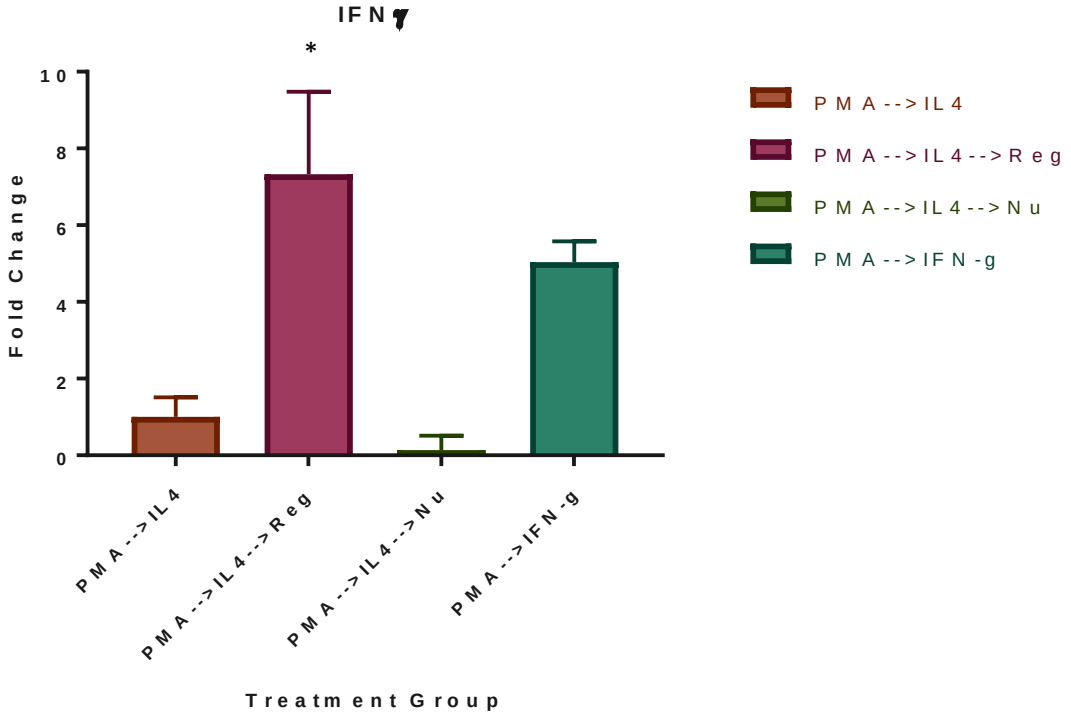

Figure 15: IFN-γ mRNA Expression in Drug-Treated THP-1 Cells. Expression of IFN-γ mRNA from RT-qPCR. Data is normalized to the average of triplicate reactions measuring GAPDH. Error bars represent standard deviation. * = $p<0.05$; **=$p<0.01$; ***=$p<0.001$ compared to control IL-4 group based on multiple t tests using Holm-Sidak for multiple comparisons.

We also examined whether drug treatment affected suppressive IL-10 mRNA expression. However, neither Reg nor NU altered IL-10 mRNA levels; all four groups of macrophages had similar expression of IL-10 across the board (**Figure 16**).

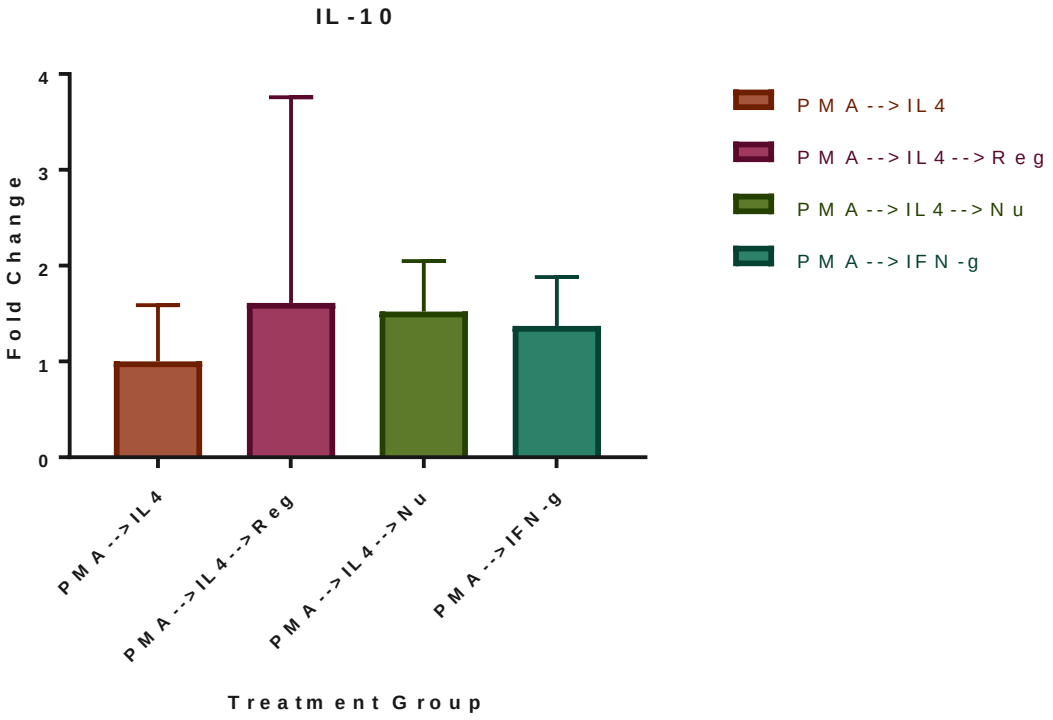

Figure 16: IL-10 mRNA Expression in Drug-Treated THP-1 Cells. Expression of IL-10 mRNA from RT-qPCR. Data is normalized to the average of triplicate reactions measuring GAPDH. Error bars represent standard deviation. * = $p<0.05$; **=$p<0.01$; ***=$p<0.001$ compared to control IL-4 group based on multiple t tests using Holm-Sidak for multiple comparisons.

Treatment with Reg/NU decreases arginase activity in mouse MDSCs

To determine the effects that Reg and NU have on MDSC arginase activity, we took unsorted splenocytes derived from B16 tumor-bearing mice, treated with drug for 48 hours, and then performed the same arginase activity assay as described above. For these samples, the lysates were incubated with arginine for 2 hours. In addition to the full undiluted sample, a 1/10 dilution was made. For the undiluted samples, untreated splenocytes had a high arginase activity level at 11.47 units/L (**Figure 17**). Eight micromolar of Reg decreased activity to 2.45 units/L. Four micromolar of Reg ablated arginase activity to 1.51 units/L while 2 µM of Reg decreased it to 1.80 units/L. NU had more of a dose-dependent effect on arginase activity with 4 µM NU decreasing arginase

levels to 2.63 units/L, 2 µM NU with a slightly higher activity of 4.25 units/L and 1 µM NU with the highest of the three with 6.41 units/L.

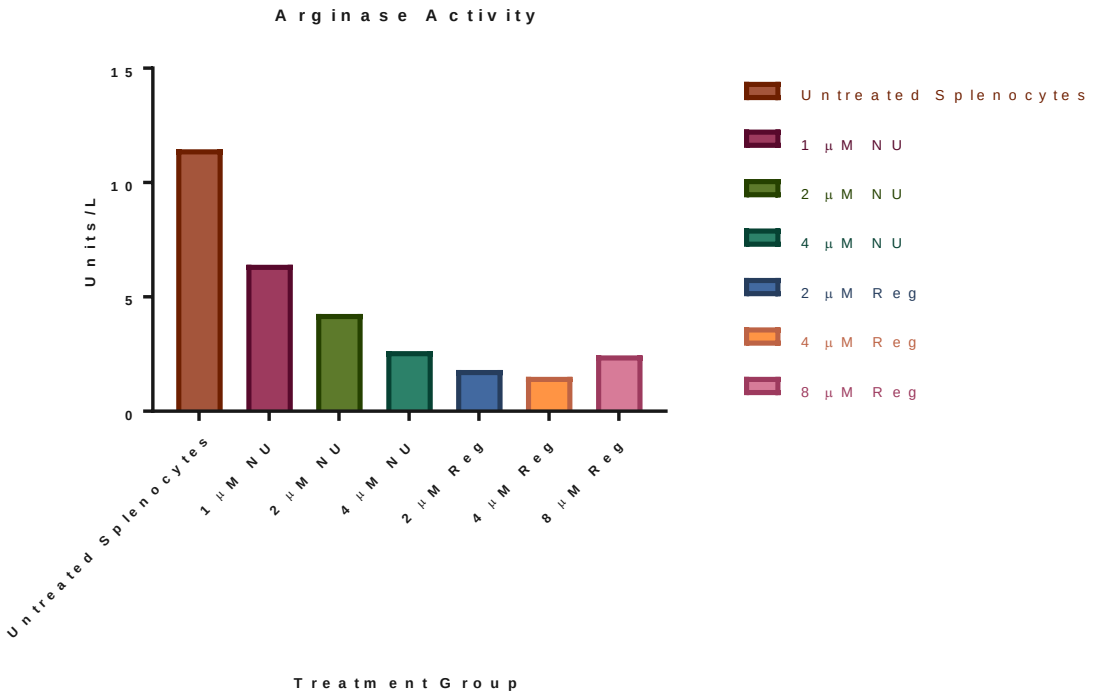

Figure 17: MDSC Arginase Activity. Arginase activity of unsorted B16 mouse splenocytes with and without drug treatment.

For the 1/10 dilution, untreated splenocytes had an activity of 13.76 units/L (**Figure 18**). At 8 µM of Reg, arginase activity was calculated at -1.2 units/L – a negative calculated level of activity was due to absorbances between the sample and sample blank wells being very similar, but slightly higher in the sample blank well. At 4 µM and 2 µM of Reg, arginase activity levels were at 4.56 and 2.98 units/L, respectively. With NU, 4 µM decreased activity to 4.65 units/L, while 2 µM and 1 µM of NU had 12.09 units/L of activity and 5.02 units of activity, respectively.

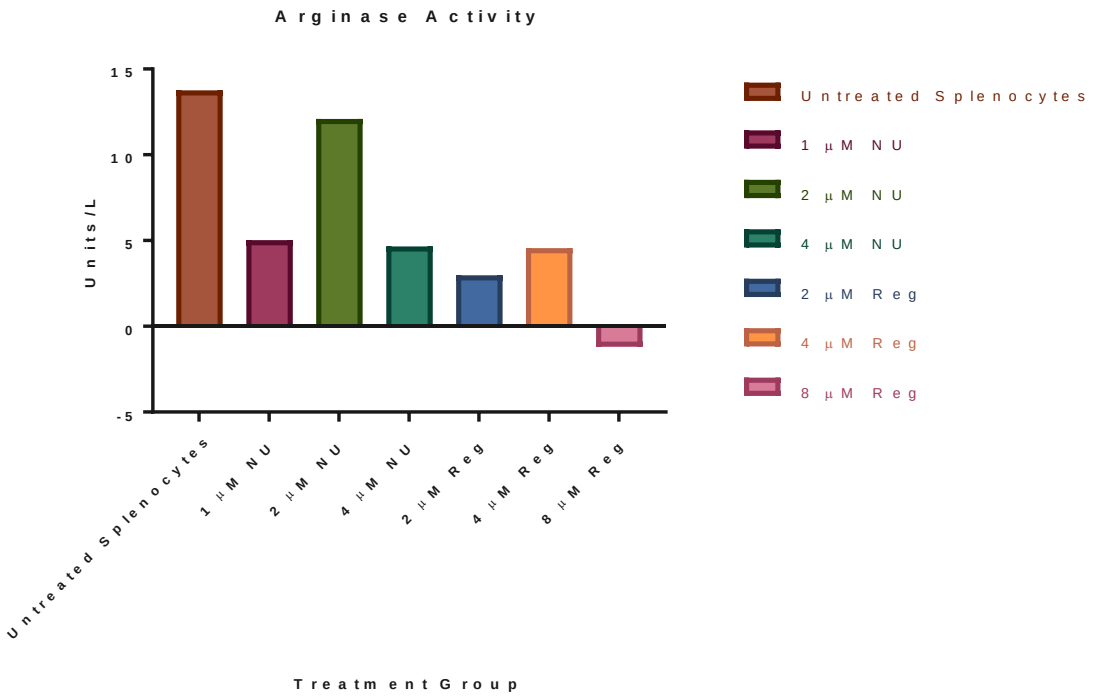

Figure 18: MDSC Arginase Activity – 1/10 Dilution. Arginase activity of unsorted B16 mouse splenocytes with and without drug treatment.

Chapter IV: Discussion and Conclusions

Both targeted therapies and immunotherapies have been great tools in treating cancer. However, both have their caveats. Though targeted therapies can be highly effective when given to patients with potent biomarkers, cancers often become resistant to these treatments over time. Immunotherapies have been clinically promising, but very few patients show robust responses. Combination therapy has been a potential approach to combat the shortcomings of both treatments to produce a robust anti-tumor response.

A previous study in our lab showed the immunomodulatory potential of two drugs, Regorafenib and NU7441 (Tsai et al., 2017). In theory, if these drugs were to work in a patient the way they have in our studies, not only would they be able to target the tumor directly, but they would also sensitize the tumor to immunotherapy treatment by allowing the expression of immunoactivating markers while decreasing immunosuppressive markers. This would lead to a more potent immune response than immunotherapy alone. However, the tumor is not a solo entity. In fact, there are many cells in the tumor microenvironment that support the tumor's growth and survival, including suppressive myeloid cells. In this thesis, we explore the effects of these two drugs on MDSCs and suppressive macrophages.

The THP-1 human monocytic cell line has long been used in research and many have produced protocols to differentiate them into macrophages, but it was crucial to confirm in our study that they can truly create a suppressive phenotype when given IL-4 with or without IL-13 like monocytes are supposed to do. Indeed, with 48 hours of PMA treatment and 48 hours of IL-4 or IL-4 + IL-13 treatment, THP-1 cells become adherent macrophages that express CD206, CD209, and IL-10. Additionally, they express CD163

and CD206 on their surface. Their suppressive ability is later seen in arginase assays where they are able to metabolize arginine.

Once we determined that THP-1 cells were able to differentiate into suppressive macrophages, we found that treating such suppressive macrophages with Reg or NU not only decreased arginase activity and similar results were found in splenocytes deriving from a B16 GM-CSF melanoma mouse model. Not only did the drugs decrease suppressive activity in splenocytes and M2s, but they were also able to increase inflammatory markers associated with the M1 phenotype. Potentially, this could mean that the drugs somehow repolarize alternatively activated macrophages into classically activated macrophages without the need for cytokine stimulation.

Though we found these immunomodulatory changes in suppressive myeloid cells, much needs to be discovered about the mechanisms by which these drugs function to make these immunologically relevant changes. NU7441 is of particular interest because it is supposed to specifically inhibit DNA-PK, which is involved in the non-homologous end joining pathway of DNA repair. Off-target effects are also an important consideration when it comes to targeted therapies. Regorafenib specifically could be a concern because kinase inhibitors can often have off-target effects due to structural similarities between kinases. Off-target effects could also explain why NU7441 has this immunomodulatory effect. By learning the mechanisms by which these drugs work as well as their off-target effects, we can better identify who would best benefit from these treatments and predict other consequences of giving patients these drugs.

This thesis primarily focused on characterizing the capacity for drug-treated myeloid cells to promote a robust anti-tumor response or, at the very least, promote a tumor

microenvironment that is less suppressive. However, suppressive myeloid cells can promote tumor growth in a T cell-independent manner. One study has shown that caspase 1 in MDSCs can directly promote tumor proliferation without T cells (Zeng et al., 2018). Though we have found that treatment with Reg or NU decreases arginase activity in MDSCs, there are several other suppressive mechanisms that MDSCs utilize to support tumor growth and some of them do not involve T cells at all. This makes it necessary to examine further whether Reg and NU affect these other mechanisms of suppression.

Overall, we have shown that two target therapies can decrease suppressive activity in M2s and MDSCs and potentially repolarize M2 macrophages into an M1 phenotype. Additional studies need to be performed to fully understand the immunomodulatory potential for these drugs. In addition to a more thorough investigation on how these drugs affect other mechanisms of suppression, it is also crucial to understand the molecular mechanisms and pathways that these drugs affect. Nevertheless, this thesis is a step in the right direction towards more potent and long-lasting immunotherapy treatment for cancer patients.

Chapter VI: Materials and Methods

Cell Culture

THP-1 cells were maintained in RPMI supplemented with 10% FBS (Gemini), 1% Pen/Strep (Gibco), and 0.05 mM β-mercaptoethanol in an upright T75 flask in an incubator set at 37º C with 5% CO_2. The cell line was obtained from ATCC or generously provided by Dr. Achsah Keegan (University of Maryland, Baltimore).

THP-1 Differentiation

THP-1 cells were seeded in 24 well culture-treated plate (Costar) with 2 mL media. Cells were then treated with 40 ng/mL of PMA for 48 hours. After PMA treatment, cells were washed twice with RPMI to remove dead and non-adherent cells. Media was then replaced, and cytokine was added. For M1 differentiation, 20 ng/mL of human recombinant IFNγ (eBioscience) was added for 48 hours. For M2 differentiation, 20 ng/mL of human recombinant IL-4 (Biolegend) was added with or without 20 ng/mL human recombinant IL-13 (Invitrogen) for 48 hours. For all studies, cells were removed using a cell scraper (Sarstedt).

Drug Treatments

NU7441 and Regorafenib were obtained from SelleckChem. All compounds were dissolved in DMSO and stored in -20º C. All experimental groups that received drug treatment were treated for 48 hours.

Flow Cytometry

All anti-human antibodies were obtained from BioLegend: CD206-Alexa700 (cat.#: 321131), CD163-APC (333609), CD8a-APC (300912). Anti-mouse CD8b-APC was obtained from eBioscience (17-0083-81). Cells were seeded at 200,000 cells/well in a 96 well v-bottom plate (Costar). Cells were stained using 200 µL of staining cocktail. Staining

cocktail contained antibodies at a 1:200 dilution in cell staining buffer (BioLegend) for 30 minutes. Cells were washed 3 times and then fixed in 100 µL of 4% paraformaldehyde (Affymetrix) for 20 minutes covered on ice. 3 more washes were performed. All analyses were performed on an LSR-II flow cytometer.

RT-qPCR

RNA was isolated using the Qiagen RNeasy Mini Kit and then converted into cDNA using the iScript Reverse Transcription Supermix from Bio-Rad, using 1 µg of RNA per reaction. qPCR was performed with iTaq Universal SYBR Green Supermix from Bio-Rad. Relative changes were calculated using ΔΔCt and normalized to GAPDH. Primers are listed below:

GAPDH forward: GTCTCCTCTGACTTCAACAGCG

GAPDH reverse: ACCACCCTGTTGCTGTAGCCAA

CD206 forward: ACGATCCGACCCTTCCTTGA

CD206 reverse: GCTTGCAGTATGTCTCCGCT

CD209 forward: CTCCATCACCGCCTGCAAAG

CD209 reverse: AGCTGTAGGAAGTTCTGCTCCTC

IL-10 forward: GGCGCTGTCATCGATTTCTTC

IL-10 reverse: GCCACCCTGATGTCTCAGTT

COX2 forward: CGGTGAAACTCTGGCTAGACAG

COX2 reverse: GCAAACCGTAGATGCTCAGGGA

CCR7 forward: CAACATCACCAGTAGCACCTGTG

CCR7 reverse: TGCGGAACTTGACGCCGATGAA

iNOS forward: GCTCTACACCTCCAATGTGACC

iNOS reverse: CTGCCGAGATTTGAGCCTCATG

IFNγ forward: GAGTGTGGAGACCATCAAGGAAG

IFNγ reverse: TGCTTTGCGTTGGACATTCAAGTC

Arginase Activity

Arginase activity was measured after drug treatment using the colorimetric test from Sigma (cat. #: MAK-112). 500,000 cells per treatment were measured in this assay. Protocol and calculations were per manufacturer's instructions that were provided in the kit.

Mouse Models

C57BL/6J and pmel (B6.Cg-Thy1/Cy Tg(TcraTcrb)8Rest/J) mice were purchased from Jackson Laboratory. Six- to eight-week old C57BL/6J mice were injected with B16 GM-CSF melanoma cells subcutaneously on the right flank. After tumors were established, the spleens were harvested. For isolation of splenocytes for both tumor and pmel mice, spleens were processed through a 100 μm cell strainer and red blood cells were removed using lysis buffer (Biolegend). Mouse MDSCs were maintained in T cell media (TCM): RPMI with 10% FBS (Gemini), 1% NEAA (Gibco), 1% Pen/Strep (Gibco), 2 mM L-Glutamine (Gibco), 0.1% Gentamicin (Gibco), and 50 μM β-mercaptoethanol. 20 ng/mL mouse GM-CSF was also supplemented to promote MDSC survival during the 48-hour drug treatment.

Mouse Proliferation Assays

Prior to coculture, pmel T cells were labeled using eFluor450 proliferation dye (eBioscience). Splenocytes that were treated with Reg or NU for 28 hours and labeled pmel cells were cocultured in a 24 well plate for 5 days at various ratios of MDSC:T cell, with T cells consistently at 500,000 cells per well. During the 5-day coculture, the cells were supplemented with 1 μg gp100 and 100U/mL recombinant mouse/human IL-2 (Biolegend). Media was changed after 2-3 days. After the coculture, cells were labeled with anti-mouse CD8b-APC and flow cytometry was performed to measure T cell proliferation.

Human Proliferation Assays

Cryopreserved and thawed PBMCs were labeled with eFluor450 and cocultured with drug-treated THP-1 macrophages in a 24 well plate at various ratios for 5 days, with T cells consistently at 500,000 cells per well. The coculture was performed in AIMV media (Gibco) supplemented with 5% human serum AB (Gemini), 1% NEAA, and 1% Pen/Strep. To activate the T cells, 50 ng/mL of anti-CD3 (Clone OKT3, eBioscience) and 100 U/mL recombinant mouse/human IL-2 were added. Media was replaced after 2-3 days. After the cocultured, cells were labeled with anti-human CD8a and then flow cytometry was performed.

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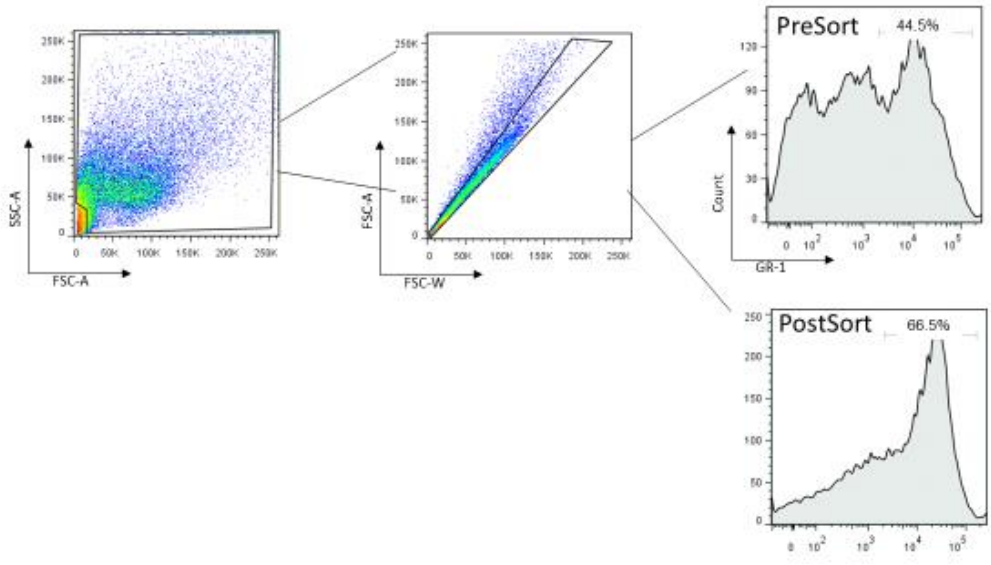

Figure 19: Mouse MDSC Selection. Flow cytometry of splenocytes deriving from B16 GM-CSF mice before and after selection using Gr-1. Figure graciously provided by Ellis Tibbs in Dr. Eduardo Davila's lab.

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