2021

Beta-Adrenergic Receptor Mediated Transcriptional Dysregulation in Hematopoeitc Stem and Progenitor Cells Leads to Bone Marrow Erythroid Suppresion in Multiple Myeloma Patients - Ex Vivo Investigations

Vimal Ravi Subramaniam

LOYOLA UNIVERSITY CHICAGO

BETA-ADRENERGIC RECEPTOR MEDIATED TRANSCRIPTIONAL DYSREGULATION IN HEMATOPOEITC STEM AND PROGENITOR CELLS LEADS TO BONE MARROW ERYTHROID SUPPRESION IN MULTIPLE MYELOMA PATIENTS - Ex VIVO INVESTIGATIONS

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN INTEGRATIVE CELL BIOLOGY

BY

VIMAL SUBRAMANIAM

CHICAGO, ILLINOIS

AUGUST 2021

TABLE OF CONTENTS

LIST OF TABLES v

LIST OF FIGURES vi

LIST OF ABBREVIATIONS viii

CHAPTER ONE: LITERATURE REVIEW 1
Statement of the Problem 1
Multiple Myeloma 1
The Sympathetic Nervous System 2
Sympathetic Nervous System in Hematological Cancers 2
Sympathetic Nervous System Role in Multiple Myeloma 3
In-vivo Murine Studies Involving Burn Injury (Prior Lab Data) 5
MAFB Expression is Increased in Burn Injury due to Adrenergic Signaling 5
Murine *MAFB* Silencing Study in Burn Injury 7
Late Erythroid and Myeloid Development in Burn Injuries from Murine Model 9
MAFB/GATA1 Axis 10

CHAPTER TWO: EXPERIMENTAL METHODS 12
BM Aspirates 12
Plating for Cultures 13
Alpha- and Beta-Adrenergic Receptor Stimulation 14
Beta Adrenergic Receptor Blockade 14
Magnetic Isolation 15
Staining With Cell Surface Markers for Flow Cytometry 16
MAFB and GATA1 Intracellular Identification and Quantification by Flow Cytometry 16
Adrenergic Receptor Incubation 17
FACS and FLOWJO Analysis 18
MAFB Gene Silencing 20
Giemsa Staining 21

CHAPTER THREE: RESULTS 22
MM Patient Samples Have a Lower Number of Total Erythroblasts Versus BM Control Samples Indicating Diminished Erythropoiesis 22
MM Patient Samples Have Greater Number of GMPs and Lower Number of MEPs Versus BM Control Samples (Day 0) 23
MM Patient Samples Have Increased MAFB Expression and Decreased GATA1 Expression Versus BM Control Samples (Day 0) 24
After Phase 1 Expansion MM Patient Samples Have Greater Number of GMPs and Lower Number of MEPs Versus BM Control Samples (DAY 6) 26
MAFB Expression is Higher and GATA1 Expression is Lower in MM Samples After Phase 1 Expansion (Day 6) 27
Myeloma Cells Do Not Seem to Influence Myeloid Bias 29
HSPCs Express All Alpha-and-Beta-Adrenergic Receptor Sub-Types 31

Alpha-Adrenergic Receptor Stimulation Does Not Cause Myeloid Bias; Beta-Adrenergic Receptor Stimulation Does 32
Alpha Adrenergic Receptor Stimulation Does Not Alter MAFB/GATA1 Expression; Beta Adrenergic Receptor Stimulation Elevates MAFB Expression and Reduces GATA1 Expression 35
MAFB Silencing in MM Samples and ISO Stimulated Control BM Leads to Recovery of MEPs and Decline in GMPs 36
MM Sample and Phase 1 Beta Stimulated Control BM Commitment Patterns Lead to Diminished Erythroblast Development and Increased Non-Erythroid Development 38
LEBs Are Diminished and Non-Erythroid Cells Are Increased in MM Versus BM Control; Beta Stimulation Has Little Effect on Erythropoietic Development 40

CHAPTER FOUR: DISCUSSION 43

REFERENCE LIST 48

VITA 55

LIST OF TABLES

Table 1. Multiple Myeloma Patient Info 12

Table 2. Alpha-Adrenergic Receptor Agonist (Phenylephrine) Does Not Affect MAFB/GATA1 Expression; Beta-Adrenergic Receptor Agonist (Isoproterenol) and Antagonist (Propranolol) Does Effect MAFB/GATA1 Expression 36

LIST OF FIGURES

Figure 1. In-Vivo Burn Murine Model. GMP Levels Increase, and MEP Levels Decrease After Burn Injury due to Increased MAFB Expression which is Under β-AR control 7

Figure 2. Ex-vivo Burn Murine Model. MAFB Silencing in Burn CMPs Improves Erythropoiesis at the Expense of Myelopoiesis 8

Figure 3. Propranolol Recovers the Erythropoietic Development that is Hampered by Burn Injury and Reduces Granulocytic Cells with Ringed Nucleus, which is Typical of Myeloid Derived Suppressor Cells (MDSCs) 10

Figure 4. Schematic of Hypothesis 11

Figure 5. Erythroblast and Progenitor Subset Gating 19

Figure 6. Increased Non-Erythroid Population and Reduced Erythroblast Population in MM Compared to Control BM (Day 0) 23

Figure 7. GMPs Increase and MEPs Decrease in MM Samples Compared to Control BM (Day 0) 24

Figure 8. MAFB Expression is Higher and GATA1 Expression is Lower in CMPs of MM Samples Versus BM Control Samples (Day 0) 26

Figure 9. GMPs are Increased and MEPs are Reduced in MM Samples Versus BM Control Samples Placed in Phase 1 Expansion (Day 6) 27

Figure 10. Phase 1 Expanded CMPs Display Higher MAFB Expression and Lower GATA1 Expression in MM samples Versus BM Control Samples (Day 6) 29

Figure 11. Myeloma Cells Do Not Seem to Influence Myeloid Bias 30

Figure 12. All Alpha-and-Beta-Adrenergic Receptor Sub-Types are Present on HSPCs 32

Figure 13. Alpha-Adrenergic Receptor Agonist (Phenylephrine) Does Not Alter GMPs/MEPs; Beta-Adrenergic Receptor Agonist (Isoproterenol) and Antagonist (Propranolol) Do Alter GMPs/MEPs 34

Figure 14. Transfection Efficiency and Validation of *MAFB* siRNA 37

Figure 15. *MAFB* Silencing Resolves/Mitigates Myeloid Bias in MM Samples and ISO Stimulated Control BM 38

Figure 16. Beta Adrenergic Stimulation During Commitment Stage Leads to Increases in NonE and Diminished Erythroid Development 40

Figure 17. MM Samples Have Increased NonE and Decreased LEBs Compared to BMC; ISO Does Not Impact Erythropoietic Development 42

Figure 18. Schematic of the Effects of Beta Adrenergic Signaling on the Myeloid Bias and Erythroid Development in MM 43

LIST OF ABBREVIATIONS

β-AR	Beta-Adrenergic Receptor
BM	Bone Marrow
BMN	Bone Marrow Niche
CMPS	Common Myeloid Progenitors
EEBs	Early Erythroblasts
EPO	Erythropoietin
GMPs	Granulocyte-Macrophage-Progenitors
GMCSF	Granulocyte-Macrophage-Colony-Stimulating Factor
Hgb	Hemoglobin
HT	Holotransferrin
HSCs	Hematopoietic Stem Cells
HSPCs	Hematopoietic Stem and Progenitor Cells
ICU	Intensive Care Unit
IL-3	Interleukin 3
ISO	Isoproterenol
LEBs	Late Erythroblasts
MDSCs	Myeloid-Derived Suppressor Cells
MEPs	Megakaryocyte-Erythrocyte-Progenitors
MM	Multiple Myeloma
PE	Phenylephrine
PROP	Propranolol
RBCs	Red Blood Cells
SCF	Stem Cell Factor

SNS	Sympathetic Nervous System
TEBs	Total Erythroblasts

CHAPTER ONE

LITERATURE REVIEW

Statement of the Problem

Anemia is a condition in which there is a lack of enough healthy red blood cells. Anemia is a pervasive side effect in multiple myeloma (MM) patients occurring in more than two thirds of all patients [1]. MM is an incurable hematological malignancy characterized by the numerous plasma B cell lesions in the bone marrow (BM). In MM patients, anemia is present with low red blood cell counts that are contributed to circumstances other than a nutrient deficiency [2]. At the time of being diagnosed, the prevalent clinical symptom in MM patients is normochromic-normocytic anemia in around 75% of patients [2]. It can be surmised then that anemia in MM stems from a lack of red blood cell (RBCs) production. Erythropoiesis is the process where hematopoietic stem cells (HSCs) develop into mature erythrocytes (red blood cells) [3]. This process is hampered in MM [4].

Multiple Myeloma

In the United States, the lifetime risk of getting MM is 1 in 132 [5]. The American Cancer Society's estimates in the United States in 2021 that about 34,920 new cases will be diagnosed with MM (19,320 in men and 15,600 in women) and about 12,410 deaths are expected to occur (6,840 in men and 5,570 in women) [6]. In a healthy BM, plasma cells are normally found inside the BM which make proteins called antibodies to fight infections. In a MM BM, plasma cells become cancerous and turn into MM cells that produce abnormal antibodies known as M-proteins [7]. When there are too many myeloma cells, they crowd out normal blood cells in

the bone marrow causing damage to bones, kidneys, and other organs [8-11]. The clinical features of MM are well remembered with the acronym CRAB which stands for calcium elevation, renal complications (elevated creatine and BUN), anemia (low Hgb and RBCs), and bone (pain, lesions, factures) [12]. The focus of this paper is on anemia.

The Sympathetic Nervous System

The human body has a host of defense mechanisms to combat itself against detrimental situations/conditions. One of the most crucial defense mechanisms is the activation of the Sympathetic Nervous System (SNS). The SNS is part of our Autonomic Nervous System and thus is activated involuntary to regulate bodily functions [13]. The SNS becomes activated upon the perceived ensuing event of harm/physiological stress [13]. This has led to people aptly naming it the body's "fight or flight response." SNS in a more detailed sense involves adrenergic signaling [14]. Adrenergic signaling is the release of neurotransmitters and the subsequent cascade of cellular events upon binding of them to their receptors [14]. The neurotransmitter norepinephrine and the hormone epinephrine are known as catecholamines that are involved in adrenergic signaling [14]. Conservatively, when a life threat/stress is sensed, it first activates the hypothalamus to send signals to the adrenal gland [15]. The adrenal gland then releases the hormone epinephrine and the neuro-transmitter norepinephrine [15]. Though this response is often a beneficial regulatory mechanism to aid our body, there are circumstances where it can do the opposite and be harmful.

Sympathetic Nervous System in Hematological Cancers

One area that has been gaining traction in the last 5 to 6 years is the correlation between the SNS and cancer. Investigations into the SNS causing adverse symptoms in hematological cancers is particularly of note due to its widening notoriety and comprehension amongst

researchers. SNS activation can be induced as a result of hematological abnormalities, in a process known as hematological stress syndrome [16]. In this process, the body perceives the hematological abnormality as a stress and thus responds by activating the SNS to combat the stress. Recent studies have shown that a diverse range of factors including psychological stress, aging, diabetes, and hematological malignancies can cause hematological abnormalities, and consequently result in the occurrence of hematological stress syndrome [17-23]. However, the influence of the SNS occurs not via an acute stress response, but through a sustained SNS activity [24]. One of the inadvertent issues in hematological malignancies due to sustained SNS activity initiates from the BM, where hematopoiesis occurs. Hematopoiesis is a process that involves the production, differentiation, and development of all types of blood cells. The BM is innervated by sympathetic nerve fibers [17]. This includes the Bone Marrow Niche (BMN) which in addition to sympathetic nerve fibers also contains Schwann cells [17,24]. The BMN is located on the perivascular space of the BM proximal to where Hematopoietic Stem Cells (HSCs) reside [25]. In the BM, the constant and enduring stream of catecholamines leads to durable alteration of the gene expression profile of the cancer and supporting stroma elements [26-29]. The fostering of HSC commitment in BMN is one aspect of the supporting stroma and as is evident may contribute to a disturbed BM environment. Along these lines, it stated that there is evidence through a multitude of epidemiologic and experimental bodies of work that a stress response induced through the SNS plays a significant part via indirectly aiding in the creation of tumor-favorable microenvironment [30-36].

Sympathetic Nervous System Role in Multiple Myeloma

One of the most lethal types of hematological cancer is MM. Much has gone into researching the SNS's role in the development and progression of MM. Recent studies have

shown that SNS input is one of the factors that modulates myeloma growth and survival [37]. In addition, pharmacological inhibition of this sympathetic input was revealed to improve survival outcomes in patients with MM [38]. Both of the above studies describe the impact of SNS on myeloma cells. However, there are other aspects involving the BM where the activation of the SNS can lead to complications during MM. A clinical trial conducted on 25 randomized MM patients found that after successful HSC engraftment following irradiation of the MM BM, that poor outcomes were persistent [39]. Gene expression analysis of mature blood cells obtained from peripheral blood extracts, indicated the upregulation of genes from myeloid lineage cells [39]. Myeloid lineage cells are cells that are derived from granulocyte-macrophage-progenitors (GMPs) during BM myelopoiesis [40]. Due to previous knowledge that the use of beta-blockers can lead to better prognosis in MM, Propranolol was administered to MM patients [38]. Propranolol is a non-specific beta-adrenergic receptor (β-AR) antagonist. Upon Propranolol administration, gene expression analysis revealed that the upregulation of genes from myeloid lineage cells seen in MM prior to Propranolol administration had been mitigated [39]. This implies that sufficient disruption of adrenergic signaling can lead to a shift away from the myeloid lineage cell bias seen in the peripheral blood of MM patients. This is reasonable as the SNS is known to have a regulatory influence on hematopoiesis [41,42]. As noted earlier, hematopoiesis occurs in the BM and the matured blood cells enter into the peripheral circulation exiting the BM. As addressed in the HCT study, blocking adrenergic signaling leads to a decrease in mature myeloid lineage cells in the peripheral blood of MM patients. Taken together, one can question if the bias towards myeloid lineage cells in the peripheral blood of MM patients stems from the effect of adrenergic signaling in the BM niche.

In-vivo Murine Studies Involving Burn Injury (Prior Lab Data)

Previous studies done by our lab investigated the effects on HSPCs due to adrenergic signaling in burn injury models. Studies on burn patients in the intensive care unit (ICU) found that 77% of patients were anemic at the time of hospital discharge [43]. Furthermore, anemic symptoms had persisted as far as 20 weeks post burn in these patients [43]. Burn injury is perceived by the body as a physiological stress and therefore induces a SNS response. This led the previous members of our lab to investigate whether the SNS was responsible for the post burn anemia seen in burn injuries. An *in-vivo* study was conducted on mice to test the correlation between adrenergic signaling (SNS response) and anemia in burn injuries [44]. Mice were either administered a burn or no burn. After administration, the mice were injected once daily for seven days with saline-control or propranolol-β-AR antagonist. BM samples were collected on post burn day 3 and 7. Collected aspirates were sorted using magnetic isolation (method outlined in Experimental Methods) to isolate LIN NEG population to examine HSPCs. HSPCs were then surface stained to identify GMPs and megakaryocyte-erythrocyte-progenitors (MEPs) and intracellular incubated with FITC tagged anti-MAFB to determine MAFB expression (method outlined in Experimental Methods). A separate model was done in conjunction of subcutaneous injections using a Alzet pump instead to dispense a continuous dose of saline-control or propranolol-β-AR antagonist for 14 days [44]. Quantifications for both models were done using FACS and FLOWJO-software for various parameters (method outlined in Experimental Methods).

MAFB Expression is Increased in Burn Injury due to Adrenergic Signaling

Transcription factors play a critical role in a complex and highly orchestrated process of directing HSCs towards lineage commitment by regulating lineage-specific gene expression,

proliferation, and differentiation. Importantly, the regulation of differentiation by transcription factors is not merely via a "presence or absence of expression" mechanism but by a dose-dependent effect. MAFB (v-maf musculoaponeurotic fibrosarcoma oncogene homolog B) is a key orchestrator of lineage commitment and differentiation of HSPCs [45]. It plays a pivotal role as it is required for most myeloid-derived cells [45,46]. It was revealed that MAFB was expressed at higher levels in BM progenitors of burned mice versus sham mice (Figure 1A,B) [44]. In addition, propranolol administration to burned mice effectively decreased MAFB expression demonstrating up-regulation of MAFB in burn injury is due to adrenergic signaling (Figure 1A,B) [44]. It was also discovered amongst hematopoietic stem and progenitor cells (HSPCs), there was a myeloid bias at the expense of erythroid progenitors in burned mice resulting in enhanced development of GMPs and reduced development of MEPs. Upon administration of propranolol, the higher number of GMPs and lower number of MEPs were reversed in burned mice (Figure 1C,D) [44]. Through these studies it was established that the high MAFB expression in HSPCs after burn injury skews their lineage bias towards a myeloid commitment [44]. Furthermore, it was found that this increase in MAFB expression was due to adrenergic signaling [44].

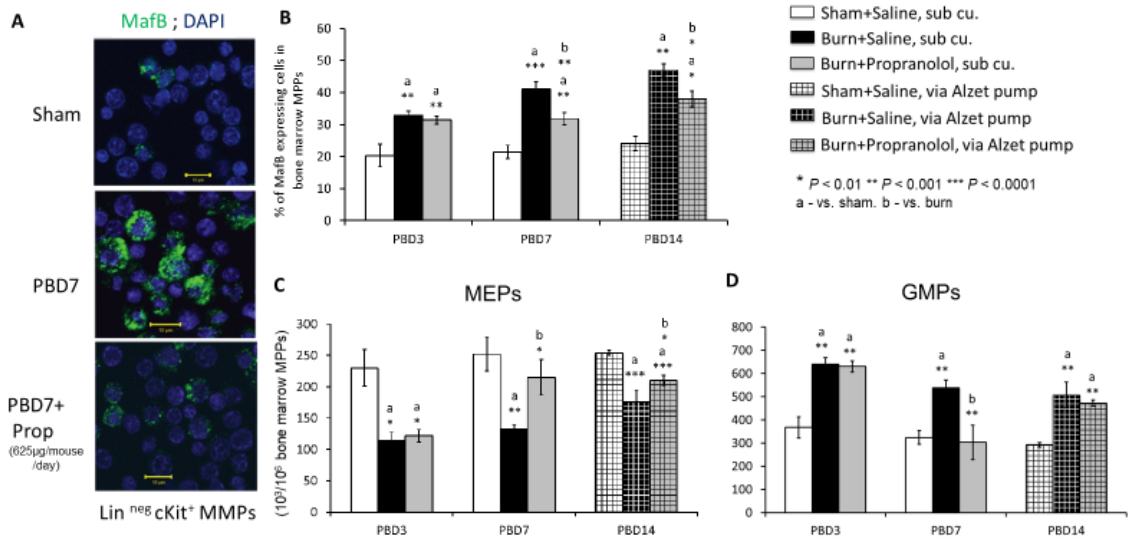


Figure 1. In-Vivo Burn Murine Model. GMP Levels Increase, and MEP Levels Decrease After Burn Injury due to Increased MAFB Expression which is Under β-AR Control. Burned and not burned mice were injected once daily for seven days with saline-control or propranolol-β-AR antagonist. BM samples were collected on post burn day 3 and 7. A separate model was done in conjunction of subcutaneous injections using a Alzet pump instead to dispense a continuous dose of saline-control or propranolol-β-AR antagonist for 14 days. **(A)** Fluorescent Imaging was used to visually outline differences in MAFB expression between collected BM samples of sham, post burn day 7 without propranolol, and post burn day 7 with propranolol. MAFB expression was determined through FITC-conjugated primary antibody. A DAPI nuclear stain was applied to identify healthy HSPCs. HSPCs from sham sample showed minimal positive MAFB expression. HSPCs from post burn day 7 sample showed increased positive MAFB expression compared to sham, but expression returned to near sham levels when propranolol was administered. **(B)** MAFB expression increased during post burn day 3 and 7, but effective blockage of β-AR did not take effect till day 7. Continuous 14-day study confirms day 7 analysis of increased MAFB expression in burn versus sham with propranolol effectively lowering MAFB expression in burn mice. **(C)** MEPs are reduced in post burn day 3 and 7 compared to sham. Treatment with propranolol shows recovery of MEP population during post burn day 7. Findings were reaffirmed through 14-day study. **(D)** GMPs are increased in post burn day 3 and 7 compared to sham. Treatment with propranolol shows reduction in GMP population during post burn day 7. Findings were reaffirmed through 14-day study. This figure was provided by Dr. Muthumalaiappan with consent to use in this thesis [44].

Murine MAFB Silencing Study in Burn Injury

In conjunction to *in-vivo* studies an *ex-vivo* silencing study was conducted on the transcription factor MAFB (Figure 2) [44]. Silencing of MAFB was done in common myeloid progenitors (CMPs) which are the highest order of HSPCs and thereby differentiate into GMPs

and MEPs. Samples were harvested bilaterally from femurs of day 7 sham and burned mice. Sorted CMPs were obtained through flow cytometry and placed in culture with myelo-erythroid growth factor cocktail. Knock down of MAFB was performed a day later through MAFB siRNA transfection (method outlined in Experimental Methods). After transfection CMPs were extended in culture for six more days. Incubation with anti-MAFB allowed for measurement of MAFB expression using fluorescent imaging and FACS. It was identified that high MAFB in CMPs acts as a transcriptional activator of MCSF-R (monocyte/macrophage colony stimulating factor 1 receptor) and CD11B (integrin alpha M), which are both critical for myeloid lineage commitment (Figure 2A,B) [44]. Additionally, MAFB was found to repress the erythroid lineage marker CD71 i.e. transferrin receptor, demonstrating reduced erythroid differentiation (Figure 2C,D) [44].

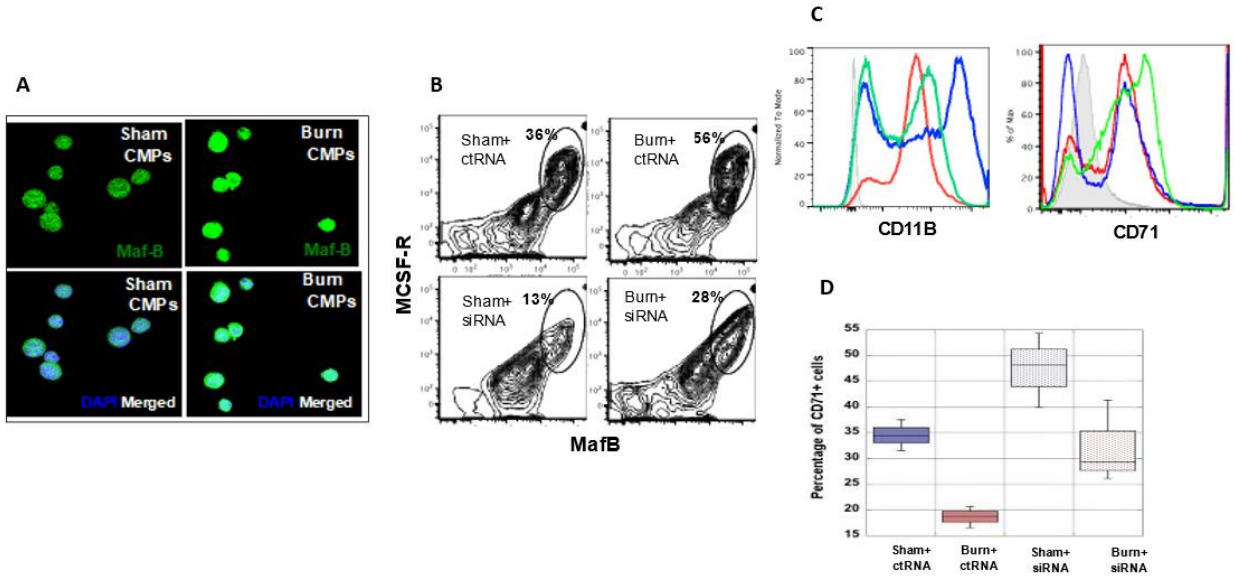


Figure 2. Ex-Vivo Burn Murine Model. MAFB Silencing in Burn CMPs Improves Erythropoiesis at the Expense of Myelopoiesis. CMPs were collected from post burn day 7 mice and day 7 sham mice. Knock down of MAFB in CMPs was done the next day and extended in culture for 6 more days. Control samples of both sham and burn which did not receive siRNA transfections were also placed in cultures. On day 7 various parameters were measured such as MCSF-R, CD11B and CD71. **(A)** On day 0 before transfection with siRNA of MAFB, CMPs were incubated with anti-MAFB and fluorescent imaging was done to obtain initial readings of MAFB expression in CMPs. **(B)** MCSF-R is increased in burn compared to sham. MAFB silencing results in lowered MCSF-R expression in both sham and burn CMPs. **(C)** CD71 expression is increased and CD11B expression is decreased upon knock down of MAFB in CMPs. **(D)** Of the CMP population, CD71 expressing myeloid progenitors increase when MAFB is silenced. This figure was provided by Dr. Muthumalaiappan with consent to use in this thesis [44].

Late Erythroid and Myeloid Development in Burn Injuries from Murine Model

To observe ongoing effects of adrenergic signaling on erythroid and myeloid maturation (erythrocyte/reticulocyte/erythroblast, monocyte, neutrophil development) harvested BM samples and smears from sham, burn, and propranolol treated burned mice were examined (Figure 3) [44]. Ter119 and CD71 are essential erythroid markers that can be used to identify the later stages of erythroid development. Harvested BM samples from mice on post burn day 7 (PBD7) were probed with antibodies of TER119 and CD71 and then subsequently read using FACS. Results conveyed diminished erythrocyte population in burn mice compared to sham that was recovered upon administration of propranolol (Figure 3A) [44]. Imaging of BM smears reaffirmed this by showing fewer reticulocytes and more MDSCs in burn mice compared to sham (Figure 3B) [44]. Treatment with propranolol for 6 days after burn injury then replenished early erythroblasts and reticulocytes with a significant reduction in MDSCs (Figure 3B) [44].

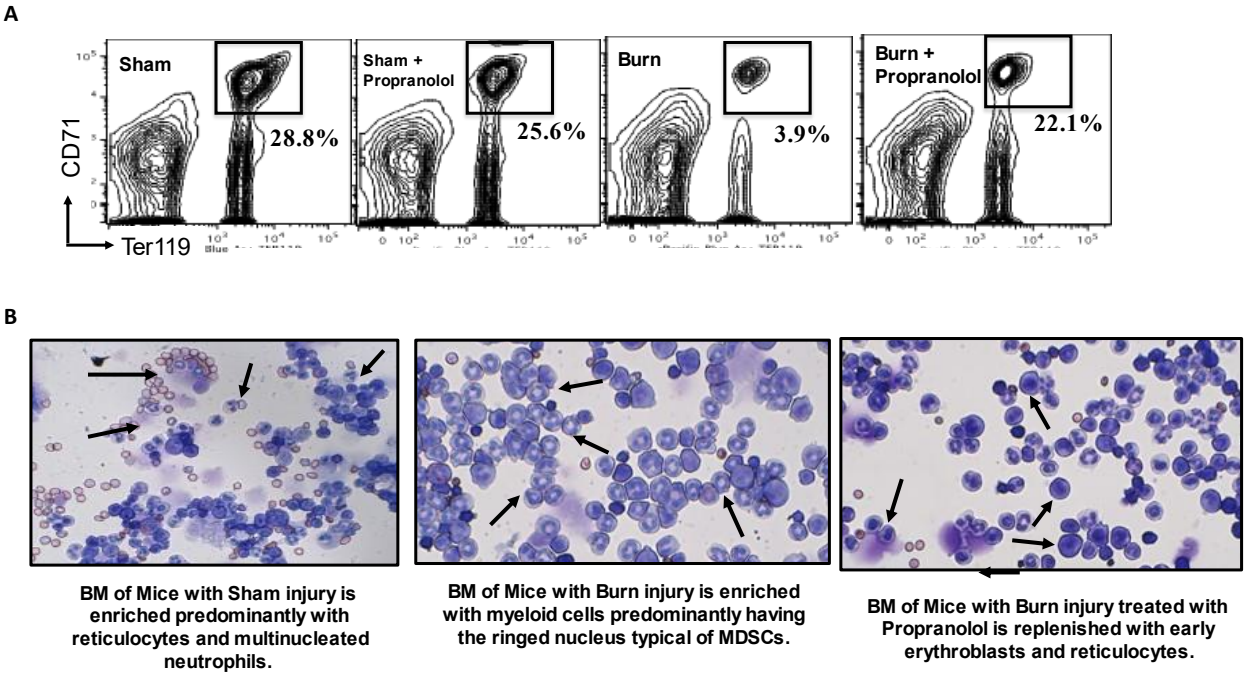


Figure 3. Propranolol Recovers the Erythropoietic Development that is Hampered by Burn Injury and Reduces Granulocytic Cells with Ringed Nucleus, which is Typical of Myeloid Derived Suppressor Cells (MDSCs). (A) CD71 and Ter119 positive cells represent mature erythrocytes which are the fewest in burn mice, but increase in number upon propranolol administration. **(B)** Representation of eluted BM smear from sham mice, post burn day 7 burn mice, and burn mice treated with propranolol. Compared to sham, burn shows fewer reticulocytes and more MDSCs (ringed nucleus). Administering propranolol for six days after burn injury replenished early erythroblasts and reticulocytes with a significant reduction in MDSCs. This figure was provided by Dr. Muthumalaiappan with consent to use in this thesis [44].

MAFB/GATA1 Axis

The in vivo and ex vivo studies transitioned the lab to look at other transcription factors that may be perturbed due to increased MAFB expression. GATA1 (globin transcription factor 1) plays an important role in managing hematopoietic lineages. GATA1 is a transcription factor that promotes erythropoiesis and is essential for HSPCs to differentiate towards erythroid lineage [47]. MAFB on the other hand acts as a repressor to the protein ETS1 which interacts with and promotes GATA1 [48-50]. It was determined in CMPs that high expression of MAFB due to adrenergic signaling caused GATA1 a crucial erythroid developmental factor to decrease in

expression [51]. The higher MAFB and lower GATA1 expression steers CMPs away from the erythroid lineage and towards the myeloid lineage [51]. Thus, these CMPs will exhibit a myeloid bias. The expression of MAFB does not completely negate GATA1 expression in CMPs. Instead, the axis between the two transcription factors leans toward the expression of one more than the other. Which direction this MAFB/GATA1 axis is leaning determines how the CMPs will differentiate. If the MAFB/GATA1 axis is more favored towards MAFB expression than those CMPs will differentiate into GMPs. On the other hand, if the MAFB/GATA1 axis is more favored towards GATA1 expression than those CMPs will differentiate into MEPs.

In this study we aimed to determine if the insights gained from a HCT study, could be related to findings that our lab discovered previously in burn injury [39,44,51]. In other words, does the increase in myeloid lineage cells in the peripheral blood of MM patients originate in the BM. If so, then is the dysregulation in the balance between transcription factors MAFB and GATA1 in progenitor cells responsible for this myeloid bias.

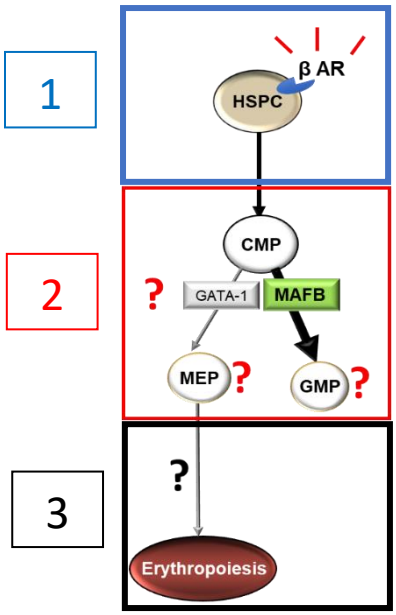


Figure 4. Schematic of Hypothesis. [1] What impact does Beta adrenergic stimulation on HSPCs **[2]** have towards CMP differentiation due to the MAFB/GATA1 axis **[3]** and how does this effect late-stage erythropoiesis.

CHAPTER TWO

EXPERIMENTAL METHODS

BM Aspirates.

MM Samples: In collaboration with Shanmugam Lab, we received mononuclear cells from bone marrow aspirates of multiple myeloma patients shipped from Emory University, Atlanta, GA (stored at -80°C in freezer). Specifications about MM patients of each aspirate were provided by Emory (Table 1).

Table 1. Multiple Myeloma Patient Information. 11 MM patients with associated IDs received from Emory. Plasma cell morphology percentages detail the amount of myeloma cells present within samples. Hemoglobin (Hgb) levels for MM samples have a range between 7.9 to 14.2. A Hgb level below 14 indicates a low Hgb that is indicative of erythropoietic arrest.

MM #	ID	Date received	Plasma cell (%) morphology	Disease stage	Cytogenetics	Hgb(g/dL) at biopsy
MM #1	PS10001774	3/3/2021	60	Progressive	t14;16), +1q, +3, +7, +9, +11, +17	11.5
MM #2	PS10002019	3/3/2021		Progressive	Not available	10.9
MM #3	PS10002001	2/4/2021	60	Newly diagnosed	+1q, +3, +7, +9, +11, -13, +14, +16	10.9
MM #4	PS10001844	10/22/2019	80	Newly diagnosed	t(11;14), +1q, -13, del(17p)	7.9
MM #5	PS10001922	12/30/2020	5	Newly diagnosed	t(11;14) +1q	11.4
MM #6	PS10001702	9/20/2018	80	Progressive	t(4;14), -1p, +1q, +3, +7, +11	9.3
MM #7	PS10001891	5/6/2021	20	relapsed	t(14;16), -1p, +1q, -13, del(17p)	11.6
MM #8	PS10002039	5/6/2021	70	relapsed	. +7, +9, +11-	11.2
MM #9	PS10002029	3/31/2021	15	newly diagnosed	t(11;14), +1p, +1q, -13-	13.4
MM #10	PS10002013	2/19/2021	30-40	relapsed	t(11;14), del(13q)	14.2
MM #11	PS10001653	4/15/2021	15-20	relapsed	-1p, +3, +7, +9, +11, +14, -17	11.5
Mean±SEM						11.25±0.5
Range						(7.9-14.2)

BM Controls: Bone marrow (BM) aspirates from four unidentified healthy donors of age>18 years and <45 years were obtained as an excess portion of the main aspirate not related to this study from Loyola Health Sciences Transplant Center. Samples were stored at 80ºC in 70% freezing media consisting of 90% fetal bovine serum with 10% dimethyl sulfoxide at $4X10^6$ cells per 1.0 mL in cryotubes until day of experiments. To isolate the mononuclear cells

from total BM aspirates of control subjects, we followed the same Ficoll method used by Emory for their collection of MM mononuclear cells. Frozen BM aspirates were brought to 37ºC in a warm bath and then distilled/washed with PBS solution. BM cells resuspended in PBS were then carefully layered over Ficoll solution and centrifuged for 35 minutes at 500xg in room temperature (20-22°C). The buffy coat monolayer containing mononuclear cells were collected for all experiments.

Plating for Cultures.

Phase 1 culture: An aliquot of thawed BM control and MM samples were washed with IMDM and counted to determine seeding density and appropriate culture dish. Cells were plated in 6 well plates for a seeding density of 300,000 to 1,000,000 cells per well or in 12 well plates when yield was less than 300,000 cells per well. Cells were then placed in Serum Free Expansion Medium (SFEM) containing; GMCSF (Granulocyte-macrophage colony-stimulating factor), SCF (Stem cell factor), IL-3 (Interleukin 3 - pluripotent hematopoietic colony-stimulating factor) and incubated at 37°C with 5% CO2 to foster the survival/proliferation of HSPCs. The mononuclear cells contain a heterogenous population including monocytes, neutrophils, lymphocytes, erythroblasts and HSPCs. These mature cells that are still maintained with lineage cocktail will be sorted out during day 6 (end of Phase 1) extraction.

Phase 2 culture: After completion of Phase I, samples are extended into Phase II cultures to measure early and late erythroblast development. Phase II cultures last from day 6 to day 16. Cells collected from Phase I are placed in a growth cocktail consisting of SCF (Stem Cell Factor), HT (Holo-Transferrin), EPO (Erythropoietin), and SFEM (Serum Free Expansion Medium). Similarly, to Phase 1 culture cells were counted to determine seeding density when plating. 100,000 to 300,000 cells were seeded per well in 12 well plates.

-SFEM: STEM CELL TECHNOLOGIES (Catalog #: 9650)

-SCF: STEM CELL TECHNOLOGIES (Catalog #: 78062.1)

-GMCSF: R&D Systems, Inc. (Catalog #: 7954-GM-020/CF)

-IL-3: STEM CELL TECHNOLOGIES (Catalog #: 78146

-EPO: STEM CELL TECHNOLOGIES (Catalog #: 78007.1)

-HT: Sigma Aldrich (Catalog #: T4132-100MG)

-IMDM (+L-Glutamine, +25mM HEPES, −Phenol Red, +1% Pen/Strap, +10% FBS, +1% Plasmocin, +1% Amphotericin): Gibco by Life Technologies (Catalog #: 31980097)

Alpha- and Beta-Adrenergic Receptor Stimulation.

Phenylephrine is an alpha-adrenergic receptor agonist and Isoproterenol is a beta-adrenergic receptor agonist [56,57]. Each were separately added at 1pM, 1nM, and 1μM to different wells in Phase 1 and subsequent Phase 2 cultures. Cells were stimulated every other day throughout cultures.

-Phenylephrine (Phenylephrine hydrochloride): SIGMA Life Science (Catalog #: P6126-10G)

-Isoproterenol (Isoprenaline hydrochloride): SIGMA Life Science (Catalog #: I5627-5G)

Beta Adrenergic Receptor Blockade.

Propranolol, a beta-adrenergic receptor antagonist was added to wells in phase 1 cultures. After 30 to 45 minutes which is enough time for effective blockage of beta receptors, Isoproterenol was added to wells.

-Propranolol (Propranolol Hydrocholoride): SIGMA Life Science (Catalog #: P0884-5G)

Magnetic Isolation.

LIN NEG: Thawed BM cells and Phase 1 cultured cells on day 6 were processed to identify and study the phenotype of MMPs using a combination of magnetic isolation and flow cytometry methods. Biotinylated Anti-human CD3, Biotinylated Anti-human CD14, Biotinylated Anti-human CD19, Biotinylated Anti-human CD20 were incubated with cultured cells along with Anti-Biotin microbeads. Those cells with microbeads attached are mature cells. Those cells that do not have the microbeads attached will elute through the column giving us our lineage negative cells (Lin NEG). We use the Lin NEG cells to isolate HSPCs.

Myeloma cell Isolation: CD138 (Syndecan-1), is a heparin sulphate proteoglycan that controls tumor cell survival, growth, adhesion and bone cell differentiation in MM [52]. The CD138 surface marker was used to isolate myeloma cells from MM samples. BM cells are incubated with biotinylated antibodies of CD138 and subsequently with Anti-Biotin microbeads. The collected sample that passes through column will have no myeloma cells ($CD\ 138^{-}$). Myeloma cells retained in column are flushed with 1.0 mL of buffer and then reseeded into various control BM cells to examine effects of myeloma cells on various parameters.

-MS columns: Miltenyi Biotec (Catalog #: 130-042-201)

-Biotinylated Microbeads: Thermo Fisher Scientific Inc (Catalog #: 11047)

-Anti-Biotin CD3 antibody: Thermo Fisher Scientific Inc (Catalog #: 13-0037-80)

-Anti-Biotin CD14 antibody: Thermo Fisher Scientific Inc (Catalog #: 13-0149-80)

-Anti-Biotin CD19 antibody: Thermo Fisher Scientific Inc (Catalog #: 13-0199-80)

-Anti-Biotin CD20 antibody: Thermo Fisher Scientific Inc (Catalog #: 13-0209-82)

-Anti-Biotin CD138 antibody: Miltenyi Biotec (Catalog #: 130-119-926)

Staining with Cell Surface Markers for Flow Cytometry.

Lineage negative cells ($CD3^{-}$ $CD14^{-}$ $CD19^{-}$ $CD20^{-}$) that flow through the column are incubated with Anti-CD34, Anti-CD38, Anti-CD45RA, and Anti-CD123 to determine the cell surface expression of $CD34^{+}$, $CD38^{+}$, $CD45RA^{+/-}$, $CD123^{+/-}$ cells and are purified (flow sort). Among the Lin ^{neg} cells, $CD34^{+}$ $CD38^{+}$ HSPC population are selected and re-gated with CD45RA, and CD123 to distinguish the $CD34^{+}$ $CD38^{+}$ $CD45RA^{+/-}$ $CD123^{+}$ common myeloid progenitors (CMPs), $CD34^{+}$ $CD38^{+}$ $CD45RA^{-}$ $CD123^{-}$ MEPs and $CD34^{+}$ $CD38^{+}$ $CD45RA^{+}$ $CD123^{-}$ GMPs.

Thawed BM cells and Phase 2 cells enriched for erythroblasts, are identified using differential expression of CD71 and CD235a to distinguish between early erythroblasts (EEBs/$CD71^{+}CD235a^{-}$), late erythroblasts (LEBs/$CD71^{+}CD235a^{+}$), and non-erythroblasts (Non-Eryth/ $CD71^{-}CD235a^{-}$).

-V450 conjugated CD45RA antibody: BD Biosciences (Catalog #: 560362)

-APC conjugated CD34 antibody: Thermo Fisher Scientific Inc (Catalog #: CD34-581-05)

-APC conjugated CD38 antibody: Thermo Fisher Scientific Inc (Catalog #: 47-0381-80)

-PeCy7 conjugated CD123 antibody: Thermo Fisher Scientific Inc (Catalog #: 25-1239-42)

-APC conjugated CD235a antibody: Thermo Fisher Scientific Inc (Catalog #: MA5-17697)

-PE conjugated CD71 antibody: Thermo Fisher Scientific Inc (Catalog #: 12-0711-82)

MAFB and GATA1 Intracellular Identification and Quantification by Flow Cytometry.

Purified HSPCs are first fixed and permeabilized using a perm buffer solution and cytofix/cytoperm solution. After permeabilization and fixation cells are incubated with Anti-

GATA1 for 48 hours and Anti-MAFB for 24 hours to determine the intracellular expression patterns of MAFB and GATA1. Cells are washed in a perm wash solution.

-FITC conjugated MAFB antibody: US Biological Life Science (Catalog #: 038061)

-PE conjugated GATA1 antibody: US Biological Life Science (Catalog #: 030259)

-Cytofix/Cytoperm Solution: BD Biosciences (Catalog #: 554714)

-Perm Buffer Solution: BD Biosciences, diluted with DI-water at 10% (Catalog #: 554714)

-Perm Wash Solution: BD Biosciences, diluted with DI-water at 10% (Catalog #: 554714)

Adrenergic Receptor Incubation.

Purified HSPCs are first fixed and permeabilized using a perm buffer solution and cytofix/cytoperm solution. After permeabilization and fixation cells are incubated with primary adrenergic receptor antibodies for 24 hours. After 24-hour incubation, cells are washed with a perm wash solution and then incubated with subsequent FITC conjugated secondary antibodies for 3 hours.

-Primary unconjugated BETA1 adrenergic antibody: Santa Cruz Biotechnology, Inc. (Catalog #: sc-47778)

-Primary unconjugated BETA2 adrenergic antibody: Santa Cruz Biotechnology, Inc. (Catalog #: sc-81577)

-Primary unconjugated BETA3 adrenergic antibody: Santa Cruz Biotechnology, Inc. (Catalog #: sc-518080)

-Primary unconjugated ALPHA1 adrenergic antibody: Santa Cruz Biotechnology, Inc. (Catalog #: sc-100291)

-Primary unconjugated ALPHA2 adrenergic antibody: Santa Cruz Biotechnology, Inc. (Catalog #: sc-390430)

-Secondary FITC conjugated antibody for ALPHA1/BETA1&2 (Anti-Goat): Santa Cruz Biotechnology, Inc. (Catalog #: sc-2356)

-Secondary FITC conjugated antibody for ALPHA2/BETA3(Anti-Rabbit): Santa Cruz Biotechnology, Inc. (Catalog #: sc-2359)

-Cytofix/Cytoperm Solution: BD Biosciences (Catalog #: 554714)

-Perm Buffer Solution: BD Biosciences, diluted with DI-water at 10% (Catalog #: 554714)

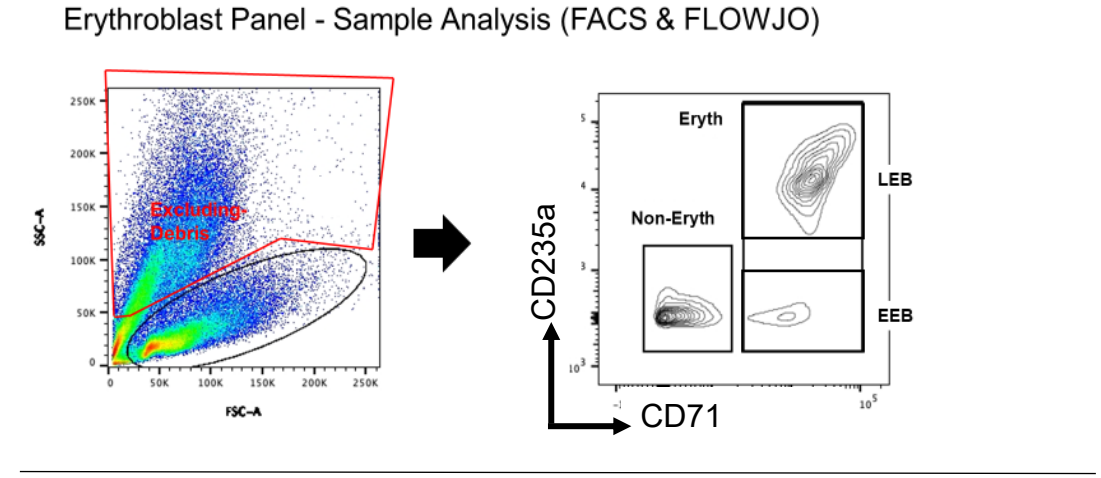
-Perm Wash Solution: BD Biosciences, diluted with DI-water at 10% (Catalog #: 554714)

FACS and FLOWJO Analysis.

FACS stands for fluorescent-activated cell sorting and is used to separate our heterogenous samples of cells into subpopulations. Upon completion of cell surface and intracellular antibody staining techniques, samples were taken to the FACS core facility for sample interrogation. Quantitative and qualitative data on fluorophore-labeled cell surface receptors and intracellular molecules are achieved using multi-channel detectors. Compensation controls and unstained samples were run to calibrate instrument setting for each experiment. The FLOWJO software was used to analyze and quantify our FACS data. FACS data was first gated by excluding debris and then gated for distinct subsets in both erythroblast (Figure 5A) and HSPC (Figure 5B) sample analyses. For erythroblast panels: Non-Eryths were gated as $CD71^{-}CD235a^{-}$, EEBs were gated as $CD71^{+}CD235a^{-}$, and LEBs were gated as $CD71^{+}CD235a^{+}$. For HSPC panels: CMPs were gated as $CD34^{+}CD38^{+}CD45RA^{+/-}CD123^{+}$, GMPs were gated as $CD34^{+}CD38^{+}CD45RA^{+}CD123^{-}$, and MEPs were gated as

$CD34^{+}CD38^{+}CD45RA^{-}CD123^{-}$. Geometric mean intensity was used to determine MAFB and GATA1 expression in CMPs.

A



B

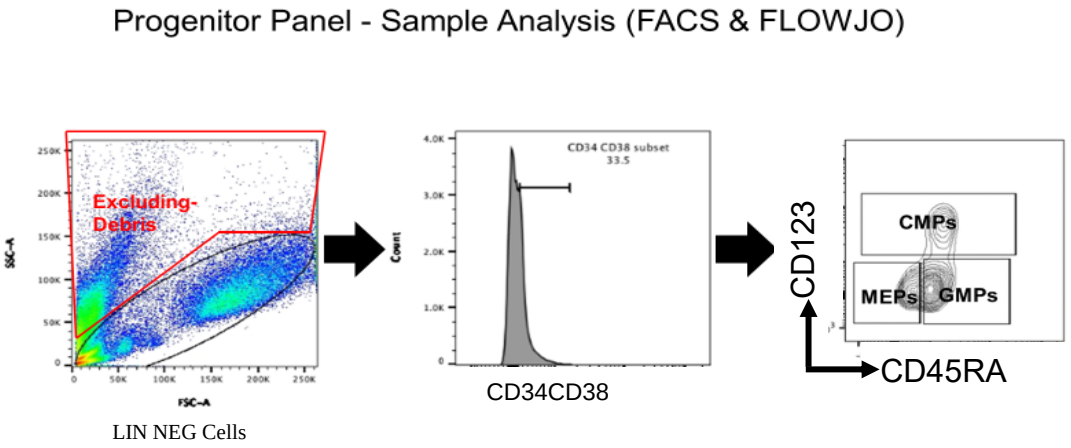


Figure 5. Erythroblast and Progenitor Subset Gating. (A) Erythroblast panel: total cells were excluded for debris on forward and side scatter and then gated for CD71 (x-axis) and CD235a (y-axis) expression to determine Non-Eryth/LEB/EEB populations. (B) Progenitor panel: total cells were excluded for debris on forward and side scatter and then gated for positive CD34 and CD38 expression followed by subsequent gating of CD45RA (x-axis) and CD123 (y-axis) expression to determine CMP/GMP/MEP populations.

MAFB Gene Silencing.

Phase 1 cultured BM controls and MM samples were left in growth factor cocktail for one day to increase proliferating cells for optimal *MAFB* silencing using siRNA. On the day of transfection, medium was aspirated out and cells were resuspended in Opti-MEM. To silence *MAFB* a lipid-based transfection medium (X-tremeGENE) was used to allow uptake of the siRNA into live cells. *MAFB* siRNA was mixed with transfection reagent and added to cells of interest one drop at a time. A non-specific FITC labelled scrambled RNA was used as a control in parallel wells. The FITC scramble will not silence *MAFB,* but instead will determine transfection efficiency to help discern on the specificity of *MAFB* silencing. Four hours after transfection, cells were replenished with Phase 1 growth cocktail and allowed to culture for seven days. Transfection efficiency was checked on day 3 through FACS by comparing MAFB expression of the control (scrambled RNA) to samples that received siRNA. CMPs/GMPs/MEPs were analyzed by FACS on day 7 after Phase 1 culture was complete. Flow cytometry was followed as explained previously to determine HSPCs, CMP, GMP and MEP development with and without *MAFB* siRNA.

-Control Scramble (FITC Fluorescein Conjugate)-A 10 µM: Santa Cruz Biotechnology, Inc (Catalog #: sc-36869)

-*MAFB* siRNA (h) 10 µM: Santa Cruz Biotechnology, Inc (Catalog #: sc-35839)

-Opti-MEM™ I Reduced Serum Medium: Thermo Fisher Scientific Inc (Catalog #: 31985062)

-X-tremeGENE™ siRNA Transfection Reagent: Millipore Sigma (Catalog #: 4476093001)

Giemsa Staining.

Cytospin fixed and permeabilized cells in IMDM at 100 rpm for 5 minutes. After cytospin, dipped slides in methanol for 1 minute. Once 1 minute of methanol dip has passed, dipped slides in May-Grunwald stain for 10 minutes. After 10 minutes, slides are dipped in Tris (25mM pH 7.4) for 2 minutes and subsequently dipped slides in Giemsa stain for 10 minutes. Lastly, the slides were rinsed with Milli-Q water and let dry vertically in a slide holder for 30 minutes before viewing under scope.

-Giemsa Stain: Sigma Aldrich, diluted 1ml in 38ml of Milli-Q water (Catalog #: G5637)

-Tris (25mM): Sigma Aldrich, 3.03g Trizma hydrochloride in 1L Milli-Q water adjusted to 7.4pH (Catalog #: 648311)

-May-Grunwald Stain: Sigma Aldrich (Catalog #: MG500)

-IMDM (+L-Glutamine, +25mM HEPES, −Phenol Red, +1% Pen/Strap, +10% FBS, +1% Plasmocin, +1% Amphotericin): Gibco by Life Technologies (Catalog #: 31980097)

CHAPTER THREE

RESULTS

MM Patient Samples Have a Lower Number of Total Erythroblasts Versus BM Control Samples Indicating Diminished Erythropoiesis

Erythropoiesis is split between three phases: the commitment phase, the proliferation phase, and the maturation phase [53]. During the commitment phase HSCs commit towards MEPs, and then progress towards the proliferation phase where they develop into EEBs [53]. Lastly, during the maturation phase EEBs mature into LEBs and eventually erythrocytes [53]. Due to our aspirates containing isolated MNCs only, we cannot measure erythrocyte numbers. However, we measured the erythroblast population and in doing so grasp how erythropoiesis is occurring in these samples. Samples were analyzed for their erythroblast populations immediately after thawing from frozen aspirates and designated as day 0 samples. Day 0 analysis of the erythroblast population compared MM samples with control BM (Figure 6). When comparing the two: a contour plot of MM sample had a lower number of erythroid cells versus control BM (Figure 6A). The contour plot has CD71 on the x-axis and CD235a on y-axis to determine erythroblast population (Figure 6A). Upon further analysis, eleven MM samples and three control BM samples were quantified per one million BM MNCs for their total erythroblast populations (Figure 6B). The MM samples displayed a significantly lower total erythroblast population when compared to the control BM samples. When looking at the different subsets of our erythroblast population we can examine the EEBs and LEBs. Quantification per one million BM MNCs of both EEBs and LEBs shows that LEBs are lower in a MM sample compared to a

control BM sample (Figure 6C). From our day 0 analysis of the erythroblast population, we can confirm that there is diminished erythropoiesis in our MM samples. The source of the erythroblast population is the MEPs, which bring into question what their status is.

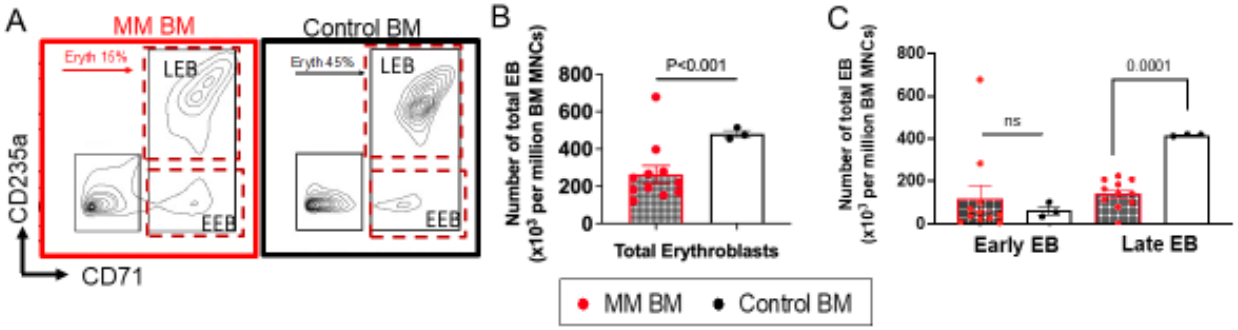


Figure 6. Increased Non-Erythroid Population and Reduced Erythroblast Population in MM Compared to Control BM (Day 0). Samples were stained for FACS the same day that they were processed. **(A)** Right contour plot (CD71/CD235a) of a control BM sample shows an erythroid population of 45% and left contour plot (CD71/CD235a) of a MM sample shows an erythroid population of 15%. **(B)** Quantification per one million BM MNCs examining the total erythroblast population of three BM control samples (n=3) and eleven MM samples (n=11) show a significantly reduced amount of total erythroblast in MM samples compared to BM controls. Unpaired t test with Welch's correction assigned a two-tailed P value between control BM and MM samples for total erythroblasts: <0.001. **(C)** EEBs (Early EB) and LEBs (Late EB) encompass the erythroblast population. When looking at these subsets per one million BM MNCs, we find that LEBs are significantly reduced in MM samples compared to BM controls. Unpaired t test with Welch's correction assigned a two-tailed P value between control BM and MM samples for EEBs: ns and LEBs: 0.001.

MM Patient Samples Have Greater Number of GMPs and Lower Number of MEPs Versus BM Control Samples (Day 0)

MM and control BM samples were analyzed for CMPs. GMPs, and MEPs immediately after thawing from frozen aspirates. Samples were designated as day 0 samples and LIN-NEG population was isolated, and surface stained with conjugated antibodies for CMPs, GMPs, and MEPs. Contour plots for myeloid and erythroid progenitors (Lin^{neg} $CD34^{+}CD38^{+}$ $CD123^{+/-}$ $CD45RA^{+/-}$) of a control BM sample compared against a MM sample demonstrates that the

percentage of GMPs increases from 13% in control BM to 52% in MM sample (Figure 7A). MEPs alternatively, decreased from 64% in a control BM sample to 19% in a MM sample (Figure 7A). When examining with more samples and quantifying per one million HSPCs we find a similar trend of MM samples containing a higher number of GMPs and lower number of MEPs when compared to control BM samples (Figure 7B).

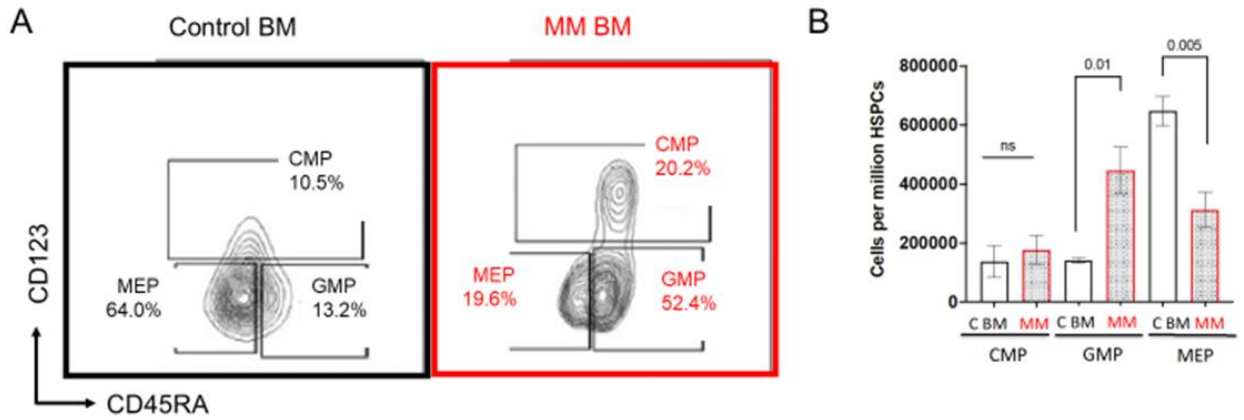


Figure 7. GMPs Increase and MEPs Decrease in MM Samples Compared to Control BM (Day 0). (A) Day 0 $CD34^{+}CD38^{+}$ cells from lineage negative cells were gated for CD45RA on X-axis and CD123 on Y-axis in contour plots from FACS. MM sample had 52.4% of GMPs / 19.6% of MEPs / and 20.2% of CMPs in HSPC subset compared to 13.2% GMPs / 64.0% MEPs / and 10.5% CMPs in BMC. **(B)** Distribution of bone marrow progenitors represented in bar graphs. Three Day 0 BM Control samples (n=3) were used for control metric and five Day 0 MM samples including three MM samples that were average amongst several trials (n=5) were used for MM sample aggregate. Unpaired t test with Welch's correction assigned a two-tailed P value between control BM and MM samples for CMPs: ns, GMPs: 0.01, and MEPs: 0.005.

MM Patient Samples Have Increased MAFB Expression and Decreased GATA1 Expression Versus BM Control Samples (Day 0)

After surface staining Day 0 samples of BM controls and MM samples were incubated with anti-MAFB and anti-GATA1 after permeabilizing and fixing the cells. Geometric mean intensities of FITC-conjugated MAFB antibody and PE conjugated GATA1 antibody was measured in CMPs of the MM samples and compared to the BM control samples (Figure 8). CMPs were used for measurements due to the influences MAFB and GATA1 have in

determining whether CMPs differentiate into GMPs (high MAFB/low GATA1) or MEPs (high GATA1/low MAFB). Contour plots ($CD34^{+}CD38^{+}$) overlayed control BM samples with MM samples to show expression patterns. Produced overlay of MAFB plot (MAFB on x-axis) signifies MM samples having most of its CMPs towards the right of the plot indicating positive expression, while control BM samples have most of its CMPs toward the left indicating negative expression (Figure 8A). When comparing absolute geometric mean intensities of MAFB: MM samples gave us a mean expression of 2,500 versus 1024 for our control BM samples (Figure 8A). Alternatively, produced overlay of GATA1 plot (GATA1 on x-axis) signifies MM samples having most of its CMPs towards the left of the plot indicating negative expression, while control BM samples have most of its CMPs toward the right indicating positive expression (Figure 8B). When comparing absolute geometric mean intensities of MAFB: MM samples gave us a mean expression of 7,650 versus 12,570 for our control BM samples (Figure 8B). When normalizing our MAFB and GATA geometric mean intensities towards our control BM we find that MAFB is increased and GATA1 is decreased in MM samples compared to control BM samples (Figure 8C). There is a fine balance and tuning between the transcription factors MAFB and GATA1. Thus, the most apt way to view the changes in MAFB and GATA1 between different conditions is as a ratio. The ratio of MAFB:GATA1 for the BM control samples was 12:1 while the MM samples was 1:3. This clearly denotes that the MAFB:GATA1 ratio increases in MM samples compared to BM control samples.

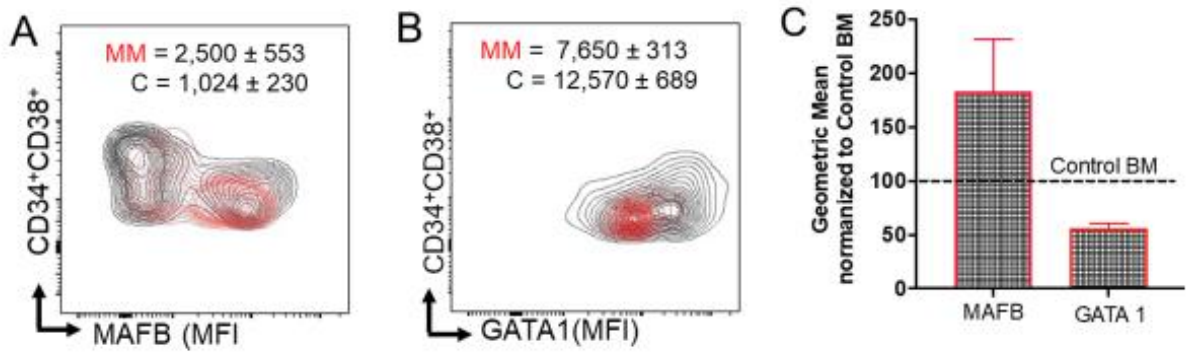


Figure 8. MAFB Expression is Higher and GATA1 Expression is Lower in CMPs of MM Samples Versus BM Control Samples (Day 0). (A) Day 0 representative overlay of intracellular expression of MAFB in CMPS from MM (red) and Control BM (Black). Lin^{neg} $CD34^{+}CD38^{+}$ $CD123^{+}$ $CD45RA^{-}$ (CMPs) from six MM samples (n=6) and three BM control samples (n=3). Numbers indicate absolute geometric mean expression followed by standard error of measurement (SEM). There is more positive expression of MAFB in CMPs of MM samples as opposed to BM control samples. **(B)** Day 0 representative overlay of intracellular expression of GATA 1 in CMPS from MM (red) and Control BM (Black). Lin^{neg} $CD34^{+}CD38^{+}$ $CD123^{+}$ $CD45RA^{-}$ (CMPs) from three MM samples (n=3) and three BM control samples (n=3). Numbers indicate absolute geometric mean expression followed by standard error of measurement (SEM). Most of the GATA1 expression is negative in MM samples indicating lower expression of GATA1 in MM samples compared to BM controls. **(C)** Bar graphs of the geometric mean fluorescent intensity of MAFB and GATA 1 normalized to Control BM as 100 (arbitrary set). MAFB = 174; GATA1 = 54 in MM versus 100 in Control BM. Statistical analysis was done using Wilcoxon Signed Rank Test to assign a two-tailed P value between normalized MAFB MM samples (0.0012) and between normalized GATA1 MM samples (0.2500).

After Phase 1 Expansion MM Patient Samples Have Greater Number of GMPs and Lower Number of MEPs versus BM Control Samples (DAY 6)

Conclusions drawn from Day 0 samples can only tell us details at a certain time-point. In order to confirm a myeloid bias is the reason for increase in GMPs and decrease in MEPs as seen in day 0 analysis we must determine the commitment pattern of the HSCs in MM samples. In addition, the environment/situation vary amongst patient samples and controls. For these reasons we place our samples in phase 1 culture. The phase 1 culture maintains HSPCs in same culture conditions ex-vivo and allows us to delineate the HSPCs commitment profile in MM. These samples were denoted as day 6. Day 6 analysis showed similar profiles to day 0 analysis of

CMPs, GMPs, and MEPs for MM samples. Contour plots for myeloid and erythroid progenitors (Lin^{neg} $CD34^{+}CD38^{+}$ $CD123^{+/-}$ $CD45RA^{+/-}$) of a control BM sample compared against a MM sample demonstrates that the percentage of GMPs increases from 11% in control BM to 59% in MM sample (Figure 9A). MEPs alternatively, decreased from 65% in a control BM sample to 20% in a MM sample (Figure 9A). When examining with more samples and quantifying per one million HSPCs we find that while CMPs had no significant difference, GMPs were significantly increased, and MEPs were significantly decreased in MM samples compared to control BM samples (Figure 9B).

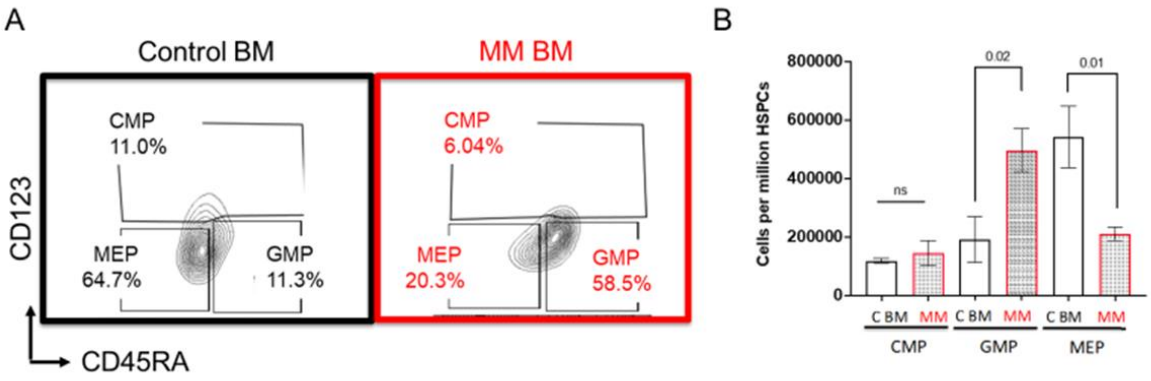


Figure 9. GMPs are Increased and MEPs are Reduced in MM Samples Versus BM Control Samples Placed in Phase 1 Expansion (Day 6). (A) Day 6 $CD34^{+}CD38^{+}$ cells from lineage negative cells were gated for CD45RA on X-axis and CD123 on Y-axis in contour plots from FACS. MM sample had 58.5% of GMPs / 20.3% of MEPs / and 6.04% of CMPs in HSPC subset compared to 11.3% GMPs / 64.7% MEPs / and 11.0% CMPs in BMC. **(B)** Distribution of bone marrow progenitors represented in bar graphs. Two Day 6 BM Control samples (n=2) were used for control metric and three Day 6 MM that were average amongst several trials (n=3) were used for MM sample aggregate. Unpaired t test with Welch's correction assigned a two-tailed P value between control BM and MM samples for CMPs: ns, GMPs: 0.02, and MEPs: 0.01.

MAFB Expression is Higher and GATA1 Expression is Lower in MM samples After Phase 1 Expansion (Day 6)

As with day 0, day 6 samples were similarly incubated with anti-MAFB and anti-GATA1. Geometric mean intensity of MAFB and GATA1 was measured in CMPs of day 6

samples (Figure 10). Contour plots ($CD34^{+}CD38^{+}$) overlayed control BM samples with MM samples to show expression patterns. Produced overlay of MAFB plot (MAFB on x-axis) signifies MM samples having most of its CMPs towards the right of the plot indicating positive expression, while control BM samples have most of its CMPs toward the left indicating negative expression (Figure 10A). When comparing absolute geometric mean intensities of MAFB: MM samples gave us a mean expression of 2,522 versus 1485 for our control BM samples (Figure 10A). Inversely, produced overlay of GATA1 plot (GATA1 on x-axis) signifies MM samples having most of its CMPs towards the left of the plot indicating negative expression, while control BM samples have most of its CMPs toward the right indicating positive expression (Figure 10B). When comparing absolute geometric mean intensities of MAFB: MM samples gave us a mean expression of 7,899 versus 24,094 for our control BM samples (Figure 10B). When normalizing our MAFB and GATA geometric mean intensities towards our control BM we find that MAFB is increased and GATA1 is decreased in MM samples compared to control BM samples (Figure 10C). Looking again at MAFB and GATA1 expression as a ratio we find the ratio of MAFB:GATA1 for the BM control samples is 16:1 while the MM samples is 1:3. This conveys that there is a increase in the ratio of MAFB:GATA1 expression in MM samples compared to BM control samples.

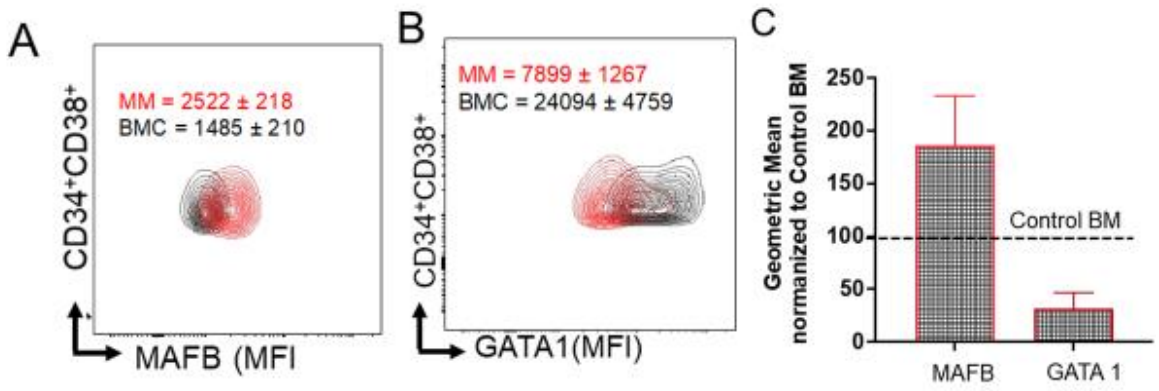


Figure 10. Phase 1 Expanded CMPs Display Higher MAFB Expression and Lower GATA1 Expression in MM samples Versus BM Control Samples (Day 6). (A) Day 6 representative overlay of intracellular expression of MAFB in CMPS from MM (red) and Control BM (Black). Lin^{neg} $CD34^{+}CD38^{+}$ $CD123^{+}$ $CD45RA^{-}$ (CMPs) from four MM samples (n=4) and two BM control samples (n=2). Numbers indicate absolute geometric mean expression followed by standard error of measurement (SEM). There is more positive expression of MAFB in CMPs of MM samples as opposed to BM control samples. **(B)** Day 6 representative overlay of intracellular expression of GATA 1 in CMPS from MM (red) and Control BM (Black). Lin^{neg} $CD34^{+}CD38^{+}$ $CD123^{+}$ $CD45RA^{-}$ (CMPs) from four MM samples (n=4) and two BM control samples (n=2). Numbers indicate absolute geometric mean expression followed by standard error of measurement (SEM). Most of the GATA1 expression is negative in MM samples indicating lower expression of GATA1 in MM samples compared to BM controls. **(C)** Graphical quantification of the four MM samples and two BM control samples for MAFB and GATA1. BM control samples were set at an arbitrary geometric mean expression of 100 and MM samples were normalized against that giving an averaged expression of 190.9 for MAFB and averaged expression of 28.5 for GATA1. Statistical analysis was done using Wilcoxon Signed Rank Test to assign a two-tailed P value between normalized MAFB MM samples (0.1250) and between normalized GATA1 MM samples (0.1250).

Myeloma Cells Do Not Seem to Influence Myeloid Bias

To determine whether myeloma cells contribute to myeloid bias of HSPCs, we isolated the myeloma cells from MM samples and reseeded them into control BM. Both MM samples without myeloma cells present and reseeded control BM were placed in phase 1 culture to identify commitment potential (i.e. myeloid bias). Day 0 MM sample and control BM sample was analyzed to determine effective surface markers for myeloma cell extraction. Literature denotes that CD138 and CD38 are used as markers for malignant plasma cells [54]. Contour plot with gating on CD138 (x-axis) and CD38 (y-axis) defining our myeloma population demonstrate

that 28% of MM samples fall within this context while less than 1% of control BM have cells that are $CD138^{+}CD38^{+}$ (Figure 11A). In addition, control BM was mostly negative for CD138 with most of the cell population lying to the left of the contour plot (Figure 11A). $CD138^{+}$ $CD38^{-}$ are still myeloma cells [55]. With justification of $CD138^{+}$ cells being present in mostly MM samples, it was used to pull entire myeloma population. When myeloma ($CD138^{+}$) cells were reseeded into BM controls and carried through phase 1 culture we found that there was no significant difference in CMPs, GMPs, and MEPs between it and intact BM controls (Figure 11B). Myeloid and erythroid progenitors were quantified per one million HSPCs. MM samples that had their myeloma ($CD138^{+}$) cells extracted were similarly placed in phase 1 culture and denoted as MM $CD138^{-}$ samples. When quantifying per one million HSPCs, myeloid bias persisted in MM samples without the presence of myeloma ($CD138^{+}$) cells as compared to BM controls (Figure 11C). This resulted in an increase in GMPs and decrease in MEPs in MM $CD138^{-}$ samples compared to BM control samples (Figure 11C).

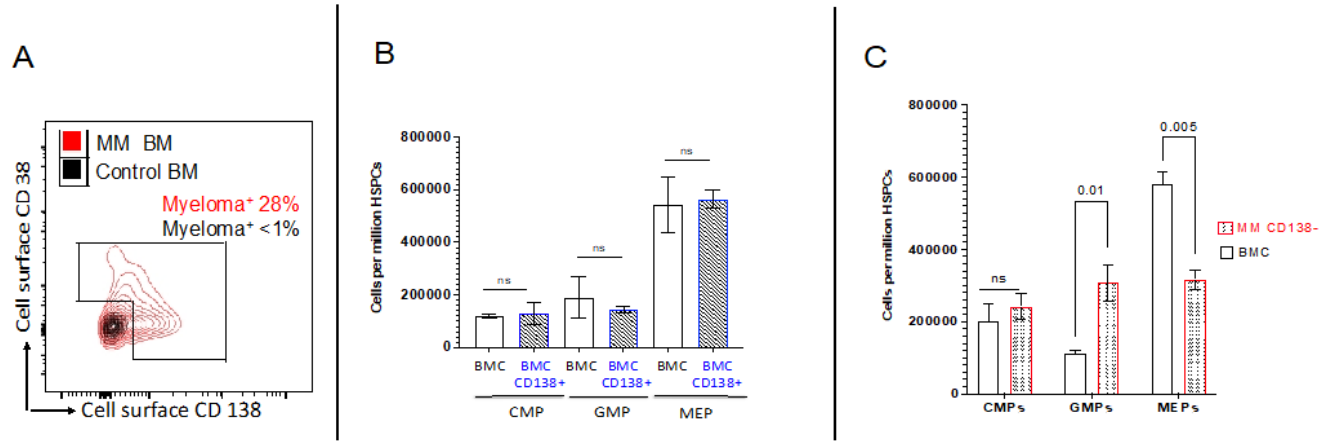
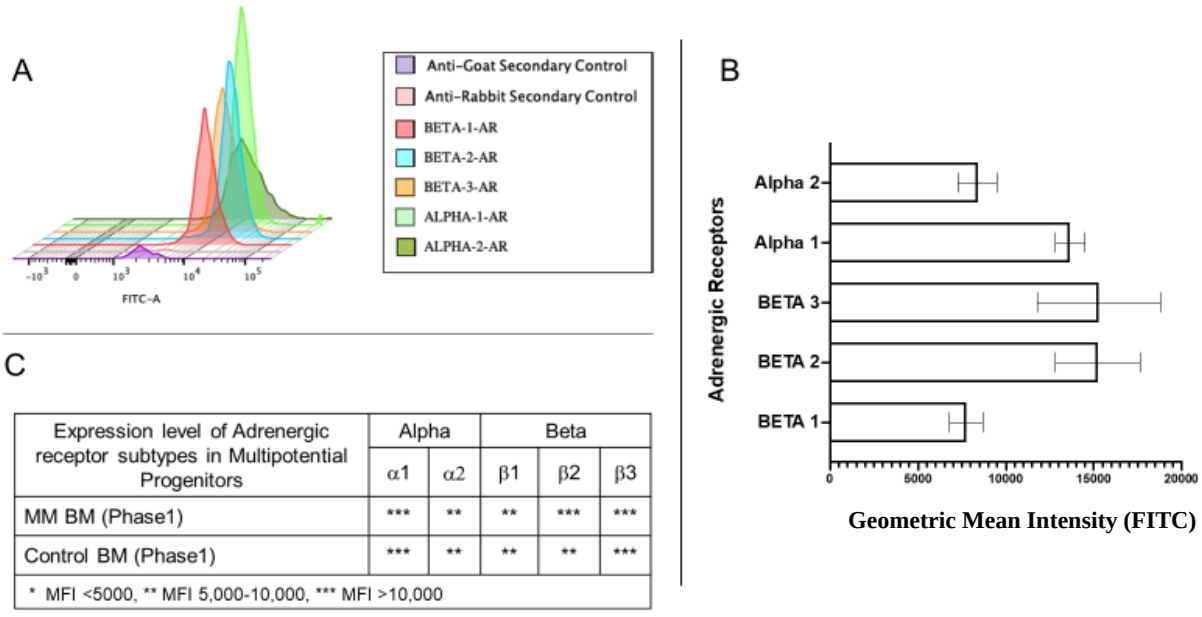


Figure 11. Myeloma Cells Do Not Seem to Influence Myeloid Bias. (A) Contour plot of day 0 MM sample and day 0 control BM sample gated for CD138 and CD38. Myeloma sample (red) has cells that are positive for both CD38 and CD138 and this accounts for around 28%. Control BM sample (black) has cells that are mostly negative for both CD38 and CD138 and this accounts for less than 1%. **(B) CMP/GMP/MEP BM control vs. BM control with CD138+ cells**. Before phase 1 culture CD138 positive cells were reseeded into two (n=2) BM Controls (BMC) and compared with two (n=2) BMCs that did not get reseeded. No significant difference was observed in CMPs, GMPs, and MEPs between BMC with CD138 positive cells and BMC without CD138 positive cells. Unpaired t test with Welch's correction assigned a two-tailed P value between control BM and control BM with CD138+ cells for CMPs: ns, GMPs: ns, and MEPs: ns. **(C) BM control vs. MM samples with CD138- cells**. CD138 positive cells were isolated from five MM samples and compared to three BMCs. CMPs when comparing the two types of samples did not have a significant difference. GMPs and MEPs did display a significant difference between the two types of samples indicating that myeloid bias is maintained in MM samples without the presence of CD138 positive cells. Unpaired t test with Welch's correction assigned a two-tailed P value between control BM and MM samples with CD138- cells for CMPs: ns, GMPs: 0.01, and MEPs: 0.005.

HSPCs Express All Alpha-and-Beta-Adrenergic Receptor Sub-Types

HSPC subset from Lin^{neg} population was used to determine presence of Alpha- and Beta-adrenergic receptor sub-types. Histogram overlay of HSPCs from control BM sample confirms positive expression of all Alpha- and Beta-adrenergic receptor sub-types (Figure 12A). Positive expression was determined based off secondary antibody control which acts as a negative control. All receptor subtypes where on the right of secondary controls on the histogram plot, denoting positive expression. Geometric mean intensity was measured in all adrenergic receptor sub-types in HSPCs from a MM sample and control BM samples (Figure 12B). Alpha2-and-Beta1-adrenergic receptors have mean intensities between 5000 and 10,000 while Alpha1-Beta2-Beta3-adrenergic receptors mean intensities above 10,000 (Figure 12B). Comparing adrenergic receptor expression of HSPCs from control BM samples to the MM sample, we find that expression patterns are similar between the two (Figure 12C).



C

Expression level of Adrenergic receptor subtypes in Multipotential Progenitors	Alpha		Beta		
	α1	α2	β1	β2	β3
MM BM (Phase1)	***	**	**	***	***
Control BM (Phase1)	***	**	**	**	***
* MFI <5000, ** MFI 5,000-10,000, *** MFI >10,000					

Figure 12. All Alpha-and-Beta-Adrenergic Receptor Sub-Types are Present on HSPCs. Phase 1 HSPCs incubated with primary AR antibodies and then attached with secondary FITC conjugated antibodies. **(A)** Histogram overlay of HSPCs from control BM show positive expression for all AR sub-types. **(B) Geometric Mean Receptor Expression**. Bar graph of geometric mean intensity of different Alpha-and-Beta-AR subtypes of HSPCs cumulatively from control BM samples and MM sample. Alpha1-AR (n=3), Alpha2-AR (n=2), Beta1-AR (n=3), Beta2-AR (n=2), Beta3-AR (n=3). **(C)** Chart conveying AR expression of HSPCs between control BM sample and MM sample. Similar expression patterns of AR subtypes exist between control BM and MM sample (comparing Mean fluorescent intensity-MFI).

Alpha-Adrenergic Receptor Stimulation Does Not Cause Myeloid Bias; Beta-Adrenergic Receptor Stimulation Does

Control BM samples placed in phase 1 cultures were treated with phenylephrine (PE) and isoproterenol (ISO) at concentrations of 1pM, 1nM, and 1μM. PE is an Alpha-adrenergic receptor agonist and ISO is a Beta-adrenergic receptor agonist [56,57]. CMPs, GMPs, and MEPs were examined per one million HSPCs to determine effect of Alpha- and Beta-adrenergic receptor stimulation on myeloid bias (increased GMPs/decreased MEPs). In terms of Beta-adrenergic receptor stimulation: increasing concentrations of ISO directly correlated to GMP levels and inversely correlated to MEP levels (Figure 13A). As ISO concentration increased,

GMPs increased and MEPs decreased (Figure 13A). On the other hand, in terms of Alpha-adrenergic receptor stimulation: increasing concentrations of PE did not seem to influence GMP or MEP levels (Figure 13B). PE dosage response kept GMPs and MEPs relatively unchanged from no PE being dispensed to PE being dispensed at highest concentration of 1μM (Figure 13B). Beta-adrenergic receptor stimulation resulted in CMPs having a myeloid bias, but Alpha-adrenergic receptor stimulation did not affect CMP differentiation. To confirm the effects of Beta-adrenergic receptor stimulation on myeloid bias, propranolol was used. Propranolol is a non-specific Beta-adrenergic receptor antagonist that binds to the receptor and blocks/prevents it from being stimulated [58]. Various dosages of propranolol were administered to control BM samples placed in phase 1 culture and then subsequent dosages of ISO were added after. Propranolol was administered at two orders higher than ISO concentration, except for at 1μM where it was administered at equal parts (1μM). This was compared to control BM samples receiving no propranolol. Propranolol treatment led to slight decreases in GMPs and recovery of MEPs during all conditions (Figure 13C). At the picomolar and micromolar condition, GMPs were significantly reduced, and at picomolar and nanomolar MEPs were significantly elevated (Figure 13C). Beta-adrenergic receptor stimulation is influential to myeloid bias as both blocking and stimulating the receptor has an impact on the bias. Stimulating the receptor leads to the bias and blocking the receptor negates the bias.

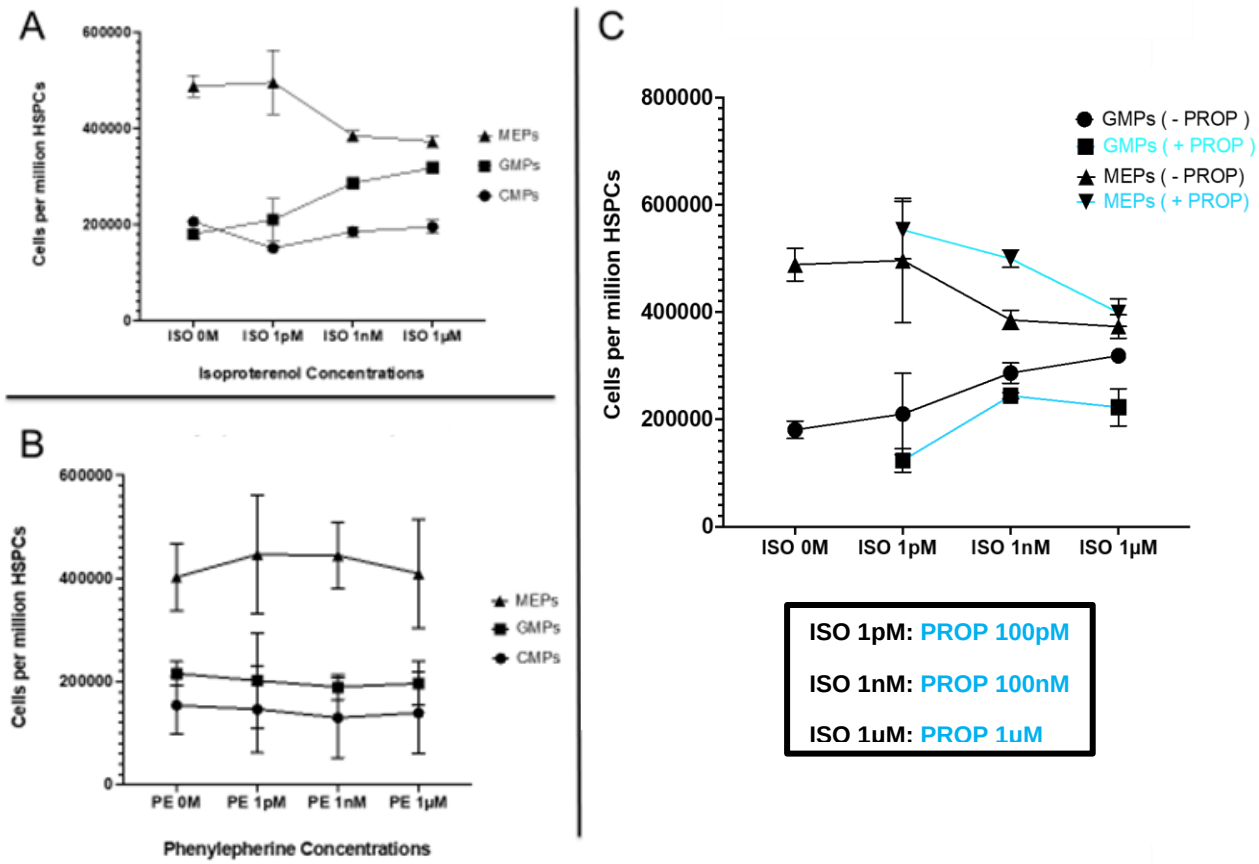


Figure 13. Alpha-Adrenergic Receptor Agonist (Phenylephrine) Does Not Alter GMPs/MEPs; Beta-Adrenergic Receptor Agonist (Isoproterenol) and Antagonist (Propranolol) Do Alter GMPs/MEPs. (A) CMP/GMP/MEP Isoproterenol Dosage Response. 4 trials (n=4) where conducted using 1pM, 1nM, and 1µM of isoproterenol (ISO) added to control BM samples in phase 1 cultures. ISO has little effect on CMPs and seems to increase GMPs and decrease MEPs with increasing concentrations. **(B) CMP/GMP/MEP Phenylephrine Dosage Response**. 2 trials (n=2) where conducted using 1pM, 1nM, and 1µM of phenylephrine (PE) added to control BM samples in phase 1 cultures. PE has little effect on CMPs, GMPs and MEPs with increasing concentrations. **(C) GMP/MEP Propranolol Study**. 4 trials (n=4) were conducted on control BM samples placed in phase 1 cultures. 100pM, 100nM, and 1µM of propranolol (PROP) was administered to cells in separate wells. One hour later cells were stimulated with 1pM, 1nM, and 1µM of ISO in corresponding wells matching PROP concentrations. This was compared to cells being stimulated with ISO alone. The addition of propranolol effectively blocked ISO stimulation and led to a decrease in GMPs and increase in MEPs in all conditions.

Alpha Adrenergic Receptor Stimulation Does Not Alter MAFB/GATA1 Expression; Beta Adrenergic Receptor Stimulation Elevates MAFB Expression and Reduces GATA1 Expression

Control BM samples were placed in phase 1 culture and tested with four different conditions to confirm effects of adrenergic stimulation on MAFB and GATA1 expression. The first condition involved no stimulation or blockage of adrenergic receptors. The second condition involved stimulation with 1μM of Alpha-adrenergic receptor agonist phenylephrine. The third condition involved stimulation with 1μM of Beta-adrenergic receptor agonist isoproterenol. The last condition involved blockage of Beta-adrenergic receptors using the antagonist, propranolol at 1μM followed by stimulation with isoproterenol at 1μM. All conditions were incubated with anti-MAFB and anti-GATA1 after completion of phase 1 to determine MAFB/GATA1 expressions based on adrenergic receptor activity. Geometric mean intensities were measured for MAFB and GATA1 in CMPs and normalized to no stimulation condition which was arbitrarily defined as 100. Phenylephrine condition did not change much from the no stimulation condition giving values of 101 for MAFB and 99 for GATA1 (Table 2). Isoproterenol condition did change from no stimulation condition by increasing MAFB by 25% and decreasing GATA1 by 50% (Table 2). Lastly, the propranolol condition reduced MAFB expression by 32% and elevated GATA1 expression by 44% compared to isoproterenol condition (Table 2). When looking at MAFB:GATA1 ratio in the isoproterenol condition it is 1:2.5. Effective blockage of isoproterenol stimulation using propranolol results in MAFB:GATA1 ratio improving to 1:1. This is similar to no stimulation being present.

Table 2. Alpha-Adrenergic Receptor Agonist (Phenylephrine) Does Not Affect MAFB/GATA1 Expression; Beta-Adrenergic Receptor Agonist (Isoproterenol) and Antagonist (Propranolol) Does Effect MAFB/GATA1 Expression. MAFB and GATA1 geometric mean intensity/mean fluorescent intensity (MFI) was measured in Lin^{neg} $CD34^{+}CD38^{+}$ $CD123^{+}$ $CD45RA^{-}$ (CMPs) from control BM in four condition: (1) no stimulation, (2) phenylephrine-Alpha adrenergic receptor stimulation, (3) isoproterenol-Beta adrenergic receptor stimulation, and (4) propranolol-Beta adrenergic receptor blockage followed by isoproterenol-Beta adrenergic receptor stimulation.

Control BM MNCs in PHASE 1 cocktail (+GMCSF/SCF/IL3) from Days 0-6 with a- and b- adrenergic stimulation	Common Myeloid progenitors (CMP)	
	MAFB (MFI)	GATA1 (MFI)
No stimulation	100	100
+ Phenylephrine 1µM (Alpha AR)	101	99
+ Isoproterenol 1 µM (Beta AR)	125	50
+ Propranolol 1 µM (Beta blocker) +1µM Isoproterenol	93	94

Expression levels of MAFB and GATA1 in no stimulation BM were arbitrarily defined as 100

MAFB Silencing in MM Samples and ISO Stimulated Control BM Leads to Recovery of MEPs and Decline in GMPs

MAFB was silenced in MM samples using a lipid-based siRNA of *MAFB* along with a transfection reagent. This was compared to MM samples were *MAFB* was not silenced to determine if MAFB was the cause of myeloid bias (increase in GMPS/decrease in MEPs) seen in MM samples. Transfection efficiency was determined to be around 37% (Figure 14). This was measured through comparing the MAFB expressing subset of a MM sample with and without siRNA after 3 days of phase 1 culture (Figure 14). The *MAFB* siRNA used was also validated from previous studies done by the lab [44]. Contour plots of myeloid and erythroid progenitors (Lin^{neg} $CD34^{+}CD38^{+}$ $CD123^{+/-}$ $CD45RA^{+/-}$) of *MAFB* silenced MM sample versus not silenced

MM sample covey GMP percentages out of HSPC population decreasing from 46% in not silenced to 15% in silenced. Inversely, MEP percentages out of HSPC population increased from 30% in not silenced sample to 47% in silenced sample (Figure 15A). Including more MM samples, myeloid and erythroid progenitors quantified out of one million HSPCs, showed a similar trend (Figure 15B). GMPs were reduced and MEPs were elevated when *MAFB* was silenced in MM samples (Figure 15B). To see the effects of *MAFB* silencing on Beta-adrenergic receptor stimulation, a control BM sample was silenced and then stimulated with isoproterenol. This was quantified out of one million HSPCs and compared to a control BM sample receiving no stimulation and to a control BM sample receiving stimulation but no *MAFB* silencing (Figure 15C). Silencing *MAFB* in isoproterenol stimulated control BM led to a decrease in GMPs and an increase in MEPs compared to when *MAFB* was not silenced in stimulated control BM (Figure 15C).

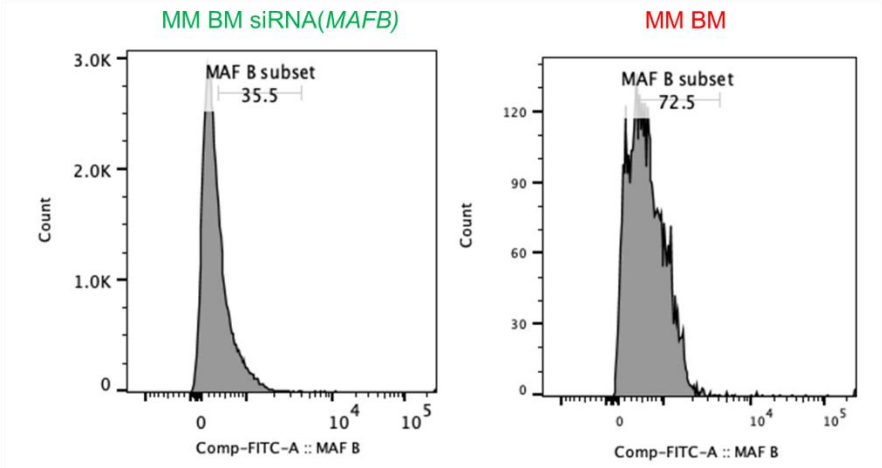


Figure 14. Transfection Efficiency and Validation of *MAFB* siRNA. 3 days after MM sample was transfected with *MAFB* siRNA in phase 1 culture, cells were incubated with FITC conjugated MAFB antibody and then taken to FACS to measure MAFB expression. MAFB subsets contain cells that are expressing MAFB. Subtracting the MAFB subset of the MM sample that did not receive the *MAFB* siRNA (72.5%) from the MM sample that did receive the *MAFB* siRNA (35.5%) we find a difference of 37%. This percentages entails the validation and efficiency of the *MAFB* siRNA that was used for this experiment. 37% of cells were successfully transfected using this *MAFB* siRNA.

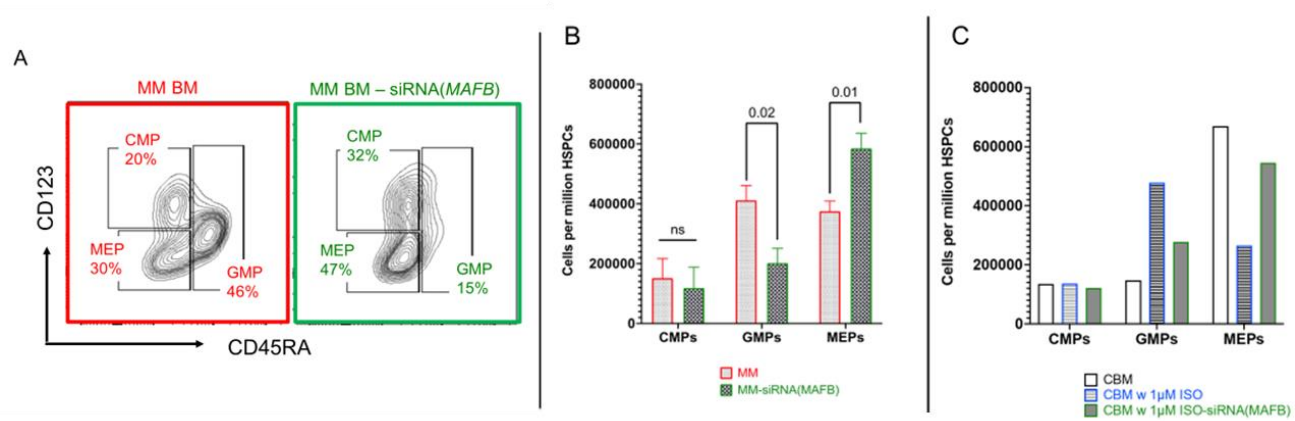


Figure 15. *MAFB* Silencing Resolves/Mitigates Myeloid Bias in MM Samples and ISO Stimulated Control BM. (A) After phase 1 completion, $CD34^{+}CD38^{+}$ cells from lineage negative cells were gated for CD45RA on X-axis and CD123 on Y-axis in contour plots from FACS. MM sample had 46% of GMPs / 30% of MEPs / and 20% of CMPs in HSPC subset compared to 15% GMPs / 47% MEPs / and 32% CMPs in MM sample with silenced *MAFB*. **(B) CMP/GMP/MEP MM *MAFB* Silencing Study**. Two *MAFB* silencing trials were comprised of a total of four non-silenced MM samples (n=4) and four silenced MM samples (n=4). Unpaired t test with Welch's correction assigned a two-tailed P value between MM samples with and without *MAFB* silencing for CMPs: ns, GMPs: 0.02, and MEPs: 0.01. **(C) CMP/GMP/MEP CBM w ISO MAFB Silencing Study**. One *MAFB* silencing trials was comprised of a non-silenced CBM sample (n=1), a non-silenced CBM sample stimulated with ISO (n=1) and a silenced CBM sample stimulated with ISO (n=1).

MM Sample and Phase 1 Beta Stimulated Control BM Commitment Patterns Lead to Diminished Erythroblast Development and Increased Non-Erythroid Development

During phase 1 culture a control BM sample was stimulated with 1μM of isoproterenol. Another control BM sample was reseeded with $CD138^{+}$ cells from MM sample before the start of phase 1 culture. A control BM sample and MM sample were also placed in phase 1 culture. All four conditions were then carried for ten extra days into phase 2 culture after completion of six-day phase 1 culture. Besides phase 2 growth cocktail consisting of SFEM, EPO, HT, and SCF nothing was added to phase 2 cultures. On day 16 cells from all 4 conditions were collected for FACS and giemsa stained for image analysis. Giemsa staining for the control BM condition displays cells that: have colony formation, are not over-crowded, and mostly erythroblasts

(Figure 16A). Giemsa staining for the MM condition displays that: myeloma cells are present, colony formation is sparse, and there are very few erythroblasts (Figure 16A). Giemsa staining for the control BM with $CD138^+$ myeloma cells displays that: myeloma cells are present, colony formation still present but not as defined as control BM condition, and that erythroblasts are present at slightly less density than control BM condition (Figure 16A). Lastly, giemsa staining for the control BM with 1μM of isoproterenol displays that: cells are over-crowded, and that there are many other cells besides erythroblasts though erythroblasts are still present (Figure 16A). The image analysis is reaffirmed from contour plots using CD71 and CD235a to set appropriate gates for non-erythroid cells ($CD71^-CD235a^-$), EEBs ($CD71^+CD235a^-$), and LEBs ($CD71^+CD235a^+$). Non-erythroid cells were the most prevalent in the MM sample and the control BM with 1μM isoproterenol sample where it accounted for 20% and 30% of all MNCs from phase 2 culture (Figure 16B). Control BM and control BM reseeded with $CD138^+$ (myeloma) cells were drastically less, as non-erythroid cells accounted for 6% and 4% of all MNCs from phase 2 culture (Figure 16B). The LEB percentages for the four conditions gave an opposite trend from the non-erythroid percentages. LEBs were the most prevalent in the control BM sample and the control BM sample reseeded with $CD138^+$ (myeloma) cells where it accounted for 50% and 42% of all MNCs from phase 2 culture (Figure 16B). LEBs for MM sample and the control BM with 1μM isoproterenol sample accounted for 20% and 30% of all MNCs from phase 2 culture (Figure 16B). EEB percentages had only an 8% difference between the four conditions ranging from 5% to 13% (Figure 16B).

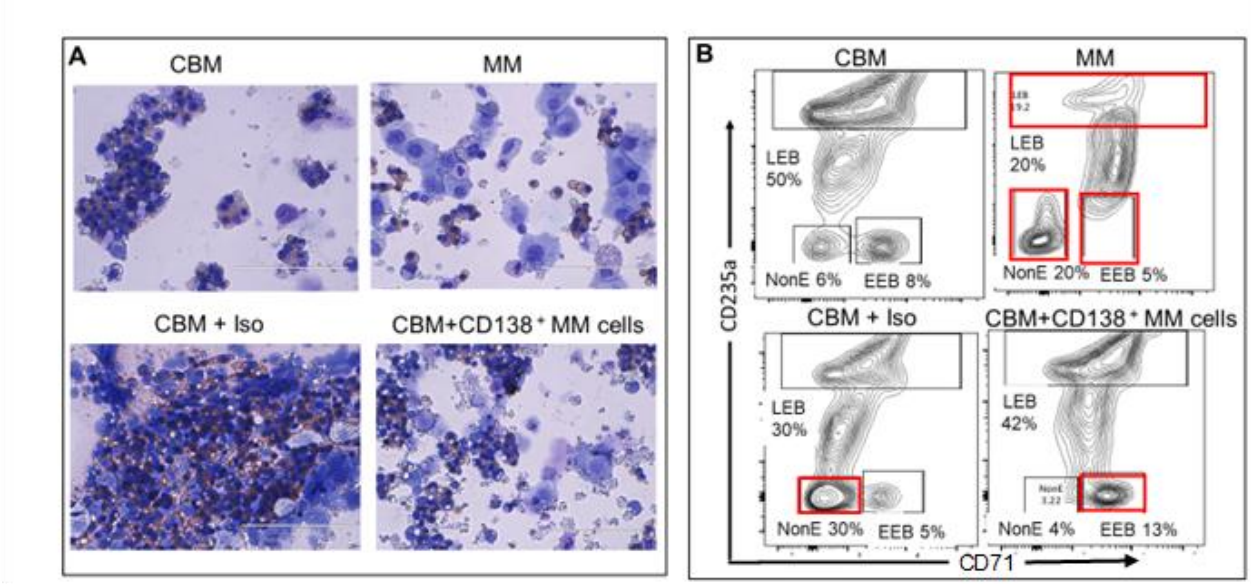


Figure 16. Beta Adrenergic Stimulation During Commitment Stage Leads to Increases in NonE and Diminished Erythroid Development. ISO and $CD138^+$ myeloma cells were added to separate CBM at the start of phase 1 culture. Along with these two conditions a CBM sample and MM sample were also placed in phase 1 culture. Once phase 1 culture was completed, cells were carried into phase 2 culture (SFEM/EPO/SCF/HT). Nothing was added in phase 2 culture. **(A) Giemsa staining of the four conditions**. CBM: Colony formation, not over-crowded, mostly erythroblasts (pink/blue, small, round cells); CBM + ISO: Over-crowded, many other cells besides erythroblast though erythroblast still present; MM: myeloma (big translucent, blue/purple) cells present, very few erythroblasts (pink/blue, small, round cells), not much colony formation; CBM + MM: myeloma (big translucent, blue/purple) cells present, colony formation still present but not as defined as CBM, erythroblasts (pink/blue, small, round cells) are present at slightly less density than CBM. **(B) Contour plots of all four conditions gating for $CD71^{+/-}$ and $CD235a^{+/-}$.** Non-erythroblasts (NonE) percentages were based off phase 2 MNC population and gated as $CD71^{-}CD235a^{-}$. NonE for CBM: 6%; MM: 20%; CBM + ISO: 30%; CBM + $CD138^+$(myeloma) cells: 4%. Late-erythroblasts (LEB) percentages were based off phase 2 MNC population and gated as $CD71^{+}CD235a^{+}$. LEBs for CBM: 50%; MM: 20%; CBM + ISO: 30%; CBM + $CD138^+$(myeloma) cells: 42%. Early-erythroblasts (EEB) percentages were based off phase 2 MNC population and gated as $CD71^{+}CD235a^{-}$. EEBs for CBM: 8%; MM: 5%; CBM + ISO: 5%; CBM + $CD138^+$(myeloma) cells: 13%.

LEBs Are Diminished and Non-Erythroid Cells Are Increased in MM Versus BM Control; Beta Stimulation Has Little Effect on Erythropoietic Development

A control BM sample and three MM samples were extended into phase 2 culture after completion of phase 1 culture. After phase 2 completion on day 16, cells were collected and processed for FACS. Contour plot of a BM control sample and MM sample used $CD71^{+/-}$ and

$CD235a^{+/-}$ for gating parameters. Based off phase 2 MNCs, the BM control sample had 6% non-erythroid cells ($CD71^{-}CD235a^{-}$), close to 9% EEBs ($CD71^{+}CD235a^{-}$), and 51% LEBs ($CD71^{+}CD235a^{+}$) (Figure 17A). The MM sample inversely, had 39% non-erythroid cells, close to 8% EEBs, and 20% LEBs (Figure 17A). To examine the effect of Beta-adrenergic stimulation on erythropoietic development and not just commitment, isoproterenol was added to MM samples only during phase 2 culture. This was compared to MM samples that did not receive isoproterenol stimulation. Quantifications of BM control, MM sample, and MM sample with isoproterenol stimulation during phase 2 were calculated per one million MNCs. A BM control sample compared with MM samples demonstrates that there is no significant difference in EEBs (Figure 17B). LEBs are significantly reduced, and non-erythroid cells are significantly increased in MM samples compared to a BM control sample (Figure 17B). When comparing MM samples with and without isoproterenol phase 2 stimulation we find that there is no significant difference between non-erythroid cells, EEBs, and LEBs (Figure 17B). This indicates that Beta-stimulation does not contribute to distinctions seen in MM samples and therefore has little effect on erythropoietic development.

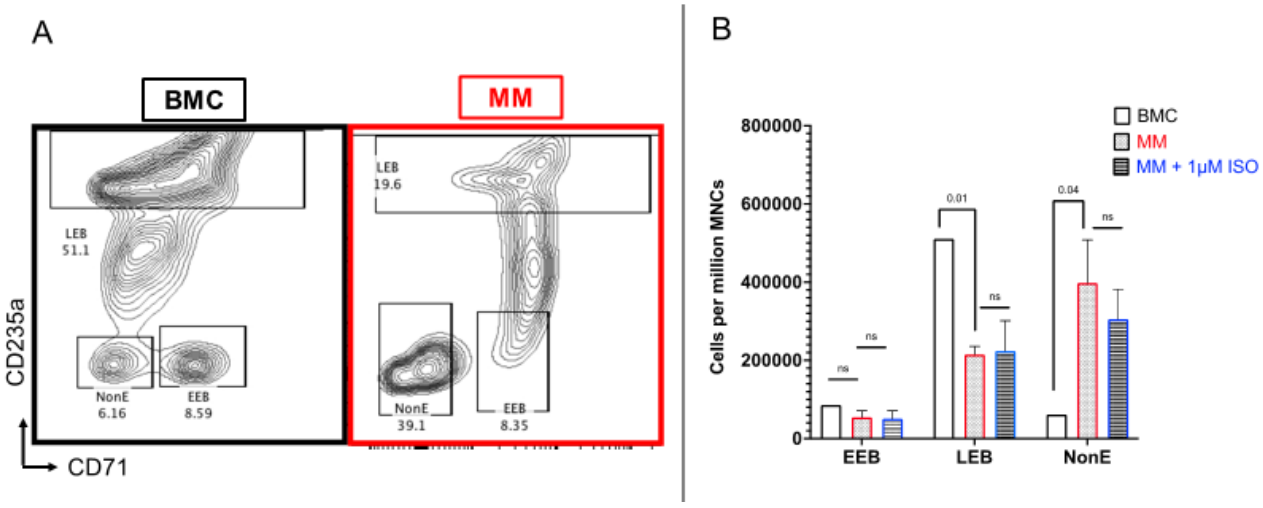


Figure 17. MM Samples Have Increased NonE and Decreased LEBs Compared to BMC; ISO Does Not Impact Erythropoietic Development. (A) Contour plots of BMC sample and MM sample with gating for $CD71^{+/-}$ and $CD235a^{+/-}$. Non-erythroblasts (NonE) percentages were based off phase 2 MNC population and gated as $CD71^{-}CD235a^{-}$. NonE for CBM: 6%; MM: 40%. Late-erythroblasts (LEB) percentages were based off phase 2 MNC population and gated as $CD71^{+}CD235a^{+}$. LEBs for CBM: 51%; MM: 20%. Early-erythroblasts (EEB) percentages were based off phase 2 MNC population and gated as $CD71^{+}CD235a^{-}$. EEBs for CBM: 9%; MM: 8%. **(B)** Quantification per one million BM MNCs examining NonE, EEBs, and LEBs of one BM control samples (n=1), three MM samples (n=3), and three MM sample with ISO stimulation during phase 2 (n=3). MM samples with 1μM ISO were stimulated every other day throughout phase 2 culture. MM samples with and without phase 2 ISO was conducted over two trials. Unpaired t test with Welch's correction assigned a two-tailed P value between control BM and MM samples for EEBs: ns, LEBs: 0.01, and NonE: 0.04. Unpaired t test with Welch's correction also assigned a two-tailed P value between MM samples and MM samples + 1μM for EEBs: ns, LEBs: ns, and NonE: ns.

CHAPTER FOUR

DISCUSSION

The previous results were gathered while attempting to elucidate the role of the transcription factors MAFB and GATA1 in causing a myeloid bias in MM due to adrenergic signaling. The progress gained in this study allowed us to validate the hypothesis that diminished erythropoiesis in MM is due to the stimulation of adrenergic receptors on HSPCs during early commitment (Figure 18). This results in the increase of the transcription factor MAFB, which restricts HSPCs differentiating into MEPs (Figure 18). With reduced MEP production, subsequent erythrocyte production will also be reduced as a result (Figure 18).

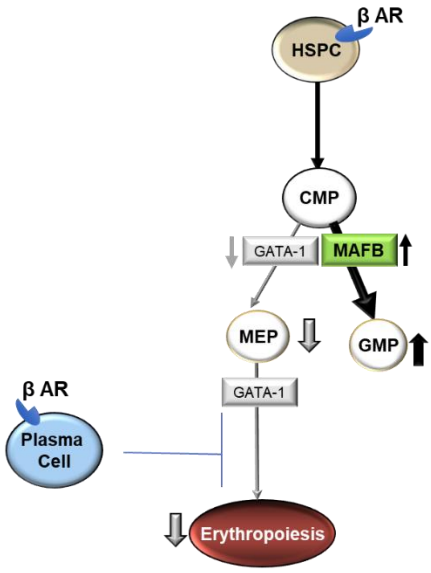


Figure 18. Schematic of the Effects of Beta Adrenergic Signaling on the Myeloid Bias and Erythroid Development in MM. BM niche HSPCs express Beta-adrenergic receptors in MM and their stimulation leads to dysregulation of MAFB/GATA1 axis causing a myeloid bias. This myeloid bias results in decreased erythroid development. Also, contributing to diminished erythroid development in MM is the myeloma cells.

In day 0 analysis of the erythroblast population, the presence of a reduced total erythroblast population was followed by a reduced number of LEBs. Taking into account the low levels of hemoglobin (below 14 g/dL) along with day 0 analysis, there is an indication of erythropoietic arrest. It is well documented that erythropoiesis is diminished in MM [4]. There is evidence based on derived models and protein/nutrient measurements that this occurs at the developmental stage in MM due to disrupted erythropoietic islands and reduced erythropoietin and iron levels [59-61]. The formation of erythropoietic islands, and efficient erythropoietin and iron levels are vital for erythropoietic development [62-65]. Issues in erythropoietic development are not alone in contributing to diminished erythropoiesis. The commitment stage may also be compromised. With this in my mind, there was a need to verify if this issue was stemming from the BM. Thus, we examined the myeloid and erythroid progenitor population at day 0 as well. The decreased MEP population matched our claims and seemed to arise due to an increased GMP population. With the source of erythroblasts being MEPs, this indicates a possible cause for the reduced erythroblast numbers. In accordance, despite varying progressions and degrees of myeloma present within MM patients, the occurrence of a low number of MEPs resulting from the increased GMP population was consistent and displays the severity of a possible myeloid bias in leading to diminished erythropoiesis in MM patients. At this point we can only theorize whether a commitment issue stemming from a myeloid bias was the cause of the increased GMPs and decreased MEPs. Further analysis was needed to confirm this claim.

As noted earlier, our lab previously found that the myeloid bias in burn injury was due to the dysregulation of the MAFB/GATA1 axis [51]. MAFB is elevated while GATA1 is reduced leading to an increase in GMPs and decrease in MEPs. Having seen this same trend in our day 0 GMPs and MEPs we then analyzed MAFB and GATA1 expression. As expected MAFB was

elevated and GATA1 was reduced. This Indicates a reason for the increase of GMPs and decrease of MEPs in MM samples versus BM control as seen from day 0 HSPC analysis. Day 6 HSPC analysis allowed us to confirm whether a myeloid bias was present in our MM samples. The same trend between day 0 and day 6 of higher GMPs and lower MEPs in MM samples versus control BM samples indicates that phase 1 expanded early myeloid progenitors' signatures are maintained during commitment stage. In correlation with a higher number of GMPs in MM samples, MAFB expression was increased denoting a mechanism for myeloid bias relationship. The lower MEPs in MM samples compared to BM control, corresponds with reduced GATA1 expression. This denotes the essential balance between the two transcription factors for erythroid commitment.

Myeloma cells produce GMCSF which is essential for GMP production [66]. Thus, it can be reasoned if the myeloid bias that we see in our MM samples is due to the contributions of the $CD138^{+}$ myeloma cells and not due to adrenergic receptor stimulation. We were able to conclude that $CD138^{+}$ myeloma cells did not cause the myeloid bias seen in the MM samples. This gives more reason to adrenergic receptor stimulation as being the primary cause for the myeloid bias, but this needs to be proven. Hence, receptor stimulation studies were conducted. By confirming the presence of adrenergic receptors on HSPCs from both control BM samples and MM samples, we can surmise that adrenergic receptor stimulation can occur in these cells. Through Alpha-and-Beta adrenergic receptor stimulation using isoproterenol and phenylephrine we found that the stimulation of Beta receptors resulted in a myeloid bias. We were able to confirm this, using the non-specific Beta-Blocker: Propranolol. Studies conducted on MAFB and GATA1 demonstrate that the myeloid bias from isoproterenol stimulation is mediated by the dysregulation of MAFB: GATA1 ratio. MAFB expression was elevated and in turn GATA1 expression was reduced when

isoproterenol was added to control BM samples, thus begetting a dysregulation in the MAFB: GATA1 ratio. Propranolol mitigated the effects from isoproterenol stimulation on MAFB and in turn GATA1 expression. Inversely, phenylephrine did not affect MAFB or GATA1 expression. Overall, these experiments convey that the commitment pattern and the transcription profile in control BM samples with a myeloid bias are predominately orchestrated by Beta-stimulation.

The connection between Beta-adrenergic signaling and MAFB expression in causing a myeloid bias is correlated through our data up to this point. Silencing *MAFB* in isoproterenol stimulated control BM demonstrates that the myeloid bias is dependent on the increase in MAFB expression. Applying this to our MM samples requires *MAFB* to also be silenced to determine if the myeloid bias persists. Silencing *MAFB* led to the dissipation of the myeloid bias seen in previous MM samples. This establishes that the increase in MAFB expression is the reason for the myeloid bias in our MM samples, as it leads to the dysregulation in the MAFB: GATA1 ratio/axis. We derived that MAFB expression increases due to Beta-adrenergic receptor stimulation. In summation, Beta-adrenergic receptor stimulation results in an increase in MAFB expression which decreases GATA1 expression (dysregulation MAFB/GATA1 axis), leading to a myeloid bias in MM.

We have analyzed the impact that Beta-adrenergic signaling has towards erythropoietic commitment, but we have yet to investigate the given impact on erythropoietic development. When doing so, we found that while Beta-adrenergic receptor stimulation impedes erythropoiesis during the commitment stage it does not during the developmental stage. The lingering effects of the commitment stage though, do influence erythropoiesis at the developmental stage. Increased non-erythroid cells in MM and isoproterenol stimulated control BM likely stems from the increased GMPs that resulted from Beta-adrenergic signaling. Additionally, the reduction of

LEBs likely stems from the decrease in MEPs that came about due to Beta-adrenergic signaling. This can give a notable reason for the high incidence of anemia in MM patients and a likely target for treatment.

Further applications of this study can be used to investigate the increased non-erythroid population in MM and how this may foster the BM cancer environment. The non-erythroid population may contain a high number of MDSCs. As such, the SNS modulation of erythropoiesis and myelopoiesis is a concerning issue in MM patients. This is because GMPs are precursors to MDSCs which help foster myeloma cell survival and proliferation [67]. More research into this area can greatly benefit MM patients and hopefully improve survival rates.

REFERENCE LIST

1) Bouchnita A, Eymard N, Moyo TK, Koury MJ, Volpert V. Bone marrow infiltration by multiple myeloma causes anemia by reversible disruption of erythropoiesis. Am J Hematol. 2016 Jun;91(4):371-8. doi: 10.1002/ajh.24291. Epub 2016 Feb 9. PMID: 26749142.

2) Bruns I, Cadeddu RP, Brueckmann I, et al. Multiple myeloma-related deregulation of bone marrow-derived CD34(+) hematopoietic stem and progenitor cells. *Blood*. 2012;120(13):2620-2630. doi:10.1182/blood-2011-04-347484

3) Nandakumar SK, Ulirsch JC, Sankaran VG. Advances in understanding erythropoiesis: evolving perspectives. *Br J Haematol*. 2016;173(2):206-218. doi:10.1111/bjh.13938

4) Liu L, Yu Z, Cheng H, et al. Multiple myeloma hinders erythropoiesis and causes anaemia owing to high levels of CCL3 in the bone marrow microenvironment. *Sci Rep*. 2020;10(1):20508. Published 2020 Nov 25. doi:10.1038/s41598-020-77450-y

5) Howlader N, Noone AM, Krapcho M, Miller D, Bishop K, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review, 1975-2014, National Cancer Institute. Bethesda, MD, https://seer.cancer.gov/csr/1975_2014/, based on November 2016 SEER data submission, posted to the SEER web site, April 2017.

6) SEER Lifetime Risk (Percent) of Being Diagnosed with Cancer by Site and Race/Ethnicity: Both Sexes, 18 SEER Areas, 2012-2014 (Table 1.15) National Cancer Institute, Bethesda, MD. Accessed at https://seer.cancer.gov/csr/1975_2014/results_merged/topic_lifetime_risk.pdf on January 5, 2018.

7) Kyle RA, Gertz MA, Witzig TE, et al. Review of 1027 patients with newly diagnosed multiple myeloma. *Mayo Clin Proc*. 2003;78(1):21-33. doi:10.4065/78.1.21

8) Faiman BM, Mangan P, Spong J, Tariman JD; The International Myeloma Foundation Nurse Leadership Board. Renal complications in multiple myeloma and related disorders: survivorship care plan of the International Myeloma Foundation Nurse Leadership Board. *Clin J Oncol Nurs*. 2011;15 Suppl(Suppl):66-76. doi:10.1188/11.CJON.S1.66-76

9) Mundy GR, Bertolini DR. Bone destruction and hypercalcemia in plasma cell myeloma [published correction appears in Semin Oncol 1986 Dec;13(4):lxiii]. *Semin Oncol*. 1986;13(3):291-299.

10) Roodman GD. Pathogenesis of myeloma bone disease. *Leukemia*. 2009;23(3):435-441. doi:10.1038/leu.2008.336

11) Hussein MA. Multiple myeloma: most common end-organ damage and management. *J Natl Compr Canc Netw*. 2007;5(2):170-178. doi:10.6004/jnccn.2007.0017

12) Nakaya A, Fujita S, Satake A, et al. Impact of CRAB Symptoms in Survival of Patients with Symptomatic Myeloma in Novel Agent Era. *Hematol Rep*. 2017;9(1):6887. Published 2017 Feb 23. doi:10.4081/hr.2017.6887

13) Alshak MN, M Das J. Neuroanatomy, Sympathetic Nervous System. [Updated 2020 Jul 27]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-.

14) Lohse, M.J. The ins and outs of adrenergic signaling. *J Mol Med* **93,** 955–962 (2015). https://doi.org/10.1007/s00109-015-1323-x

15) McCorry LK. Physiology of the autonomic nervous system. *Am J Pharm Educ*. 2007;71(4):78. doi:10.5688/aj710478

16) Reizenstein P. The haematological stress syndrome. *Br J Haematol*. 1979;43(3):329-334. doi:10.1111/j.1365-2141.1979.tb03759.x

17) Fielding C, Méndez-Ferrer S. Neuronal regulation of bone marrow stem cell niches. *F1000Res*. 2020;9:F1000 Faculty Rev-614. Published 2020 Jun 16. doi:10.12688/f1000research.22554.1

18) Powell ND, Sloan EK, Bailey MT, et al. Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via β-adrenergic induction of myelopoiesis. *Proc Natl Acad Sci U S A*. 2013;110(41):16574-16579. doi:10.1073/pnas.1310655110

19) Heidt T, Sager HB, Courties G, et al. Chronic variable stress activates hematopoietic stem cells. *Nat Med*. 2014;20(7):754-758. doi:10.1038/nm.3589

20) Sudo K, Ema H, Morita Y, Nakauchi H. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med*. 2000;192(9):1273-1280. doi:10.1084/jem.192.9.1273

21) Jin J, Wang X, Wang Q, et al. Chronic psychological stress induces the accumulation of myeloid-derived suppressor cells in mice. *PLoS One*. 2013;8(9):e74497. Published 2013 Sep 18. doi:10.1371/journal.pone.0074497

22) Hanoun M, Zhang D, Mizoguchi T, et al. Acute myelogenous leukemia-induced sympathetic neuropathy promotes malignancy in an altered hematopoietic stem cell niche. *Cell Stem Cell*. 2014;15(3):365-375. doi:10.1016/j.stem.2014.06.020

23) Busik JV, Tikhonenko M, Bhatwadekar A, et al. Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. *J Exp Med*. 2009;206(13):2897-2906. doi:10.1084/jem.20090889

24) Bunimovich YL, Keskinov AA, Shurin GV, Shurin MR. Schwann cells: a new player in the tumor microenvironment. *Cancer Immunol Immunother*. 2017;66(8):959-968. doi:10.1007/s00262-016-1929-z

25) Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014;505(7483):327-334. doi:10.1038/nature12984

26) Katayama Y, Battista M, Kao WM, et al. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell*. 2006;124(2):407-421. doi:10.1016/j.cell.2005.10.041

27) Lutgendorf SK, DeGeest K, Sung CY, et al. Depression, social support, and beta-adrenergic transcription control in human ovarian cancer. *Brain Behav Immun*. 2009;23(2):176-183. doi:10.1016/j.bbi.2008.04.155

28) Powell ND, Sloan EK, Bailey MT, et al. Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via β-adrenergic induction of myelopoiesis. *Proc Natl Acad Sci U S A*. 2013;110(41):16574-16579. doi:10.1073/pnas.1310655110

29) Hanoun M, Maryanovich M, Arnal-Estapé A, Frenette PS. Neural regulation of hematopoiesis, inflammation, and cancer. *Neuron*. 2015;86(2):360-373. doi:10.1016/j.neuron.2015.01.026

30) Reiche EM, Nunes SO, Morimoto HK. Stress, depression, the immune system, and cancer. *Lancet Oncol*. 2004;5(10):617-625. doi:10.1016/S1470-2045(04)01597-9

31) Spiegel D, Giese-Davis J. Depression and cancer: mechanisms and disease progression. *Biol Psychiatry*. 2003;54(3):269-282. doi:10.1016/s0006-3223(03)00566-3

32) Palesh O, Butler LD, Koopman C, Giese-Davis J, Carlson R, Spiegel D. Stress history and breast cancer recurrence. *J Psychosom Res*. 2007;63(3):233-239. doi:10.1016/j.jpsychores.2007.05.012

33) Satin JR, Linden W, Phillips MJ. Depression as a predictor of disease progression and mortality in cancer patients: a meta-analysis. *Cancer*. 2009;115(22):5349-5361. doi:10.1002/cncr.24561

34) Kissane DW, Grabsch B, Clarke DM, et al. Supportive-expressive group therapy for women with metastatic breast cancer: survival and psychosocial outcome from a randomized controlled trial. *Psychooncology*. 2007;16(4):277-286. doi:10.1002/pon.1185

35) Andersen BL, Yang HC, Farrar WB, et al. Psychologic intervention improves survival for breast cancer patients: a randomized clinical trial. *Cancer*. 2008;113(12):3450-3458. doi:10.1002/cncr.23969

36) Lutgendorf SK, Sood AK, Antoni MH. Host factors and cancer progression: biobehavioral signaling pathways and interventions. *J Clin Oncol*. 2010;28(26):4094-4099. doi:10.1200/JCO.2009.26.9357

37) Cheng Y, Sun F, D'Souza A, et al. Autonomic nervous system control of multiple myeloma. *Blood Rev*. 2021;46:100741. doi:10.1016/j.blre.2020.100741

38) Hwa YL, Shi Q, Kumar SK, et al. Beta-blockers improve survival outcomes in patients with multiple myeloma: a retrospective evaluation. *Am J Hematol*. 2017;92(1):50-55. doi:10.1002/ajh.24582

39) Knight JM, Rizzo JD, Hari P, Pasquini MC, Giles KE, D'Souza A, Logan BR, Hamadani M, Chhabra S, Dhakal B, Shah N, Sriram D, Horowitz MM, Cole SW. Propranolol inhibits molecular risk markers in HCT recipients: a phase 2 randomized controlled biomarker trial. Blood Adv. 2020 Feb 11;4(3):467-476. doi: 10.1182/bloodadvances.2019000765. PMID: 32027744; PMCID: PMC7013267.

40) Kawamoto H, Minato N. Myeloid cells. *Int J Biochem Cell Biol*. 2004;36(8):1374-1379. doi:10.1016/j.biocel.2004.01.020

41) Hanoun M, Zhang D, Mizoguchi T, et al. Acute myelogenous leukemia-induced sympathetic neuropathy promotes malignancy in an altered hematopoietic stem cell niche. *Cell Stem Cell*. 2014;15(3):365-375. doi:10.1016/j.stem.2014.06.020

42) Arranz L, Sánchez-Aguilera A, Martín-Pérez D, et al. Neuropathy of haematopoietic stem cell niche is essential for myeloproliferative neoplasms. *Nature*. 2014;512(7512):78-81. doi:10.1038/nature13383

43) Posluszny JA Jr, Gamelli RL. Anemia of thermal injury: combined acute blood loss anemia and anemia of critical illness. *J Burn Care Res*. 2010;31(2):229-242. doi:10.1097/BCR.0b013e3181d0f618

44) Hasan S, Johnson NB, Mosier MJ, Shankar R, Conrad P, Szilagyi A, Gamelli RL, Muthumalaiappan K. Myelo-erythroid commitment after burn injury is under β-adrenergic control via MafB regulation. Am J Physiol Cell Physiol. 2017 Mar 1;312(3):C286-C301. doi: 10.1152/ajpcell.00139.2016. Epub 2016 Dec 28. PMID: 28031160; PMCID: PMC5401945.

45) Carolina Vicente-Dueas, Ines Gonzalez-Herrero, Idoia Garcia-Ramirez, Isidro Sanchez-Garcia MAFB (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B) Atlas Genet Cytogenet Oncol Haematol. 2015;19(7):453-457.

46) Pittet MJ, Nahrendorf M, Swirski FK. The journey from stem cell to macrophage. *Ann N Y Acad Sci*. 2014;1319(1):1-18. doi:10.1111/nyas.12393

47) Winston Y Lee, Olga K Weinberg GATA1 (GATA binding protein 1 (globin transcription factor1)) Atlas Genet Cytogenet Oncol Haematol. 2020;24(05):197-200.

48) Seth A, Robinson L, Thompson DM, Watson DK, Papas TS. Transactivation of GATA-1 promoter with ETS1, ETS2 and ERGB/Hu-FLI-1 proteins: stabilization of the ETS1 protein binding on GATA-1 promoter sequences by monoclonal antibody. *Oncogene*. 1993;8(7):1783-1790.

49) Sieweke MH, Tekotte H, Frampton J, Graf T. MafB represses erythroid genes and differentiation through direct interaction with c-Ets-1. *Leukemia*. 1997;11 Suppl 3:486-488.

50) Sieweke MH, Tekotte H, Frampton J, Graf T. MafB is an interaction partner and repressor of Ets-1 that inhibits erythroid differentiation. *Cell*. 1996;85(1):49-60. doi:10.1016/s0092-8674(00)81081-8

51) Johnson NB, Posluszny JA, He LK, Szilagyi A, Gamelli RL, Shankar R, Muthumalaiappan K. Perturbed MafB/GATA1 axis after burn trauma bares the potential mechanism for immune suppression and anemia of critical illness. J Leukoc Biol. 2016 Oct;100(4):725-736. doi: 10.1189/jlb.1A0815-377R. Epub 2016 Mar 18. PMID: 26992433; PMCID: PMC6608024.

52) Kawano Y, Fujiwara S, Wada N, et al. Multiple myeloma cells expressing low levels of CD138 have an immature phenotype and reduced sensitivity to lenalidomide. *Int J Oncol*. 2012;41(3):876-884. doi:10.3892/ijo.2012.1545

53) Dzierzak E, Philipsen S. Erythropoiesis: development and differentiation. *Cold Spring Harb Perspect Med*. 2013;3(4):a011601. Published 2013 Apr 1. doi:10.1101/cshperspect.a011601

54) Flores-Montero J, de Tute R, Paiva B, et al. Immunophenotype of normal vs. myeloma plasma cells: Toward antibody panel specifications for MRD detection in multiple myeloma. *Cytometry B Clin Cytom*. 2016;90(1):61-72. doi:10.1002/cyto.b.21265

55) Aljabry, M.S. CD38 negative anaplastic plasma cell myeloma with (14;16) translocation: a diagnostic dilemma for highly aggressive neoplasm: case report. *J Hematopathol* **12,** 27–30 (2019). https://doi.org/10.1007/s12308-018-0338-y

56) Dean II JS, Reddivari AKR. Alpha 1 Receptor Agonists. [Updated 2020 Aug 10]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-.

57) Szymanski MW, Singh DP. Isoproterenol. [Updated 2021 May 1]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-.

58) Prichard BN. Propranolol and beta-adrenergic receptor blocking drugs in the treatment of hypertension. *Br J Clin Pharmacol*. 1982;13(1):51-60. doi:10.1111/j.1365-2125.1982.tb01336.x

59) Bouchnita, Anass & Eymard, Nathalie & Koury, M & Moyo, T. (2015). Normal erythropoiesis and development of multiple myeloma. ITM Web of Conferences. 5. 10.1051/itmconf/20150500008.

60) Beguin Y. Erythropoiesis and erythropoietin in multiple myeloma. *Leuk Lymphoma*. 1995;18(5-6):413-421. doi:10.3109/10428199509059639

61) VanderWall K, Daniels-Wells TR, Penichet M, Lichtenstein A. Iron in multiple myeloma. *Crit Rev Oncog*. 2013;18(5):449-461. doi:10.1615/critrevoncog.2013007934

62) Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. *Blood*. 2008;112(3):470-478. doi:10.1182/blood-2008-03-077883

63) Schoener B, Borger J. Erythropoietin. In: *StatPearls*. Treasure Island (FL): StatPearls Publishing; January 25, 2021.

64) Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. *Clin Chem*. 2003;49(10):1573-1578. doi:10.1373/49.10.1573

65) Kim A, Nemeth E. New insights into iron regulation and erythropoiesis. *Curr Opin Hematol*. 2015;22(3):199-205. doi:10.1097/MOH.0000000000000132

66) Galea HR, Cogné M. GM-CSF and IL-12 production by malignant plasma cells promotes cell-mediated immune responses against monoclonal Ig determinants in a light chain myeloma model. *Clin Exp Immunol*. 2002;129(2):247-253. doi:10.1046/j.1365-2249.2002.01929.x

67) De Veirman K, Menu E, Maes K, et al. Myeloid-derived suppressor cells induce multiple myeloma cell survival by activating the AMPK pathway. *Cancer Lett*. 2019;442:233-2
doi:10.1016/j.canlet.2018.11.002

VITA

Vimal Subramaniam was born in Bolingbrook, IL on March 21st, 1993. He attended the University of Illinois-Urbana Champaign where, in June 2015, he earned his Bachelors degree. Upon graduating, Vimal entered into Loyola University Chicago's Bioethics program. After completion of his Master degree in Bioehics, Vimal matriculated into the Loyola University Chicago Integrative Cell Biology Graduate Program to begin his graduate education under the mentorship of Dr. Muthumalaiappan where he began investigating the role of MafB and GATA1 on the myeloid bias that is persistent in Multiple Myeloma.

After completion of his Master's, Vimal hopes to enter Medical School where he hopes to pursue a career in Hematology-Oncology.