LOSS OF SIMPL INCREASES TNFα SENSITIVITY DURING HEMATOPOIESIS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Biochemistry and Molecular Biology
Indiana University

October 2008

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

This thesis is dedicated to my wife, Heather Benson, for her endless love, support and encouragement throughout our marriage and particularly during my thesis process; and to my parents Errol and Lessa Benson for constant love and a solid foundation upon which I continue to build to this day.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Maureen Harrington, for providing guidance towards the completion of my thesis and for developing me into a complete scientist. Dr. Harrington spent endless hours guiding me through the inner workings of running a lab, and many more critically thinking about scientific questions. From the moment I met Dr. Harrington, her passion for research was evident, and that passion was instilled in me. I would like to thank Harrington Lab members, Erin Breese, Yong Luo, and Angelia Lockett for also providing guidance and mentorship.

I would like to thank members of my committee, Dr. Harrington, Dr. Mark Goebl, Dr.Wade Clapp, and Dr. David Skalnik for their guidance and encouragement throughout this training process. I especially would like to thank Dr. Wade Clapp, the Clapp Lab, and Dr. Feng-Chun Yang for their wonderful guidance, instruction, and dedication to this process, along with the reagents needed for the accomplishment of the bone marrow studies.

I would like to acknowledge other individuals and institutions at Indiana University that contributed support to my research including Dr. Rueben Kapur, Dr. Hal Broxmeyer, the Indiana University Cancer Center Flow Cytometry Resource Facility - especially Susan Rice and Lizz Scaletta - and the Goebl Lab.

I would like to thank my family for constant support and understanding throughout this difficult pursuit, even as I became recluse as research intensified.

Most importantly, I would like to thank my wife and inspiration, Heather Lynette Benson, who was my strength when I was empty. She provided unconditional love, constant hope and objective guidance. I cannot imagine arriving at this point without her.

ABSTRACT

Eric Ashley Benson

LOSS OF SIMPL INCREASES TNFα SENSITIVITY DURING HEMATOPOIESIS

The innate and adaptive immune responses are critical for host survival. The TNFα/NF-κB signaling pathway is a major regulator of the immune response. The TNFα/NF-κB signaling pathway has also been proposed to play a role in the regulation of hematopoiesis. In the TNFα signaling pathway, full induction of NF-κB (specifically the p65 subunit) dependent transcription is regulated by a co-activator SIMPL. The biological significance of SIMPL in TNFα dependent responses is poorly understood. To study SIMPL *in vitro* and *in vivo* in mammalian cells, a knockdown system utilizing shRNA (short hairpin RNA) was used. Analysis of hematopoietic progenitor cells infected with a retrovirus encoding the SIMPL shRNA was used to study the role of SIMPL in hematopoiesis. The ability of progenitor cells lacking SIMPL to grow and differentiate was not compromised. In contrast in the progenitors cells lacking SIMPL, TNFα mediated inhibition of colony formation was significantly enhanced. These growth inhibitory effects of SIMPL were not due to an increase in apoptosis.

The enhanced inhibitory affects were specific for TNFα and not found in other common hematopoietic inhibitors (TGF-β1 and IFNγ). Results of this work reveal that SIMPL is a component of the hematopoiesis that is required for TNFα dependent effects upon myeloid progenitors.

Maureen Harrington, Ph.D., Chair

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ABBREVIATIONS

7-AAD	7-Amino-actinomycin D
AML	Acute myeloid leukemia
AP1	Activator protein 1
BAF3	Pro B-cell line
BFU-E	Blast forming unit-erythrocyte
BIR	Baculoviral IAP Repeat
BrdU	5-bromo-2-deoxyuridine
CAPS	10 mM 3-cyclohexylamino-1-propanesulphic acid
CD3	Cluster of differentiation 3
CFU-GEMM	Colony forming unit-granulocyte erythrocyte macrophage megakaryocyte
CFU-S	Colony forming unit-spleen
cIAP	Cellular inhibitor of apoptosis
c-kit	Stem cell factor receptor
Con A	Concanavalin A
c-Rel	V-rel reticuloendotheliosis viral oncogene homolog with additional C-terminal residues
CSF	Colony stimulating factors
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

Epo	Erythropoietin
FACS	Fluorescence activated cell sorter/scan
FBS	Fetal bovine serum
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HEK 293	Human embryonic kidney epithelial cells
HPC	Hematopoietic progenitor cell
HPP	High proliferative potential colony
HSC	Hematopoietic stem cell
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IKK	IκB kinase
IKKα	IκB kinase alpha
IKKβ	IκB kinase beta
IKKγ	NEMO
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-8	Interleukin-8
IMDM	Iscove's Modified Dulbecco's Medium
IRAK-1	Interleukin-1 receptor associated kinase 1
IκB	Inhibitor of κB
IκBα	Inhibitor of κB alpha

IκBβ	Inhibitor of κB beta
IκBγ	Inhibitor of κB gamma
KFT3	C-kit, flt3 ligand, thrombopoietin, interleukin-3
LDMNC	Low density mononuclear cell
Lin	Lineage markers
LN2	Liquid nitrogen
LPP	Low proliferative potential colony
LPS	Lipopolysaccharide
LTBMC	Long term bone marrow culture
LTC	Long term culture
LTC-CFC	Long term culture colony forming cell
LTC-IC	Long term culture initiating cell assays
LTα	Lymphotoxin-α
M-CSF	Macrophage colony-stimulating factor
mdr1	Multiple drug resistance gene 1
MDS	Myelodysplastic syndrome
MEKK3	Mitogen-activated protein kinase kinase kinase 3
MPP	Multipotential Progenitors
mSCF	Mouse stem cell factor
mTNFα	Mouse TNFα
NEMO	Nuclear factor kappaB essential modulator
NES	Nuclear export sequence
NF-κB	Nuclear Factor-κB

NLS	Nuclear localization signal
NOD/SCID	Non-obese diabetic-severe combined immunodeficient
p100	NF-κB2
p105	NF-κB1
p50	NF-κB1
p52	NF-κB2
p65	Rel A
PBS	Phosphate buffered saline
PI	Propidium iodide
PMA	Phorbolmysteric acid
PWMSCM	Poke weed mitogen spleen conditioned media
RA	Refractory Anemia
RAEB	RA with excess blasts
RAEB-T	RAEB in transformation
RelA	V-rel reticuloendotheliosis viral oncogene homolog A
RelB	V-rel reticuloendotheliosis viral oncogene homolog B
RHD	Rel homology domain
rhIL-3	Recombinent human IL-3
RIP1	Receptor interacting protein 1
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT	Room temperature
Sca	Stem cell antigen

ShRNA	Short hairpin RNA
SIMPL	signaling molecule that associates with mPLK
SRBC	sheep red blood cells
TACE	TNF alpha converting enzyme
TAD	Transactivation domain
TGF-β1	Tumor growth factor-Beta
TNFα	Tumor necrosis factor alpha
TNF-RI	Type I TNF receptor
TNF-RII	Type II TNF receptor
Tpo	Thrombopoietin
TRADD	TNF RI-associated death domain
TRAF2	TNF receptor-associated factor-2

I. Background and significance

Hematopoiesis is the process of normal replication and maturation of all blood cell varieties in a host. The innate immune response is critical for normal host defense, with the TNFα/NF-κB signaling pathway playing a major role. The TNFα/NF-κB pathway also plays a less well understood role in the control of hematopoiesis. Full transcriptional induction of NF-κB controlled genes, specifically hetero- or homodimers of NF-κB that contain p65, in the TNFα pathway requires the transcriptional co-activator SIMPL. Insight into the function of SIMPL in TNFα signaling events have been gained using immortalized cell lines; however the role of SIMPL in a more physiological setting has not been studied. The goal of my thesis project was to test the hypothesis that SIMPL plays an integral role in the control of steady-state hematopoiesis.

A. The Immune response

Mammals and multicellular organisms have the ability to react to pathogens. For mammals, this reaction requires an immune system, which can be divided into the innate and the acquired immune responses. The acquired immune response has specificity, and the ability to remember pathogens and to discriminate between self and non-self. The acquired immune response includes B- and T-lymphocytes. In contrast, the innate immune response is a non-specific response against pathogens including microbes or macromolecules. It does not have the ability to remember a pathogen. The innate immune response consists of physical barriers like the skin and mucous membranes, tears, granular and phagocytic cells and circulating factors like antibodies (IgM) and complement [6]. For example, during an innate immune response activated basophils and

eosinophils release granules containing metabolites (i.e. acid phosphatase, cathepsin, major basic protein); neutrophils phagocytose and release granular metabolites (i.e. collagenase, lysozyme, anti-bacterial basic proteins, myeloperoxidase) [7]; activated monocytes phagocytose pathogens/debris and release the cytokine tumor necrosis factor-α (TNFα) and other soluble mediators. TNFα is also released by epithelial cells in response to pathogen activation in the respiratory and gastrointestinal tracts [8]. TNFα is a pro-inflammatory cytokine that acts locally, depending on the local microenvironment, to stimulate production of various cytokines (granulocyte monocyte-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), and chemokines, (interleukin-8; IL-8). Cytokines and chemokines recruit and enhance neutrophil and monocyte function and increase the expression of adhesion molecules on endothelial cells permitting the attachment and margination of phagocytic cells into tissues [5, 9]. TNFα promotes tissue repair by stimulating production and release of angiogenic factors. Systemically, TNFα elicits fever production and acute phase protein production. Thus, TNFα is a vital component of the host defense [5].

B. TNFα signaling

TNFα does share ~28% homology at the amino acid level with lymphotoxin-α (LTα) [10]. TNFα is primarily produced by macrophages, but also produced by fibroblasts, epithelial cells, T- and B-cells, while LTα is made by natural killer cells, B- and T-cells. However, the action(s) of LTα in humans is generally undefined. In general, TNFα is a pro-inflammatory mediator with the ability to stimulate cell differentiation and to a lesser extent apoptosis. TNFα can be found as both soluble and membrane bound

forms. Soluble TNFα (along with LTα) signals mainly through the type I TNF receptor (TNF-RI) and binds with a lower affinity to the type II TNF receptor (TNF-RII). TNF-RI is found in most tissues whereas TNF-RII expression is more restricted and is found primarily in cells of the immune and hematopoietic system. Soluble TNFα starts as a transmembrane, homotrimer that is cleaved by the metalloprotease, TNF alpha converting enzyme (TACE) to release a 51 kDa soluble trimer of TNFα. Membrane bound TNFα is an uncleaved transmembrane homotrimer and is needed to fully activate signaling through TNF-RII. TNF-RI and RII share 25% identity in their extracellular domains and are single membrane spanning receptors [5]. Classically, the TNFα controlled immune response occurs through activation of NF-κB (Nuclear Factor-κB) controlled gene expression which is mediated in large part through TNF-RI [5, 11].

C. NF-κB, a transcription factor

The transcription factor NF-κB, through controlling the expression of growth factors and cytokines, plays a critical role in regulating embryonic development, immune and inflammatory responses [9, 11]. In mammals, NF-κB is found as a homo- or heterodimer composed of members of the rel family consisting of RelA (p65), p50/p105 (NF-κB1), c-Rel, RelB, and p52/p100 (NF-κB2). NF-κB contains a 300 amino acid rel homology domain (RHD), located at the amino-terminus that allows for protein-protein interactions, DNA binding, and it also contains a nuclear localization signal. Only p65, relB, and c-rel contain a transactivation domain (TAD) which is located in the carboxyl-terminus; p50 and p52 lack a TAD. The TAD recruits co-activators and displaces repressors while promoting gene transcription [11]. NF-κB activity is regulated primarily

by cytoplasmic retention and removal from DNA. Members of the IκB (Inhibitor of κB) family bind to and mask the nuclear localization signal in NF-κB (discussed in more detail later). The IκB family consists of seven members including, IκBα, IκBβ, IκBγ which contain five to seven ankyrin repeats (33 amino acids) that bind the RHD of NF-κB [11].

D. NF-κB activation

Initially, NF-κB is bound to IκB which inhibits NF-κB DNA binding activity and nuclear retention, by constantly shuttling the NF-κB /IκB complex in and out of the nucleus. The classic model for NF-κB activation involves two IκB kinases (IKKs): IKKα and IKKβ plus IKKγ (NEMO) a non-catalytic protein that acts as a scaffold. IKKα and/or IKKβ phosphorylate IκBα and IκBβ on two amino terminal serine residues releasing bound NF-κB. Phosphorylated IκB is then recognized by a SCF (skp, cullin, βTrCP1/2) ubiquitin ligase and is targeted for degradation by the proteasome. Released NF-κB either enters or is retained in the nucleus and binds to DNA. The transactivation domain of one of the rel dimers initiates transcription. TNFα, along with a number of other cytokines can signal for changes in gene expression through NF-κB. Current models predict that the signaling pathways downstream of cytokines/chemokines/growth factors known to activate NF-κB converge upon and activate the IKKs. TNF RI induction of NF-κB appears to be IKKβ dependent. How the IKKs are activated is not well understood (complete review [11]).

E. TNF-RI activation of NF-κB

Classically, TNFα, through TNF-RI, activates the NF-κB heterodimer composed of p65 and p50. Upon TNFα binding to cell surface TNF-RI receptors, TNF-RI oligomerizes. The cytoplasmic tail of TNF-RI contains a death domain that allows for protein-protein interactions. Oligomerized TNF-RI binds TRADD (TNF RI-associated death domain protein) which in turn binds TRAF2 (TNF receptor-associated factor-2), RIP1(receptor interacting protein 1) and/or MEKK3 (Mitogen-activated protein kinase kinase kinase 3) leading to activation of downstream signaling events culminating in the activation of the IKK complex [12, 13]. TRAF2 is not required for TNF-RI dependent activation of NF-κB, but is required for activation of the transcription factor AP1 and TNFα dependent apoptosis [11]. RIP1 is a serine/threonine kinase required for TNF-RI activation of NF-κB but interestingly its kinase activity is not required [5, 11]. As mentioned previously, activated IKKβ phosphorylates IκBα. Non-phosphorylated IκBα blocks the NLS (nuclear localization signal) of p65, the NF-κB component which contains the TAD. The p50 NLS is exposed and the NF-κB/IκBα complex shuttles in and out of the nucleus due to IκBα's nuclear export sequence (NES). This limited nuclear import of p65/p50 may account for the basal transcriptional activity of the complex. Phosphorylated IκBα releases NF-κB allowing it to trans-locate into the nucleus, and subsequent upregulation of κB controlled genes; phospho-IκBα is ubiquinated and degraded [11].

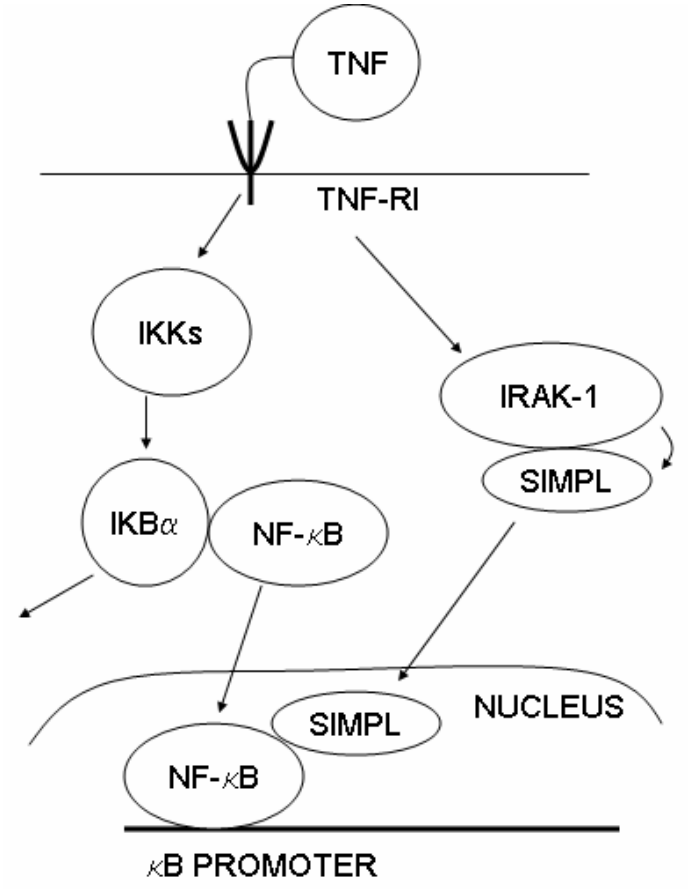


Figure 1. TNFα sigaling to NF-kB and SIMPL co-activation.
TNFα activates NF-kB through the IKKs and SIMPL is required for full activation of NF-kB through a yet clarified alternate pathway.

As schematically depleted shown in Figure 1, full activation of NF-κB controlled genes through TNF-RI requires a transcriptional co-activator called SIMPL (signaling molecule that associates with mPLK) which was discovered in the Harrington lab [14, 15]. SIMPL is a p65 specific co-activator that contains a carboxyl terminal nuclear localization signal. Interleukin-1 receptor associated kinase 1 (IRAK-1, also known as mouse pelle like kinase mPLK) is a serine/threonine kinase with an amino-terminal death domain that is also involved in full activation of NF-κB transcriptionally contolled genes. IRAK-1 can be detected in TNF-RI containing complexes and can phosphorylate SIMPL [16]. Analysis of cells derived from IRAK-1 null mice have demonstrated a requirement for IRAK-1 in TNF-RI activation of NF-κB [17].

F. SIMPL

As mentioned earlier, SIMPL was identified in the Harrington lab. A SIMPL mutant (ΔSIMPL) that lacks the first 80 amino acids was generated to study the role of SIMPL in TNFα signal transduction. Using ΔSIMPL, we determined that TNFα dependent signaling through TNF-RI to NF-κB was dependent on SIMPL [15]. IKKα and IKKβ induction of NF-κB activity was significantly diminished in the presence of the Δ SIMPL mutant, and catalytically inactive IKKα or IKKβ diminished wild type SIMPL activity. Over-expression of ΔSIMPL induced apoptosis showing a critical role for its ability to signal to NF-κB. SIMPL signaling was shown to be TNFα specific in NF-κB reporter assays. Further study revealed that SIMPL functions as a specific p65 co-activator in the TNF-RI pathway [14, 15].

SIMPL is the focus of this study, which is aimed at determining the role of SIMPL in TNFα driven physiological responses. Thus far SIMPL has been shown to be involved in TNF-RI activation of NF-κB, but a physiological/phenotypic role for SIMPL has not been elucidated. There is evidence for TNFα and NF-κB involvement in blood cell development and differentiation (hematopoiesis) which intuitively involves the cells of the immune system. NF-κB, specifically p65, has been shown to be necessary for normal, steady-state, blood cell development [18, 19]. Thus, focusing on the role of SIMPL in hematopoiesis represents an ideal starting point for exploring the physiological role of SIMPL.

G. Role of TNF-RI dysregulation, a role for TNF-RII

TNFα induces apoptosis understand certain conditions. Signaling through TNF-RII usually enhances a TNF-RI signal and only in a limited manner signals for NF-κB activation independent of TNF-RI [20]. As discussed above TNFα binds to TNF-RI leading to oligomerization of TNF-RI and binding of TRADD, TRAF2 and other signaling pathway components, culminating in activation of the IKK complex. TNF-RI normally inhibits apoptosis through TRAF2 dependent recruitment of the anti-apoptotic proteins including cellular inhibitor of apoptosis 1 and 2 (cIAP1 and cIAP2). cIAP1/2 each contain a caspase inhibitory Baculoviral IAP Repeat (BIR) domain. The TNF-RI/TRAF2/cIAP complex protects TNF-RI from receiving or triggering apoptotic signals. Interestingly, TNF-RII promotes apoptosis by competitively sequestering cIAP1 and cIAP2 TNF-RII bound TRAF2, thus allowing for caspase-8 mediated initiation of apoptosis. Specifically, cIAPs block the ability of activated caspase-8 from converting

pro-caspase-3, through cleavage, to activate caspase 3. The activated caspase-3 both activates caspase-8 through cleavage of the pro form, and activates apoptosis. Caspase-8 also leads to the release of cytochorome c from the mitochondria. TNF-RII co-stimulation increases TNF-RI dependent caspase-8 activation. The carboxyl terminal domains of cIAP1 and cIAP2 each contain a RING finger E3 ubiquitin ligase enabling cIAP1 to ubiquinate and target TRAF2 for degradation TRAF2 (Figure 2). Thus, cIAPs have a pro-apoptotic function when TNFα binds to TNF-RII. As cellular levels of TRAF2 are depleted, the competition between the TNF receptors for TRAF2 increases. This mechanism provides a viable way to control a dysregulated TNFα/ NF-κB signal. The TNFα/ NF-κB pathway has a number of NF-κB induced feedback inhibitors and activates the expression of a number of anti-apoptotic genes [5, 21]. If NF-κB cannot signal due to a loss of component in the pathway, then potentially, TNF-RII will bind cellular anti-apoptotic factors. The diminished production of cIAPs will lead to a net loss in cellular anti-apoptotic activity and apoptosis would be predicted to occur. Thus, SIMPL acting as a co-activator in this pathway will have a major role in the control of the TNFα induced NF-κB (p65/p50) signal and its' effects can evaluated by studying the effect of SIMPL upon induction of apoptosis.

H. Basics of hematopoiesis

Hematopoiesis, most often studied in animal models, in particular mice, is an enormous field. I will discuss hematopoiesis in general here and focus in the results and

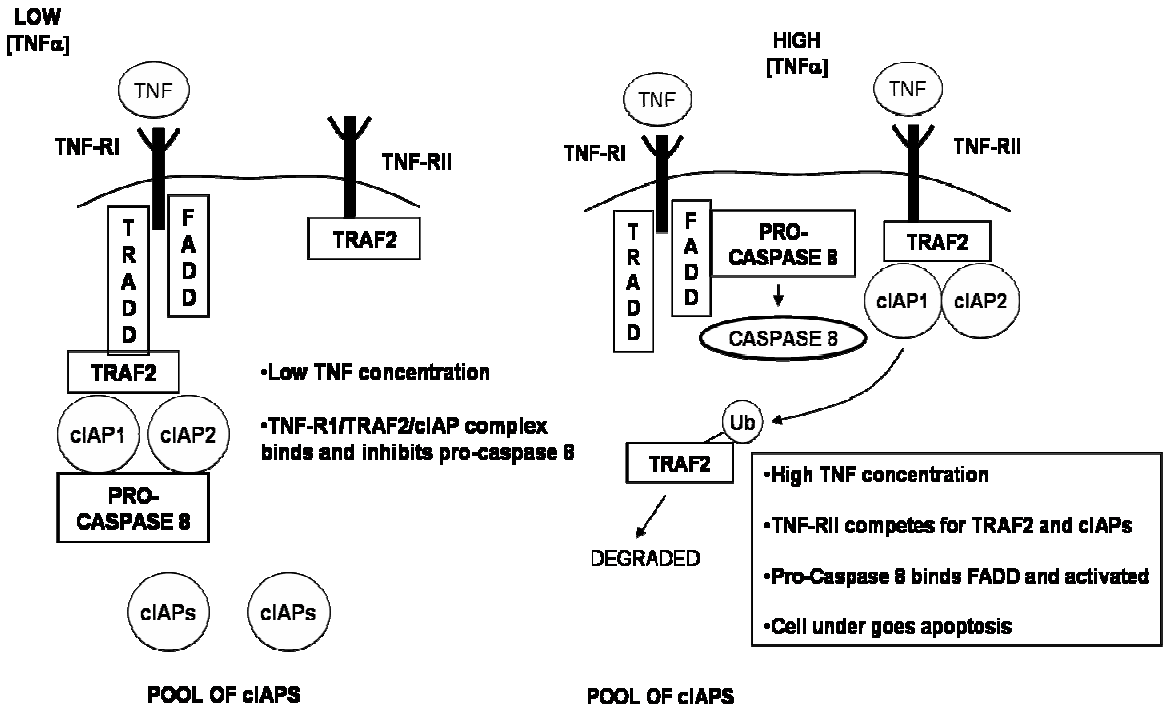


Figure 2. Dose dependent effects of TNFα.
Low dose TNFα leads to an increase cIAP gene expression that blocks an apoptotic response. High dose TNFα elicits activation of TNF-RI and TNF-RII leads to activation of an apoptotic response.

(adapted from [5])

discussion sections on specific parts of hematopoiesis as information on SIMPL is elucidated experimentally.

Hematopoiesis is the process of development and formation of blood cells throughout the life of the animal. On any given day, approximately 2×10^{10} white blood cells and 2×10^{11} red blood cells are turned over. Blood cell development starts with hematopoietic stem cells (HSCs), which are pluripotent cells that have the ability to self-renew and proliferate. HSCs have the potential to develop into any of the mature circulating hematopoietic cell types including neutrophils, basophils, erythrocytes, megakaryocyte, mast cells, dendritic cells, macrophage/monocytes, and lymphocytes. In a simplified view, during development, HSCs can differentiate into Multipotential Progenitors (MPP) that retain the ability differentiate into any blood cell type given the right growth factors, microenviroment, and/or cytokines. In contrast to HSCs, MPPs lack the ability to indefinetly self-renew. The first major choice for a HSC is whether one daughter cell should differentiate into either a common lymphoid or a common myeloid precursor (progenitor) [3, 22] (Figure 3).

Since my studies focused on myeloid development (occurs mostly in bone marrow), the rest of this discussion will focus on the control of myeloid hematopoiesis. Lymphocyte development and differentiation will be briefly discussed later. Most of the knowledge regarding the process of blood cell differentiation has been, and continues to be, gained from work done using an *in vitro* colony progenitor assay. In this assay freshly isolated bone marrow cells are plated at a single cell density in a semi-solid

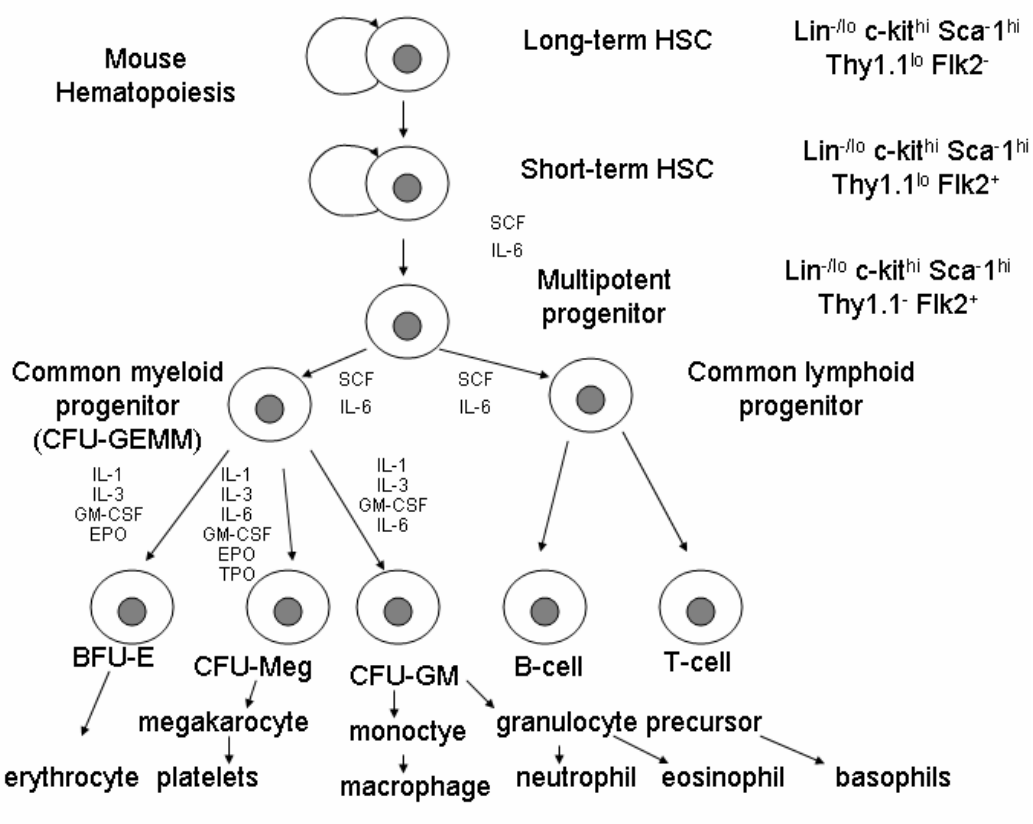


Figure 3. Mouse Hematopoiesis.
Highlights the flow of differentiation starting with the bone marrow stem cell.

Adapted from [3, 4]

matrix in the presence of a cytokine/growth factor cocktail. Seven to fourteen days later, the numbers of colonies (>50 cells) are quantitated. Each colony is derived from one progenitor or HSC. The common myeloid progenitor can give rise to colony forming unit-granulocyte erythrocyte macrophage megakaryocyte (CFU-GEMM) colonies (which was named for the differentiated cell types formed in the colony). Therefore CMPs can form every myeloid cell type (Figure 3). Thus, the colony assay is a way to identify the type of progenitors present in bone marrow. Hematopoietic stem cells and MPPs can also be identified by the presence of cell surface markers. For example, c-kit, the receptor for stem cell factor (also known as steel factor, SCF) is a common marker for HSCs and MPPs. Other cell surface makers are also used to further distinguish HSCs and MPPs (as will be elucidated later). Also, HSCs and MPPs can be given colony stimulating factors (CSFs) that can force the precursors down specific pathways in order to study the development of specific hematopoietic cell types like differentiated myeloid cell types [3, 22-25]. Therefore, SIMPL that is proposed to be involved in hematopoiesis can be exquistely explored by using the various combinations of variables including multiple growth factors, and hematopoeitic cell types. The outcomes to these experiments will be visualized as disruptions in hematopoeisis, including from disruption in colony formation, growth inhibition, apoptosis, pressure selection, and developmental problems.

I. Evidence for TNF-RI/NF-κB involvement in differentiated hematopoietic cell types

As described previously, SIMPL is critical for full activation of NF-κB (p65/p50) allowing for enhanced transcription of p65/p50 controlled genes through TNF-RI.

Therefore, evidence that shows involvement of the TNFα/NF-κB pathway in hematopoiesis implicates a role for SIMPL in hematopoiesis. I will present evidence for this involvement beginning with the most differentiated cell types and work background to the immature precursors including HSCs. Analysis of mice lacking TNFα/NF-κB signaling pathway components has confirmed the role of this pathway in the generation of more differentiated hematopoietic cell types. These mature cell types include B and T cells, macrophages, monocytes, neutrophils, and red blood cells.

When TNFα is knocked-out in mice a number of problems occur. TNFα deficient mice fail to develop normal class switching from IgM to IgG in B-cells, and do not develop germinal centers in the spleen when presented with a T-cell dependent antigen such as sheep red blood cells (SRBC) [26]. This demonstrates a role for TNFα in B and T-cell lymphopoiesis. Mice lacking TNF-R1 and/or TNFα die after an oral injection with *Toxoplasma gondii* infected macrophages; B-cell interactions are known to be necessary for the response to *T. gondii*. However, TNF-RII knockout mice do not die after the same challenge; highlighting a TNF-RI dependent involvement in this immune responses [5]. Originally RelA (p65) knockout mice were thought die from poor liver development at embryonic age 15-16 [9]. Mice lacking both TNF-RI and RelA, survive to day 10 (after birth), at which point, they died from immature neutrophil invasion of the liver; highlighting an inherent defect myelopoiesis with the loss of RelA (p65). This result suggests that p65 protects the developing embryo from TNFα induced apoptosis. These data highlight two additional points that are frequently over looked. One, based upon inference is that TNFα levels are present during early embryogenesis. Two, lack of TNF-RI leads to immature neutrophil invasion of the liver in the absence of pathogenic

challenge, thus hinting at a role for the TNF-RI pathway in the regulation of steady-state hematopoiesis [27]. TNFα through p65 controls a number of growth factors necessary for differentiation. Expression of the GM-CSF along with M-CSF, IL-2 and G-CSF, genes are under p65 control and their protein products, are important for hematopoietic differentiation and development (i.e. neutrophils and macrophages) [28-31]. GM-SCF is a good example; analysis of the p65 knockout revealed that production of granulocyte-macrophage colony stimulating factor (GM-CSF) required TNFα induction of p65 to induce expression of GM-CSF above basal levels [9]. Another study confirmed that GM-CSF is a TNFα responsive gene and that the GM-CSF gene promoter contains NF-κB sites [26, 32].

RelA (p65) has been linked to the normal development of T and B cells [33]. T- and B-cells derived from the p65 knock-out mice have a decreased poliferative ability upon challenge with various stimuli including Con A, anti-CD3, anti-IgM, lipopolysaccharide (LPS) and phorbol mysteric acid (PMA) [34]. B-cells from RelA-/- mice treated with TNFα have an increased sensitivity to apoptosis. Mice lacking RelA(p65) and NF-κB1(p50) (the heterodimer activated through TNF-RI) do not generate B and T lymphocytes [33]. Thus, mature hematopoietic B and T cells, neutrophils and macrophages require both functional TNF-RI and p65.

J. NF-κB (p65/p50) involved with terminal precursors of differentiated cell types

Terminal precursors are mature blast cells that can differentiate into a limited range of differentiated cells (as illustrated in Figure 4). The p65/p50 complex is the

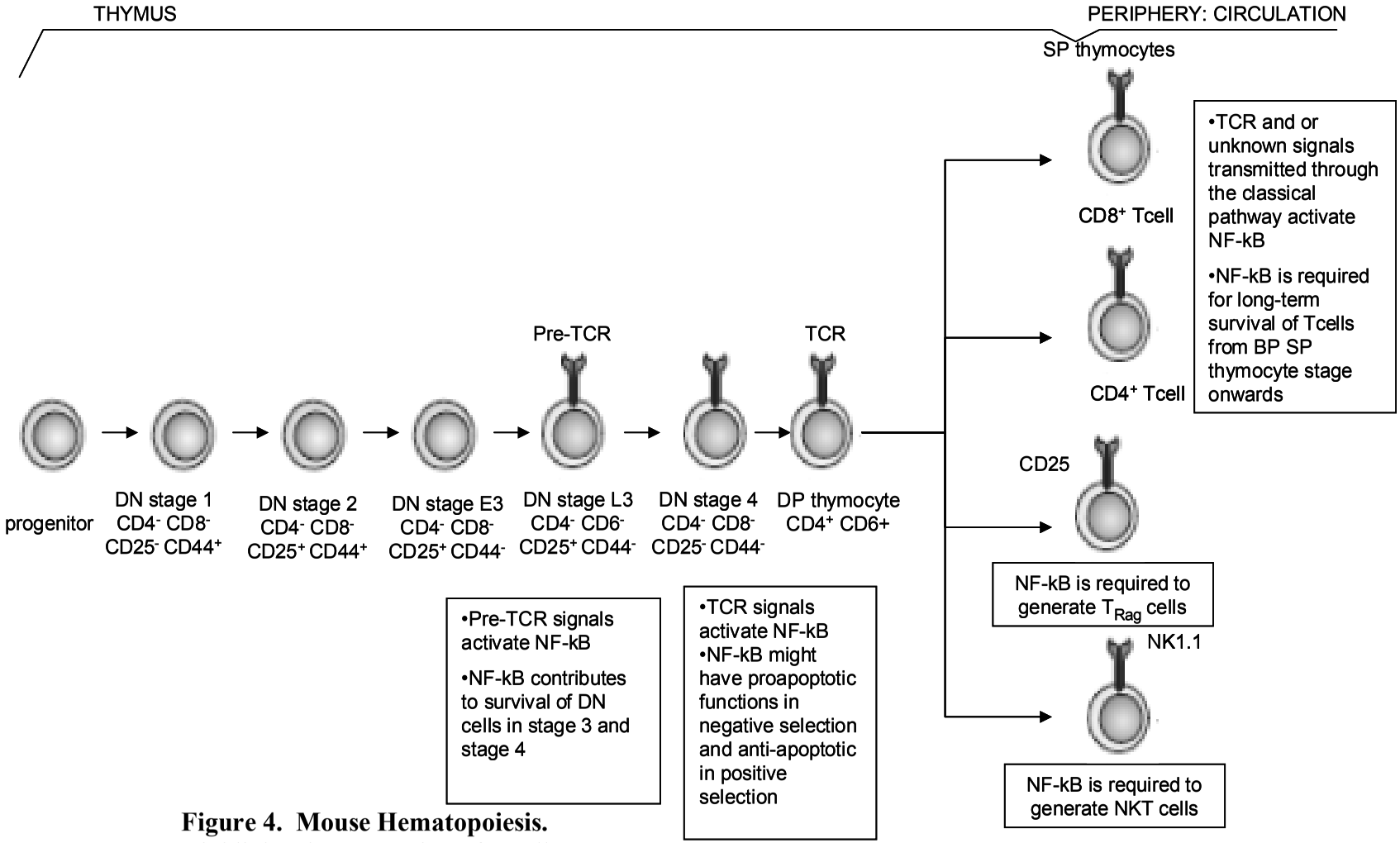


Figure 4. Mouse Hematopoiesis.
Highlights the maturation of T cells
Adapted from Sibenlist et al. 2005

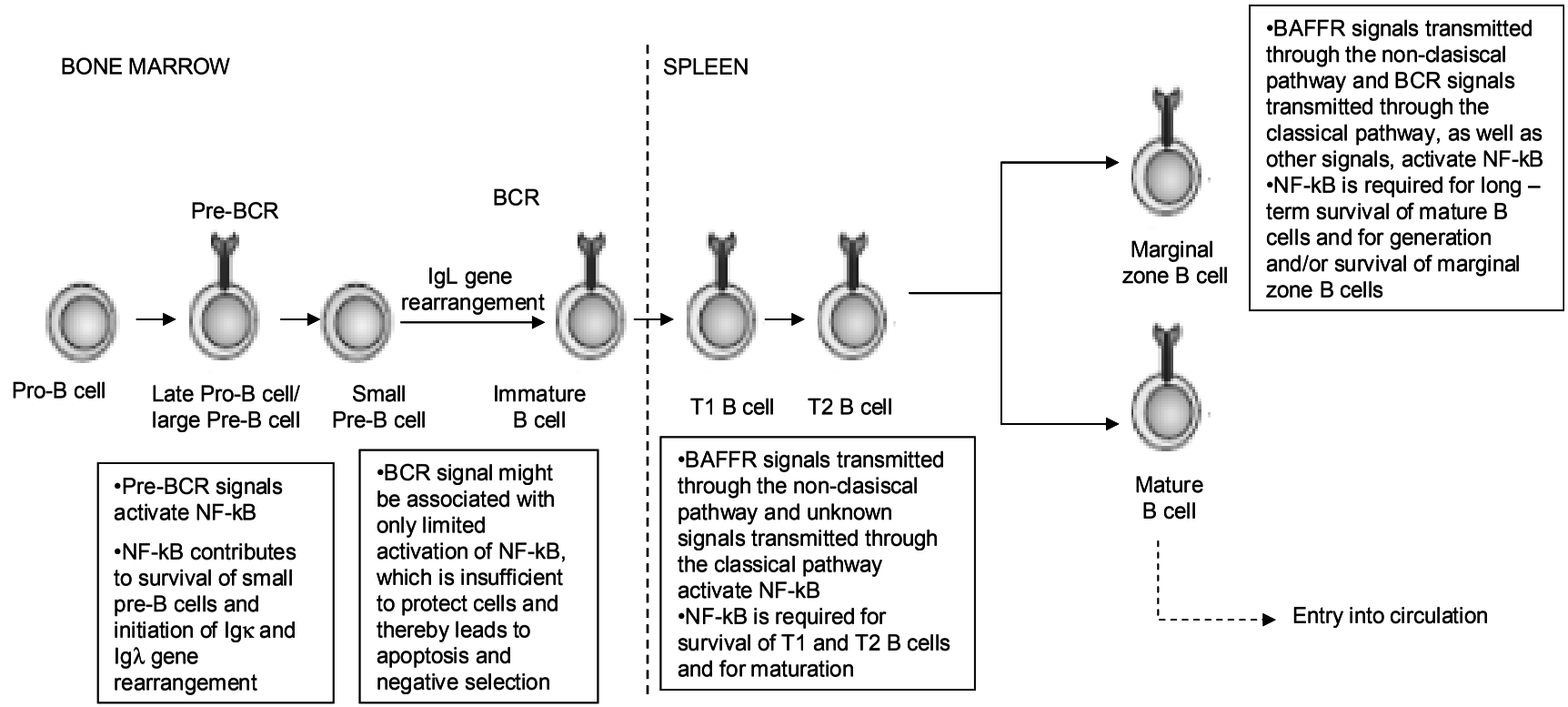


Figure 5. Mouse Hematopoiesis
Highlights the maturation of B cells
Adapted from Sibenlist et al. 2005

predominant form of NF-κB in pre-B cells [34]. Schematics of the expression pattern of NF-κB involvement in T and B cell differentiation are presented in Figures 4 and 5. RelA (p65) and p50 are also involved in normal erythropoiesis. BFU-E are the mature blasts that eventually terminally differentiate into red blood cells. RelA (p65) and p50 have high expression levels and are localized in the nucleus of day 10 blast forming unit-erythrocyte (BFU-E) cultures. There is a decline in cellular p65 and p50 levels by day 14. These data highlight the involvement of p65/p50 in early erythrocyte formation. Mechanistically this may reflect the fact that the c-myc, and c-myb genes, which are important in erythroid proliferation contain NF-κB binding sites in their promoters. Taken together, p65 along with TNFα are intimately involved in every facet of mature terminally differentiated hematopoietic cell development.

K. Understanding TNFα in immature progenitor and hematopoietic stem cells

1. Overview

The more immature hematopoietic cells require a more extensive explanation of their ability to form many hematopoietic cells types. Therefore a more extensive review of TNFα involvment in early immature precursor will be provided. Insight into an understanding of hematopoietic stem and progenitor cells reveals TNFα's global effects on hematopoiesis. Insight into the global effects of TNFα will highlight specific conditions that SIMPL may modulate. TNFα can potentially inhibit hematopoietic stem cells or hematopoietic progenitor cells at 3 critical stages: survival, proliferation and differentiation. Insight into the role of TNFα will be best described by separating the

roles of proliferation, survival, and differentiation. Blocking survival is inhibiting the HSC or progenitor from living; cell death can occur through apoptosis or necrosis. Inhibiting proliferation entails blocking the ability of immature precursors to expand and possibly, in the case of the HSC, self renew. Expansion is not necessarily differentiation and should be considered different from self-renewal. Blockage of differentiation inhibits the immature cell type from becoming a more mature cell type that will have increasingly limited options for expansion and functionality.

TNFα's effects on immature hematopoietic precursors have been studied in both human and mouse model sysems, and an overview of both is provided. Key to understanding studies in the role TNFα plays in hematopoiesis, human TNFα activates human TNF-RI and TNF-RII in a concentration dependent manner. Soluble mouse TNFα (mTNFα) activates mouse TNF-RI and TNF-RII to the same extent that soluble human TNFα activates human TNF-RI and TNF-II. Human TNFα binds to and activates mouse TNF-RI; mouse TNFα does not bind to either human TNF-RI or TNF-RII.

In the human hematopoietic system HSCs can be defined by the cell surface markers CD34 and CD38; HSCs are $CD34^+$ and $CD38^-$; by itself $CD34^+$ alone defines the immature progenitor which contains a small subset of HSCs.

In mouse hematopoietic populations, HSCs are defined as $lin^-sca^+ckit^+$ while HPCs defined as $lin^-sca^-ckit^+$. HSCs and HPCs are the two most important subsets of hematopoietic cells because they can give rise to all the myeloid and lymphoid cells. In the literature conclusions on hematopoiesis have been drawn using cells expressing a combination of these markers, and these studies will be described herein to gain insight into influence TNFα has on the hematopoietic system.

The advantage of using mice is the ability to the remove a gene of interest (i.e. TNFα) to better define the role of the gene product. The myeloid microenvironment involves cytokines, chemokines, growth factors, and cell-cell interactions (stromal layers). Each can be studied in the mouse system by gene manipulation. A second advantage of mice is that *in vivo* tests can be performed that cannot occur in humans. Thirdly, there is a complete range of organ systems to study the phenotypic impact of manipulating hematopoietic system. Thus in mouse models, the roles of TNFα in the microenvironment and the role of cell type specificity has been and can be more easily explored. A disadvantage is the wealth of information caused from all the potential ways to explore the effects of TNFα in mouse models makes a comprehensive study, as was done by Dybedal and coworkers [35] for TNFα in human early hematopoiesis, difficult. The comprehensive picture of TNFα on hematopoiesis presented below is derived from several different publications.

2. TNFα inhibits proliferation of precursor hematopoietic cells

A comprehensive paper that explored TNFα effects on survival and proliferation (at two of the three possible sites of inhibition) of human HSCs was done by Dybedal and co-workers [35]. To promote the survival, self-renewal and expansion of human HSCs, serum-free media supplemented with a mix of cytokines which included: c-kit (KL), flt3 ligand (FL), thrombopoietin (Tpo), and interleukin-3 (IL-3) was used. From here on this media is referred to as KFT3. These liquid culture conditions allow the human HSCs to maintain normal function, thus the HSCs can still proliferate and differentiate when placed in a differentiation assay. The serum-free condition eliminates exogenous

cytokine contamination. By using the KFT3 combination in the presence or absence of human TNFα, the authors showed that TNFα inhibited human hematopoietic stem cell proliferation but not their survival. To show this, human HSCs from cord blood or bone marrow were incubated with KFT3 and TNFα (20 ng/mL; 5 or 8 days) and were compared to KFT3 without TNFα. The clonal proliferation of TNFα treated HSCs from cord blood or bone marrow were diminished by ~65% and 67% respectively. The growth inhibition by TNFα could be caused by subpopulations of HSCs dying or by all the HSCs not growing. To answer this question the authors examined the ability of the HSCs to clonally proliferate at the single cell level in the presence of TNFα. Surprisingly, every single cell could form a clone, except the clonal size was greatly reduced when compared by cell number. In this experiment the colonies were not made up of differentiated cell types because factors were not given to support differentiation or mature cell growth. More specifically KFT3 treated HSCs clonal population ranged from 50 cells $\leq$10%, 10-50%, and 50-100% coverage of a dish, while those treated with KFT3 and TNFα ranged from 3- 9 cells, 10-49 cells and 50 cells $\leq$10% coverage [35]. In parallel to the human HSC experiment, mouse HSCs ($lin^-sca^+c\text{-}kit^+$) placed as single cells per well and treated with IL-3, SCF and mTNFα, TNFα inhibited clonal expansion as compared to control (non-TNFα treated) [36]. Similarly in 12 day single cell liquid culture expansion assays done with a mix population of mouse HSCs and HPCs (lin^-sca^+) and even more mature HPCs (lin^-sca^-), clonal proliferation was inhibited with mTNFα as compared to control [37]. These data reveal that HSCs can divide at least once in the presence of TNFα before becoming sensitive to the growth inhibitory effects of TNFα, and that progenitor clonal proliferation can also be inhibited.

Dybedal and co-workers used an *in vivo* method to test if human TNFα treated human HSCs proliferative clones were functionally capable of bone marrow reconstitution. In these experiments the goal is three part. One is to confirm that TNFα inhibits proliferation; two is to determine if the proliferation is reversible; and three is to begin to address HSC functionality. The inability of the TNFα treated HSCs to reconstitute bone marrow would confirm a loss of proliferation, suggest inhibition of differentiation, and question self-renewal. Briefly, these experiments consisted of using sub-lethally irradiated NOD/SCID (non-obese diabetic-severe combined immunodeficient; NON/SCID) mice engrafted with the human HSCs. NOD/SCID mice are ideal for these studies as they lack T and B cells that would normally recognize human cells as foreign. Sub-lethal irradiation of the mice should limit proliferation and differentiation and inhibit currently cycling mouse HSCs; the host HSCs will eventually begin to divide again as the low dose of irradiation is not lethal. This is advantageous because comparisons of growth between different treated groups of human HSCs can be competitively compared to the mouse HSC (normalize for growth). In the experiment, the engrafted HSCs had been cultured (to allow for clonal expansion) for 2 or 8 days in KFT3 in the presence or absence of TNFα. The cells were then transplanted into sub-lethally irradiated NOD/SCID mice. 6 weeks later, the NOD/SCID mice bone marrow was harvested and analyzed by fluorescence activated cytometry using mouse versus human specific cell surface markers.

In the first group of transplant experiments, only 1 out of 9 mice that received human HSCs treated *in vitro* with TNFα (20 ng/mL) for at least 5 days were reconstituted and in that one animal only 1.1% of the blood cells were human HSCs. In the non-TNFα

group all 9 mice were reconstituted, with 25% of the cells consisting of human HSCs. However, if the human HSCs were expanded for only 2 days (a time frame too short for HSCs to proliferate in culture), instead of 5 days and exposed to TNFα, 6 out of 8 mice were reconstituted with percentage of engraftment similar to control. It was hypotheisze and confirmed that 2 day TNFα exposure was not effecting HSC because they were not proliferating. They found that HSCs given Tpo, which prevents proliferation and promotes survival, were impervious to the 5 day exposure with TNFα. In the human studies, no increase in apoptosis was detected, as determined by annexin V and 7-AAD staining, in the TNFα treated cells. These results hint at a cell cycle regulation of HSCs by TNFα, which as been seen in mice. In mouse studies using BrdU incorporation and propidium iodide staining, both $lin^{-}sca^{+}c\text{-}kit^{+}$ (HSCs) and $lin^{-}sca^{-}c\text{-}kit^{+}$ (HPCs) populations were shown to be arrested at the G1/G0 stage of cell cycle (50% mTNFα treated vs. 18% control HSCs and 40% mTNFα treated vs. 24% control SPCs) [36]. These data along lend further support to the overall hypothesis that TNFα can function as a cell cycle inhibitor [38-41].

Assessing the data reveals that TNFα requires proliferation for it to have an effect on hematopoietic precursors. Further, the effects of TNFα are dependent not only on concentration, but also length of exposure. For example, after short term exposure to TNFα, HSCs to retain normal function; normal function is the ability to reconstitute bone marrow which requires survival, proliferation, and differentiation of HSCs.

Others have found that the effect(s) of TNFα is reversible, but in those studies the HSCs were exposed to short term TNFα for 2 days. But taken with the above data these HSCs probably did not proliferate. Dybedal and co-workers found that the effect of the

5 day exposure to TNFα on HSC engraftment ability could not be reversed with a subsequent 2 day treatment with anti-TNFα before transplantation. Of the 5 mice transplanted for each condition, only one given the TNFα antibody was successfully reconstituted, and only 0.1% of those transplanted cells were derived from the human HSCs. These data suggest strongly that the HSCs were not maintaining their pluripotent nature in the presence of TNFα.

Overall, these data suggest there is a natural balance or gradient of TNFα that must be maintained in order to neither irreversibly inhibit HSC proliferation nor allow for uncontrolled expansion. Uncontrolled expansion has been shown for HSCs derived from TNFα-/- mice that are grown on stromal cells, the TNFα-/- derived HSCs live 4 times longer than wild type HSCs. Intringuingly, near irreversible HSC proliferation occurs in diseases that expose bone marrow to chronically high levels of TNFα such as myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and aplastic anemia.

3. TNFα promotes differentiation of hematopoietic precursors

a. Overview

The TNFα treated HSCs are not undergoing apoptosis, but the cell cycle is inhibited suggesting that hematopoietic differentiation is being affected through inhibition of proliferation. Differentiation requires HSCs and HPCs to mature through rounds of proliferation followed by narrower choices of differentiation then the more mature cells proliferate and differentiate further. Each subsequent round of maturation leads to a decreased ability to proliferate and fewer options of cell types to mature into. Thus, differentiation tests the functionality of a precursor. For illustration, expansion of

HSCs without differentiation to mature cell types leaves the host pancytopenic (anemic, leukopenic, thrombocytopenic). However, differentiation has two contexts that need to be explained and explored. In the first scenario, explained thus far, the HSC or HPC undergoes clonal expansion in the presence of TNFα (5 or more days), and then the expanded clones are put into a methylcellulose differentiation/proliferation colony assay. This would test if the HSC or HPC has already differentiated thus either limiting the amount of proliferation and/or limiting the type of mature cell type it can become. The second scenario, is one in which TNFα is added to HSCs or HPCs at the same time the cells are placed into a methylcellulose differentiation/proliferation colony assay. Colony counts in methylcellulose differentiation/proliferation colony assay are not made until 7-14 days (depending on specific experiment) after plating. Assuming the previous work on TNFα was correct, then TNFα would not take effect for 2 days (for HSCs) and not be potentially irreversible for the first 5 days. Thus, in the second scenario, it would be expected for the HSCs or immature HPCs to expand/proliferate and differentiate before TNFα starts to inhibit the proliferation or expansion.

b. *First scenario: Understanding differentiation by pre-treating HSCs and HPCs with TNFα before placement into proliferation/differentiation Assay*

For scenario one, Dybedal and co-workers followed the expression of the early percursor cell surface marker CD34, which identifies a mix of HSCs and early HPCs in human hematopoietic cells. Human $CD34^+$ cells were cultured in KFT3 with or without TNFα (20 ng/mL) and followed for 2, 5, and 13 days for expression of lineage markers by fluorescence activated cell sorting (see methods). At 2 days, the TNFα treated and the

non-TNFα groups looked similar. At 5 days, in the TNFα treated group the level of expression of the progenitor marker CD34 was down. By 12 days, CD34 was completely gone in the TNFα group; while in the control group of the expanded cells, 17% were lin^- $CD34^+$ and 40% were $CD34^+$. Without CD34 human hematopoietic cells are considered mature and at the end-stages of differentiation [35].

For more functional differentiation experiments, Dybedal and co-workers utilized long term culture initiating cell assays (LTC-IC) followed by LTC-colony forming cell (LTC-CFC) assays. In these experiments HSCs were expanded in liquid culture for 5 to 7 days in media containing KFT3 with and without TNFα (2 or 20 ng/mL). The HSCs were then placed on a murine/mouse fibroblast stromal layer cell line (1:1 mix of M210B4 and sl/sl) that produce high amounts of IL-3, G-CSF, Flt3 ligand in long term culture (LTC) media (supplemented with hydrocortisone 21-hemisuccinate) for 6 weeks. Then, the culture was evaluated by taking adherent and non-adherent cells and putting them into a differentiation/proliferation colony assay containing only human growth factors (rhIL-3, rhG-CSF, rhSCF, rhFl3 ligand, rhGM-CSF), so only human and not mouse cells could grow. It was shown that a 5 day exposure to TNFα inhibited the ability of HSCs to form LTC-CFC (colonies) by 98% compared to wild type control. Further, engrafted human HSCs in mice exposed to 5 days of high dose TNFα intravenously, and placed into the colony assays were equally as inhibited compared to control.

In attempt to test this hypothesis, (Rusten et al 1994) [42] argue TNFα induced colony inhibition of HSCs grown in TNFα for up to 4 days could be reversed by washing off the TNFα. The problem with the Rusten study was that HSCs were not given any

cytokines other than TNFα. Therefore they were not forced to proliferate, and TNFα would not have an opportunity to work in proliferating cells [42].

c. Second Scenario: Understanding differentiation by concurrent addition of TNFα with myelopoietic colony formation

In the second scenario, $lin^-sca^+ckit^+$ HSCs are plated directly into methylcellulose differentiation conditions with mTNFα. TNFα inhibits the ability of mouse HSCs to form highly proliferative colonies (HPP; large sized colonies with a dense core of cells {which usually contain >50,000 cells [43]}; greater than 0.5mm in diameter), but did not inhibit the low proliferative potential (LPP) colonies. This inhibition was mTNFα concentration dependent with higher levels of mTNFα causing greater inhibition of the number of highly proliferative colonies [36]. Similarly, human $CD34^+$ cells, plated in methylcellulose differentiation/proliferation assay, HPP colony formation was similarly inhibited in a hTNFα dependent manner [42]. However, the effects of TNFα varied depending upon the other cytokines present. Human HPP and LPP colonies numbers were inhibited in $lin^-sca^-ckit^+$ HPCs exposed to hTNFα in the presence of G-CSF and SCF. But HPP colonies were not inhibited when HPCs were given IL-3, SCF, and TNFα, while HPP colony formation was inhibited when HSCs were given the same cytokines. In fact, HSC colony formation inhibition by mTNFα was not growth factor specific. Thus, in the second scenario, it would be expected for the HSCs $lin^-sca^+ckit^+$ to expand/proliferate and differentiate before TNFα could start to inhibit the proliferation or expansion. Therefore TNFα treated HSCs can form LPP colonies, but only limited HPP colonies. Hematopoietic progenitor cells proliferation is regulated by TNFα in a growth

factor specific manner. The difference of regulation of the HPCs (both treated and control received SCF) to the different growth factors G-CSF versus IL-3 may simply be a matter of kinetics and colony type type. As far as kinetics are concerned, IL-3 has been shown to induce rapid expansion as well as differentiation [44]. This expansion could occur more rapidly in mature HPCs since they are actively cell cycling compared to HSCs. Therefore expansion could be over before TNFα could take effect. With G-CSF, first, the expansion and proliferation is probably more insidious. It takes longer to differentiate into neutrophils, the cell type most influenced to expand/proliferate with G-CSF. As far as cell type is concerned, IL-3 produces more CFU-GM cell types which are more immature that than the CFU-G colonies G-CSF would preferentially produce [45]. It has already been shown in this introduction that TNFα has intimate and direct regulation of mature hematopoietic cells, especially granulocytic neutrophilic cell types [41, 46-48].

Analysis of cells derived from TNFα-/- animals should be the antithesis of these differentiation results, and it is. Mouse $lin^{-}sca^{+}ckit^{+}$ (HSC) and $lin^{-}sca^{-}ckit^{+}$ (HPC) cells derived from TNFα-/- animals were plated in GM-CSF differentiation/proliferation assays, formed more than 4 times more CFU-GM than wild type. As measured by injection of $lin^{-}sca^{+}ckit^{+}$ HSCs into lethally irradiated mice and counting colonies on the spleen (CFU-S), the number of multipotent progenitors/HSCs were also increased in mouse TNFα-/- derived bone marrow as compared to bone marrow derived from wildtype animals.

4. Summary

Of the three possible ways, survival, proliferation, and/or differentiation, to effect immature hematopoietic precursors, the role of TNFα is to inhibit proliferation. When bone marrow derived from TNFα-/- animals is compared to bone marrow derived from wild type animals, it becomes apparent that a steady state level of TNFα is present in bone marrow, and it prevents large increases in proliferation of HSCs. It also is clear that HSCs and HPCs have to be protected from chronic exposure to TNFα that could permanently inhibit their proliferation potential. Direct exposure to TNFα does allow for differentiation of HSCs, though it may only be a few hundred cells versus the tens of thousands that would normally occur due to reduction of proliferation. This protection of these HSCs and early HPCs is probably a niche, which is an asymmetric compartment that creates a special regulatory microenvironment for cells housed inside. Finally, taken together these data highlight that chronic exposure of HSCs to high levels of TNFα as seen in certain hematopoietic disorders (ie. acute myeloid leukemias, MDS, aplastic anemia) could cause diminished proliferation of normal HSCs. Therefore, the extent of inhibition of proliferation depends on: the concentration of TNFα, the time exposed to TNFα, and the cell cycle state of the cell.

L. Receptor specific role of TNF-RI in hematopoietic immature precursors

TNFα has differing effects on hematopoiesis when TNF receptor 1 (TNF RI; p55; CD120a) and TNF receptor 2 (TNF RII; p75; CD120b) dependent responses are compared. Understanding of the TNF-RI receptor will require comparative subtraction from the action of TNFα. Since soluble TNFα activates both TNF-RI (with higher

affinity) and TNF-RII in a dose dependent manner, TNF-RI signals for only part of the total response. TNF-RI is undoubtly vital to understand because SIMPL is a specific member of the TNF-RI/NF-κB signal cascade. The research on TNF-RI and TNF-RI-/- mice has not been as thoroughly studied as TNFα, so the available relevant knowledge will be gathered from multiple publications.

1. Role of TNF-RI on Proliferation

Proliferation is the expansion of HSCs or HPCs without maturation. In a study by Zhang and co-workers [36], they explored which TNFα receptor affected proliferation and looked at the ability of more mature progenitors to proliferate. However, to date, no one has looked at the effects of long term culturing of HSCs in TNFRI-/- as has been done for TNFα-/-.

Zhang and co-workers examined 3 month old TNF-RI-/- mice and they found, in comparison to wild-type mice, a 1.6-fold increased proliferation of lin- cells and significant proliferative increases in HSC (lin-sca^{+}ckit^{+} 12.2% vs. 4.8%; respectively) and HPC (lin^{-}sca^{-}ckit^{+}; 31% to 23% respectively) populations [36]. Other authors claim only slight differences in TNF-RI-/- mouse hematopoiesis, but these apparent non-differences are due to incomplete exploration of only fully differentiated/mature hematopoietic subpopulations [47, 49].

The cell cycle inhibition at G1/G0 induced by TNFα is signaled for through TNF-RI [36]. Mouse HSCs and HPCs, derived from TNF-RI-/- plated in liquid suspension culture with either IL-3 and SCF (lin^{-}sca^{+}ckit^{+}) or G-CSF and SCF (lin^{-}sca^{-}ckit^{+}) and treated with mTNFα, failed to arrest in G1 like cells derived from wild type mice treated

with mTNFα. These determinations were made by following 5-BrdU incorporation assay and propidium iodide staining [36]. These data suggests that TNF-RI makes a valuable contribution to the inhibition of proliferation through cell cycle arrest of proliferating HSCs or HPCs.

2. Role of TNF-RI on Differentiation

a. TNF-RI specific influences on the First Scenario

The influence of TNFα on hematopoietic cell differentiation has been examined under the two different scenarios described previously (section K3). Remember in scenario one, proliferation of HSCs or HPCs occurs in the presence of TNFα for a given time, and then differentiation of the treated cells in assessed by placing the expanded cells in methylcellulose culture with cytokines in the absence of TNFα that supports rounds of expansion and maturation.

Dybedal and co-workers isolated $CD34^+$ $CD38^-$ human HSCs, expanded them in KFT3 (c-kit ligand, Flt3 ligand, Thrombopoietin, IL-3) and either wildtype hTNFα, a specific TNF-RI activator or a specific TNF-RII activator, for 5 days. Cells were then plated in a LTC-IC assays for 6 weeks and then into the proliferation differentiation colony assay. The TNF-RI specific activator (hTNFα containing mutations Arg32 to Trp and Ser86 to Thr; wild type binding activity [50]) and wildtype TNFα caused a severe inhibition of LTC-CFC colony proliferation. The specific TNF-RII activator, with hTNFα mutations Asp143 to Asn and Ala145 to Arg, and a binding activity about 5 to 10

fold less than wild type hTNFα, had a limited effect, very little inhibition, on LTC-CFC colony growth.

As described previously, in long-term cultures, bone marrow cells are harvested and co-cultured on a stromal layer with cytokines. In a series of experiments by Rogers and co-workers [51], mouse bone marrow cells were treated with hTNFα (0 U/mL to 200 U/mL) in a concentration dependent manner (activates only TNF-RI in mice) for the duration of their growth on the stromal cell layer (for up to five weeks). At 2, 3, 4 or 5 weeks, $4x10^4$ of the cells in suspension were replated in a progenitor colony differentiation/proliferation assay or were injected into the tail veins of mice to determine if they could repopulate the spleen (the colony forming unit-spleen, CFU-S). A CFU-S represents mouse HSC and very immature HPCs. In the proliferation/differentiation colony assay, growth of mature HPC derived colonies CFU-GEMM, CFU-GM and CFU-M were nearly completely inhibited when pre-treated with hTNFα in a dose dependent manner (up to 200 U/mL). At the highest dose, hTNFα (200 U/mL) partially inhibited HPP colony formation. The HPP (high proliferative potential) colonies represent immature precursor HSCs and immature HPCs which have a high capacity to expand/proliferate. In contrast, hTNFα enhanced the growth of CFU-S (HSCs and immature) [51]. Based on the hTNFα dependent expansion of CFU-S, TNF-RI cell cycle inhibition is reversible, TNF-RII causes the irreversible effects of long-term exposure to TNFα, in the expansion of immature precursors.

b. TNF-RI influences on the Second Scenario

In the second scenario, as described earlier, HSCs or HPCs are plated directly in methylcellulose culture with cytokines in presence of TNFα that supports rounds of proliferation and maturation.

In continuation of the above hTNFα experiment, mouse bone marrow cells added directly to a differentiation colony assay with maximum dose of hTNFα (200 U/mL) HPP colony growth was not inhibited. These data confirm that TNF-RI inhibition of proliferation still requires TNF-RII to be fully effective in an enviroment of multiple cell type interactions (*in vivo* or in a differentiation/proliferation colony assay). Again, the antithesis to activation of TNF-RI is seen in the analysis of TNF-RI-/- mice. Analysis of mouse HSCs ($lin^-sca^+c\text{-}kit^+$) derived from TNF RI-/- mice had increased formation of HPP colonies when grown in IL-3 and SCF as compared to control [36], and increase CFU-GM formation [49]. Plus, the inhibition of HPP ($lin^-sca^+c\text{-}kit^+$) and HPC ($lin^-sca^-c\text{-}kit^+$) colony formation by mTNFα is blocked [36].

3. Summary

TNF-RI is likely responsible for inhibition of proliferation caused by TNFα seen in immature precursors, but TNF-RI alone is probably not responsible irreversible inhibtion of proliferation due to long exposure to TNFα.

M. Exploration of TNFα through examination of diseases

A quote from Pikarsky and co-worker that appeared as a Letters to Nature discussing the TNFα/NF-κB pathway most appropriately summarizes the potential for

using modulators of TNFα for therapy. "It has been proposed that anticancer research might be more effective if aimed at eradicating the cause or the signaling context of abnormality rather than just treating the end result…disrupting the signaling context of the evolving tumor may be a more realistic objective [52]." This sentiment is shared by others in the field [53].

Acute myeloid leukemia (AML) is ideal for the study of the effects of dysregulation of TNFα in disease. Other diseases with TNFα dysregulation have a corresponding pattern of effects that can be predicted through understanding the TNFα/NF-κB pathway.

2. AML- Role of dysregulated TNFα/NF-κB system

Acute myeloid leukemia (AML) is a TNFα linked hematopoietic disease characterized by the inability of immature myeloid blasts cells to differentiate, leading to rapid loss of life within 1-3 years for those over 60 years old [54]. The early death is possibly due to older individuals not able to endure the negative effects of chemotherapy.

Understanding the role of TNFα in acute myeloid leukemia will allow for the identification of stage specific treatments and better predictions of the effects of the treatments. AML cells and patients own immune system produce high levels of TNFα that promotes hematopoietic blast cell growth while inhibiting normal hematopoiesis. TNF RI and TNF RII are also upregulated in AML cells of stages M4 and M5 [55, 56]. The TNF-RI activated transcription factor, NF-κB (p65/p50 deterodimer) is upregulated and constitutively active in AML, usually as a result of a mutation at the level of the IKKs (for review Figure 1) [57, 58]. In AML, the TNF RI/p65 signaling pathway

component IKKβ is constitutively active, due to constitutive phosphorylation which leads to higher than normal NF-κB activity [58]. NF-κB activation is sustained in spite of increased expression of the NF-κB controlled IκBα gene. This is most likely the result of continual phosphorylation of IκBα by IKKβ leading to its degradation. These results indicate that in AML conditions for preferential survival of transformed leukemic cells exist. Constitutive p65 activity in AML leads to increased expression of the inhibitors of apoptosis that include: XIAP, survivin (normally expressed only in primitive precursors and only in G2/M), cIAP1, cIAP2, cFLIP, Bcl-2, and Bcl-xL [59-61]. Furthermore the high levels of TNFα enhance p65 induced anti-apoptotic activity [58]. However, high dose TNFα inhibits HSC and early progenitor proliferation, and induces apoptosis among more differentiated hematopoietic cell types. Thus TNFα appears to have different effects upon normal versus AML-derived hematopoietic cells. Dysregulation of TNFα in AML patients may be the main contributing factor to the progression towards the disease, since, chronic TNFα exposure can cause clonogenic pressure selection. This type of effect is also seen in patients with rheumatoid arthritis who are also characterized by high levels of TNFα [62]. The high TNFα levels are directly linked to a 2-fold increase in the development of malignant lymphoma (non-hodgkins and hodgkins lymphoma) in patients with rheumatoid arthritis [63]. High TNFα also causes detriment in hairy cell leukemia, chronic lymphocytic leukemia, and acute lymphocytic leukemia [64-66]. Acute myeloid leukemic cells thrive in high TNFα levels which can inhibit normal HSCs [67].

Supernates from AML cultures prevents hematopoiesis in normal LTBMCs, and the inhibition can be partially blocked with anti-TNFα [67]. These data indicate that autocrine production of TNFα by AML cells can have profound effects upon AML cell

growth/differentiation capacity. TNFα up regulates c-kit expression (primitive marker) in AML cells, while decreasing c-kit expression in non-neoplastic normal hematopoietic cells [36, 68]. AML activation of NF-κB also allows for other ways in which the leukemic cells can evade chemotherapies. NF-κB controls the expression of the multiple drug resistance gene (mdr1) which encodes P-glycoprotein. The P-glycoprotein is a transporter that controls the removal of anti-cancer drugs from cells [59]. In multiple myeloma p65 levels are increased and have been shown to bind directly to and elicit nuclear relocalization of the reverse transcriptase component of telomerase, which by promoting survival would enhance transformation and clonal expansion [59].

3. Myelodysplastic Syndrome

Myelodysplastic Syndrome (MDS) is a collection of TNFα linked diseases in which hematopoietic cells are functionally and morphologically aberrant. The bone marrow cellularity in these patients is either normal or hypercellular with peripheral pancytopenia (global loss of all peripheral blood cell types). This condition is also known as ineffective hematopoiesis [1, 2, 53, 69, 70]. MDS may be the most prevalent clonal hematopoietic neoplastic disease in adults in the Western world and is thought to be linked to the aging process [53]. MDS is linked to impaired hematopoietic cell differentiation, and can progress from less severe Refractory Anemia (RA) to most severe AML [1, 53]. This is a disease of accumulating mutations over time, and elevated TNFα and sTNF-RI (soluble TNF-RI) levels are often present. Levels of TGF-β1, another inhibitor of hematopoietic differentiation are normal in MDS [2]. As explained previously, a mutation in the TNFα pathway which would support clonal development of

precursors, can create the breeding ground for mutations leading to the inevitable neoplastic transformation. Along with these changes in severity seen in Table 1 changes in TNF RI and TNF RII levels hint at the role of TNFα signaling in neoplastic transformation. In refractory anemia, early stage of MDS, TNF RI expression is significantly increased. Refractory anemia is a difficult to manage anemia that currently is only successfully treated through blood transfusions. As the disease transforms into RA with excess blasts (RAEB), the level of TNF RII expression increases [1]. The TNF RII increase is linked to increased apoptosis and fas signaling [71]. Thus, increased pressure on differentiated cells to survive while precursor cells still have more than one way to survive. Multiple survival mechanisms (see AML) of precursor cells is a reason myeloid leukemias are highly drug resistant [53, 59-61]. TNFα levels are highest in RA along with high amounts of apoptosis Table 1 [2, 69]. Fas L, an inducer of apoptosis is increased in RA with excess blasts (RAEB) and seems to mark the transition into severe disease which is evidenced by a rapid expansion of blasts [2]. To show that TNFα was the cause of the hematopoietic stress in MDS patients' bone marrow, administration of anti-TNFα to MDS cultured cells promoted differentiation of HSCs and early precursors especially of the myeloid committed cell subpopulation (CFU-Meg, CFU-GM) [2, 70]. However, the total $CD34^+$ cell number did not change. These data are supportive of the chronic inhibition of proliferation caused by TNFα exposure on $CD34^+$ cell populations as mentioned earlier. In MDS patients cA2 anti-TNFα antibody treatment also decreased activated $CD4^+$ and $CD8^+$ T-lymphocyte populations which are also thought to contribute to MDS and TNFα release [70]. These data again highlight the pleotropic effects of TNFα.

	mRNA levels		Protein pg/mL	
MDS Categories	TNF-RI	TNF-RII	TNFα levels	FasL
Refractory Anemia (RA)	Increased ↑↑	upregulated	14.7 ↑↑	4.5
RA with Excess of Blasts (RAEB)	Same as control	Increased ↑	9.4 ↑	40.5
RAEB in transformation (RAEB-T)	Same as control	Increased ↑	10.3 ↑	17
Acute myeloid leukemia (AML)	Same as control	Increased ↑↑		

Table 1. Increasing levels of MDS severity and relation to TNF-RI and TNF-RII levels

This is a relative adaptation of two papers. Highlighting the different levels of mRNA levels of TNF-RI and TNF-RII and protein levels of TNFα and Fas Ligand relative to the different progressively severe stages of MDS. The arrows are relative indicators of increase.

Adapted from [1, 2]

For pleotropic mechanisms balance is the key to normal homeostasis. Take hypotension as an example. Hypotension (too little blood pressure) is due to a number of causes and can kill just as easily as hypertension (too much blood pressure). Sometimes the end stages of either disease can mimic the other. For example, hypertension can lead to heart failure and the heart failure leads to decreased cardiac output and thus hypotension. Hypotension in one part of the body can cause the heart to increase cardiac output (hypertension) and constrict blood vessels to increase blood pressure hypertension). Relative to TNFα, too much TNFα can block HSC proliferation and inhibit and/or induce apoptosis of terminally differentiating mature cells. Under normal conditions, this normal self-regulating biological response inhibits any out of control HSC proliferation and allows for controlled terminal cell differentiation for immune reactions. Too little TNFα will allow an increase in HSC expansion, but inhibits their function and prevents normal terminal cell activation. Prevention of normal terminal differentiation will cause a build up of upstream precursors promoting clonogenic expansion. Thus with another insult (such as constitutive activation of IKKβ allowing for differentiation, inhibiting apoptosis of blasts) preferential blast formation could occur [see above]. However, a low TNFα scenario generally will not occur as the low TNFα would lead to a diminished ability to effectively fight infection. But if given enough time like in MDS these affects would likely occur (review earlier sections).

N. Hypothesis

Our hypothesis is that decreasing SIMPL activity may be a better alternative than anti-TNFα therapies. Loss of SIMPL should bypass the need for inhibiting NF-κB

activators like IKKβ, as activation of these two arms of the TNF-RI pathway appear to occur independently [14, 15]. Inhibition IKKβ is not advantageous because a large number of other cytokines link to IKKβ to activate NF-κB. As a start toward determining if inhibition of SIMPL is a viable option for treatment of hematopoietic disorders I tested the hypothesis that loss of SIMPL led to decreased myelopoiesis in a TNFα dependent manner. This was best characterized by examining CFU-GM colony formation. Further, loss of SIMPL, like over activation of TNFα, does not induce apoptosis, but instead inhibits differentiation of myelopoietic precursors. Loss of SIMPL allows for normal colony growth when not treated with TNFα. In theory, this allows SIMPL to mediate hematapoietic cells with a dyregulated TNFα signaling, without harming hematopoietic cells with a normal response to TNFα, as other therapies would do. In addition, hematopoietic cells would still have basal NF-κB activity due to SIMPL being in separate pathway that is not necessary for NF-κB nuclear localization nor DNA binding.

II. Methods

A. Plasmid constructs

RNAi-Ready pSIREN-RetroQ-ZsGreen shRNA expression vector purchased from (BD Bioscience, San Diego, CA). Flag-tagged SIMPL generates the full length SIMPL (259 amino acids) expressed in pFLAG-CMV-2 expression vector (Sigma Aldrich, St. Louis, MO).

B. Antibodies

SIMPL antibody was created by immunizing rabbits with full length recombinant SIMPL protein and kindly affinity purified by Maureen A. Harrington (Indiana University). IRAK-1 antibody and Anti-FLAG® M2 monoclonal antibody (F3165) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-mouse IgG F(ab')$_2$ fragment specific-HRP was from Upstate Biotechnology (Lake Placid, NY; 115-36-006). Goat anti-mouse IgG F(ab')$_2$ fragment specific-HRP was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The following fluorochrome-labeled antibodies used for flow cytometry were from BD Bioscience Pharmingen: (BD Biosciences, San Diego, CA), R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD117 (c-kit) monoclonal antibody (clone: 2B8, isotype: IgG$_{2b}$) (553355), allophycocyanin (APC)-conjugated rat anti-mouse CD117 (c-kit) monoclonal antibody (clone: 2B8, isotype: IgG$_{2b}$) (553356), APC mouse lineage antibody cocktail, with isotype control CD3e, CD11b, CD45R/B220, erythroid cells, and Ly-6G and Ly-6C (respective clones: 145-2C11, M1/70, RA3-6B2, TER-119, RB6-8C5; respective isotype: Arm. Hamster

IgG1, Rat IgG_{2b}, Rat IgG_{2a}, Rat IgG_{2b}, Rat IgG_{2b}) (558074), R-Phycoerythrin (R-PE)-conjugated rat anti-mouse Ly-6G and Ly-6C (Gr-1) monoclonal antibody (clone: RB6-8C5, isotype: Rat IgG_{2b}) (553128), PE-Cy7-conjugated rat anti-mouse CD11b (integrin α_m chain, Mac-1 α chain) monoclonal antibody (clone: M1/70, isotype: Rat (DA) IgG_{2b}) (552850), PE-Cy7 rat anti-mouse Ly-6A/E (Sca-1) (clone: D7, isotype: Rat (LEW) IgG_{2b}) (558162), R-Phycoerythrin (R-PE)-conjugated rat IgG_{2b} monoclonal immunoglobulin isotype control (553989), PE-Cy7-conjugated rat IgG_{2a} monoclonal immunoglobulin isotype control (552784), PE-Cy7-conjugated rat IgG_{2b} monoclonal immunoglobulin isotype control (552849), purified rat anti-mouse CD16/CD32 (FCγIII/II receptor) monoclonal antibody (Mouse BD Fc Block™).

C. Cytokines, Growth Factors, Chemokines

The following cytokines were from Sigma-Aldrich, Inc. (Sigma-Aldrich, St. Louis, MO) and reconstituted in 0.22 μM filtered 0.5% BSA in PBS: recombinant human Tumor Necrosis Factor-α (cat. #T6674), recombinant mouse Tumor Necrosis Factor-α (cat. #T7539). The following growth factors and cytokines were from PeproTech Inc. (PeproTech, Rocky Hill, NJ): recombinant murine granulocyte macrophage-colony stimulating factor (GM-CSF) (cat. #315-03), recombinant murine stem cell factor (SCF) (cat. #250-03), recombinant murine granulocyte- colony stimulating factor (G-CSF) (cat. #250-05), recombinant murine IL-3. recombinant Human TGF-beta1 (mammalian derived) (TGF-β1) (cat. #100-21), recombinant Murine IFNγamma (IFNγ) (cat. #315-05).

D. Cell culture and transfection

Mouse embryo fibroblast (C3H10T½; ATCC® number CCL-226™) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro®) containing 10% fetal bovine serum (FBS) supplemented with 1% L-glutamine and 1% penicillin/streptomycin. Human embryonic kidney epithelial cells (HEK 293; ATCC® number CRL-1573™) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro®; Mediatech, Inc.; Herndon, VA) containing 10% FBS supplemented with 1% L-glutamine and 1% penicillin/streptomycin. Plasmids were transfected with Fugene® 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN) using manufacturer's protocol. The BAF3 pro-B cell line was donated by Hal Broxmeyer (Indiana University). Phoenix GP MMULV-based retroviral packaging cell line (ATCC® number 3514) was kindly donated by Rueben Kapur (Indiana University) and grown in media supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin on 100 mm tissue culture dishes coated in 0.1% gelatin (dissolved in water; autoclaved). Phoenix GP cells were frozen (LN_2) in large batches in 10% DMSO in growth media. A thawed tube of cells was used only once except to expand and then they were re-frozen in LN_2. Phoenix GP cells were split 1:5 and were not allowed to grow past 80% confluence in order to maintain viral transduction capability (avoid long term cell passage). All cells were maintained at 37ºC in an atmosphere containing 5% carbon dioxide and normal oxygen unless otherwise stated. The Transfection protocol for Phoenix GP cells is described later.

E. Mice

8 week old C57BL-6 female mice ($CD45.2^+$) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Eight week old B6.SJL-PtrcaPep3b/BoyJ (B6.BoyJ) ($CD45.1^+$) were purchased from The Jackson Laboratory (Bar Harbor, MA).

F. Bone marrow cells and LDMNC separation

C57BL-6 mice 8-12 week old were euthanized by carbon dioxide inhalation followed by cervical dislocation. The tibia, femur and flat bone of the pelvis were harvested and placed in Iscove's Modified Delbeco media (IMDM; Invitrogen; Carlsbad, CA) adjusted to contain 1% penicillin/streptomycin and 1%glutamine (both included in every media except serum free) and 2% fetal bovine serum. Bones were separated, cleaned of flesh and the bone ends were cut with surgical scissors. The bone marrow was flushed out with IMDM containing 2% FBS using a 1 mL syringe with a 16g needle. The bone marrow was resuspended by suctioning into and out 5 mL syringe with a 23g needle.

Low density mononuclear cells (LDMNCs) the bone marrow population containing immature precursors: differentiated progenitors, multipotential progenitors and hematopoietic stem cells were separated out of the whole bone marrow using Histopaque 1119 (Sigma-Aldrich; contains polysucrose and sodium diatrizoate; 2.5 mL). Histopaque 1119 was added slowly to the bottom of the 5 mL tube containing the whole bone marrow cells. Histopaque creates a density gradient separating low density mononuclear cells in the interphase (buffy coat) while most of the granulocytes and red blood cells are collected in the tube bottom. Gradients are created by centrifugation (700 x g, 30 min, no brake). The interphase was carefully removed, collected and the cells

were resuspended in 10 mL of IMDM supplemented with 2% FBS and centrifuged (~500 x g). The media was removed and pellets were resuspended in ~5 mL IMDM supplemented with 20% FBS. Resuspended LDMNCs were counted via Coulter Counter (Beckman-Coulter) with RBCs lysed (Zap-OGLOBIN II lytic reagent; Beckman-Coulter) or counted by eye with a hemocytometer using a non-inverted phase contrast microscope. Only nucleated cell numbers were recorded. The LDMNCs cells were plated on 100 mm petri dishes at a density of $4x10^6$ cells/mL media that was supplemented with 100 ng/mL SCF (stem cell factor), 200 units/mL IL-6 to support cell survival and proliferation. Cultures were incubated under standard conditions (37ºC, 5% CO_2 and normal oxygen).

G. Retroviral packaging of plasmid and collection

The retroviral plasmid (RNAi-Ready pSIREN-RetroQ-ZsGreen shRNA expression vector) contains a ψ (psi) packaging gene required to package the plasmid into a retrovirus. The retrovirus was created in the HEK 293 based Phoenix GP MMULV-based retroviral packaging cell line by transfecting in the pSIREN plasmid along with a plasmid containing the envelop gene and a plasmid containing the gag/pol genes (kindly provided by Ruben Kapur, Indiana University) using the Calcium Phosphate Transfection Kit (Invitrogen, 44-0052) following a modified protocol. Briefly, before starting, a collection media was made that consists of 500 ml high glucose media (4.5 g) DMEM (Invitrogen), 55 mL FBS, and 2% penicillin/streptomycin which was adjusted to pH7.9 with 10N NaOH. Next it was adjusted to contain 1% Hepes buffered solution (1M) (Invitrogen, 15630-080) and the pH was readjusted to pH7.9 with 10N NaOH. The media was sterile filtered (0.2 μm sterile filter) and stored at 4ºC. One tube of frozen

Phoenix GP cells (see culturing technique above) was thawed and plated in late afternoon. The next morning the media was replaced to remove DMSO; by 24hr, the cells were 70-80% confluent and were split 1:5 into 5 x 100 mm tissue culture dishes. 24 hours later, for each 70-80% confluent Phoenix GP dish a transfection mix was made. The transfection mix consisted of 10 µg Gag/pol plasmid DNA, 3 µg Env plasmid DNA, 15 µg pSIREN plasmid DNA plus 36 µL 2M $CaCl_2$ adjusted to a total volume of 300 µL with sterile water. Mixtures were made in 1.5 ml microcentrigue tubes. Next, to each tube, 300 µl of a 2 X Hepes buffered saline solution was added and air was bubbled in for 30 seconds with a pipet (blow air out and move pipet frequently). Mixtures were then incubated 30 min at room temperature. The 600 µl transfection mix was added to 9 ml of collection media (in a 15 ml conical tube) and 1 µL/mL chloroquine (50 mM stock; Sigma-Aldrich, St. Louis, MO; C-6628) Chloroquine stabilized the DNA for transfer across membrane. Media covering the Phoenix GP cell monolayers was replaced with 10 ml collection media containing the DNA mixtures. Media was added gently and incubated cultures were returned to a (37ºC incubator). The next morning, media on the Phoenix GP cell monolayers was replaced with 8 mL fresh collection media and cultures were then incubated for 4-8 hours at 37ºC. The media was then replaced with 9 mL fresh collection media and incubated at 32ºC at 5% CO_2/95% air. After 24 hours, the collection media (containing the retrovirus) was collected centrifuged (1000 rpm, 5 min, RT) to pellet any cells and all but the last ~1 mL of media was transferred to a new tube. The retrovirus containing media was aliquoted and frozen (-80ºC). The media was replaced with 9 mL fresh collection media and the process was repeated twice more with media collected at 48 and 72 hour time points.

H. Retroviral transductions

All adherent cell lines were grown in normal tissue culture conditions and were transduced with the MMULV-based retrovirus at 50-80% confluency in 8 μg/mL polybrene (stored at 4ºC for one month) in respective growth media. Dishes were centrifuged (2000 rpm, 30 min., 32ºC) and then incubated at (37ºC, 30 min). Twenty-four hours later, the media was replaced with fresh growth media and incubated at (37 ºC). For non-adherent cell lines, after the 30 min incubation, the cells were collected by centrifugation, resuspended in fresh growth media and incubated at (37ºC).

LDMNCs were grown for 2-3 days in the pre-stimulation conditions stated above (SCF plus IL-6). The day before or the day of transduction with retrovirus, 100 mm petri dishes were coated with Retronectin (Takara; 64 μL 5 ml PBS). To generate control cells for use in establishing parameters for FACsorting, 35 mm petri dishes were coated with Retronectin (Takara, Japan; 10 μL in 1 mL PBS). Dishes were incubated for 2 hrs, at room temperature or overnight at 4ºC; retronectin was removed and PBS supplemented with 2% BSA (~3-5 mL; Sigma) was added for 30 min to bind excess fibronectin fragments (based upon manufacturers recommendation). The 100mm petri dishes were pre-treated with 4 to 8 mL of virus containing shRNA and centrifuged (2000 rpm, 30 min, 32ºC). Prestimulated cells were scraped from dishes and pelleted (1500 rpm, 5 min, room temperature). From virally pre-treated dishes, virus was removed and 7 mL of fresh virus containing media supplemented with SCF (100ng/mL), IL-6 (200units), and Polybrene (4μg/mL; increases viral transduction by neutralizing charges in lysosomes of host cells). The pelleted LDMNCs were re-suspended (~1.05 mL/100 mm pre-

stimulation petri dish), and 1 mL of cell containing solution was plated into each virus pre-treated dish (at least one SIMPL and one scramble shRNA) with an additional 0.1 mL added to 35 mm retronectin petri dish (Non-GFP control to setup FAC parameters). All dishes were then centrifuged (2000 rpm, 30 min, 32ºC) and placed at (30 min, 37ºC, 5% CO_2). Then virus was removed, 8 mL of fresh media (IMDM with 20% FBS, mSCF (100ng/mL), hIL-6(25 ng/mL) was added and cultures were returned to a 37ºC, 5% CO_2 incubator. Depending on the amount of virus availability, a second virus transduction was performed 24 hrs later by removing media and repeating steps above starting at the step at which 7 ml of fresh virus containing media is added to the cultures.

I. Fluorescence activated cell sorter and analyzers

Susan Rice and Lizz Scaletta (Indiana University Cancer Center Flow Cytometry Resource Facility) supervised the analysis and performed the fluorescence activated cell sorting. Cells were sorted on one of (Becton Dickinson (BD) three fluorescence cytometry cell sorters: FACStar Plus, FACSVantage, or the FACSAria. Analysis was on one of three fluorescence cytometry cell analyzers, FACSCalibur (2 total) or the FACScan. For Fluorescence Activated cell sorting/analysis, cells are labeled with an antibody conjugated to a fluorochrome that emits light (fluorescence) at a specific wavelength when struck by the light from a laser of a specific wavelength. Thus, the fluorochromes can be discriminated based on their excitation or emission spectra (either the wavelength of light required to cause fluorescence or the wavelength of light emitted from the fluorochrome). For all FAC sorting/analysis experiments, BD Bioscience

antibodies and fluorochromes were used. For all experiments, the BD products come with protocols that were followed or modified as indicated.

J. Staining for c-kit$^+$ cells containing shRNA

LDMNCs transduced with shRNA containing retroviruses and control (non-transduced) were scraped from their dishes, transferred to fresh 15 mL tubes, pelleted by centrifugation (1500 rpm, 5 min, room temp), and supernates were discarded. Pellets were resuspended in PBS containing 0.5% BSA (~1 mL), transferred to a 1.5 mL tube, centrifuged (1500 rpm, 5 min, room temp), and supernates were discarded. The cell pellets were resuspended in PBS containing 0.5% BSA (150 µL). For non-transduced controls, 20 µL of cells were added to marked 1.5 ml tubes (isotype control, APC lineage cocktail isotype control, APC conjugated c-kit) containing PBS with 0.5% BSA (130 µL), 3 total tubes (one unstained control). For the GFP only controls, ~4 µL of unstained scramble shRNA LDMNCs were transferred to a fresh tube containing PBS with 0.5% BSA (~ 600 µL). All tubes were treated with Mouse FC Block (5 µL for control tubes, 10 µL for shRNA) (5 min, 4ºC). ShRNA LDMNCs were incubated with the APC-c-kit antibody (10 µL, 4ºC, 25 min., in dark). The control LDMNCs tubes were incubated in APC lineage isotype control (contains APC-c-kit antibody isotype control) and APC-c-kit antibody into respective tubes (5 µL each, 4ºC, 25 min, in dark). PBS containing 0.5% BSA (600 µL) was added to each tube (wash), and then cells were pelleted (1500 rpm, 5 min, room temp, in dark). All samples were resuspended in PBS containing 0.5% BSA (control sample 400 µL, shRNA 800 µL). PBS containing 0.5% BSA was added to the

shRNA samples and they were filtered to remove cell clumps (final volume ~1.5 to 2 mL).

K. Apoptosis Assay

Annexin V conjugated to APC (BD Biosciences, San Diego, CA; cat. #550474) along with 7-amino-actinomycin (7-AAD) (BD Biosciences, San Diego, CA; cat. #559925), and Annexin V binding buffer (BD Biosciences, San Diego, CA; cat. #556454) was used for the apoptosis assay using the manufacturers protocol and a fluorescence activated cell analyzer to quantitate the staining. APC was used because the pSIREN plasmid is linked to GFP. During apoptosis, plasma membrane localized phosphatidylserine located on the intracellular leaflet translocates to extracellular leaflet of the plasma membrane. Annexin V binds to the extracellular phosphatidylserine and is identified by the APC fluorochrome via FAC analysis. 7-AAD binds to DNA and only enters cells that have disrupted membranes. In these experiments, briefly, up to 100,000 experimental cells were washed with PBS containing 0.5% BSA (filtered) then resuspended in 100 µL of 1 x binding buffer in 1.5 mL tubes that contained 5 µL each of APC Annexin V and 7-AAD. One color control cells (non-pSIREN GFP transfected/transduced) were setup following the same procedure using either Annexin V or 7-AAD. Cells were mixed by finger tapping, incubated (4ºC, 15 min), and then 400 µL of 1 x binding buffer was added just before analysis. All solutions were ice cold.

L. Growth inhibition assay

A defined number of c-kit$^+$ cells containing shRNA (scramble or SIMPL) were mixed with a defined number of c-kit$^+$ cells (non-transduced) from the same sorting pool, (SIMPL shRNA transduced LDMNCs sorted for c-kit$^+$ shRNA$^+$ and c-kit$^+$ shRNA$^-$ populations), along with c-kit$^+$ (control) cells, and plated in media (varied based on experiment). Then the cells (based on the experiment) were collected by either scraping or pipetting supernant (at experimentally determined time points) into a tube, collected by centrifugation (1500 rpm, 5 min, room temp), and washed with ice cold PBS containing 0.5% BSA. All cells were resuspended in ice cold PBS containing 0.5% BSA and subjected fluorescence activated cell analysis. These experiments were done to determine growth ability of shRNA cells as compared to non-GFP c-kit$^+$ cells and to compare SIMPL to scramble shRNA containing c-kit$^+$ cell growth.

M. Progenitor (differentiation and proliferation) colony assay for c-kit$^+$, whole bone marrow and spleen

The c-kit$^+$ cells (1000 to 2000 cells/mL), whole bone marrow cells (50,000 nucleated cells/mL), or spleen cells (~200,000 LDMNCs to 200,000 c-kit$^+$ cells) containing shRNA (scrambled or SIMPL) were placed in a methylcellulose mix (4 mL final volume in 15 mL tube) containing an experimentally determined amount of cytokines with common starting products (Table 2). The methylcellulose mix (95 mL) was pre-made with all cytokines (for a given experiment) except for the variable cytokines and frozen in aliquots (3.8 mL; extra 200 μL for cells, variable cytokine(s), and serum free media IMDM). Each condition was plated in triplicate (1 mL final volume/35

	Individual Triplicate 4mL
Fetal Bovine serum (30%)	1.2mL
Penicillin/Streptomycin 2%	80µL
B-mercaptoethanol 11mL/10mL filtered 0.22µM	20µL
IMDM(serum free)	460µL
Methylcelluolose	1.6mL
Glutamine 1%	40µL

Table 2. Proliferation/Differentiation Assay Base Contents

mm petri dish), and six 35 mm dishes were placed in a 150 mm petri dish with autoclaved water (~3 mL in a lidless 35 mm petri dish). Each 150 mm petri dish was incubated ($37^{o}C$, 5% CO_2) for 7 days unless noted in experiment. The dishes were counted for (depending on experiment) colony number, size and/or type.

N. Whole bone marrow cellularity assay using transplanted animals.

Bone marrow cells (100,000 cell) from transplanted (3 x 100,000 cells for each group) and control mice (100,000 cells/antibody) were added to PBS containing 0.5% BSA (~1 mL, wash) in a 1.5 ml tube, pelleted by centrifugation (1500 rpm, 5 min, room temperature) and the supernates were discarded. The pellets were resuspended in PBS containing 0.5% BSA (150 µL). One tube of transplanted cells (100,000 cells) was left unlabeled to assess GFP (ZsGreen) percentage by fluorescence activated cell analysis. A modified BD Bioscience protocol provided with antibodies was used to characterize the cells (modification: no BD FC Block used as there was not enough available for all the samples). PE conjugated rat anti-mouse CD117 (c-kit), APC mouse lineage antibody cocktail, and PE-Cy7 rat anti-mouse Sca-1 or PE-Cy7-conjugated rat anti-mouse CD11b (Mac-1 α chain) and PE conjugated rat anti-mouse Gr-1 antibodies (5 µL each antibody) were added to tubes containing the cells from the transplanted animals (2 x 100,000 cell tubes). Whole bone marrow from control mice was used for one color antibody labeling (5 µL antibody/tube) to setup the FAC analyzer.

O. Real-time PCR

Real-time PCR was performed by Sonal Sanghani (Indiana University).

P. Cell Lysates

For whole lysates, adherent cell monolayers in growth media were scraped up with a rubber policeman, pipetted into a tube, and centrifuged (<1000 RPM, 5 min, R.T.). The cell pellets were washed with PBS. Pellets were then resuspended in immunoprecipitation (IP) lysis buffer (10 mM HEPES, pH 7.4, 5mM EDTA, 150 mM NaCl, 1% Triton X-100) with protease inhibitor (Complete, mini protease inhibitor cocktail tablets, Roche, Indianapolis, IN) by pipetting up and down until a precipitate formed. Then, lysate was immediately vortexed (30 sec) and put on ice (20 min) with vortexing (10 sec/5 min on ice), and then centrifuged (20 min, 14,000 rpm, 4^{o}C) to remove cell debris. Supernant was used immediately for IP or direct use in a western used immediately or stored 20^{o}C.

Q. Protein Assay

Protein assays were performed using the Bio-Rad protein assay (cat. # 500-006) (Bio-Rad Laboratories Inc, Hercules, CA) based upon the manufacturer's recommendations. A standard curve was created using bovine serum albumin (BSA; 1 mg/mL) in 0.25 M Tris (pH 8.0) added (2, 6, 10, 14 or 17 µL) to 1.5 mL microcentrifuge tubes containing 0.25 M Tris (pH 8.0; 800 µL). The cell lysates (2 µL) were added to 800 µL 0.25 M Tris. All samples were mixed by vortexing and collected by centrifugation (14,000 rpm, up to top speed and down). To all samples, 200 µL of the Bio-Rad protein assay solution was added and samples were quickly centrifuged again. Samples were transferred to cuvettes just before reading the absorbance of protein in a spectrophotometer at the 595 wavelength. The standard curve was setup by plotting

protein concentration versus absorbance in an Excel sheet. A best fit line was created and the equation of the line (y = mx + b; x = absorbance, y = protein concentration, slope of the line, b = point line meets x axis) was used to determine sample protein concentration from corresponding sample absorbance.

R. Immunopreciptation

Equal amounts of protein (as determined by protein assay) were added to a total volume of 900 µL of IP lysis buffer (10 mM HEPES, pH 7.4, 5mM EDTA, 150 mM NaCl, 1% Triton X-100). Lysates were incubated (1h, 4ºC, on a nutator) in 100 µL of a 10 % protein-A sepharose suspension (Sigma-Aldrich, St. Louis, MO) and 2 µg of rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) to reduce non-specific binding. Samples were pelleted (14,000 RPM, 10 sec, 4ºC) and the supernate was transferred to fresh tube. Lysates were incubated (4ºC, overnight, on a nutator) with protein-A sepharose and 2 µL (2 µg) rabbit anti-SIMPL antibody. The next morning, the SIMPL bound protein-A sepharose complex was collected by centrifugation (14,000 rpm, 10 sec, 4ºC). Supernates were removed by pipetting and washed four times with 500 µL IP wash buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100). Following removal of final wash buffer, 40 µL of Laemmli buffer (Bio-Rad, Hercules CA) containing β-mercaptoethanol was added to the protein-A sepharose and samples were either analyzed immediately or stored at -80ºC. All steps were performed at 4ºC or on ice.

S. Western Blot analysis

Lysates and immunocomplexes resuspended in Laemmli buffer containing β-mercaptoethanol were boiled (10 min) and loaded on a 12.5% SDS-polyacrylamide gel containing a 5% acrylamide stacker. After electrophoretic separation, proteins were transferred (20V, 4ºC, overnight or at 30V, room temperature, 2 hours) in CAPS (10 mM 3-cyclohexylamino-1-propanesulphic acid, pH 11) buffer onto an Immobilon-P membrane (prepared by incubating in methanol for 15 seconds, 2 min in water, and then at least 5 min in CAPS buffer). Following transfer the membrane was blocked in 5% (w/v) non-fat dry milk [dissolved in PBS containing tween-20 (PBS-T); 30 min] (Sigma-Aldrich, St. Louis, MO). Next, the membranes were incubated with primary antibody PBS-T containing 5% milk (1 hr 30 min) followed by 3 x 10 minute washes in PBS-T. The membranes were then incubated with horeseradish peroxidase (HRP) conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in 5% milk (1 hr) followed by 3 x 10 minute washes in PBS-T. To visualize proteins detected by the antibodies ECL™ or ECL Plus™ Western Blotting Detection Reagent (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was added to the membrane followed by exposure to film.

T. Lineage cell depletion by magnetic cell sorting

The day of fluorescence activated cell sorting, the LDMNCs transduced with retrovirus containing the shRNA were run through magnetic cell sorting using a mouse lineage cell depletion kit (Miltenyi Biotec, Auburn, CA, 130-090-858). The procedure was done according to the manufacturers protocols and both the LS and LD columns

were used for sorting. LS columns produced ten times more cells than the LD columns. LS columns were impratical due to the high number of cells that contaminated the population that needed to be fluorochrome-conjugated lineage depleted. After lineage depletion, the cells were counted, incubated in Mouse BD Fc Block™ (5 min, 4ºC, 1 µL/million cells) followed by the APC-lineage cocktail, PE-Cy7 Sca-1 and PE-c-kit antibody according to BD Protocol with proper isotype controls (BD Bioscience, San Diego, CA). Left over lineage positive cells containing the scrambled shRNA were used for GFP positive cells. Single stain controls were performed same as above. Cells were taken to the FAC sorting facility to sort for $lin^-sca^+c\text{-}kit^+$ and $lin^-sca^-c\text{-}kit^+$ populations. Thus, it was verified each time that the LDMNCs were lineage depleted.

U. Transplant experiment

After two weeks rest, 8 to 10 week old C57BL-6 female mice (22 mice over one month) were irradiated, and kindly intravenously (lateral tail vein) transplanted by Yan (Indiana University) with $c\text{-}kit^+$ (250,000 cells/300 µL) cells containing the SIMPL (9 mice) or the scrambled shRNA (13 mice). 4 months post-transplant, all the mice were alive, and ready to be analyzed. Whole bone marrow from 2 x tibias, 1 x femur, and 2 x pelvic flat bones were harvested (modified method F: whole bone marrow flushed in 20% FBS IMDM media). Whole blood cells from a portion of spleen (cut and weighed) were harvested by homogenizing the spleen in a nylon cell strainer (70µm; BD Bioscience, Bedford, MA) and flushing with ~5 mL IMDM media containing 20% FBS. Spleen cells then treated like bone marrow cell (method E). Lymph nodes (intestinal and superficial), liver chunk, spleen chunk, femur, heart, lung chunk and kidney were harvested and

placed in 10 % Buffered Formaldehyde. (Fisherbrand, Pittsburgh, PA) for histological examination.

V. Competitive repopulation

c-kit$^+$ cells (250,000 c-kit$^+$cells) containing either the scrambled or the SIMPL shRNA from 2 x transplanted 4 month old C57BL-6 (CD45.2) female mice were mixed with congenic whole bone marrow (500,000 nucleated) derived from B6.SJL-PtrcaPep3b/BoyJ (B6.BoyJ) (CD45.1$^+$) mice. Cell mixtures were transplanted into 6 x C57BL-6 (8 week old) female mice (followed method U.).

II. Results

A. Develop a suitable model system to characterize the physiological role of SIMPL

1. Zebrafish (Preliminary) SIMPL knockdown results

SIMPL morphilino (diminshes SIMPL in zebrafish) causes rapid expansion then inhibition of red blood cells within the first 48 hours of zebrafish development. This preliminary work (data not shown) was done by Erin Breese. This result caused us to look further into SIMPL's effect on normal hematopoiesis.

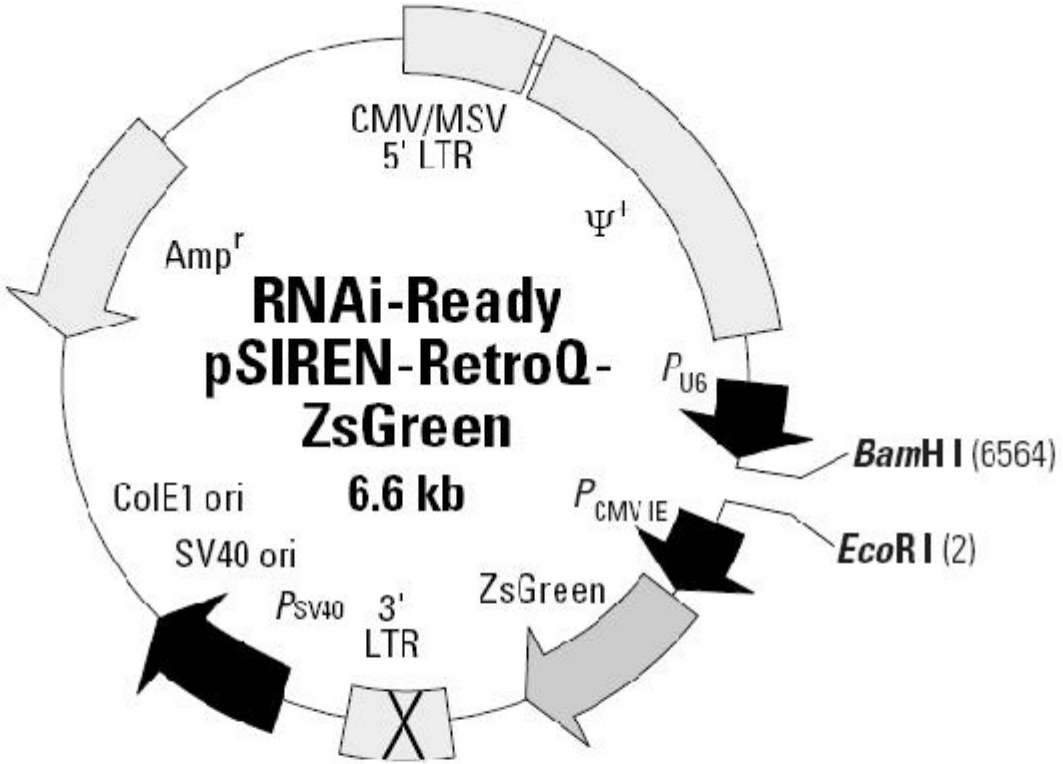
2. SIMPL knockdown system created to model effects of SIMPL on hematopoiesis

a. *ShRNA generation and construction*

ShRNA (short hairpin RNA) to SIMPL was designed using the Clontech siRNA Sequence Selector program. The program generates a list of siRNA of 19 nucleotide length using pre-set criteria and gives their position within the open reading frame and GC content. SIMPL siRNA were generated against the mouse SIMPL sequence deposited in GenBank (AF093135). The identified siRNAs were subject to Blast search to ensure specificity. Three siRNAs dispersed through the SIMPL sequence were selected from the list of 31. The siRNA to the 5'most part of the SIMPL mRNA is thought to prevent translation the most by inhibiting its start, but we decided to try a variety of locations. The first sequence (sequence 1) was near the 5' at position 48 (CTGGTTCCCTGGGCTGA, GC content 68.42%); the next (sequence 4) at position 326 (TGTAACTGTGACAAAGAAC, GC content 36.84%), and the third (sequence 18) at

position 557 (AGCTCAGGAAGTCTGTGAC, GC content 52.63%). An additional SIMPL siRNA was designed using accession number (NM_022986) at position 664 (GCCAAACGGATGATCATCA, GC content 47%) because only SIMPL sequence 18 successfully knocked down SIMPL. The shRNAs were designed using siRNA Hairpin Oligonucleotide Sequence Designer by Clontech which converts the siRNA sequence into shRNA by making a sense strand (listed above) followed by a hairpin loop sequence (TTCAAGAGA) followed by the antisense sequence. A MluI restriction site was added in order to test for proper ligation into a vector. A Bam HI (5' of top strand) and Eco RI (3' of top strand) overhangs were added to each end. A complementary single strand was designed for each shRNA. The oligonucleotides were commercially generated and subject to PAGE purification (Sigma Genosys). Sequence similarity of the three murine SIMPL siRNA sequences was compared with the human SIMPL (accession number: XM_059729) sequence. Sequence 1 and 18 (SIMPL 18 shRNA) were 100% identical and sequence 4 was ~88.9% identical. The additional siRNA at position 664 (SIMPL 664 shRNA) was made after SIMPL 18 was confirmed.

To generate subclonable fragments, a top and bottom strand of each oligo was annealed and the shRNA was ligated into a RNAi-Ready pSIREN-RetroQ-ZsGreen (Figure 6) and into RNAi-Ready pSIREN-RetroQ (contains a puromycin marker) vector. The plasmid was used directly for transfection, and alternatively was used to generate a retrovirus for retroviral delivery, and visualized by FACS due to the co-expressed ZsGreen (GFP-like) fluorescent marker. A scrambled ShRNA (negative control) plasmid was also generated and tested in parallel as explained below. The ligated shRNA plasmids were transformed into DH5α bacteria and transformants were selected for



6560 10 20 30 40

5'-TGGAAAGGACGAGGATCC[...shRNA oligo cloning site...]GAATTCTAGTTATTAATAGTAATCAATTACGGGGTCATTA-3'

*Bam*H I *Eco*R I

3'-ACCTTTCCTGCTCCTAGG[...shRNA oligo cloning site...]CTTAAGATCAATAATTATCATTAGTTAATGCCCCAGTAAT-5'

Figure 6. GFP shRNA retroviral plasmid vector.

growth on liquid broth agar plates supplemented with ampicillin. Colonies were picked, and amplified in liquid culture. Glycerol stocks of all colonies were made. Plasmids were isolated using a miniprep protocol and all plasmids were analyzed on an agarose gel either as supercoiled plasmid or linearized with the restriction endonuclease MluI [72]. One representative clone for each SIMPL shRNA (1, 4, 18, and 664) was expanded and Qiagen Maxiprep® columns were used for plasmid purification.

b. SIMPL shRNA knocks down mRNA and protein levels

As seen in Figures 7-12, SIMPL shRNA knocks down protein levels in multiple cell types and shown to knock down SIMPL mRNA in MEFs as determined by real-time PCR. Two different shRNA constructs (SIMPL 18 and 664) were shown to knock down SIMPL protein levels. Additionally, SIMPL shRNA specifically knocks down SIMPL shRNA and not IRAK-1 protein (another member in the TNF-RI pathway).

Used as a quick screen to identify effective shRNA, only SIMPL 18 shRNA diminished Flag-wt levels as compared to scramble control (Figure 7). Optimization of the amount SIMPL 18 shRNA added, led to effective diminishment of SIMPL levels below detection by antibody in Western blots (Figure 8). Any shRNA does not completely degrade all protein. SIMPL 18 shRNA knocks down SIMPL levels in a hematopoietic pro-B cell line (Figure 9). In Figure 9, SIMPL migration forms two bands. In data not shown, the same amount SIMPL antibody was run on the gel without SIMPL lysate, probed with the same antibodies and light chain was not detectable. Thus, the lower SIMPL band is not light chain. SIMPL 664 shRNA also knocks down endogenous SIMPL levels to highlight specificity of SIMPL shRNA (Figure 10). In Figure 11C,

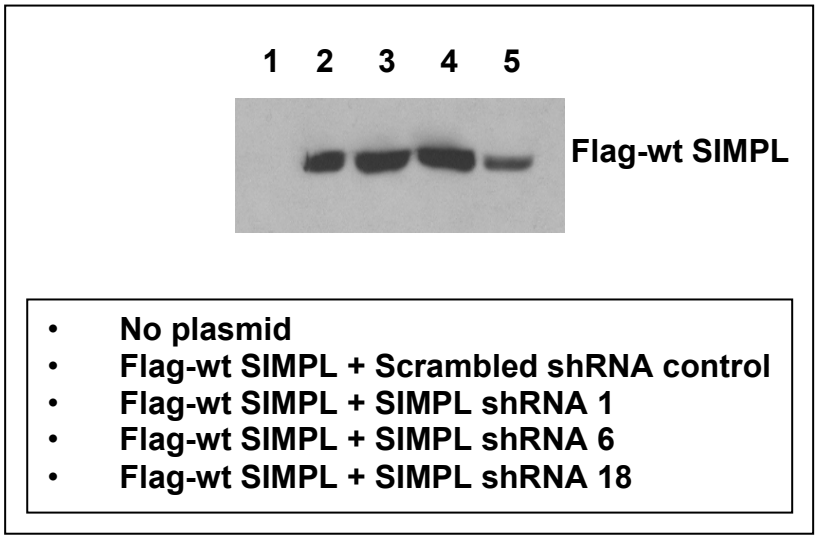


Figure 7. SIMPL 18 shRNA knocks down SIMPL levels. Flag-wildtype (wt) SIMPL (1 μg) was co-expressed with shRNA (4 μg) in HEK 293 cells for 96 hours. Cells from transfected and wildtype HEK 293 were harvested, whole cell lysates were generated and ran on SDS-PAGE. Western was probed with anti-FLAG antibody.

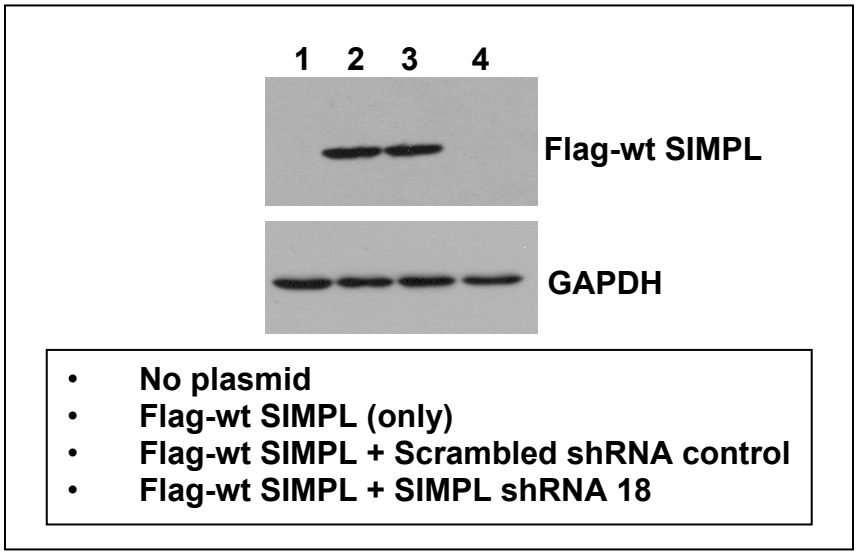


Figure 8. Optimized level of SIMPL 18 shRNA knocks down SIMPL levels.
Attempt to get maximal level of knockdown of Flag-wildtype SIMPL. Flag-wildtype (wt) SIMPL (0.25 μg) was co-expressed with shRNA (6 μg) in HEK 293 for 96 hours. Cells from transfected and wildtype HEK 293 were harvested, whole cell lysates were generated and ran on SDS-PAGE. Western was probed with anti-FLAG antibody.

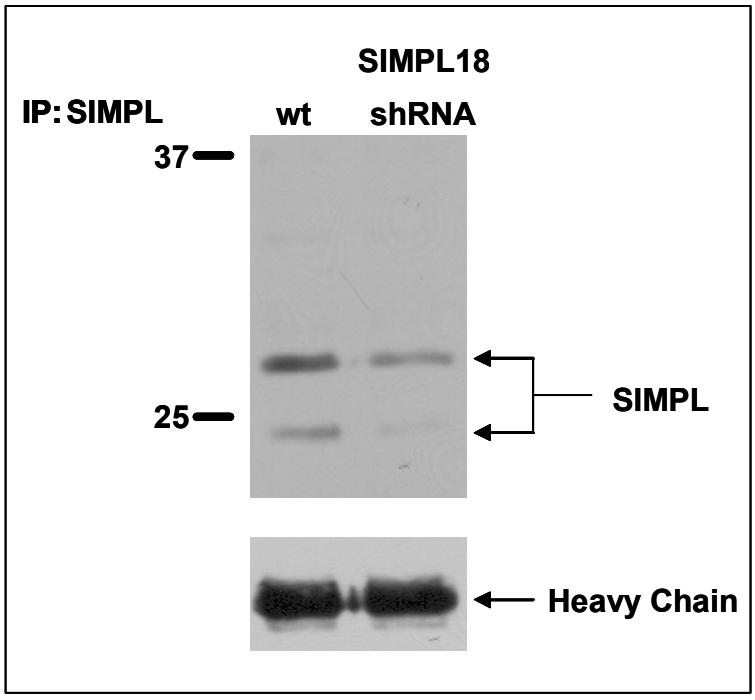


Figure 9. Transduced SIMPL 18 shRNA knocks down endogenous SIMPL levels in BAF3.
Baf3 (Pro-B cell line) was transduced with SIMPL 18 shRNA and FAC sorted (>90% pure for shRNA). Cells from transduced and wildtype BAF3 were harvested, immunocomplex was generated using purified rabbit anti-SIMPL antibody. Lysates with complexs were ran on SDS-PAGE. Western was probed with purified rabbit anti-SIMPL antibody.

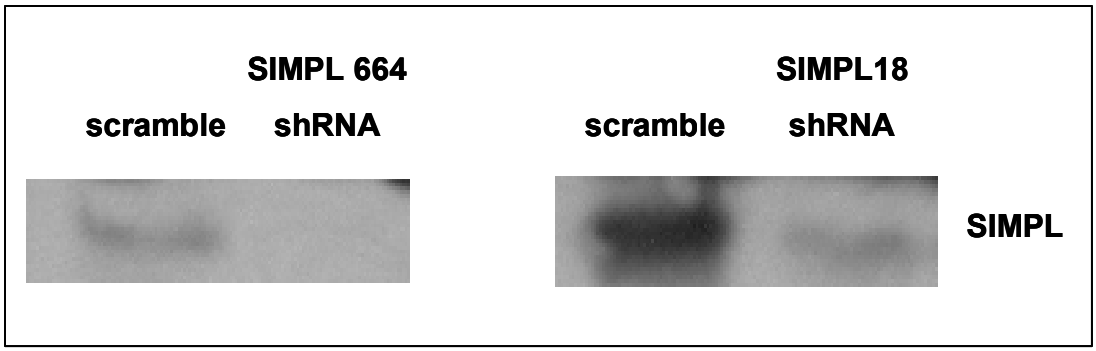


Figure 10. SIMPL 18 and SIMPL 664 shRNA knocks down endogenous SIMPL levels.
ShRNA was transfected into HEK 293. The cells were harvested, run on SDS-PAGE, and the western blot probed with purified rabbit anti-SIMPL antibody.

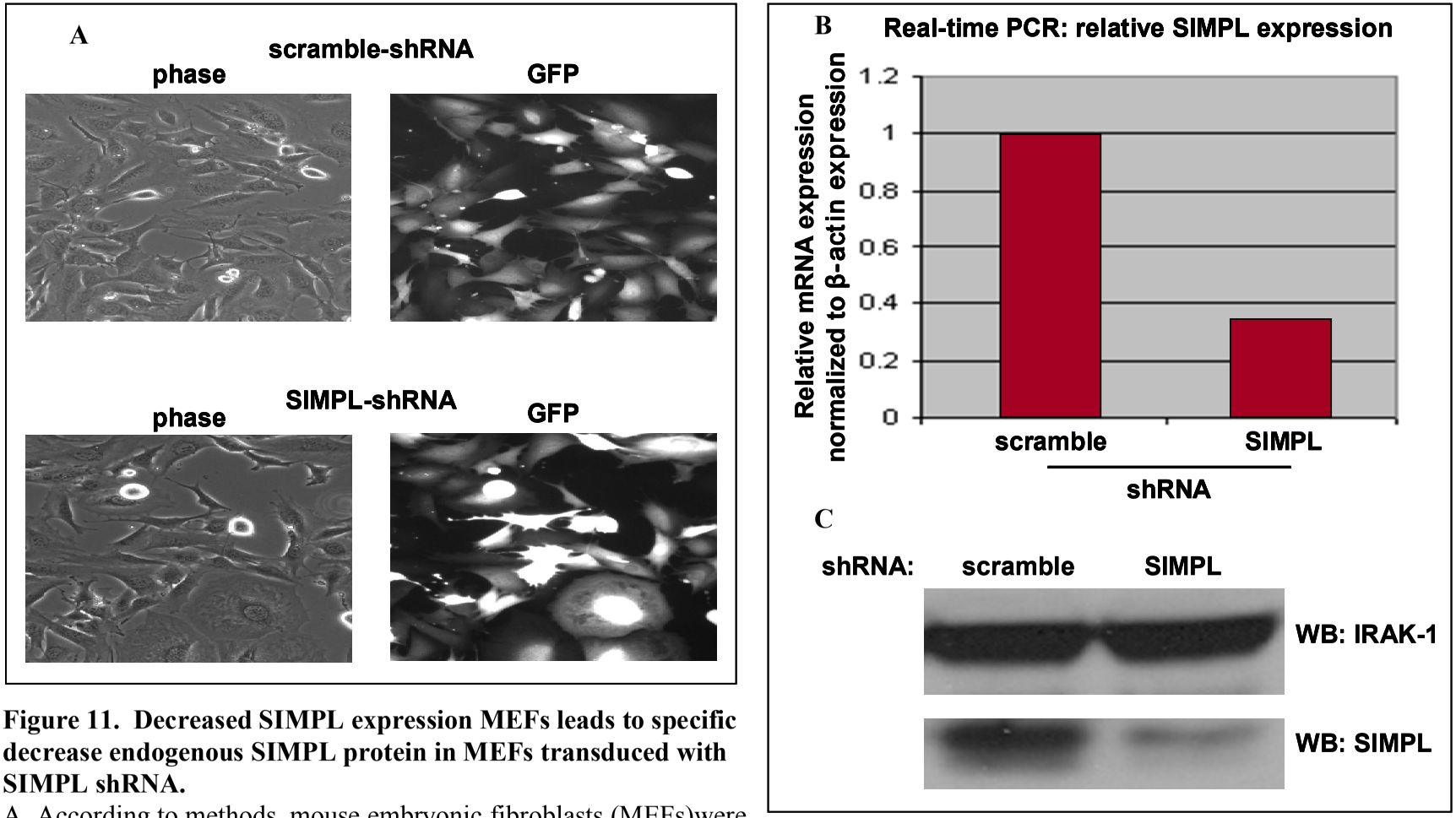


Figure 11. Decreased SIMPL expression MEFs leads to specific decrease endogenous SIMPL protein in MEFs transduced with SIMPL shRNA.

A. According to methods, mouse embryonic fibroblasts (MEFs)were transduced with shRNA (produces GFP) . B. The mRNA expression was measured by real-time PCR kindly done by Sonal Sanghani (Indiana University). C. Same MEFs were harvested and run on SDS-PAGE. The western blot was probed for rabbit anti-SIMPL and rabbit anti-IRAK-1.

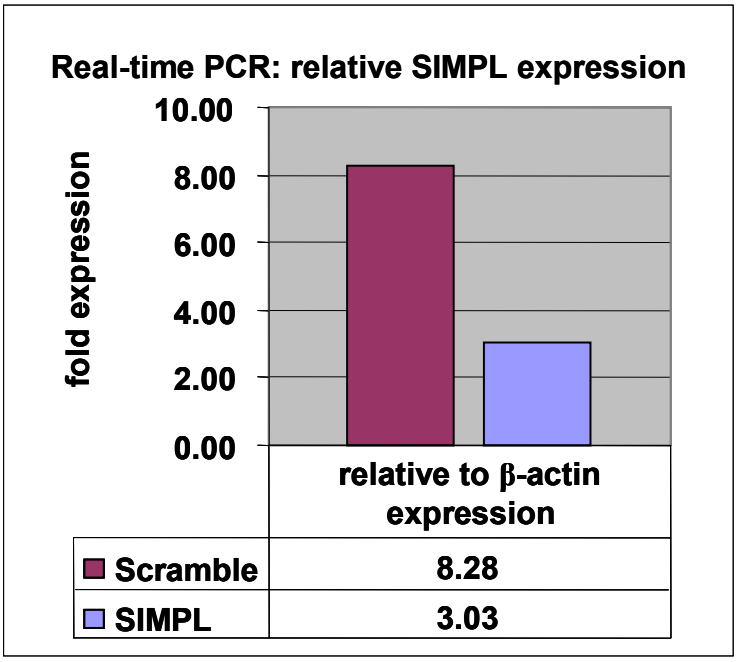


Figure 12. Decreased SIMPL expression in lin$^-$sca$^-$c-kit$^+$ cells containing SIMPL shRNA.
According to methods, mouse bone marrow cells were transduced with shRNA and sorted for lin$^-$sca$^-$c-kit$^+$/shRNA$^+$ cells using FAC sorting. The mRNA expression was measured by real-time PCR kindly done by Sonal Sanghani (Indiana University).

SIMPL shRNA (SIMPL 18 shRNA from here on) diminished endogenous levels of SIMPL in MEFs while not blocking IRAK1 an upstream component in TNFα pathway (Figure 1). This demonstrates SIMPL shRNA specificity to knock down SIMPL directly and not secondarily by reducing levels of an upstream protein in the same pathway. Direct reduction of SIMPL mRNA by SIMPL shRNA leads to a similar reduction in SIMPL protein (Figure 11A and B). SIMPL mRNA decreased to 30% with SIMPL shRNA as compared to scramble control (Figure 11B). Endogenous SIMPL mRNA was decreased six fold in HPCs ($lin^-sca^-c\text{-}kit^+$) containing SIMPL shRNA (Figure 12). These data authenticate SIMPL shRNA's capability to reduce SIMPL levels definitively in multiple cell types including hematopoietic. Accordingly, SIMPL shRNA was prepared for use in our hematopoietic paradigm.

B. Loss of SIMPL Sensitizes CFU-GM cells to TNFα

1. Mouse TNFα in PWMSCM inhibits CFU-GM colonies with loss of SIMPL

We wanted to confirm that mTNFα, in presence of a natural complement of cytokines using poke weed mitogen spleen conditioned media (PWMSCM), could inhibit colony formation in a HPCs colony assay. Bone marrow from 8 to 12 week old C57BL-6 mice was harvested from the tibia, femur and pelvis (flatbone). The low density mononuclear cells (LDMNCs) were separated from the remaining bone marrow entire cell population using Histoplaque 1119. LDMNCs represent an immature subpopulation of bone marrow containing HPCs and HSCs. To expand this population for later FACsorting, LDMNCs were plated at 4×10^6 cells/mL in media containing murine stem

cell factor (mSCF) and human interleukin-6 (hIL-6) (normal signaling on mouse receptors) for two days. These conditions maintains survival and proliferation of early HPCs and HSCs, and inhibit the differentiation or maintainance of differentiated cells.

The LDMNCs were transduced with retrovirus containing scrambled shRNA, and sorted for c-kit$^+$ and GFP$^+$ populations at the IU Cancer Center Flow Cytometry Resource Facility (see methods). The scrambled c-kit$^+$/GFP$^+$ population was plated (1000 to 2000 cells/mL) in a progenitor colony assay containing PWMSCM, mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL), with an increasing gradient of mTNFα (0, 1, 5, 10 ng/mL). Each colony represents growth, proliferation, and differentiation from one HPC or HSC. A c-kit$^+$ cell type represents only 10% of whole bone marrow, 1% of which are HSCs (lin$^-$sca$^+$c-kit$^+$). The results are given as percent control to normalize for variation between biological reproduced experiments.

Mouse TNFα inhibits colony growth as shown by the decrease in the percent CFU-GM colonies expressing scrambled shRNA with increasing concentrations of mTNFα. The peak inhibition occuring at 10 ng/mL and this change was significant at 10 ng/mL (Figures 13-14). Therefore, in the presence of PWMSCM mTNFα inhibits normal progenitor growth in a dose-dependent manner (activates TNF-RI and TNF-RII).

Under the same conditions, c-kit$^+$/GFP$^+$ progenitors expressing SIMPL shRNA showed an enhanced inhibition of CFU-GM growth over scrambled in the presence of a natural TNF-RI and TNF-RII activator with PWMSCM and mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL) (Figures 13-14).

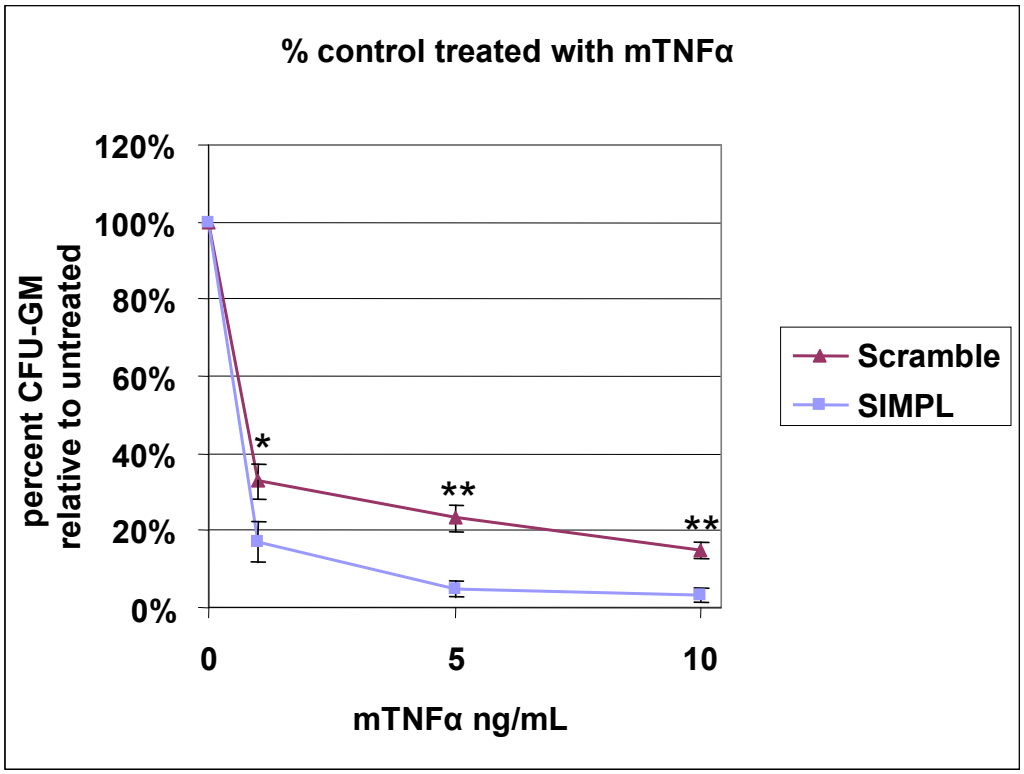


Figure 13. Loss of SIMPL significantly inhibited total colony growth at 1, 5, and 10 ng/mL mTNFα.
Scrambled and SIMPL shRNA c-kit^{+} cells were plated into a colony assay containing mGM-CSF, mIL-3, mSCF, PWMSCM with the indicated concentrations of mTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of mTNFα by the total colonies at the given concentration mTNFα for each group. P-values were from Scrambled compared to SIMPL. N = 3; *$p<0.05$ **$p<0.006$

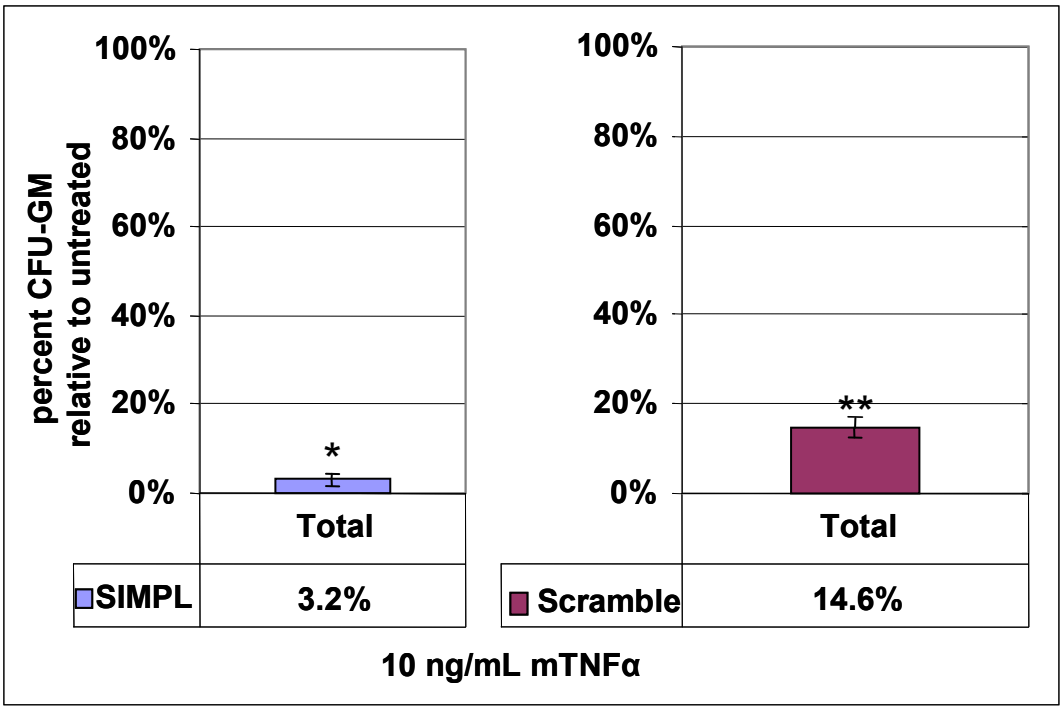


Figure 14. SIMPL and scramble shRNA c-kit$^+$ colonies growth were significantly inhibited by mTNFα. Compared 0 ng/mL to 10 ng/mL mTNFa to determine if any inhibition occurred within each group. Scrambled and SIMPL shRNA c-kit$^+$ cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF and PWM with a concentration gradient of mTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of mTNFα by the total colonies at the given concentration mTNFα for each group. One tailed TTEST comparing within SIMPL or Scramble comparing 0 ng/mL to 10 ng/mL mTNFa CFU-GM colony formation. n = 3; *$p<1.19x10^{-7}$ **$p<1.18x10^{-6}$

2. Human TNFα in PWMSCM inhibits CFU-GM colony formation with loss of SIMPL. PWMSCM effects on TNF-RI dependent progenitor proliferation

We next tested whether the observed effects were specific to a certain TNFα receptor. Other authors have shown that TNF-RI activation through hTNFα or an TNF-RI agonist does not inhibit HPC growth in a differentiation colony assay [51]. In these experiments, the influence of PWMSCM on TNFα effects mediated through TNF-RI on the growth of GM progenitors was also examined.

Using the experimental design as previously mentioned (III. B), c-kit$^+$ scrambled shRNA HPCs were placed in a colony assay containing increasing concentrations of hTNFα (0, 0.1, 1, 5, 10 ng/mL) along with PWMSCM. In the presence of hTNFα there was not a significant decrease in CFU-GM colony number/growth over the range of hTNFα (Figures 15-16). At maximum dose of hTNFα (10 ng/mL), scramble shRNA colony formation was decreased but the effect was not statistically significant (87.6% $\pm$ 18.1%). Therefore, the ability of normal progenitors to form CFU-GM colonies is not inhibited by specific TNF-RI activation in the presence on PWMSCM.

To determine if TNF-RI activation inhibited the growth of c-kit$^+$ SIMPL shRNA progenitors, the same experiment was performed as in III.B. Surprisingly, SIMPL shRNA progenitors colony growth (CFU-GM formation) was inhibited in response to increasing concentrations of the TNF-RI activatior, hTNFα, with maximal inhibition occuring at 10 ng/mL hTNFα (Figures 15-16). This inhibition was significantly more than that observed for progenitors containing scramble shRNA. Therefore, activation of

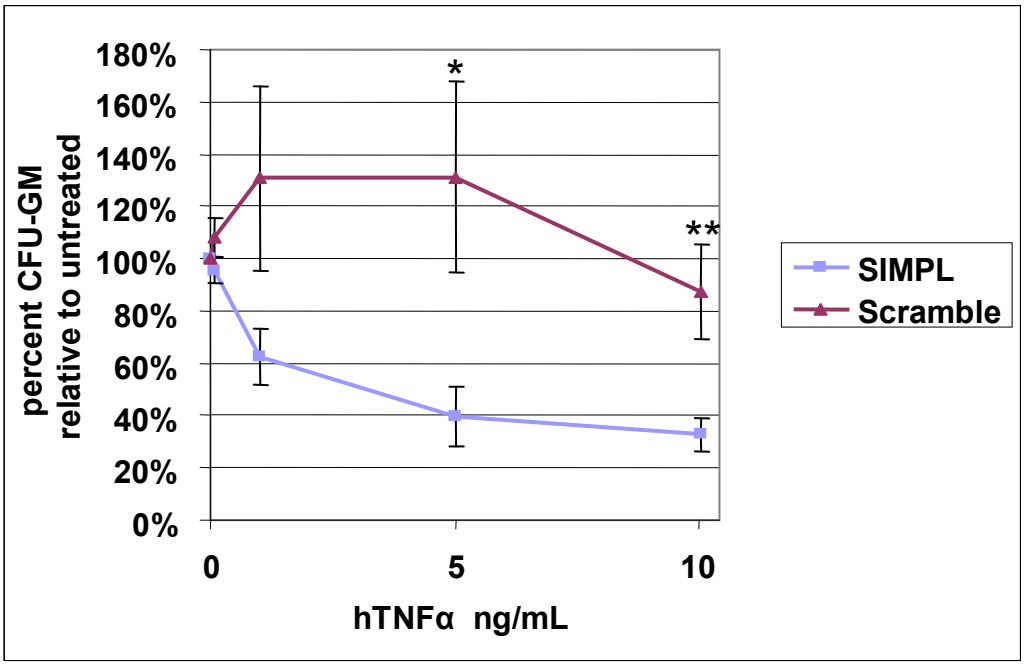


Figure 15. Loss of SIMPL significantly inhibited CFU-GM at 1, 5, and 10 ng/mL hTNFα.
Scrambled and SIMPL shRNA c-kit^{+} cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF, PWMSCM with the indicated concentrations of hTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of hTNFα by the total colonies at the given concentration hTNFα for each group. P-values were from Scrambled compared to SIMPL. n = 3; *p<0.04, **p<0.03

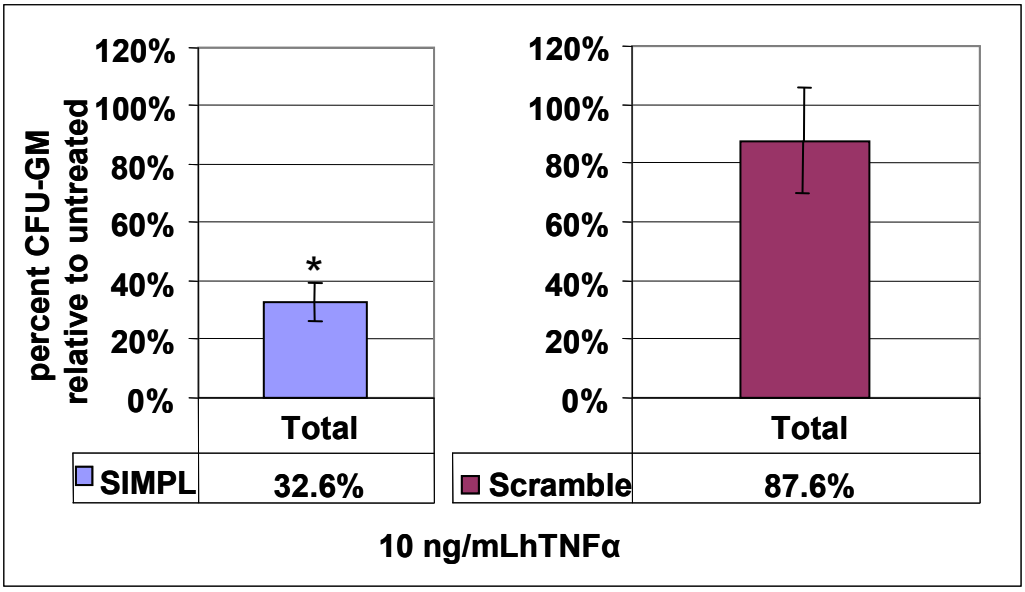


Figure 16. SIMPL shRNA c-kit$^+$ colonies growth were significantly inhibited by hTNFα in PWM with Epo, but Scramble was not. Compared 0 ng/mL to 10 ng/mL hTNFα to determine if any inhibition occurred within each group.

Scrambled and SIMPL shRNA c-kit$^+$ cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF, PWM and Epo with a concentration gradient of hTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of hTNFα by the total colonies at the given concentration hTNFα for each group. One tailed TTEST comparing within SIMPL or Scramble comparing 0 ng/mL to 10 ng/mL hTNFα CFU-GM colony formation. n = 3; *$p<0.0003$

TNF-RI in progenitors with decreased levels of SIMPL, in the presence of PWMSCM inhibits expansion and differentiation of CFU-GM progentiors.

3. To explore the effects of TNFα on subsets, CFU-GM colonies were subcategorized

Colony growth was subcategorized based on number of cells in the colonies and size of the colonies. High proliferative potential colonies (HPP) are colonies greater than 0.5 mm, with a dense core/nucleus of cells. The presence of these colonies indicates that the plated cell (that leads to the formation of the colony) was an early hematopoietic precursor, which has the ability to vastly proliferate/expand and differentiate. Low proliferative potential colonies (LPP) are colonies greater than 50 cells that did not meet both criteria for HPP (dense core, >0.5 mm). Normally, HPP and LPP separation is made at 10 or 14 days after the start of a colony assay, and is usually done in the presence of limited cytokines or PWMSCM; however, the conditions of no PWMSCM and mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL) leads to rapid expansion after 7 days with many HPPs colonies over 1 mm in size. Therefore in these experiments HPP and LPP colonies were counted at 7 days.

In data not shown, $ckit^+$ cells containing scramble shRNA were plated in mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL) in presence or absence of PWMSCM with increasing doses of mTNFα. HPPs were much higher in the absence of PWMSCM after 7 days. Amazingly, the HPPs in the PWMSCM group did not recover by 14 days, while the HPPs in the absence of PWMSCM after 14 days were too big to accurately count. Other (data not shown) samples repeat this same trend in the absence of PWMSCM. Therefore, this system utilizes a 7 day count for HPPs when given mSCF

(100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL) without PWMSCM. HPP and LPP subcategorization was not available all CFU-GM experiments.

4. Loss of TNF-RI specific inhibition of CFU-GM with diminished SIMPL in the absence of PWMSCM

Since we observed TNF-RI specific inhibition of CFU-GM colony formation of c-kit$^+$ cells containing SIMPL shRNA in PWMSCM, as a next logical step, PWMSCM was removed from the experimental conditions. This enabled us to see if TNF-RI activation (hTNFα) was sensitizing c-kit$^+$ SIMPL shRNA containing progenitors or if there was something else in the PWMSCM that could be causing this inhibition. The colony assays were setup the same as above with mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL) except PWMSCM was not added to the colony assay mix.

The growth of c-kit$^+$ cells containing scrambled shRNA was slightly inhibited (Figures 17-18 similar to B1) over the hTNFα concentration range. At the maximum hTNFα dose a slight but significant inhibition occurred in the low proliferative potential (LPP) and total colony populations (down to 89.8% $\pm$ 8.2% and 89.9% $\pm$ 6.2% repectively). Thus, on normal progenitors, TNF-RI activation has a slight, at best, effect on CFU-GM formation.

CFU-GM colony formation of c-kit$^+$ cells containing SIMPL shRNA was only slightly inhibited by the maximum dose of hTNFα (10 ng/mL) (LPP down to 80.6% $\pm$ 3.7%; total colony down to 81.5% $\pm$ 4.1% repectively) (Figure 18). There was no increased inhibition over colony formation of c-kit$^+$ cells containing scrambled shRNA (Figure 17) at any concentration of hTNFα. This immediately indicated that something

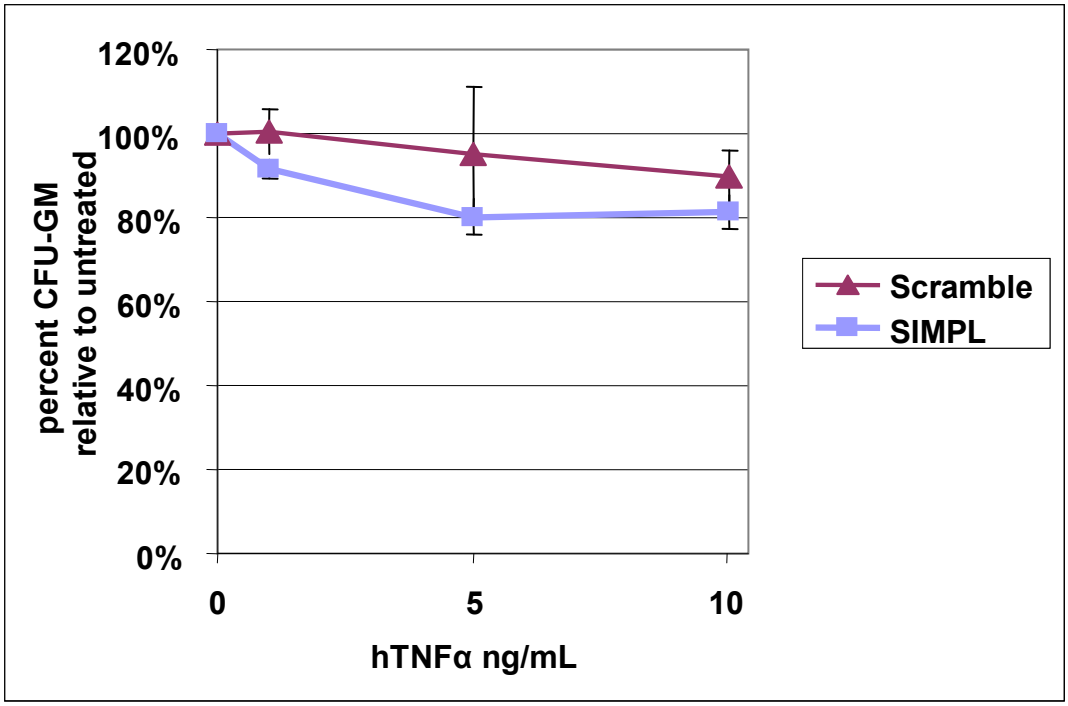


Figure 17. Loss of SIMPL has no effect upon hTNFα dependent inhibition of CFU-GM formation.
Scrambled and SIMPL shRNA c-kit^{+} cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF with the indicated concentrations of hTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of hTNFα by the total colonies at the given concentration hTNFα for each group. P-values were from Scrambled compared to SIMPL. NO PWMSCM was used in this experiment. n = 3; no significant changes occurred

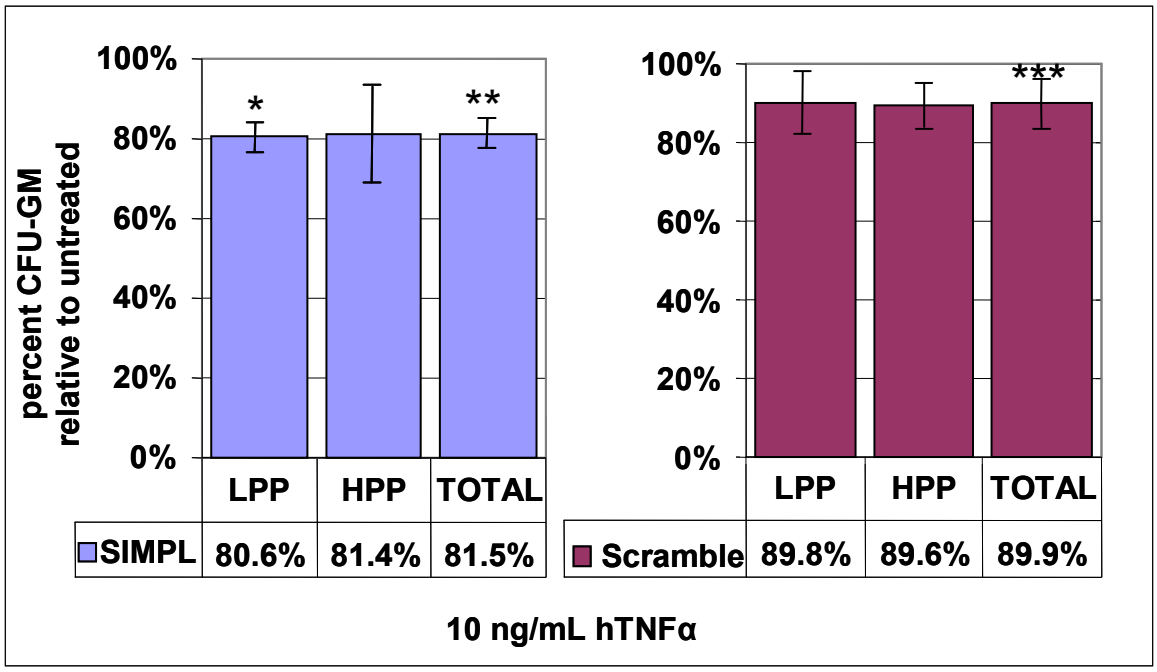


Figure 18. SIMPL LPP and Total colony formation were significantly inhibited by hTNFα along with Scramble shRNA c-kit$^+$ total colony growth. Compared 0 ng/mL to 10 ng/mL hTNFα to determine if any inhibition occurred within each group with no PWM.
Scrambled and SIMPL shRNA c-kit$^+$ cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF with a concentration gradient of hTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of hTNFα by the total colonies at the given concentration hTNFα for each group. One tailed TTEST comparing within SIMPL or Scramble comparing 0 ng/mL to 10 ng/mL hTNFα CFU-GM colony formation. n = 3; *$p<0.005$ **$p<0.0007$ ***$p<0.05$

in the poke weed mitogen spleen conditioned media enhances the ability of a TNF-RI activator to inhibit colony growth in progenitors with diminished levels of SIMPL in a concentration dependent manner.

C. Isolation of the cause of TNF-RI inhibition of CFU-GM in PWSCM sensitization in PWMSCM by removal of PWMSCM and addition of various growth factors

1. Mouse TNFα without PWMSCM causes inhibition of CFU-GM colony formation with loss of SIMPL

To determine if activation of the TNF-RII receptor along with the TNF-RI re-establishes inhibition of c-kit$^+$ scramble HPC colony growth in the absence of PWMSCM, a repeat of experiment represented in Figure 17 was performed with mTNFα (0, 1, 5, 10 ng/mL).

Colony formation by scramble shRNA ckit$^+$ cells was only slightly but not significantly inhibited in the presence of 10 ng/mL TNFα (Figures 19-21). Thus, in the absence of PWMSCM, mTNFα did not cause the sweeping inhibition of normal progenitor growth seen earlirer.

Colony formation by SIMPL shRNA c-kit$^+$ cells was significantly inhibited in a dose dependent manner by mTNFα (0, 1, 5, 10 ng/mL) as compared to c-kit$^+$ scrambled shRNA cells, with significant inhibition over scramble occuring at 10 ng/mL mTNFα. Mouse TNFα did re-sensitize the c-kit$^+$ progenitors expressing the SIMPL shRNA to TNFα. This inhibition of the ability to form CFU-GM by SIMPL shRNA c-kit$^+$ cells more closely mimicked hTNFα in the presence of PWMSCM than mTNFα in

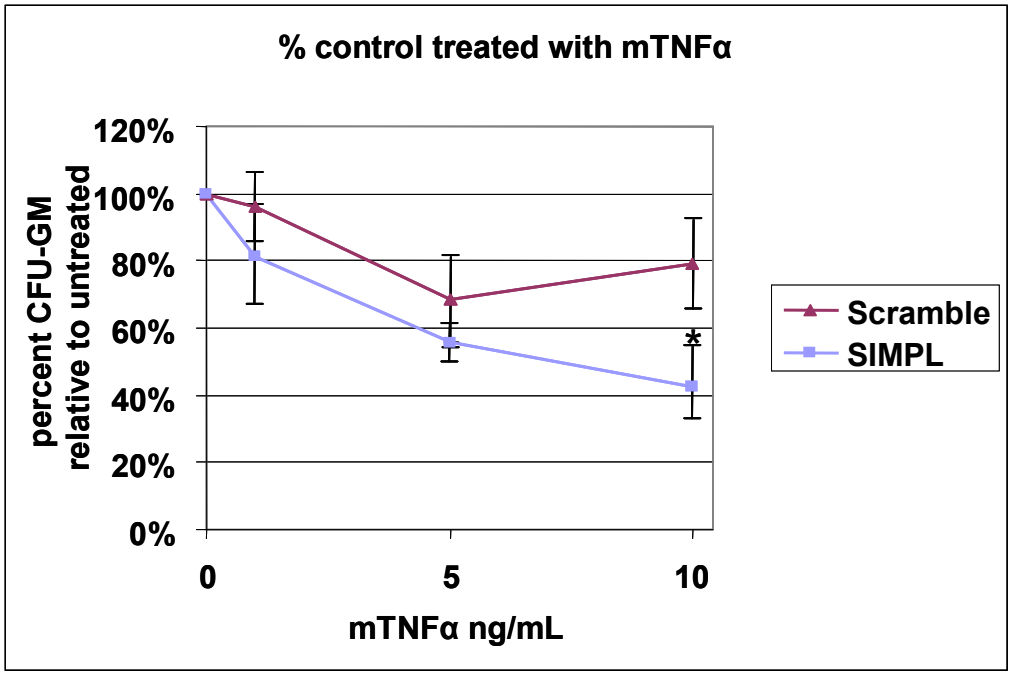


Figure 19. SIMPL shRNA c-kit$^+$ LPP and total colonies showed significant inhibition of colony growth compared to scrambled shRNA control at 10 ng/mL mTNFα without PWM.
Scrambled and SIMPL shRNA c-kit$^+$ cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF, with a concentration gradient of mTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of mTNFα by the total colonies at the given concentration mTNFα for each group. P-values were from Scrambled compared to SIMPL. n = 5; *p<0.04

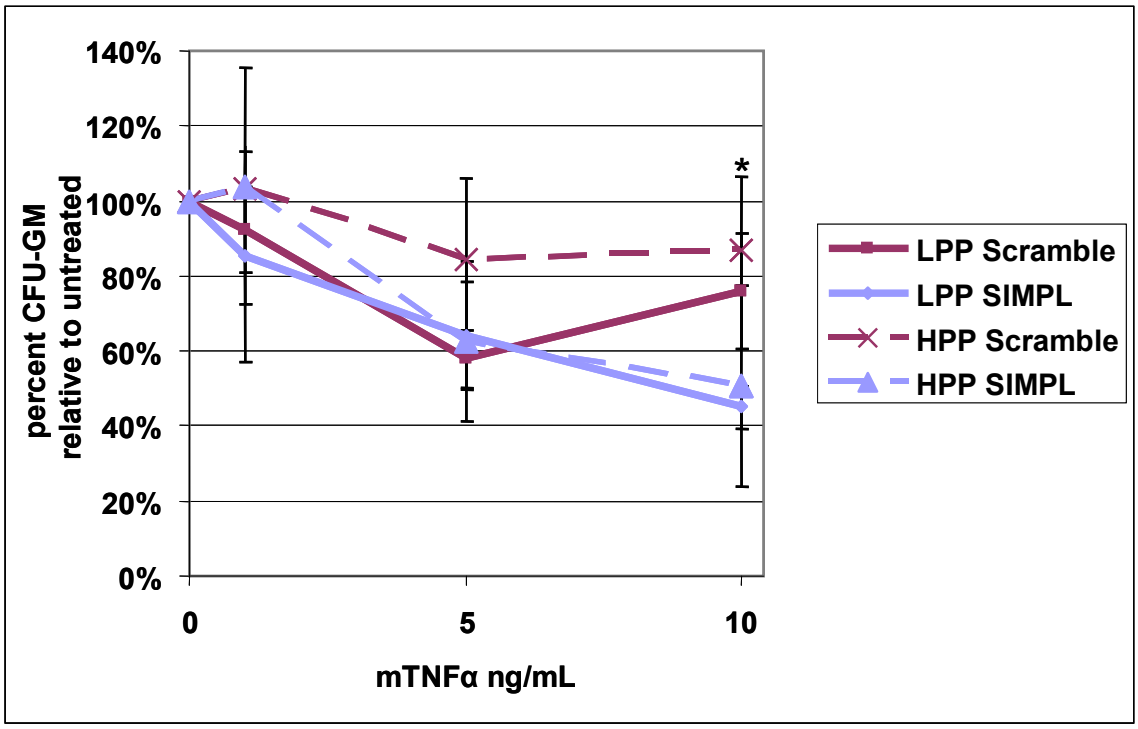


Figure 20. SIMPL shRNA c-kit$^+$ LPP and total colonies showed significant inhibition of colony growth compared to scrambled shRNA control at 10 ng/mL mTNFα without PWM.
Scrambled and SIMPL shRNA c-kit$^+$ cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF, with a concentration gradient of mTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies at a given concentration mTNFα into 0 ng/mL mTNFα colony number for each group. P-values were from scrambled LPP or HPP compared to SIMPL LPP or HPP colonies, respectively. n = 5; $p<0.04$

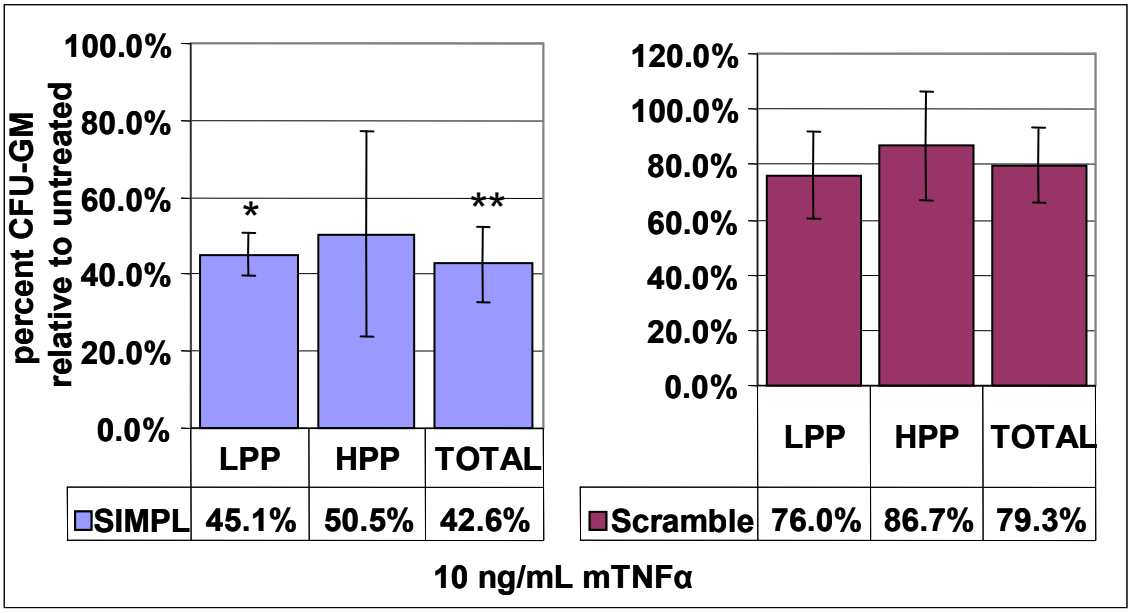


Figure 21. SIMPL shRNA LPP and Total colony growth was significantly inhibited at maximum dose mTNFα (10 ng/mL), while scramble shRNA c-kit^{+} colony growth was not. Compared 0 ng/mL to 10 ng/mL mTNFa to determine if any inhibition occurred within each group.

Scrambled and SIMPL shRNA c-kit^{+} cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF with a concentration gradient of mTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of hTNFα by the total colonies at the given concentration hTNFα for each group. One tailed TTEST comparing within SIMPL or Scramble LPP, HPP, total CFU-GM colony formation comparing 0 ng/mL to 10 ng/mL hTNFa. n = 5; *$p<3.7x10^{-6}$ **$p<0.0003$

PWMSCM. Thus for inhibition of CFU-GM by SIMPL shRNA c-kit$^+$ cells, hTNFα plus PWMSCM approximately equals mTNFα. Surprisingly, significant inhibition of LPPs did occur in the scramble shRNA group at 5 ng/mL mTNFα (down to 57.9% $\pm$ 7.9%) (Figure 20). This result highlights the balance of activation between TNF-RI and TNF-RII that occurs in a concentration dependent manner with mTNFα. It has been shown that activation of the TNFα receptors is dependent on the concentration of TNFα; (soluble TNFα has a higher affinity for TNF-RI) [73]. Therefore, these results highlight an ideal activation of TNF-RI and TNF-RII leading to inhibition of CFU-GM growth at 5 ng/mL, but the inhibition disappears at a higher TNFα concentration (10 ng/mL) due most likely to activation of TNF-RII.

2. Neither of the two other important inhibitors (IFNγ or TGF-β1) of hematopoiesis cause inhibition of CFU-GM colony formation with knock down SIMPL levels

To show that decreased SIMPL levels specifically increases TNFα inhibition of colony formation, I tested two major pleotropic inhibitors of hematopoiesis, IFNγ and TGFβ.

a. Interferon gamma (IFNγ) does not decrease CFU-GM with SIMPL shRNA

To determine if mIFNγ was the PWMSCM component that enables the combination of TNF-RI plus loss of SIMPL to inhibit CFU-GM colony formation, mouse IFNγ was added in a dose dependent manner (0, 1, 5, 10 ng/mL) with mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL) without PWMSCM in the standard colony assay with c-kit$^+$ scrambled progenitors. The concentration of mIFNγ

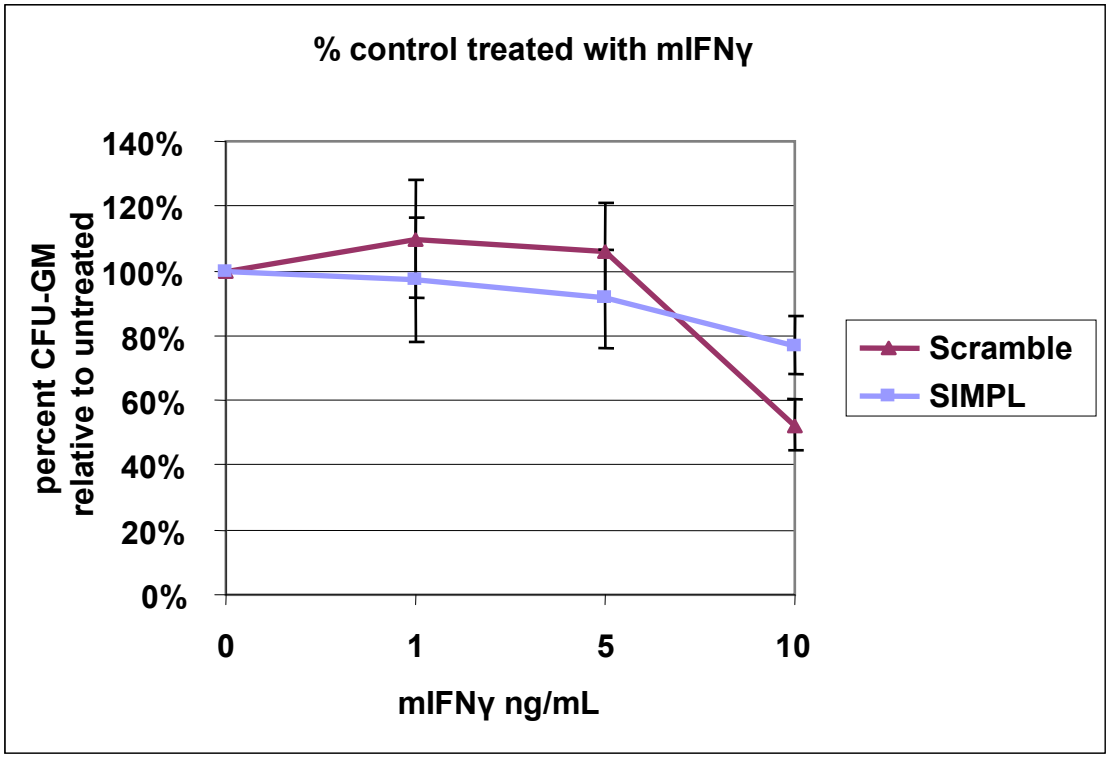


Figure 22. Loss of SIMPL has no effect upon mIFNγ dependent inhibition of CFU-GM formation.
Scrambled and SIMPL shRNA c-kit^{+} cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF with the indicated concentrations of mIFNγ. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of mIFNγ by the total colonies at the given concentration mIFNγ for each group. P-values were from Scrambled compared to SIMPL. NO PWMSCM was used in this experiment. n = 3; No significant changes occurred

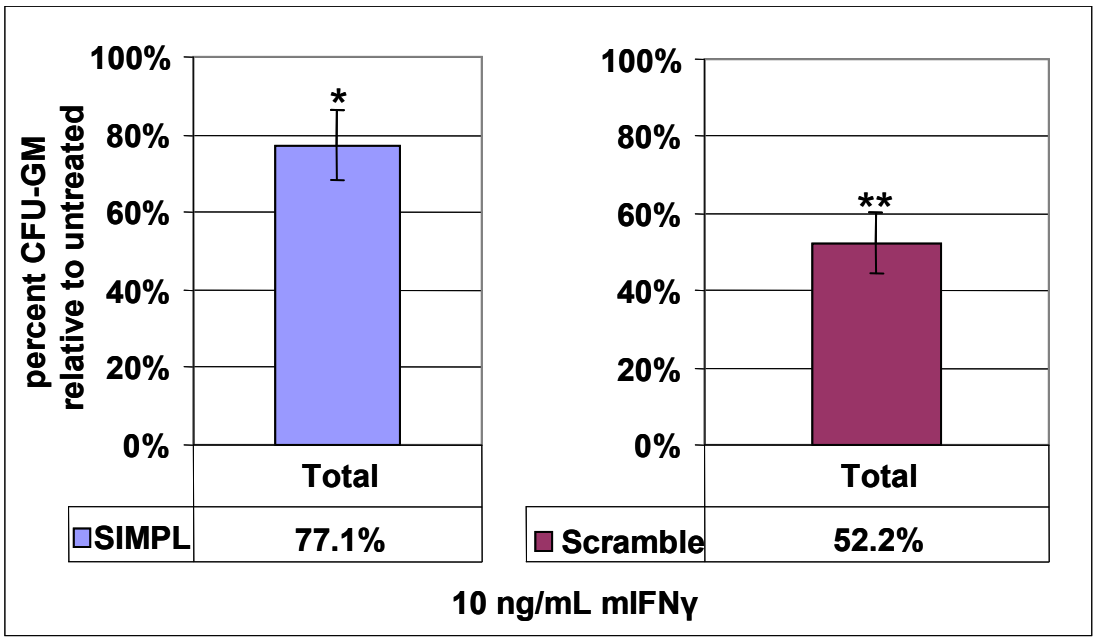


Figure 23. SIMPL and scramble shRNA c-kit$^+$ colonies growth were significantly inhibited by mIFNγ. Comparing SIMPL or Scramble 0 ng/mL to 10 ng/mL to determine if any inhibition occurred with mIFNγ.
Scrambled and SIMPL shRNA c-kit$^+$ cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCFwith a concentration gradient of mIFNγ. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of hTNFα by the total colonies at the given concentration hTNFα for each group. One tailed TTEST comparing within SIMPL or Scramble comparing 0 ng/mL to 10 ng/mL mIFNγ CFU-GM colony formation. n = 3; *$p<0.04$ **$p<0.002$

needed for the colony assay was determined based on an assay performed by Broxymeyer and co-workers [74]; however, in their colony assays mIFNγ in inhibited colony formation in the presence of PWMSCM. C-kit$^+$ HPC expressing the scrambled shRNA were inhibited in a dose dependent manner with increasing concentration of mIFNγ, and significantly inhibited (52.2% $\pm$ 7.8%) when examined at the maximum dose given (10 ng/mL) (Figures 22-23). Therefore mIFNγ inhibits normal progenitor colony formation in a dose dependent manner in the absence of PWMSCM.

C-kit$^+$ HPCs expressing the SIMPL shRNA were not inhibited to a greater extent by mIFNγ than c-kit$^+$ cells containing scramble shRNA.

b. Tumor growth factor-Beta 1 (TGF-β1) not decrease CFU-GM with SIMPL

To determine if TGF-β1 was the missing PWMSCM component that enables the combination of TNF-RI plus loss of SIMPL to inhibit progenitor formation, human TGF-β1 (which has normal activity in mouse cells) was added in a dose dependent manner (0, 0.005, 0.05, 5 ng/mL) with mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL) without PWMSCM in the standard colony assay with c-kit$^+$ scrambled progenitors. Human TGF-B1 concentrations was based on Broxymeyer et al. [75, 76], with the caveat that they performed their assays in the presence of PWMSCM.

Formation of CFU-GM by c-kit$^+$ HPCs containing the scrambled shRNA were inhibited with increasing concentrations of TGF-β1. At the maximum dose of TGF-β1, the LPP and total colony were sigficantly inhibited (60.7% and 62.9% respectively) (Figures 24-25). Therefore TGF-β1 inhibits normal progenitor colony formation in a dose dependent manner in the absence of PWMSCM.

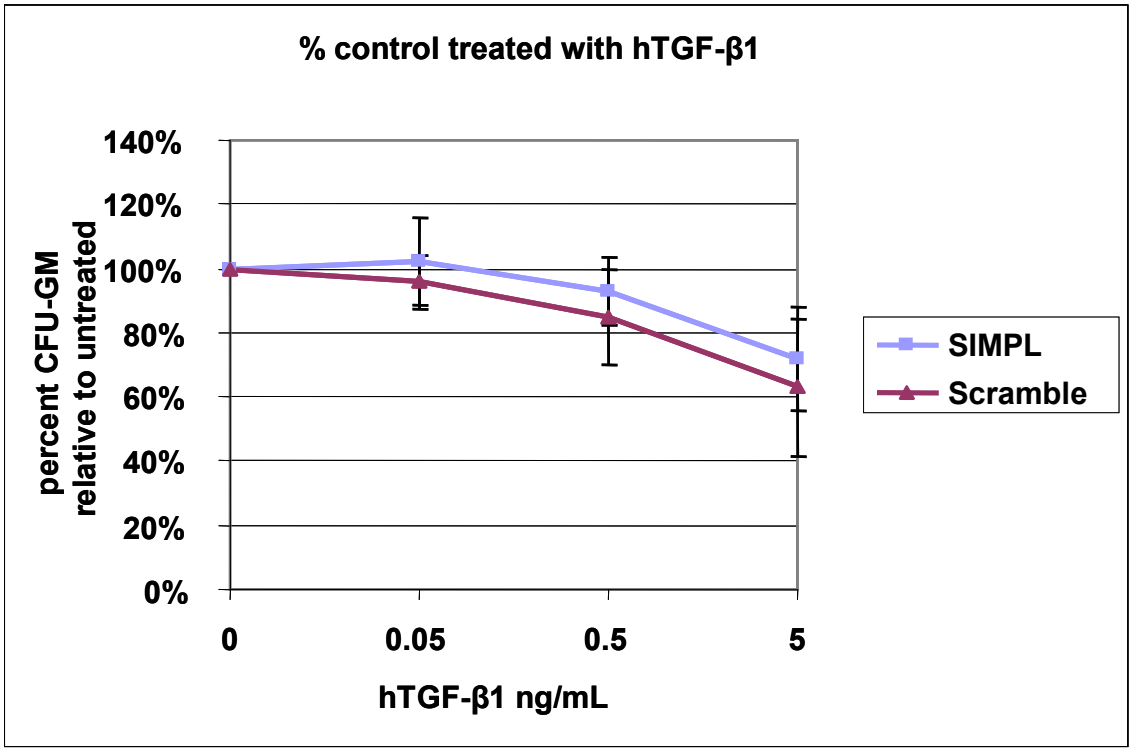


Figure 24. Loss of SIMPL has no effect upon hTGF-β1 dependent inhibition of CFU-GM formation.
Scrambled and SIMPL shRNA c-kit^{+} cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF with the indicated concentrations of hTGF-β1. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of hTGF-β1 by the total colonies at the given concentration hTGF-β1 for each group. P-values were from Scrambled compared to SIMPL. Total CFU-GM colony formation presented because no significant difference occurred when comparing SIMPL to Scramble LPP or HPP colonies. (NO PWMSCM was used in this experiment). n = 4

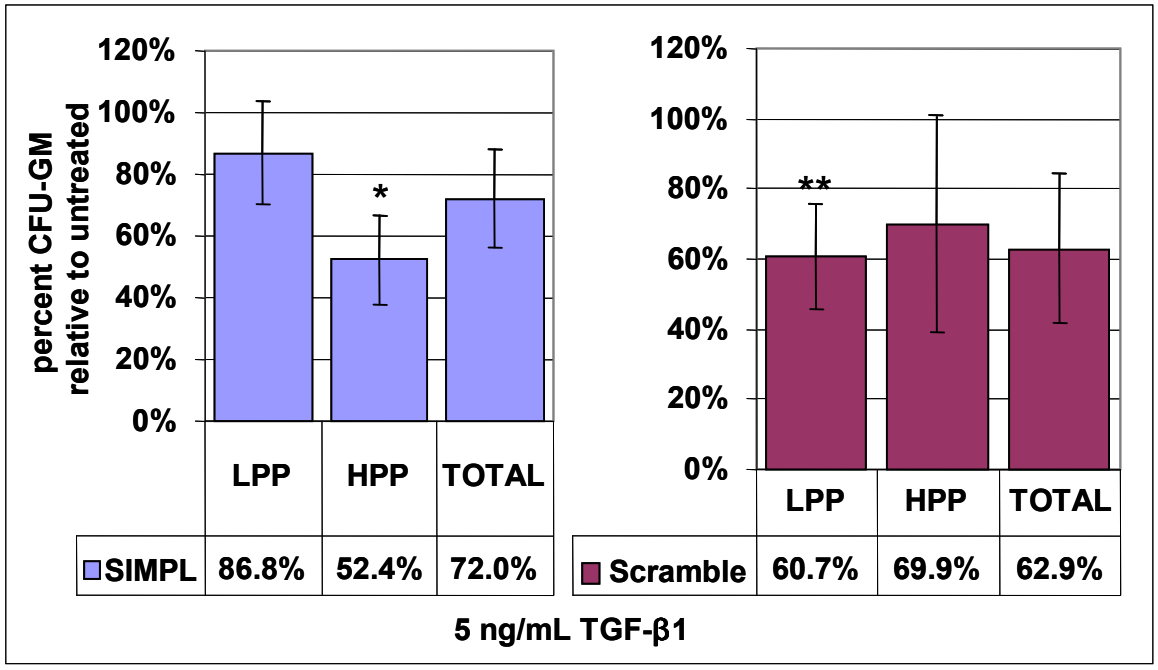


Figure 25. For SIMPL, HPP colony growth was significantly inhibited by hTGF-β1, however scramble shRNA c-kit$^+$ LPP colony growth was significantly inhibited. Compared 0 ng/mL to 10 ng/mL hTGF-β1 to determine if any inhibition occurred within each group.
Scrambled and SIMPL shRNA c-kit$^+$ cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF with a concentration gradient of hTGF-β1. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of hTGF-β1 by the total colonies at the given concentration hTGF-β1 for each group. One tailed TTEST comparing within SIMPL or Scramble comparing 0 ng/mL to 5 ng/mL hTGF-β1 CFU-GM colony formation. n = 4; *$p<0.01$ **$p<0.03$

While CFU-GM colony formation of c-kit$^+$ cells containing the SIMPL shRNA was actually not statistically significantly inhibited any further than the CFU-GM assays performed with the c-kit$^+$ scrambled shRNA cells. Still, at the maximum dose of TGF-B1 (5 ng/mL), HPP and total colony numbers (52.4% and 72% respectively) were significantly inhibited in the presence of SIMPL shRNA (Figures 24-25). Therefore, expression of the SIMPL shRNA in c-kit$^+$ cells does not cause a significant change in inhibition of CFU-GM (total, LPP or HPP) colony formation as compared to scrambled control c-kit$^+$ cells. However, expression of SIMPL shRNA does cause a significant decrease in HPP formation preferentially over LPPs at the maximum TGF-β1 dose, while normal progenitors show a preferential decrease in LPP colonies when compared to untreated within respective groups. As mentioned in the statistics section, since there was no evidence for enhanced inhibition of SIMPL shRNA over scrambled shRNA for other cytokines, a two tailed Ttest was used to determine significant difference of either inhibition or growth advantage.

3. p65 controlled erythropoietin does not cause further inhibition through TNF-RI with PWMSCM

Red blood cell growth and differentiation is a major component of myelopoiesis. As mentioned in the introduction, TNFα has been shown to decrease red blood cell colony formation. Addition of erythropoietin to the colony assay cocktail of PWMSCM, mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL), did not lead to increased inhibition of colony number with increasing concentrations of hTNFα (0, 0.1, 5, 10 ng/mL). Colony growth was comparable to Figure 15 with no significant decrease

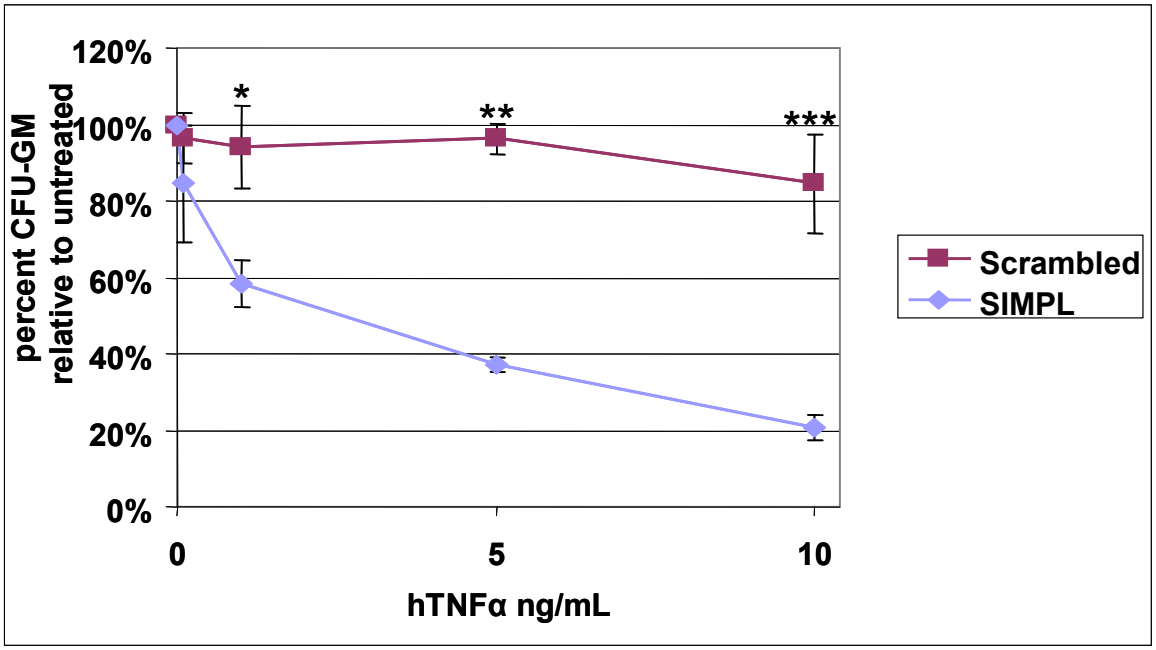


Figure 26. Loss of SIMPL significantly inhibited total colony formation at 1, 5, and 10 ng/mL hTNFα.
Scrambled and SIMPL shRNA c-kit^{+} cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF, PWMSCM, EPO with the indicated concentrations of hTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of hTNFα by the total colonies at the given concentration hTNFα for each group. P-values were from Scrambled compared to SIMPL. n = 3; *p<0.03 **p<0.0002 ***p<0.005

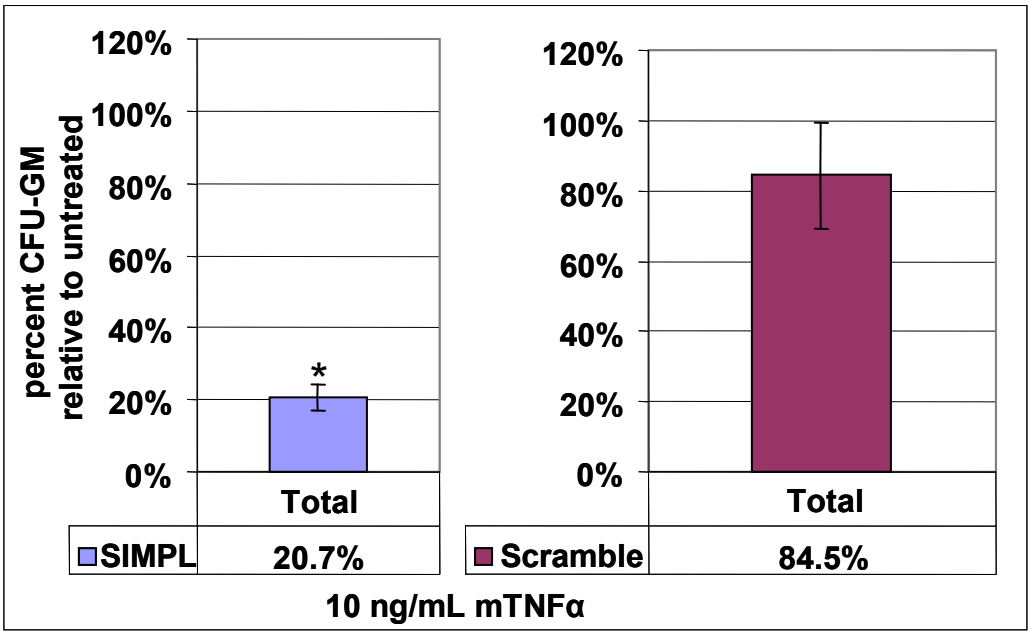


Figure 27. SIMPL shRNA c-kit$^+$ colonies growth were significantly inhibited by hTNFα in PWM and Epo, but scrambled was not. Comparing SIMPL or Scramble 0 ng/mL to 10 ng/mL to determine if any inhibition occurred with hTNFα. Scrambled and SIMPL shRNA c-kit$^+$ cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF, PWM and Epo with a concentration gradient of hTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of mTNFα by the total colonies at the given concentration mTNFα for each group. One tailed TTEST comparing within SIMPL or Scramble comparing 0 ng/mL to 10 ng/mL mTNFa CFU-GM colony formation. n = 3; *$p<2x10^{-5}$

of colony formation of c-kit$^+$ cells containing scramble shRNA in the presence of hTNFα (Figures 26-27). Colony formation was similarly inhibited in the SIMPL shRNA c-kit$^+$ samples compared to scramble (Figures 26-27). The inhibition was comparable to Figure 15. Human TNFα inhibited SIMPL shRNA containing c-kit$^+$ cell colony growth in the presence of erythropoietin and PWMSCM, with maximal inhibition occuring at 10 ng/mL hTNFα. Therefore, erthropoietin is not an additional factor stimulating or limiting increased inhibition of HPCs containing the SIMPL shRNA.

4. Lack of progenitor growth not caused by increasing levels of GM-CSF, GM-CSF activation of p65 is possibly not SIMPL dependent

GM-CSF is another growth factor found in PWMSCM. It also is an important factor the CFU-GM formation. GM-CSF expression is upregulated by p65, and GM-CSF can induce p65 activation. Logically with SIMPL functioning as a TNF-RI specific co-activator, GM-CSF could potentially modulate SIMPL activity indirectly through p65 activation. GM-CSF (data not shown) was added in various concentrations to a colony assay with scrambled and SIMPL c-kit$^+$ HPCs. SIMPL shRNA had no effect on colony inhibition. These experiments were a biological n = 1, and were not explored further.

D. Loss of SIMPL allows normal CFU-GM colony formation in the absence of TNFα

Without the addition of TNFα, CFU-GM colony formation from c-kit$^+$ cells containing knock downed SIMPL levels grow the same as scrambled control c-kit$^+$ cells (Figure 28). CFU-GM colony numbers were compiled from various experiments (n = 13) grown in the same conditions of mGM-CSF, mIL-3, and mSCF. This highlights that

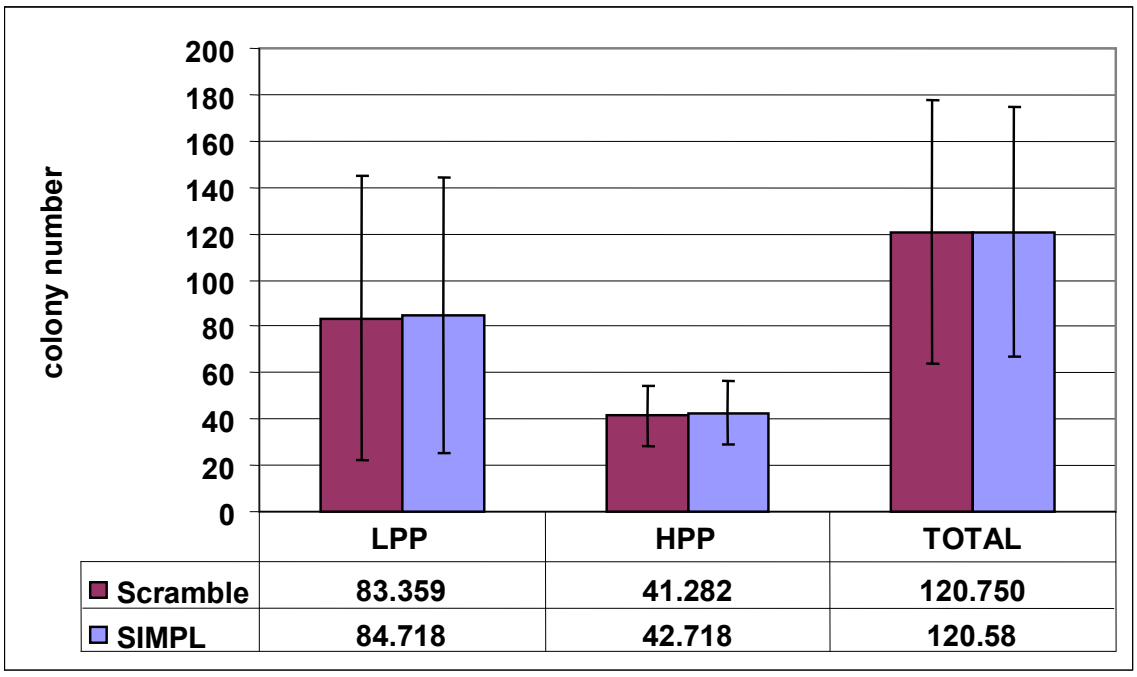


Figure 28. Loss of SIMPL has no effect upon untreated CFU-GM formation without PWMSCM.
Scrambled and SIMPL shRNA c-kit+ cell were plated into a colony assay containing mGM-CSF, mIL-3, mSCF. Total colony number shown. P-values were from LPP, HPP, and Total Scrambled compared to SIMPL colony numbers. n = 13; No significant change occurred.

the total number of CFU-GM from c-kit$^+$ cells with loss of SIMPL were not significantly different from CFU-GM from c-kit$^+$ cells with scrambled control shRNA (120.8 to 120.6 respectively). Additionally the CFU-GM sizes and proliferative potential as represented by HPP and LPP were also not significantly different when comparing CFU-GM colony formation of c-kit$^+$ cells containing SIMPL shRNA to scrambled control (HPP 42 vs. 41 and LPP 85 vs. 83 respectively).

E. TNF-RI inhibition of CFU-GM colony formation of c-kit$^+$ cells with diminished SIMPL not caused by apoptosis from analysis of various apoptosis conditions

A series of apoptosis assays were performed using various concentrations of hTNFα and growth factors. C-kit$^+$/shRNA containing bone marrow cells were sorted and 100,000 cells/mL along with c-kit$^+$ only control bone marrow cells were plated under the various conditions described in more detail below. At the end of the experiment, all cells in the dish (adherent plus non-adherent) or non-adherent only were collected, pelleted by centrifugation, and incubated in a buffer containing annexin V and 7-AAD. The FACs analysis was performed with a one color controls and the experimental c-kit$^+$/shRNA bone marrow were analyzed. Though each individual apoptotic experiment was performed one time, when viewed together an obvious trend forms. SIMPL shRNA containing cells have no increase in apoptosis over scrambled control.

1. *No increased apoptosis of progenitors with knock down SIMPL in presence of cytokine that allows for differentiation*

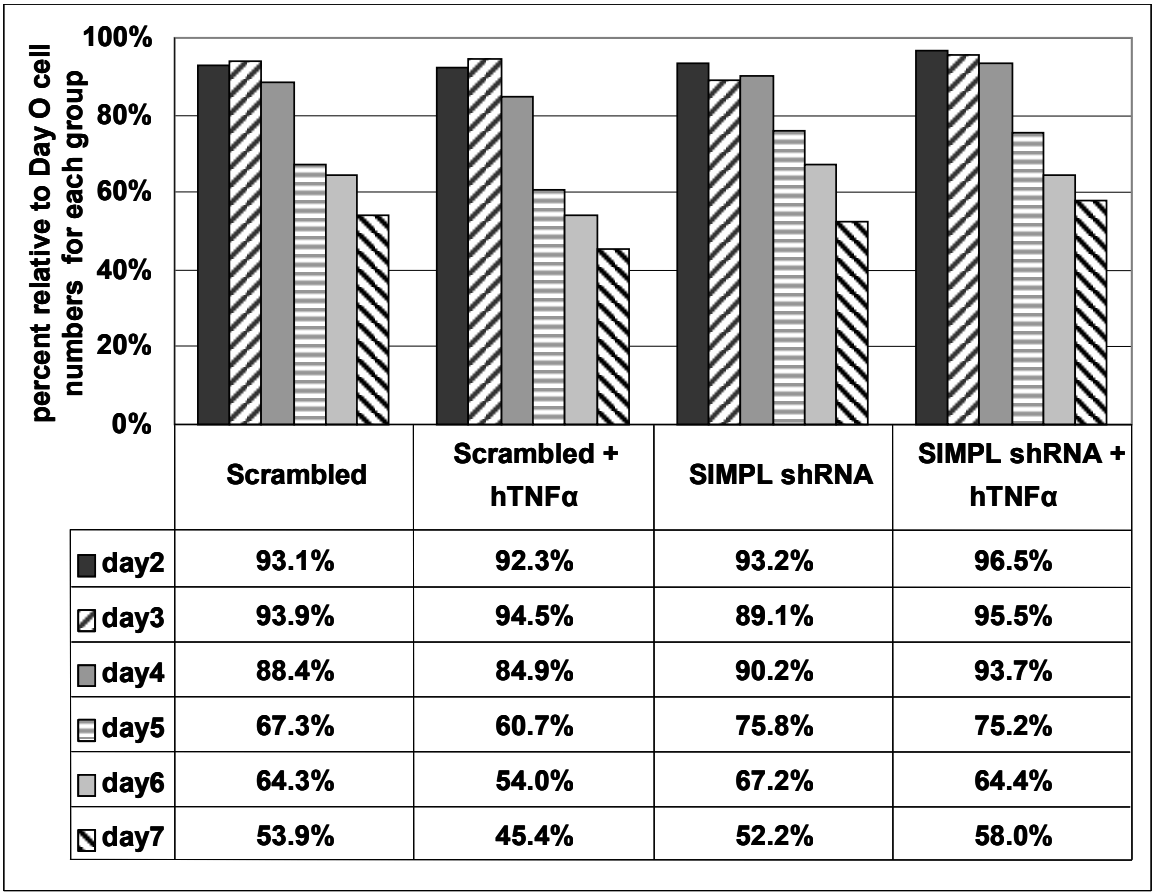
a. *Suspension cells*

C-kit$^+$ cells (140,000 cells/well) containing the shRNA (scramble or SIMPL) were placed in (6 well dishes; ~35 mm dish) conditions of differentiation with and without hTNFα (10 ng/mL). The liquid culture (3 ml total volume) consisted of: mSCF, 200 ng/mL; mGM-CSF, 10 ng/mL; mIL-3 (20 ng/mL); 5% PWMSCM, 20% FBS, IMDM, 1% glutamine, 1% pen/strep. Two days later, the suspension cells (200 μL) were collected and analyzed by FACS. The remaining cells were analyzed everyday for 6 days.

Over the course of 7 days, no increase of apoptosis occurred in the c-kit$^+$ cells containing SIMPL shRNA as compared to scrambled shRNA containing c-kit$^+$ cells in the presence or absence of the maximum dose of hTNFα (10 ng/mL) (Figure 29).

b. *Total cells (adherent and suspension cells)*

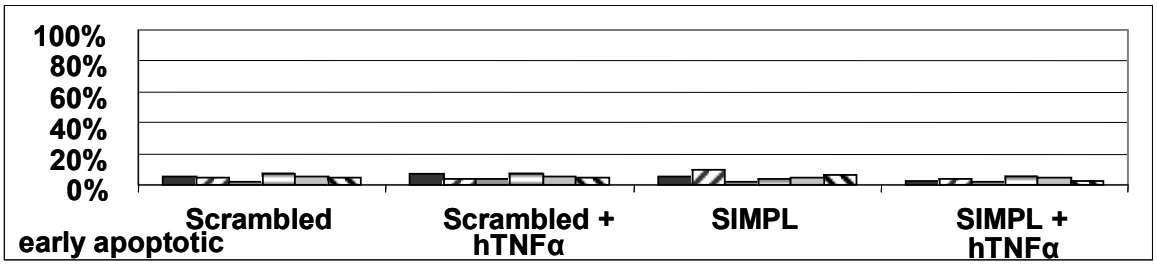
C-kit$^+$ cells containing scrambled shRNA or SIMPL shRNA (100,000 cells/ well) were put in (2 x 6 well dishes; ~35 mm dish) conditions of differentiation with mIL-3, mGMCSF, mSCF, PWMSCM, 1% penicillin/streptomycin, 1% glutamine, 20% FBS, and with or without hTNFα (5ng/mL). Twenty-four hours after plating all cells were collected and were analyzed by FACs 48 hours laters the same procedure was repeated on the second set of cultures.

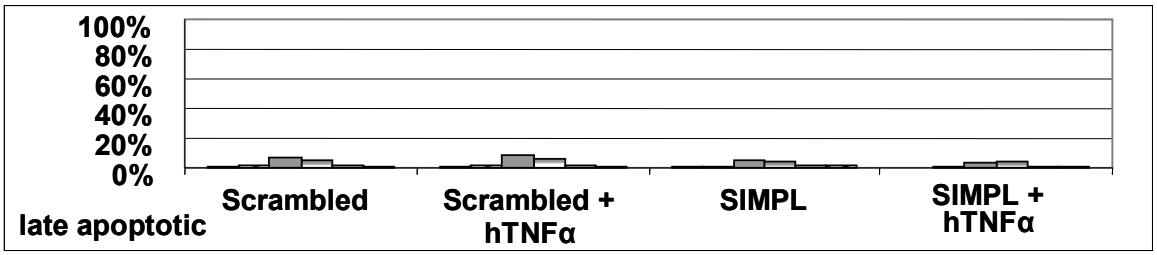


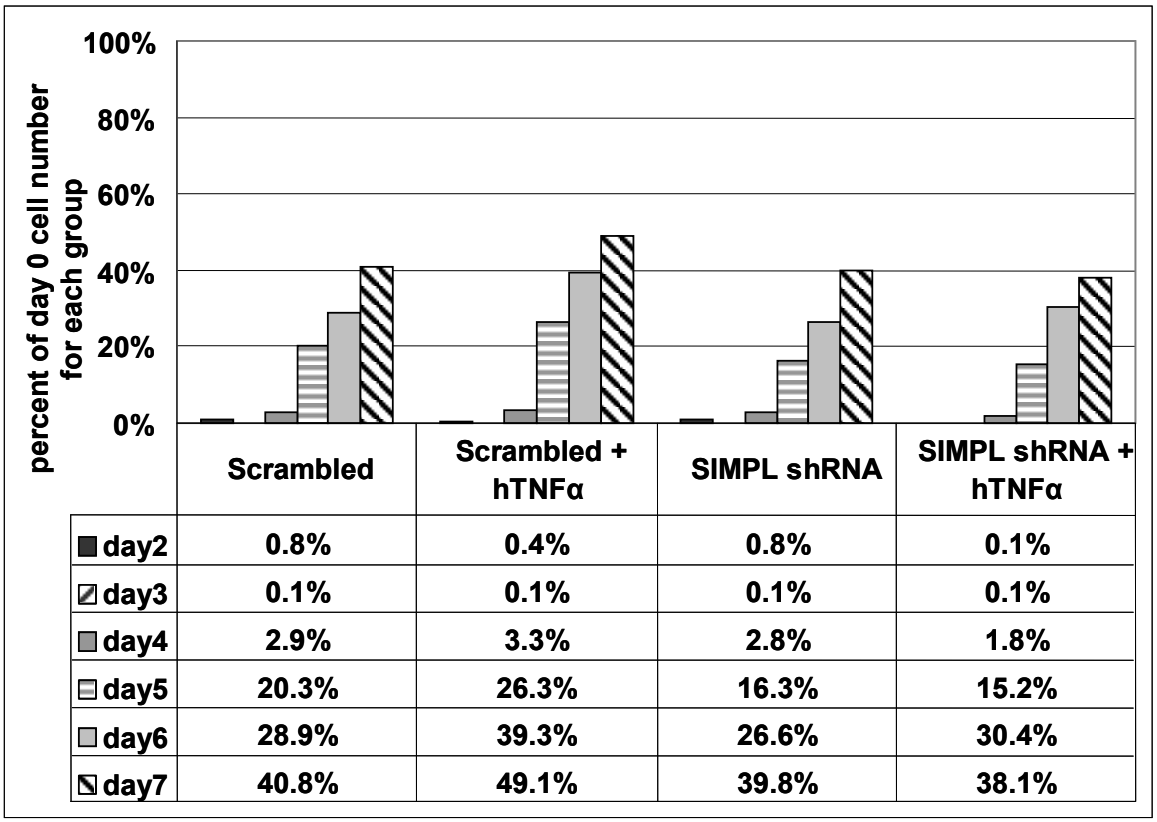
	Scrambled	Scrambled + hTNFα	SIMPL shRNA	SIMPL shRNA + hTNFα
day2	93.1%	92.3%	93.2%	96.5%
day3	93.9%	94.5%	89.1%	95.5%
day4	88.4%	84.9%	90.2%	93.7%
day5	67.3%	60.7%	75.8%	75.2%
day6	64.3%	54.0%	67.2%	64.4%
day7	53.9%	45.4%	52.2%	58.0%

non-apoptotic

Figure 29. No apparent difference in apoptosis on c-kit$^+$ SIMPL shRNA containing cells compared to c-kit+ scramble containing cells. Scrambled and SIMPL shRNA c-kit$^+$ cells (~140,000 cells in 3 mL) were plated in wells containing mIL-3 (20 ng/mL), mGMCSF (10 ng/mL), mSCF (100 ng/mL), PWMSCM, penicillin/streptomycin (1%), glutamine (1%), FBS (20%), and hTNFα (10 ng/mL). Suspension cells (150 µL) were taken each day and labeled with APC conjugated annexin V and 7-AAD, and analyzed by Flow cytometry. n = 1







necrosis

Figure 29. (Contiuned) No apparent difference in apoptosis on c-kit$^+$ SIMPL shRNA containing cells compared to c-kit$^+$ scramble containing cells.

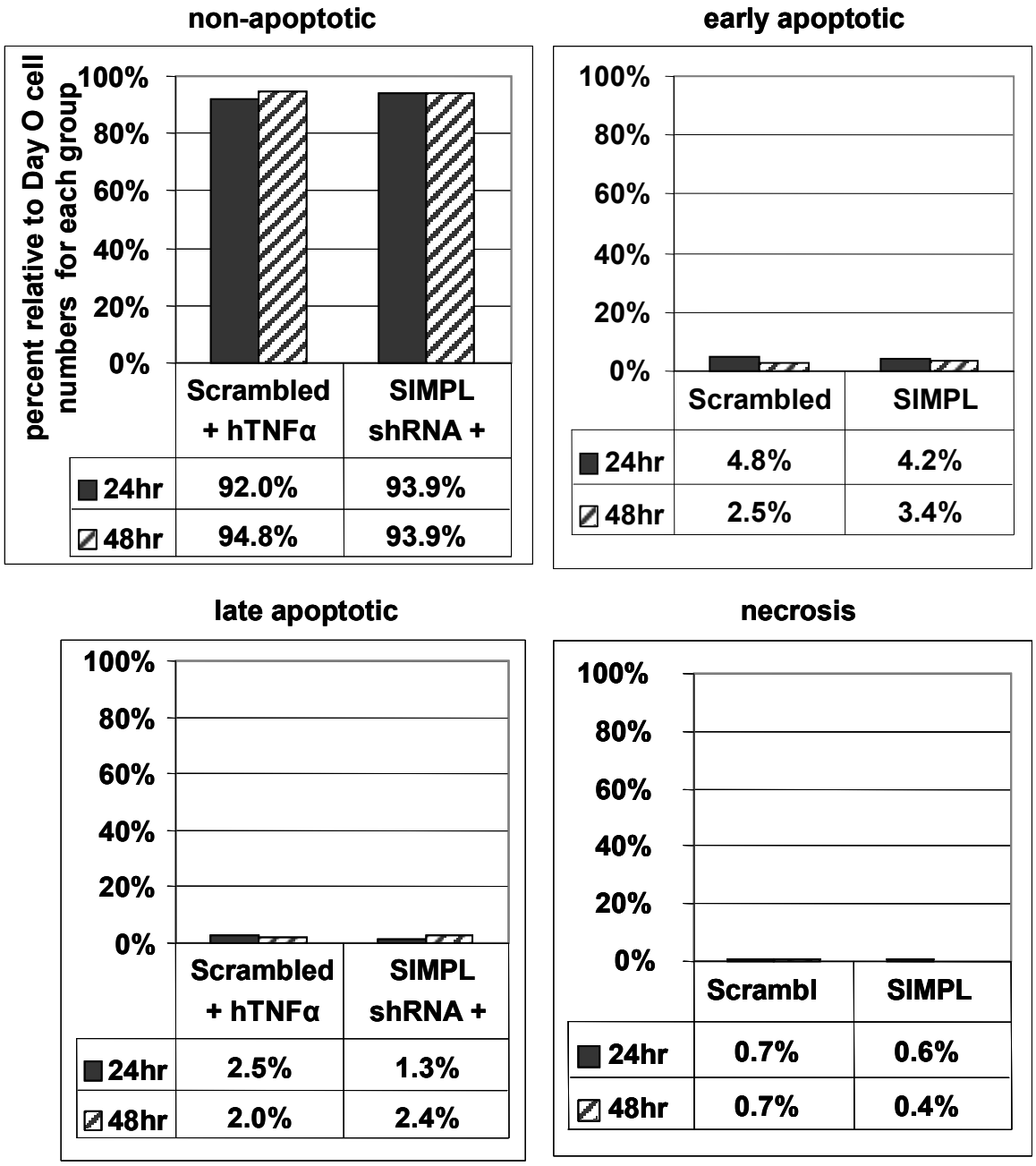


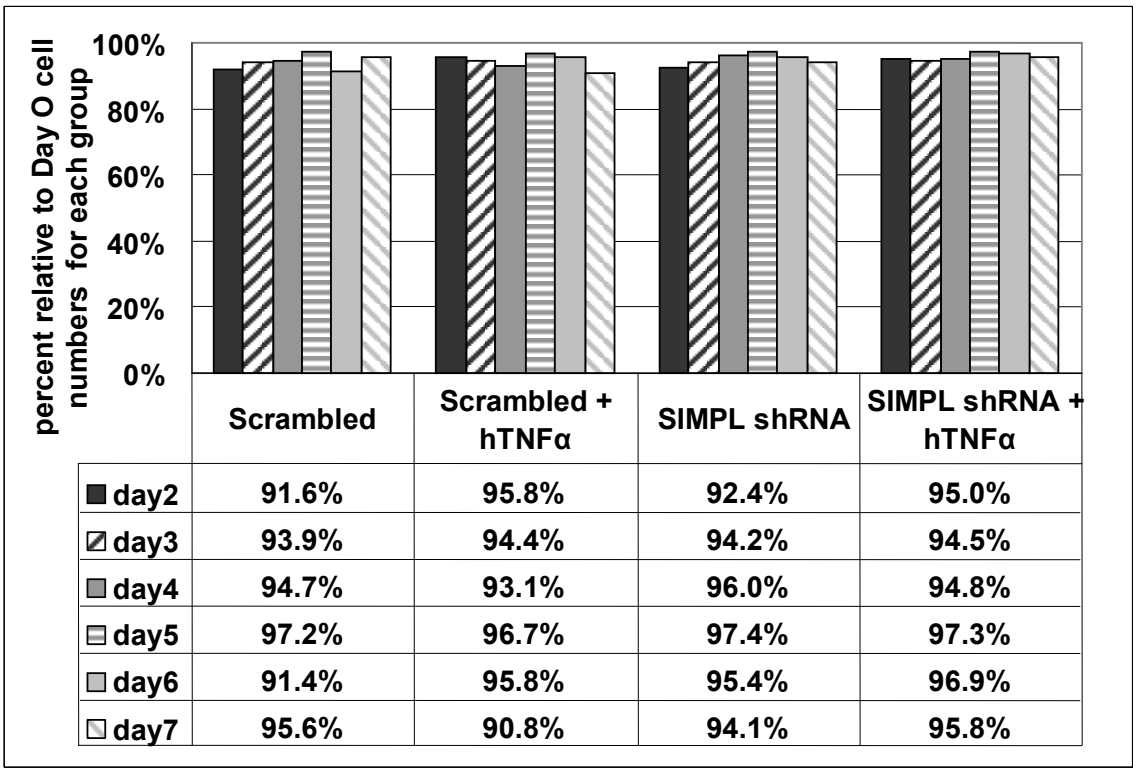
Figure 30. No apparent difference in apoptosis on c-kit$^+$ SIMPL shRNA containing cells compared to c-kit$^+$ scramble containing cells. Scrambled and SIMPL shRNA c-kit$^+$ cells (100,000 cells) were plated in wells containing mIL-3, mGMCSF, mSCF, PWM penicillin/streptomycin, glutamine, FBS, and hTNFα (5ng/mL) The cells were scraped from wells, stained with APC conjugated annexin V and 7-AAD, and analyzed by Flow cytometry. n = 1

Over the course of 48 hours, the c-kit$^+$ cells containing SIMPL shRNA did not have an increase in apoptosis as compared to the scrambled shRNA containing c-kit$^+$ cells in the presence or absence of hTNFα (5 ng/mL) (Figure 30).

Therefore, neither suspension nor adherent with suspension cells showed an increase in apoptosis caused by activation of TNF-RI by hTNFα.

2. *No increase in apoptosis of LDMNCs with diminished SIMPL supplied with cytokines that allow only proliferation/expansion*

The next logical step was to examine if differences in apoptosis may occur at the level HPC maintainence (proliferation/expansion with blockage of differentiation (Figure 31). C-kit$^+$ cells containing scrambled or SIMPL shRNA (140,000 cells in 3 mL) were plated in wells (2 x 6 well dishes; ~35 mm dish) containing mSCF (100 ng/mL), and hIL-6 (25 ng/mL) 1% penicillin/streptomycin, 1% glutamine, 20% FBS, and hTNFα (10 ng/mL). Over the course of 7 days, no increase of apoptosis occurred in the c-kit$^+$ cells containing SIMPL shRNA did not have an increase in apoptosis over scrambled shRNA containing c-kit$^+$ cells in the presence or absence of the maximum dose of hTNFα (10 ng/mL). Therefore, neither a mix of differentiation cytokines, or a mix low density monuclear cell (LDMNC) proliferation cytokines caused increased apoptosis of SIMPL shRNA ckit$^+$ over scramble shRNA c-kit$^+$ cells.



	Scrambled	Scrambled + hTNFα	SIMPL shRNA	SIMPL shRNA + hTNFα
day2	91.6%	95.8%	92.4%	95.0%
day3	93.9%	94.4%	94.2%	94.5%
day4	94.7%	93.1%	96.0%	94.8%
day5	97.2%	96.7%	97.4%	97.3%
day6	91.4%	95.8%	95.4%	96.9%
day7	95.6%	90.8%	94.1%	95.8%

non-apoptotic

Figure 31. No apparent difference in apoptosis on c-kit$^+$ SIMPL shRNA containing cells compared to c-kit$^+$ scramble containing cells in pre-stimulation conditions.

Scrambled and SIMPL shRNA c-kit$^+$ cells (140,000 cells in 3 mL) were plated in wells containing mSCF (100 ng/mL), and hIL-6 (25 ng/mL) (pre-stimulation condition, penicillin/streptomycin, glutamine, FBS (20%), and hTNFα (10 ng/mL). Suspension cells (150 μL) were taken each day and labeled with APC conjugated annexin V and 7-AAD, and analyzed by Flow cytometry. Biological n = 1

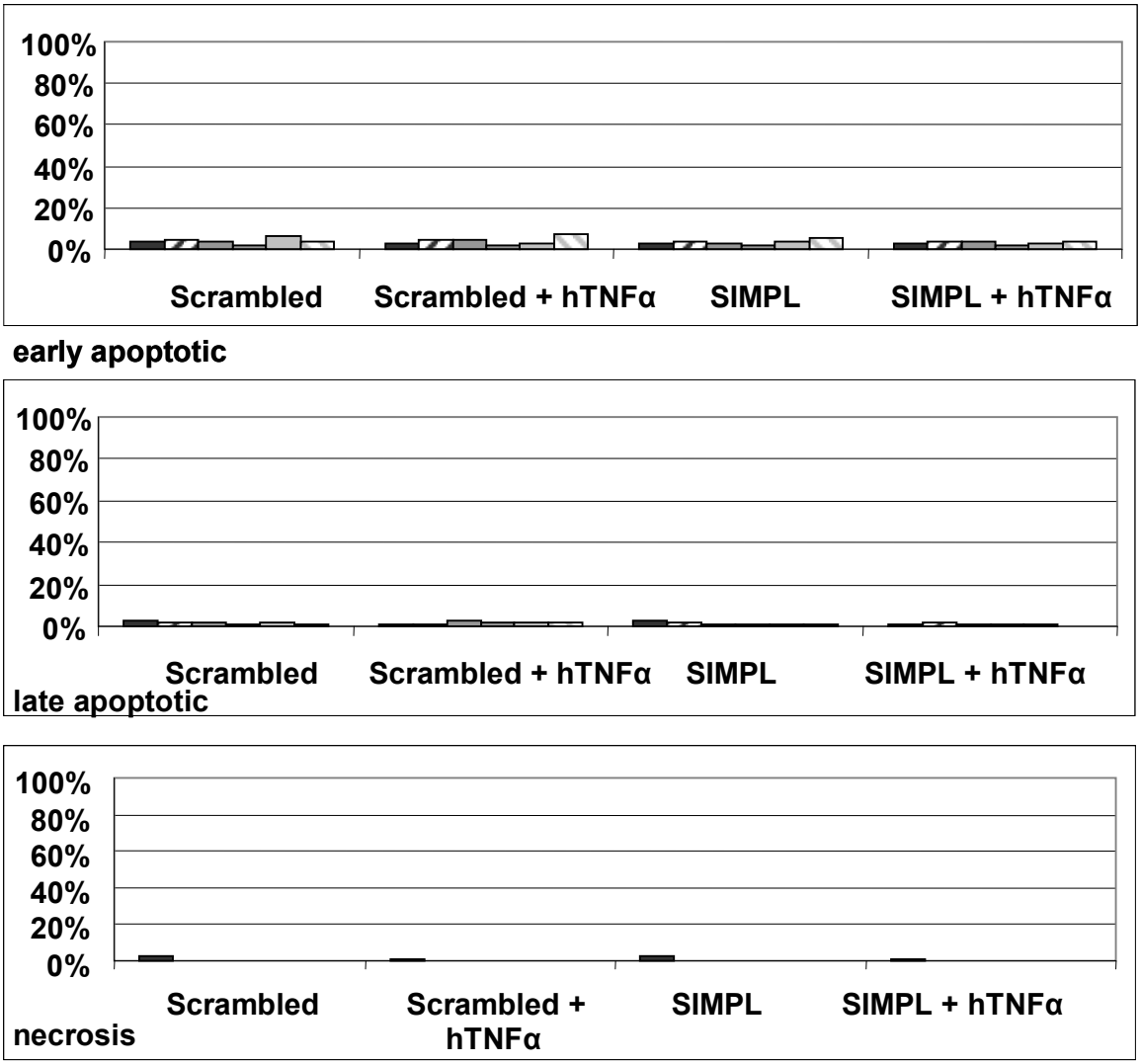


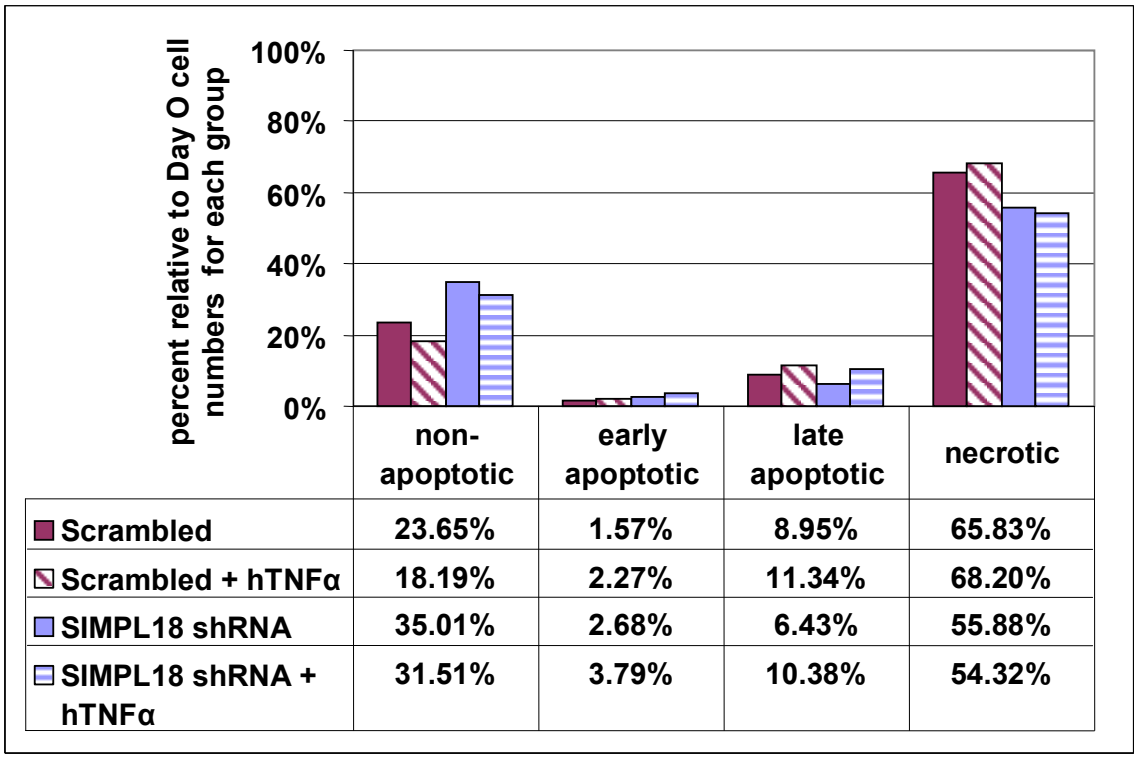
Figure 31. (Continued) No apparent difference in apoptosis on c-kit$^+$ SIMPL shRNA containing cells compared to c-kit$^+$ scramble containing cells in pre-stimulation conditions.

3. No increased apoptosis of LDMNCs with loss of SIMPL in SFM used to induce apoptosis

A positive induction of cell death was needed to show if stress caused an increase of apoptosis in the SIMPL shRNA c-kit$^+$ cells. C-kit$^+$ cells expressing either scrambled or SIMPL shRNA (100,000 cells) were plated in wells (2 x 6 well dishes; ~35 mm dish) containing serum free media, BSA (0.5%) , penicillin/streptomycin, glutamine with hTNFα (10 ng/mL) for 48 hours (Figure 32). After 48 hours, there were no change in apoptosis, or necrosis between SIMPL shRNA and scramble groups.

F. C57BL-6 mice transplanted with HSCs with knock down levels of SIMPL have TNFα induced sensitivity

Lethally irradiated mice were transplanted with c-kit$^+$/shRNA (SIMPL or scramble) containing cells. Over the course of a month, 22 mice were transplanted with the expectation of having at least 6 mice from each group (SIMPL or scramble shRNA) survive 4 months post transplantation. Six mice were analyzed for each of the various categories examined. Categories examined were bone marrrow number and cellularity (lin, sca, c-kit and gr-1 and Mac-1). Along with colony formation potential of the bone marrow and spleen in the presence and absence of mTNFα was explored. The ability of whole bone marrow and c-kit$^+$ cells to repopulate the spleen called colony forming unit spleen (CFU-S) was also examined, but was not successful. Another category was apoptotic state of the bone marrow of SIMPL compared to scramble shRNA.



	non-apoptotic	early apoptotic	late apoptotic	necrotic
Scrambled	23.65%	1.57%	8.95%	65.83%
Scrambled + hTNFα	18.19%	2.27%	11.34%	68.20%
SIMPL18 shRNA	35.01%	2.68%	6.43%	55.88%
SIMPL18 shRNA + hTNFα	31.51%	3.79%	10.38%	54.32%

Figure 32. No apparent difference in apoptosis on c-kit$^+$ SIMPL shRNA containing cells compared to c-kit$^+$ scramble containing cells in serum free media.
Scrambled and SIMPL shRNA c-kit$^+$ cells (100,000 cells) were plated in wells containing serum free media containing BSA (0.5%), penicillin/streptomycin, glutamine with hTNFα (10 ng/mL). The cells were scraped from wells (total cell population) after 48 hours, stained with APC conjugated annexin V and 7-AAD, and analyzed by Flow cytometry. n = 1

1. Significant decreases in the growth of bone marrow progenitors in SIMPL knockdown group versus scrambled control in a TNFα concentration dependent manner.

Whole bone marrow was flushed from 1 femur, 2 tibia, and 2 pelvic flatbone from all mice. 50,000 cells/mL were plated into the colony assay along with mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL) and mTNFα (0, 1, 5, 10 ng/mL). Colony formation is reported as percent change from control (no mTNFα). There was a significant reduction in colony formation of cells isolated from animals that received the SIMPL shRNA in a mTNFα concentration dependent manner. CFU-GM colony formation from mice transplanted with c-kit$^+$ cells containing SIMPL shRNA were significantly decreased at 5 and 10 ng/mL mTNFα compared to CFU-GM colony formation from mice transplanted with c-kit$^+$ cells containing scrambled shRNA. Also, total colony formation from mice transplanted with c-kit$^+$ cells containing scramble shRNA was not significantly inhibited at 10 ng/mL mTNFα, which was similar to *in vitro* results (Figures 33-35).

2. No change in the number of colonies formed without the addition of mTNFα in the SIMPL compared scrambled shRNA groups.

When the colony growth was compared in the absence of mTNFα in the SIMPL shRNA transplant and scrambled transplant groups, no change in the potential of colony formation was found (Figure 36). This was also seen *in vitro* (Figure 28).

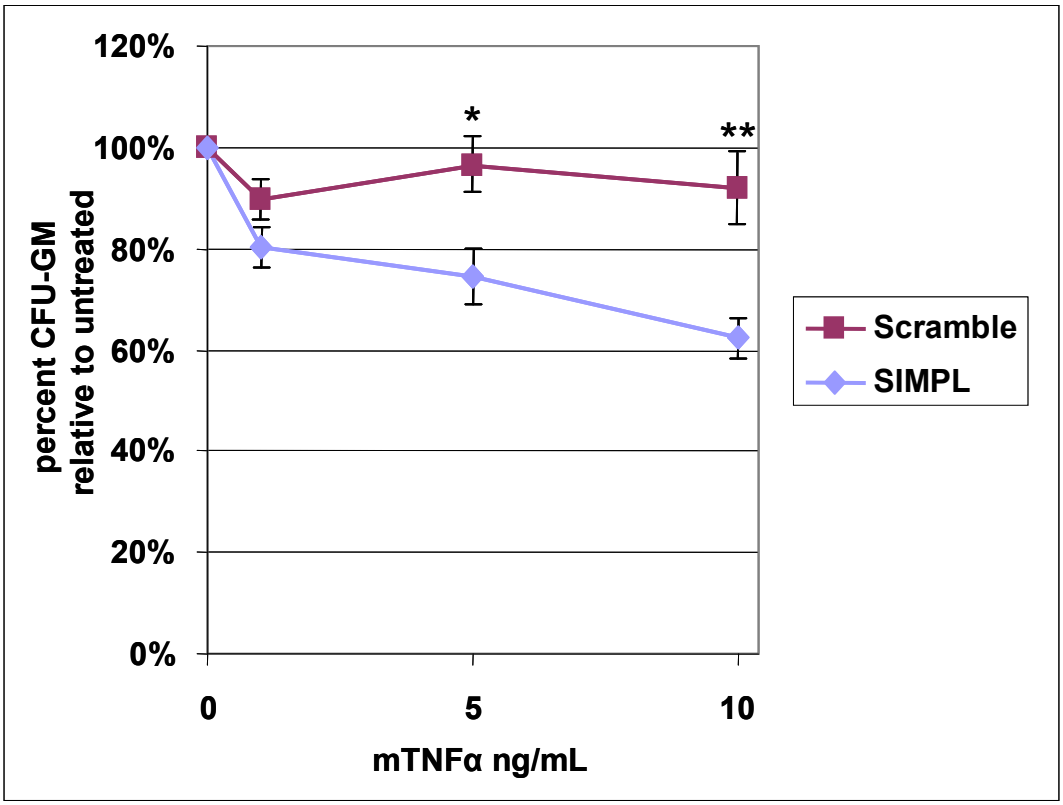


Figure 33. SIMPL shRNA whole bone marrow colonies showed significant difference from scrambled shRNA control at 5 and 10 ng/mL mTNFα.
Scrambled and SIMPL shRNA cells (50,000 cells/mL) were plated into a colony assay containing mGM-CSF, mIL-3, mSCF with a concentration gradient of mTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of mTNFα by the total colonies at the given concentration mTNFα for each group. P-values were from Scrambled compared to SIMPL. n = 6; *p<0.02 **p<0.04

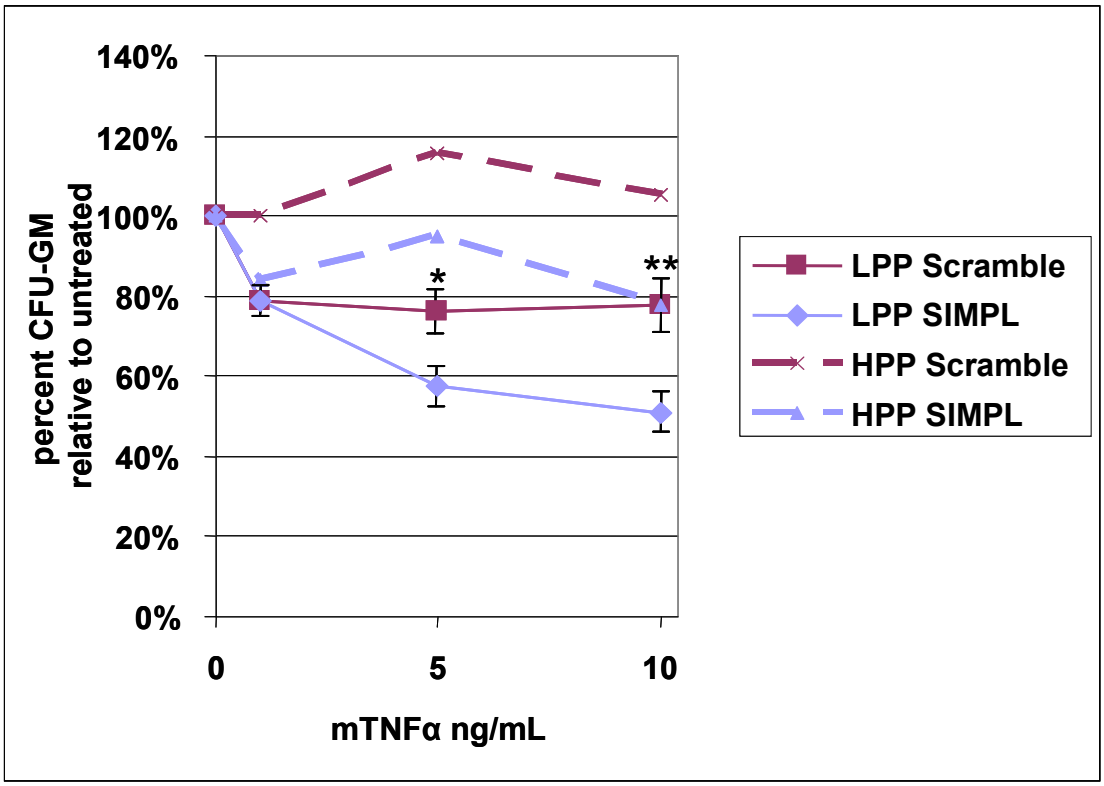


Figure 34. SIMPL shRNA whole bone marrow colonies showed significant difference from scrambled shRNA control at LPP 5 and 10 ng/mL mTNFα.

Scrambled and SIMPL shRNA cells (50,000 cells/mL) were plated into a colony assay containing mGM-CSF, mIL-3, mSCF with a concentration gradient of mTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies at a given concentration mTNFα into 0 ng/mL mTNFα colony number for each group. P-values were from scrambled LPP or HPP compared to SIMPL LPP or HPP colonies, respectively. n = 6; *p<0.04 **p<007

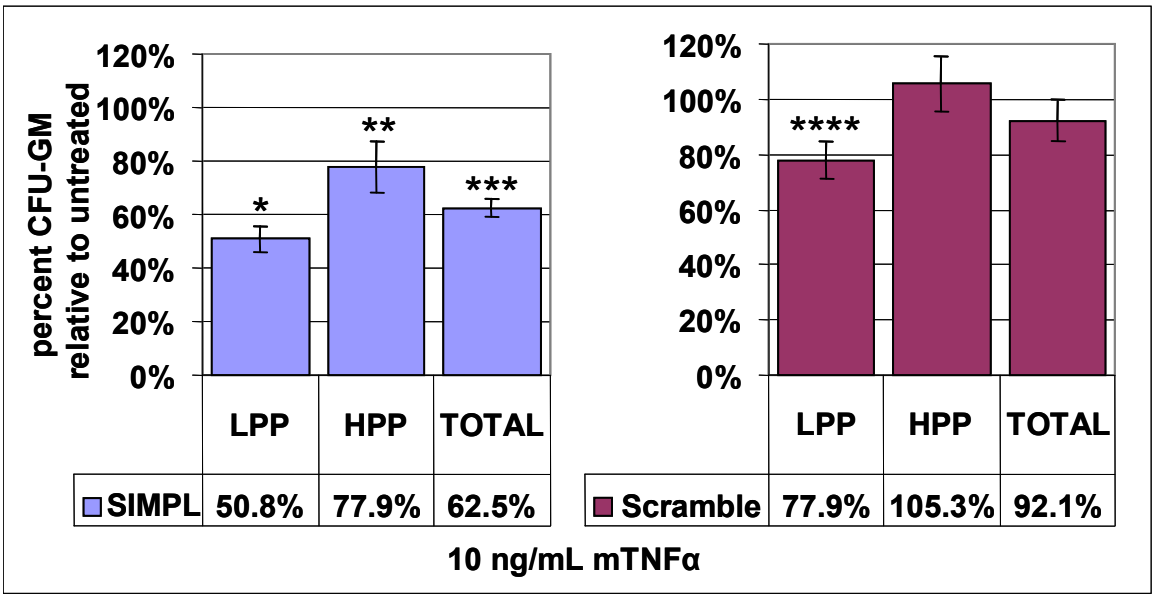


Figure 35. SIMPL LPP, HPP and Total whole bone marrow had significant inhibition by mTNFα while scramble whole bone marrow only LPP was significantly inhibited, Comparing SIMPL or Scramble 0 ng/mL to 10 ng/mL to determine if any inhibition occurred with mTNFα with no PWM.
Scrambled and SIMPL shRNA cells (50,000 cells/mL) were plated into a colony assay containing mGM-CSF, mIL-3, mSCF with a concentration gradient of mTNFα. Percent of CFU-GM relative to untreated determined by dividing the total number of colonies in the absence of mTNFα by the total colonies at the given concentration mTNFα for each group. One tailed TTEST comparing within SIMPL or Scramble LPP, HPP, total CFU-GM colony formation comparing 0 ng/mL to 10 ng/mL mTNFa *p<4x10-7 **p<0.03 ***p<5x10-7 ****p<0.004

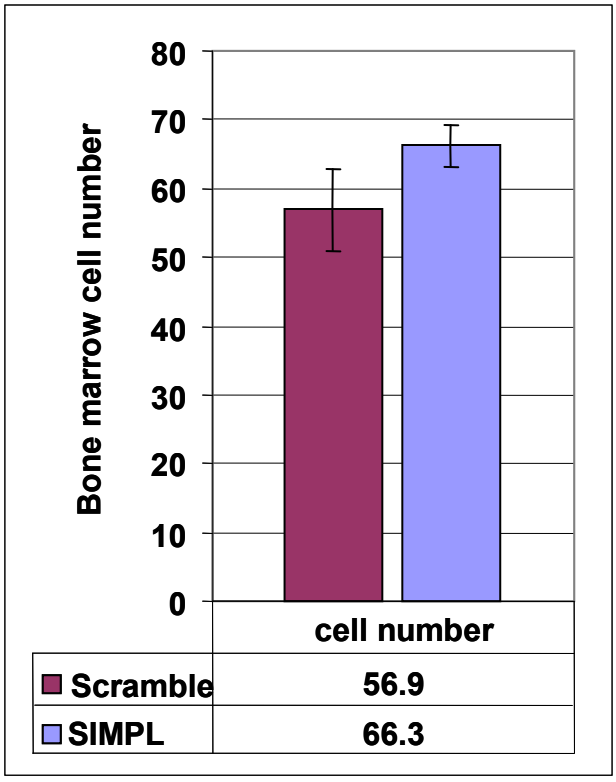


Figure 36. SIMPL and scramble whole nucleated bone marrow had no significant change in the total number of cells.
Whole bone marrow containing scrambled and SIMPL shRNA cells were flushed from the tibia, femur, and pelvis. The combined nucleated bone marrow cell population was counted. Biological n = 6

3. Number of cells in the bone marrow, and type of cells from mice with SIMPL shRNA were similar to control.

As seen in Table 3, the total number of bone marrow cells and cellularity were the same in the SIMPL and scramble shRNA groups. Cellularity was tested by taking 200,000 whole bone marrow cells for each mouse. 100,000 cells were combined with a flurochrome (APC) linked lineage cocktail, PE Cy7 linked Sca-1, and PE-linked c-kit and analzyed by fluorescence activated cell analysis. A separate 100,000 cells was combined with fluorochrome linked Gr-1, and Mac-1, which are markers for granulocytes (neutrophils, eosonophils, basophils) and macrophages respectively. In neither groups were there statistically significant changes in HSC ($lin^-sca^+ckit^+$) or in HPCs ($lin^-sca^-ckit^+$). Differentiated hematopoietic markers Gr-1 and Mac-1 revealed no statistically significant changes amoung the two groups.

4. SIMPL knockdown underwent a slight, but significantly decreased amount of early apoptosis.

The SIMPL knock down whole bone marrow cells demonstrated slight but significant decrease in early apoptsis compared to scramble shRNA whole bone marrow. 100,000 whole bone marrow cells were combined with annexin V and 7-AAD and analyzed by FACs. Late apoptosis, necrosis and non-apoptotic populations were not significantly different (Figure 37).

	Scramble		*SIMPL*	
Fraction	***% of BM***	***SEM***	***% of BM***	***SEM***
Lin^- Only	3.660	0.651	5.410	1.979
Lin^-Sca^+	0.468	0.060	0.422	0.109
$Lin^-c\text{-}kit^+$	0.260	0.054	0.390	0.087
$Lin^-Sca^+c\text{-}kit^+$	0.203	0.134	0.047	0.010
Lin^- Total	4.592	0.751	6.268	1.980

	Scramble		*SIMPL*	
Fraction	***% of BM***	***SEM***	***% of BM***	***SEM***
$MAC\text{-}1^+$	1.878	0.129	1.757	0.131
$GR\text{-}1^+$	14.648	1.542	13.107	1.904
$MAC\text{-}1^+/GR\text{-}1^+$	33.437	1.959	26.002	4.727

Table 3. No change in bone marrow cellularity comparing mice transplanted with scramble or SIMPL shRNA.
Total bone marrow of 4 month old mice transplanted with shRNA was analyzed by FAC sorting as explained in Methods.

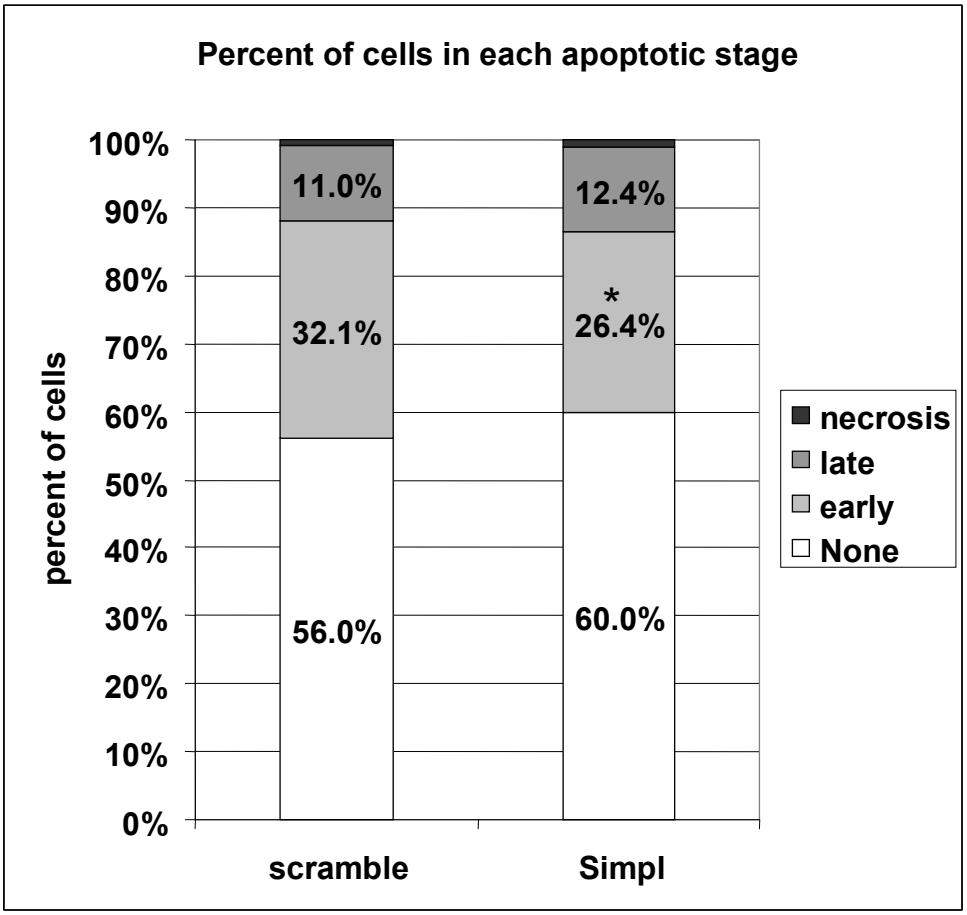


Figure 37. No apparent difference in apoptosis on SIMPL shRNA containing cells compared to scramble containing cells.
Apoptosis assay of whole bone marrow cells containing either SIMPL or scramble shRNA. P-values were from Scrambled compared to SIMPL with in same apoptotic groups. n = 6; *P<0.03

5. One outlier in the SIMPL shRNA group had expanded HSC and immature precursor populations with decreased apoptosis (data not shown)

This outlier could possibly represent HSCs that lack the ability to differentiate, but still have the ability to expand. Thus, this might be a possible pre cancerous state. But with only one outlier, these are only speculations.

6. GFP equally shutoff in SIMPL and scramble groups

The GFP gene (ZsGreen) on pSIREN retroviral plasmid is under the control of a different promotor (CMV) than the shRNA construct (Pol III) Over long term expression of GFP as in this transplant, it was not unexpected for GFP to be silenced. GFP silencing does not necessarily have effect on the shRNA expression. The SIMPL and scramble shRNA transplant groups both had significant loss of GFP protein levels as compared to a non-GFP control analyzed by FACs. The two groups compared together showed no difference in the amount of GFP silencing between SIMPL and scramble shRNA whole bone marrow (Figure 38).

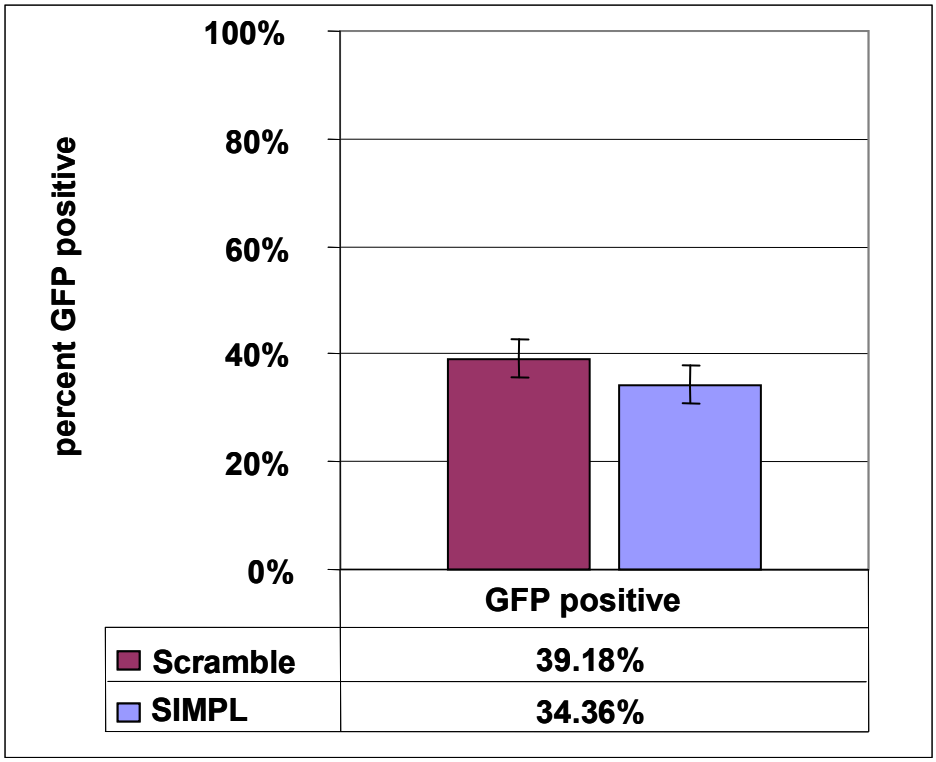


Figure 38. SIMPL and scramble whole nucleated bone marrow had no significant change in % GFP positive cells. Whole bone marrow containing scrambled and SIMPL shRNA cells were flushed from the tibia, femur, and pelvis. Results were analzyed by FACS biological n = 6

IV. Discussion

The long term objective of my thesis work was to determine if inhibition of SIMPL is a viable option for treatment of hematopoietic disorders. To test this hypothesis, it was necessary to determine SIMPL's role in myelopoiesis. Through changes in red blood cell development seen in Zebra fish with decreased SIMPL levels, it was clear SIMPL plays a part in steady state erythropoiesis and presumably hematopoiesis. To understand the role of this p65 specific co-activator, SIMPL, in hematopoiesis, a thorough review of its two main components (TNFα and p65) was necessary. Selection of TNFα was needed because it initiates the SIMPL specific signaling cascade, and p65 is a direct effecter of TNFα and is directly linked to SIMPL. The introduction reviewed TNFα and p65's involvement in every stage of progression in hematopoiesis; my data will prove SIMPL's involvement in myelopoiesis and connects it to the previous evidence presented. The discussion below highlights the potential for loss of SIMPL to diminish a dysregulated TNFα response without inhibiting normal functions of myelopoiesis.

A. Myelopoietic, CFU-GM, colonies with loss of SIMPL behave similarly to CFU-GM exposed to high dose TNFα

Loss of SIMPL sensitizes CFU-GM to TNFα. CFU-GM colony formation is diminished in a dose dependent manner with increasing TNFα concentrations [36, 37, 43]. CFU-GM with decreased amounts of SIMPL, after *in vitro* introduction of SIMPL shRNA in c-kit$^+$ cells, were also inhibited by increasing doses of mTNFα, and further, the colony formation was inhibited significantly more than in response to mTNFα alone,

42.6% to 79.3% respectively (Figure 21). Thus, the loss SIMPL sensitizes the myelopoietic cells to TNFα, acting like high dose TNFα. Analysis of mouse bone marrow reconstituted with SIMPL shRNA for ~5 months represents *in vivo* exposure to diminished SIMPL levels (Figure 33). After harvesting the bone marrow, CFU-GM colony formation from c-kit$^+$ cells with decreasesd SIMPL levels were still inhibited in a mTNFα dose dependent manner compared to mTNFα alone (62.5 to 92% respectively). These results contend that inhibition of SIMPL acts like high dose TNFα. This may seem counter intuitive for use as a therapy for a dysregulated TNFα disorder, like AML. However, loss of SIMPL restores normal TNFα function. In AML, like other cancers [59, 64], the anti-apoptotic transcription factor p65/50 is constitutively active. High dose TNFα usually inhibits the NF-κB pathway by activating expression of inhibitors of p65/p50 (IkB). However in a dysregulated response this inhibition will classically be blocked by: constitutively activated IKKβ, mutation of NF-κB, and/or IκBα [59, 64]. SIMPL is a gatekeeper that acts as a co-activator downstream of the putative sites of mutation when diminished, inhibits up regulation of NF-κB [77]. Thus, inhibiting TNFα induced anti-apoptotic signaling, apoptosis occurs because of the cumulative mutations that cancer cells accrue [52, 59, 78, 79]. However, this does not explain the selective advantage of knocking down SIMPL over anti-TNFα therapies.

B. Loss of SIMPL allows for normal CFU-GM colony formation in the absence of TNFα highlighting the advantage of pathway specific therapy

Reduction of SIMPL in c-kit$^+$ cells alone (i.e. in the absence of TNFα) does not significantly inhibit CFU-GM colony formation as compared to scrambled control

(Figure 28). When 13 separate experiments with SIMPL shRNA compared to scrambled control weere pooled no significant difference in CFU-GM colony number, size, or proliferation potential was detected. This result is similar to CFU-GM derived from TNF-RI-/- mice; in the absence of TNFα stimulation, there are no significant differences in CFU-GM as compared to control [49]. Thus, CFU-GM colonies with diminished SIMPL levels maintained basal NF-κB activity allowing for colony formation. This highlights the advantage of SIMPL inhibition over anti-TNFα therapies. In the face of a dysregulated TNFα pathway mentioned above, anti-TNFα inhibits multiple TNFα pathways needed for normal hematopoieitc function including ERKS, JNK and p38 [60, 80-82]. Highlighting the complex nature of TNFα, HSCs isolated from TNFα-/- mice produce 3-fold higher amounts of CFU-GM colonies compared to wildtype [48]. The loss of SIMPL, allows for basal activity of NF-κB in non-cancerous cells and functioning of the other TNFα signaling pathways. *In vivo*, loss of SIMPL did not effect HSC, HPC, nor mature differentiated myelopoietic cells, as revealed in the SIMPL shRNA mouse bone marrow transplant experiments (Table 3). This is in contrast to the TNFα-/- and TNF-RI-/- results; which revealed lineage with increased levels of the HSC, and HPCs in the bone marrow [36, 48, 49]. Therefore, in concurrence with TNF-RI-/- data, loss of SIMPL does not inhibit normal CFU-GM colony formation in the absence of TNFα, and unlike either TNFα-/- or TNF-RI, knock down of SIMPL did not effect bone marrow formation. This demonstrates a select advantage of knocking down SIMPL over anti-TNFα therapy, the latter affects bone marrow formation and lineage as shown from the TNFα-/- results.

C. Similar to high dose TNFα, decrease CFU-GM formation from loss of SIMPL is not caused by apoptosis

TNFα induces apoptosis when there is inhibition of the TNF-RI to p65/p50 pathway which does not include SIMPL (Figure 1). To induce apoptosis by TNFα requires the addition of protein synthesis inhibitors (cycloheximide) or a proteasome inhibitors (MG132 or lactacystin) to block production of anti-apoptotic factors or IκBα breakdown respectively [73, 83, 84]. Normal TNFα activation does not cause apoptosis, and similarly, loss of SIMPL alone does not induce apoptosis more than control cells exposed to TNFα (Figures 29-32). Our data show a trend that independent of the permutation (including changes in time exposed to TNFα, dose of TNFα, or cytokine mixture) loss of SIMPL did not cause apoptosis. Thus, inhibition of colony formation seen in cells containing lower SIMPL protein levels is through cell cycle arrest. Others have shown that TNFα inhibits colony formation by cell cycle arrest at G1/S phase [36].

In data not shown, loss of SIMPL on myelopoietic cell proliferation was explored but the results were inconclusive. In these experiments, known numbers of c-kit+ only or c-kit+ cells containing SIMPL shRNA were plated, and the change in growth was compared to scrambled shRNA containing c-kit+ cells in the presence or absence of hTNFα using non-transduced c-kit+ cells as a competitive control. Under these conditions, the SIMPL shRNA containing c-kit+ cells could be compared in the presence or absence of hTNFα to the scrambled results. Unfortunately, the results of these experiments were inconsistent, and many additional replicates are needed for any solid conclusions to be drawn. The preliminary data suggest that the growth of cells expressing the SIMPL shRNA, maintained under proliferative conditions (hIL-6 and

mSCF) was inhibited to a greater extent than cells expressing the scrambled shRNA. However, these conditions were not reproduced consistently. Therefore, we only confirmed that CFU-GM colony growth inhibition by c-kit$^+$ cells containing SIMPL shRNA was not from apoptosis, and only, highly likely caused by cell cycle arrest. Thus, we next focused on which TNFα receptors mediated the response.

D. CFU-GM colony inhibition from the loss of SIMPL requires both TNF-RI and TNF-RII

CFU-GM colony formation from c-kit$^+$ cells with diminished SIMPL levels was not significantly inhibited by hTNFα, the TNF-RI specific activator in mice, over control (Figure 17). Thus signaling through the TNF-RI receptor alone does not allow inhibition of colony growth when SIMPL is knock downed. This result was confirmed in two additional experiments. One, significant inhibition of CFU-GM colony growth was seen in the SIMPL shRNA c-kit$^+$ cells treated with mTNFα, which binds both TNF-RI and TNF-RII. Two, when SIMPL shRNA cells are grown with hTNFα and poke weed mitogen spleen conditioned media (PWMSCM) [see review below], CFU-GM with loss of SIMPL were significantly inhibited (20.7% to 84.5% control). This highlights that TNF-RI alone does not cause inhibition and that the addition of a TNF-RII activator is necessary. However, the data reveals that only a small amount of TNF-RII activation is necessary if TNF-RI is activated by hTNFα. In cells derived from TNF RI-/- mice, colony formation is not inhibited with addition of mTNFα (TNF RI and TNF RII activator) [36]. Unlike TNF-RI-/- bone marrow, TNF-RII-/- bone marrow has a similar hematopoietic makeup to wildtype mice [49]. Highlighting that TNF-RII alone does not

cause inhibition. Additionally, culturing wild type mouse hematopoietic progenitors under proliferation/differentiation assay conditions in the presence of hTNFα (TNF-RI specific activator in mice), has no effect upon CFU-GM colony proliferation [51]. My data (Figure 13 compared to Figure 15) illustrates that loss of SIMPL sensitizes the TNF-RI receptor. Note in Figure 15, TNF-RI and TNF-RII activation by mTNFα, mediates a significant reduction of CFU-GM in both groups. However, TNF-RI activation with PWMSCM causes a large reduction in CFU-GM colonies generated from c-kit$^+$ cells SIMPL knock down colonies that is not seen with control cells. This data with the addition of previous studies proves loss of SIMPL sensitizes CFU-GM to TNFα through TNF-RI, but TNF-RII is also required.

In review, PWMSCM contains a host of additional cytokines produced by T and B lymphocytes, macrophages, and stromal cells necessary for normal hematopoietic interactions. The interactions involve regulation and maintenance (checks and balances) of normal growth, survival, proliferation, and differentiation. The interactions and combinations of cytokines (additive, synergistic, inhibitory) and growth factors are still under intense investigation. The lack of knowledge on various interactions can be disadvantageous when exploring the role of an individual specific cytokine or growth factor. For example, taking a cytokine or growth factor out of a natural background can produce results not seen *in vivo* due to complex interactions. However, the biggest strength of PWMSCM is that it closely mimics an *in vivo* complement of cytokines. Thus, bone marrow cells treated with PWMSCM would likely see an array of cytokines that they may find in an *in vivo* setting. Thus, CFU-GM colony formation from c-kit$^+$ cells containing SIMPL shRNA is inhibited by TNFα through TNF-RI and notable TNF-RII

activation, but TGF-β1 and IFNγ, two additional pleotropic cytokines that inhibit hematopoiesis, are also found in PWMSCM [74-76].

E. Neither TGF-β1 nor IFNγ sensitizes CFU-GM colonies with diminished SIMPL levels

CFU-GM colony formation of c-kit$^+$ cells containing knock down SIMPL was not significantly inhibited by either TGF-β1or IFNγ (Figures 22-24). However, both TGF-β1 and IFNγ did cause inhibition of colony growth shown in Figures 23 and 25 [74-76, 85]. Complex interactions in PWMSCM cannot be discounted. However, individually TGF-β1 and IFNγ do not cause inhibition CFU-GM numbers in the face of diminished SIMPL levels in a manner similar to that observed with TNFα. Additionally PWMSCM contains erythropoietin (EPO), GM-CSF, and G-CSF. The genes encoding these proteins are regulated by p65 through TNF-RI (see introduction).

F. EPO and G-CSF do not allow further inhibition of colonies with diminished SIMPL levels while GM-CSF highlights SIMPL's specificity to TNFα

The addition EPO or G-CSF with TNF-RI did not further reduce colony formation of the c-kit$^+$ cells containing SIMPL shRNA. In presence (Figures 26-27) and absence (data not shown) of PWMSCM the addition of EPO to CFU-GM assays performed with SIMPL shRNA c-kit$^+$ cells did not cause additional colony in the presence of hTNFα. Interestingly, this same experiment done in the presence of low oxygen tension (5% O_2), led to an increase in the inhibition of scramble shRNA containing colonies, but not to a level less than that observed for the SIMPL shRNA containing colonies (data not shown; biological n=1).

In mouse HPCs the addition of G-CSF as the only cytokine to a colony proliferation/differentiation assay allows TNF-RI activation to signal for a decrease in CFU-G (more differentiated than CFU-GM) colony number [43]. Thus, TNF-RI activation has cytokine specificity. So, G-CSF was added without PWMSCM in the hope that G-CSF plus hTNFα would inhibit CFU-GM colonies when cells were expressing the SIMPL shRNA. In data not shown, G-CSF (10 ng/mL) was added to the proliferation/differentiation colony assay without PWMSCM and with hTNFα and mSCF (biological n = 1) or with mSCF, mIL-3, and GM-CSF (biological n = 2). G-CSF alone produced no colonies (data not shown). Again, no difference of inhibition occurred between groups (SIMPL and scramble shRNA) and overall hTNFα did not cause inhibition at the highest concentration.

GM-CSF is important because GM-CSF up regulates its own transcription through activation of p65 (see introduction). In data not shown (n = 1), addition GM-CSF without TNFα in the presence or absence of PWMSCM did not cause a greater inhibition of colonies containing SIMPL shRNA compared to scramble. These trends support that SIMPL p65 co-activation is specific to TNFα pathway and not other activators of p65. Again highlighting TNF-RI and TNF-RII's requirement, additional cytokines, like G-CSF, did not allow activation ofTNF-RI only to induce colony inhibition in response to low levels of SIMPL.

V. Conclusion

Loss of SIMPL enhances TNFα dependent inhibition of colony proliferation in a direct dose-dependent manner. Additionally, loss of SIMPL alone still allows for normal CFU-GM colony formation most likely because of basal NF-κB activity. TNF-RI activation, by TNFα, dose-dependently inhibits colonies containing SIMPL shRNA, and this inhibition requires at least background activation of TNF-RII. Increasing concentrations of mouse TNFα (activates both TNF-RI and TNF-RII), as well as increasing doses of a TNF-RI activator with a set amount of TNF-RII activation, inhibits colonies containing SIMPL shRNA. This inhibition was not caused by apoptosis, and most likely caused by inhibition of proliferation through cell cycle arrest. Growth arrest due to loss of SIMPL is specific to TNFα, and is not observed in TGF-β1 or IFNγ treated cultures, the other two major inhibitors of hematopoiesis. Additionally, activation of p65 by a cytokine other than TNFα did not inhibit CFU-GM colonies with reduced SIMPL levels over control. These conclusions support the hypothesis that loss of SIMPL is potentially a better alternative than anti-TNFα therapies due to increased specificity. Specifically, my data supports that SIMPL is specific to the TNFα pathway. Thus, loss of SIMPL targets TNFα induced p65, allowing p65 activation by other pathways to continue. This would allow for diminished side effects. Next, SIMPL targets NF-κB downstream of the many pre-cancerous/cancerous mutations of the TNF-RI/NF-κB pathway [14, 77]. Finally, loss of SIMPL would still allow TNFα to signal through ERKS, JNK and p38, which should also allow for reduced side effects. In summary, the data presented shows that better understanding of a specific signaling pathway allows for the ability to identify specific cures, while limiting potential side effects.

VI. Future Directions

To understand the efficacy of SIMPL as a treatment for hematopoietic disorders, future studies should continue to explore loss of SIMPL's ability to limit a dysregulated TNFα system and minimize side effects, and the potential to create a cancer model. Currently, a mouse SIMPL-/- model, already in development, would be the best determent of SIMPL's advantage as a therapy. With SIMPL-/-, the lack of apoptosis of hematopoietic precursors and CFU-GM proliferation/ differentiation assays in the presence of TNFα could confirm our previous data. Next, SIMPL-/- HSC's and HPCs can be used to confirm growth inhibition with the addition of TNFα compared to wild type by a variety of methods. First, a propidium iodide cell cycle assay using flow cytometry could identify the stage in the cell cycle when growth arrest appears [36, 49]. Second, SIMPL-/- HSCs could be FAC sorted as single cells per well with TNFα to measure diminished ability of the cells to proliferate compared to wild type [35]. Third, the SIMPL-/- HSCs or HPCs in the presence of TNFα could be placed in a gene expression array to establish increased expression of cell cycle regulators as compared to wild type. Next, efficacy of SIMPL-/- to inhibit a dysregulated TNFα pathway could be replicated by using over expression of IKKβ, or p65 and treating with TNFα, SIMPL should inhibit the TNFα induce p65 activation of anti-apoptotic factors. Apoptosis should be increased in SIMPL-/- as compared to wild type.

It is necessary to show that loss of SIMPL leads to minimal side effects due to its specificity to the TNF/NF-κB pathway. It would be expected for SIMPL-/- mice to live to birth, like the TNFα-/- and TNF-RI-/- mice; demonstrating that loss of SIMPL is not embryologically lethal like p65-/-. Analyzing SIMPL-/- bone marrow lineage, we should

be able to determine if SIMPL-/- are similar wild type, and placing the HSC's into colony assays without TNFα, SIMPL-/- should form normal CFU-GM colonies. SIMPL-/- mice could be crossed with TNF-RI-/- mice to determine if the CFU-GM growth inhibition is reversed in response to mTNFα. This confirms SIMPL's specificity to the TNF-RI signaling pathway as compared to TNF-RII. Lastly, a gene array of SIMPL-/- treated with TNFα would be needed to determine if SIMPL up regulates p65/p50 induced gene expression or only a subset. This would allow for more finite understanding of the effects that loss of SIMPL might acquire.

SIMPL-/- may provide a long term cancer model. SIMPL-/- mice treated with TNFα or LPS (causes release of TNFα) periodically could put hematopoiesis under clonogenic stress due to diminished ability to proliferate. Thus, the model would create pressure on HSCs and early HPCs to mutate to allow for proliferation. This is advantagous because there are no current myelodyplasia syndrome (MDS) mouse models that this SIMPL-/- mouse may model. Additionally, competitive repopulation assays could be done in the short term to evaluate SIMPL -/- HSCs ability to reconstitute mouse bone marrow. TNFα-/- and TNF-RI-/- show decreased ability to repopulate bone marrow as compared to wildtype. If SIMPL-/- HSCs also have diminished capacity to reconstitute bone marrow, SIMPL-/- HSCs could be losing the ability to be regulated or function properly, either of the two would put it in a pre-cancerous state. This should act as a screening tool for the potential of cancer model.

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CURRICULUM VITAE

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Education

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1997-2001	B.S. with Honors in Chemistry and Molecular Biology Graduated Magna Cum Laude Florida Agricultural and Mechanical University, Tallahasse, FL
1993-1997	Dipoloma O'Fallon Township High School, O'fallon, IL

Honors & Activities

2006	Invited Speaker "Determining a Biological Role for SIMPL in Hematopoiesis," University of Indianapolis
2005	Jack Davis Award – Best student seminar, Indiana University School of Medicine
2000-2001	President of the Chemistry Club, Florida Agricultural and Mechanical University
1999-2000	Vice-President of the Chemistry Club, Florida Agricultural and Mechanical University
1997-2001	Member of Jr SNMA (Student National Medical Association), Florida Agricultural and Mechanical University
1998	Student Government Volunteer, Florida Agricultural and Mechanical University
1997-1998	Biology Club, Florida Agricultural and Mechanical University

Work Experience

2000	Summer Internship: Eli Lilly and Company, Indianapolis, IN Research Subject: Exploratory Organic Synthesis Supervisor: Mr. James Aikins Date of Research: May-August

1999	Ten Week Internship: Engineering Research Center for Particle Science and Technology, University of Florida, Gainesville, Fl Research Subjects: Enhancing NMR Signals with Hyperpolarized Xenon 129 Gas, and MRI Scans with Hyperpolarized Xenon 129 on complex polymers Supervisor: Dr. C. Russell Bowers Graduated Student Mentor: Tony Zook Date of Research: June-August
1998-1999	BIONR Tutor in Chemistry
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Publications

Vig-Varga E., **Benson E.A.**, Limbil T.L., Allison B.M., Goebl M.G., Harrington M.A., Alpha-Lipoic Acid Modulates Ovarian Surface Epithelial Cell Growth. Gynecologic Oncology, received 3 Nov, 2005, accepted 2006.