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# Eliminating Acute Myeloid Leukemia Stem Cells by Targeting the Niche Microenvironment: Co-Inhibition of TNF/IL1- JNK and NF- $\kappa$ b

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LOYOLA UNIVERSITY CHICAGO

ELIMINATING ACUTE MYELOID LEUKEMIA STEM CELLS

BY TARGETING THE NICHE MICROENVIRONMENT:

CO-INHIBITION OF TNF/IL1-JNK AND NF- $\kappa$ B

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR BIOLOGY AND BIOCHEMISTRY

BY

ANDREW GUERIN VOLK

CHICAGO, ILLINOIS

MAY 2015

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To my family

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## LIST OF ABBREVIATIONS

AML	Acute Myeloid Leukemia
CAF	Cancer Associated Fibroblast
CFU	Colony Forming Unit
CML	Chronic Myeloid Leukemia
ES	Embryonic Stem cell
HSPC	Hematopoietic Stem and Progenitor Cell
IL	Inter-Leukine (cytokine)
iPS	inducible Pluripotent Stem cell
JNK	Jun N-terminal Kinase
LC	Leukemia Cell
LSC	Leukemia Stem Cell
MLL	Mixed Lineage Leukemia
MRD	Minimal Residual Disease
NF- $\kappa$ B	Nuclear Factor kappa light chain enhancer of activated B cells
PB	Peripheral Blood
PCR	Polymerase Chain Reaction
PLC	Pre-Leukemia Cell
TNF	Tumor Necrosis Factor alpha
TNFR	Tumor Necrosis Factor Receptor
WT	Wild Type

## ABSTRACT

Leukemia Stem Cells (LSCs) from Acute Myeloid Leukemia (AML) require the activity of the transcription factor NF- $\kappa$ B to maintain stemness and drive tumor formation. Blocking NF- $\kappa$ B can preferentially eliminate LSCs *in vitro* with minimal effects on healthy Hematopoietic Stem and Progenitor Cells (HSPCs), making NF- $\kappa$ B a compelling target for anti-leukemia therapies. However, blocking NF- $\kappa$ B *in vivo* can only extend survival for a short period of time before transplanted mice succumb to the disease. I propose this is due to components of the *in vivo* niche supporting LSC survival and compensating for the inhibition of NF- $\kappa$ B.

I observed patients with partially differentiated blast-like AML (subtypes M3, M4, and M5) have significantly elevated levels of pro-inflammatory cytokines TNF $\alpha$  and IL-1 $\alpha/\beta$  (TNF and IL1) circulating in their peripheral blood. Further study revealed these cytokines are primarily produced by LSCs because such cells express several times more of TNF and IL1 than their healthy tissue counterparts. I found that TNF and IL1 stimulate the growth and expansion of LSCs while inducing cell death in HSPCs. Also, LSC-conditioned media alone is sufficient to drive apoptosis/necroptosis in HSPCs that can be prevented by blocking TNF and IL1, suggesting a mechanism for hematopoietic repression commonly observed in AML cases.

TNF and IL1 drive their pro-inflammatory effects on target cells through activation of cellular signaling networks. Both TNF and IL1 are potent activators of NF-

$\kappa$ B in almost all cells studied, connecting the ability of these cytokines to drive LSC growth with the need of NF- $\kappa$ B for survival. In addition, TNF and IL1 also stimulate activation of JNK signaling which operates in parallel to NF- $\kappa$ B in LSCs and HSPCs. I found that JNK stimulation results in cell death in HSPCs by subsequent inactivation of pro-survival Bcl-2 family proteins by phosphorylation. However, LSCs convert JNK-mediated cell death signals into proliferation/survival signals by both limiting the signal duration to <60 min through dephosphorylation by Map Kinase Phosphatase 5, and also by upregulating JNK's nuclear target c-Jun. Such short duration of JNK activation correlates to activation of JNK's nuclear targets without activating the death signal.

I determined that concurrent inhibition of NF- $\kappa$ B and JNK has two major effects: (1) combined inhibition specifically targets LSCs *in vitro* and *in vivo*, and (2) the toxicity in healthy HSPCs due to loss of NF- $\kappa$ B signaling is mediated by JNK, making the combined treatment protective. I can substantially increase survival in AML-transplanted mice if they are treated with combination of NF- $\kappa$ B and JNK inhibition *in vivo*. I can further extend survival in leukemic mice when I treat with blockers to upstream pro-inflammatory cytokines: anti-TNF, anti-IL1, and NF- $\kappa$ B inhibitor. These data provide a strong rationale to treat AML patients by combined inhibition of both TNF/IL1- JNK and NF- $\kappa$ B signaling.

## CHAPTER 1

### INTRODUCTION

Leukemia Stem Cells (LSCs), like their healthy tissue counterparts, are a subset of Leukemia Cells (LCs) capable of maintaining tumor homeostasis, are contextually undifferentiated, and can remain in a relatively slow-cycling state until signaled to proliferate. LSCs possess the ability to regenerate the leukemia tumor.<sup>1,2</sup> While the leukemic burden can be almost entirely eliminated (clinically: complete remission) in most patients when treated with chemotherapeutic drugs, some LSCs remain due to their intrinsic apoptosis resistance and niche-based protection. This renders these cells immune to traditional chemotherapies, making these LSCs the population of cells widely considered to be responsible for minimal residual disease (MRD) following therapy.<sup>3</sup> Cells expanded from the LSCs retained in the MRD population are essentially pre-selected for chemotherapeutic resistance. Therefore, in order to eliminate MRD and prevent relapse, we need to develop therapies that will target LSCs at the very core of what makes a LSC a stem cell, not just reducing blast percentage by killing differentiated progenitor and blast cells.<sup>4,5</sup> If we could block the stem cell maintenance machinery components from LSCs, then we should be able to more effectively reduce the risk of disease relapse. My studies suggest I can accomplish this through manipulating pro-inflammatory signaling in the leukemic stem cell niche.

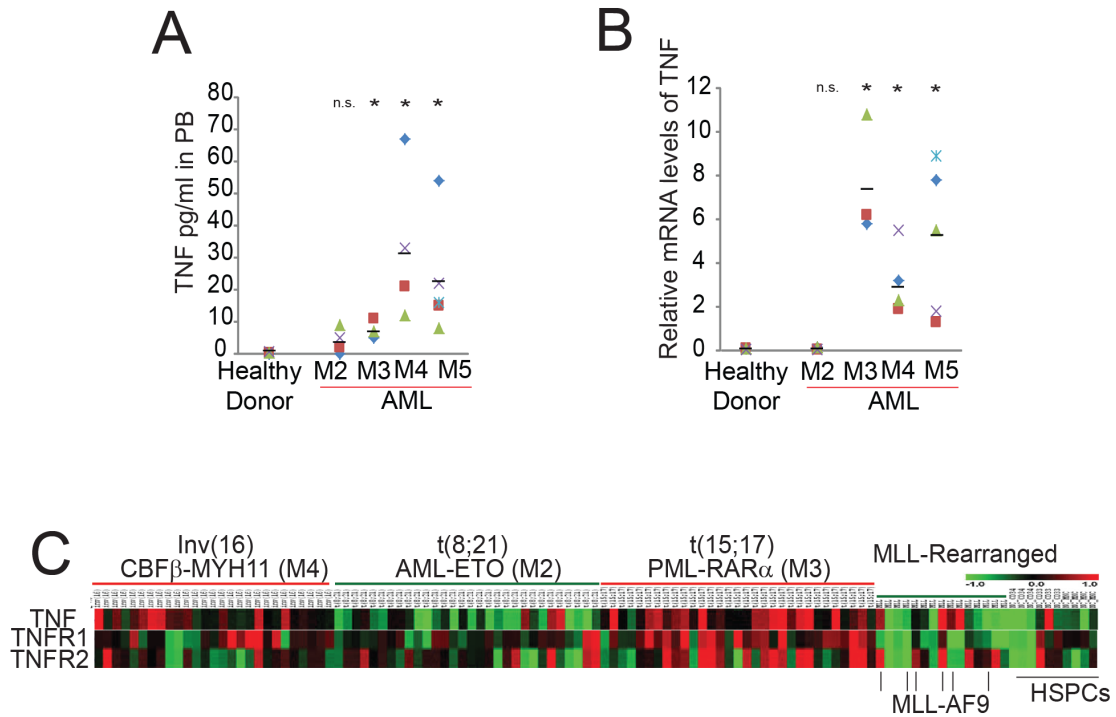
All stem cells have a home, which is a specialized microenvironment called the “niche.” The niche provides all the extrinsic support stem cells require to block differentiation, ensure cell survival, and maintain self-renewal.<sup>6,7</sup> LSCs also reside in a niche, and although the LSC niche is located in the bone marrow along with the HSPC niche, its microenvironment is fundamentally altered.<sup>8</sup> Unlike the hematopoietic stem cell (HSC) niche, the LSC niche is pro-inflammatory and does not sustain normal hematopoiesis. This alteration is critical for sheltering LSCs from chemotherapy and retaining the necessary stem cell maintenance processes.<sup>9</sup> Identifying these and other key differences between the healthy HSC niche microenvironment and the LSC niche microenvironment will provide us with the necessary tools to block LSC functions while protecting HSPCs.

A primary function of normal HSC niches is to protect the HSCs from excessive inflammatory stimuli. By working with collaborators, we determined LSCs produce large amounts of inflammatory cytokines, Tumor Necrosis Factor alpha (TNF) and Interleukin-1 (IL-1), which they secrete into their niche. Normally, these inflammatory cytokines induce differentiation, apoptosis and necroptosis in normal tissue cells.<sup>10,11</sup> In AML patients, elevated levels of TNF are associated with higher leukocyte counts, lower complete remission rates, and poorer prognoses.<sup>12</sup> I found that some patients with AML have high levels of circulating TNF in their blood serum when compared to healthy donors. While normal levels of TNF in healthy individuals with no major immune activity should be approximately 12 pg/mL, I found some patients had circulating TNF as high as 65 pg/mL. This indicates an acute induction of inflammatory reactions in the

body. I measured serum of patients from the M2, M3, M4, and M5 subgroups of AML and found the increase in circulating TNF was primarily in patients from M3 (APL), and M4/5 (monocytic) AMLs (**Figure 1A**). Many cancer patients can exhibit inflammation (elevated TNF, IL1, IL6, TGF-B) due to the massive amounts of cell death caused by tumor formation and immune response or the effects of chemotherapy. Therefore I sought to determine if the observed TNF levels were being produced by the tumor cells themselves, or by macrophages/immune cells/stromal cells. To do this, I isolated AML blasts (CD34+) from the peripheral blood of these patients and performed qRT-PCR analysis to determine if TNF mRNA was being produced. I found that tumor cells from these patients had a 5-6 fold increase in TNF production (**Figure 1B**). Large scale analysis of samples from AML patients from collaborator at University of Chicago corroborated this data, showing an increase in TNF and receptors in M3, M4, and MLL-Rearranged M5 AMLs (**Figure 1C**). This validates my selection of MLL-AF9 as a fusion gene to transform my mouse leukemia cells because MLL-AF9 AMLs are characterized as M4 and M5.

However, while we observed elevated TNF levels in these patients, we did not understand the role TNF played in affecting LSC survival. To test this, I stimulated LCs with an increasing gradient of exogenous recombinant Tnf, from levels resembling normal physiological state (0.01 ng/mL) to acute inflammatory disease (50 ng/mL) and measured colony formation. I found that this stimulation increased the colony formation rate of LCs in a dose-dependent manner, while increasing doses of Tnf reduced the

**Figure 1. Several subtypes of AML produce TNF**



**A)** TNF concentrations in peripheral blood of AML patients as shown by ELISA from individual patient samples. **B)** TNF expression in LC isolated from individual AML patient samples as shown by qRT-PCR assay. Horizontal bars in **A** and **B** indicate mean levels. \* indicates  $p < 0.05$  as determined by student's T test when compared to healthy donor control. **C)** cDNA microarray analysis of TNF and TNF receptors (R1 and R2) in AML patient samples. t(8:21), t(15:17), and inv(16) are AML subtypes M2, M3, and M4, respectively. MLL leukemias include MLL-AF4 (B-ALL) and MLL-AF9 (AML-M5). CD34<sup>+</sup> HSPCs, CD33<sup>+</sup> myeloid progenitors, and/or MNCs from healthy donors were used as controls.

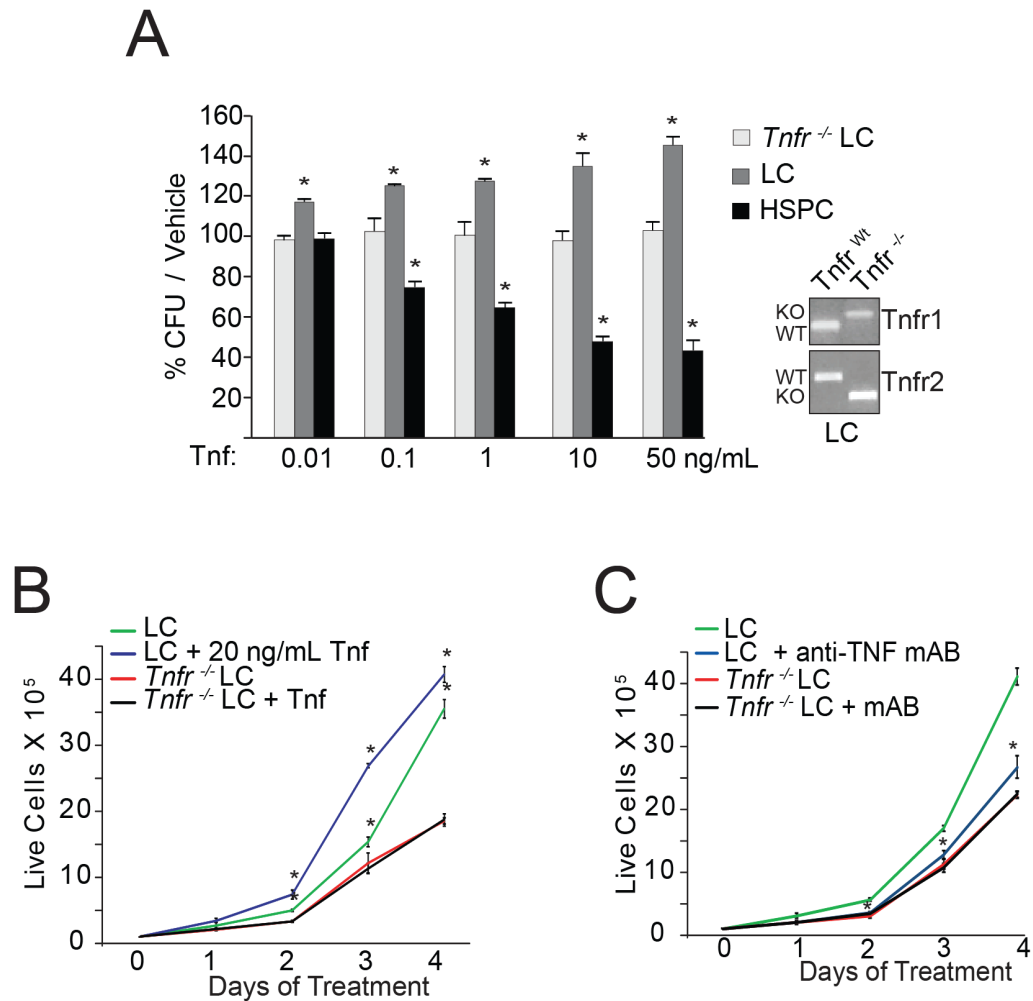
**A** and **B** were acquired by collaborators Drs. Jing Li and Xingyu Li at Shanghai Normal University, while **C** was acquired from collaborator Dr. Jianjun Chen at University of Chicago



colony formation capacity of hematopoietic stem and progenitor cells (HSPCs, mixture of HSC and progenitors). *Tnfr<sup>-/-</sup>* LCs deficient for both Tnf receptors (Tnfr1a/1b, *Tnfr<sup>-/-</sup>* hereafter) were used as negative controls because they lack the Tnf receptors and should not respond to Tnf stimulation (**Figure 2A**). Tnf receptor deletion was confirmed genetically by PCR of tail cell DNA. Wild type and knockout alleles are denoted.

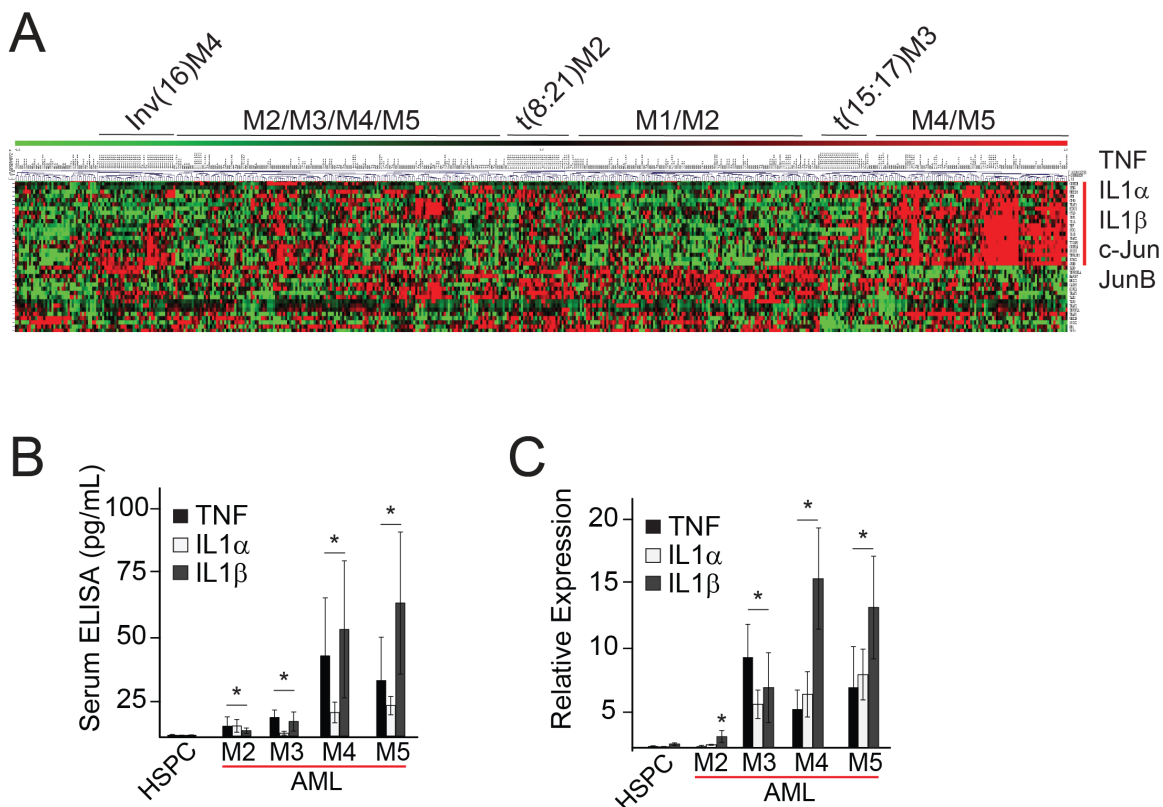
I then asked if the increase in colonies upon Tnf stimulation was directly Tnf-dependent. To answer this, I grew LCs in suspension culture and treated with exogenous Tnf every day for four days. I found that this treatment was able to significantly increase the growth rate of LCs when compared to vehicle treated control cells as control. *Tnfr<sup>-/-</sup>* LCs were also used as controls, and showed a reduced growth rate when compared to their *Tnfr<sup>+/+</sup>* (*WT* hereafter) counterparts (**Figure 2B**).

I determined LCs produce their own endogenous Tnf, and this endogenous Tnf could affect the growth rate of *WT* LCs. To account for this, I treated LCs in suspension culture with an anti-TNF monoclonal antibody (mAB) to neutralize soluble Tnf. This treatment slowed the growth of the LCs to the rate of *Tnfr<sup>-/-</sup>* LCs, suggesting that the higher rate of cell growth in *WT* LCs versus *Tnfr<sup>-/-</sup>* LCs is due to TNF (**Figure 2C**). Vehicle treated LCs, *WT* and *Tnfr<sup>-/-</sup>*, were used as controls. These TNF-expressing AMLs were later found to be highly sensitive to NF- $\kappa$ B and JNK inhibition treatment (**Figure 39**). However, I found that some patient samples were sensitive to co-inhibition of NF- $\kappa$ B and JNK, but did not express TNF (**Figure 42**). Therefore, I looked at other pro-inflammatory cytokine expression in AMLs using a large-scale microarray analysis of

**Figure 2: TNF promotes LC growth**

**A)** CFU from LCs and HSPCs were treated with indicated doses of Tnf in methylcellulose. The numbers of CFU in each cell type in Tnf-treated groups were normalized to CFU number in vehicle treated group. Genotype results are shown for *WT* and *Tnfr*<sup>-/-</sup> LCs. **B and C)** LCs and *Tnfr*<sup>-/-</sup> LCs were treated with Tnf (B) or anti-TNF mAB (C) in suspension culture daily for four days. Daily live cell counts were measured by trypan blue exclusion assay. Values shown are mean  $\pm$  SD from three independent trials. \* indicates  $p < 0.05$  when compared to vehicle treated control

**Figure 3: Many different types of AML produce IL1 and TNF**



**A)** 580 different human AML samples were analyzed by cDNA array for up and downregulation of various factors compared to internal controls. Green indicates low expression, red indicates high expression. Different AML subtypes are indicated. Genes listed to the right of the vertical red bar are upregulated along with TNF in M4/5 AML.

**B)** Serum samples taken from newly diagnosed AML patients analyzed for TNF, IL1 $\alpha$ , and IL1 $\beta$  by ELSIA. HSPCs from healthy donors were used as negative controls.

**C)** Relative expression of mRNA levels compared to HSPC expression of TNF as control. cDNA levels were normalized to GAPDH. \* indicates  $p < 0.05$  when compared to HSPC control as determined by one-way ANOVA with Bonferonni post-hoc test.

cDNA array data in **A** was generated by Dr. Jianjun Chen at University of Chicago. Patient data from **B** and **C** was generated by Dr. Jing Li at Shanghai Normal University, Shanghai, China.

581 patients from a collaborator. I found that in addition to TNF, the same AML subtypes that express TNF also expressed an additional pro-inflammatory cytokine, IL1 $\alpha$  and IL1 $\beta$  (IL1 hereafter) (**Figure 3A**). These patients expressed both cytokines in their peripheral blood (**Figure 3B**), and the cytokine was determined to be coming from the leukemia tumors (**Figure 3C**). To study the pro-survival effects of IL1 signaling in LCs and HSPCs, I treated LCs and HSPCs in parallel with increasing doses of exogenous IL1, and measured colony formation. I found that even with treatments at 1 ng/mL, colony formation was significantly enhanced in both *WT* LCs and *Tnfr*<sup>-/-</sup> LCs. Further increases in IL1 treatment concentration drove a dose-dependent CFU increase in both cell types. This suggests that IL1 is operating as a Tnf-independent survival signal (**Figure 4A**). Vehicle treated cells were used as controls. HSPC colony formation increased with the dose of IL1 in a dose-dependent manner (**Figure 4B**). I thought this might be due to enhanced colony formation of progenitor cells following IL1 stimulation, but that the stem cells may be negatively affected.

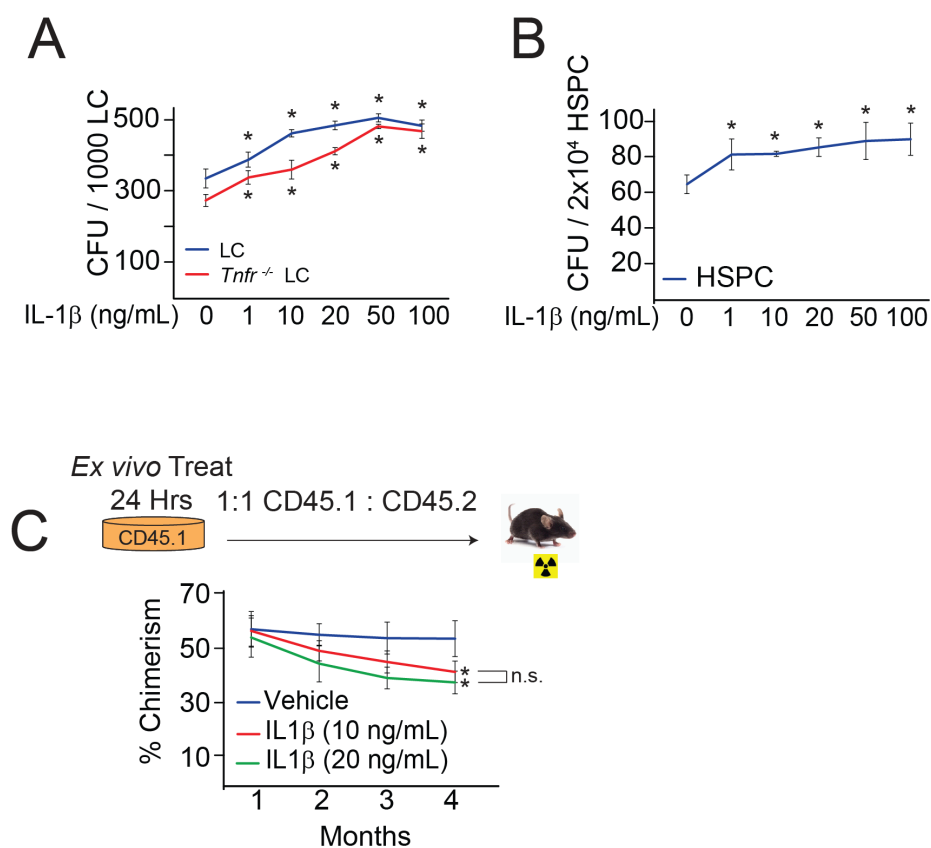
To test this, I stimulated HSPCs *ex vivo* with two doses of IL1. If this treatment affected HSPCs, then the treated cells would have impaired engraftment and reconstitution of hematopoiesis *in vivo*. This turned out to be correct. HSPCs treated with the low dose of IL1 had repressed ability to reconstitute lethally-irradiated mice when compared to vehicle-treated control. High dose-treated HSPCs showed the lowest reconstitution ability (**Figure 4C**). Vehicle-treated HSPCs were used as controls, and exhibited stable reconstitution during the competitive transplantation assay.

I determined that TNF drives a powerful survival signal that is augmented by IL1 signaling, operating in parallel. that functions parallel to NF- $\kappa$ B through activation of JNK signaling. These pro-inflammatory cytokines function in an autocrine manner to support LSC survival and self-renewal while also functioning in a paracrine manner to induce apoptosis/necroptosis in neighboring HSPCs. This effectively clears HSPCs from the LSC niche and may account for hematopoietic repression observed in AML patients.

I found that, conversely, blocking IL1 in LCs can repress colony formation. Blocking IL1 and Tnf together was able to accomplish the largest amount of CFU repression in LCs. This further confirms my hypothesis that LSCs use TNF and IL1 as critical survival signals. They accomplished this by first treating LCs with an IL1 receptor antagonist to block IL1 in combination with a monoclonal antibody against Tnf. They saw a dose-dependent decrease in CFUs with increasing doses of IL1 receptor antagonist (IL-1Ra, **Figure 5A**). They confirmed this by performing similar experiments in *Tnfr1a<sup>-/-</sup>1b<sup>-/-</sup>* (*Tnfr<sup>-/-</sup>* hereafter) LCs treated with an increasing dose of IL-1Ra and found similar results, suggesting that these effects are specific (**Figure 5B**). Finally, they confirmed the specificity of the IL-1Ra by knocking down the IL1 receptor by shRNA (**Figure 5C**) and treating with anti-Tnf antibody, measuring CFUs. Vehicle treated cells expressing a control shRNA were used as control (**Figure 5D**).

Based on these observations, I proposed that LSCs produce pro-inflammatory cytokines in order to both promote their own survival and proliferation as well as repress normal stem cell function.

**Figure 4. Exogenous IL1 enhances CFUs of LCs, promotes HSPC repression**



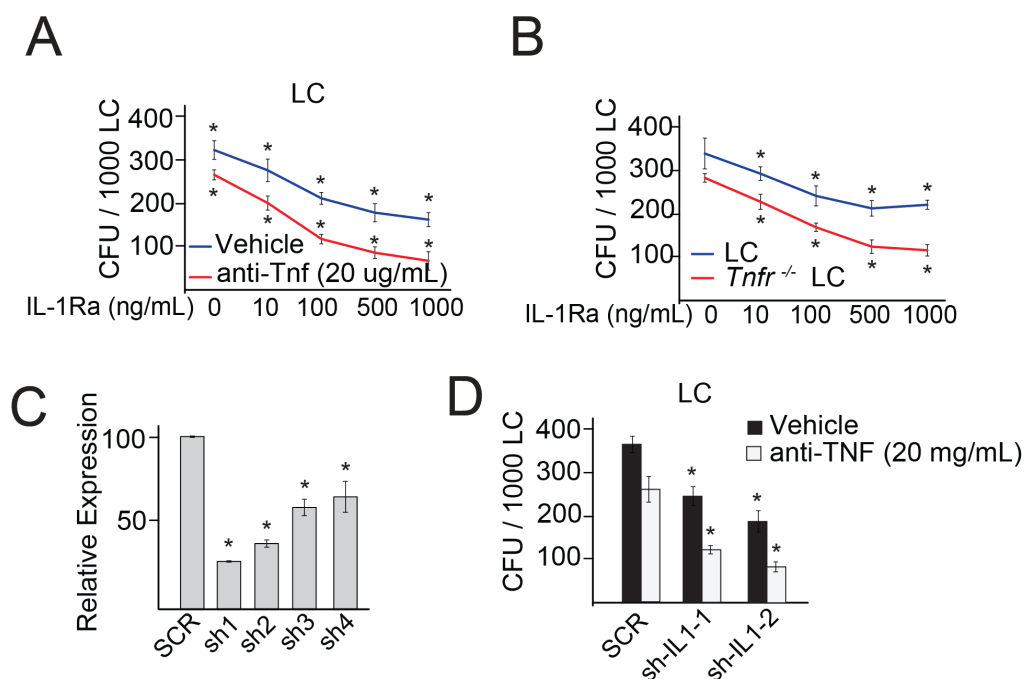
**A)** *WT* and *Tnfr*<sup>-/-</sup> LCs were treated with indicated doses of exogenous IL1 in methylcellulose, and colonies were counted seven days later. Vehicle-treated LCs of each genotype were used as negative controls. Results shown are mean ± SD from three independent trials. **B)** *WT* HSPCs were treated with indicated doses of IL1 in methylcellulose, and colonies were counted ten days later. Vehicle-treated HSPCs were used as negative controls. Results shown are mean ± SD from three independent trials. **C)** CD45.1 HSPCs were treated *ex vivo* with indicated doses of IL1 and competitively transplanted 1:1 with vehicle-treated CD45.2 HSPCs in lethally-irradiated recipient mice. Chimerism was measured monthly. Results shown are mean ± SD from n=5 recipient mice for each condition. \* indicates p<0.05 as determined by one-way ANOVA with Bonferonni post-hoc test.

These results were obtained by Dr. Jing Li at Shanghai Normal University, Shanghai, China.

LSCs are dependent on NF- $\kappa$ B, a transcription factor, for maintaining survival and self-renewal.<sup>13,14</sup> Blocking NF- $\kappa$ B *in vitro* can preferentially eliminate LSCs while having minimal effects on HSPCs. This creates a promising target for anti-leukemia therapies. However, *in vivo* NF- $\kappa$ B inhibition in leukemia-transplanted mice only minimally extends their survival, suggesting that some component of the *in vivo* leukemic niche is stimulating a compensatory survival signal to NF- $\kappa$ B. I determined that LSCs secrete pro-inflammatory cytokines TNF and IL-1 into the niche microenvironment, reorganizing the niche to support LSC survival and self-renewal. These cytokines are responsible for stimulating NF- $\kappa$ B-independent survival signals mediated through the JNK-AP1 survival pathway in LSCs and cell death in HSPCs through activation of the JNK-Bid/Bad/Bax apoptosis pathway. By blocking TNF/IL-1-mediated JNK signaling, I can re-sensitize LSCs to NF- $\kappa$ B inhibition *in vivo*, driving the disease into complete remission. The goal of this project is to determine how to improve elimination of LSCs by adding to the effects of NF- $\kappa$ B inhibition. *In vivo* experiments show NF- $\kappa$ B inhibition alone is insufficient to clear LSCs. In fact, NF- $\kappa$ B inhibition *in vivo* can only extend the lifespan of transplanted mice by <1 week. This is in stark contrast to the complete elimination of LSCs *in vitro* with a similar treatment. I propose that LSCs can escape NF- $\kappa$ B inhibition *in vivo* by utilizing TNF/IL1-dependent, NF- $\kappa$ B-independent survival signaling. I will accomplish this goal by examining two aims (**Figure 6**).

In the first aim of this dissertation, I explored how LSCs escape the effects of NF- $\kappa$ B inhibition *in vivo* through endogenously secreted TNF. In LCs, I found TNF acts as an

**Figure 5. Co-inhibition of Tnf and IL1 represses LC CFU ability**



**A)** *WT* and *Tnfr<sup>-/-</sup>* LCs were treated in parallel with indicated doses of IL1 receptor antagonist (IL-1Ra) in methylcellulose. Colonies were read seven days following treatment. **B)** *WT* LCs were treated with anti-TNF and IL-1Ra alone or in combination in methylcellulose. Colonies were read seven days following treatment. **C)** LCs were transduced with shRNA-expressing retroviral vectors targeted to IL1 receptor. Knockdown was confirmed by qRT-PCR analysis. **D)** IL-1 knockdown LCs were treated with vehicle or anti-TNF and seeded for CFU assay in methylcellulose. \* indicates  $p < 0.05$  as determined by one-way ANOVA with Bonferonni post-hoc test. Results shown are mean  $\pm$  SD, and are indicative of three independent trials of experimental replicates.

These results were obtained by Dr. Jing Li at Shanghai Normal University, Shanghai, China.

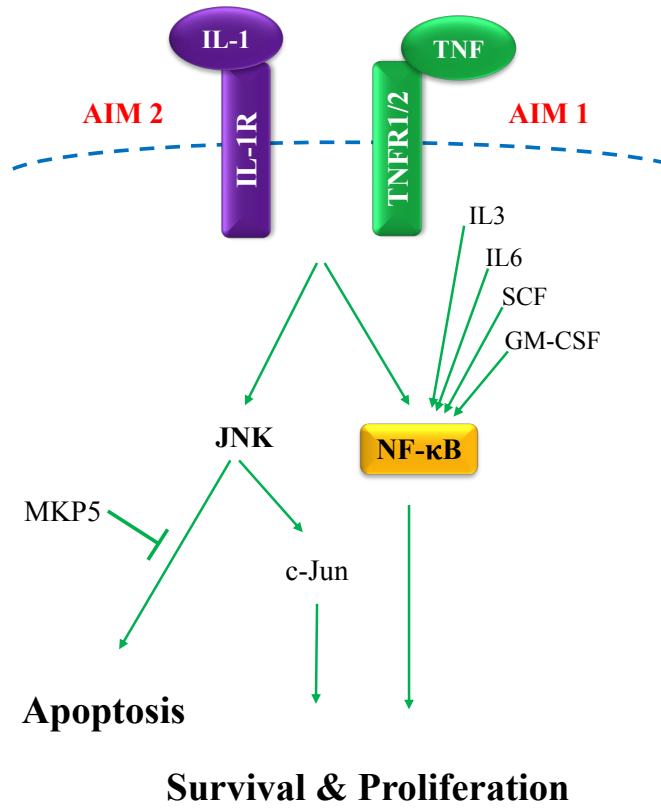


autocrine survival signal by activation of JNK as a survival signal. They accomplish this by strict regulation of JNK through TNF receptor endocytosis, MKP5-mediated dephosphorylation of JNK, and upregulation of nuclear substrate c-Jun. All of these factors contribute to allow LCs to escape TNF-mediated cell death and instead use the cytokine as an autocrine survival and proliferation signal.

In the second aim of this dissertation, I explored how other pro-inflammatory cytokines contribute to NF- $\kappa$ B-independent survival of LCs. I determined that TNF alone did not explain the pro-inflammatory survival phenotype observed. Therefore I concluded that there were additional pro-inflammatory cytokines secreted by LCs and used as autocrine survival signals. I determined that LCs also secrete pro-inflammatory IL1, and that IL1 signaling augments the TNF-mediated survival signal through weak activation of similar pro-survival signaling molecules JNK, NF- $\kappa$ B, ERK, and c-JUN.

I conclude that by blocking both TNF and IL1 together in LSCs can I maximally eliminate NF- $\kappa$ B-independent survival signaling. The inhibition of these pro-inflammatory cytokines might also assist in re-organizing the LSC niche *in vivo* to a state that is more similar to the normal healthy homeostatic hematopoietic niche. Mice xenografted with AML and treated with TNF and IL1 blockers are highly sensitive to NF- $\kappa$ B inhibition, with complete disease remission in some mice.

Figure 6. Flow chart diagram of dissertation specific aims.



## CHAPTER 2

### LITERATURE REVIEW

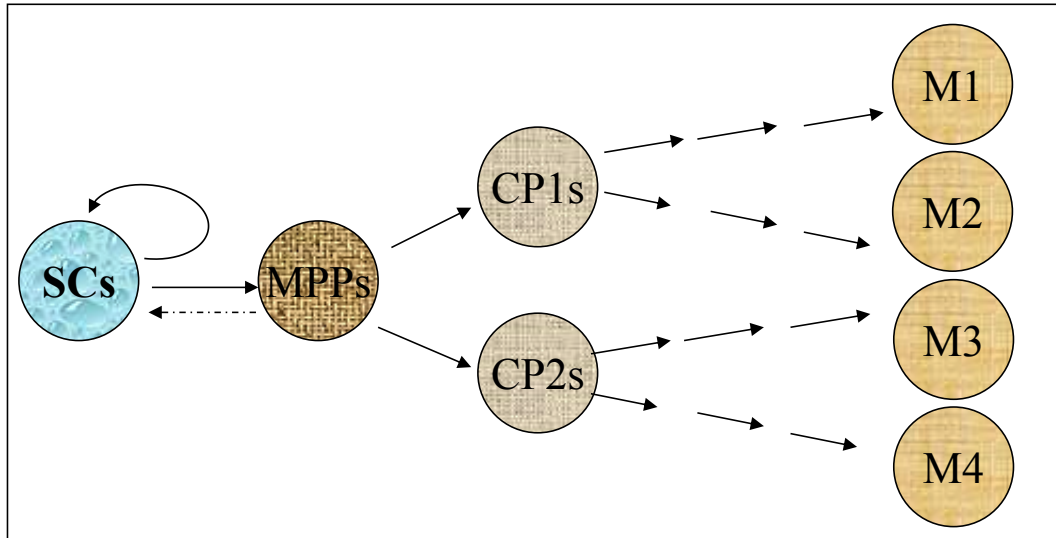
#### **Stem Cell Overview**

Complex organisms are made up of many different tissues, all cooperating with each other to carry out the everyday tissue demands of life. Each tissue, in turn, is made up of many different cell types, all cooperating with each other to carry out the function of the tissue. Each cell within the tissue originates from a common ancestor cell called a “stem cell.” Stem cells are a special subset of cells found within each tissue that are responsible for maintaining homeostasis and function of their tissue by (1) remaining dormant until signaled to divide, and (2) differentiating into the appropriate types of cells. These stimuli can include acute signals such as inflammation released during physical trauma, which will stimulate stem cells residing in the bulge of the hair follicle in the epidermis to differentiate into new melanocytes and keratinocytes to heal the wound, as well as more long-term homeostasis signals such as the blood stem cells reacting to low oxygen at high altitude by differentiating into large numbers of oxygen-carrying red blood cells.<sup>15-18</sup>

Stem cells were first characterized in the early 1960’s as specialized cells called “Hematopoietic Stem Cells,” that were located somewhere in the bone marrow, and were capable of dividing and regenerating blood cells.<sup>19</sup> Since then, scientists have found similar types of cells in almost every major tissue in the body.<sup>20-23</sup> These stem cells all

share common features: they are undifferentiated, they remain quiescent until activated, and they can differentiate into any cell type from their tissue upon proper stimulation.<sup>24,25</sup> These cells are so powerful at tissue regeneration that, such as the case of hematopoietic tissue, a small number of stem cells (< 30) are sufficient to completely repopulate the tissue in mice.<sup>26</sup> Stem cells process along a one-way path from a single undifferentiated cell to a completely differentiated tissue effector cell in stages, from stem cell, to progenitor cell, to committed progenitor (**Figure 7**). With each division, the progenitor cells become slightly more differentiated. A fertilized oocyte called the zygote and their early daughter cells (morulas stage) can generate, with enough divisions, every cell in the body and the extraembryonic tissues (including the placenta). This type of cell is called “totipotent.” The early embryonic (blastocyst stage) cells produced by totipotent stem cells will form all somatic cells (ectoderm, endoderm and mesoderm) and germline cells of the developing embryo. These cells are called pluripotent stem cells. Pluripotent cells can form any cell of the embryo, but are not capable of creating the placenta. As the pluripotent cells divide, they eventually create each tissue layer, and within it, each individual tissue.<sup>27</sup> While most cells will become mature differentiated effector cells, a small subset of cells will retain some stemness and will maintain tissue homeostasis throughout the life of the organism. These are called somatic stem cells or multi-potent stem cells. With each division, these somatic stem cells will become partially more differentiated, progressing from stem cell to multipotent progenitor cell, to common progenitor cell, and finally to completely differentiated effector cell (**Figure 7**).

**Figure 7: Stem cell differentiation paradigm**



Hierarchy of stem cells (SCs) and their progeny: Multipotent progenitors (MPPs), committed progenitors (CPs), and mature (M) cells.

Figure adapted from Breslin and Volk, et al. 2014.

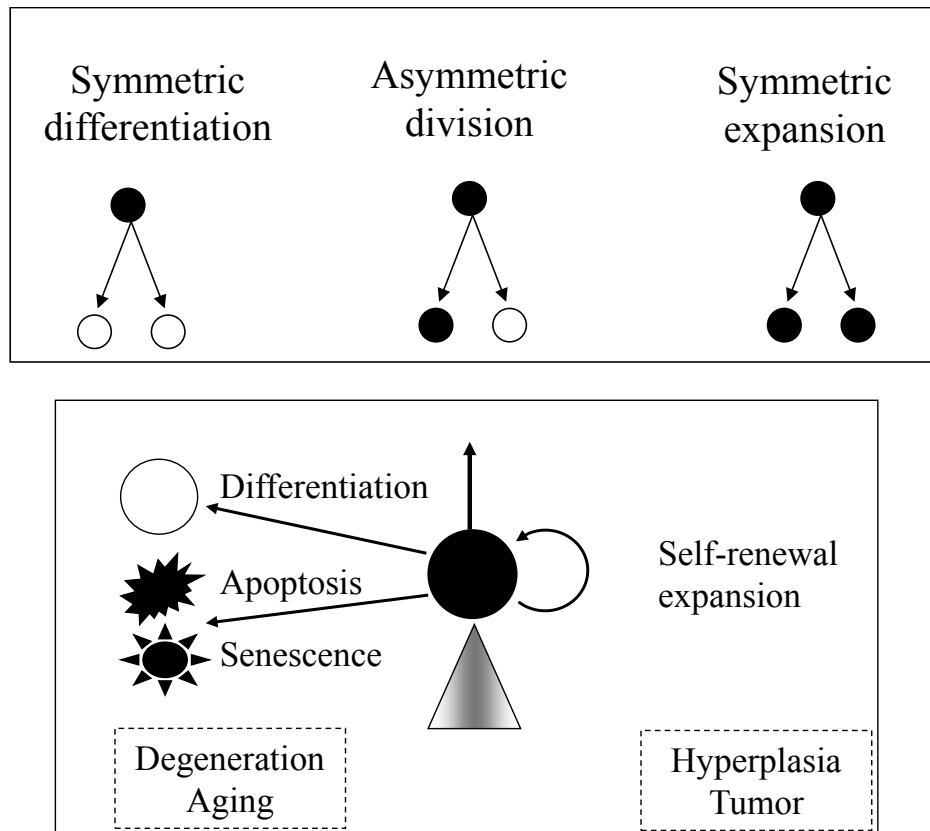
The final type of stem cell is called the germ-line stem cell, and is responsible for

When a stem cell is signaled to divide, there are three possible outcomes: symmetric expansion (self-renewal expansion), asymmetric division (also known as self-renewal maintenance), and symmetric differentiation. Each outcome is determined by factors related to tissue homeostasis. Symmetric expansion is a situation in which a single stem cell divides into two undifferentiated stem cells. This process is commonly seen in situations calling for an increase in stem cell number and will continue until a sufficient number of stem cells is reached, such as observed in the rapidly growing tissues of post-natal mammals, or in post-irradiated immune-depleted mice.<sup>28,29</sup> Asymmetric division is a process where one stem cell will divide and create a copy of itself and a multipotent progenitor cell. The stem cell re-enters quiescence and the multipotent progenitor cell goes on to divide into tissue-specific effector cells. This occurs in tissue homeostasis where cell numbers are stable, and is widely considered the default program for stem cell division.<sup>30-35</sup> Symmetric differentiation is a process in which one stem cell divides and differentiates into two multipotent progenitor cells without replenishing the lost stem cell. This is useful if there are proportionally too many stem cells or if the demand for tissue effector cells is especially high. However, this situation can also occur to the detriment of the organism if the normal machinery for maintaining stem cells is somehow broken **(Figure 8)**.

### **Stem Cell Self-Renewal**

Self-renewal is a cell-division process unique to stem cells, where one stem cell divides to create a multipotent progenitor cell and another stem cell. Self-renewal allows for tissue regeneration without exhausting the stem cell pool. There are three general

**Figure 8. Stem cell division and fate paradigms**



Undifferentiated stem cells (black filled circles) can divide through three different programs. Symmetric differentiation produces two partially differentiated MPPs (white circles, far left), asymmetric division produces one SC and one MPP (middle), and symmetric expansion produces two SCs (far right).

The fate a stem cell undergoes upon stimulation is determined by a balance of cell autonomous machinery, and is highly regulated (bottom).

Figure adapted from Breslin and Volk, et al. 2014.

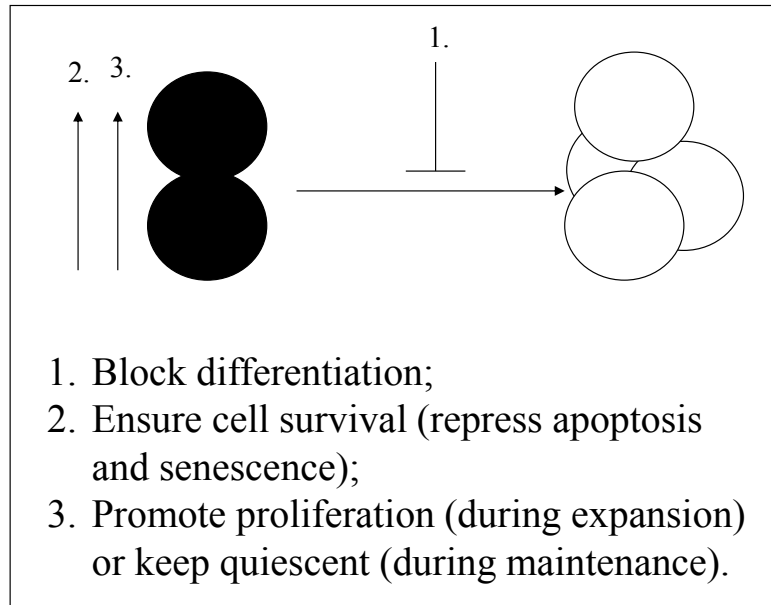
processes common to all self-renewing stem cells: (1) mechanisms that block differentiation in order to maintain the undifferentiated state, (2) mechanisms that ensure cell survival by blocking apoptosis and senescence, and (3) mechanisms to promote proliferation during expansion and quiescence during maintenance (**Figure 9**).

There is a key core transcriptional network responsible for self-renewal in pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. These factors are Oct4, Nanog, Klf2 and Sox2, which normally function as a complex. These factors promote self-renewal by up-regulating proliferation/survival genes and down-regulating a collection of genes involved in apoptosis, senescence, and differentiation. In addition, these pluripotent factors also maintain their own levels by binding to their regulatory region in an auto-regulatory manner. Furthermore, these pluripotent factors can reset the epigenetic landscape in differentiated mature tissue cells, play an indirect role in opening the regulatory region of self-renewal related genes including themselves. Adding these factors in any mature effector cells can actually reprogram and dedifferentiate those cells back to a pluripotent stem cell state. Such reprogrammed stem cells are called iPS cells.<sup>36-39</sup>

There is also a key core transcriptional network responsible for self-renewal in each type of somatic multipotent stem cell. Such a core transcriptional network is formed by tissue specific transcriptional factors, which ensure self-renewal by promoting proliferation and blocking differentiation at same time preventing stress-related apoptosis or senescence. Differentiation blockage has been determined to be one of the key



**Figure 9. Factors that drive stem cell self-renewal.**



In order to self-renew, stem cells need to block differentiation, ensure cell survival, and promote proliferation/keep quiescent.

Figure adapted from Breslin and Volk, et al. 2014.

mechanisms for stem cell self-renewal and such mechanism seems tissue specific. In some tissues, comprised of only one cell type, blocking stem cell differentiation can be achieved with few factors. For examples, BMP-Bam signal in *Drosophila* germline stem cells and Notch-CSL signal in satellite muscle stem cells, are sufficient to block differentiation and ensure self-renewal.<sup>40-47</sup> However, some somatic stem cells reside in tissues with diverse lineages of effector cells need (such as hematopoietic and nervous systems), and the machinery to maintain the undifferentiated state is significantly more complicated. Blocking differentiation appears to be the most important factor, though, in maintaining adult tissue stem cell self-renewal.<sup>48-58</sup> Multiple signals and factors are normally involved in maintaining the undifferentiated state in stem cells. This might explain why it is so difficult to expand hematopoietic and nerve stem cells *in vitro*; it might also explain why so many self-renewal factors have been identified in these tissue stem cells. However, there are many self-renewal factors are required for the self-renewal of almost all types of tissue stem cells. For example, 1) Polycomb proteins Bmi1, EZH1 and EZH2 regulate self-renewal of stem cells by repressing P15-P16/P19/P21 -mediated apoptosis and senescence;<sup>59-69</sup> 2) HIF1 $\alpha$  and the FoxO family of transcription factors regulate self-renewal by preventing ROS-stress –induced cell cycle arrest and apoptosis;<sup>70,71</sup> 3) many different tissue stem cells block intrinsic apoptosis through upregulation of Bcl-2 and Bcl-xL, and c-FLIP to prevent extrinsic apoptosis (Overexpression of such anti-apoptosis genes has been shown to actually increase the stem cell pool in most tissues studied);<sup>72-76</sup> 4) Tissue stem cells also upregulate DNA-repair proteins to prevent the accumulation of DNA damage;<sup>71,77,78</sup> 5) Many stem cells

also express telomerase, a protein responsible for maintaining telomere length. With each trip through the cell cycle, chromatid telomeres are shortened by 20-50 bp due to end-replication deficiency generated through subsequent divisions.<sup>79-84</sup>

Quiescence is a defining characteristic of adult tissue stem cells. They spend the majority of their lives being quiescent, which is essentially a dormant state where they are not actively dividing, and have a very low level of protein production. Both *in vitro* and *in vivo* studies have shown stimulation of stem cells into rapid proliferation will compromise self-renewal and ultimately exhaust the stem cell pool. This is likely due to an inability to complete all tasks of the self-renewal machinery (DNA damage repair, telomere lengthening, replacing chromatin modifications), and driving either apoptosis/death of the stem cell or differentiation into an effector cell. In support of this, almost all tissue stem cells are slow-cycling when they replicate.<sup>72,85-88</sup> Some studies even suggest slow cycling is one of the most important mechanisms utilized by adult stem cells to retain multipotency and maintain self-renewal.<sup>89-92</sup> The slow-dividing nature of stem cells also allows for ample time between divisions to repair any accumulated DNA damage before it can trigger the apoptosis machinery. Although these are all cell-intrinsic mechanisms for maintaining self-renewal, stem cells grown outside of ideal environments will still differentiate and eventually die. Stem cells require a special microenvironment called a niche to properly maintain themselves and tissues.

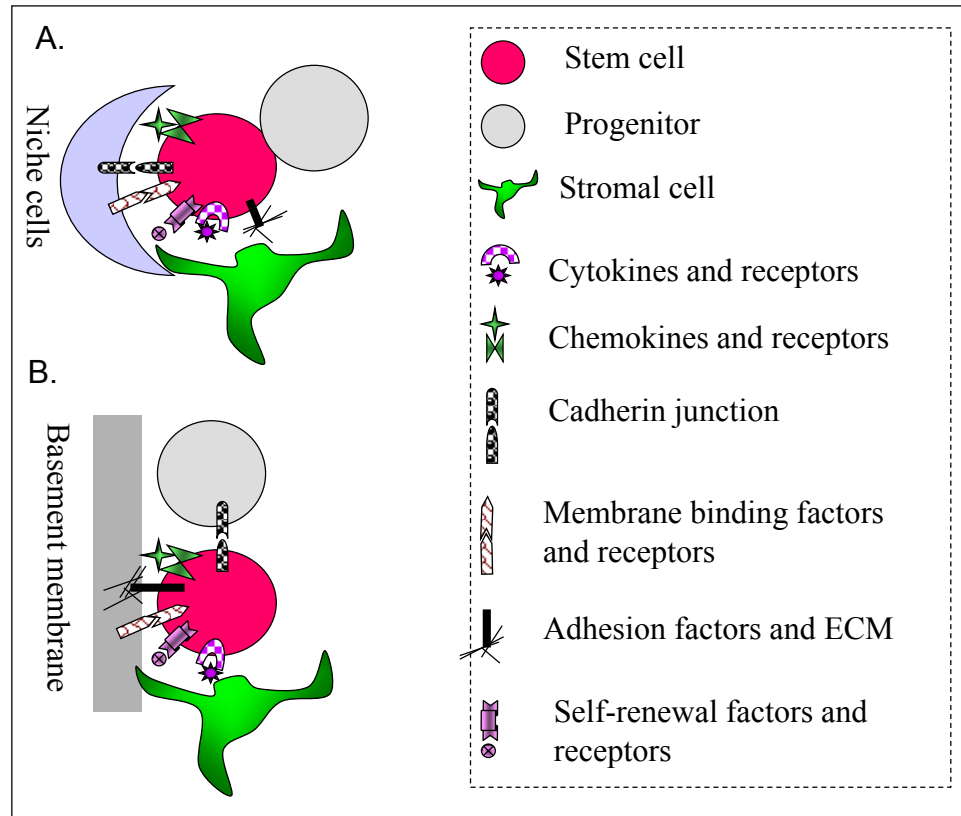
### **Stem Cell Niche Overview**

The stem cell niche is the home of the stem cell, and is composed of two major components: stationary/cellular (support cells, extracellular matrix, contact-dependent

signals), and secreted/extracellular (soluble cytokines, oxygen concentration, **Figure 10**). This microenvironment is responsible for enriching the stem cell, and maintaining it in an undifferentiated, multipotent state by promoting self-renewal, blocking differentiation and preventing apoptosis/senescence.<sup>6,7</sup> In fact, many tissues have two identified niches: one a quiescent niche, and the other an active niche. Stem cells residing in the quiescent niche tend to be dormant, but stem cells in the active niche tend to proliferate and readily differentiate into mature effector cells upon induction. The dynamic state of stem cells (quiescent vs. active) can be regulated by movement of the stem cells between the two niches (**Figure 11**).<sup>93-95</sup>

The stationary components of the stem cell niche are specialized cells that anchor stem cells through cadherins and integrins, and communicate self-renewal signals through contact-dependent signaling molecules. Some of the best studied contact-dependent niche signals include development-related factors such as Notch ligands, Wnts, BMPs, membrane-bound Kit, and Hedgehog.<sup>96-102</sup> Many of these factors are cell surface mediated signals that can only operate in *trans*, suggesting that the stem cell must be in direct contact with the niche cell to maintain undifferentiated state and promote self-renewal. These contact-dependent signals also provide spatial context for mitotic spindle alignment during replication. When the stem cell divides during self-renewal, one cell will remain in contact with the niche and the other cell, not in contact with the niche, will differentiate into a multipotent progenitor. In the *Drosophila* ovary, germ line stem cells interact with cap cells (niche cells) via E-cadherins.<sup>103,104</sup> The self-renewal signal is augmented by a gradient of self-renewal mediating factors (produced by the cap cells)

**Figure 10. Proposed model for the cellular and non-cellular components of the stem cell niche**



The stem cell niche is formed by stationary components which provide a stable physical residence for the stem cells, together with other support cells, and extracellular components. Based on the type of stationary component, cellular or noncellular, two types of stem cell niche have been proposed. (a) The stationary components of the cellular niche are specialized niche cells, and (b) the stationary component of the noncellular niche is a specialized ECM called the basement membrane.

Figure adapted from Breslin and Volk et al. 2014.

such as Unpaired and BMP. Disruption of E-cadherins in the germ line stem cells causes detachment and chemotaxis away from the cap cell and cytokine gradient, and results in differentiation due to loss of the self-renewal signal leading to eventual depletion of the stem cell pool. SNO cells (Spindle shaped N-cadherin<sup>+</sup> Osteoblastic) in the hematopoietic niche express cell-membrane bound SCF, Jagged, and Angiopoietin-1, which work together to restrict proliferation and maintain stemness.<sup>105-109</sup> Studies in rat mammary epithelial stem cells show deletion of integrin signaling between the stem cell and the basement membrane (a specialized component of the extracellular matrix) causes disorientation of the mitotic spindle resulting in loss of self-renewal during replication and eventual depletion of the stem cell pool.<sup>110,111</sup>

In addition to stationary components of the stem cell niche, there are also support cells that accompany the stem cell and provide support by augmenting the existing niche signals. The support cells also play a role in effecting the migration and trafficking of stem cells between quiescent and active niches. An example of such a cells is the CAR cell (CXCL-12 Abundant Reticular) in the HSPC niche. These CAR cells secrete SDF and IL6, which act as chemottractants for the HSPCs, drawing them strongly to the bone marrow and increasing their affinity for the vascular niche. This cell is hypothesized to chaperone HSPCs from the quiescent osteoblastic bone marrow niche to the active vascular niche.<sup>105</sup> In addition to CAR cells, two additional types of support cells have been identified in the bone marrow niche: CD146<sup>+</sup> osteoprogenitors and Nestin<sup>+</sup> mesenchymal stem cells.<sup>112,113</sup> HSPCs in the vascular niche are thought to be actively cycling and producing progenitor

**Figure 11. Active and quiescent niches regulate stem cell self-renewal**

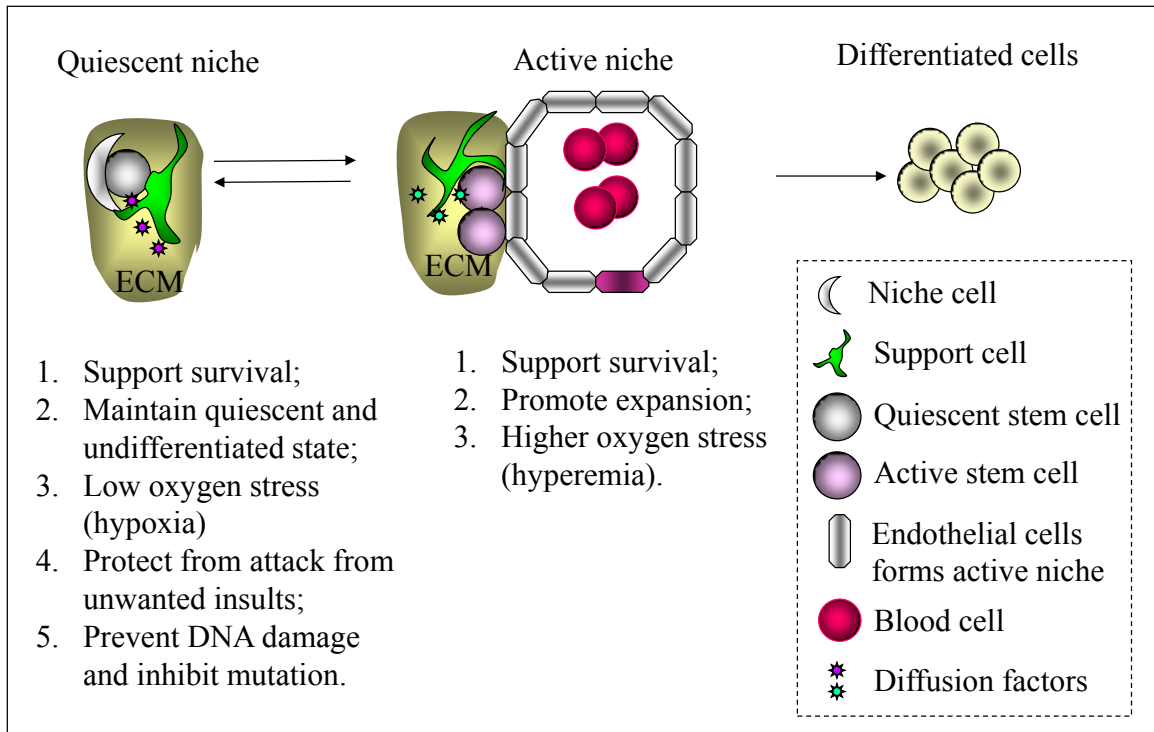


Figure adapted from Breslin and Volk, et al. 2014.

cells. In the *Drosophila* ovary, the somatic stem cell is a support cell component of the germline stem cell niche that produces progeny, migrates away from the cap cell (niche) during development, and is always in contact with the germline stem cell. The somatic stem cell produces progeny (some of which become more niche cells), and its direct contact with the germline stem cell and progenitor cells is required for complete differentiation and formation of functioning follicles.<sup>114-117</sup>

Some non-neural stem cell niches have been identified that have requisite sympathetic nervous stimulation. In the HSPC niche, nonmyelinating schwann cells (part of the nervous system) innervate the osteoblasts and stimulate them to provide critical growth factors such as TGF- $\beta$  and CXCL-12 which support the niche and promote stem cell dormancy and self-renewal. Denervation of the hematopoietic niche causes a sharp reduction in osteoblastic tendrils and mobilization of HSCs.<sup>118</sup>

The extracellular components of the stem cell niche are all the soluble factors contained in the microenvironment, as well as some of the extracellular matrix components responsible for orientation and location signaling during replication and self-renewal. These components are frequently secreted by classic niche cells, but some are long-distance paracrine signals produced from cells that are far away from the stem cell niche or even delivered from the peripheral circulation.

The extracellular matrix (ECM) consists of a complex network of interlocking molecules including glycosaminoglycans and hyaluronic acids, as well as proteins including collagens, fibronectins, and laminins. These proteins are produced both by stem cells and niche cells and immerse the stem cell in niche ECM-mediated signaling through



surface receptors such as integrins and CD44.<sup>119-126</sup> Focal adhesion signaling is critical for directing stem cell migration and anchoring, while integrins are implicated in correctly aligning the mitotic spindle during cell division, which is responsible for proper cell polarity driving asymmetric self-renewal division.<sup>31,33,35</sup>

In addition to the extracellular matrix, there is a broad array of soluble cytokines in the niche microenvironment. These cytokines are responsible for induction of quiescence or proliferation, and self-renewal or differentiation. The HSPC niche is one of the best studied models for soluble cytokine-mediated stem cell maintenance. Some identified soluble factors responsible for maintaining quiescence and self-renewal are thrombopoietin (TPO), SCF, and SDF1 in the bone marrow niche. Mice deficient in these cytokines have stem cell activation and depletion as well as mobilization of stem cells away from the niche microenvironment. Other cytokines are responsible for driving differentiation in response to some tissue need, including IL6, IL3 and G-CSF. Most of these niche factors drive critical survival machinery that prevents senescence or apoptosis/necroptosis in response to inflammatory stimuli such as TNF $\alpha$ , IL1 $\alpha/\beta$ , and interferons.<sup>127-133</sup>

The role of oxygen is also well-understood in the stem cell niche. High oxygen content, as seen near the vasculature, induces cell stress-driven stem cell proliferation and differentiation. Low oxygen content supports low cell stress and quiescence.<sup>134-138</sup> The HSPC niche is thought to be composed of two separate niches (quiescent osteoblastic niche, and active vascular niche), each with a different stem cell activity and ambient oxygen level. The vascular niche is near the blood vessels, is highly perfused with fresh

oxygen, and is the hypothesized location of actively dividing stem cells. The osteoblastic niche is further away from the vasculature and has a lower oxygen content. Here, HSPCs are not stressed by oxygen levels and remain quiescent. However, some studies also show that the space between the vascular niche and the osteoblastic niche is only one cell-width in distance, suggesting that these two may share the factors which regulating the dynamic of quiescence and proliferation of stem cells.<sup>139</sup>

A dynamic balance of these cytokines exists in the stem cell niche, forcing any external stimuli to overcome the quiescence and survival signals provided by the niche before the stem cell exits and divides. This is how the niche can both promote stem cell quiescence and self-renewal, yet at the same time provide the tissue with a steady source of appropriate numbers of progenitor cells to maintain tissue homeostasis. Deviation from these signals causes stem cells to either expand and become exhausted or divide uncontrollably. If a mutation occurs during this cellular expansion that represses cell death or further promotes cycling and accumulates more mutations (**Figure 12**).

### **Acute Myeloid Leukemia Overview**

Acute myeloid leukemia (AML) is a diffuse tumor of malignantly transformed blood stem and progenitor cells from the myeloid lineage. This tumor is composed of partially differentiated non-functioning blast cells. There are many known transforming events in AML, including point mutations and genetic deletions. While most AMLs are karyotypically normal, some AMLs can have two chromosomes fused together, creating a fusion gene. Fusion genes are formed when double strand break repair results in two genes from different chromosomes being fused together, though deletions/inactive

mutation of tumor suppressors and chromosome amplifications /active mutation of oncogenes are also common. These types of leukemia are characterized by the cell type in which the mutations occur. Acute Promyelocytic Leukemia (APL) is caused by a fusion between PML-RAR $\alpha$ . Other common fusion genes and their associated subtypes are: RUNX1-ETO (M2 subtype of AML), and CBFb-MYH11 (M4 subtype of AML). Other genetic abnormalities are also common in AMLs, including deletions on chromosomes 5, 9, and 13, as well as trisomy of chromosomes 8 and 21. Overexpression of fusion genes and deletions/triplications of these portions on chromosomes generates a pro-survival bias for these cells, and promotes self-renewal and uncontrolled proliferation. However, roughly half of AMLs are cytogenetically normal, meaning they do not express any detectable fusion genes.<sup>140</sup>

AML has a distinct disease progression. The disease is diagnosed by bone marrow samples containing > 30% leukemic blasts (FAB morphologic diagnosis and classification), and characterized by flow cytometric analysis of cell surface proteins as well as cytogenic techniques (molecular diagnosis and classification). AML prognosis is highly dependent on age of onset, with 60-85% of patients <65 y.o.a. achieving complete remission (as determined by <5% blasts in bone marrow) and 30% achieving long-term survival (> 5 years following treatment). However, if the patient is >65 y.o.a., their chance of achieving complete remission is 40-55% and only 10-20% achieve long-term

**Figure 12. Niche factors that regulate stem cell self-renewal.**

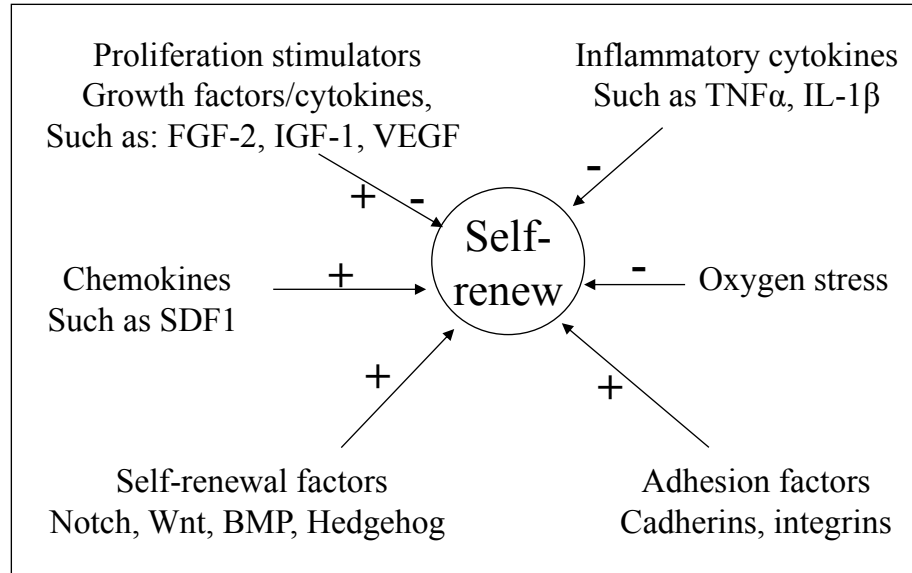


Figure adapted from Breslin and Volk, et al. 2014.

survival. Clinical treatment of AML is often composed of a “7+3” regimen of seven days of cytarabine plus three days of anthracycline treatments. This treatment protocol eliminates blasts from the bone marrow and peripheral blood. Some patients also receive a stem cell transplantation consolidation therapy following induction chemotherapy, depending on the disease karyotype, prognosis, and age of onset. In late-stage AML, the blast percentage is so high that the blood can no longer carry sufficient oxygen for proper tissue function or platelets for hemostasis, rendering these patients hypoxemic and coagulopathic.

AMLs are diagnosed and characterized within eight different subgroups, according to the French-American-British (FAB) subtyping system, with increasing integers indicating the level of relative maturation of the predominating blasts or the predominating cytogenetic abnormality. M0 is observed as undifferentiated AML, M1 is an AML with minimal maturation, M2 has evidence of maturation (40% of such AML are caused by translocation of the *RUNX1* and *ETO* genes). M3 is also known as Acute Promyelocytic Leukemia (APL), >95% of M3 AML is caused by translocation and fusion of the *PML* and *RAR $\alpha$*  genes, and will be discussed in this review later. M4 is an acute myelomonocytic leukemia. >30% is caused by fusion of *CBF $\beta$*  and *MYH11* genes due to *inv(16)*. M5 is an acute monocytic leukemia and includes AMLs caused by fusion of the *MLL* gene to partner genes (such as *AF9* and *ENL*, although *MLL* fusions are also detected in other AML subtypes as well as Acute Lymphoblastic Leukemias and Mixed Lineage Leukemias), and M6 is a relatively rare occurrence of acute erythroid leukemia.

M7 is an AML formed of predominantly transformed megakaryocytes (acute megakaryocytic leukemia), and is also relatively rare.

Successful treatment options are limited for AML. There is currently only one subtype of AML that can be reliably put into long-term remission: Acute Promyelocytic Leukemia (APL), which is caused by chromosomal translocations between chromosomes 15 and 17 in myeloid progenitor cells, resulting in the fusion of two genes: PML and RAR $\alpha$ . This type of leukemia is successfully treated by a combination of All-Trans Retinoic Acid (ATRA) and Arsenic Tri-oxide (AsO<sub>3</sub>). APL patients treated with ATRA and AsO<sub>3</sub> have a 95% long-term remission rate.<sup>141</sup> Current consensus mechanism of such treatment is AsO<sub>3</sub> induces apoptosis and ATRA degrades the fusion gene protein product and induces differentiation to functioning granulocytes.

However, there is always a small population of leukemic cells that can evade induction/remission chemotherapy.<sup>142,143</sup> Because these treatments cause selective pressure on the AML cells, they will remove all the sensitive cells and leave behind any cells that have some kind of mutation or dysregulated gene that allows them to survive. As a result, any future treatment with similar drugs will be ineffective at removing this disease. This phenomenon is the basis of resistance, and in cancer is called Minimal Residual Disease (MRD). Cells from the MRD population are the primary reason for relapse and the eventual death of the AML patient. Because leukemic MRD cells are capable of repopulating the tumor after chemotherapy, it was commonly believed that MRD cells are enriched “Leukemia Stem Cells” (LSCs).<sup>144</sup>

## Leukemia Stem Cells

An increasing body of evidence suggests that neoplastic cells found in many tumor types are functionally heterogeneous in their ability to initiate tumor growth as determined by *in vivo* transplantation in mouse models.<sup>1,2</sup> This suggests that tumors, like normal tissues, are composed of a heterogeneous hierarchy of different cell types, with some cells able to produce more tumor cells (stem cells), and partial differentiated progenitor and blast cells. These cancer stem cells share some characteristics of normal tissue stem cells: they are slow dividing, relatively resistant to cell death, and can maintain tumor homeostasis through differentiation into tumor progenitor cells. These characteristics make cancer stem cells very difficult to treat because almost all conventional chemotherapy drugs target actively dividing cells and induce cell death via DNA damage. In addition, cancer stem cells might have powerful machinery in place to prevent cell death and repair DNA damage.<sup>145-150</sup>

It is generally believed that cancer stem cells (CSCs) are initiated from tissue stem cells which have self-renewal capacity. However, CSCs could also arise from mutated versions of progenitor cells in any stage of differentiation if self-renewal ability can be obtained.<sup>151-153</sup> Genetic deletion of the APC gene in intestinal crypt stem cells ultimately leads to expansion of the cell population into a malignant tumor and a variety of intestinal cancers.<sup>154</sup> These studies suggest a stem cell origin of CSCs. Committed myeloid progenitors or granulocyte-macrophage progenitors from Bcr-Abl<sup>+</sup> chronic myeloid leukemia patients in blast crisis phase of the disease commonly express high levels of active  $\beta$ -catenin and have self-renewal capability.<sup>155,156</sup> Many leukemic fusion

genes such as *MLL-AF9* and *MOZ-TIF2* can induce leukemia development from myeloid progenitors. These studies suggest progenitor origin of CSCs. In certain cancers, genetic mutations and niche signaling collaborate to induce the development of CSCs by dedifferentiation. For example, chronic inflammation in the liver (due to viral infection or cirrhosis) and colon induces a perpetual state of wound-healing and can lead to mutations and activations of genes that can impart self-renewal and dedifferentiation in previously terminally-differentiated hepatocytes and intestinal epithelia through genetic instability and downregulation of tumor suppressor p53 (**Figure 13**).<sup>157-161</sup>

LSCs, like any other stem cell population, are a specialized set of cells that are capable of regenerating a similar type of leukemia upon transplantation. They are able to repopulate leukemia tumor in leukemia remission patients after withdrawing chemotherapeutic drugs. LSCs are a fascinating cell type to study because they arise from healthy blood stem and progenitor cells, and reside in the HSPC niche, but they hijack the niche and secrete inflammatory factors that repress the healthy blood stem cells. LSCs were first identified as  $\text{lin}^- \text{CD34}^+ \text{CD38}^-$  AML cells that could regenerate leukemias in xenograft animal models.<sup>1,2,162,163</sup>

LSCs, like all other stem cells, require factors to promote self-renewal, block differentiation, and prevent apoptosis/senescence. One of a well-studied self-renewal factor in LSCs is the polycomb repressor complex component BMI-1, which is responsible for repressing senescence and cell cycle inhibition genes *p16<sup>Ink4a</sup>* and *P19<sup>Arf</sup>*.

BMI-1 has been regulated by many different mechanisms, including transcriptional, translational and post-translational. For example, a recent study suggests

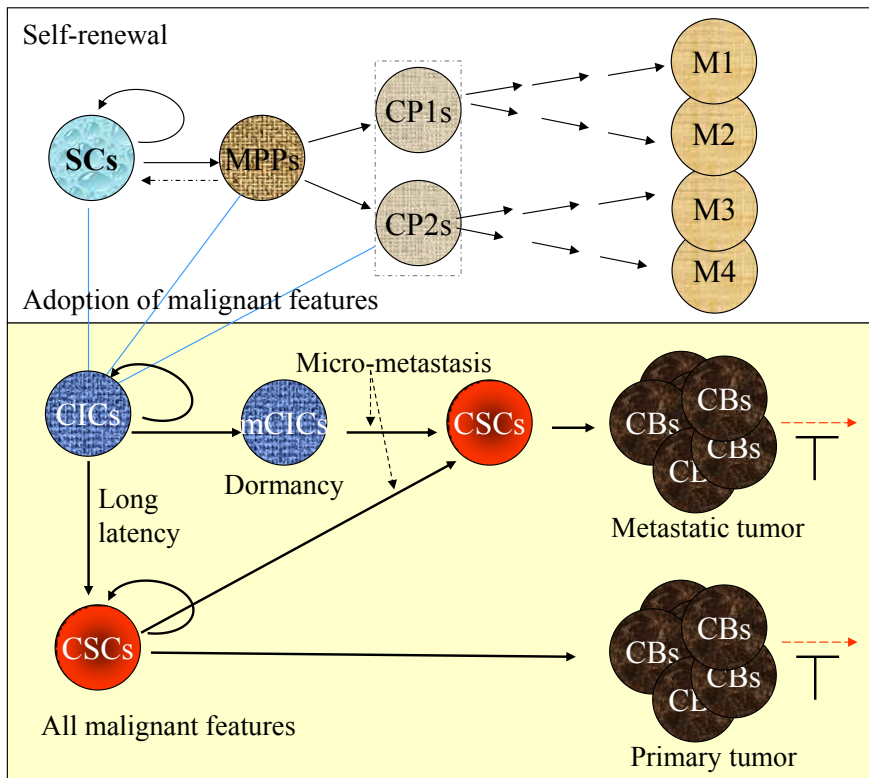


that BMI1 activity is negatively regulated by AKT phosphorylation at serine residues 251, 253, and 255. Such phosphorylation of BMI1 is linked to proliferation and differentiation of LSCs, leading to reduction of the stem cell pool and regression of the tumor.<sup>164</sup>

Activated  $\beta$ -catenin, through the Wnt signaling pathway, travels to the DNA and forms trimeric complexes at the regulatory regions of proliferation and survival genes with the AP1 family transcription factors Jun and Fos. Together, the expression of these genes block senescence and maintain the undifferentiated status of LSCs.<sup>165</sup> NF- $\kappa$ B is another critical transcription factor required for maintaining LSC survival through the upregulation of antiapoptotic genes like c-FLIP, Bcl-2, Bcl-xL, and anti-necroptotic genes like cIAP1/2. Recent studies show that NF- $\kappa$ B is even required for function of oncoproteins involved in MLL-rearranged AMLs, contributing to the building evidence that NF- $\kappa$ B activity is critical for LSC function.<sup>13</sup>

Integrins have been recently identified as critical mediators of LSC survival and self-renewal. LSCs deficient in integrin beta3 have defects in engraftment and survival due to reduction in intracellular kinase SYK activity. This results in loss of powerful antiapoptotic and dedifferentiation signals, and the exhaustion of the LSC pool.<sup>166</sup>

**Figure 13. Model for cancer stem cell development.**



Model of cancer initiating cell (CIC) and incorporation of the cancer stem cell (CSC). CICs arise from SCs, MPPs, or CPs, and adopt malignant features that transform them with cancerous features. CICs give rise to CSCs, and CSCs within heterogeneous tumors give rise to differentiated cancer blasts (CBs). This process is similar to the differentiation hierarchy of normal tissue stem cells.

Figure adapted from Breslin and Volk, et al. 2014.

### NF- $\kappa$ B overview

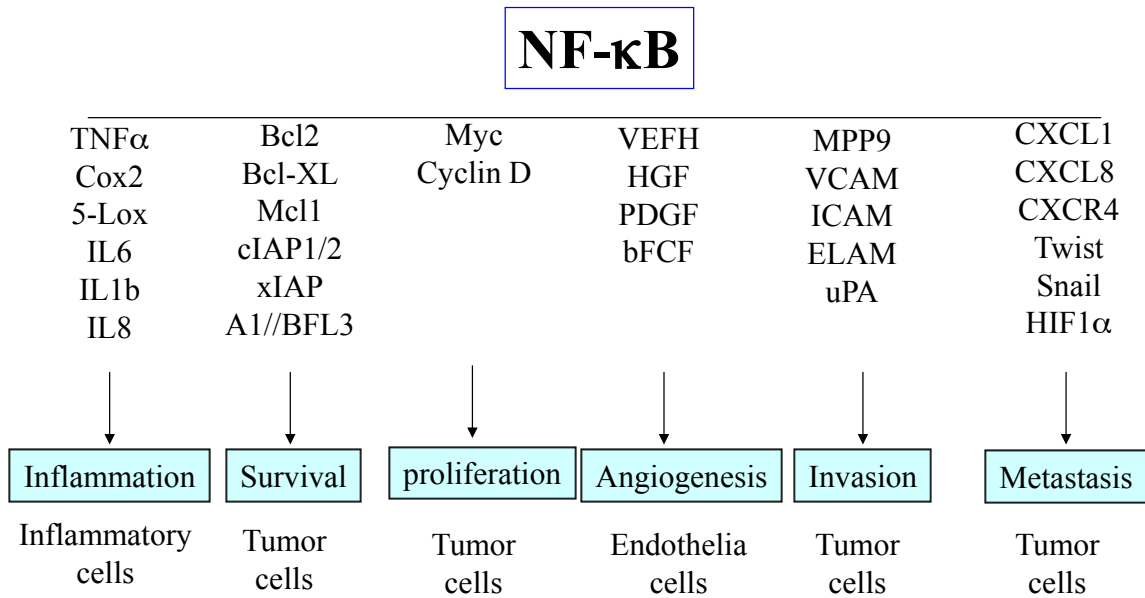
Nuclear Factor kappa B (NF- $\kappa$ B) is a major mediator of immunity, inflammation, tissue regeneration, and cancer signaling.<sup>167-169</sup> It is a member of a transcription factor family composed of many different subunits that form dimers and translocate to the nucleus to drive target gene expression (**Figure 14**). Canonical NF- $\kappa$ B is comprised of p65-RelA, c-Rel and P105/50-NF- $\kappa$ B1, while non-canonical NF- $\kappa$ B is comprised of precursor p100/p52-NF- $\kappa$ B2 and Rel-B. Most studies identify canonical NF- $\kappa$ B for being critical in maintaining LSC function in AML, while non-canonical NF- $\kappa$ B functions in many types of B lymphocytic malignancies. In AML, NF- $\kappa$ B is primarily stimulated by inflammatory cytokines like TNF and IL1, as well as by the toll-like receptor (TLR) family responsible for detecting pathogen-associated molecular patterns (called PAMPs) and tissue damage-associated molecular patterns (named DAMPs). The standard paradigm for NF- $\kappa$ B activation begins with surface receptor activation (TNFr1/2, IL1r, TLRs), and recruitment of scaffolding proteins (such as TRAF2/5) to the intracellular portion of the receptor. These scaffolding proteins are poly-ubiquitinated by E3 ubiquitin ligases in the cIAP family to form K63 type of poly-ubiquitin chains. Both TAB2/TAK1 and IKK (containing IKK $\alpha/\beta/\gamma$ ) complexes are recruited to these chains, where TAK1 is activated by an auto-phosphorylation mechanism which in turn activates the IKK complex by phosphorylating IKK $\beta$ . I $\kappa$ B $\alpha$ 's function is to bind NF- $\kappa$ B and obscure the nuclear localization signal, effectively sequestering NF- $\kappa$ B in the cytoplasm. After phosphorylation, I $\kappa$ B $\alpha$  is polyubiquitinated (K48 type) and targeted for destruction by the proteasome.<sup>170</sup> This interaction is so strong that NF- $\kappa$ B can be artificially blocked by

overexpression of a super repressor mutation of I $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ SR), which has both IKK phosphorylation serine sites mutated to alanine.

NF- $\kappa$ B, once freed from the I $\kappa$ B $\alpha$  suppressor protein, translocates to the nucleus to activate target genes. The transactivation domain of the NF- $\kappa$ B protein binds to other NF- $\kappa$ B subunits, and travels to specific sequences in the promoter regions of target genes, where the DNA-binding domain of the two subunits interacts with regulatory regions of target genes, most of which happen to be Bcl-2 family proteins, c-FLIP antiapoptotic proteins, or TNF family inflammatory proteins. The cellular signaling results of NF- $\kappa$ B-activated gene products are complex, because NF- $\kappa$ B can activate both pro-apoptotic inflammatory cytokines and anti-apoptotic proteins. Pro-inflammatory cytokines induce NF- $\kappa$ B activity, and NF- $\kappa$ B induces pro-inflammatory cytokine production, resulting in a feed-forward loop or induces expression of negative regulator of NF- $\kappa$ B (such as I $\kappa$ B $\alpha$  and A20) to turn off signal in a feed-back loop. This results in a cycle of NF- $\kappa$ B activity upon stimulation with Tnf, as observed in mouse embryonic fibroblasts, of peaks every 30-60 minutes. This is attributed to the degradation and replenishment of I $\kappa$ B $\alpha$  repressor subunits.<sup>171</sup>

Healthy HSPCs have a low level of NF- $\kappa$ B activity, making them prone to destruction by inflammatory cytokine stimulation. Although these pro-inflammatory cytokines can induce a low-level of NF- $\kappa$ B, it is not enough to overcome the pro-apoptotic/necroptotic signals imparted by these cytokines. LSCs, on the other hand, have a very high basal level of NF- $\kappa$ B activity, rendering them mostly immune to

**Figure 14. Activation paradigm of NF- $\kappa$ B.**



any cell death mediated by inflammatory cytokine stimulation, while capitalizing on the pro-survival and proliferative effects of NF- $\kappa$ B.<sup>172</sup>

These characteristics of NF- $\kappa$ B make it an attractive target for many different types of cancers with elevated NF- $\kappa$ B activity. In fact, several *in vitro* studies show that NF- $\kappa$ B activity is critical for maintaining LSCs. Inhibition of NF- $\kappa$ B activity can completely eliminate LSCs *in vitro* before toxicity is seen in healthy HSPCs.<sup>14</sup> However, because NF- $\kappa$ B mediates a powerful anti-apoptotic signal in most tissues studied through upregulation of several different families of survival genes, generating a clinically-effective inhibitor is challenging.<sup>4,5,173</sup> The best molecules available for clinical inhibition focus on either blocking IKK activity or preventing degradation of the I $\kappa$ B $\alpha$  repressor subunit through proteasome inhibitors.<sup>3,5,174</sup> These treatments have been approved and are standard of care for some cancers like Multiple Myeloma. In AML, the observed effects of NF- $\kappa$ B inhibition *in vitro* appear to vanish *in vivo*, suggesting that there are additional signaling pathways activated *in vivo* that can compensate for NF- $\kappa$ B inhibition in LSCs. I propose that these signals are provided by the LSC niche.

### **Leukemia Stem Cell Niche**

AML patients often have repressed hematopoiesis. In my studies, I found that patients with AML have significantly elevated serum levels of inflammatory cytokines circulating in their peripheral blood, and that these inflammatory cytokines are being produced by the leukemia cells. These inflammatory cytokines act in a paracrine manner to induce cell death in neighboring HSPCs, and act in an autocrine manner to drive survival and proliferation in the LSCs.<sup>172</sup> Additionally, many of the standard-of-care

chemotherapies are designed to induce apoptosis and necroptosis in rapidly dividing leukemic blast cells. Ours and other's studies suggest the LSCs are resistant to these therapies because they change the healthy HSPC niche into a leukemia promoting environment.

The dependence of a niche for the growth of disease stem cells is well demonstrated in a pre-leukemic neoplasm called Myelodysplastic Syndrome (MDS). MDS cells are a myeloid neoplasm that reconfigure the bone marrow niche, ultimately displacing and rendering ineffective the resident HSPCs. MDS is characterized by refractory anemia due to insufficient bone marrow hematopoiesis. Approximately 10% of MDS patients progress to leukemia due to the expansion of mutant blasts. In *in vivo* murine studies, MDS cells isolated from patients cannot be xenografted into mice without concurrent transplantation of mesenchymal stromal stem cells from the same patient. These studies suggest that the disease mesenchymal stem cells promote MDS stem cell self-renewal through activated secretion of niche factors such as N-cadherin, IGFBP2, VEGFA, and LIF. Without MDS mesenchymal stromal cells, MDS stem cells are unable to engraft in recipients, suggesting these stromal cells are a critical component of the CSC niche.<sup>175</sup>

### **LSCs reorganize the hematopoietic stem cell niche**

LSCs utilize a complex signaling network to integrate various survival and self-renewal signals from the niche in order to promote their own survival, self-renewal, and block differentiation. Many of these self-renewal factors are similar to common stem cell self-renewal factors (i.e. Bmi1, Wnt/B-catenin, SCF, and Bcl-xL). In fact, LSCs can

secrete the chemotractant SCF that can attract both LSCs and healthy HSPCs to the malignant LSC niche. This causes death and repression of the HSPC population in patients, which can be reversed by inhibition of LSC-secreted SCF.<sup>176</sup> Osteopontin (OPN), a cytokine secreted by osteoblasts in the bone marrow, also acts as a chemotractant to anchor blood stem cells in the niche. While this is advantageous for maintaining HSPCs, LSCs also utilize OPN as a pro-survival/self-renewal signal. Following cytotoxic chemotherapy, LSCs retreat to the OPN-rich HSPC niche, where OPN induces quiescence and apoptosis-resistance. Blocking OPN in this system induces proliferation and detachment from the niche, and can synergize with cytotoxic chemotherapies.<sup>177</sup> Additionally, LSCs have the ability to change the HSPC niche microenvironment through secretion of pro-inflammatory cytokines and conversion of vascular endothelia into vascular tissue-associated AML cells and mesenchymal stromal cells (MSCs) into Cancer-Associated Fibroblasts (CAFs). This pro-inflammatory microenvironment is toxic to healthy HSCs.

#### **MSCs and vascular endothelia support LSCs in the reorganized niche**

LSCs also have been shown to localize to the vascular hematopoietic niche, due to their innate ability to survive in an oxygen-reactive environment.<sup>178</sup> They accomplish this by converting neighboring vascular endothelia cells into vascular tissue-associated AML cells through the upregulation of V-CAM and CD105. Through this mechanism, LSCs recreate the vascular niche into a supportive microenvironment. It should be noted that LSCs have also been identified outside of the bone marrow niche and in the peripheral



blood and spleen, suggesting that LSCs have the ability to survive and self-renew outside of the niche.

Following therapies, LSCs can also induce local MSCs to form Nestin<sup>+</sup> CAFs. CAFs are characterized as large, spindle-shaped mesenchymal cells that are characteristically similar to fibroblasts.<sup>179,180</sup> In leukemias, studies suggest that LSCs secrete pro-inflammatory cytokines which recruit Nestin<sup>+</sup> MSCs to the leukemic niche and differentiate them into CAFs. These CAFs then support the LSCs by secretion/surface expression of additional pro-inflammatory and LSC-supportive molecules such as TGF- $\beta$ , Wnt, and Notch.<sup>180,181</sup>

By inducing local MSCs to become CAFs cells, LSCs convert the low-oxygen/non-inflammatory healthy hematopoietic niche into a malignant/high-oxygen/pro-inflammatory leukemic niche. This leukemic niche promotes the survival of LSCs in the presence of cytotoxic chemotherapies. Therefore, treatments are needed that can evict LSCs from the niche and restore the niche to its original healthy form. Some studies in mice suggest that displacing the LSCs from their niche can accomplish such a task, and allow for repopulation with healthy HSPCs and long term remission from the disease.<sup>182</sup> Making the leukemic niche inhospitable to LSCs is the focus of our studies in this dissertation, and represents the leading solutions for removing LSCs, MRD, and providing AML patients with true long-term remission.

### **Pro-inflammatory cytokines support LSCs in the reorganized niche**

Pro-inflammatory cytokines act in a paracrine manner to induce cell death and differentiation in healthy HSPCs, effectively clearing the niche for expansion and colonization by LSCs. The LSCs also have mechanisms in place to convert the usual death signals from the pro-inflammatory cytokines into survival signals. This way, the pro-inflammatory cytokines can act in an autocrine manner to drive survival and self-renewal of the LSC. Some of these critical factors in leukemogenesis are IL6, IL3, TNF, and IL1.

IL6 is one of the major pro-inflammatory cytokines secreted by immune cells in the body. IL6 stimulation normally drives multipotent progenitor cell differentiation into the granulocyte lineage. In Bcr-Abl chronic myeloid leukemia (CML), the blast cells secrete pro-inflammatory IL6 to reorganize the niche in order to promote survival and proliferation of CML stem cells. CML stem cells are different from the differentiated CML blasts in that they are tyrosine kinase inhibitor resistant.<sup>183,184</sup> Although seemingly independent of tyrosine kinase activity, these CML stem cells appear to be dependent on a positive feed-forward loop revolving around IL6 secreted by granulocytes for survival and proliferation. CML blasts secrete IL6, which drives multipotent progenitor cells towards the granulocyte/macrophage lineage and inhibits other lineage differentiation. IL6 drives the proliferation of CML progenitors into leukemic granulocytes, which in turn, produce more IL6, thus continuing the process. This process has been implicated as a critical component for driving Bcr-Abl fusion leukemias.<sup>185</sup>

IL3 is another pro-inflammatory cytokine secreted by activated T and mast cells, and is implicated in LSC self-renewal. IL3 receptor, CD123 (IL3  $\alpha$ -subunit), is overexpressed on LSCs and progenitors, and clinically correlates with lower survival and poor prognosis in AML patients.<sup>186,187</sup> Inhibition of the IL3 signal has been shown to block survival/proliferation and homing of LSCs to their niche, as well as induces an immune reaction against the LSCs, making targeting the IL3 receptor an interesting therapeutic strategy for LSC-specific treatments. Several groups are currently working on IL3-mediated therapies by develop anti-CD123 antibody or chimeric antigen receptor T cells specific for CD123.<sup>188-190</sup>

TNF is a pleiotropic cytokine that induces a variety of cellular responses, including cell survival, proliferation, and inflammatory cytokine production. TNF can also stimulate both survival and death signals within the same cell, depending on the context. TNF-stimulated survival is mainly mediated by formation of a complex that consists of TRADD, TRAF2/5, cIAP1/2 and RIP1 (Complex I). TNF induces activation of its receptors, TNFR1 and/or R2, stimulating Complex I formation. Complex I induces the activation of TAK1 (a member of the MAP3K family of enzymes) through a K63-linked polyubiquitin-dependent mechanism.<sup>191-202</sup> TAK1 then induces the activation of downstream signaling pathways, including IKK-I $\kappa$ B-NF- $\kappa$ B, MKK4/7-JNK-AP1 and MKK3/6-p38.<sup>192,203-209</sup> Activation of IKK-I $\kappa$ B-NF- $\kappa$ B signaling pathway protects the cell from apoptosis/necroptosis by inducing expression of survival genes (Bcl2, Bcl-xl, Mcl1, Survivin, cIAP1/2, and cFLIP).<sup>192,203-206,210-214</sup> MKK3/6-p38 signaling will not be discussed here because I found that inactive p38 promotes the colony-forming ability of

both normal hematopoietic stem/progenitor cells (HSPCs) and *MLL-AF9*-transduced murine LCs.

The TNF-induced death signal is mediated by the apoptotic complex (Complex II), a necroptotic complex (necrosome) or a ROS production-related complex.<sup>215-230</sup> Apoptotic Complex-II consists of RIP1/FADD/Caspase-8/cFLIP.<sup>215,216,220-230</sup> FADD is a scaffolding protein required for efficient TNF-induced caspase-8 activation, while cFLIP is a caspase-8 homologue that negatively regulates caspase-8 activity by forming a catalytically inactive heterodimer with caspase-8.<sup>215,216,231-235</sup> The necroptotic complex contains RIP1/RIP3 and induces cellular necroptosis through a mechanism that is currently not understood.<sup>227-229,236-238</sup> Recent studies suggest that the apoptotic and necroptotic signals are dependent on each other: they form another complex called the Ripoptosome which contains both Complex II and the Necrosome (RIP1/RIP3/MLKL).<sup>236</sup> Activated caspase-8 (Complex II) inhibits necroptosis by inducing the cleavage of RIP1/RIP3. Thus upon ripoptosome formation, whether the cell undergoes apoptosis or necroptosis is determined by the level of enzymatic activity of Caspase-8.<sup>215,216,220,231,239</sup> TNF can also induce the formation of a TRADD/RIP1/NOX1/RAC1 complex which leads to an increase in ROS production and JNK signal-mediated cell death.<sup>240</sup> The role of JNK in caspase-8-mediated apoptosis and RIP3-mediated necroptosis is still a mystery.

Our lab found that TNF stimulates distinct signals in HSPCs and LCs. In HSPCs, in addition to NF- $\kappa$ B-mediated survival signaling, TNF induces two parallel death signals: JNK/RIP1/RIP3-dependent necroptosis and JNK1/Caspase-8-dependent type-II

apoptosis.<sup>241</sup> TNF-induced necroptosis can be prevented by treatment with Necrostatin-1 (Nec1, a RIP1 inhibitor) or *RIP3* deletion, whereas TNF-stimulated apoptosis can be repressed by JNK1 inactivation or *Bcl2* over-expression and can be converted to necroptosis by caspase-8 inhibition. The remaining HSPCs seem resistant to TNF treatment. In LCs, JNK signal promotes survival/proliferation.

### **TNF and Acute Myeloid Leukemia**

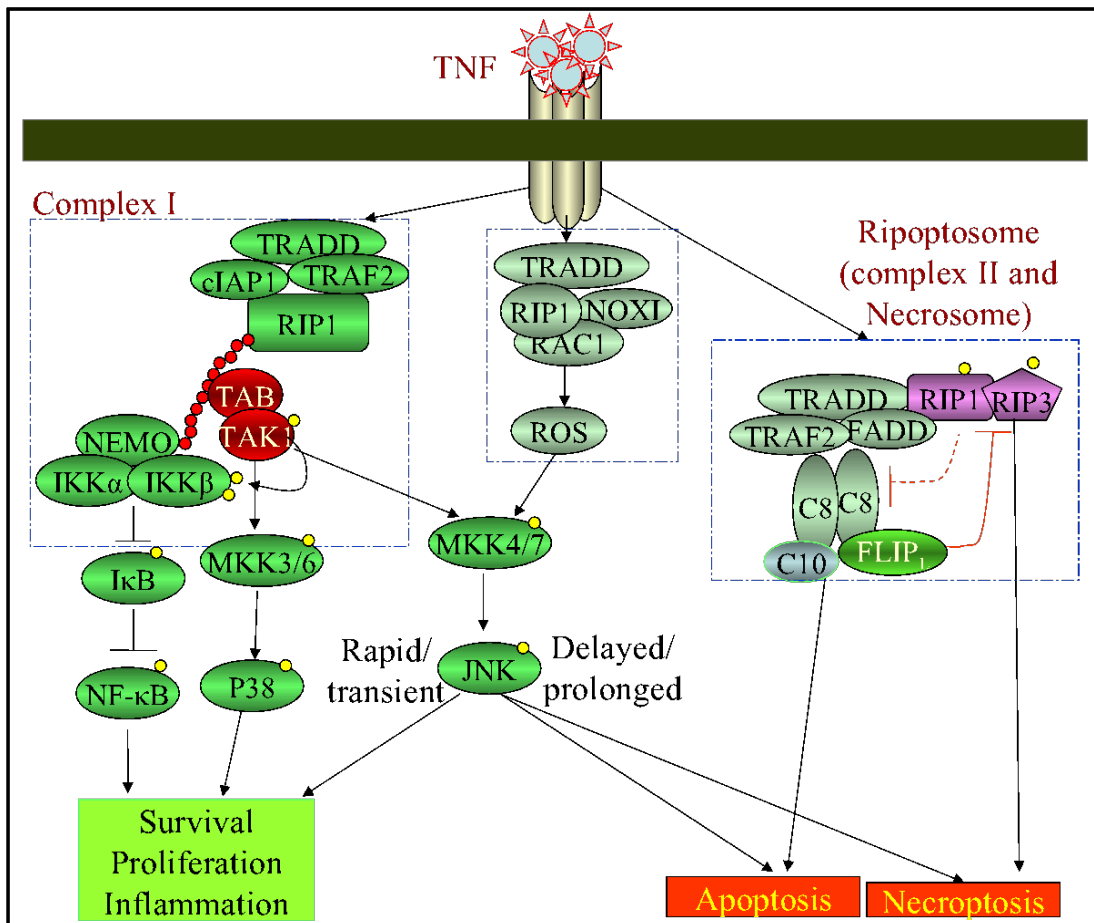
TNF is significantly increased in most tumor tissues, being produced by both tumor cells and tumor-infiltrating hematopoietic cells.<sup>242-245</sup> Early studies suggested that TNF suppresses tumor activity by inducing apoptosis and/or necroptosis in tumor cells.<sup>246-249</sup> However, most cancer cells are resistant to TNF-induced death signaling.<sup>246,247,250</sup> Animal model studies suggested a tumor-promoting activity for TNF.<sup>251-255</sup> JNK-AP1 signaling induced by TNF plays an essential role in carcinogen-induced skin, liver and colon cancers.<sup>256-264</sup> Inhibition of TNF signaling using soluble receptors and neutralizing antibodies has become a potential treatment for some malignancies. In fact, TNF blockers are currently standard of care for multiple myeloma.<sup>265</sup>

In hematopoietic tissues, TNF-represses normal hematopoiesis by inducing death signals in HSPCs. Increased TNF levels were detected in patients with bone marrow failure, including aplastic anemia and myelodysplastic syndromes (MDS), suggests that such hematopoietic repressive activity of TNF might contribute to the cytopenic phenotype of these patients.<sup>143,266-275</sup> The further increase in TNF levels during disease progression in MDS patients implies that TNF might be involved in leukemic

transformation of the mutant HSPCs and disease progression.<sup>271,276-278</sup> In support of this notion, TNF promotes the clonal evolution of leukemic cells in *FancC*-mutant HSPCs.<sup>279</sup> Increased levels of TNF are detected in the PB and BM of most human leukemia patients and are correlated to higher WBC counts and poorer prognosis.<sup>12</sup> Our studies suggest that TNF promotes LC growth *in vitro* and leukemogenesis *in vivo*. Inactivation of TNF can inhibit the colonigenic growth of TNF-expressing LCs *in vitro* and repress leukemia development in transplanted mice.<sup>172</sup>

IL1 $\alpha$ / $\beta$  (IL1) is another pleiotropic pro-inflammatory cytokine that has similar pro-survival/pro-death activity as TNF. IL1, like TNF, is initially produced as a pro-enzyme in most leukocytes and endothelial cells, and is responsible for initiating canonical inflammatory response pathways in most cells studied. IL1 pro-enzyme is cleaved by catalytically active Caspase-1, which is only rendered functional after stimulation of the NLRP1 inflammasome. This protein complex is normally activated by interferon stimulation, IL1/TNF receptor activation, or toll-like receptor activation. These inflammatory factors (and others from different cancers: IL-23, VEGF-A, CSF-1, S100A8/9, TGF-B, and lysyl oxidase) are produced by LSCs and surrounding stromal/epithelial/immune cells and act not just as chemottractants for cancer stem cells, but also for formation of myeloid derived suppressor cells (MDSCs) that can downregulate immune surveillance.<sup>242,280-284</sup> MDSCs accomplish this by inhibiting both adaptive and innate anti-tumor immunity. In the tumor stem cell niche microenvironment,

Figure 15. Pleiotropic effects of TNF stimulation on target cells.



hypoxia alters the MDSC function and drives differentiation towards Tumor-Associated Macrophages (TAMs) through activation of Hif1- $\alpha$ . TAMs can also differentiate from normal healthy monocytes, and have a higher immuno-suppressive capability than MDSCs by blocking T and NK-cell mediated tumor surveillance. TAMs also secrete large amounts of pro-inflammatory cytokines IL-1 $\beta$ , TNF, IL4, and IL6, all of which promote the expansion of LSCs while repressing normal healthy HSPC survival and self-renewal.<sup>144</sup>

### **LSC self-renewal**

LSCs employ many of the same components of the self-renewal machinery that normal tissue stem cells utilize, and use them in a similar manner. This includes cell cycle regulation and anti-senescence machinery like Bmi1, differentiation and lineage control proteins like Wnt/ $\beta$ -catenin, and survival machinery like Bcl-xL and c-FLIP. LSCs from MLL-rearranged leukemias also require other genes from the homeobox group (Hox genes) to maintain self-renewal. In fact, simple over-expression of Hoxa9 in hematopoietic progenitor cells from mice is sufficient to generate leukemia.<sup>285</sup>

Jun N-terminal Kinase (JNK) is one of the major mediators of leukemogenesis in several models studied. In *Fancc*-knockout mice, TNF and JNK are required for clonal evolution of the disease, and these animals develop leukemia in a JNK-dependent manner.<sup>279</sup> In Bcr-Abl-induced CML, TNF-induced JNK1 signaling is required for activation of critical survival genes.<sup>286</sup> This is in contrast to its most well-known role of activating cell death via the intrinsic/mitochondrial apoptosis pathway. Other known pathways requiring JNK activity for survival and proliferation is the complimentary



proliferation response in hepatocytes following resection of a portion or chemical/inflammation induced damage of the liver.<sup>287-289</sup> Under this condition, TNF/JNK signaling causes the death of a large number of hepatocytes, but stimulates others to reform the tissue, while NF- $\kappa$ B acts as a tumor suppressor by repressing TNF-JNK activity.<sup>290</sup> TNF/JNK also promotes a proliferative signal in epidermal cells by upregulating the positive regulator of cell cycle CDK4 and downregulating negative cell cycle regulators like P16<sup>Ink4a</sup>.<sup>289,291</sup> These signals are required for epidermal neoplasias following NF- $\kappa$ B inhibition.

The most well-studied target of JNK is the transcription factor c-Jun. JNK phosphorylates c-Jun at the N-terminus (hence its name: Jun N-terminal Kinase). Phosphorylation by JNK causes c-Jun to form a dimer with either a member of the Fos family (c-Fos, FosB, and splice variants Fra-1 and Fra-2), or another member of the Jun family (JunB, Jun D). Together, this dimer is called AP1, and translocates to the nucleus to act as an activator of target genes along with other transcription factors from the LEF and  $\beta$ -Catenin family. Many genes are regulated by c-Jun including Myc/Myb, NF- $\kappa$ B family proteins, and CD42, to name a few (**Figure 16**).

JNKs are members of the MAPK family variant called SAPK (stress-activated protein kinases). Three isoforms are known: JNK1, 2 and 3. JNK1 and JNK2 are ubiquitously expressed while JNK3 is primarily expressed in neuronal and heart tissues. Like other MAPK family members, JNK is activated by a MAP3K-MAP2K-MAPK three-component complex signal cascade. So far, More than 14 MAP3Ks for the JNK signal have been identified (**Figure 16**).<sup>208,292-295</sup> Although the physiological relevance of

MAP3Ks in JNK activation has yet to be determined, some studies suggest that different MAP3Ks might stimulate different levels and duration of JNK, generating different biologic functions.<sup>296-298</sup>

Both pro-apoptotic and pro-survival/proliferative activities for JNK signal have been reported. In many types of cells, upon TNF stimulation, JNK promotes a mitochondrion-dependent cell apoptosis by phosphorylation-dependent inactivation of several pro-survival Bcl-2 family members, or phosphorylation-dependent activation of the pro-apoptotic BH3-only subgroup of the Bcl2 family of proteins.<sup>11,299-305</sup> JNK also induces the cleavage of Bid to a specific fragment termed “jBid”, and up-regulates pro-apoptotic genes such as Puma and Bax (Figure 2). The relationship between JNK and Caspase-8 in apoptosis as well as the relationship of JNK and RIP1/3 to necroptosis remain to be determined. Some studies suggested that Caspase-8 can function upstream of JNK. However, in many types of cells, JNK induces apoptosis through stimulating Caspase-8 activity by inducing the degradation of cFLIP, the key negative regulator of Caspase-8, through the proteosomal degradation pathway. This is accomplished by phosphorylating the E3 ubiquitin ligase responsible for marking cFLIP for degradation by polyubiquitination, ITCH.<sup>306</sup>

The pro-survival and proliferative activities of the JNK signal are mediated by transcriptional mechanisms. Several transcription factors, including AP-1 (cJun, ATF2, JunD, JunB, cFos), Elk-1, c-Myc, NFAT4, HSF-1, androgen receptor, RXR $\alpha$  and RAR $\alpha$ , have been identified as nuclear substrates of JNK. AP-1 is the key transcriptional regulator of JNK signaling, regulating the expression of survival and proliferation-related

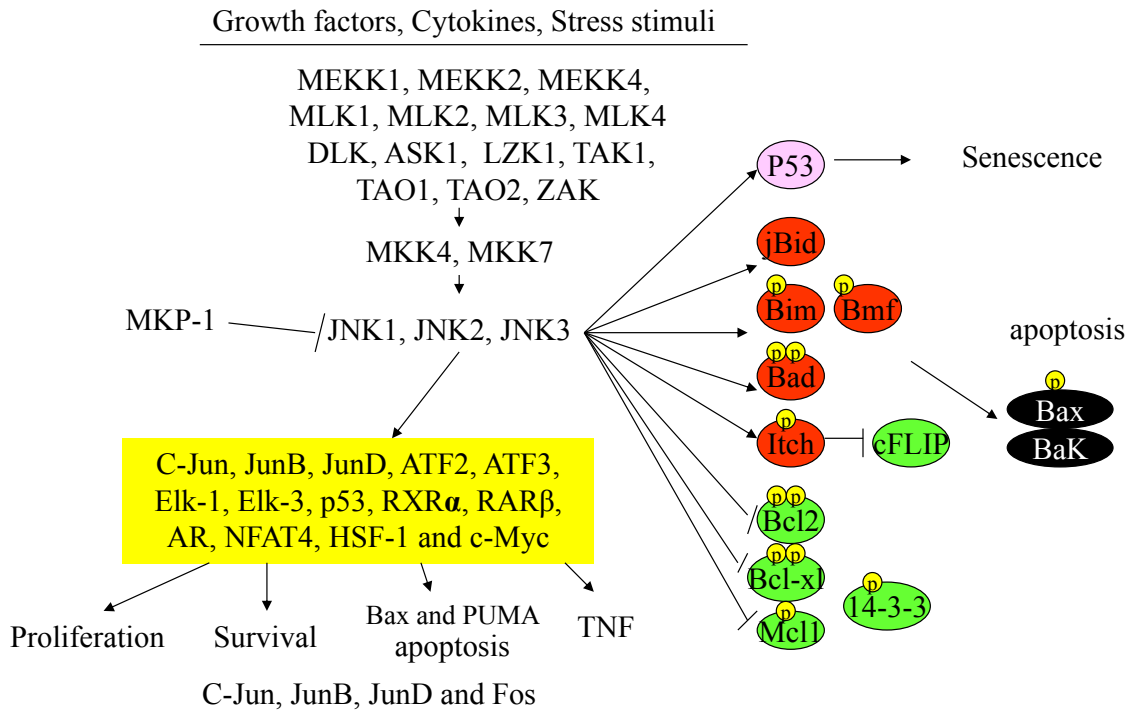
genes including c-Jun, cyclin D, c-Myc, ATF2, JunD and JunB.<sup>307,308</sup> Despite intensive study, the molecular mechanism by which JNK determines which pathway to activate (apoptosis or survival/proliferation) is still largely unknown.

### **JNK Signaling and Leukemia Development**

Sustained JNK activation has been reported in many types of AML.<sup>309</sup> It was found that JNK-AP1 signaling coordinates with AKT/FOXO signaling in maintaining AML cells in an undifferentiated state.<sup>309</sup> In a Bcr-Abl-induced leukemia/lymphoma model, JNK1-AP1 signaling is activated by the fusion enzyme, and is required for leukemia development by mediating a key survival signal.<sup>310</sup> In Fanconi's anemia, JNK is required for TNF-induced leukemic clone evolution of *FancC*-mutant HSPCs.<sup>279,311</sup> These studies suggested that the JNK signal promotes leukemia development and progression by inducing proliferation and survival activities. Thus, this signal could be a potential target for anti-leukemia therapy. However, several previous studies suggested that JNK might mediate cell death signaling induced by chemotherapeutic drugs such as arsenic trioxide and some other agents in certain leukemic cells. These studies suggested that inhibition of JNK might result in drug-resistance.<sup>312-315</sup> However, these studies are mainly based on evaluating the ratio of dead cells after short-term treatment. I found that inhibition of JNK can significantly sensitize TNF-expressing LCs to the toxicity of several chemotherapeutic drugs and NF- $\kappa$ B inhibitors with fewer effects on non-TNF-expressing LCs, as shown by colonogenic assays.<sup>172</sup> I propose that JNK inhibition might attenuate the acute cytotoxicity of chemotherapeutic drugs and NF- $\kappa$ B inhibitors on partially

differentiated non-colonigenic LCs but promote the elimination of undifferentiated colonigenic LCs (enriched with leukemic progenitors and LSCs).

**Figure 16. JNK is stimulated by upstream factors to drive downstream responses.**



## CHAPTER 3

### METHODS

#### **Mice and genotyping**

All experiments using mice were performed according to the guidelines of Loyola University Medical Center and were approved by the Loyola University Institutional Animal Care and Use Committee. *Tnfr1*<sup>-/-2</sup> mice (B6.129S-TnfrsflaTnfrsflb) were purchased from the Jackson Laboratory. BM hematopoietic cells from *Rip3*<sup>-/-</sup> (Rip3-knockout) mice were provided by Dr. Vishva M. Dixit of the Department of Immunology, Genentech, 1 DNA Way, South San Francisco, California 94080, USA.

Genotypes of all mice were determined by PCR assay using primers listed below (5'-3'):

<i>Tnfr1</i> -1	GGATTGTCACGGTGCCGTTGAAG	WT = 120 bp
<i>Tnfr1</i> -2	TGACAAGGACACGGTGTGTGGC	Mut = 155 bp
<i>Tnfr1</i> -3	TGCTGATGGGGATACATCCATC	
<i>Tnfr1</i> -4	CCGGTGGATGTGGAATGTGTG	
<i>Tnfr2</i> -1	CCGGTGGATGTGGAATGTGTG	WT = 257 bp
<i>Tnfr2</i> -2	AGAGCTCCAGGCACAAGGGC	Mut = 160 bp
<i>Tnfr2</i> -3	AACGGGCCAGACCTCGGGT	
<i>Rip3</i> -1	GGCTTTCATTGTGGAGGTAAGCTGAGA	WT = 280 bp
<i>Rip3</i> -2	GAACCCGTGGATAAGTGCACCTTGAAT	Mut = 320 bp

### Generation of murine leukemia cell lines:

CD117<sup>+</sup> HSPCs were isolated from *wild-type* (*WT*), *Tnfr*<sup>-/-</sup> (knockout of both Tnfr 1 and 2), and *Rip3*<sup>-/-</sup> mice and infected with MA9-neo-expressing retrovirus. Infected cells were selected with G418 for 2 weeks in 4-cytokine medium (10ng/ml IL-3, 25ng/ml IL6, 100ng/ml SCF and 20ng/ml GM-SCF) to generate MA9-immortalized cells (pre-leukemic cells or PLC in the text) with different genetic mutations. Such PLC were transplanted into lethally- irradiated recipient mice together support bone marrow cells to generate leukemic mice. *WT*, *Tnfr*<sup>-/-</sup>, and *Rip3*<sup>-/-</sup> LCs isolated from spleens and BM of the corresponding leukemic mice were used in our studies.

To generate NF-κB signal-inactivated *MA9-LC*, *WT-LC* or *Tnfr*<sup>-/-</sup>, LC generated from the above experiments were infected with IκBαSR-GFP-expressing virus and selected by FACS; these were used in our inhibitor treatment studies. IκBαSR is a mutant form of IκBα with S32A/S36A substitutions. The protein product of IκBαSR is stable and has more effective NF-κB inhibitory ability than does WT IκBα. To generate AP1-repressed *MA9-LC*, *WT-LC* generated from the above experiments were infected with DN-AP1-GFP-expressing virus. Infected LC were purified by FACS and subsequently used in our studies.

### Reagents

Rip-1 inhibitor Necrostatin (Nec1) was purchased from Santa Cruz Biotechnology. Recombinant murine-IL-3 (rm-IL-3), rm-IL-6, rm-SCF and rm-GM-CSF were purchased from eBioscience (San Diego). TNFα was purchased from BD Biosciences. BAY11-7085 and SP600125 small molecule inhibitors were purchased from

Millipore. LY294002 was purchased from LC Laboratories. JNKi I peptide and negative control were obtained from Millipore/Calbiochem. Cell lysis buffer (10x) was obtained from Cell Signaling, and supplemented with proteinase inhibitors and phosphatase inhibitors (Roche Diagnostics). Anti-TNF monoclonal antibody used for blocking TNF signaling in culture was obtained from Amgen Inc. Anti- $\beta$ -Actin, GAPDH, Bcl-2, and Bcl-xL antibodies were obtained from Santa Cruz Biotechnology. Anti-I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$ , P65, p-P65, Jnk, p-Jnk, Erk, p-Erk, p38, p-p38, Akt, p-AktS473, p-AktT308, Mcl-1, xIAP1, Cyclin D, Il-1 $\beta$  primary and requisite secondary antibodies were also obtained from Cell Signaling. c-FLIP antibody was obtained from Assay Gate. Tri-reagent used for RNA extraction was purchased from Sigma Aldrich. TNF antibody was purchased from Novus biologicals.

### **Cell culture**

All cells were incubated at 37°C., 100% humidity, and 5% CO<sub>2</sub>. c-Kit<sup>+</sup> HSPC from indicated genotypes of mice were enriched by *EasySep* mouse CD117 Positive Selection Kit (StemCell Technologies). HSPC were cultured in 6-well plates in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 100 ng/mL rmSCF, 50 ng/mL rmIL-6, 20 ng/mL rmIL-3, and 20 ng/mL rmGM-CSF. All murine LC generated in this study were cultured in such medium. Human leukemic cell lines were cultured following instructions from ATCC.

### **Cell counting**

Cell number was determined by trypan blue exclusion using 0.4% trypan blue (Gibco). At each 24-hour interval, all cells were harvested from the plate, centrifuged, re-



suspended in 1 mL of complete medium, and a sample was taken for counting. All cell counting was performed using trypan blue exclusion as visualized in a hemocytometer under 40× magnification. After counting, cells were re-suspended in a total of 3 mL medium and re-plated. This was repeated every 24 hours for 4 repetitions.

### **Colony-Forming Unit assay**

Indicated genotypes of murine LC or BM cells were seeded into *MethoCult GF M3434*<sup>®</sup> (StemCell) at 1000 cells/mL (LC) or 20,000 cells/mL (BM HSPC), incubated at 37°C, 100% humidity, and 5% CO<sub>2</sub> for 7 days (LC) or 10 days (HSPC cells). Numbers of colonies were counted according to the manufacturer's instructions. Clusters of cells containing >50 cells were counted as colonies. Triplicated experiments were performed in all of our studies. All data were verified by three individual experiments.

Human AML cell lines were seeded into *MethoCult* base medium without cytokines at 1000 cells/mL and incubated and read as murine LC. Primary AML patient samples were seeded into *MethoCult 4035 Optimum* without EPO and incubated at 37°C, 100% humidity, and 5% CO<sub>2</sub>. Colonies were read 14 days following seeding. Clusters of cells containing >50 cells were counted as colonies. Triplicated experiments were performed in all of our studies. All data were verified by three individual experiments.

### **Cell death assay**

HSPC or LC were incubated in medium with or without inhibitors for 24 hours. Cells were then stained with allophycoyanin-conjugated Annexin V in Annexin binding buffer following the manufacturer's instructions (BD Biosciences). Cell death was determined by the percentages of Annexin V<sup>+</sup> cells.

### **Detection of nuclear localization of NF- $\kappa$ B by Amnis Imagestream-X**

Cells were fixed and permeabilized using *BD Fix/Perm* kit as described by the manufacturer. Intracellular staining was performed using p65 antibodies (Cell Signaling), Alex-647 secondary (e-Biosciences) and DAPI. Nuclear localization of p65 was determined using a similarity dilate algorithm determined by *Imagestream Ideas* software and analyzed per 5000 cells. Significant differences were determined by mean and standard deviation values provided by *Imagestream Ideas* and compared student's t-test.

### **Retroviral infection**

High titer retrovirus was produced by co-transfecting Phoenix cells with a retroviral vector containing the indicated genes together with packaging vectors using *Calphos Mammalian Transfection Kit* (Clontech). Retroviral supernatants were harvested 24 and 48 hours after transfection. Retroviral plasmids MSCV-GFP and MSCV-Cre-GFP were kindly provided by Dr. Chao Niu of the University of Southern California, MSCV-MLL-AF9-neo was kindly provided by Nancy Zeleznik-Le of Loyola University Chicago, MSCV-I $\kappa$ B $\alpha$ aa-GFP was generated by sub-cloning the I $\kappa$ B $\alpha$ aa gene from pBabe-I $\kappa$ B $\alpha$ aa-puro plasmids (kindly provided by Dr. Mitchel Denning, Loyola University Chicago) into the MSCV-IRES-GFP vector. pMieg-DN-AP1 was obtained through Addgene. Viruses were generated using these retroviral vectors. LC or c-kit<sup>+</sup> HSPC were transduced with such virus-expressing genes of interest by spinoculation at 32°C., 2000 rpm for 4 hours. Cells were then incubated using standard culture conditions as described above, and GFP% of representative samples were read by flow cytometry every 24 hours. This assay allowed us to evaluate the functions of genes on cell growth

with both internal control (GFP<sup>-</sup> non-transduced cells) and external control (MSCV-GFP infection). Transduced cells were also purified by FACS for CFU assay.

### **Western Blot analysis**

LC/HSPC ( $10^6$ ) were plated in 6-well plates for 5 hours in RPMI-1640 medium supplemented with 10% FBS. FBS was present at all times during experiments, and did not show any effect on the measured signals. Growth factors were removed during this phase to reset the cell signaling to an unstimulated, basal rate. After 5 hours of growth factor withdrawal, inhibitors were added if required for 1.5 hrs. Following inhibition, cells were stimulated if required for 10 minutes and removed from the plates then lysed using cell lysis buffer (Cell Signaling) supplemented with protease and phosphatase inhibitor cocktails (Roche). Contents were resolved on 10% SDS-PAGE and visualized following transfer to nitrocellulose membranes.

### ***Ex vivo* transplantation**

$10^4$  LC (CD45.2<sup>+</sup>) were plated in each well in a suspension culture and treated with indicated doses of BAY11-7085, SP600125, anti-TNF antibody (Amgen) in indicated combinations for 12 hours. All cells in each well were harvested and mixed with  $10^6$  support BM cells (CD45.1<sup>+</sup>) then equally transplanted into 5 lethally-irradiated recipient mice (CD45.1<sup>+</sup>). Mice were monitored for leukemia development by observing for symptoms: hunched body, significant weight loss, or hind-limb paralysis. Leukemia was confirmed by examining CD45.2<sup>+</sup> LC in PB, spleen and BM, as well as liver and kidney infiltration.

### ***In vivo* transplantation and treatment**

$2 \times 10^4$  LC were transplanted into sub-lethally irradiated C57BL6/J mice with  $2 \times 10^5$  support BM cells via tail vein injection. Twenty days after transplantation, mice were treated with 10mg/kg *InVivo*MAb anti m-TNF $\alpha$  or IL-1 receptor antagonist (*BioXcell*) and 10 mg/kg BAY11-7085 individually or in combinations every other day for 10 days. Mice were monitored for leukemia development by lethargy, paralysis, significant weight loss and/or enlarged abdomen. Leukemia was verified after the mice were sacrificed by examining infiltration of LC in livers, lungs and spleens.

### **shRNA knockdown**

LC were transduced with retrovirus-expressing shRNAs (Origene) specifically targeted to c-Jun (TG501139), Mkp5 (1: TG514083; 3: TG514782; 5: TG513100), or IL-1 receptor (TG501076). The transduced cells were selected for one week using puromycin to obtain stably transduced cells. Knockdown efficiency was examined by Western blot (c-Jun), or RT-PCR (Mkps). Scrambled *shRNAs* were transduced and studied in parallel as controls.

### **Primary human samples**

Peripheral blood samples from AML patients were obtained from the clinic at Loyola University Medical Center in accordance with the IRB protocol. Leukemic blasts in PB of all patients were 30-90% when samples were collected. Samples were processed for mono-nuclear cells (MNC) by Ficoll-paque gradient centrifugation. A portion of MNC was used for RNA extraction and TNF expression analysis; another fraction of MNC was plated in *StemSpan* serum-free medium (StemCell) supplemented with

recombinant human SCF (100 ng/mL), Flt-3L (100 ng/mL), TPO (20 ng/mL), IL-6 (20 ng/mL), and IL-3 (20 ng/mL). Cytokines were obtained from Humanzyme. Following overnight culture,  $3 \times 10^5$  cells from each sample were harvested and treated with the indicated doses of BAY11-7085, SP600125, TNF, Enbrel®, or Anakinra® and plated in methylcellulose (StemCell) for CFU assay. Colonies were read after 14 days. In addition, serum was collected from the same patients for examination of TNF and IL-1 $\beta$  levels.

### **Statistical analysis**

Significant differences were determined by student's t test or one-way ANOVA with Bonferonni post-hoc test, where appropriate, unless otherwise noted. Log-rank test was used to determine statistical significance in Kaplan-meier survival curves using in vivo murine experiments or human AML patient data from UCSC Cancer Genome Browser analysis. \* or # indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$  when compared to vehicle-treated control or indicated groups. Other p values are indicated.

## CHAPTER 4

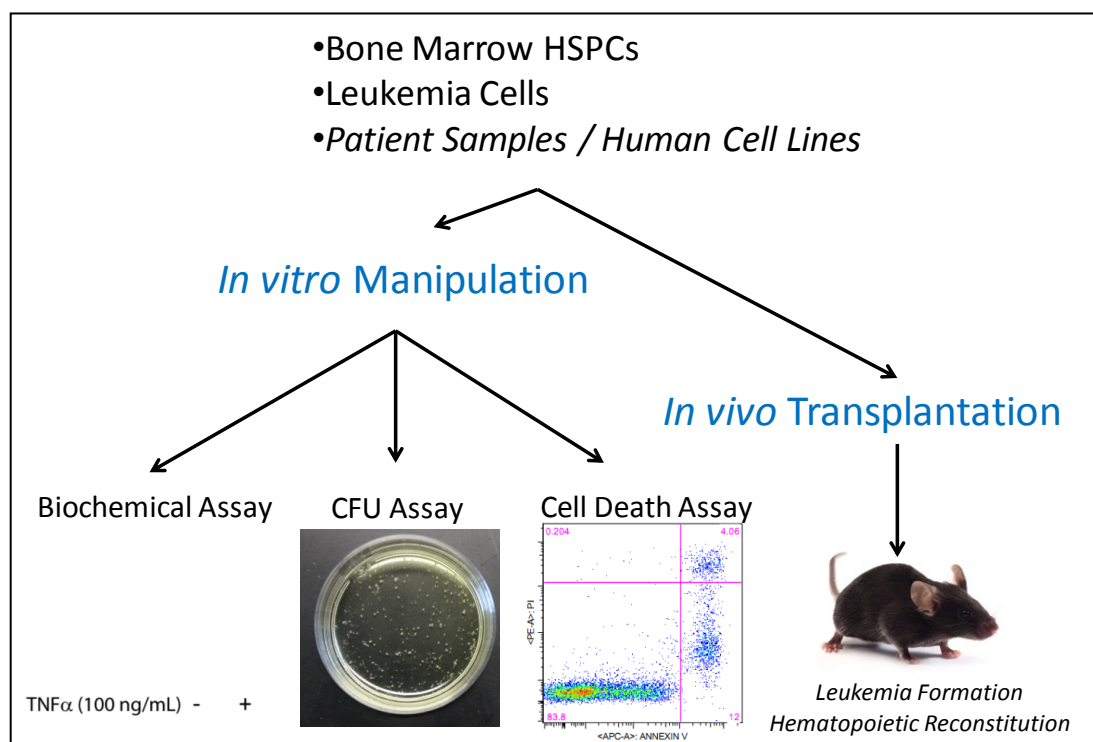
### RESULTS

#### **Rationale and experiment design**

The goal of this project is to determine TNF/IL1-mediated, NF- $\kappa$ B independent survival signals that allow LCs to escape NF- $\kappa$ B inhibition *in vivo*, and understand how LSCs use pro-inflammatory cytokines to further their own survival by reorganizing the niche. My studies are based on a comparative model where I treat malignant cells (LCs, AML cell lines, primary human patient samples) in parallel with normal/healthy cells (HSPCs, Peripheral Blood Stem Cells from healthy donors, Bone marrow aspirates from healthy donors). Each of these cells were collected, cultured, and treated according to the needs of the experiment and read by one of several assays: hematopoietic functional assay (colony forming units in methylcellulose), cell death assay (Annexin V/PI stain read by flow cytometry), and biochemical assays to determine the functions of specific proteins (western blot analysis).

Once I determined the mechanisms of TNF/IL1-mediated, NF- $\kappa$ B independent survival through the above-mentioned methods, I confirmed my findings by transplantation of LCs in to irradiated recipient mice. The mice were treated with various inhibitors/drugs to confirm what I found *in vitro* is similar *in vivo* (**Figure 17**).

**Figure 17. General experiment design**



Most experiments in this dissertation will follow the same basic experiment design: HSPCs and LCs will be treated in parallel with some sort of stimulation/inhibition, and effects will be measured in vitro by biochemical assay, CFU assay, or cell death assay. All mechanistic findings will be confirmed by in vivo transplantation and treatment, and confirmation in human AML cell lines or primary patient samples.

**Aim1: Determine if TNF-JNK acts as an NF- $\kappa$ B-independent survival signal in LSCs and contributes to chemoresistance to NF- $\kappa$ B inhibition *in vivo***

**Cell concentration determines NF- $\kappa$ B inhibitor efficacy *in vitro***

NF- $\kappa$ B, a key transcriptional regulator of inflammatory cytokine stimulated signaling, plays a central role in the development and progression of inflammation-associated cancer. In AML, TNF and NF- $\kappa$ B promotes LSC function in a feed forward manner. Basal NF- $\kappa$ B activity is undetectable in unstimulated normal CD34<sup>+</sup> hematopoietic stem/progenitor cells (HSPCs), but is constitutively activated in CD34<sup>+</sup>CD38<sup>-</sup> LSCs isolated from almost all AML patients. Inactivation of NF- $\kappa$ B signaling selectively eradicates LSCs *in vitro* especially when combined with chemotherapy drugs without significant influence on the survival and growth of normal HSPCs. These studies, however, use relatively purified LSCs cultured in low density (<3x10<sup>3</sup> cells/mL), suggesting that NF- $\kappa$ B signaling might be an optimal target of anti-AML therapy.<sup>4,14,316,317</sup> However, when I cultured high density AML cells in *in vitro* culture (5x10<sup>5</sup> cells/mL), the therapeutic effects of NF- $\kappa$ B inhibitor on clonogenic leukemic progenitors and LSCs was significantly attenuated, suggesting factors secreted by AML cells might provide protection to LSCs. The inhibitor we used (BAY11-7085, BAY hereafter) is a small molecule that recognizes the phosphorylation sites on NF- $\kappa$ B inhibitory protein, I $\kappa$ B $\alpha$ . This was observed both *in vitro* in methylcellulose on leukemic progenitors (**Figure 18A**), and during *ex vivo* transplantation assaying effects on LSCs (**Figure 18B**). By analyzing the expression of genes encoding inflammatory factors in microarray data of 90 human AML samples, I initially predicted that TNF might be one of these secreted factors. In Aim 1, I confirmed this by demonstrating that TNF prevents

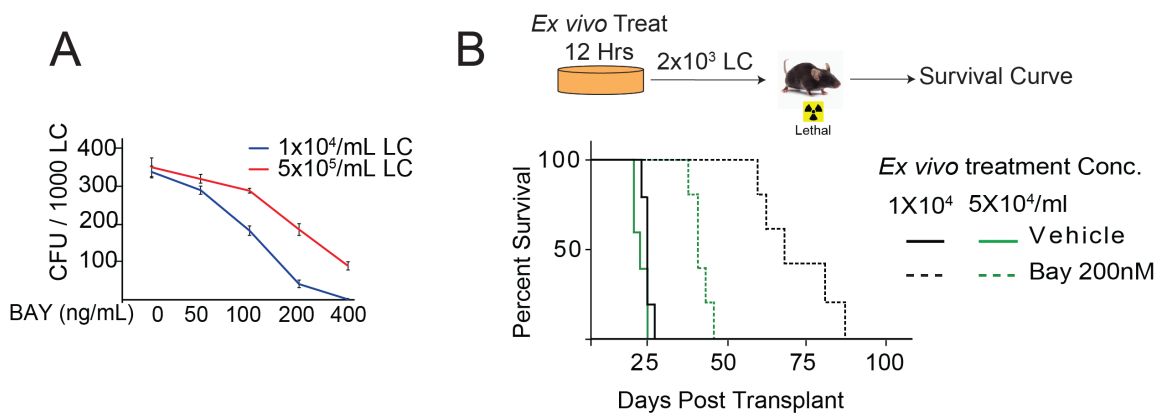


LCs from NF- $\kappa$ B inhibitor treatment through stimulating JNK signaling. At the end of Aim 1, I concluded that there are additional pro-inflammatory cytokines involved beyond Tnf providing anti-apoptotic protection to LSCs in the presence of NF- $\kappa$ B inhibition. In Aim 2, I determined IL1 is another factor which is normally expressed in TNF-expressing AML samples, and functions in parallel to TNF. I concluded that inhibition of both TNF/IL1-JNK and NF- $\kappa$ B signaling might be a better treatment for AML patients whose cancer cells express inflammatory cytokines.

### **Development of experimental models: murine and human AML models**

TNF stimulation in most healthy tissues induces apoptosis and necroptosis via the Caspase 8 extrinsic apoptosis pathway and the RIP1/RIP3 necroptosis pathway. However, tumors from AML patients appear to be producing TNF and not undergoing cell death. In order to study this phenomenon, I used a mouse model and a collection of human AML cell lines. I also developed murine leukemia cells (LCs) by sequential transplantation. First I isolated c-Kit<sup>+</sup> HSPCs from bone marrow and immortalized them through spinoculation with MLL-AF9-expressing MSCV retrovirus (I also used Runx1-Eto and Cbfb-Myh11 fusion genes as TNF non-expressing and expressing LCs<sup>172</sup>). These MLL-AF9-expressing cells are now considered pre-leukemia cells (PLCs), and are virtually indistinguishable from their fully transformed counterparts (LCs) *in vitro*. These cells were transplanted  $2-5 \times 10^6$  cells into lethally-irradiated recipient mice and collected the spleen and bone marrow after leukemia disease development 3-4 months following transplantation. These collected cells are fully transformed leukemia cells (LCs), and

**Figure 18. Cell concentration determines NF- $\kappa$ B inhibitor efficacy *in vitro***



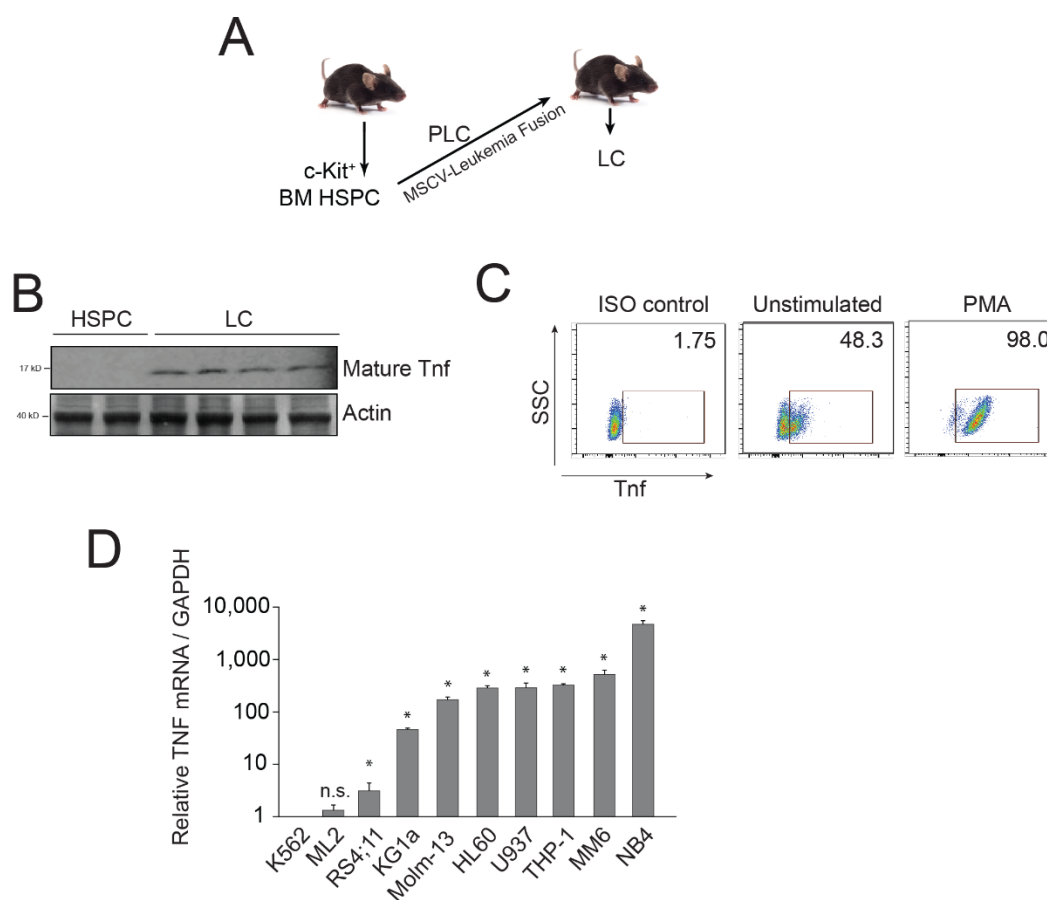
**A)** LCs were cultured and treated overnight at the indicated concentration with an increasing dose of BAY.  $1 \times 10^3$  live cells per condition were plated in methylcellulose and read for CFUs. Results shown are mean  $\pm$  SD and representative of three independent trials. **B)** Indicated concentrations of LCs were cultured for 12 hours in suspension culture and treated with vehicle or BAY, then  $2 \times 10^3$  live LCs were transplanted into lethally-irradiated mice.

have the ability to generate leukemias in transplant mi development 3-5 weeks following transplantation. We used these cells (LCs) for all studies in this dissertation (**Figure 19A**).

These LCs produce mature Tnf, making them ideal for studying the mechanisms of survival in TNF-producing AMLs (M3, M4, and M5). I confirmed Tnf production by western blot of lysed cells, using an antibody that recognizes mature, cleaved Tnf (app 17 kDa). Protein levels were normalized to actin. Murine HSPCs do not produce mature Tnf and were used as negative controls. (**Figure 19B**). I further confirmed the presence of mature Tnf in LCs by intracellular labeling analyzed by flow cytometry. LCs were either unstimulated or treated with PMA (phorbol 12-myristate 13-acetate) to stimulate protein production, and staining gates were set to LCs stained with an antibody isotype as a control (**Figure 19C**).

Any data determined from a murine model must be confirmed in a human (or humanized) disease model before I can derive any clinically relevant meaning. Therefore, I also utilized several human AML cell lines with a wide range of TNF expression to confirm anything found in the murine model in human AML cells. TNF levels were normalized to CML blast crisis cell line K562 (Bcr-Abl+), which showed the lowest levels of endogenous TNF production. Therefore I concluded that all of our human AML cell lines are producing at least some level of TNF higher than HSPCs, with some producing more than others. I found the highest levels of TNF production in the APL

**Figure 19: Development of TNF-expressing murine and human AML models**



**A)** HSPCs were isolated from *WT* and *Tnfr<sup>-/-</sup>* mice and transduced with a leukemia fusion-MSCV retrovirus to produce PLC. PLCs were transplanted into recipient mice to generate LC. LC were isolated from spleens and bone marrow of leukemic mice and used for most studies in this dissertation. **B)** LCs produce TNF as shown by western blot analysis of four separate transplantations of LCs into recipient mice, and were compared to HSPCs from two non-transplanted mice. **C)** Intracellular TNF was measured in LC by intracellular staining and read by flow cytometry with and without PMA stimulation. Gating was set by comparison to isotype control. **D)** Relative mRNA levels of TNF produced in established human AML cell lines. Values shown are mean  $\pm$  SD based on three independent trials. \* indicates  $p < 0.05$  as determined by students T test, compared to K562.

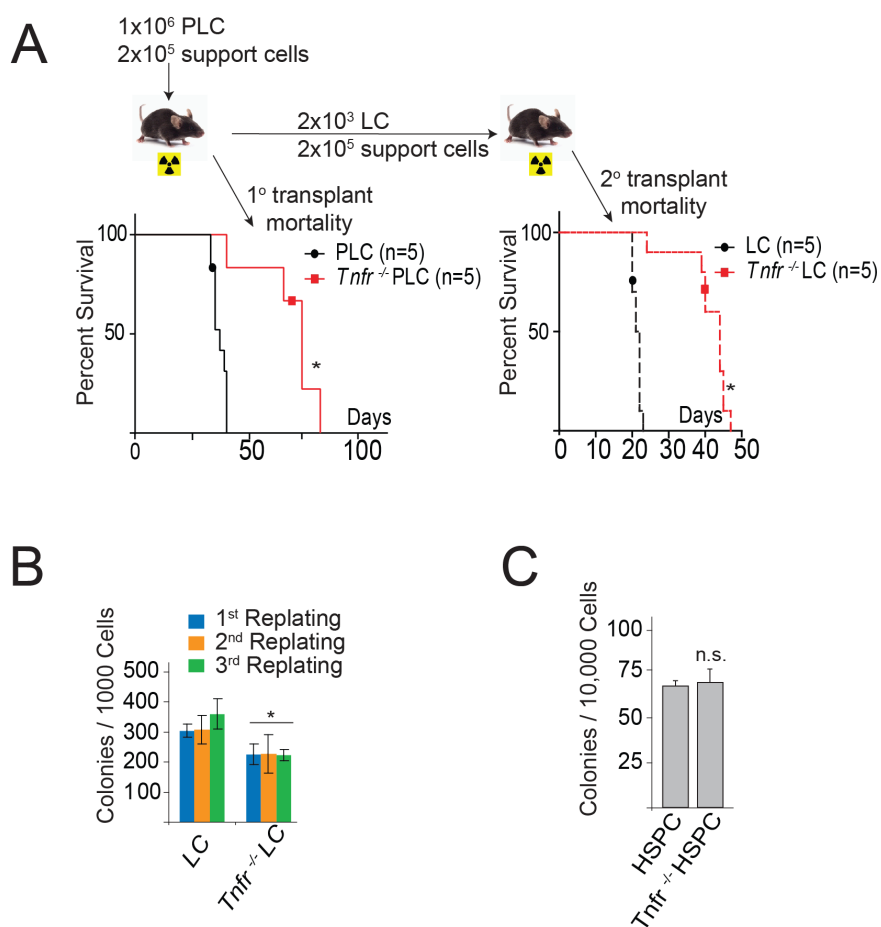
(PML-RAR $\alpha$ ) cell line, NB4. This supports observations in APL patients, and our observations in cDNA data from AML patient databases that APL tumors produce very large amounts of endogenous TNF (**Figure 19D**).

### **Tnf receptor deletion drives growth defects in LCs**

The previous data showed addition of exogenous Tnf stimulated the growth of LCs, and removal of TNF repressed the growth. To further study this, I serially transplanted *WT* and *Tnfr<sup>-/-</sup>* PLCs and LCs into lethally-irradiated recipient mice and found a distinct defect in leukemogenesis of *Tnfr<sup>-/-</sup>* PLCs/LCs when compared to their *WT* counterparts, with time to disease doubled in both cases. This effect was seen following transplantation of both PLCs and LCs (**Figure 20A**).

Further comparison of *Tnfr<sup>-/-</sup>* and *WT* LCs revealed that although *Tnfr<sup>-/-</sup>* LCs do not show any replating defects, they do have a reduced total ability to form colonies during a serial replating assay when compared to *WT* LCs. The total colony numbers of *Tnfr<sup>-/-</sup>* LCs were compared to *WT* LCs for control (**Figure 20B**). A similar effect was not observed in HSPCs, suggesting that Tnf stimulation does not play as important a survival role in these cells as in LCs. *Tnfr<sup>-/-</sup>* HSPCs were compared to *WT* HSPCs as control (**Figure 20C**). These data suggest that Tnf plays an important survival role, and that elimination of Tnf-dependent signaling can significantly prolong the duration until disease occurrence in xenografted mice. This in combination with the reduction in colony-formation *in vitro* would suggest blocking Tnf could be a possible anti-leukemia therapeutic.

**Figure 20: *Tnf* receptor deletion drives growth defects in LCs**



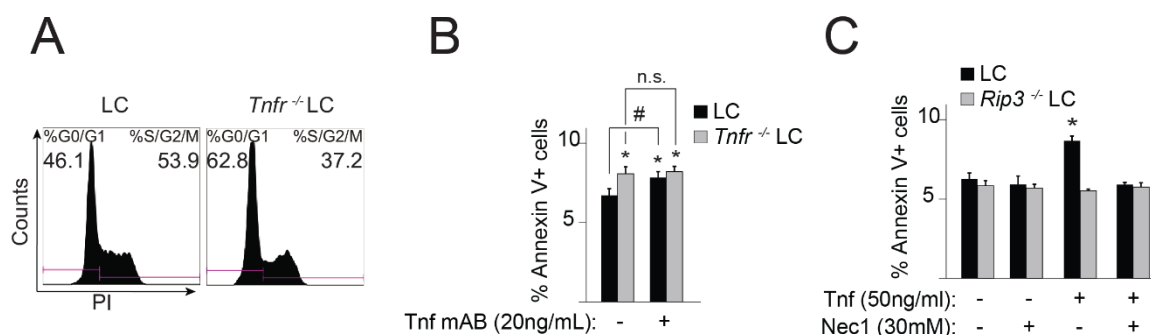
**A)** Leukemogenic capacity of *WT* LCs/PLCs and *Tnfr*<sup>-/-</sup> LCs/PLCs as compared with in vivo transplantation. Numbers of recipient mice are indicated. **B)** Serial colony forming ability was compared between *WT* and *Tnfr*<sup>-/-</sup> LCs. *WT* LCs were used as control. **C)** CFU/10,000 cells shown for *WT* and *Tnfr*<sup>-/-</sup> HSPCs. Colony formation of *WT* HSPCs was used as controls. Values shown (**B and C**) are mean ± SD from three independent trials. \* indicates p<0.05 when compared to vehicle treated/*WT* control.

### **Tnf promotes LC survival and proliferation**

Stimulation of LCs with exogenous Tnf promotes colony formation and increase in cell number, while blocking Tnf through genetic deletion of its receptors or neutralization with a monoclonal antibody slows their growth and limits colony formation. To understand this mechanism, I measured the proliferation rates of *WT* and *Tnfr<sup>-/-</sup>* LCs through PI stain assay, and measured the percentage of cells in G0/G1 vs S/G2/M via DNA content. When cells proliferate, they need to replicate the DNA. Propidium Iodide (PI) is a stoichiometric DNA dye that binds to DNA and fluoresces. The fluorescent signal is directly related to the amount of DNA present. I found that in addition to the colony defect observed in **Figure 20**, *Tnfr<sup>-/-</sup>* LCs also have lower proliferation than their *WT* counterparts as noted by a decrease in the percentage of cells in S/G2/M phase as determined by Propidium Iodide staining (**Figure 21A**).

If Tnf drives an important survival signal, then removal of the Tnf signal should also remove the survival signal and induce some degree of cell death. I tested this by neutralizing soluble Tnf or genetic deletion of Tnf receptors. Either of these actions increased cell death by a slight but significant value. Cell death was measured by Annexin V stain and compared to vehicle treated *WT* LCs as control (**Figure 21B**).

I also found that stimulation with exogenous Tnf also drives necroptotic cell death in a small population of LCs, because this cell death can be completely reversed by co-treatment with a Rip1k inhibitor (Necrostatin-1, Nec1), or by genetic deletion of Rip1k's necroptosome binding partner Rip3 (**Figure 21C**). *WT* LCs treated with vehicle were used as controls.

**Figure 21: Tnf promotes LC survival and proliferation**

**A)**  $1 \times 10^6$  LC of indicated genotype were grown in suspension in 6-well plates, fixed and permeabilized, then stained with saturating levels of PI. DNA content was measured by flow cytometry, and gates were set with G0/G1 indicating all values  $\leq 2n$  DNA, and S/G2/M indicating all values  $\geq 2n$  DNA. Results shown are indicative of three independent trials. **B)** LC of indicated genotypes were treated overnight with 20 ng/mL Tnf mAB and assayed for cell death by Annexin V stain. *Tnfr*<sup>-/-</sup> LC were used as controls. **C)** Indicated genotypes of LCs were treated overnight with exogenous Tnf (50 ng/mL) and/or Necrostatin-1 (Nec1, 30 mM). Cell death was measured by Annexin V stain by flow cytometry. Results shown are mean  $\pm$  SD. \* indicates  $p < 0.05$  when compared to vehicle treated WT LC control as analyzed by students t test. # indicates  $p < 0.05$  of *WT* LCs treated with Tnf mAB when compared to *WT* LCs treated with vehicle.



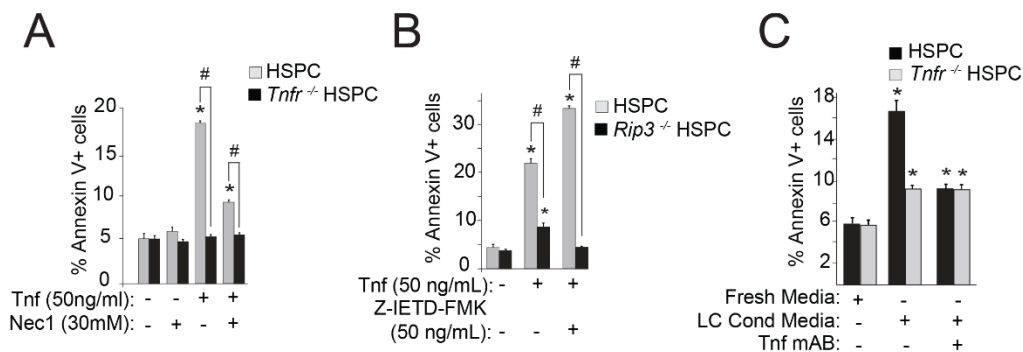
### **Tnf produced by LCs induces apoptotic and necroptotic cell death in HSPCs**

Tnf's normal role in most tissues studied is to induce apoptosis and necroptosis through the extrinsic (complex 2) apoptosis pathway. I confirmed this in HSPCs by treating HSPCs with a combination of exogenous Tnf and Nec1, and measured cell death by Annexin V stain. HSPCs showed a significant increase in cell death when treated overnight with Tnf that was partially rescued by Nec1 treatment, suggesting that the cell death induced by Tnf was primarily necroptotic (can be rescued by Nec1), with some apoptosis (cannot be rescued by Nec1) (**Figure 22A**). *WT* HSPCs treated with vehicle and *Tnfr*<sup>-/-</sup> HSPCs were used as negative controls.

To measure the direct activation of Caspase-8-mediated apoptosis following Tnf stimulation, I treated *WT* and *Rip3*<sup>-/-</sup> HSPCs in parallel with Tnf and a Caspase-8-specific inhibitor Z-IETD-FMK. *Rip3*<sup>-/-</sup> HSPCs are unable to undergo necroptosis upon stimulation, and co-treatment with Caspase-8 inhibitor in these cells was able to completely prevent any Tnf-mediated cell death. This suggests that Tnf induces a combination of apoptotic and necroptotic cell death in HSPCs. Without Caspase-8's catalytic inhibition of Rip1/Rip3-mediated necroptosis, the *WT* HSPCs underwent a significant increase in cell death (**Figure 22B**). *WT* HSPCs treated with vehicle were used as controls.

AML patients commonly show significant hematopoietic repression in later stages of the disease. I propose that this is due to LCs secreting pro-inflammatory cytokines,

**Figure 22: Tnf produced by LCs induces apoptosis and necroptosis in HSPCs**

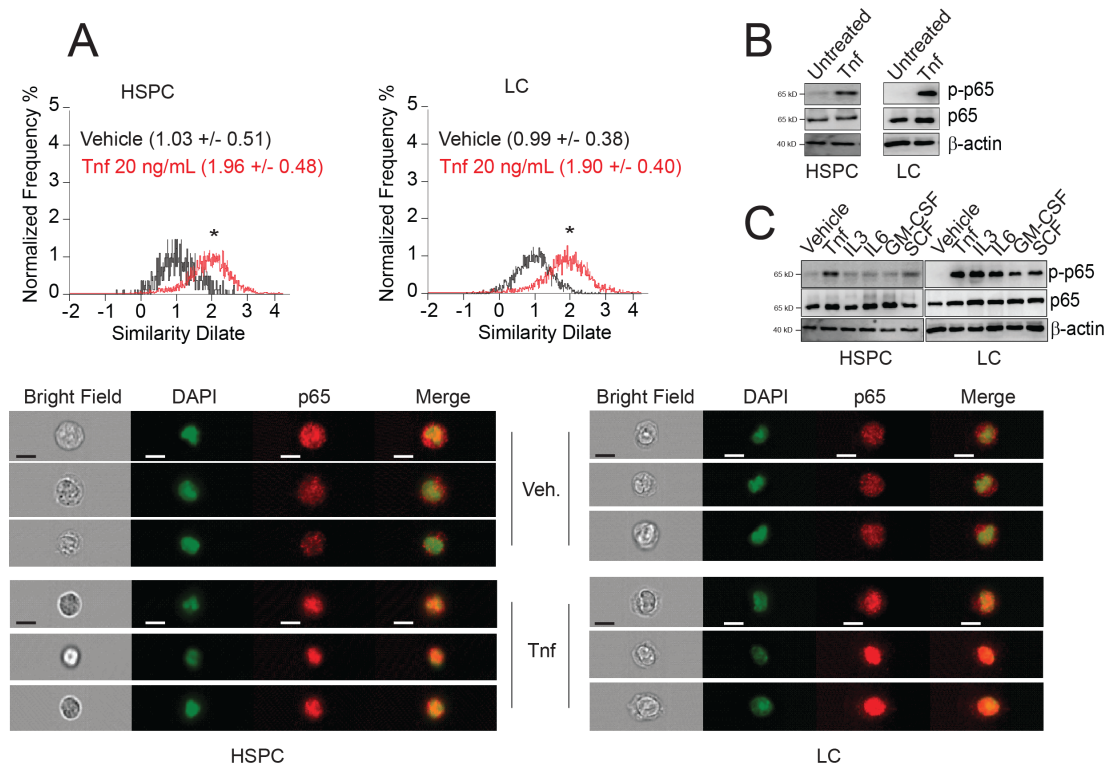


**A)** *WT* HSPCs and *Tnfr*<sup>-/-</sup> HSPCs were cultured in medium with or without exogenous Tnf. Nec1 was used to block necroptosis. **B)** HSPCs from *WT* (*Rip3* WT) and *Rip3*<sup>-/-</sup> mice were cultured with or without Tnf. Z-IETD-FMK was used to inhibit Caspase 8 activity. Cell death was examined 24 h after Tnf treatment. **C)** Tnf secreted by LCs is sufficient to induce cell death in HSPC. *WT* and *Tnfr*<sup>-/-</sup> HSPCs were incubated in fresh medium or 1:5 conditioned/fresh medium from LC culture and treated with or without anti-TNF mAb. \* indicates  $P < 0.05$  when compared with vehicle treated control (A–C and G–M) or *WT* control (D–F) as determined by Student's t test analysis. # indicates  $P < 0.05$  significant difference when compared with indicated conditions. Values shown are mean  $\pm$  SD analysis from three independent trials, unless otherwise noted.

thereby inducing cell death and depleting the healthy stem cell population. To test this, I conditioned media with LCs, diluted 1:5 in fresh media, and grew HSPCs overnight. Annexin V was measured the following day. I found that cytokines secreted by LCs were sufficient to induce cell death in HSPCs. The effect was mostly removed by neutralizing soluble Tnf, or by use in *Tnfr<sup>-/-</sup>* HSPCs, suggesting this effect is directly due to Tnf (**Figure 22C**). Because this effect could not be completely neutralized, I proposed that there may be additional pro-inflammatory cytokines secreted by LCs in addition to Tnf. Such a cytokine would promote LC proliferation and induce cell death in HSPCs. I identified that this cytokine is IL-1, and will be discussed later.

#### **Tnf stimulates NF- $\kappa$ B activity in HSPCs and LCs**

NF- $\kappa$ B is the best-studied effector of Tnf-mediated survival signaling in most tissues studied. LSCs require NF- $\kappa$ B activity for survival and self-renewal. The standard method of measuring NF- $\kappa$ B activity is nuclear localization of subunits and activation of target genes. I measured nuclear localization of NF- $\kappa$ B subunit p65 following stimulation with Tnf by Imagestream Analysis and found that Tnf does induce nuclear localization in both HSPCs and LCs, suggesting Tnf can induce NF- $\kappa$ B activity (**Figure 23A**). Results were compared to vehicle treated cells as negative controls. Phosphorylation of p65 subunits by IKK $\beta$  occurs following release and degradation of the I $\kappa$ B $\alpha$  repressor subunits, and can also be used as a marker for NF- $\kappa$ B activity (**Figure 23B**). Untreated HSPCs and LCs were used as controls.

**Figure 23: Tnf induces NF- $\kappa$ B activity in HSPCs and LCs**

**A)** Tnf stimulation of NF- $\kappa$ B activity in both LC and HSPC as shown by p65 (NF- $\kappa$ B1) nuclear localization (values in parenthesis are mean similarity dilate peak, sample size = 5,000 cells) as determined by ImageStreamX. Horizontal scale bars indicate 10  $\mu$ m. **B)** Tnf stimulation of p65 phosphorylation in HSPCs and LCs. **C)** HSPC and LC stimulated with individual hematopoietic cytokines show different patterns of p65 phosphorylation. \* indicates  $p < 0.05$  when compared to vehicle treated controls as determined by Student's t test two-tailed analysis. All results shown are indicative of three independent trials.

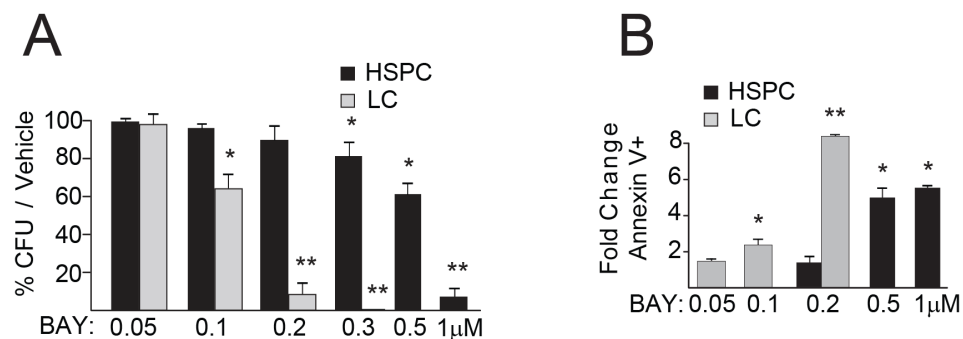
NF- $\kappa$ B is highly upregulated in LCs when compared to HSPCs. I observed that a main reason for this is that HSPCs appear to only be able to activate NF- $\kappa$ B when stimulated by Tnf (and slight activity when stimulated with SCF). However, LCs are able to translate stimulation by all hematopoietic cytokines into NF- $\kappa$ B activity, suggesting a mechanism for elevated NF- $\kappa$ B activity in these cells (**Figure 23C**).

#### **LCs are more sensitive to NF- $\kappa$ B inhibitor *in vitro* than HSPCs**

A 2001 study by Guzman et al. found that NF- $\kappa$ B is constitutively expressed in LSCs, and that NF- $\kappa$ B activity is critical for maintaining LSC properties. This activity appears confined to LSCs, as HSPCs do not express high levels of NF- $\kappa$ B. Therefore this is a therapeutic window where, *in vitro*, one can kill LSCs before having an adverse effect on HSPCs. These observations were made in  $\text{lin}^- \text{CD34}^+ \text{CD38}^-$  human AML cells. Although our murine AML model (MLL-AF9 LCs) fits the human disease model by expressing pro-inflammatory cytokines TNF and IL1, I needed to confirm that these cells would also be sensitive to NF- $\kappa$ B inhibition. To accomplish this, I utilized a small molecule BAY11-7085 (BAY hereafter). BAY is a membrane-permeable ATP analogue that can bind to the I $\kappa$ B $\alpha$  repressor subunit of NF- $\kappa$ B, preventing its phosphorylation and subsequent degradation by the proteasome.

I tested BAY inhibitor treatment in HSPCs and LCs in parallel in both methylcellulose culture and suspension culture. I found that LCs reduced their CFUs in a dose dependent manner in response to increasing BAY concentrations (**Figure 24A**). 90% of LC colonies were removed when treated with 200 nM BAY, with no effects on HSPCs. Vehicle-treated cells from each type were used as negative controls.

**Figure 24. LCs are more sensitive than HSPCs to NF- $\kappa$ B inhibition *in vitro***



**A and B)** HSPCs and LCs were treated in parallel with increasing doses of BAY11-7085 (BAY) inhibitor in methylcellulose or suspension culture, respectively. **A)** CFUs were read seven days following seeding. **B)** Cell death was measured by Annexin V staining after overnight treatment with BAY, and read by flow cytometry. \* indicates  $p < 0.05$  when compared with vehicle treated control, \*\* indicates  $p < 0.01$  as determined by Student's t test analysis. Values shown are mean  $\pm$  SD analysis from three independent trials.

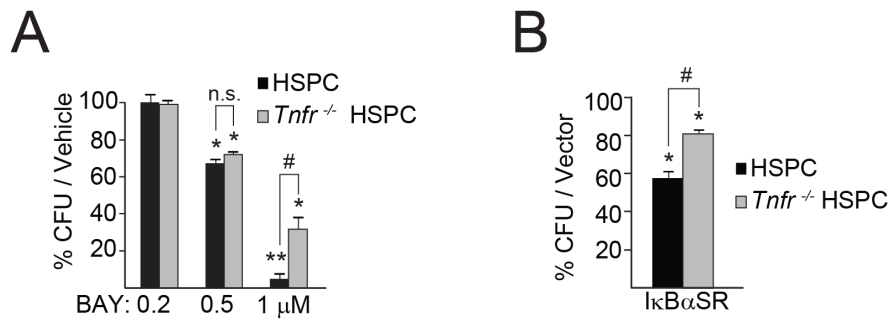
I confirmed that the drop in colony number was due to an increase in cell death by treating HSPCs and LCs in parallel in suspension culture overnight with increasing doses of BAY inhibitor. There was an eight-fold increase in cell death in LCs without any significant change in HSPCs (**Figure 24B**).

### **Tnf receptor deletion protects HSPCs from NF- $\kappa$ B inhibitor effects**

In most tissues studied, NF- $\kappa$ B promotes a strong survival signal that can counteract cell death caused by pro-inflammatory cytokine stimulation by upregulation of anti-apoptotic genes. HSPCs behave in a similar fashion. I tested this by treating *WT* and *Tnfr<sup>-/-</sup>* HSPCs in parallel with a gradient of BAY NF- $\kappa$ B inhibitor. If HSPCs underwent TNF-mediated cell death in the presence of the inhibitor, then there would be a drop in colonies and an increase in Annexin V staining that could be reversed by inhibition of the Tnf signal. In this case, I deleted both Tnf receptor genes (*Tnfr1<sup>-/-</sup>Tnfr2<sup>-/-</sup>*, *Tnfr<sup>-/-</sup>* for simplicity). As expected, I found that *Tnfr<sup>-/-</sup>* HSPCs are more resistant to CFU reduction driven by high-dose treatment with BAY (**Figure 25A**).

In order to confirm the specificity of the BAY inhibitor, I spinoculated HSPCs (and LCs in the future) with an MSCV retrovirus constitutively over-expressing a super-repressor mutant of the NF- $\kappa$ B repressor subunit I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ SR. I $\kappa$ B $\alpha$ SR binds all the free NF- $\kappa$ B subunits in the cytoplasm, but cannot be degraded due to mutation of the IKK phosphorylation sites (Serine to Alanine). This construct is able to completely block NF- $\kappa$ B activity and has been used experimentally in cells from several tissues including skin, liver, and intestine. I found HSPCs expressing I $\kappa$ B $\alpha$ SR showed a reduction in CFU that was rescued by Tnf receptor deletion (**Figure 25B**). This confirmed that the activity

**Figure 25. Tnf receptor deletion protects HSPCs from NF- $\kappa$ B inhibitor effects**



*WT* and *Tnfr*<sup>-/-</sup> HSPCs were treated in parallel with increasing doses of BAY (**A**), or expressing I $\kappa$ B $\alpha$ SR (**B**) in methylcellulose culture. Results shown are mean  $\pm$  SD. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$  when compared to vehicle treated cells by Student's t test. # indicates  $p < 0.05$  when compared to indicated conditions by Student's t test.



of the BAY inhibitor was due to specific inhibition of NF- $\kappa$ B. Vehicle-treated/vector-expressing cells were used as the negative control.

### **Tnf receptor deletion sensitizes LCs to NF- $\kappa$ B inhibitor effects**

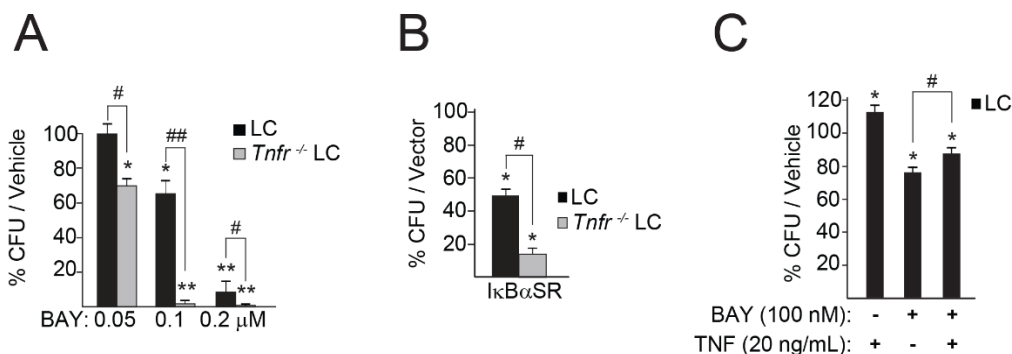
I attempted to confirm our results from BAY and I $\kappa$ B $\alpha$ SR experiments in HSPCs by performing similar experiments in LCs. However, I found that while Tnf receptor deletion protected HSPCs from NF- $\kappa$ B inhibition, the opposite was the case with LCs. By blocking the Tnf signal, I actually sensitized LCs to NF- $\kappa$ B inhibition by treatment with BAY (**Figure 26A**). Vehicle treated cells were used as negative controls.

I confirmed the specificity of BAY in LCs by overexpressing I $\kappa$ B $\alpha$ SR on a retrovirus and measuring similar results. I found that, like BAY, I $\kappa$ B $\alpha$ SR also affected the *Tnfr*<sup>-/-</sup> LCs more than the *WT* LCs. It should be noted that *WT* LCs have a greater reduction in CFUs when overexpressing the I $\kappa$ B $\alpha$ SR than *WT* HSPCs. Overexpression of I $\kappa$ B $\alpha$ SR reduced CFU by 90% in *Tnfr*<sup>-/-</sup> LC (**Figure 26B**).

These results suggest that Tnf may be an NF- $\kappa$ B-independent death signal in HSPCs and an NF- $\kappa$ B-independent survival signal in LCs. If Tnf drives an NF- $\kappa$ B-independent survival signal, then treatment with additional exogenous Tnf should rescue some of the CFU reduction due to NF- $\kappa$ B inhibition. I found this to be the case, with a slight but significant rescue of colonies in *WT* LCs (**Figure 26C**).

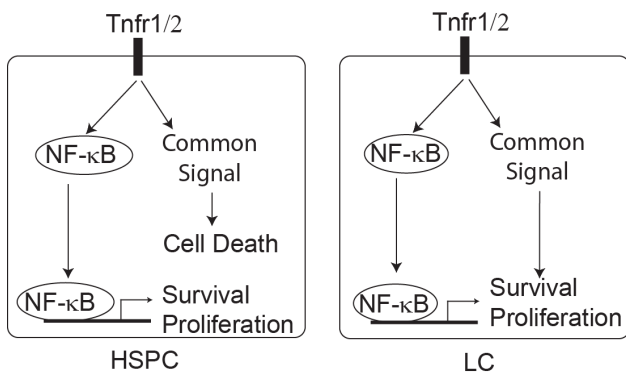
This led us to question what this signal could be. The previous two experiments suggest that there is a common signal between the two cell types that is independent of NF- $\kappa$ B, stimulated by Tnf, and acting differently between the two cell types. Such a

**Figure 26. Tnf receptor deletion sensitizes LCs to NF- $\kappa$ B inhibitor effects**



**A)** *WT* LCs and *Tnfr*<sup>-/-</sup> LCs were treated in parallel with increasing doses of BAY in methylcellulose for CFU assay. Vehicle treated cells were used as negative control. **B)** *WT* LCs and *Tnfr*<sup>-/-</sup> LCs were spinoculated with IκBαSR-expressing retrovirus and plated in methylcellulose. Cells transduced with empty vector are used as negative controls. **C)** *WT* LCs were treated with indicated doses of BAY, or exogenous Tnf and plated in methylcellulose. Values shown are mean  $\pm$  SD from three independent trials. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$  when compared to vehicle/vector control as determined by Student's t test. # indicates  $p < 0.05$ , ## indicates  $p < 0.01$  when compared to indicated condition as determined by Student's t test.

**Figure 26 Model:**



Common signal between HSPCs and LCs drives cell death in HSPCs and cell survival/proliferation in LCs.

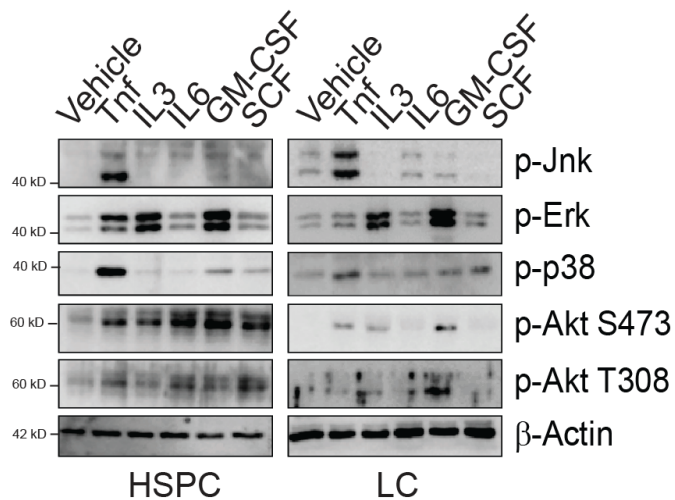
signal would be a survival mediator in LCs and a death mediator in LCs

**(Figure 26 Model).**

### **JNK is stimulated by Tnf in LCs**

I next determined which Tnf-mediated signal is responsible for driving NF- $\kappa$ B-independent cell survival/proliferation in LCs, and cell death in HSPCs. To do this, I treated HSPCs and LCs in suspension culture with each of the various hematopoietic cytokines used in culture: IL3, IL6, Granulocyte/Macrophage-Colony Stimulating Factor (G-CSF), Stem Cell Factor (SCF), and Tnf. Following 15 minutes of stimulation, cells were lysed and contents analyzed by western blot analysis for activation of several known survival signals in hematopoietic cells, involved in the SAPK, MAPK, and AKT pathways. I tested the activity of p-Jnk, p-Erk, p-p38, p-Akt<sup>S473</sup>, and p-Akt<sup>T308</sup>. Vehicle treated cells were used as negative controls, and total protein levels were normalized to GAPDH, a metabolic protein.

I found Tnf stimulation activated Jnk, Erk, p38, and Akt<sup>S473</sup> (PI3k activation pathway) in both HSPCs and LCs. It should be noted that while Erk, p38, and Akt can be stimulated by multiple cytokines, only Jnk appears to be Tnf-mediated. IL3 and SCF appear to be able to repress Jnk phosphorylation, but this mechanism was not pursued further. IL6 and G-CSF are known activators of Jnk in many tissues, and also activated Jnk in our experiments. p38, a Mapk, was also activated in a similar pattern to Jnk in both HSPCs and LCs. However, p38 was more strongly activated by GM-CSF and SCF than Jnk. p38's role in Tnf-mediated leukemogenesis will be measured later. Erk and Akt were

**Figure 27: Jnk is primarily stimulated by Tnf in HSPCs and LCs**

HSPCs and LCs were treated in suspension for 15 minutes with indicated hematopoietic cytokines. Cells were lysed and contents resolved by SDS-PAGE and assayed by western blot analysis. Protein levels were normalized to  $\beta$ -Actin for loading control. Vehicle treated HSPCs and LCs were used as negative control.

both activated by multiple cytokines, and were not particularly strongly activated by Tnf stimulation. Erk and Akt were activated by IL3 and GM-CSF (**Figure 27**).

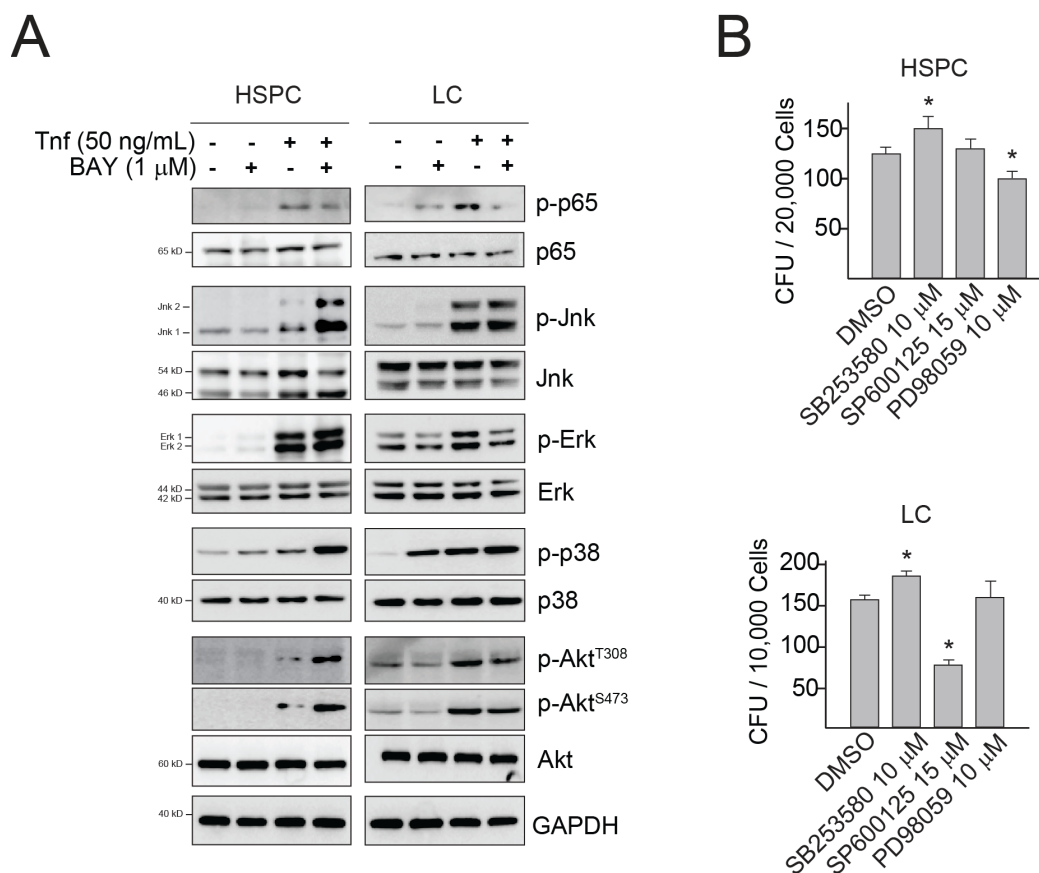
**Jnk is activated by Tnf, independently of NF- $\kappa$ B, promotes LC colony formation**

Our previous studies showed that there is a Tnf-mediated and NF- $\kappa$ B independent signal that is a survival mediator in LCs but a cell death mediator in HSPCs. When studying the effects of different hematopoietic cytokine stimulation, I determined Jnk and p-38 were primarily activated by Tnf, making these two proteins possible contenders. To test which of these survival signals fit the criteria, I treated HSPCs and LCs in parallel in suspension culture first with BAY for two hours, and then added Tnf for 15 minutes. Any NF- $\kappa$ B-independent/Tnf-dependent signals would be activated upon Tnf stimulation and remain activated or increase in activation in the presence of BAY.

I found Erk and AKt were repressed by BAY in LCs, suggesting these signals require NF- $\kappa$ B for activity. These were different in HSPCs and LCs, with BAY treatment enhancing their respective activities, suggesting a possible compensatory mechanism in HSPCs. Jnk and p-38 were active in the presence of Tnf and BAY (**Figure 28A**).

To determine which of these mediate survival signals, I treated HSPCs and LCs in parallel with small molecule inhibitors specific for each protein: (SB253580, p38; SP600125, Jnk; PD98059, Erk). Erk inhibitor had no observed effect on LC CFUs. p38 inhibition actually increased the CFUs of LCs while decreasing CFUs in HSPCs, suggesting p38 represses cell death in LCs and promotes cell death in HSPCs, which would make it a poor choice for anti-leukemia therapies. Jnk inhibition increased HSPC CFUs and decreased LC CFUs, making this a survival signal in LCs and a death signal in

**Figure 28. Jnk is a Tnf-dependent, NF- $\kappa$ B-independent survival signal**



**A)** HSPCs and LCs were pre-treated with BAY for two hours, followed by stimulation with Tnf for 15 minutes. Cells were lysed, contents resolved by SDS-PAGE, and analyzed by western blot. Protein levels were normalized to GAPDH for loading control. Vehicle treated cells were used as negative controls. **B)** HSPCs and LCs were treated in parallel with p38 inhibitor (SB253580), Jnk inhibitor (SP600125), and Erk inhibitor (PD98059). Total number of colonies is shown. Values shown are mean  $\pm$  SD. \* indicates  $p < 0.05$  when compared to vehicle treated control by Student's t test.

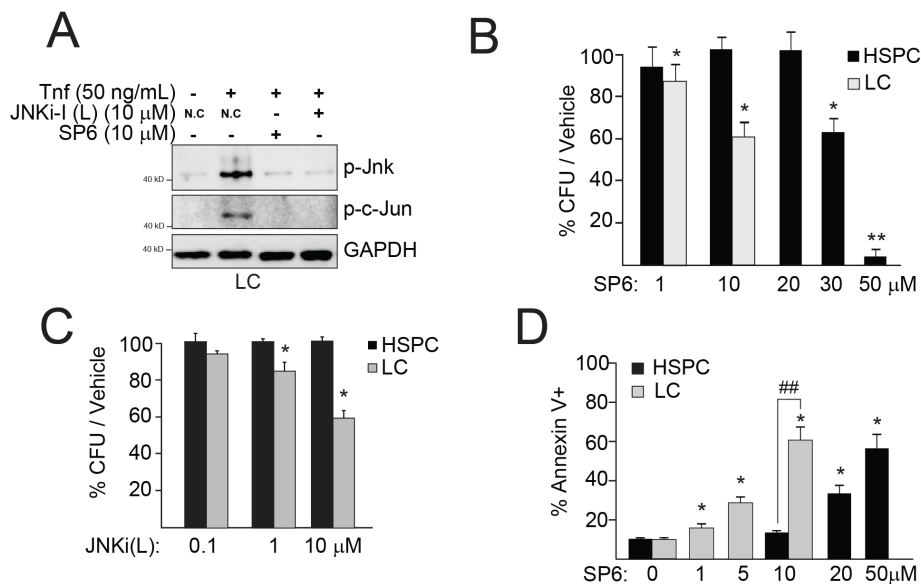
HSPCs, suggesting Jnk is the Tnf-mediated, NF- $\kappa$ B survival signal in LCs, and death signal in HSPCs (**Figure 28B**).

### **Jnk inhibition drives cell death in LCs**

The previous study suggested Jnk functioned as a survival signal in LCs. To test this, I used two different Jnk inhibitors: a small molecule inhibitor (SP600125, SP6 hereafter), and a Jnk binding domain peptide (aa sequence: GRKKRRQRRRPPRKRPTTLNLFQVPRSQDT) that interferes with upstream SAPKK activation of Jnk. Both of these inhibitors were able to completely block Tnf-mediated Jnk phosphorylation and subsequent c-Jun phosphorylation (**Figure 29A**). Cells treated with a control peptide (aa sequence: GRKKRRQRRRPP) were used as negative control.

Treatment with SP6 in HSPCs and LCs showed that Jnk inhibition promotes cell death in LCs at a much lower concentration than in HSPCs in a dose dependent manner (**Figure 29B**). I noted that at high concentrations, SP6 can cause cell death in HSPCs. However, SP6 has complete Jnk inhibition at 10  $\mu$ M. Any effects observed at concentrations above 10  $\mu$ M could be attributed to non-specific toxicities. Cells treated with vehicle were used as negative controls.

I confirmed the effects of SP6 in HSPCs and LCs by treating both cell types in parallel with another Jnk inhibitor, which was a peptide mimetic of Jnk-binding domain, JNKi-I(L). This peptide also showed a dose-dependent effect on LCs, suggesting a specific activity is due to blocking Jnk. At high concentrations (10  $\mu$ M), I observed a large decrease in CFUs of LCs without any decrease in HSPC CFUs (**Figure 26C**). Cells

**Figure 29: Jnk inhibition promotes cell death in LCs**

**A)** LCs were treated in suspension culture with indicated JNKi-I(L) or SP6 for 2 hours before stimulation with Tnf for 15 minutes. Cells were lysed and contents resolved by SDS-PAGE and Jnk/c-Jun activity was determined by western blot. Negative control (n.c.) peptide was used as negative control. **B)** HSPCs and LCs were treated in parallel with increasing doses of SP6 inhibitor in methylcellulose. Vehicle treated cells were used as negative controls. **C)** HSPCs and LCs were treated in parallel with increasing doses of JNKi-I(L) peptide in methylcellulose. Negative control-peptide treated cells were used as negative control. **D)** HSPCs and LCs were treated in suspension culture with increasing doses of SP6 overnight. Cell death was measured the following day by Annexin V staining and read by flow cytometry. Vehicle treated cells were used as negative control. **A** is representative of three independent trials. Values shown are mean  $\pm$  SD. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$  by Student's t test.



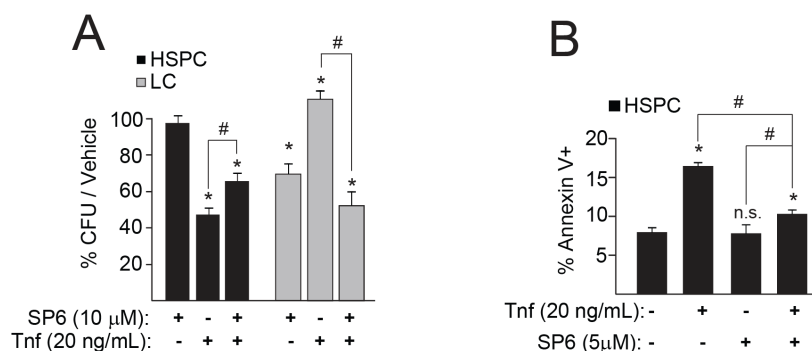
treated with a control peptide that has key JNK binding domain interaction amino acids mutated were used as negative controls.

Finally, I attributed the reduction in CFUs to an induction of cell death by treating HSPCs and LCs in parallel in suspension culture with increasing doses of SP6. At 10  $\mu$ M, 70% of LCs were Annexin V positive, while no significant increase in HSPC cell death was observed (**Figure 29D**). Vehicle treated cells were used as controls.

### **Jnk is a Tnf-mediated survival signal in LCs, death signal in HSPCs**

I identified that Jnk functions as a survival signal in LCs by treating LCs with a Jnk inhibitor and reducing the colony formation. Next, I needed to determine if Jnk was acting as a Tnf-mediated (to be specific) survival signal in LCs, and a Tnf-mediated death signal in HSPCs. To accomplish this I treated HSPCs and LCs in parallel with SP6 or exogenous Tnf individually or in combination. If Jnk acted as a Tnf-mediated survival signal, then CFUs gained by Tnf stimulation could be prevented by treatment with SP6. On the other hand, if Jnk acted as a Tnf-mediated death signal, then loss of CFUs due to Tnf stimulation could be rescued by co-treatment with SP6. I found that Tnf treatment of HSPCs, in agreement with our earlier data, caused a 50% drop in CFUs that was partially rescued by co-treatment with SP6, suggesting that Jnk functions as a Tnf-mediated death signal. However, in LCs, co-treatment of SP6 and Tnf reduced CFUs by as much as 50%, suggesting Jnk functions as a Tnf-mediated survival signal (**Figure 30A**). Vehicle treated cells were used as control.

**Figure 30: Jnk is a Tnf-mediated survival signal in LCs, death signal in HSPCs**



**A)** HSPCs and LCs were treated in parallel with SP6 and Tnf individually or in combination in methylcellulose culture and plated for CFU assay. Numbers of CFUs were normalized to vehicle-treated HSPCs and LCs, respectively. **B)** HSPCs were treated overnight with Tnf or SP6 individually or in combination in suspension culture. The following day, cell death was measured by Annexin V assay and read by flow cytometry. Vehicle treated cells were used as negative controls. Values shown are mean  $\pm$  SD from three independent trials. \* indicates p < 0.05 when compared to vehicle control as measured by Student's t test. # indicates p < 0.05 when compared to indicated groups as measured by Student's t test.

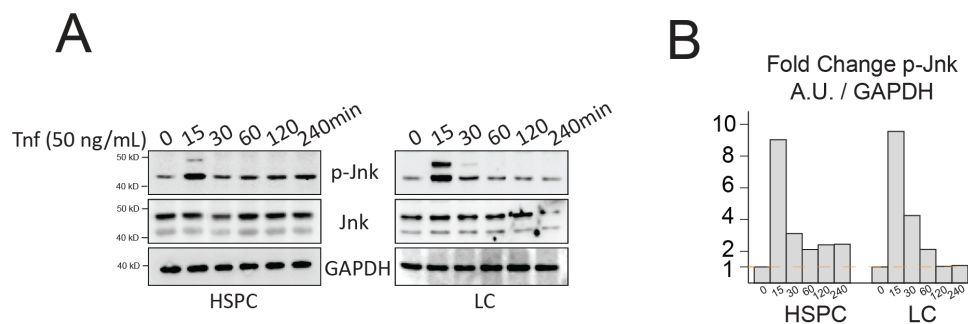
The reduction in HSPC CFUs following Tnf stimulation appeared to be almost entirely due to induction of cell death, and more specifically Jnk-mediated cell death. This is because co-treatment of Tnf and SP6 in HSPCs almost completely prevented all Tnf-mediated cell death in these cells (**Figure 30B**). Vehicle treated HSPCs were used as control.

### **Tnf-mediated Jnk activation is attenuated in LCs**

Jnk activation can play opposing roles in tissues depending on the nature of its activity. For example, the pro-survival function of Jnk is related both to its duration of activity (< 2 hrs) as well as its nuclear substrates in the Jun/Fos AP-1 family of transcription factors. This results in activation of survival and proliferative genes through the AP1 transcription factors binding to appropriate target genes. In opposition, the pro-apoptosis function of Jnk is, in turn, related to its duration of activity (> 2 hrs) as well as its cytoplasmic substrates. When phosphorylated by Jnk, pro-apoptotic Bim, Bad, Bax, Bak, and Bid increase in affinity for the puncturing the mitochondrial membrane and also prevent Bcl-2 family antiapoptotic genes from protecting the mitochondria. This results in release of cytochrome C leading to activation of executor caspases and apoptosis.

I hypothesized that because Tnf-stimulated Jnk is acting as a survival signal in LCs and death signal in HSPCs, that there would be stringent regulation of Jnk phosphorylation in LCs (<2 hrs). To test this I treated cytokine-starved HSPCs and LCs in suspension culture with exogenous Tnf for a time course experiment extending from 15 minutes to 240 minutes (4 hours). I found that both HSPCs and LCs have the most activity of Jnk 15 minutes after stimulation. But when LCs are able to return to a basal,

**Figure 31: Tnf-mediated Jnk activity is attenuated in LCs**



**A)** HSPCs and LCs were stimulated with exogenous Tnf for indicated time (in minutes). Jnk activity was determined by western blot analysis for phosphorylated Jnk. Protein levels were normalized to GAPDH for loading control. Vehicle treated cells were used as control. **B)** Pixel analysis was performed using p-Jnk blots and normalizing the arbitrary units to GAPDH for each. Fold change in p-Jnk levels were compared to vehicle (0 min) treated cells. Results shown are representative of three independent trials.

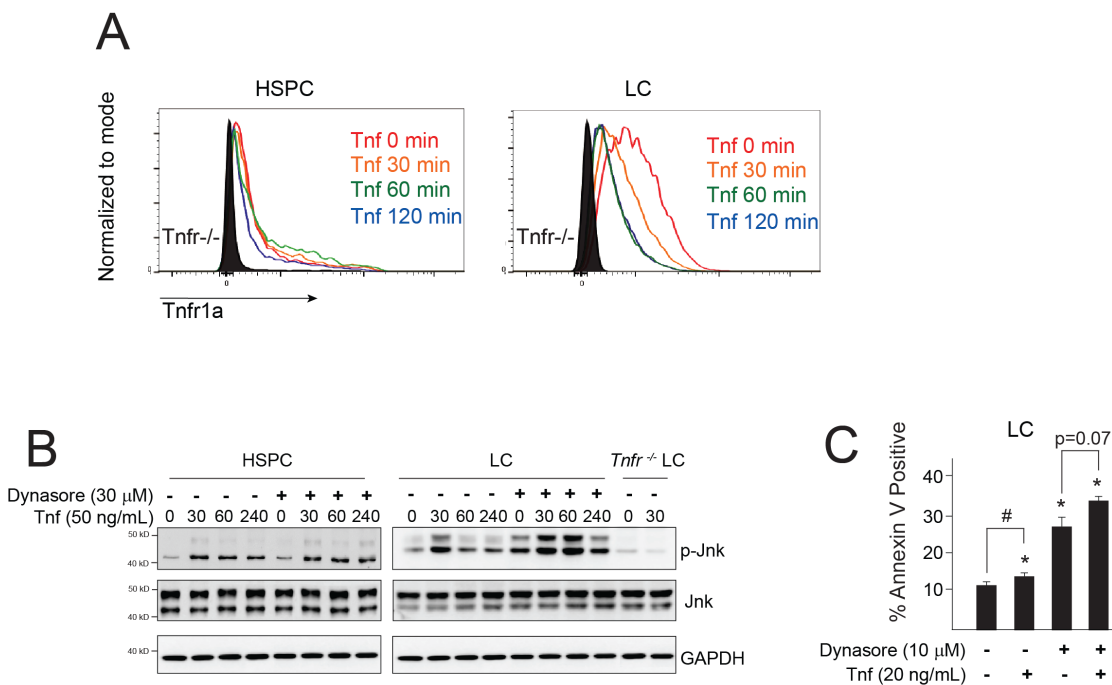
unstimulated level of Jnk phosphorylation after 60 minutes, HSPCs have Jnk activity that persists up to 4 hours following stimulation (**Figure 31A**). Total signal was analyzed using pixel analysis and normalized to GAPDH strength as internal loading controls (**Figure 31B**). Vehicle treated cells were used as negative control.

### **LCs attenuate Jnk signal strength through Tnf receptor endocytosis**

There are two possible mechanisms for attenuating the Jnk signal duration: LCs can either endocytose the Tnf receptor thereby removing the stimulus, or LCs can directly dephosphorylate Jnk with a Map Kinase Phosphatase (Mkp). I tested the first possibility by treating HSPCs and LCs with Tnf for a timecourse and measured surface expression of Tnf receptor. If Tnf stimulation drove endocytosis of the receptor, then the mean fluorescence intensity should decrease, and the curve should shift to the left (this antibody recognizes a different sequence on the extracellular portion of the receptor than binds the ligand, so I can be confident that ligand binding does not displace the antibody, or vice versa). I found that LCs do endocytose the receptor in response to ligand binding when compared to HSPCs (**Figure 32A**). HSPCs and LCs lacking the Tnf receptor were used as negative control.

Tnf receptors are endocytosed via the classic clathrin-mediated pit mechanism. The final step of clathrin-dependent endocytosis is the cinching and sealing of the internalized vesicle by an ATP-dependent protein called dynamin, which can be inhibited by a small molecule called Dynasore. I expected to be able to prolong the Jnk signal in LCs by blocking endocytosis, but I found that the signal was only slightly prolonged (still active at 60 minutes). However, I did note that the signal of phosphorylated Jnk was

**Figure 32: LCs attenuate Jnk signal strength through Tnf receptor endocytosis**



**A)** HSPCs and LCs were treated with exogenous Tnf (20 ng/mL) for indicated times, then stained for Tnfr1a expression and read by flow cytometry. *Tnfr*<sup>-/-</sup> HSPCs and LCs were used as negative controls. Results shown are indicative of three independent trials. **B)** HSPCs and LCs were treated with exogenous Tnf (for indicated time in minutes) and Dynasore individually or in combination. Phosphorylated Jnk was measured by western blot analysis. Results shown are indicative of three independent trials. **C)** LCs were treated with Dynasore or Tnf alone or in combination overnight in suspension culture. Cell death was measured the following day by Annexin V staining and read by flow cytometry. Results shown are mean  $\pm$  SD. \* indicates  $p < 0.05$  when compared to vehicle control as determined by Student's t test. # indicates  $p < 0.05$  between indicated groups. Other p values are noted.

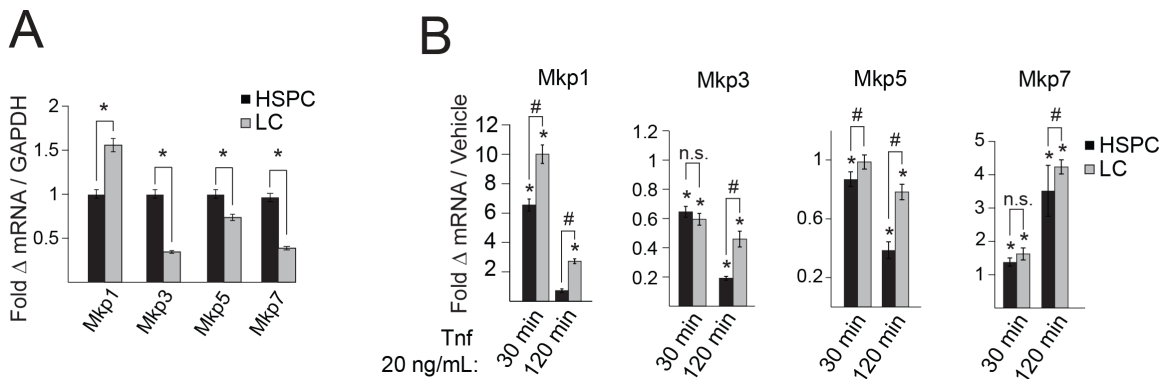
significantly strengthened (**Figure 32B**). I then found blocking endocytosis did enhance overall cell death in LCs, but addition of exogenous Tnf did not further enhance cell death (**Figure 32C**). I concluded from these results that LCs are utilizing receptor endocytosis to regulate the Jnk signal, but only by controlling the signal strength.

### **Mkps are stably expressed in LCs in response to Tnf**

The previous figure showed how LCs can endocytose the Tnf receptor in order to limit the signal strength of Jnk. This mechanism did not have a major effect on the Jnk signal duration though, and LCs were still able to return Jnk phosphorylation to an unstimulated level after one hour. Therefore, there must be additional mechanisms in place to limit Jnk activity. These are the Map Kinase Phosphatases (Mkp). Mkps are poorly understood dual-specificity proteins that can dephosphorylate and inactivate the map kinases. Mkps 1, 3, 5, and 7 were shown to have some interactions with Jnk, therefore I hypothesized that one of these Mkps were responsible for regulating Jnk in LCs. Mkps are regulated at the level of transcription, so I first assayed for total transcripts of each Mkp (1, 3, 5, and 7). Results displayed are normalized to each condition in HSPCs. I found that only Mkp1 is upregulated in LCs (**Figure 33A**).

Mkp transcription changed in response to Tnf stimulation. I hypothesized that Tnf would stimulate necessary regulation mechanisms for Tnf targets, and I found that Tnf stimulation did increase Mkp1 and 7 transcription in both HSPCs and LCs. Tnf drove a repression of Mkp3 and 5 transcription in HSPCs (**Figure 33B**). Even though Tnf drove changes in Mkp7 transcription levels, Mkp7 was not significantly changed between

**Figure 33: Mkps are stably expressed in LCs during Tnf stimulation**



**A)** HSPCs and LCs were analyzed by qRT-PCR to determine relative transcription of Mkps. Results shown are normalized to levels in HSPCs for each type of Mkp. Results shown are mean  $\pm$  SD from three independent trials. \* indicates  $p < 0.05$  when compared to HSPC transcription level, as determined by Student's t test. **B)** HSPCs and LCs were treated with Tnf for indicated times, and Mkp transcription at each time was assayed by qRT-PCR. Values shown are mean  $\pm$  SD from three independent trials, and are normalized to vehicle treated cells as control. \* indicates  $p < 0.05$  when compared to vehicle treated cells, # indicates  $p < 0.05$  when compared to indicated groups, both determined by Student's t test (n.s. = non significant).



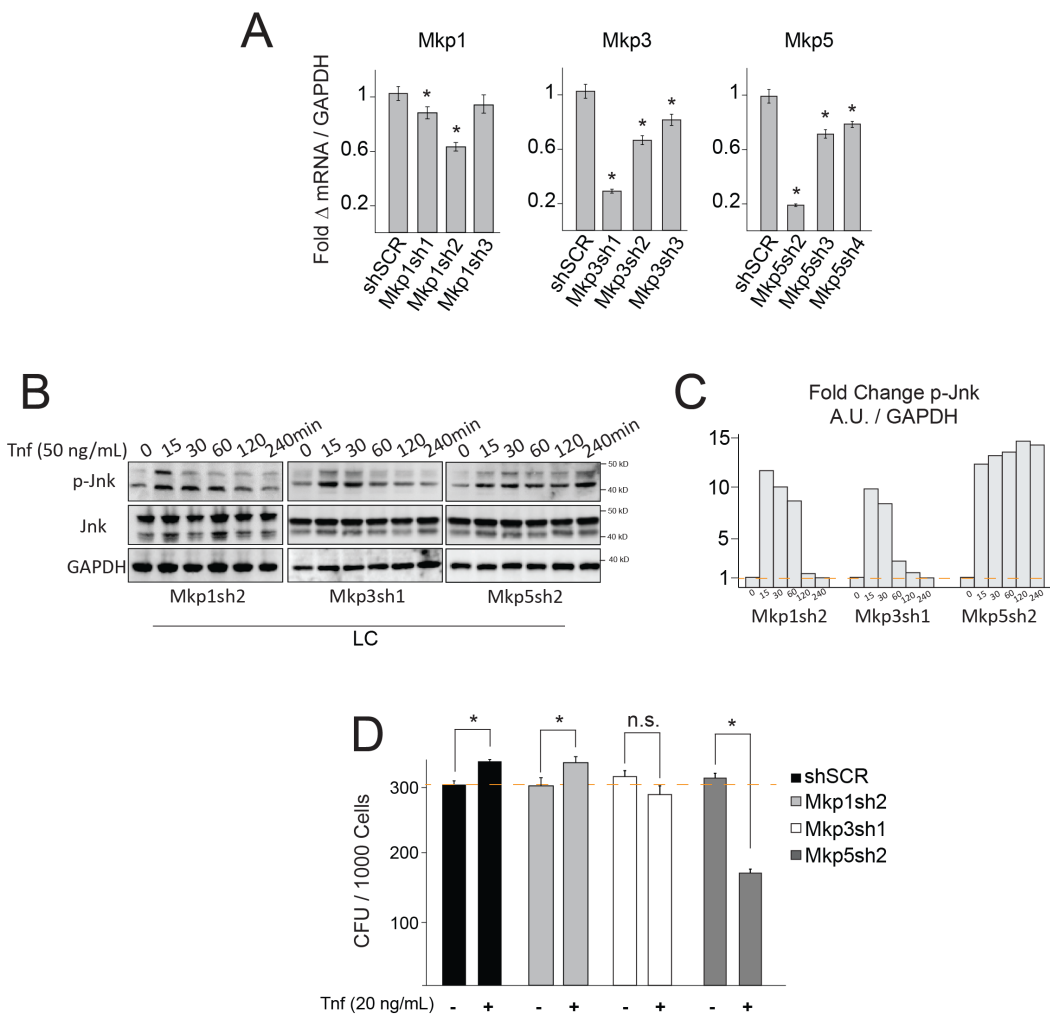
HSPCs and LCs, and was not further studied. HSPCs displayed a significant drop in Mkp3 and 5 levels during the course of Tnf treatment, suggesting that Tnf can repress expression of these genes. However, Mkp3 and 5 levels were not changed in LCs during the course of Tnf stimulation.

### **Mkp5 regulates Jnk activity in LCs**

I determined that several Mkps are either upregulated or maintained differentially in LCs than in HSPCs in response to Tnf stimulation (Mkp1/3/5). In order to determine which of these is responsible for Jnk regulation in response to Tnf in LCs, I knocked down Mkp1, 3, and 5 in LCs using shRNA constructs, and measured knockdown by qRT-PCR (**Figure 34A**). Scrambled shRNA was used as negative control, and the best knockdown was used for future experiments.

I were expecting that knockdown of Mkp1 would prolong p-Jnk because it is upregulated in LCs compared to HSPCs, and it also increases during Tnf stimulation. However, I found that Mkp1 knockdown only slightly prolonged Jnk activation duration. Mkp5 knockdown, however, completely eliminated LCs ability to regulated p-Jnk. In these cells, Tnf stimulation perpetually activated Jnk (**Figure 34B**). Quantification of the p-Jnk signal was performed by pixel analysis and normalized to GAPDH loading control. Arbitrary units shown are relative to p-Jnk signal from vehicle-treated cells (**Figure 34C**).

Next I asked if prolonging Jnk activity in LCs would convert Jnk from a Tnf-mediated survival signal to a death signal. I tested the knockdowns of Mkp1, 3, and 5 and

**Figure 34: Mkp5 regulates Tnf-mediated Jnk activity in LCs**

**A)** shRNA knockdown of indicated Mkps in LCs. Values are normalized to scrambled shRNA transduced control. **B)** LCs expressing each Mkp knockdown shRNA construct were treated with Tnf for indicated time periods, lysed, and contents resolved by SDS-PAGE and analyzed by western blot. Results shown are representative of three independent trials. **C)** Quantification of western blot results in **B** by pixel analysis. Arbitrary units are set for each shRNA by vehicle treated cells and normalized to GAPDH. **D)** LCs expressing indicated shRNAs were treated with Tnf in methylcellulose for colony forming unit assay. Total number of colonies is shown. Values shown are mean  $\pm$  SD from three independent trials. \* indicates  $p < 0.05$  when compared to vehicle treated cells, unless otherwise noted, by Student's t test.

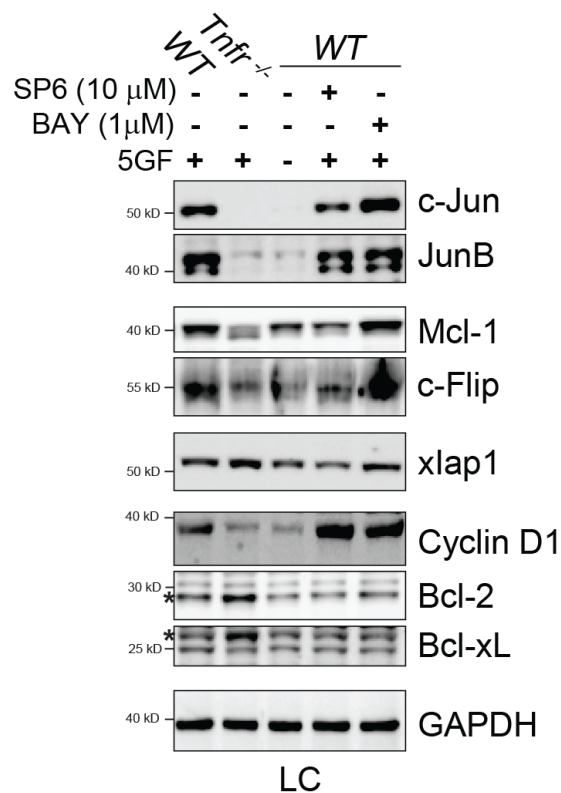
found that Mkp5 knockdown was able to both prolong the Jnk signal in the presence of Tnf as well as cause reduction in CFUs. Mkp1 also slightly prolonged the Jnk signal, but apparently not enough to convert it to a death signal. These cells increased CFUs upon Tnf stimulation (**Figure 34D**).

### **Jnk promotes Tnf-mediated LC survival by promoting Mcl-1 and c-Flip expression**

I determined that Jnk is acting as an NF- $\kappa$ B independent, Tnf-dependent survival signal regulated by Mkp5 in LCs. As a SAPK protein, Jnk drives target gene expression indirectly through phosphorylation of nuclear substrate transcription factors in the AP1 (Jun/Fos) family. In order to determine which survival genes were Jnk mediated, Tnf-dependent, and NF- $\kappa$ B independent, I grew *WT* and *Tnfr<sup>-/-</sup>* LCs in culture media without cytokine stimulation for four hours to reset the internal protein machinery of the cell to a basal rate. Then I pre-treated cells with either BAY or SP6 to block NF- $\kappa$ B or Jnk, respectively, then added back all four hematopoietic cytokines (IL3, IL6, GM-CSF, SCF) plus Tnf. I called this condition “5 growth factors” (5GF). Any signals that were Tnf-mediated would be downregulated in *Tnfr<sup>-/-</sup>* LCs grown with 5GF, and any signals that were Jnk mediated would remain downregulated in the presence of SP6 once 5GF were added back.

I found that Tnf-mediated survival genes were from the Jun family (c-Jun, JunB, positive controls), Mcl-1, c-Flip, and Cyclin D1. Bcl-2 and Bcl-xL were upregulated in *Tnfr<sup>-/-</sup>* LCs, suggesting a compensatory mechanism for maintaining cell survival without Tnf. Cyclin D1 levels returned following SP6 and BAY treatment, suggesting that it is a

**Figure 35: Jnk promotes Tnf-mediated LC survival by reinforcing Mcl-1 and c-Flip expression**



WT and *Tnfr*<sup>-/-</sup> LCs were grown in cytokine-free medium for four hours to reset the internal protein machinery of the cell to a basal rate. Then I pre-treated cells with either BAY or SP6 to block NF-κB or Jnk, respectively, then added back all four hematopoietic cytokines (IL3, IL6, GM-CSF, SCF) plus Tnf. Results shown are representative of three independent trials. \* indicates specific binding of Bcl-2/Bcl-xL antibody, other bands are non-specific.

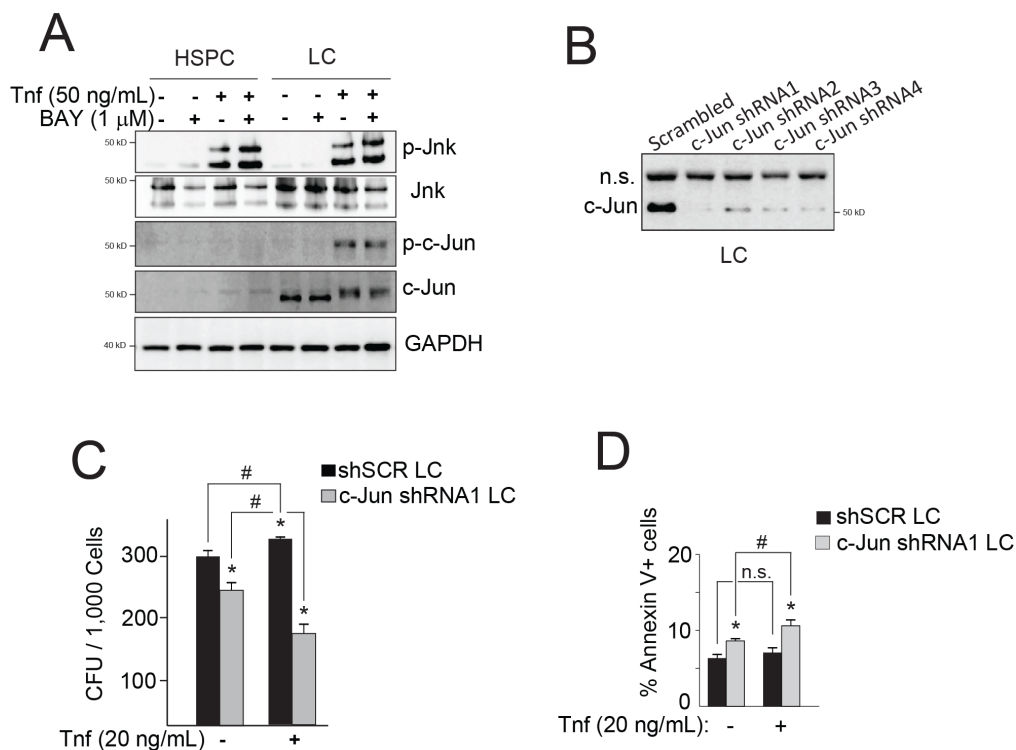
Tnf-mediated, NF- $\kappa$ B and JNK-independent cell cycle genes. Mcl-1 and c-Flip levels returned to normal in the presence of BAY with the addition of 5GF media condition, but were repressed with SP6 treatment, suggesting these are Jnk-mediated survival genes. xIAP1 was also repressed following Jnk treatment but was not downregulated in *Tnfr*<sup>-/-</sup> LCs, suggesting this protein is Jnk-mediated but Tnf-independent (**Figure 35**). LCs grown in 5GF culture medium were used as controls.

### **Jnk mediates its pro-survival signal through c-Jun**

Jnk's primary nuclear substrate for driving survival and proliferation signaling is c-Jun. I compared c-Jun expression between HSPCs and LCs and found LCs express a much higher level of c-Jun protein than HSPCs. Also, Tnf drives Jnk-mediated c-Jun phosphorylation in an NF- $\kappa$ B-independent manner because treatment with BAY did not repress either Tnf-mediated Jnk phosphorylation or c-Jun phosphorylation (**Figure 36A**). Vehicle treated cells were used as controls.

In order to determine c-Jun's function in driving Tnf-mediated LC survival, I performed a shRNA knockdown experiment. LCs were spinoculated with c-Jun shRNA expressing retrovirus, selected, and contents resolved by SDS-PAGE and western blot analysis. I found that c-Jun shRNA1 provided us with the best knockdown. Non-specific binding was used as loading control (**Figure 36B**).

If c-Jun functioned as a Tnf-mediated survival signal, then removing it via shRNA knockdown should also eliminate Jnk's nuclear target and convert it to a death signal. I found this to be the case as c-Jun knockdown cells had a significant reduction in

**Figure 36. Jnk mediates its pro-survival signal through c-Jun**

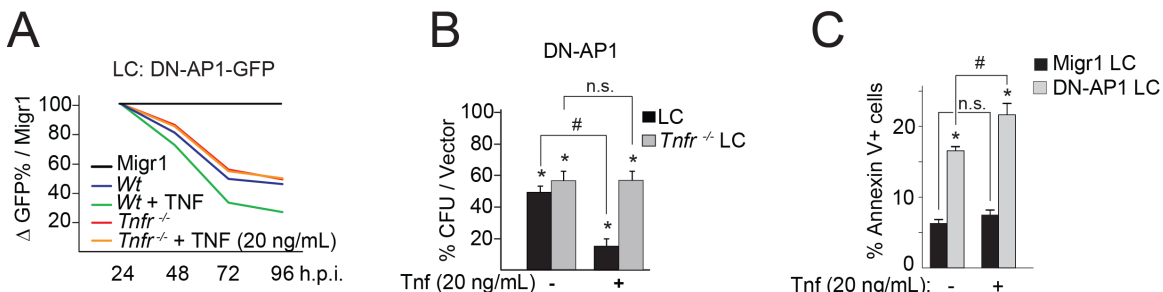
**A)** HSPCs and LCs were treated in parallel with exogenous Tnf and BAY individually or in combination. Cells were lysed and contents resolved by SDS-PAGE and analyzed by western blot. Vehicle treated cells were used as negative controls. Results shown are representative of three independent trials. **B)** LCs were spinoculated with c-Jun shRNA-expressing constructs and selected by puromycin for one week. Knockdown efficiency was measured by western blot. Scrambled shRNA-expressing retrovirus was used as negative control. Results shown are indicative of three independent trials. **C)** shSCR and c-Jun shRNA1 expressing LCs were treated with exogenous Tnf in methylcellulose and CFUs measured. Results shown are mean  $\pm$  SD from three independent trials. **D)** shSCR and c-Jun shRNA1 expressing LCs were treated with exogenous Tnf in suspension culture overnight and measured for cell death by Annexin V stain and read by flow cytometry. Results shown are mean  $\pm$  SD from three independent trials. \* indicates  $p < 0.05$  when compared to vehicle treated cells as measured by Student's t test. # indicates  $p < 0.05$  when compared to indicated groups as measured by Student's t test.

CFUs when treated with exogenous Tnf in methylcellulose (**Figure 36C**), as well as a significant induction of apoptosis as measured by Annexin V stain following overnight stimulation with exogenous Tnf (**Figure 36D**). In both cases, LCs expressing a scrambled shRNA and treated with vehicle were used as negative controls. The amount of cell death induced was slight but significant, though I noted that it did not seem large enough to explain the significant drop in CFUs following Tnf stimulation. I propose that this is due to additional members of the Jun family compensating for loss of c-Jun. To account for this, I turned to a dominant negative form of c-Jun (TAM67).

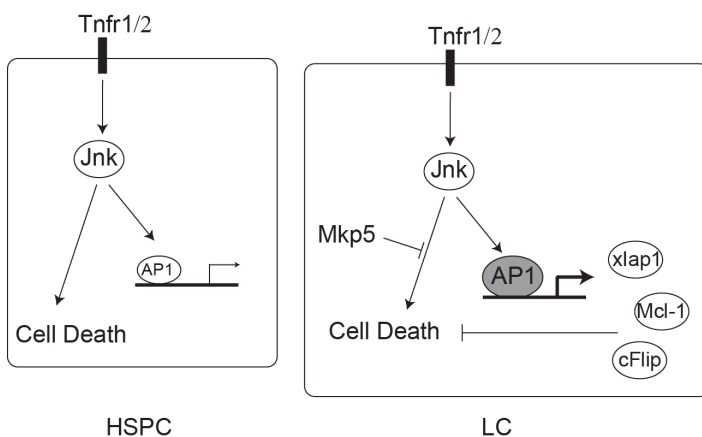
#### **Dominant negative AP-1 abrogated Tnf-mediated survival in LCs**

I spinoculated LCs with a dominant negative AP-1 (TAM67), which contains the necessary structures to interact with and be phosphorylated by SAPKs (Jnk1/2), then bind other AP-1 family members. TAM67 (DN-AP1, hereafter) lacks a DNA-binding domain, preventing the transcription factors from binding to target genes. This molecule can block the activity of all Jun family members (c-Jun, JunB, and JunD). Overexpression of this molecule resulted in significant defects in overall cell growth compared to non-transduced cells as determined by change in GFP% over time (**Figure 37A**). Addition of Tnf enhanced this effect. It should be noted that DN-AP1 had an effect in *Tnfr<sup>-/-</sup>* cells, which do not express high levels of c-Jun or JunB. I expect that this is due to JunD compensating, but did not perform any experiments to this end. Non-transduced cells were used as internal control.

The slight reduction in CFUs and increase in Annexin V observed by treating c-Jun knockdown LCs with Tnf was significantly enhanced by blocking the entire

**Figure 37. Dominant negative AP-1 abrogated Tnf-mediated survival in LCs**

**A)** *WT* and *Tnfr<sup>-/-</sup>* LCs were spinoculated with DN-AP1 expressing GFP retrovirus in suspension culture and were observed daily by flow cytometry for reduction in % of GFP-expressing cells. Tnf was also added to the culture media in test groups. Vehicle treated cells were used as negative control, non-transduced cells were used as internal control. Results shown are representative of three independent trials. **B)** *WT* and *Tnfr<sup>-/-</sup>* LCs were spinoculated with DN-AP1 expressing retrovirus and treated with exogenous Tnf in methylcellulose for CFU assay. Results shown are normalized to vehicle treated cells expressing empty vector. **C)** LCs were spinoculated with DN-AP1 expressing retrovirus and treated with Tnf in suspension culture overnight. The following day, cell death was measured by Annexin V staining as read by flow cytometry. Vehicle treated cells expressing empty vector were used as negative control. \* indicates  $p < 0.05$  compared to vehicle treated cells by Student's t test. # indicates  $p < 0.05$  compared to indicated test group as determined by Student's t test.

**Figure 37 model. LC conversion of Tnf-mediated Jnk signaling to a survival signal**



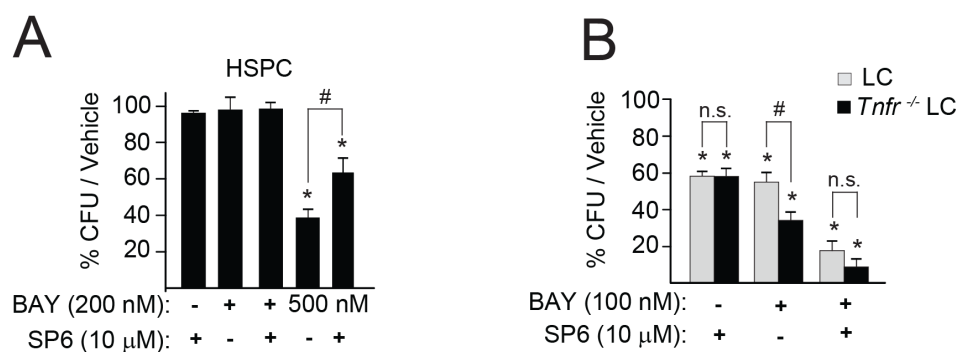
transcription factor family with DN-AP1. I found that overexpression of DN-AP1 combined with exogenous Tnf treatment reduced CFUs in LCs by 80% (**Figure 37B**, compared to 40-50% with c-Jun only knockdown), and increased Annexin V staining by 20% (**Figure 37C**, compared to 11% with c-Jun only knockdown). These suggest that there are additional AP1 family transcription factors activated in addition to c-Jun that need to be accounted for when trying to block Tnf-mediated survival signaling in LCs and LSCs.

### **Co-inhibition of NF- $\kappa$ B and Jnk is protective in HSPCs, synergistic in LCs**

I identified Jnk as a critical Tnf-driven pro-survival signal in LCs, but a pro-death signal in HSPCs. This presents an interesting option for an anti-cancer therapeutic target: blocking Jnk would induce cell death in LCs while protecting HSPCs against cell death, and I may be able to further sensitize LCs to NF- $\kappa$ B inhibitor effects *in vivo* by blocking the compensatory pathway. I started by measuring the effects of co-inhibition of NF- $\kappa$ B and Jnk *in vitro* by combination treatment in methylcellulose and measuring change in CFUs. I found co-treatment of HSPCs with BAY and SP6 at therapeutic doses had no effect on the cells. In fact, the toxicity of over-dosing HSPCs with BAY was partially rescued by co-treatment with SP6 (**Figure 38A**). Vehicle treated HSPCs were used as negative control.

I then tested the combination therapy in *WT* and *Tnfr<sup>-/-</sup>* LCs. I found that treatment with an even lower dose of BAY (100 nM vs 200 nM) than originally determined was sufficient to induce >90% reduction in CFUs, as long as co-treated with SP6 (**Figure 38B**). As expected, both genotypes of LCs were equally sensitive to SP6 treatment, and

**Figure 38. Co-inhibition of NF- $\kappa$ B and Jnk is protective in HSPCs, synergistic in LCs**



**A)** HSPCs were treated with indicated concentrations of BAY and SP6 alone or in combination in methylcellulose. Vehicle treated HSPCs were used as negative controls. **B)** LCs were treated with indicated concentrations of BAY and SP6 alone or in combination in methylcellulose. Vehicle treated LCs were used as negative controls. Values shown are mean  $\pm$  SD from three independent experiments. \* indicates  $p < 0.05$  when compared to vehicle treated controls as determined by Student's t test. # indicates  $p < 0.05$  when compared to indicated groups by Student's t test.

the *Tnfr*<sup>-/-</sup> LCs were more sensitive to the low-dose BAY treatment. By inhibiting Jnk using SP6, I were able to reduce colonies by >90% in both cell types.

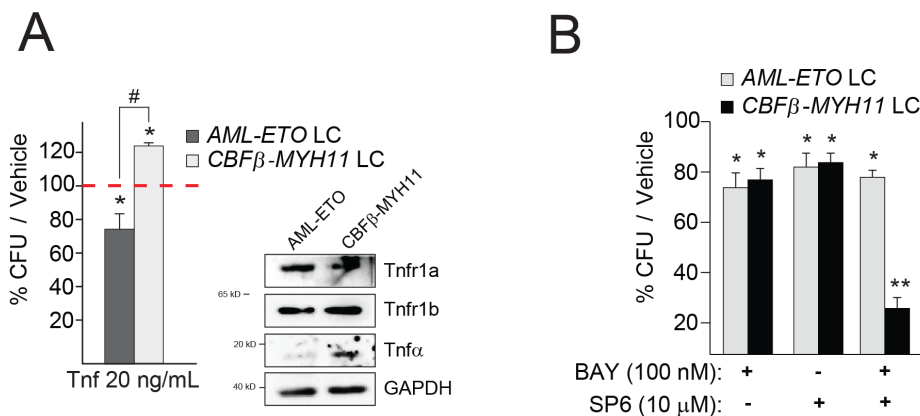
Interestingly, combination treatment of *Tnfr*<sup>-/-</sup> LCs also reduced CFUs further than BAY inhibitor alone, suggesting that there may be additional Jnk-mediated, Tnf-independent survival signaling. This could be due to x-IAP, as determined in **Figure 35**. Vehicle treated LCs from each genotype were used as negative controls in this experiment.

#### **Co-inhibition of NF-κB and Jnk is most effective in Tnf-expressing LCs**

Our murine model of AML that I have been using up to this point is based on expression of the MLL-AF9 fusion gene, which only accounts for a small percentage of diagnosed AMLs. I also generated LCs from other leukemia fusions: AML-ETO (M2 subtype) and CBFβ-MYH11 (M4 subtype) LCs. Just as observed in human AMLs, the AML-ETO LCs did not express mature Tnf, while the CBFβ-MYH11 LCs did. Additionally, treatment with exogenous Tnf drove an increase in CFUs in the Tnf-expressing LCs, but reduced the CFUs in the non-Tnf-expressing LCs (**Figure 39A**). Vehicle treated cells were used as negative control. I confirmed Tnf and Tnf receptor expression in both types of leukemia cells by western blot analysis.

I then tested the two additional murine leukemia cell types with the combination treatment, BAY and SP6. The combined inhibition of NF-κB and Jnk only had a minimal effect on AML-ETO LCs (non-Tnf-expressing LCs), while the combined inhibition had a major effect on CBFβ-MYH11 LCs (**Figure 39B**). These data suggest that although the

**Figure 39. Co-inhibition of NF- $\kappa$ B and Jnk is most effective in Tnf-expressing LCs**



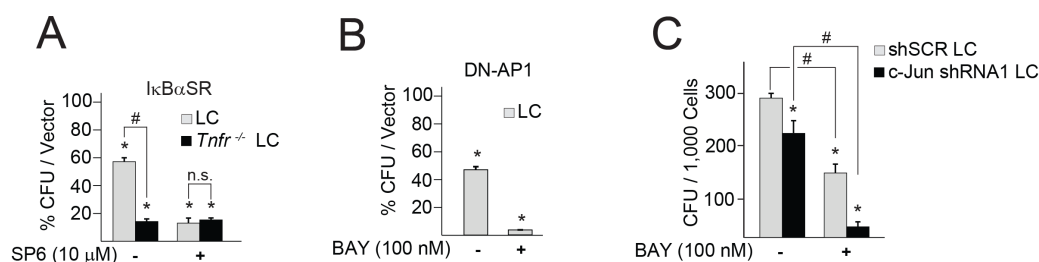
**A)** AML-ETO and CBF $\beta$ -MYH11 LCs were treated with Tnf in methylcellulose for CFU assay. Tnf and receptor expression was measured by western blot analysis of lysed cells.

**B)** AML-ETO and CBF $\beta$ -MYH11 LCs were treated with BAY or SP6 alone or in combination at indicated doses in methylcellulose for CFU assay. Results are normalized to vehicle treated cells. All results shown are mean  $\pm$  SD. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$  when compared to vehicle treated cells as determined by Student's t test. # indicates  $p < 0.05$  when compared to indicated experiment groups as determined by Student's t test.

inhibition of NF- $\kappa$ B and Jnk can have an effect on most AMLs, it is most effective in AMLs that express Tnf. Vehicle treated cells were used as controls.

### **Confirmation of inhibitor specificity in LCs**

A major concern when working with small molecule inhibitors in live cells is the possibility of non-specific or off-target effects of the drug. This can be tested for by co-treatment with a genetic inhibition of the same target. For example, the specificity of the BAY inhibitor can be measured by co-treatment with DN-AP1 to block Jnk/c-Jun or c-Jun shRNA, or SP6 can be co-treated in I $\kappa$ B $\alpha$ SR-expressing cells. If the end result of the inhibitor and genetic inhibition is the same as two inhibitors, then I can conclude that the effects I observe are due to direct interactions. I performed these experiments in *WT* and *Tnfr<sup>-/-</sup>* LCs expressing I $\kappa$ B $\alpha$ SR treated with or without SP6 (**Figure 40A**), *WT* LCs expressing DN-AP1 treated with or without BAY (**Figure 40B**), and *WT* LCs expressing c-Jun shRNA treated with or without BAY (**Figure 40C**). In each case, the genetic modification augmented the inhibitor's effects. This suggests that the effects I observe with the combination drug therapies are specific, and not due to off-target effects. Cells expressing empty vector or scrambled shRNA (where appropriate) and treated with vehicle were used as negative controls.

**Figure 40. Confirmation of inhibitor specificity in LCs**

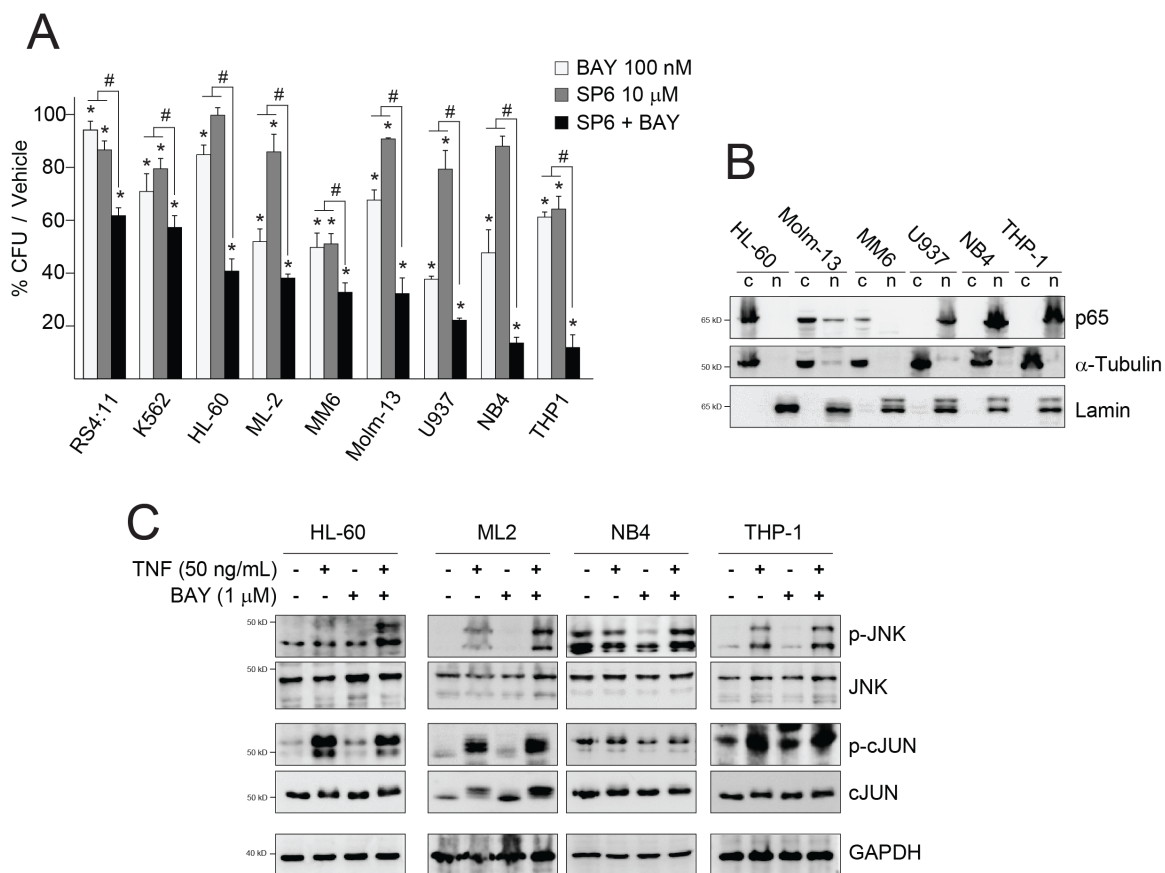
**A)** *WT* LC and *Tnfr*<sup>-/-</sup> LC were transduced with I $\kappa$ B $\alpha$ SR-GFP. GFP<sup>+</sup> cells were sorted and plated in methylcellulose with or without SP6 treatment. Values are normalized to vector-only transduced control cells. **B)** LCs were transduced with DN-AP1-GFP. GFP<sup>+</sup> cells were sorted and plated in methylcellulose with or without BAY treatment. **C)** LCs were transduced with c-Jun-shRNA1; GFP<sup>+</sup> cells were sorted and plated in methylcellulose with or without BAY treatment. shSCR transduction was used as control. Values shown are mean  $\pm$  SD from three independent experiments. \* indicates  $p < 0.05$  as determined by Student's t test when compared to vehicle treated vector-expressing control cells. # indicates  $p < 0.05$  when compared to indicated experiment groups as determined by Student's t test.

### **Co-inhibition of NF- $\kappa$ B and JNK is synergistic in TNF-expressing human AML**

To study whether combination treatment using both NF- $\kappa$ B and JNK signaling inhibitors is also additive in eliminating human LC, I compared the responses of human AML cell lines to the treatments. By incubating the LC with NF- $\kappa$ B and JNK inhibitors individually or in combination for 12 hours, and then seeding for CFU to examine the reduction of clonogenic LC, I observed additive inhibition effects of the two inhibitors in almost all AML cell lines (**Figure 41A**). I noted that the sensitivity of AML cell lines to such additive inhibition effects are correlated to levels of TNF expression (**Figure 19D**). Cell lines RS4:11 (B-ALL), K562 erythroid LC and ML-2 that had a reduced response also express lower levels of TNF, whereas the TNF-expressing cell lines, including HL-60 (M2/3), ML-2 (M5), Molm-13 (M5), U937 (M5), NB4 (M3), and THP-1 (M5), showed exceptional sensitivity to the combined inhibitor treatment. Results shown are normalized to colonies formed by vehicle-treated cells.

The most sensitive human AML cell lines to the combination therapy showed constitutive NF- $\kappa$ B activity, as determined by p65 localization to the nucleus, while the least sensitive cell lines did not have constitutive NF- $\kappa$ B activation (**Figure 41B**). In addition, I found that, although all the sensitive cell lines showed TNF-stimulated NF- $\kappa$ B-independent JNK-c-JUN activation (**Figure 41C**), the most sensitive AML cell lines (U937, NB4 and THP1) had increased basal levels of NF- $\kappa$ B and JNK-c-JUN activities as shown by elevated nuclear p65 and total p-JNK/p-c-JUN. Vehicle-treated cells were used

**Figure 41. Co-inhibition of NF- $\kappa$ B and JNK is synergistic in TNF-expressing human AML cell lines**



**A)** CFU from human leukemic cell lines treated with indicated doses of SP, BAY or combination. Results shown are mean  $\pm$  SD from three independent experiments. \* indicates  $p < 0.05$  when compared to vehicle treated control, as determined by Student's *t* test. # indicates  $p < 0.05$  when compared to indicated experiment groups as determined by Student's *t* test. **B)** Nuclear fractionation analysis showing NF- $\kappa$ B activity in AML cell lines. Results shown are representative of three independent trials. **C)** Several TNF-high expressing AML cell lines were treated with TNF and BAY individually or in combination. Levels of p-JNK and p-cJUN were examined by Western blotting. Results shown are representative of three independent trials.



as negative controls, and protein levels were normalized to  $\alpha$ -Tubulin/Lamin (**Figure 41B**) or GAPDH (**Figure 41C**) as loading control.

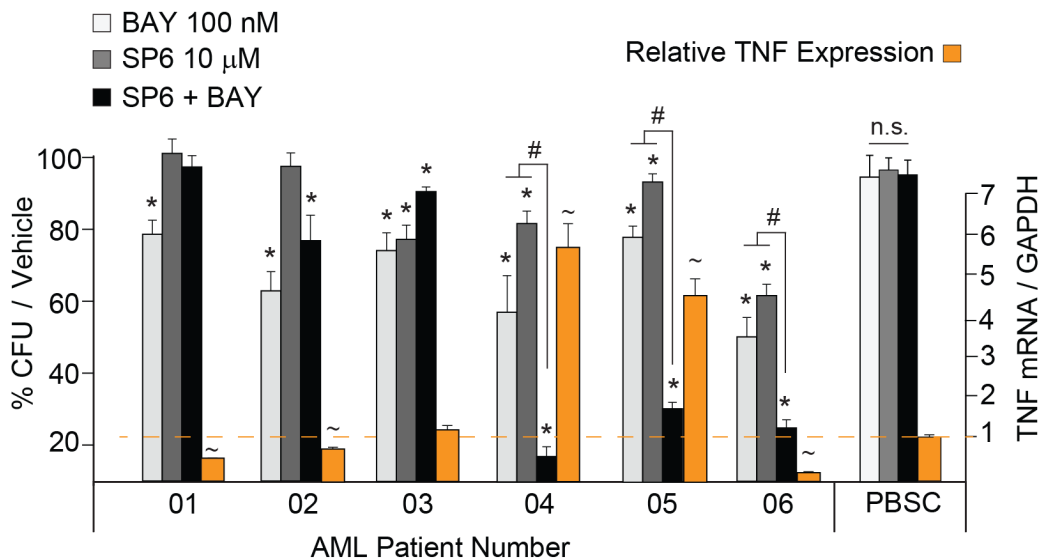
### **Co-inhibition of NF- $\kappa$ B and JNK is synergistic in TNF-expressing primary AML patient samples**

The gold-standard of testing novel drug efficacy is to use tumor samples excised directly from newly-diagnosed patients. I collaborated with the clinicians at the hematology oncology department at Loyola University Medical Center to collect peripheral blood and bone marrow aspirate samples from newly diagnosed AML patients. Upon receipt of these samples, nucleated cells were purified using Ficoll-paque centrifugation and cultured as stated in the methods section. In healthy patients, colony-forming cells are rarely found in the peripheral blood, so I used Peripheral Blood Stem Cells (PBSCs) isolated from donors following G-CSF therapy to mobilize HSCs from the niche for collection.

I found that while all but one sample were somewhat sensitive to combined NF- $\kappa$ B and JNK inhibition, 3/6 were highly sensitive with CFU reductions of >80%. Two of these samples expressed high levels of Tnf, while the third did not. At the time I proposed that this sample (patient #6) may express high levels of IL1. This has since been confirmed (**Figure 42**).

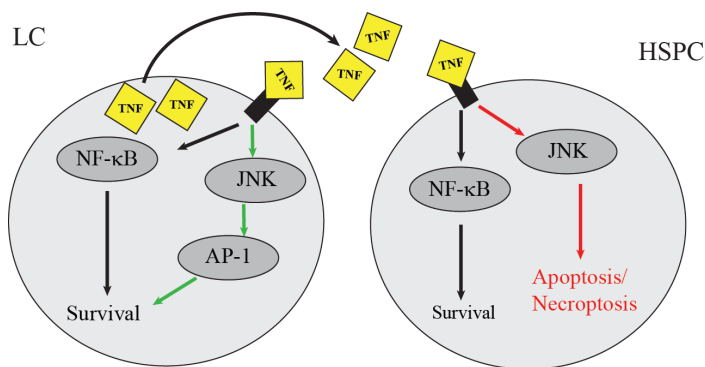
These data, taken together suggest that some AMLs use TNF as an autocrine survival signal by activation of JNK as a pro-survival/proliferation marker. AMLs also use secreted TNF as a paracrine signal to reorganize the niche and eliminate HSPCs, which use JNK as a pro-death signal (**Figure 42 model**).

**Figure 42. Co-inhibition of NF- $\kappa$ B and JNK is synergistic in TNF-expressing primary AML patient samples**



Mononuclear cells from AML patient samples isolated from the peripheral blood were isolated and cultured overnight in patient sample medium.  $1 \times 10^4$  live cells were plated in methylcellulose along with indicated treatments and read for CFU assay. Remaining cells were lysed and RNA isolated for qRT-PCR. Results shown are normalized to vehicle treated cells, and are mean  $\pm$  SD from three independent trials. \* indicates  $p < 0.05$  when compared to vehicle treated cells, # indicates  $p < 0.05$  when compared to indicated experiment groups, and ~ indicates  $p < 0.05$  when compared to TNF mRNA levels of PBSCs, all determined by Student's t test.

**Figure 42 model.**

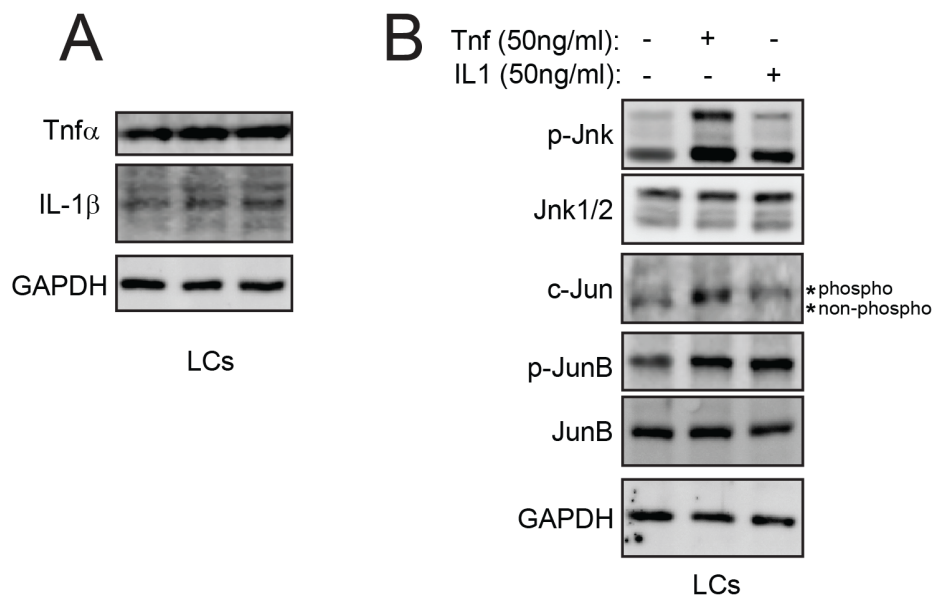


**Aim 2. Determine IL1 contribution to TNF-independent, NF- $\kappa$ B independent survival and chemoresistance of LSCs to NF- $\kappa$ B inhibition.**

**IL1 stimulates Jnk and Jun family members' activation in LCs**

When I saw that some patient samples that do not express TNF were sensitive to NF- $\kappa$ B and JNK inhibition treatments, I suggested there might be additional pro-inflammatory cytokines operating similarly to TNF, but in a parallel fashion. This would account for some of the AML patient samples that responded to NF- $\kappa$ B and JNK inhibition, but did not express TNF. To find out which pro-inflammatory cytokines could be producing such an effect, I turned to the resources available by my collaborators. They determined that the same patients that express high levels of TNF also express high levels of IL1 (**Figure 3**). My collaborators also found that LSCs, in addition to producing endogenous IL1, respond to exogenous IL1 stimulation by promoting survival and proliferation (**Figure 4**). I confirmed that the mouse MLL-AF9 LCs produced both Tnf and IL1 by western blot analysis by looking at tumors isolated from three separate xenograft transplants of LCs into mice (**Figure 43A**). If IL1 was operating in parallel to TNF, then I would expect to see activation of JNK and subsequent Jun family members. To test this, I stimulated LCs with exogenous IL1 and Tnf and measured the phosphorylation events on Jnk, c-Jun, and JunB. I found that while Tnf is a strong inducer of Jnk and c-Jun, IL1 is a weak inducer of Jnk and c-Jun. JunB was equally activated by both Tnf and IL1 (**Figure 43B**). This suggests that without Tnf, LCs are able to secrete IL1 to activate similar signaling pathways. Therefore, blocking Tnf alone in LCs will not be enough to inhibit NF- $\kappa$ B-independent survival.

**Figure 43. LCs produce IL1, which weakly activates Jnk, c-Jun, and JunB**



**A)** LCs from three separate xenografts were isolated from mice, lysed, and analyzed for Tnf and IL1 expression by western blot analysis. GAPDH was used as loading control. Mature IL1 is the lower (strongest) band resolved, the upper band is non-specific. **B)** LCs were stimulated with either Tnf or IL1 and contents were resolved for western blot analysis. c-Jun phosphorylation can be determined by upward shift, and respective bands are notated. GAPDH was used as loading control.

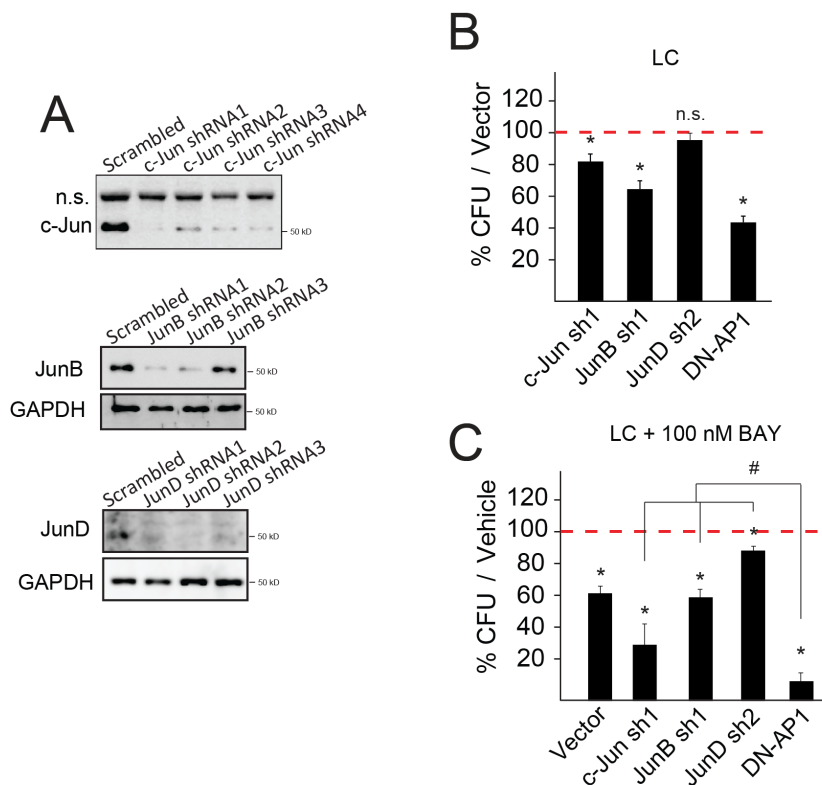
### **c-Jun, JunB, and JunD contribute to LC resistance to NF- $\kappa$ B inhibition**

When I screened through pro-inflammatory genes that were upregulated in AML patient tumors, I found that the Jun family of proteins (c-Jun, JunB, JunD) were highly expressed. While c-Jun is a well-described tumor promoter in most tissues studied, JunB is widely considered to be a tumor suppressor.<sup>308</sup> In order to understand the functions of these proteins further, I did shRNA knockdown experiments and measured effects on colony formation in LCs. I were able to stably knockdown each individual Jun family member in LCs (**Figure 44A**). Total knockdown was compared to scrambled shRNA-transduced control, and protein loading was normalized to GAPDH, except for c-Jun knockdown which was normalized to a non-specific band.

I found that knockdown of c-Jun resulted in a decrease in LC CFUs, but that JunB knockdown resulted in a further decrease in CFUs, suggesting that JunB may be acting as a tumor promoter parallel to c-Jun in these cells. JunD knockdown did not have a significant effect on the CFU ability of LCs. However, blocking the entire family of transcription factors with a c-Jun dominant negative (DN-AP1) had the most profound effect on CFU reduction (**Figure 44B**). CFUs were normalized to vector/scrambled shRNA controls.

When treated with NF- $\kappa$ B inhibitor (BAY), LCs expressing knockdown of any Jun family member showed a significant decrease in CFUs when compared to vehicle treatment. LCs expressing c-Jun shRNA were most susceptible to BAY treatment, but both JunB and JunD knockdown had a significant decrease in colonies as well. The most

**Figure 44. Jun subunits c-Jun, JunB, and JunD contribute to LC survival and resistance to NF- $\kappa$ B inhibition**



LCs were transduced with indicated shRNAs. Knockdown was measured by western blot. **B)** LCs expressing indicated shRNAs were plated in methylcellulose for CFU assay. Results shown are relative to vector-expressing control. **C)** LCs expressing indicated shRNAs were treated with BAY in methylcellulose for CFU assay. Results shown are relative to vehicle-treated cells expressing each shRNA. \* indicates  $p < 0.05$  when compared to control (Vector in **B**, Vehicle in **C**), # indicates  $p < 0.05$  when compared to indicated test groups, as determined by one-way ANOVA with Bonferonni post-hoc test.

effective transgene was DN-AP1, suggesting that the different Jun family members might contribute to certain degree of LC growth and are able to compensate for loss of another **(Figure 44D)**.

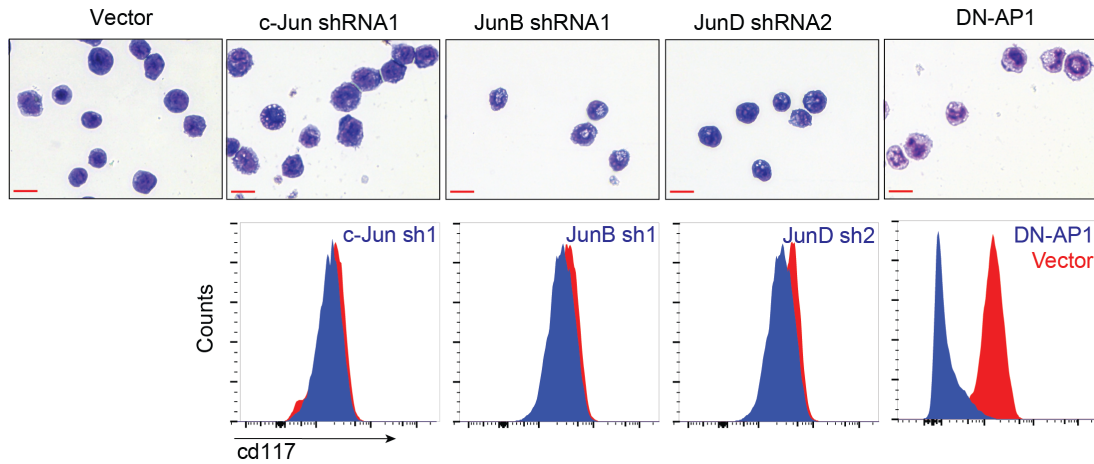
### **Jun family subunits cooperate to prevent differentiation in LCs**

While knocking down each of the Jun family proteins, I observed that functional repression of all family proteins of the AP1 family transcription factors (through transduction with DN-AP1) resulted in a more significant effect and nearly complete differentiation of LCs into granulocytes.

Because functional repression of all AP1 family of transcription factors resulted in differentiation, I wanted to know which family member was responsible for blocking differentiation. To study this in detail, I assayed the differentiation status of LCs with each individual Jun family member knocked down. DN-AP1 drove complete differentiation into granulocytes, as evidenced by multi-nuclear morphology, stain resistance, and cytoplasmic granule formation. Knockdown of each individual Jun family member only induced partial differentiation the LCs, and failed to induce cytoplasmic granule formation when compared to vector-transduced LCs **(Figure 45, top row)**. I did notice a proliferation defect in JunB-deficient cells, but did not investigate further. Vector-transduced LCs were used as negative control.

I then found that knockdown of each Jun family member only resulted in a slight decrease in cd117 surface expression (common stem cell marker for murine hematopoietic cells, SCF receptor). However, functional repression of the entire family resulted in complete loss of cd117 surface receptor, suggesting a more complete

**Figure 45. Jun family subunits cooperate to prevent differentiation in LCs**



LCs were spinoculated with indicated constructs via retroviral transduction and plated in suspension culture for 7 days. After this time, LCs were assayed for morphology by cytopsin followed by Wright-Giemsa stain. Differentiation is characterized by increase in cytoplasm:nucleus ratio, change in counter-stain affinity (light purple vs. dark purple), and presence of granules in the cytoplasm. Red scale bars indicate 20  $\mu\text{m}$ . Additionally, LCs were assayed for cd117 surface marker stain. Results shown are representative of three independent trials.



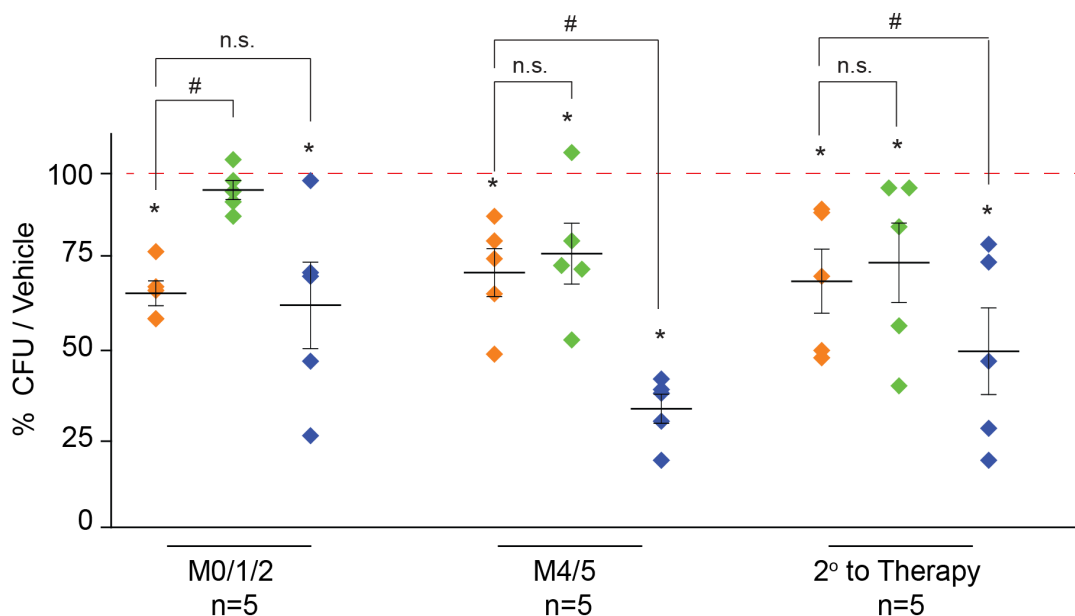
differentiation (**Figure 45, bottom row**). Surface receptor expression was compared to vector control-expressing cells. Addition of vector did not change the cd117 levels compared to untransduced cells. Taken together, these data suggest that multiple members of the Jun family of transcription factors can compensate for each other.

#### **TNF/IL1 inhibition sensitizes AML M4/5 to NF- $\kappa$ B inhibition**

I determined, in murine AML models, that co-inhibition of pro-inflammatory cytokines Tnf and IL1 can resensitize LSCs to NF- $\kappa$ B inhibition *in vitro* and *in vivo*.

I collected tumor samples from newly-diagnosed AML patients' peripheral blood or bone marrow at Loyola University Medical Center and isolated mononuclear cells by Ficoll-Paque gradient centrifugation, and treated in methylcellulose culture with either NF- $\kappa$ B inhibitor (BAY), combination of anti-TNF and anti-IL1 (Etanercept® and Kinaret®), or all three together. I found that M0/1/2 (non-pro-inflammatory cytokine-expressing) were least susceptible, and combined treatment was not any more effective than BAY alone. However, M4/5 AMLs (pro-inflammatory cytokine-expressing) and non-classified AMLs generated secondary to therapy for other cancers were more sensitive to the combination treatment (**Figure 46**). Results shown are normalized to number of colonies grown in vehicle-treated patient samples. Further study is necessary to determine the correlation of the effects of combined treated to TNF and IL1 expression.

**Figure 46. TNF/IL1 inhibition sensitizes AML M4/5 to NF-κB inhibition**



**BAY (100 nM)**

**anti-TNF (Etanercept® 25 ug/mL) + anti-IL1 (Kinaret®, 100 ng/mL)**

**BAY + anti-TNF + anti-IL1**

Primary patient samples were collected and mononuclear cells isolated by Ficoll-Paque gradient centrifugation, then cultured overnight in suspension culture.  $1 \times 10^4$  live cells were plated in methylcellulose containing the indicated treatments and colonies read 14 days later. Values shown are the % CFU normalized to vehicle treatment for each sample. Horizontal bars represent mean  $\pm$  SD. \* indicates  $p < 0.05$  when compared to vehicle treatment, # indicates  $p < 0.05$  when compared to indicated groups, both determined by one-way ANOVA with Bonferonni post-hoc test.

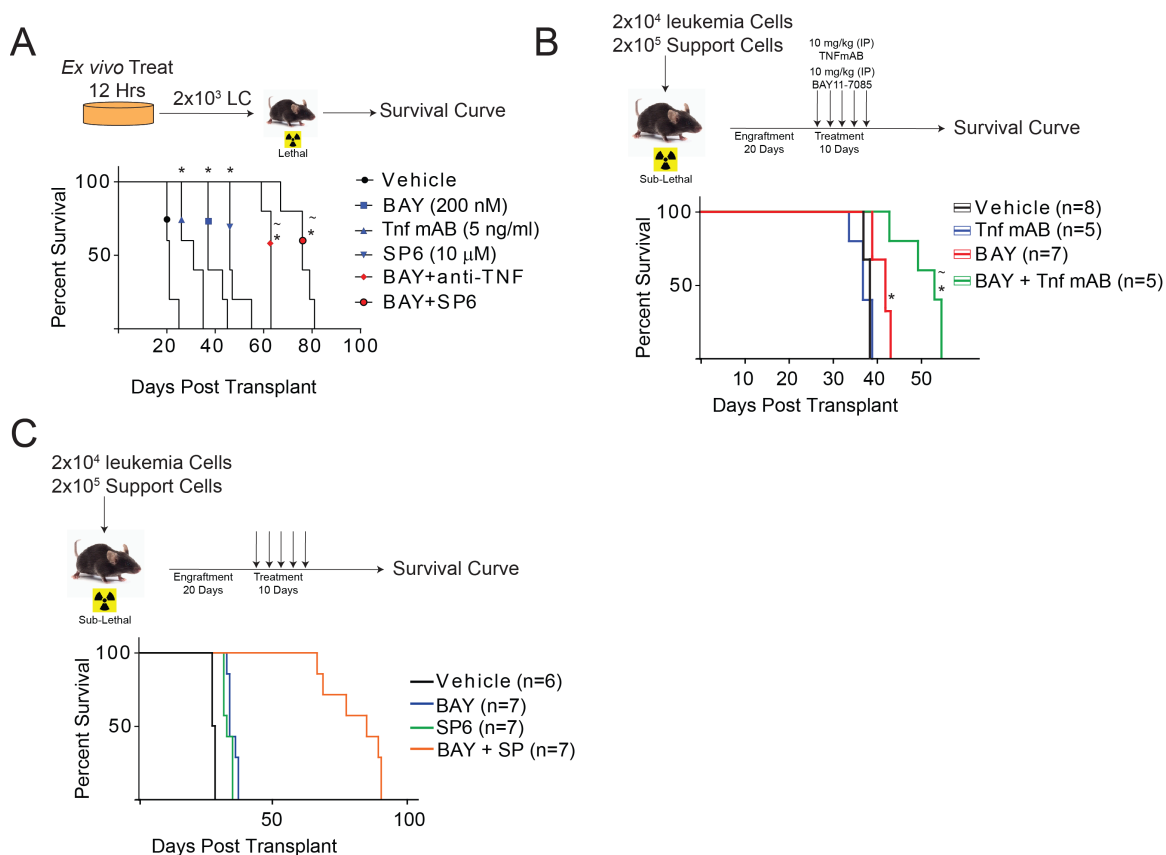
## CHAPTER 5

### DISCUSSION

Increased NF- $\kappa$ B activity has been detected in a variety of types of human cancers including nearly all types of hematopoietic malignancies. However, mutations of the key regulators of NF- $\kappa$ B signaling have been detected only in some B cell lymphomas and multiple myeloma. In these B lymphocytic malignancies, abnormal activation of NF- $\kappa$ B signaling is the result of active mutations of upstream regulatory components which are less dependent on cytokine stimulation. Inhibition of NF- $\kappa$ B signaling in these malignancies has been demonstrated to be an effective treatment option, inducing disease remission and significant improvement in patient survival.<sup>318,319</sup> In AML, there is generally a lack of identified mutations in the components of NF- $\kappa$ B signaling, suggesting enhanced NF- $\kappa$ B activity might be induced by micro-environmental factors. Our studies suggest that TNF, produced by LC, promotes growth of these cells through autocrine stimulation of NF- $\kappa$ B and JNK-AP1 as parallel proliferation/survival signals. Therefore, the anti-leukemic effects of NF- $\kappa$ B inhibition in AML *in vivo* are likely compensated by TNF activating anti-apoptotic genes operating through JNK. Additionally, I showed that paracrine TNF released from LC represses the growth of normal HSPC, suggesting a link to the hematopoietic repression observed in AML patients. Therefore, I speculate that co-inhibition of both TNF-JNK and NF- $\kappa$ B signals

might be a more comprehensive treatment for TNF-expressing AML by synergistically repressing the growth of LC and simultaneously protecting HSPC. If this is the case, then we would be able to eliminate LSCs *in vitro* by inhibiting NF- $\kappa$ B and TNF/JNK. To test this, our collaborators performed an *ex vivo* treatment experiment where they treated LCs with NF- $\kappa$ B inhibitor (BAY), JNK inhibitor (SP6), or Tnf mAB alone or in combination before transplanting the surviving cell into mice. This kind of experiment allows us to know if our treatment is toxic to leukemia initiating stem cells (LSCs). They determined that indeed, blocking NF- $\kappa$ B and Jnk together was able to eliminate stem cells because the tumors xenografted into mice were significantly delayed in forming diseases (**Figure 47A**). Additionally, they xenografted untreated LCs into mice and treated the mice following engraftment. Blocking Tnf alone *in vivo* was able to extend survival of xenograft mice, but not for a profoundly longer period of time (**Figure 47B**). This was most likely due to IL1 acting in parallel, and unaffected by the Tnf blocking antibody treatment. Therefore, we needed to block all downstream signaling by directly inhibiting both NF- $\kappa$ B and Jnk together. To test this, our collaborators treated MLL-AF9 LC-xenografted mice with either BAY or SP6 alone or in combination and found that the combination treatment was very successful in prolonging survival in mice (**Figure 47C**). These results prompted our investigation into Tnf-independent pro-inflammatory signaling in LCs, and ultimately led us to IL1.

Our studies show that IL1 stimulation appears to functionally similar and parallel to Tnf stimulation in LCs. This suggests that these two pathways can be activated and compensate for NF- $\kappa$ B inhibition in LCs. To test this hypothesis, I inactivated both Tnf

**Figure 47. Co-inhibition of NF- $\kappa$ B and Jnk eliminates LSCs *in vitro* and *in vivo***

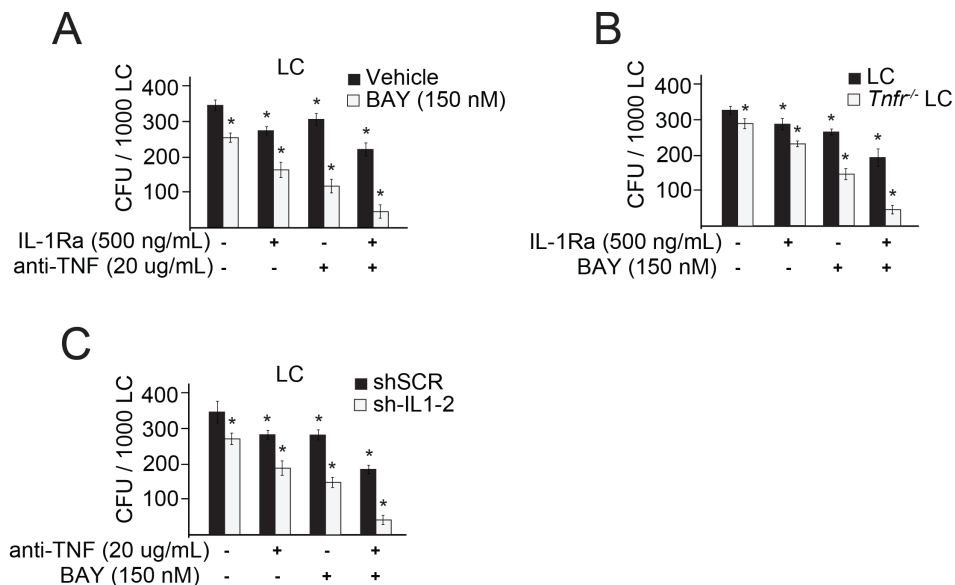
**A)** LCs purified from mice were plated in suspension culture, and treated for 12 hours. Following treatment,  $2 \times 10^3$  live cells were isolated and transplanted back into lethally-irradiated transplant mice. 5 mice were used for each group. **B)**  $2 \times 10^4$  LCs were engrafted for 20 days in sub-lethally irradiated mice. After engraftment, mice were treated with indicated treatments every other day for 10 days. **C)**  $2 \times 10^4$  LCs were engrafted for 20 days in sub-lethally irradiated mice. After engraftment, mice were treated with indicated treatments every other day for 10 days. Mice received BAY (10 mg/kg IP), SP6 (30 mg/kg IP), alone or in combination. Single treatment survival was significantly enhanced when compared to vehicle-treated mice, and combination treatment survival was also significantly enhanced. \* indicates  $p < 0.05$  when compared to vehicle survival by Log-Rank test, ~ indicates  $p < 0.05$  when compared to BAY treated mice by Log-Rank test.

These experiments were performed by collaborators Drs. Jing Li and Xinyu Li at Shanghai Normal University, Shanghai, China

and IL1 signaling through either pharmacologic inhibition of the ligand or genetic inactivation of the receptors through knockout or knockdown, followed by treatment with NF- $\kappa$ B inhibitor (BAY).

Inhibition of Tnf or IL1 using anti-TNF and IL-1Ra in combination with BAY was significantly better at reducing CFUs in LCs than single treatment of any one compound (**Figure 48A**). Vehicle-treated LCs were used as negative controls. I confirmed the specificity of the treatments by treating *WT* and *Tnfr<sup>-/-</sup>* LCs in parallel with IL-1Ra and BAY. The *Tnfr<sup>-/-</sup>* LCs treated with IL-1Ra and BAY reduced CFUs at a similar rate as *WT* LCs treated with anti-TNF, IL-1Ra, and BAY. This suggested that the inhibition and effects of IL-1Ra were specific for IL1, and that anti-TNF was specific for Tnf receptors (**Figure 48B**). Vehicle-treated cells from each genotype were used as negative controls. Finally, I inactivated the IL1 signaling pathway by using an shRNA specific for IL1 receptor. In these cells, inactivation of IL1 receptor via shRNA synergized with anti-TNF and BAY in methylcellulose and significantly reduced the CFU ability of LCs (**Figure 48C**). Vehicle-treated cells expressing scrambled shRNA on a retrovirus were used as negative controls.

I proposed that LSCs secrete pro-inflammatory cytokines in order to promote their own survival (autocrine effect) and to reorganize the niche to support themselves (paracrine effect). This allows LSCs to protect themselves from NF- $\kappa$ B inhibition *in vivo*. If this is indeed the case, then co-inhibition of both pro-inflammatory cytokines Tnf and IL1 can re-sensitize transplanted mice to NF- $\kappa$ B inhibition *in vivo*. To test this my collaborators performed two experiments: an *ex vivo* treatment and transplantation to

**Figure 48. IL1 receptor inactivation sensitizes LCs to NF- $\kappa$ B inhibition**

**A)** *WT* LCs were treated with either IL-1Ra, anti-TNF, or BAY alone or in combination as indicated in methylcellulose. Colonies were read seven days after treatment. **B)** *WT* and *Tnfr*<sup>-/-</sup> LCs were treated with IL-1Ra or BAY alone or in combination, as indicated. Colonies were read seven days after treatment. **C)** LCs expressing scrambled shRNA or IL-1 shRNA were treated with anti-TNF or IL-1Ra alone or in combination in methylcellulose. Colonies were read seven days following treatment. Results shown are mean  $\pm$  SD from three independent trials. \* indicates  $p < 0.05$  when compared to vehicle treated controls as determined by one-way ANOVA with Bonferonni's post-hoc test.

These results were generated Dr. Jing Li at Shanghai Normal University, Shanghai, China.

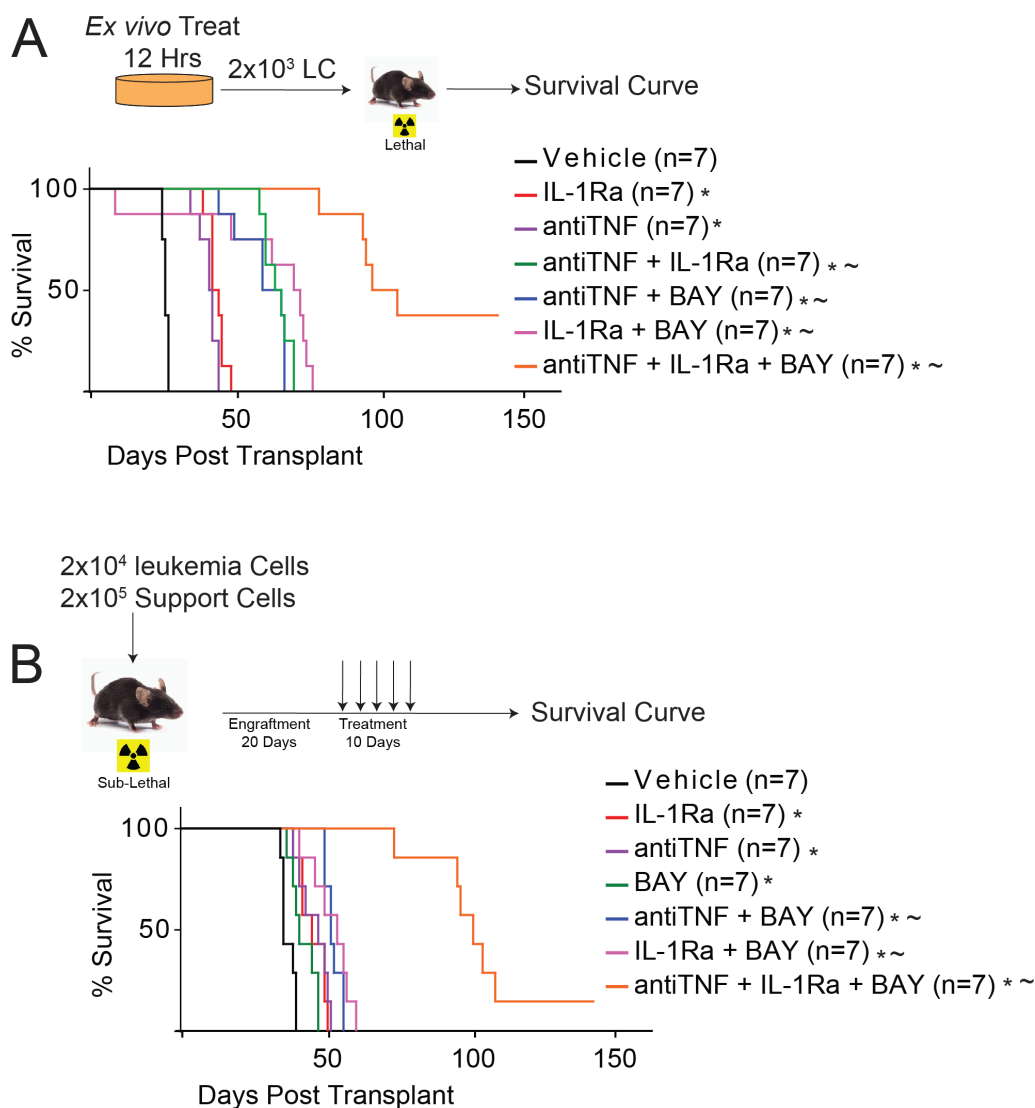
determine if the treatment would eliminate LSCs, and *in vivo* engraftment and treatment to determine if I could return the niche to its original anti-inflammatory state and resensitize LSCs to NF- $\kappa$ B inhibition.

*Ex vivo* treatment of *WT* LCs with anti-TNF, IL-1Ra, or BAY alone or in all combinations revealed that blocking one of any signal eliminated some LSCs in the *in vitro* stage. Combination of any two treatments was better, and almost complete elimination of LSCs was observed when all three signals (Tnf, IL1, and NF- $\kappa$ B) were blocked *ex vivo* (**Figure 49A**). Vehicle treatment studies were used in parallel as negative controls.

*In vivo* engraftment of leukemia followed by ten days of treatments with indicated inhibitors showed similar results. However, in this model, single inhibition of Tnf, IL1, and BAY was not nearly as effective as in the *ex vivo* experiment, suggesting LSCs use both pro-inflammatory cytokines in parallel to reorganize the *in vivo* niche and promote their own survival. However, combination therapy of all three factors (Tnf, IL1, and BAY) was able to triple survival time of transplanted mice, and even induce complete remission in some (**Figure 49B**).

The *in vivo* engraftment and *ex vivo* treat-and-transplant studies suggest that pharmacological inhibition of pro-inflammatory cytokines can resensitize LSCs to NF- $\kappa$ B inhibitor treatment *in vivo*. I confirmed our inhibitor specificity by repeating the experiments in **Figure 49** using *Tnfr<sup>-/-</sup>* LCs instead of *WT* LCs and treating with combinations of IL-1Ra and BAY. I confirmed that the *Tnfr<sup>-/-</sup>* LCs exhibit a significant delay in leukemia formation when compared to *WT* LCs. Interestingly, *ex vivo* treatment



**Figure 49. Inhibition of IL1, Tnf, and NF- $\kappa$ B eliminates LSCs *in vivo***

**A)** *WT* LSCs were treated *ex vivo* with indicated compounds (IL-1Ra: 500 ng/mL, anti-TNF: 20  $\mu$ g/mL, BAY: 150 nM) and live cells were transplanted into recipient mice. **B)** LSCs were transplanted into sub-lethally irradiated mice and allowed to engraft for. Mice were treated every other day (IP injection) with indicated drugs (IL-1Ra: 100mg/kg, anti-TNF: 10 mg/kg, BAY: 10 mg/kg). Results are shown as % survival over time. \* indicates  $p < 0.05$  compared to vehicle treated control, ~ indicates  $p < 0.05$  compared to BAY-treated group as determined by log-rank test.

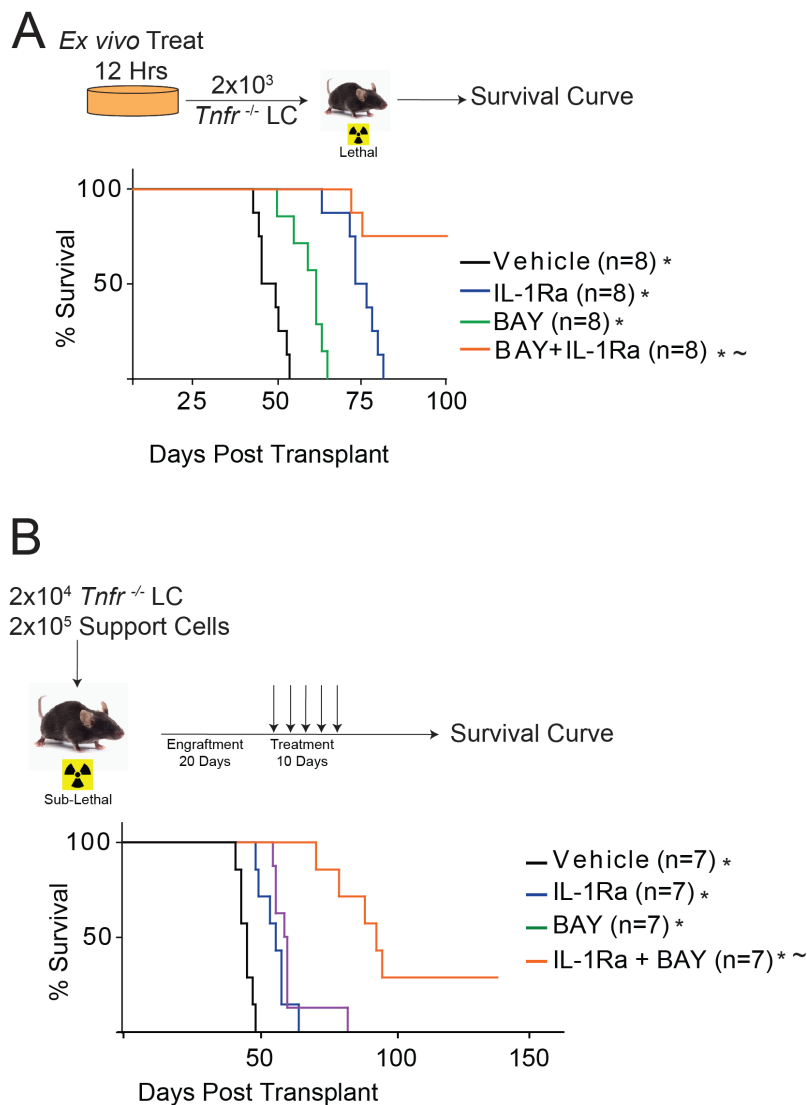
Results obtained by Dr. Jing Li at Shanghai Normal University, Shanghai, China.

of *Tnfr*<sup>-/-</sup> LCs with BAY + IL-1Ra prevented disease development in 6/8 transplant mice (**Figure 50A**), which was better than 2/7 *ex vivo*-transplanted mice with *WT* LCs treated with anti-TNF, IL-1Ra, and BAY (**Figure 50A**). This suggests that LSCs may have a way to utilize Tnf-mediated signaling internally, or in a manner that cannot be blocked by anti-TNF treatment during *in vitro* assays, but could also be due to the efficiency of inhibition of the anti-Tnf antibody. Vehicle treated *Tnfr*<sup>-/-</sup> LCs were used as controls.

*In vivo* engraftment of *Tnfr*<sup>-/-</sup> LCs in sub-lethally irradiated mice treated with IL-1Ra and BAY showed similar rates of survival to mice transplanted with *WT* LCs and treated with anti-TNF/IL-1Ra/BAY (**Figure 50B**). This suggests that combined inhibition of both pro-inflammatory cytokine signaling (Tnf and IL1) can remove the NF- $\kappa$ B independent parallel survival signals and resensitize LSCs to NF- $\kappa$ B inhibition. Most of these mice survived for approximately 100 days, which was comparable to mice transplanted with *WT* LCs receiving the triple-inhibition treatment. Vehicle treated mice transplanted with *Tnfr*<sup>-/-</sup> LCs were used as negative control.

In skin and liver tissues, TNF-induced tissue damage is primarily mediated by JNK signals inducing apoptosis/necroptosis. Normally, TNF-induced damage in these tissues is prevented by NF- $\kappa$ B. NF- $\kappa$ B signal inactivation results in severe inflammation-related tissue damage due to the excessive sensitivity of signal-inactivated cells to TNF-JNK-induced death signaling.<sup>287,289,290,320-324</sup> The tumor-promoting effects of TNF in these tissues are also mediated by JNK signaling through a “complementary proliferation” and/or senescence repressing mechanisms.<sup>287,289,290,320-324</sup> NF- $\kappa$ B functions

**Figure 50. Genetic elimination of Tnf receptors further sensitizes LSCs to IL1 and NF- $\kappa$ B inhibition *in vivo***



**A** *Tnfr*<sup>-/-</sup> LCs were treated *ex vivo* with indicated compounds (IL-1Ra: 500 ng/mL, BAY: 150 nM) and  $2 \times 10^3$  live cells were transplanted into lethally irradiated mice. **B**) *Tnfr*<sup>-/-</sup> LCs were transplanted into sub-lethally irradiated mice and allowed to engraft for 20 days. Mice were treated every other day (IP injection) with indicated drugs (IL-1Ra: 100mg/kg, BAY: 10 mg/kg). Results are shown as % survival over time. \* indicates  $p < 0.05$  compared to vehicle treated control, ~ indicates  $p < 0.05$  compared to BAY-treated group as determined by log-rank test.

Results were obtained Dr. Jing Li at Shanghai Normal University, Shanghai, China.

as a tumor suppressor in these tissues by repressing TNF-JNK activity. Therefore, blocking the TNF-JNK signal can largely repress both tissue damage and tumor generation in NF- $\kappa$ B signal-inactivated animals. These studies provide a strong empirical basis for our combination treatment approach consisting of inhibition of both signals simultaneously. Doing so should not only enhance the anti-leukemic effects but may also reduce the side-effects in other tissues usually induced by NF- $\kappa$ B inhibitors.

The two contradictory activities of TNF-JNK signaling, pro-death and pro-survival/proliferation, in normal/benign tissue cells and malignant cells have been reported in other systems. In Fanconi's anemia, JNK is required for TNF-induced leukemic clonal evolution of *Fancc*-mutant HSPC by inducing apoptosis in mutant cells and stimulating proliferation/survival activities in LC<sup>279</sup>. In *Drosophila*, TNF represses tumor growth of *scribble* (a tumor suppressor)-mutant cells by stimulating a JNK-mediated death signal. However, via JNK, TNF promotes tumor progression and metastasis in *scribble*-mutant cells when either oncoprotein RAS or Notch is expressed.<sup>325</sup> In skin epidermis, via the Tnfr1-Jnk2-Ap1 signaling pathway, TNF promotes epidermal cell proliferation by up-regulating the cell cycle positive regulator CDK4 and down-regulating cell cycle negative regulators such as P16<sup>Ink4a</sup>. Such signals which prevent growth restraint and the induction of senescence are required for epidermal neoplasia induced by NF- $\kappa$ B-inactivation, chemicals, UV irradiation or oncogenic Ras.<sup>289,320,321</sup> In hepatocytes, TNF-JNK induces apoptosis in large numbers of cells and stimulates compensatory proliferation in remaining cells, which are required for chemical or genetic lesion (such as Nemo deletion)-induced hepatocellular carcinoma.<sup>290,322-324</sup>

These studies suggest that a combination of NF- $\kappa$ B and JNK inhibitors might also be useful in the treatment of these cancers. However, in certain types of cancer cells, the pro-survival activities of JNK might be dependent upon NF- $\kappa$ B signaling activity. In such cells, inactivation of the NF- $\kappa$ B signal will convert the pro-survival activity of JNK signaling to pro-death activity.<sup>326</sup> Therefore, to best use such combination treatment in cancer therapy, it is important to distinguish whether or not the pro-survival/proliferative activity of JNK is NF- $\kappa$ B signal dependent.

Evidence also suggests that whether a cell undergoes death or proliferation/survival fate in response to TNF-JNK signaling is determined by the duration of JNK activity. Elongated JNK activity (>2 hours) induces cell death (as shown in HSPC), whereas limited JNK activity (<2 hours) promotes cell proliferation/survival (as shown in LC). I found that the duration of JNK in murine LC is primarily limited by MKP5 which is independent of NF- $\kappa$ B signal. Thus experiments to determine whether expression of MKP5 is elevated in primary human LC will be necessary in future studies. Such studies will allow us to evaluate if inactivation of MKP5 could be a treatment for AML patients.

The inflammatory reaction has been described as a very important component of the tumor environment in many solid tumors. In these tumor tissues, chemokine(s) secreted by malignant cells induce(s) the infiltration of many types of hematopoietic/immune cells (such as macrophages, myeloid-derived suppressor cells, NK cells and T/B-lymphocytes) to the tumor tissues. Most of the tumor-infiltrating hematopoietic/immune cells stimulate a persistent cycle of damage and repair in tumor

tissue to generate a tumor-promoting inflammation by producing tumor supporting cytokines including TNF, IL-1, and IL-6. Thus, such cells and the inflammatory cytokines and signals generated by them have been proposed to be critical targets for anti-tumor therapy.<sup>244,251,256,282,327-332</sup> However, almost all of the previous studies evaluate the anti-tumor activity by targeting these cytokines and their signaling pathways individual. In fact, signals stimulated by these cytokines are not independent. Most of these cytokines share some common signaling pathways and function compensatory. Thus inactivation one cytokine stimulated signaling most case is not sufficient to repress tumor growth due to the compensation of signaling stimulated by other cytokines. Our studies suggest that removing all the tumor promoting cytokines might be necessary for maximally eliminating tumor cells especially for tumor stem cells.

I found that the expression of TNF and IL1 are elevated in many M4/M5 subtypes of AML cells. Early studies suggested that IL1 is an autocrine survival factor in many types of AML cells. Also, significantly elevated IL1 levels in serum of many AML patients and are correlated to poor prognosis. IL1 promotes the colony forming ability of AML cells<sup>333,334</sup> by stimulating the activation of downstream signaling and/or the secretion of hematopoietic cytokines such as GM-CSF in AML cells and stromal cells.<sup>330,331,335</sup> By blocking these pro-inflammatory cytokines, IL1 antagonists, sIL-R or IL-1Ra repress the growth and CFU of AML cells in a dose-dependent fashion.<sup>336-338</sup> Recent studies demonstrated that IL1RAP, a well-known coreceptor of IL-1R1 is highly expressed in many AML patient samples<sup>339,340</sup> and might be involved in LSC self-renewal.<sup>336,337,338,339</sup> In addition, elevated phospho-IRAK1, a hallmark of activated IL1

signaling, was detected in advanced MDS and AML patient samples, which has also been described as an anti-AML target. However, the role of IL-1 in AML development and progression *in vivo* have not been evaluated.

This production of pro-inflammatory cytokines is most likely caused by the driver mutations (fusion genes, chromosomal deletions, or other point mutations) because non-transduced/transformed HSPCs do not produce such cytokines. Once one of these driver mutations is introduced into the HSPCs do pro-inflammatory cytokine production and secretion begin. Additionally, one of the major functions of NF- $\kappa$ B is to drive pro-inflammatory cytokine production. By relying on NF- $\kappa$ B, LSCs need to find a way to at least compensate for the pro-death signals generated by TNF and IL1. My studies show that LSCs not only compensate, but can capitalize on these pro-inflammatory cytokines due to activities of JNK and nuclear substrates from the AP-1 family.

We found that treatment of LCs with a TNF blocker was not sufficient to sensitize these cells to NF- $\kappa$ B inhibition. This could be due to two different mechanisms: (1) there are additional pro-inflammatory cytokines being produced that can function parallel to TNF, and (2) TNF and its receptor are synthesized by the same cells, allowing for both receptor and ligand to be present inside the same sorting vesicle following synthesis by the Golgi. I was able to account for the first option in this dissertation by screening for additional pro-inflammatory cytokines. However, the second option is also possible. This may be why the anti-TNF mAB treatment did not work *in vitro* when the cells are sufficiently spread out enough to prevent paracrine signaling. By expressing both TNF and its receptor on sorting vesicles as they travel from Golgi to the cell surface, LSCs can

benefit from TNF signaling without having to utilize anything at the cell surface. This type of intracellular signaling was shown in 2005 using HEK 293 cells transduced with different mutant versions of TNF receptors.<sup>341</sup>

Similar to TNF, IL-1 stimulates the activation of JNK and NF- $\kappa$ B signaling. A balance of JNK and NF- $\kappa$ B signaling is absolutely required for the proper response of normal tissue cells to pro-inflammatory cytokine-stimulated reactions. Inactivation of NF- $\kappa$ B signaling results in cell death-related damage and compensatory proliferation in most tissues due to the excessive response of tissue cells to inflammatory cytokine-stimulated JNK signaling. JNK induces a pro-apoptotic activity in most normal tissues which can be converted into pro-survival/proliferation signaling by oncogenic mutation(s), thereby promoting tumor development and progression. I found that most tumors from all AML subtypes (M0-M7) express some degree of IL1, M4 and M5 subtypes of AML are particularly highly pro-inflammatory as shown by increased IL1 and TNF expression. Here, I report that combined inhibition of JNK and NF- $\kappa$ B signaling might be a better treatment for TNF-expressing AML, but I further demonstrated that such therapy might also represent an useful therapeutic strategy for IL1 expressing AML. Although our *in vitro* study suggested that the TNF/IL1 low-expressing AML cells respond less to JNK and NF- $\kappa$ B inhibitor treatment, I predict that our combined inhibitor-treatment might be also benefit to these AML patients when combined with canonical chemotherapies. I found many TNF/IL1 low-expressing AML samples express Toll-like receptors (TLRs) including TLR2, 4 and 5 (Cannova et al unpublished data). The TLRs in AML cells can be activated by pathogen-associated molecular patterns (PAMPs)



during bacterial/viral infections and damage-associated molecular patterns (DAMPs) released by the dead cells during chemotherapy. Activated TLRs can stimulate JNK/NF- $\kappa$ B directly through MyD88-mediated pathway and/or indirectly by stimulating the production of inflammatory cytokines. Additionally, TNF and IL-1 can be produced by bone marrow niche cells and other tissue cells especially during chemotherapy or irradiation therapy or during AML LSC reorganization of the hematopoietic niche. This pro-inflammatory niche environment plays a critical role in leukemia drug-resistance by protecting LSCs from chemotherapy. Whether or not TNF/IL1 induced JNK/NF- $\kappa$ B signaling contribute to drug-resistance development need to be further verified.

In this study, I also identify that multiple Jun family members can compensate for loss of others. This is in contrast to the classic belief that c-Jun functions primarily as a tumor promoter while JunB functions as a tumor suppressor. Knockdown of individual family members of the AP1 transcription factors (c-Jun, JunB, and JunD) can each induce a reduction in CFU ability in LCs and can sensitize such cells to NF- $\kappa$ B inhibitor treatment. However, the best response is observed when the entire family is blocked through transduction with DN-AP1, which can bind with Fos and other AP1 family members, be phosphorylated by Jnk, but cannot bind to DNA because it lacks the DNA-binding domain. Only DN-AP1 can completely differentiate and sensitize LCs to NF- $\kappa$ B inhibitor, suggesting that multiple family members of Jun can compensate for loss of any other. This also suggests that JunB and JunD can function as tumor promoters in AML.

Multiple NF- $\kappa$ B inhibitors such as proteasome inhibitor Bortezomib and natural compound Parthenolide have been successful used in clinical for Multiple Myeloma

therapy and currently under evaluation in clinical trials for AML treatment in combination with other canonical chemotherapy drugs.<sup>342,343</sup> Unfortunately, clinical grade JNK inhibitors are not available. However, I found that in these TNF/IL-1 expressing AML, inactivation of JNK signaling can be achieved by co-inhibition of both IL1 and TNF. Our studies suggest this will have a dual role: (1) it will represses the growth of and significantly sensitize clonogenic leukemic progenitor cells and leukemogenic LSCs to NF- $\kappa$ B inhibitor treatment, and (2) protect healthy HSPCs from chemotherapeutic intervention and assist them in recreating a healthy niche microenvironment. Given that both TNF and IL-1 antagonists are commonly used in clinical for rheumatoid arthritis and autoimmune diseases and are confirmed safe, I speculate that I might able to use these FDA approved TNF/IL1 antagonists and NF- $\kappa$ B inhibitors to evaluate our novel treatment approach in AML patients.

In addition, oral and intestinal mucositis are severe pathologic conditions afflicting most patients treated with standard chemotherapy or radiation therapy.<sup>344-348</sup> TNF and IL1-stimulated inflammation has been determined as the perpetrators for such side effects. In addition, TNF/IL-1 might be also contribute to the leukemia-promoting pro-inflammatory microenvironment by inducing MRD survival and drug-resistance.<sup>349</sup> In AML patients, combined chemotherapy together with TNF/IL-1 antagonizes and NF- $\kappa$ B inhibitor might be benefit leukemia patients by preventing the severe intestinal side effects and might be also help to eliminated MRD.

### **Future Directions**

In this dissertation, I have provided evidence and support for treating AML with anti-inflammatory drugs (anti-TNF, anti-IL-1) in combination with NF- $\kappa$ B inhibition. The LSC niche is more critical for maintaining leukemia tumors during chemotherapies than for leukemia development, because LSCs have been isolated in niche-independent environments including the peripheral blood. For future studies, I would like to focus on how the LSC niche provides protection to LSCs by sheltering them during chemotherapy. To test this, I would label LCs and engraft these cells into sublethally-irradiated recipient mice, treat with chemotherapeutic agents, and perform experiments isolating factors of the LSC niche that support these cells. I know that a subset of cells will remain following therapy and reconstitute the tumor (MRD). These experiments will determine which factors are required for supporting MRD via the niche, and will focus on cellular components of reorganization like CAFs, TAMs, and vascular endothelia-supporting AML cells.

Intracellular signaling of pro-inflammatory cytokines is something I find very interesting. As a continuation of this project, I would propose to look at the ability for LCs from different FAB subtypes of AML to signal in this manner. By blocking intracellular signaling, we may find a new way of targeting LSCs.

Finally, all of the targets I identified as good anti-LSC therapies in this dissertation are currently drugable using FDA-approved medications. These drugs (Kinaret®, Etanercept®, and Velcade®) are available, well-tolerated by patients, and have approved dose levels and schedules. In the future, I would like to propose a clinical

trial and recruiting patients for treatment with TNF/IL1 blockers in combination with NF- $\kappa$ B inhibition for M4/5 AML.

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## VITA

Andrew Volk was born in Des Plaines, IL on February 28<sup>th</sup>, 1986 to Laura and Raymond Volk. He received a Bachelor of Science with a major in Medical Biology and minor in Music Performance from Baylor University in Waco, Texas in August of 2009. As an undergraduate student, Andrew began his medical studies in the clinic as an Emergency Medical Technician/ER Technician at Hillcrest Hospital, and at the bench studying Methicillin-resistant Staphylococcus aureus (MRSA) with Dr. Tamarah Adair, Ph.D. In addition to his medical studies, Andrew maintained several musical appointments during his undergraduate studies, playing handbells with the Baylor Bronze and Council Oak Bells, and clarinet/bass clarinet with Waco Symphony.

In July 2009, Andrew joined the Molecular Biology Ph.D. program at Loyola University Chicago, and later the laboratory of Dr. Jiwang Zhang, M.D., Ph.D., where he studied how hematopoietic and leukemic stem cells interact with their niche, and how this contributes to normal tissue homeostasis and disease. While a doctoral candidate, Andrew accomplished many milestones including receiving the T32 Institutional Research Training award in Experimental Immunology, being awarded an F31 Pre-doctoral Fellowship from the National Institutes of Health's National Cancer Institute (NIH-NCI), receiving the Graduate Research Mentoring Program Fellowship and a nomination for the Langerbeck Award for Excellence in Undergraduate Research Mentoring, and married a wonderful woman (his wife, Stephanie). In addition to

fellowships and awards, Andrew has published his research in several top-tier journals, presented his work at national and international conferences, and led science communication skills seminars at Loyola.

After completing his Ph.D., Andrew will continue his scientific training as a postdoctoral fellow studying hematopoiesis and disease development in Down Syndrome with Dr. John Crispino, Ph.D., at Northwestern University in January.

