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Divergent Pro-Leukemic Effects of MYD88 and TICAM-1/TRIF Mediated Toll-Like Receptor Signaling

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DIVERGENT PRO-LEUKEMIC EFFECTS OF MYD88 AND TICAM1/TRIF MEDIATED TOLL-LIKE RECEPTOR SIGNALING

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Far and away the best prize that life has to offer is the chance to work hard at work worth doing.

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LIST OF ABBREVIATIONS

4OHT	4-hydroxytamoxifen
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
AMoL	Acute Monocytic Leukemia
AOM	Azoxymethane
APL	Acute Promyelocytic Leukemia
ATRA	All-*trans* Retinoic Acid
BSA	Bovine Serum Albumin
CD11b	Cluster of Differentiation Antigen 11-Like Family Member B
CD14	Cluster of Differentiaiton Antigen 14
CD117	Cluster of Differentiation Antigen 117
SCFR	Stem Cell Factor Receptor
CFA	Colony Forming Ability/Assay
CFU	Colony Forming Unit
CML	Chronic Myeloid Leukemia
CreERT2	Fusion of Cre and Estrogen Receptor Activated by Tamoxifen
DNA	Deoxyribonucleic Acid
dsRNA	Double-stranded RNA
FACS	Fluoresence-Activated Cell Sorting

FBS	Fetal Bovine Serum
GM-CSF	Granulocyte/Monocyte Colony Stimulating Factor
IFNα	Interferon Alpha
IFNβ	Interferon Beta
IL-1	Interleukin 1
IL-3	Interleukin 3
IL-6	Interleukin 6
IL-1R	Interleukin 1 Receptor
IRAK1	Interleukin 1 Receptor Associated Kinase 1
IRAK2	Interleukin 1 Receptor Associated Kinase 2
IRAK4	Interleukin 1 Receptor Associated Kinase 4
IRF3	Interferon Regulatory Factor 3
ITGAM	Integrin Subunit Alpha M
JNK	Jun N-Terminal Kinase
LPS	Lipopolysaccharide
KD	Knockdown
KMT2A	Lysine Methyltransferase 2A
MAPK8	Mitogen-Activated Protein Kinase 8
MFI	Median Fluorescence Intensity
MLKL	Mixed Lineage Kinase Domain Like Pseudokinase
MLL1	Mixed Lineage Leukemia 1
MLL-AF9	Mixed Lineage Leukemia and ALL1-Fused Gene from Chromosome 9 fusion protein

MLLT3	Mixed Lineage Leukemia Translocated to Chromosome 3 Protein
MM6	Mono-Mac 6 Cell Line
MYD88	Myeloid Differentiation Primary Response 88
Myd88$^{\Delta/\Delta}$	Myd88 Deletion with Cre or CreERT2
Myd88$^{-/-}$	Myd88 null, derived from Myd88 knockout mice
Myd88$^{fl/fl}$	Myd88 gene intact, flanked by LoxP sites
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
PAM3CSK4	Synthetic Triacylated Lipoprotein - N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine
PRR	Pattern Recognition Receptor
RHIM	RIP Homotypic Interaction Motif
RIPK1	Receptor Interacting Serine/Threonine Kinase 1
RIPK3	Receptor Interacting Serine/Threonine Kinase 3
RPM	Rotations Per Minute
RPMI	Roswell Park Memorial Institute Medium
RNA	Ribonucleic Acid
SCF	Stem Cell Factor
shRNA	Short Hairpin Ribonucleic Acid
TANK	TRAF-Associated NF-κB Activator
TBK1	TANK-Binding Kinase 1
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline with Tween 20
TLR	Toll-like Receptor

TLR1	Toll-like Receptor 1
TLR2	Toll-like Receptor 2
TLR3	Toll-like Receptor 3
TLR4	Toll-like Receptor 4
TLR5	Toll-like Receptor 5
TLR6	Toll-like Receptor 6
TLR7	Toll-like Receptor 7
TLR8	Toll-like Receptor 8
TLR9	Toll-like Receptor 9
TLR10	Toll-like Receptor 10
TICAM1	Toll-like Receptor Adaptor Molecule 1
TIR	Toll/Interleukin 1 Receptor Homology Domain
TNFα	Tumor Necrosis Factor Alpha
TRAF3	TNF Receptor Associated Factor 3
TRAF6	TNF Receptor Associated Factor 6
TRIF	TIR Domain Containing Adaptor Inducing Interferon-Beta
UBE20	Ubiquitin Conjugating Enzyme E2 O

ABSTRACT

The goal of this study is to determine the role of Toll-like receptor (TLR) signaling in acute myeloid leukemia (AML). AML is a hematopoietic malignancy that predominately affects the elderly. The median age of AML diagnosis is 67 years old. The standard of care of AML is treatment with cytotoxic chemotherapy, which elderly patients are often unable to tolerate. To identify novel pathways as potential targets for treatment, a microarray on AML primary patient samples was performed to determine the expression of key inflammatory genes. This microarray, and other published datasets, showed that TLR are overexpressed in myelomonocytic (M4) and monocytic (M5) AML subtypes. TLRs are components of the innate immune system that sense and respond to inflammatory stimuli from infections or tissue injury. TLR signaling is mediated by one of two adaptor proteins, myeloid differentiation factor 88 (MYD88) or Toll/IL1 receptor (TIR) containing adaptor molecule 1 (TICAM-1). Inhibition of kinases downstream from these receptors inhibits MLL-AF9 leukemia cell growth. To investigate TLR signaling in the setting of AML, two models were employed. First, inducible genetic deletion of the *Myd88* gene in MLL-AF9 leukemia cells was applied. Myd88 was determined to be required for leukemia cell stemness, colony forming potential, proliferation, resistance to chemotherapy, and leukemogenesis *in vivo*. Next TICAM-1 expression was suppressed by shRNA in human and murine models and found to have similar effects, protecting leukemia cell stemness, proliferation, colony forming potential, and leukemogenesis *in vivo*. Knockdown of downstream

kinases, IL-1 receptor-associated kinase 1 for Myd88, and Tank-binding kinase for Ticam-1, failed to recapitulate these phenotypes. While the exact mechanism of Myd88 pro-leukemic function remains undetermined, TICAM-1 was shown to protect protein levels of receptor-interacting protein kinase 1 (RIPK1) and RIPK3, and TICAM-1 knockdown in human ML-2 or murine MLL-AF9 cells resulted in corresponding loss of RIPK1 and RIPK3. Together, these data indicate that TLR-associated adaptor proteins participate in pro-leukemia signaling pathways independently from TLR-associated kinases. These findings have implications for the treatment of AML and suggest that targeted disruption or inhibitions of MYD88 or TICAM-1-dependent signaling pathways might have clinical benefit.

CHAPTER ONE

INTRODUCTION AND BACKGROUND

Overview

Acute myeloid leukemia (AML) is a malignancy of the blood derived from hematopoietic progenitor cells. AML is known clinically for its heightened incidence in the elderly, diverse array of mutation events and fusion proteins, poor outcomes, and few targeted therapies. Often, AML patients of advanced age cannot tolerate doses of cytotoxic chemotherapy required to eradicate AML cells and induce remissions. As a result, overall five-year survival is 26% and falls even lower with advanced age. Compared to many other malignancies, the survival of AML has improved relatively little since modern cytotoxic chemotherapy was implemented clinically three decades ago. AML is divided into a range of subtypes either by morphological presentation or recurring genetic mutations. Depending on the subtype, it will confer a range sensitivity to different treatments, leading to positive or negative prognoses. Because of the wide range of recurring and co-occurring mutations in AML, few targeted therapies have been shown to be clinically effective. Toll-like receptors (TLRs) and interleukin 1 (IL-1) receptors (IL-1R) are transmembrane receptors for conserved molecular patterns associated with inflammation and innate immunity. Leukemia cells of acute myelomonocytic (M4) and monocytic (M5) subtypes have been shown to be protected from cell death by IL-1 in both preclinical models and primary patient samples.[1]

Additionally, AML cells of all subtypes are known to express Toll-like receptors and their associated signaling components, but monocytic and myelomonocytic subtypes demonstrate the highest expression of these pathways. I hypothesized that key TLR signaling pathways are required for leukemia development and progression. In this dissertation, the contribution of TLR signaling to AML pathogenesis from two key TLR adaptor proteins, myeloid differentiation primary response 88 (MYD88) and TLR adaptor molecule 1 (TICAM-1) will be examined.

Innate Immunity

During infection, the presence of bacteria, viruses, or tissue damage in multicellular organisms is first detected by the innate immune system. The innate immune system serves as a first line of defense against pathogens and endogenously derived tissue-damage factors and is required to initiate a signaling and transcriptional response to them. Innate immunity is mediated by pattern recognition receptors (PRRs).[2] These PRRs are expressed in most tissues throughout an organism, and primarily act in epithelial and endothelial cells to detect tissue damage or infection and initiate an inflammatory response. This generalized inflammatory response to PRR stimuli is then mediated by tissue-resident immune cells, such as macrophages, natural killer cells, and histiocytes, and circulating immune cells, such as monocytes and neutrophils, among others. Both local tissue cells and these immune cells are also known to express distinct PRR repertoires. PRRs include several groups of receptors that are known to recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Subgroups of PRRs include Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors, and retinoic acid inducible gene (RIG)-1-like receptors.[2] Innate immune receptors induce an inflammatory response to these molecular patterns that stimulate inflammatory responses mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

and other pro-inflammatory pathways. This response leads to the production of pro-inflammatory cytokines, IL-1, interleukin 6 (IL-6), tumor necrosis factor α (TNFα), and other genes. In the case of viral stimuli, type I α and β interferons are produced through NF-κB-independent signaling.[3]

These cytokine signals activate the immune response, leading to generalized, local inflammation and causing immune cells to home to the site of infection and/or tissue damage. This inflammatory response, when appropriately regulated, is vital for the recruitment of immune cells to the site of tissue infection or injury, clearing of pathogens and tissue damage, initiation of healing processes, mobilization and expansion of myeloid precursors from the bone marrow niche, and initiation of the adaptive immune response. When this response is dysregulated or chronically stimulated, it is known to contribute to the pathogenesis of various disease processes, including autoimmunity, immunodeficiency, hematopoietic dysfunction, and malignancy.[4-7] The TLRs are the best studied family of PRRs. Their mechanisms of activation, cognate ligands, regulation, and roles in disease are relatively well understood. All TLRs are required for competent innate immune function, and specific loss, mutation, or even single nucleotide polymorphism of individual TLRs or downstream mediators results in corresponding specific immune deficiencies.[8-10]

Toll-like Receptor Signaling

Toll-like receptors (TLRs) comprise a widely studied subset of pattern recognition receptors (PRRs). TLRs act by recognizing conserved molecular patterns at the cell surface or within endosomes. These molecular patterns are not specific to individual species of pathogens, but conserved across a variety of bacterial and viral infections. TLRs sense PAMPs in the setting of an infection as well as DAMPs arising from injury or chronic inflammatory states. All TLRs

have a single transmembrane domain, an N-terminal extracellular domain, and a C-terminal intracellular domain. The TLR extracellular domain interacts with PAMPs and DAMPs to initiate TLR activation, downstream signaling, and subsequent cytokine secretion in response to the infectious or damage-associated event. Toll-like receptors were the first subset of PRRs to be associated with this function.

The *Toll* gene was first identified in the *Drosophila melanogaster* model organism and shown to be required for dorso-ventral polarity in the developing fly embryo.[11] Several Toll homologs were later identified in the genomes of humans, mice, and other metazoan organisms because of their similar intracellular domains and were termed Toll-like receptors (TLRs).[12] Several TLR subfamilies are conserved across all metazoan organism genomes surveyed to date. Genomes of higher order organisms often encode homologous TLRs, but rarely have identical TLR repertoires.[13] For example, mice are known to express twelve individual TLRs, while humans have ten known TLRs. TLR1-TLR9 are expressed in both mice and humans and retain homologous functions. Despite this, mice lack the equivalent for human *TLR10*, and humans lack the equivalent genes for murine *Tlr11*, *Tlr12*, and *Tlr13*. Recent phylogenetic and structural studies have shown that significant variation of TLRs exists across vertebrate species.[13] Structural analysis of the extracellular domain has determined that TLRs fall into one of several ancestral families. The highly conserved presence and structure of extracellular domains of these TLRs across nearly all species indicates their essential function.

Though the precise ligands of TLRs were not known when TLR genes were being first identified, they were hypothesized to function as receptors, with distinct cognate ligands. The TLR extracellular domain possesses 19-25 leucine rich repeats (LRRs), which form horseshoe-like concave structures that determine ligand specificity.[14, 15] Despite lacking specific ligands for

the extracellular LRR domains, the TLR intracellular domain was shown to be homologous to that of interleukin-1 (IL-1) receptor (IL-1R).[14] The shared TLR and IL-1R intracellular domain was termed the Toll/IL-1R (TIR) homology domain.[16] This TIR domain is shared on adaptor proteins and required for the recruitment of myeloid differentiation primary response 88 (MYD88) to both IL-1R and TLRs, except for TLR3.[17-19] Because of this shared TIR domain and MYD88 recruitment, TLR and IL-1R signaling overlap to a great degree. MYD88 is an adaptor protein comprised of a C-terminal TIR domain, which allows interaction with TIR domains on TLRs and IL-1R intracellular domains via a homotypic interaction. In this manner, the downstream signaling mediators of IL-1R and TLRs were built and studied in parallel.

Toll-like Receptor Ligands.

Pathogen-associated molecular patterns. As the field of TLR study expanded in the 1990s, these receptors remained without identified ligands. Even before the discovery of TLRs, many immunologists hypothesized that an unidentified mechanism of 'innate immunity' mediated the initial inflammatory phase of infections and injuries. For some, TLRs were poised to be key mediators of the early inflammatory response, if their ligands were indeed associated with inflammation.

The first identified TLR ligand identified was lipopolysaccharide (LPS), the PAMP ligand for TLR4.[12, 20-22] Also known as endotoxin, LPS is found on the outer membrane of Gram-negative bacteria. LPS is characterized by an O-antigen, an outer and inner core domain, and a lipid component, resulting in a structure that is highly specific for Gram-negative bacterial infections. In infections with Gram-negative bacteria, TLR4 is required for LPS detection and activation of the immune system in response to it. After many decades of hypothesizing about the nature of 'innate immunity', the determination that LPS is one of the cognate ligands of TLR4 was the

first empirical finding to validate this hypothesis. This finding bound the field of TLR study to that of innate immunity and spurred the rapid identification of other TLR ligands.

Currently, TLRs are well-characterized, and each TLR has multiple PAMP and DAMP ligands (Figure 1). TLR2 forms heterodimers with either TLR1 or TLR6, resulting in ligand specificity to triacylated and diacylated lipopeptides, respectively.[23, 24] These PAMPs are associated with mycobacterial infections, such as *M. tuberculosis* and *M. leprae*, and functional TLR2 is required for an adequate immune response.[21,25] The ligand of TLR5 is bacterial flagellin.[26, 27] The TLR3 PAMP ligand is double-stranded RNA (dsRNA).[28, 29] The TLR7/8 heterodimer ligand is single-stranded RNA (ssRNA).[30] Lastly, the PAMP ligand of TLR9 is unmethylated DNA CpG fragments present in bacteria and viruses, but methylated in eukaryotes.[31] Each of these PAMPs are associated with bacterial or viral infections, and their localization and downstream signaling reflects these functions. While other TLRs sense bacterial components at the cell surface, TLR3, TLR7, TLR8, and TLR9, localize to endosomal compartments to sense viral PAMPs.[32, 33] In contrast to other TLRs, TLR10 exerts anti-inflammatory effects and has no known ligand.[34]

Under unstimulated conditions, TLRs exist either as dimers in a 'relaxed' conformation or as monomers. With the introduction of known stimuli, TLRs that exist as monomers assemble into dimers, and, once dimerized, undergo a conformational change.[27, 28, 35, 36] This conformational change extends to the intracellular domains and allows the TLRs to recruit MYD88 and TICAM-1 directly or by intermediate adaptor proteins. These events are required to transduce the extracellular signal of TLRs to the intracellular signaling compartment to activate the proinflammatory and/or interferon response.

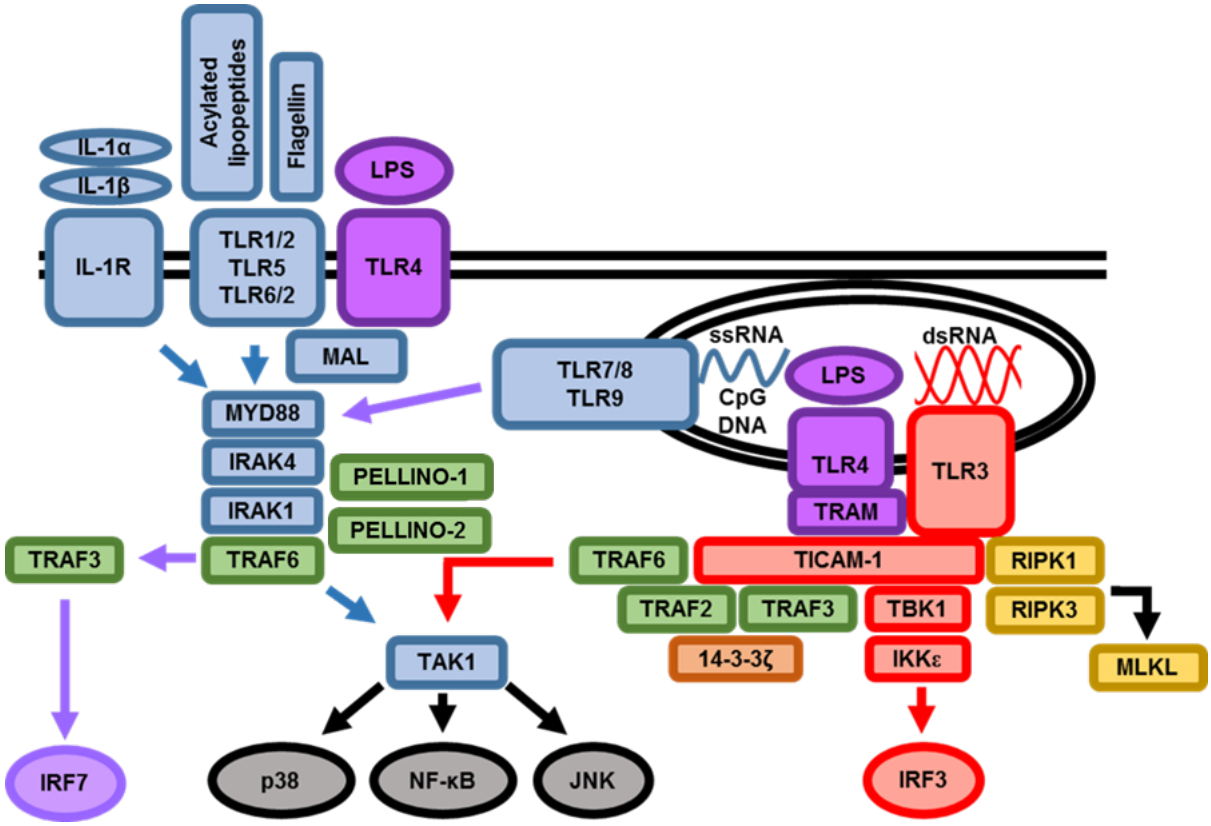


Figure 1. Toll-like Receptor Signaling. Toll-like receptor (TLR) signaling is composed of several cell surface and endosomal transmembrane receptors that function to detect inflammatory and infectious molecular patterns and induce a downstream transcriptional response through overlapping downstream signaling pathways. Endosomal TLRs and TICAM-1 mediated TLRs may induce interferon regulatory factors (IRFs) to transcribe interferon. MYD88-dependent and TICAM-1-dependent signaling result in the activation of p38, NF-κB, and JNK to generate a stress response and proinflammatory cytokines.

Damage-associated molecular pattern ligands. TLRs have been shown to recognize endogenous DAMP ligands as well as the aforementioned PAMPs. DAMPs are markers of tissue damage or inflammatory cell death. These DAMPS act on TLRs to induce an inflammatory response, which may aggravate the initial injury. For example, TLRs are well characterized as mediators of inflammation in ischemia/reperfusion injuries following DAMP release in myocardial infarctions and other ischemic events.[37] Also, TLR-DAMP stimulation occurs in a range of chronic inflammatory conditions and malignancies. In addition to PAMPs, most TLRs also recognize DAMPs. Unlike PAMP ligands, multiple DAMP ligands may stimulate individual

TLRs. For example, TLR4 is robustly stimulated by LPS, but it is also stimulated by several S100 proteins and HMGB1, both of which can indicate localized tissue damage, inflammatory cell death, or malignancy.[38, 39] Additionally, TLR4 may be stimulated by several distinct heat shock proteins (HSPs), several components of the extracellular matrix, and many others.[40, 41] TLR1-TLR2 may also be stimulated by extracellular matrix proteins, HMGB1, and HSPs.[38, 42] Lastly, nucleic acid-specific TLR9 may also detect HMGB1 and human cardiac myosin, so they are not necessarily limited to the surveillance of nucleic acids or similarly structured molecules.[43, 44] Thus, the innate immunity of TLRs may be stimulated by a range of factors, often in the absence of infection and with pathogenic consequences.

Interleukin 1 inflammasome-mediated activation and receptor stimulation. Interleukin 1 is a well-established inflammatory cytokine that acts as one of two subunits, either IL-1α or IL-1β. The generation of IL-1α and IL-1β is mediated by the inflammasome, which holds pro-IL1α/β and pro-IL18 in a primed state so that it may be rapidly cleaved and activated by caspase 1 in the presence of TLR stimulation and a second, concurrent inflammatory signal. In unstimulated conditions, caspase 1 exists in a primed pro-caspase 1 form, which is then cleaved to generate active caspase 1, which cleaves and activates IL-1α and IL-1β.[45] This second signal may consist of potassium ion efflux or reactive oxygen species, as well as other stimuli of infectious or sterile origin. Once sufficient signaling stimuli have been met, the inflammasome is activated, resulting in the cleavage of IL-1α or IL-1β into their active forms and their subsequent secretion. Once secreted, IL-1α or IL-1β are detected by nearby cells bearing the IL-1R, which requires the co-receptor IL-1R accessory protein (IL-1RAP), which forms a dimer with IL-1R and mediates the recruitment of intracellular receptor associated complex factors. Inflammasome activation and consequent IL-1α/β production is carefully regulated. A key mediator of TLR

signaling, TGF-β activated kinase-1 (TAK1), exerts a suppressive effect on the inflammasome. Genetic deletion of TAK1 results in overactivation of the inflammasome and persistence of a chronic inflammatory state.[46]

MYD88-Dependent Signaling.

Myddosome formation and IRAK activation. Downstream TLR signaling is mediated by adaptor proteins such as MYD88, which is required for signaling downstream of all TLRs except for TLR3. MYD88 may interact with TLRs directly, or indirectly via interaction with Myd88-adaptor-like protein (MAL), a membrane-associated adaptor.[47] Upon TLR stimulation, the intracellular TIR domain undergoes a conformational change required for MAL/MYD88 homotypic TIR interaction. MYD88 also possess an N-terminal death domain (DD, Figure 2), which recruits IL-1R-associated kinase 4 (IRAK4). IRAK4 is a serine/threonine kinase that is activated by phosphorylation of two of three required phosphorylation sites within its activation loop: Thr342, Thr345, and Ser346. Discovered as a homolog to the *Drosophila Pelle* gene, IRAK4 is required for downstream signaling for both IL-1R and MYD88-dependent TLR signaling.[48, 49] Upon recruitment to MYD88, IRAK4 undergoes an oligomerization-induced dimerization and trans-autophosphorylation.[50] IRAK4 then recruits IRAK1 as a substrate and phosphorylates it. This results in a cytosolic, membrane-associated receptor complex called the Myddosome. Crystallization studies indicate that each Myddosome is composed of six MYD88 proteins, four IRAK4 proteins, and four IRAK1 proteins.[51] Phosphorylation of IRAK1 leads to partial IRAK1 activation and enables IRAK1 to phosphorylate and activate Pellino-1, an E3 ubiquitin ligase. Signaling downstream of IRAK1 requires K63-linked ubiquitin chain formation mediated by E3 ubiquitin ligases factor α (TNFα) receptor associated factor 6 (TRAF6), Pellino-1, or Pellino-2.[52] These K63-linked ubiquitin chains form on IRAK1 or TRAF6. K63-linked

ubiquitin chains recruit downstream mediators and are required to induce activation of downstream TAK1-mediated signaling.

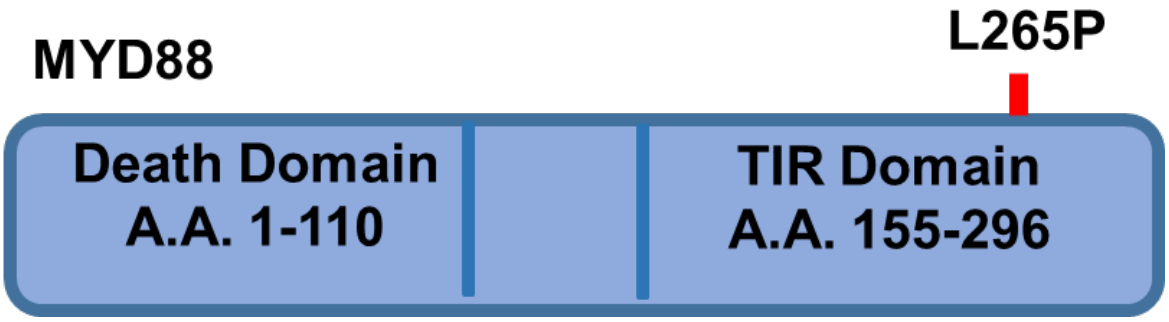


Figure 2. MYD88 Adaptor Protein Domains. MYD88 is known to have an N-terminal death domain (DD) and a C-terminal Toll/IL-1 receptor (TIR) domain. The intermediate domain is not known to have a conserved structure or function. The TIR domain is recurrently mutated in B cell lymphoma subtypes and Waldenström's macroglobinemia, a low grade malignancy of B cell origin. However, it is not known to be mutated in AML.

TRAF6 activation and-downstream TAK1-mediated signaling. The polyubiquitination of IRAK1 and TRAF6 mediates the recruitment of TAK1. TAK1 is known to interact closely with TAB1, TAB2, and TAB3. TAB1 interacts with TAK1 through its N-terminal domains, whereas TAB2 and TAB3 interact with TAK1 through its C-terminal domains and facilitate TAK1 activation by binding to K63-linked ubiquitin chains.[53] Activation of TRAF6 results in the phosphorylation and activation of the TAK1 complex upon binding to ubiquitin chains. TAK1 then phosphorylates Jun N-terminal kinases (JNK), p38, and the IKK complex, which also interacts with K63-linked ubiquitin chains via NEMO, leading to NF-κB activation.[54] The activation of these downstream signaling pathways and NF-κB transcription factor results in the transcription of key cytokines for the immune response. These cytokines include IL-1β, G-CSF, IL-6, and TNFα, among others, and are required to generate an immune response.

TICAM-1-Dependent Signaling.

Aside from MYD88-dependent signaling, a phenomenon of MYD88-independent NF-κB induction and interferon transcription occurs following LPS-TLR4 stimulation in *Myd88* knockout (*Myd88*$^{-/-}$) macrophages and dendritic cells. This LPS-TLR4-NF-κB induction demonstrates that MYD88-independent TLR signaling occurs through independent pathways.[55, 56] Two research groups discovered an additional adaptor protein, TLR adaptor molecule 1 (TICAM-1, also known as TIR Domain Containing Adaptor Inducing Interferon-β, or TRIF).[57-59] TICAM-1 mediates MYD88-independent signaling through endosomal TLR4 and TLR3. Therefore, TLR4 stimulation induces activation of both MYD88-dependent and TICAM-1-dependent signaling.[60] In contrast, TICAM-1 is the sole adaptor protein known to transduce endosomal TLR3-mediated signaling.[59, 61] Additionally, TICAM-1 has an accessory TIR-containing adaptor protein, TICAM-2 (also known as TRAM), which bridges the stimulated TLR4 receptor to TICAM-1, resulting in subcellular localization to the stimulated TLR4 receptor.[62] The TIR domains on each protein mediate interaction of TICAM-2 and TICAM-1. Homotypic interactions between TICAM-2 molecules are required for recruitment of TICAM-1 and downstream signaling.[63] TICAM-2 functions as adaptor in MYD88-dependent signaling.[64] To regulate TLR4-mediated signaling and TICAM-1 activity, TICAM-2 transcript is downregulated by mir-27a following TLR4 stimulation in injury models.[65]

Like MYD88, the TLR adaptor protein TICAM-1 has no catalytic activity of its own, and its functions are dependent upon the recruitment of other proteins. Formation of the TICAM-1 signalosome is dependent on conserved domains within the TICAM-1 protein and polymerization of TICAM-1 at the site of TLR3/4 stimulation. Unlike MYD88, TICAM-1 is known to interact with a diverse set of signaling partners via conserved domains. TICAM-1 has a

TIR domain, a RIP homotypic interacting motif (RHIM), and several TRAF-interacting domains (Figure 3). Depending on which proteins are recruited to the TICAM-1 multimer, downstream signaling can range from induction of innate immune cytokine effectors to induction of necroptotic signaling.

The TICAM1 TIR domain is required for interaction with the intracellular TIR domain of TLR3 and that of TICAM-2 in TLR4 signaling. Upon stimulation, TICAM-1 aggregates at the receptor and recruits a large set of proteins. Proteomic approaches have studied the TICAM-1-TLR complex and identified 14-3-3-zeta as a required protein for multimerization of TICAM-1 and propagation of downstream inflammatory signaling.[66] However the exact relationship between TICAM-1 and many of its other complex partners and their respective significance remains to be determined.[66]

The best studied function of TICAM-1 is its ability to induce the interferon response. TICAM-1 multimerization is required for the activation of TANK-binding kinase 1 (TBK1) and IKKε. Upon TICAM-1 stimulation, TBK1 is recruited to TICAM-1 and undergoes a transautophosphorylation-mediated activation in which recruitment to the TICAM-1 multimer is required.[67] Activation of TBK1 permits TBK1-mediated phosphorylation of IKKε. Activation of IKKε results in phosphorylation of interferon regulatory factors (IRFs). IRFs are a family of transcription factors that are required for transcription of downstream interferons following TLR3 or TLR4 stimulation. In unstimulated conditions, IRFs exist as monomers in the cytosol, but form dimers and translocate to the nucleus following IKKε-mediated phosphorylation.

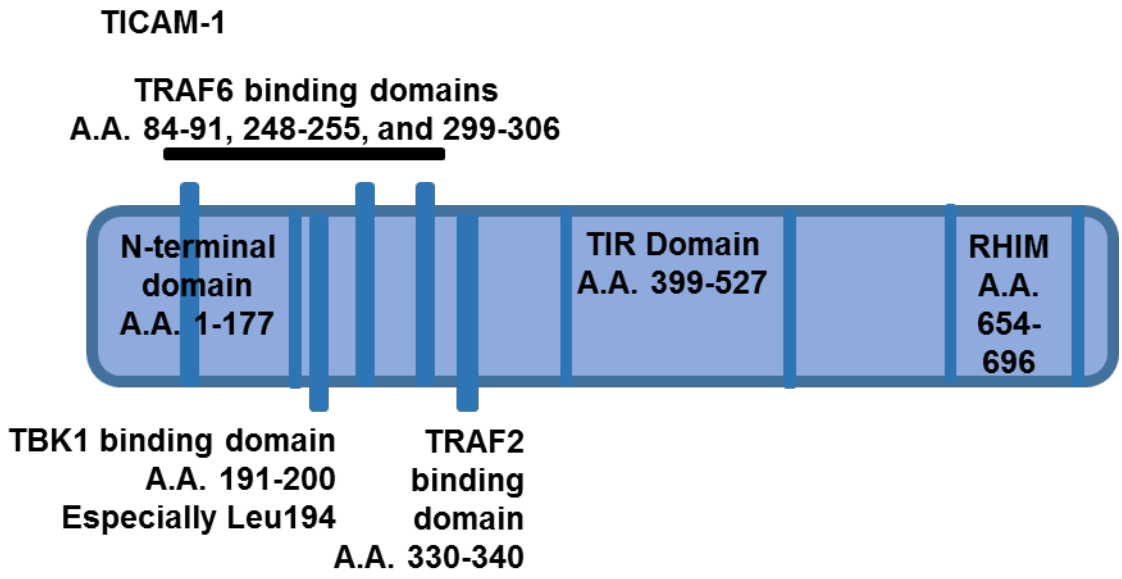


Figure 3. TICAM-1 Adaptor Protein Domains. TICAM-1 is known to have several conserved domains required for downstream signaling. TANK-binding kinase 1 (TBK1), TRAF2, and TRAF6 interact with TICAM-1 through conserved domains and are required for downstream signaling. Unlike most TLR adaptors, the TIR domain is centrally located in the protein. Lastly, TICAM-1 has a C-terminal receptor interacting protein (RIP) homotypic interaction motif (RHIM) domain, which allows it to interact with RIPK1/3 proteins.

Though precise mechanisms remain unclear, the recruitment of not only TRAF3, but also TRAF2 and TRAF6, is required to induce downstream interferon and NF-κB induction. By extension, the recruitment of these TRAFs is also required to initiate TBK1-mediated IRF induction. TICAM-1 is known to interact directly with several TRAF family members through distinct conserved TRAF-binding domains. TRAFs are known to act as E3 ubiquitin ligases to initiate construction of K63-linked ubiquitin chains and ubiquitin-dependent signaling. TICAM-1 interacts directly with TRAF3. TICAM-1 may also interact directly with both TRAF2 and TRAF6 through conserved TRAF-binding domains distinct from those required for interaction with TRAF3.[68] TRAF2 and TRAF6 polymerize with TICAM-1 upon stimulation. TRAF2/6 interaction with TICAM-1 mediates K63-linked ubiquitination and is required for the induction of downstream activation of both IRF3 and NF-κB.

As previously mentioned, MYD88-dependent and TICAM-1-dependent signaling are known to have overlapping downstream signaling mediators. Pellino-1 is a known substrate of IRAK1-mediated phosphorylation. TBK1 and IKKε directly interact with and phosphorylate Pellino-1 to activate its E3 ubiquitin ligase activity.[69] TBK1 and IKKε may also induce crosstalk with other IKK family members and are required to induce TICAM-1-mediated NF-κB activation. Importantly, TICAM-1 induces TRAF6 activity, so inhibition of MYD88-dependent signaling using inhibitors against IRAK1/4 would fail to prevent downstream signaling in the presence of TICAM-1-mediated stimuli.

TICAM-1 possesses a RIP homology interacting motif (RHIM), which permits interferon-independent signaling. RHIM domains are present in receptor interacting protein kinase 1 (RIPK1) and RIPK3.[70] Both RIPK1 and RIPK3 are serine/threonine kinases known to function in TNFα receptor-induced cell death and NF-κB induction. Both RIPK1 and RIPK3 are required for cells to undergo necroptosis, a form of cell death. In the setting of necroptosis, RIPK1 and RIPK3 interact closely via their RHIM domains. RIPK1 activates RIPK3 by phosphorylation, and RIPK3 in turn phosphorylates mixed lineage kinase like (MLKL) protein. Activation of MLKL is required for successful necroptosis, in which it is required for pore formation in the plasma membrane.[71] In the setting of TLR signaling, RIPK1 and RIPK3 are required for TICAM-1-mediated ERK and NF-κB induction after LPS stimulation.[72, 73] Necroptosis has also been reported to occur in response to TLR3 stimulation, perhaps as a mode of limiting the spread of viral infection.

Regulation of Toll-like Receptor Signaling.

Because TLRs stimulate such a strong inflammatory transcriptional response, they are carefully regulated. Shortly after the introduction of inflammatory stimuli several processes are initiated that limit or deactivate cell membrane-mediated TLR signaling, most often by ubiquitination and

degradation of key TLR signaling intermediates. For example, following TLR2 and TLR4 stimulation, MAL is phosphorylated in an IRAK1 or IRAK4 dependent manner, which permits its ubiquitination and subsequent degradation.[74] TICAM-2 is known to be downregulated by mir-27a.[65] Downstream, after IRAK1 is phosphorylated and ubiquitinated, it is rapidly degraded.[75] Further, several deubiquitinases including A20 limit both the duration and degree of the transcriptional response. A20 is known to negatively regulate multiple nodes within TLR signaling pathways. For example, it is known to interact with the C-terminal domain of NEMO to limit NF-κB activation.[76] The failure of A20 recruitment leads to an accumulated of K63 ubiquitination and prolonged NF-κB activation. When A20 function is limited by disruption of the A20-NEMO interaction, expression of nonfunctional A20, or deletion of A20, the TLR-mediated immune response is dysregulated, leading to hyperactive transcriptional responses, increased cytokine secretion, and higher incidence of autoimmunity.[76, 77]

Conversely, TLRs are known to augment their own transcription, as well as that of their adaptors. This response is thought to increase the sensitivity of the stimulated cell to other TLR ligands and amplify the immune response. TLR2 is well-known to induce its own transcription.[78]

Recent Discoveries Challenge Conventional TLR Models.

While the framework of TLR signaling was set over the past twenty years, recent studies suggest that the divisions between MYD88-dependent and MYD88-independent pathways may not be as straightforward as initially thought. For example, while TLR1/2 heterodimers and TLR4 homodimers were thought to be the only modes of dimerization, TLR2 and TLR4 are now known to form functional heterodimers in an MYD88-dependent manner.[79] Further, a small subset of genes, including *CCL4* and *CCL5*, downstream of TLR2 are induced in a TICAM-2 (TRAM)-TICAM-1-TBK1-IRF3-dependent manner.[78] TICAM-1 is also known to be induced by

MYD88-dependent signaling through TLR1/2 and TLR4, which contributes to the TICAM-1 mediated effects of both signaling pathways.[80]

These recent studies continue the illumination of the complex and overlapping network of TLR signaling. Going forward, this dissertation will examine the known functions of TLR signaling pathways in hematopoiesis, solid tumor malignancy, and hematopoietic pathology and malignancy in support of a role for TLR signaling in acute myeloid leukemia.

Hematopoiesis and the Bone Marrow Niche

Hematopoiesis is the process by which all blood cells are generated. All blood cells arise from hematopoietic stem cells (HSCs). HSCs are a multipotent cell population capable of differentiating into myeloid and lymphoid cells and regenerating both compartments. In order to generate hematopoietic progenitors and blood cells while also maintaining the HSC population, HSCs undergo asymmetric cell divisions in which one daughter cell loses HSC potential and the other daughter cell retains it. The daughter cell that loses HSC potential cannot become or generate an HSC, but instead acts as a multipotent progenitor (MPP) to maintain terminally differentiated populations of myeloid or lymphoid cells and their respective progenitors. By maintaining the quiescent HSC population while also generating a proliferative MPP population as needed for hematopoietic homeostasis, HSCs are said to have the ability to 'self-renew'. However, HSCs are not able to autonomously maintain themselves in a multipotent state, and must be maintained in an organized, specialized, and stable niche by a balance of several discrete signaling pathways.[81-83] This microenvironment protects the HSC and is comprised of both secreted cytokines and direct cell-to-cell contact with niche cells.[84, 85] In support of the goal of expanding HSCs *in vitro* for the treatment of hematologic disease by autologous transplantation, the HSC niche has been carefully studied and dissected. However, HSCs, MPPs, other

hematopoietic progenitors, and stromal cells that comprise the HSC niche all express TLR signaling, which influences the proliferation, differentiation, and repopulating potential of HSCs.

Hematopoiesis under Toll-like Receptor Stimulation.

Hematopoietic progenitors are known to respond to infectious stimuli, and this process serves various functions from embryonic development to adulthood. In the developing embryo, TLR signaling is required for the establishment of the HSC population. Sterile TLR4-MYD88-NF-κB signaling has been shown, using genetic deletion models, to be required for the emergence of embryonic HSCs from hemogenic endothelial cells of the aorto-gonad-mesenephros (AGM) region in both mouse and zebrafish models.[86] Therefore, TLR stimulation is required for the establishment of the embryonic HSC population. Local production of several inflammatory cytokines induced by TLR4 are associated with the emergence of embryonic HSCs. These cytokines include interferon γ, interferon α, TNFα, IL-3, and IL-1β.[87-91] Each of these cytokines is required for HSC emergence, and loss of any individual cytokine signal reduces the efficiency of HSPC emergence. The precise mechanism of TLR4 stimulation in this setting remains to be determined, but the controlled, localized production of endogenous TLR4-specific DAMPs is a likely possibility. Interestingly, the requirement for Toll-mediated signaling in facilitating the origin of hematopoietic cells extends into *D. melanogaster*, the organism in which it was initially discovered. In *D. melanogaster*, hemocyte proliferation and differentiation within the developing larva is regulated by the Toll/Cactus pathway.[92] The repeated implication of TLR4 in the embryonic emergence of HSPCs in distantly related model systems underlies an ancestral role for this TLR4 signaling axis in hematopoiesis.

Formerly, in the absence of infection, no TLR signaling was thought to occur in adult organisms. However, basal stimulation of hematopoietic progenitors by compounds derived from

a healthy microbiota appears to have neutral effects on the robustness of the HSC population, but results in the maintenance of bone marrow resident myeloid cell and neutrophil populations through both MYD88 and TICAM-1 dependent stimulation.[93] This stimulation by microbiota-derived PAMP stimuli is well below the threshold to elicit an inflammatory response. However, this low level of stimulation was shown to maintain a population of myeloid cells with in the bone marrow. Accordingly, the depletion of gut microbiota or deletion of MYD88/TICAM-1 results in a comparatively smaller myeloid pool within the bone marrow. These findings suggest that the presence of the gut microbiota and other tissue-specific microbiota facilitate a basal production of myeloid cells that may serve to later augment the innate immune response.

Consistent with this observation, infections of numerous etiologies have been known in humans and animal models to stimulate hematopoiesis above baseline blood cell production in a myeloid-skewed manner, resulting in production of granulocytes, monocytes, and other myeloid-derived cells, at the expense of homeostatic production of lymphocytes.[94] This phenomenon of augmented myeloid cell production and accelerated differentiation during infection is known as emergency myelopoiesis. Bone marrow hematopoietic stem and progenitor cells (HSPCs) are able to detect infection-derived PAMPs via TLRs and generate a functional response to the infectious stimuli. Induction of HSC cycling and differentiation leading to emergency myelopoiesis response may be elicited by known synthetic TLR ligands such as PAM3CSK4 or R848.[95-98] HSPCs directly respond to TLR ligands in a cell-autonomous manner after transplantation of wildtype Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ $IL7Ra^-$ HSPCs into *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, or *Myd88*$^{-/-}$ mice. Following transplantation, treatment with respective TLR ligands induces HSPC macrophage differentiation, showing that HSPCs directly respond to TLR stimulation.[99] While HSPCs may directly respond to TLR ligand stimulation, emergency myelopoiesis may also be

induced indirectly by G-CSF cytokine secretion from endothelial cells presented with LPS or by IL-6 cytokine release from stimulated HSPCs.[100, 101] Following the resolution of the infection, the stimuli are no longer present, and the bone marrow niche returns to a quiescent state to preserve HSPC functional potential.

Persistence of TLR stimuli, however, results in perpetually stimulated HSPCs, eventually reducing their hematopoietic potential. Chronic LPS stimulation of also results in the differentiation of common myeloid progenitors, but also stimulates the differentiation of common lymphoid progenitors into dendritic cells.[96] A variety of *in vivo* TLR stimulation and infection models have been shown to elicit similar myelopoiesis responses.[102-106] However, chronic *in vivo* TLR stimulation has been shown to result in defects in HSC proliferation and differentiation due to HSC exhaustion.[107] Conversely, *Myd88*$^{-/-}$, *Tlr2*$^{-/-}$, or *Tlr4*$^{-/-}$ HSPCs are marked by enhanced repopulating capacity, demonstrating that TLR signaling regulates HSC function in the absence of infection.[108] Thus, overstimulation of TLRs on HSCs is detrimental to the functional repopulating capacity of HSCs. However, TLR stimulation drives the expansion and differentiation of myeloid progenitors. This effect may influence the myeloid skew in hematopoiesis often observed in aging bone marrow microenvironments.[109]

Bone marrow microenvironments may also be disrupted by leukemia-derived cytokines to result in a myelopoiesis phenotype. In emergency myelopoiesis granulocyte/macrophage progenitor (GMP) populations are dispersed throughout the bone marrow and expand forming localized GMP clusters that produce terminally differentiated granulocytes within the bone marrow. This is due to the controlled activation of myelopoiesis by cytokine signals, including IL-1β. These cytokine signals are eventually deactivated to restore the quiescent hematopoietic niche, but, in leukemia, the pro-myelopoiesis cytokine signal is chronically activated by the

invading leukemia cells, so GMP clusters are always present.[110] Therefore, disease processes, including malignancies, activate the bone marrow niche using the same cytokine signals identified in TLR-mediated emergency myelopoiesis. These observations within a healthy hematopoietic compartment have led to the study of TLR signaling in diseases and malignancy arising from excessive TLR stimulation or dysregulation. This TLR dysregulation has been shown to be a key element of several hematological diseases, including myelodysplastic syndrome.

Toll-like Receptor Signaling in Malignancy and Disease

MYD88-Dependent Signaling in Solid Tumors.

Since the discovery and assembly of the TLR signaling pathways, they have been primarily studied in the immune system. However recent work implicates TLR signaling mediators in the development and progression of malignancy of both hematopoietic and solid tissue origin. TLR signaling was first implicated in the development of malignancy when it was determined that *Myd88* deletion reduces the incidence and slows the progress of colorectal tumors in both $APC^{Min/+}$ and colon cancer induced by the carcinogen azoxymethane (AOM).[111] Thus, Myd88 is required for intestinal tumorigenesis for both genetic and carcinogen-induced tumorigenesis. More recently, genomic analysis of a large set of colorectal cancer patients has demonstrated that single nucleotide polymorphisms (SNPs) of TLR5 correlate with activation of downstream signaling pathways and unfavorable patient survival.[112] Further, even elevated expression of TLR5, MYD88, IL-1α, and IL-6, downstream of TLR signaling, are correlated with poor survival in colorectal cancer cohorts.[113] These studies and others have established that TLR signaling promotes tumor establishment and progression, and might serve as prognostic indicators for colorectal tumors.

Since the initial discovery of MYD88 as a promoter of colorectal cancer, MYD88 and TLR signaling pathways have been studied in a range of malignancies. These include both cancers of hematopoietic origin and solid tumors, including breast cancer, brain cancer, pancreatic cancer, hepatocellular carcinoma, and many others. A relatively large number of cancers tend to express TLR2 and TLR4 highly. Despite this, the exact function of TLR signaling in solid tumor malignancy appears to be diverse and tissue-specific. TLR function has been shown to be required for tumors, tumor progression, survival, and metastases. Because diverse tumor types are derived from a wide range of tissues, often with unclear connections to the oncogenic drivers for individual tumor types. TLR signaling promotes the initiation and progression of breast cancer, and DAMP signaling has been associated with breast cancer stem cell maintenance.[114, 115] Similar findings demonstrate IRAK1 promotes cancer cell stemness in hepatocellular carcinoma.[116] TLRs have been linked to lung cancer, and IRAK4 is active and targetable by inhibitors in preclinical pancreatic adenocarcinoma models.[117-119]

However, activation of TLR signaling within certain tumors is actively suppressed because it prevents tumor progression by inducing inflammation and apoptosis. Therefore, a range of TLR modulatory agents that block or stimulate TLR signaling are being considered for potential therapeutic use. Glioblastoma multiforme, the most common brain cancer in adults, differs from other solid tumors in that it actively suppresses TLR signaling. In glioblastoma multiforme cancer stem cells, TLR4 expression and activity is reduced to protect tumor stem cell function.[120] Further, glioblastoma stem cells suppress IL-1β-induced TLR4 expression in a β-defensin-3-dependent mechanism.[121] Therefore, in these models for glioblastoma multiforme, TLR signaling, especially TLR4 signaling, is evaded and suppressed to protect the malignant

glioblastoma stem cells. However, in breast cancer subtypes, metastases have been shown to occur in a TLR4-dependent manner.

Variants of MYD88 and Toll-like Receptor Mediators Associated with Hematopoietic Disease.

MYD88 mutations in lymphomas and B cell malignancies. While overexpression of *wildtype* MYD88 is associated with a range of solid tumors, mutant variants of MYD88 are known to recur in several other malignancies. As previously described, MYD88 has two functional domains, the N-terminal DD (Figure 2), which directly interacts with IRAK4 and TRAF6, and the C-terminal TIR domain, with interacts with the intracellular domains of TLRs. MYD88 is known to be recurrently mutated in several B cell lymphoma subtypes and Waldenström's macroglobinemia.[122, 123] The recurring MYD88 mutation consists of a conversion of leucine to proline (L265P) within the TIR domain. This mutation allows MYD88 to spontaneously assemble, without upstream stimulation or interaction with TLR intracellular domains. The spontaneous assembly of L265P-mutant MYD88 results in the chronic activation of IRAK4 and IRAK1, leading to the chronic activation of NF-κB and transcription of NF-κB-regulated genes.[123] This MYD88 L265P mutation is known to occur in over 86-95% of Waldenström's macroglobinemia cases and 87% of cases of monoclonal gammopathy of undetermined significance (MGUS).[124-126] Among B cell lymphomas, 29% of activated B cell-like diffuse large B cell lymphomas (ABC-DLBCL) possess this mutation.[122] The site of the lymphoma seems to have a significant role in DLBCL with *L265P MYD88*. Clinical meta-analyses indicate 60% and 77% of central nervous system and testicular ABC-DLBCLs present with *L265P MYD88*, respectively.[127] Another subtype of DLBCL, primary cutaneous DLBCL, leg type (PC-DLBCL-LT), is known to have L265P MYD88 mutations in 59% of cases.[128]

Across several studies, the mutation is associated with advanced age and adverse outcomes.[127, 129]

Because the MYD88 L265P mutation confers spontaneous and constitutive myddosome assembly and activation of downstream IRAKs, IRAK inhibitors would be ideal agents for treating lymphomas and other diseases with such activating mutations. Currently no IRAK1/4 inhibitors are part of DCBCL or Waldenström's macroglobinemia treatment plans, though novel inhibitors of IRAKs are being developed and undergoing preclinical testing.[130] Currently one clinical trial is testing the efficacy of IRAK inhibitors against MYD88 mutant and MYD88 wildtype refractory non-Hodgkin's lymphomas.[131 131 131 131 131 130 130 128 128] Since the MYD88 L265P mutation seen in Waldenström's macroglobinemia and DCBCL is known to activate Bruton's tyrosine kinase (BTK), it is currently treated with ibrutinib, a BTK inhibitor.[132-135] However, whether targeted inhibition of IRAK1/4, downstream of activated mutant MYD88 L265P, is clinically effective remains to be determined.

Toll-like receptor signaling dysregulation in myelodysplastic syndrome. Myelodysplastic syndrome (MDS) is a hematological disorder in which hematopoiesis within the bone marrow fails to produce a sufficient number of cells to replace lost erythrocytes, immune cells, and megakaryocytes. Patients with MDS exhibit a variety of chronic cytopenias. MDS arises due to the development of clonal hematopoiesis bearing recurring MDS-related mutations. The incidence of this disease is 5.3-13.1 per 100,000 individuals and, like AML, this incidence increases to with age, eclipsing AML with an incidence of 75-162 per 100,000 people over age 65.[136] It also has a five-year survival of 35%, and median overall survival ranges from a median is 46 months, although it varies depending on the subclassification.[137, 138] A subset of MDS patients will see their disease convert into a secondary AML and face the accompanying poor

prognosis, but this is difficult to predict and transformation varies with both subtype and specific mutation events. Dysregulation and activation of TLRs and downstream innate immune signaling pathways is known to be a key element of MDS.

Several upregulated genes in MDS, identified using H3K4me3 chromatin immunoprecipitation and sequencing, belong to TLR signaling pathways.[139] Independently, MYD88 is overexpressed in 40% of MDS and correlated with poor survival compared to MDS patients without MYD88 overexpression.[140] MYD88 overexpression induces activation of downstream IRAK1/4 signaling that can be efficiently targeted by specific IRAK1/4 pharmacologic inhibitors.[141] TLR4 is upregulated in HSPCs in MDS in a TNFα-dependent manner, resulting in increased apoptosis and favoring the expansion of the dysfunctional MDS clone.[142] Upstream of TLR4, known TLR4 ligands, principally S100A8 and S100A9, are also overexpressed in certain MDS models. Overexpression of S100A8 and S100A9 results in a progressive anemia by stimulating TLR4 to cause a block in erythroid differentiation.[143] Furthermore, the presence of the TLR4 signaling axis in mesenchymal stem cell niches induces mitochondrial and genotoxic stress in HSPCs, inducing hematopoietic dysfunction and raising the risk for MDS progression to leukemia.[144]

Activating variants of TLR2 (F217S) recur in MDS in 11% of the studied cohort and inhibit the production of erythroid, megakaryocytic, and lymphoid lineages.[5] This results in a skewing hematopoietic production toward granulocytes and monocytic cells, as would be expected if TLR2 were to be chronically stimulated. The chronic TLR2 stimulation also leads to an exhaustion of the HSPC clone and worsening of the hematopoietic inefficiency in MDS. Interestingly, the expression of TLR9 has been shown to be upregulated in MDS, but relatively downregulated after conversion to AML. Like TLR9, TNFα is downregulated with MDS

conversion to AML, but TLR2 and TLR4 transcripts remain elevated compared to healthy controls.[145]

Recurring mutations in myelodysplastic syndrome lead to TLR dysregulation. MDS patients with the recurring deletion of the 5q chromosome arm (del(5q)) also lose both miR-145 and miR146a microRNAs, which are known to regulate TRAF6 expression.[146] An MDS-like phenotype may be elicited by knockdown of these microRNA or overexpression of TRAF6 in HSPCs, validating the involvement of downstream TLR mediators in MDS. The association of TRAF6 to MDS has been shown to occur through TRAF6-mediated alternative splicing of negative regulators of the Rho GTPase Cdc42, causing its activation and hematopoietic dysfunction.[147] Dysregulation of TLR signaling plays a prominent role in the pathogenesis of MDS, and future treatments may be targeted to downstream mediators of TLR signaling.

Acute Myeloid Leukemia

Development and Symptoms.

The goal of this dissertation is to determine the role of TLR signaling in acute myeloid leukemia. Acute myeloid leukemia (AML) is a malignancy that arises from hematopoietic stem and progenitor cells. In AML, hematopoietic stem/progenitor cells (HSPCs) gain mutations and/or global epigenetic alterations that accumulate over time.[148] These alterations do not directly result in leukemia, but rather, competing clonal subpopulations of HSPCs. A subset of these clonal HSPC populations then acquires sufficient genome and epigenome alterations to become leukemia precursor cells, cells that can be transformed into leukemia cells by few further alterations. This process culminates with the transformation of a leukemia precursor cell from one of these clonal populations into a leukemia initiating cell. Once this transformation occurs, the leukemia initiating cell proliferates rapidly, leading to the expansion of the leukemic clone

within the bone marrow and disruption of the homeostasis of the bone marrow niche. Accordingly, the population of leukemia blasts may be composed of several clonal populations, defined by the presence of individual mutations and/or gene expression profiles. These leukemic clonal populations demonstrate varying degrees of chemotherapy resistance and acquire new mutations with treatment, amplifying the clinical challenge of disease reoccurrence.[149]

The expansion of the leukemic clone within the bone marrow disrupts ongoing hematopoiesis, leaving critical systemic needs for erythrocytes, granulocytes, megakaryocytes and other blood cells unmet. The unmet need for these hematopoietic cells results in the development of symptoms classically associated with AML: frequent and prolonged bleeding, bruising and contusions with very minor insult, susceptibility to infection, fatigue, and anemia. If untreated, the expansion of the leukemia clone in the bone marrow will lead to death due to bone marrow insufficiency or opportunistic infections. Additionally, leukemia cells may travel throughout the body through the vasculature, seeding bone marrow niches throughout the body and invading organs, such as the lungs, liver, and spleen.

AML may occur at any age, and overall AML incidence is 1.8 cases per 100,000 individuals. However, the incidence rises to 12.2 cases per 100,000 individuals for patients over the age of 65. In the United States, 19,520 people are expected to be diagnosed with AML in 2018, accounting for 1.1% of the overall cancer burden.[150] Sadly, in 2018, approximately 10,670 people are expected to die of AML, accounting for 1.8% of cancer deaths. The increased incidence of AML occurs in a progressive manner once the patient reaches the sixth decade. Each decade thereafter increases the risk of AML development. Older patients tend to fare worse than younger patients, with the five year survival of patients aged <65 years at 43%, 65-74 years at 5.2%-13.5%, and 75-84 years at 2.0-3.0%.[151] The frequent occurrence of AML in elderly

patients poses obstacles for treatment, because, with advancing age, patients are at increased risk for development of AML with adverse cytogenic profiles, other comorbidities, poor performance status, and other predictors of adverse outcomes. Not only does the advanced patient age predispose them to poor outcomes, but it also limits treatment options for older AML patients.

Causes of AML.

AML is primarily associated with four causes: 1) age; 2) prior exposure to carcinogens or chemotherapy; 3) hereditary conditions; and 4) preexisting hematological disorders. As previously described, AML incidence increases significantly with age, which is often described as the primary risk factor for AML. Increased AML incidence with age co-occurs with other age-related hematological diseases, such as MDS, clonal hematopoiesis, skewed myelopoiesis, and increasing adiposity and inflammation within the bone marrow niche.

Prior exposure to a variety of genotoxic chemicals and stressors are associated with AML development. Occupational exposure to benzene, a widely used solvent in rubber manufacturing, oil and gasoline refineries, cleaning supplies, detergents, paints, dry-cleaning, and other materials, is a well-established as a risk factor for AML. Cigarette smoking also results in benzene exposure to the body and increased AML risk. Relative risk for AML development is increased with both increased cigarette consumption and increased duration of smoking activity.[152] Interestingly, preclinical studies have shown upregulation of TLRs and inflammatory cytokine expression in bone marrow mononuclear cells when they are exposed to cigarette smoke extract.[153] Lastly, preleukemic conditions, including MDS, have also been associated with smoking.[154, 155]

The other chemical risk factor for AML is prior exposure to DNA damaging chemotherapeutic agents, such as alkylating agents and topoisomerase II inhibitors.[156] These

treatments are applied to treat unrelated malignancies, but then result in the development of treatment-related AML. Unfortunately, due to its poor response to chemotherapy and high incidence of adverse cytogenetic factors, treatment-related AML has relatively unfavorable prognoses compared to *de novo* AML.[157] This same phenomenon is observed with therapeutic doses of radiation. Thus, genotoxic stress, whether it is occupational, habitual, or therapy-related, increases the risk of AML development.

AML is also associated with a number of hereditary conditions. Most notable among these is trisomy 21 (Down Syndrome). Patients with trisomy 21 are at greater risk for AML characterized by somatic mutations in the transcription factor *GATA1*, which coordinate with gene dysregulation specific to trisomy 21.[158, 159] Fortunately, Down syndrome patients with AML respond very well to chemotherapy. Increased risk of AML is also associated with syndromes characterized by mutation of tumor suppressor genes, including ataxia telangiectasia (*ATM*) and Li-Fraumeni syndrome (*TP53*).[160] AML is also associated with Fanconi anemia, Wiskott-Aldrich syndrome, and Diamond-Blackfan anemia and germline GATA2 and RUNX1 genes.[159, 161] Lastly, AML is known to development from preexisting hematological pathology, such as MDS as previously described. This may also occur in aplastic anemia and paroxysmal nocturnal hemoglobinuria (PNH).[162, 163]

Current AML Treatments.

For the past three decades, the standard of care for AML patients has been a 7+3 induction chemotherapy regimen. This regimen is comprised of a dose of 60-90 mg/m^2 daunorubicin or 10-12 mg/m^2 idarubicin delivered over 15 minutes on days 1-3 and a continuous, 24 hour 100 mg/m^2 dose of cytarabine on days 1-7.[164] These chemotherapy regimens yield a long-term disease-free survival of approximately 30% for patients under the age of 60. However, for

patients older than 60, only 5-10% will survive for five years or more, because the majority of those that attain remission will see their disease relapse. Furthermore, the treatment-related mortality for adults over 60 may be as high as 30%. As a result, an urgent unmet need for novel treatments persists. More tolerable treatments for AML include azacitidine, which acts as a DNA hypomethylating agent, reversing the silencing DNA hypermethylation observed in AML. Azacitidine is used to treat AML in elderly patients to avoid treatment-related toxicities and was recently shown to extend median survival by 3.8 months over conventional treatments in an older patient population.[165]

Minimal Residual Disease.

Despite the achievement of complete remission rates of 64% by combination anthracycline and cytarabine cytotoxic chemotherapy, median survival following an AML diagnosis stands at 8-9 months, with an overall five-year survival of 26%.[166] Despite achieving complete remission, certain subpopulations of leukemia cells, thought to persist in a quiescent stem-like state, survive the cytotoxic chemotherapy regimen. These leukemia stem cells (LSCs) then proliferate and regenerate the leukemia blast population, resulting in disease reoccurrence. This phenomenon is known as minimal residual disease (MRD), wherein residual leukemia cells that survive the initial chemotherapy treatment regenerate the initial disease. Because AML has such a high rate of MRD, the goal of numerous preclinical studies and clinical trials is to develop a treatment that successfully eradicates LSCs, preventing MRD.

Classification Methodologies and Subtypes of AML.

So far, AML has been described here as a singular disease. However, like many other malignancies, AML is best described as a group of diseases which are phenotypically similar, but genetically diverse. Accordingly, the two primary modes of categorizing AML subtypes rely on

the morphological phenotype of the undifferentiated AML blast cells or the recurring genetic mutations that have been observed in AML. The French, American, and British (FAB) classification system divides AML into seven discrete subtypes based on the lineage and degree of differentiation as determined by subtype morphologies as follows: M0: undifferentiated myeloblastic leukemia; M1: acute myeloblastic leukemia with minimal maturation; M2: acute myeloblastic leukemia with maturation; M3: acute promyelocytic leukemia (APL); M4: acute myelomonocytic leukemia; M4eos: acute myelomonocytic leukemia with eosinophilia; M5: acute monocytic leukemia; M6: acute erythroid leukemia; and M7: acute megakaryoblastic leukemia.[167] Of these AML subtypes, APL has the best prognosis. APL is characterized by a t(15:17) translocation, resulting in a PML-retinoic acid receptor α (RARα).[168] APL is effectively treated with a combination of all-*trans* retinoic acid (ATRA) and arsenic trioxide, both of which act to degrade the PML-RARα fusion protein.[168] These treatments yield remission rates greater than 95% and cure rates greater than 80%.[169] ATRA acts on the retinoic acid receptor to promote myeloid differentiation in both APL and non-APL cells. However, ATRA-induced differentiation is blocked in non-APL cells, and much research has been aimed at sensitizing other AML subtypes to ATRA-induced differentiation. The FAB classification was used since the 1970s, but it has since been widely replaced by the World Health Organization (WHO) classification.

The WHO classification divides AML into groups based on a subset recurring genetic mutations, with priority given to subtype-defining translocations. These include AML with t(9:11) (MLL-MLLT3/AF9), t(8:21) (RUNX1-RUNX1T1/ETO), inv(16) (CBFβ-MYH11), APL with t(15:17) (PML-RARα), and several other recurring translocations. Other categories include AML related to previous DNA-damaging chemotherapy or radiation, AML with myelodysplasia-

related changes, AML not otherwise specified, and other less commonly occurring AML groups.[170] Certain subtypes of AML respond more favorably to cytotoxic chemotherapy and thus confer favorable prognoses.

AML with MLL Rearrangement.

As previously mentioned, several well-characterized translocations occur in AML. One gene that is a frequent fusion partner in AML is the histone-lysine N-methyltransferase 2A (KMT2A) gene, also known as mixed lineage leukemia 1 (MLL1 onward). The *MLL1* gene is located at the 11q23 locus and encodes a 500kDa protein that acts as a histone methyltransferase through its numerous functional domains and binding partners. As a histone methyltransferase, MLL1 is required for epigenetic regulation of histones by removing activating methyl marks on the histone 3 tail. MLL1 acts to exert silencing of gene expression through epigenetic modification of enhancer regions. Approximately 11% of all AML patients exhibit MLL1 translocations or rearrangements with a variety of recurring fusion protein partners.[171] MLL-rearrangement occurs in 38% of M5 monocytic AML subtype, accounting for 32.7% of adult M5 AML and 52.6% of pediatric M5 AML.[171] The resulting fusion proteins are characterized by the loss of the C-terminal MLL1 exons, corresponding functional domains, and methyltransferase activity. The retention of the N-terminal KMT2A domains permits localization to normal genomic regions through intermediary proteins. Despite this, the MLL1 translocation partners, which comprise the C-terminal domains of the resulting fusion protein, yield novel activity, which allows the fusion protein to promote leukemogenesis.

The translocation of *MLL1* is known to occur recurrently in leukemia with more than 80 other genes, yielding a diverse group of fusion proteins. Fusion partners of *MLL1* frequently are members of a super-enhancer complex (SEC).[172, 173] The fusion of silencing MLL1 with SEC

components amplifies the epigenetic dysfunction inherent to MLL1 mutation. Rather than acting to downregulate epigenetic loci across the genome by removing gene expression promoting methylation, MLL1 fusions or rearrangements exert an activating effect on the genome by allowing SEC components to activate RNA polymerase II mediated gene transcription. MLL1 rearrangements are characterized by reactivation of the embryonic *HOXA* gene cluster, *MEIS1*, and other genetic programs to drive leukemia pathogenesis. In particular, HOXA9, PBX3, and MEIS1 have been shown to be required for MLL-fusion leukemic transformation and leukemia stem cell maintenance and surface, and knockdown of these genes.[174, 175] Lastly, MLL-MLLT3/AF9 (termed MLL-AF9 onward) has been widely studied in preclinical murine models. This is because retroviral expression of human MLL-AF9 cDNA efficiently transformed murine progenitor cells into a potent cell line, capable of serial *in vivo* transplantation and leukemogenesis.[176] This model system has been widely used to study the pathogenesis of MLL-AF9 leukemia *in vivo* in a preclinical setting, and will be using in this study of TLR signaling in AML.

Toll-like Receptor Signaling in AML.

Because AML cells are partially differentiated blasts, arising from stem and progenitor cells, they bear many of the cell surface markers and immune signaling pathways also found in granulocytes, monocytes, macrophages and their progenitors. These shared markers and signalings pathways are known to include TLRs and IL-1R. However, despite the knowledge that TLRs are present on AML cells, the literature is divided on their exact role, if any. Some believe that TLRs and IL-1R are required to maintain leukemia cells, and that blocking these pathways would be a viable treatment for leukemia. However, others contend that the presence

of TLRs allows leukemic blasts to be differentiated by way of a specific TLR-stimulation. Here, the current status of published literature will be reviewed as it relates to TLR signaling in AML.

Toll-like receptor signaling to induce AML blast differentiation. Several studies show that the stimulation of certain TLRs induces partial differentiation in human cell lines and primary tissues. The goal of these stimulations is to either partially mimic the terminal differentiation induced by ATRA and arsenic trioxide treatment in APL or to partially differentiate leukemic blast cells so that they become autoreactive and elicit cytotoxicity against blast cells. Both TLR7 and TLR8 synthenic agonists, imiquimod and R848 respectively, have been shown to induce differentiation of human cell lines.[177, 178] TLR7/8 stimulation by R848 suppresses colony formation and enhances nitrotetrazolium blue (NTB) signal in treated human HL60 and THP-1 cell lines through a MYD88 and p38 dependent mechanism.[177] Stimulation of human cell lines *in vitro* in a TLR7-MYD88-dependent manner by imiquimod induces cell cycle suppression and apoptosis in a subset of cell lines.[178] Stimulation of TLR7/8 by R848 also enhanced T cell activation and killing of leukemia cells when co-cultured *in vitro*. Lastly, primary AML cells may be induced to differentiate into AML-derived dendritic cells (AML-DCs) by IL4 and GM-CSF.[179] Following this differentiation, enhanced cytotoxic activity and leukemia cell killing was elicited by combined treatment with TNF-α, R848, and LPS. Thus, several possible mechanisms exist that favor TLR stimulation as a therapeutic adjunct to AML treatment.

Of note, the subcutaneous administration of TLR7 synthetic agonist 852A has been clinically tested on a small cohort of patients with AML, ALL, non-Hodgkin's lymphoma, Hodgkin's lymphoma, and multiple myeloma.[180] The 852A TLR7 agonist was intended to stimulate plasmacytoid dendritic cells to have an anti-tumor response, and, of the thirteen patients studied, resulting in 1 complete response, 1 partial response, 2 stable diseases, and 9 patients with

progressive disease. The precise effect of the TLR7 agonist on the tumor cells themselves was not studied, but observed tolerability of the prolonged drug administration and clinical effectiveness in a subset of patient merits further study to clarify the precise mechanism of TLR7 agonism.

Toll-like receptor and IL-1 receptor inhibition to treat AML cells. In contrast, several studies have indicated that TLR and IL-1R signaling protects leukemia cells and are required for their survival and pathogenesis. The most significant and thorough of these studies links MYD88-IRAK4 signaling and the epigenetic modifier MLL1, specifically in MLL1-rearranged leukemia.[181] Stimulation of MYD88 signaling activates IRAK4 and results in IRAK4-mediated activation of the ubiquitin ligase UBE20. UBE20 then ubiquitinates MLL1 on its C-terminal domain, marking it for degradation. However, this domain is lost in MLL1-rearranged leukemia, and chronic stimulation of MYD88-dependent signaling results in the selective degradation of wildtype MLL1, with no effect on mutated MLL1 fusions, which allows the MLL1-rearranged fusion protein to exert its pro-leukemia epigenetic effect without having to compete with the epigenetic silencer MLL1.

The protective effect of MYD88-dependent signaling in leukemia cells was shown by the sensitization of leukemia cells to NF-κB inhibitors after dual IL-1R and TNFR inhibition using murine *in vitro* and *in vivo* systems and primary patient samples.[182, 183] Primary AML samples may induce their own proliferation by production of IL-1 and IL-1-mediated induction of GM-CSF.[184] This protective effect was thought to occur by IL-1β-mediated induction of NF-κB transcription of pro-survival genes and complementary pro-survival pathways.

Interestingly, TLR4 is stimulation is known to result in ligand-specific responses. Known TLR4 ligands S100A8 and S100A9 are produced by a subset of AML cells in myelomonocytic

and monocytic primary patient samples as well as murine and human *in vitro* model systems.[185] When TLR4 is stimulated with S100A9, differentiation is induced by downstream ERK1/2, p38, and JNK signaling. However, stimulation with S100A8, which may be produced by leukemia cells as well, blocks this effect. The field of TLR stimulation in AML cells appears split with respect to what the precise effect of TLR signaling is on AML cells, but this study raises the possibility that the differentiation phenotypes observed in AML cells might be receptor-specific, or even ligand-specific. The presence of certain ligands might even blunt or block the pro-differentiating effect of other ligands, as with S100A8 blocking the effects of S100A9. Lastly, these findings suggest that AML blast cells are capable of modulating their own differentiation by producing these TLR4 ligands to potentially block differentiation induced by TLR4. Though TLR4 is known to have many more DAMP ligands than other TLRs, nearly every other TLR has known DAMP ligands, and their effects on AML pathogenesis remains uncharacterized.

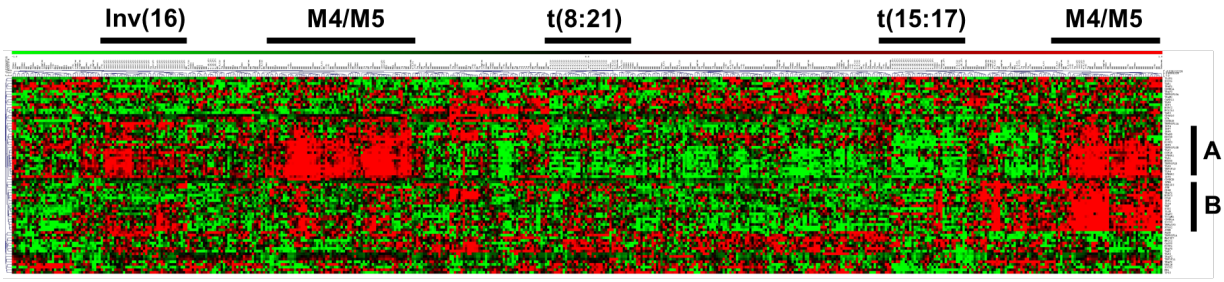
Lastly, downstream TLR signaling pathways are known to have potent pro-leukemia effects. TAK1 is known to be overexpressed in AML primary patient samples and genetic knockdown or pharmacologic inhibition of TAK1 impairs AML survival *in vitro*.[186] TAK1 inhibition exerts negative effects on NF-κB activation, which is well-known to be required for AML pathogenesis. Several combinatorial approaches have been proposed and validated in preclinical models that would integrate targeted inhibition of NF-κB with standard chemotherapy or with other targeted inhibitors of IL-1R, JNK and other pathways activated in AML.

Summation, Hypothesis and Aims

In summary, TLR signaling is an essential signaling network for functional innate immune responses. TLR signaling maintains hematopoiesis and is required for myelopoiesis during infections. Recently, TLR signaling has been shown to promote carcinogenesis in numerous

solid tumors, including breast cancer, colorectal cancers, pancreatic cancer, hepatocellular carcinoma, and others. In hematopoietic diseases, particularly MDS, TLR signaling is known to be dysregulated due to loss of suppressors of TRAF6 and other TLR mediators. MYD88 is also known to be mutated in Waldenstrom's macroglobinemia and several B cell lymphoma subtypes, leading to aberrant and constitutive NF-κB activation. TLR signaling pathways are expressed in AML cells, though their exact function remains unclear. Previous research efforts have sought to induce partial differentiation of AML cells by TLR stimulation, either alone or in combination with other agents. TLR and IL-1R signaling in AML cells is known to protect AML cells from cell death.

Recent microarray data on a large cohort of AML patients, generated by Jianjun Chen, Ph.D. in collaboration with Jiwang Zhang, M.D./Ph.D. and previously published in *Leukemia*, indicates that expression of TLR signaling factors is enhanced, specifically in M4/M5 AML subtypes (Figure 5, modified from Figure 1 in Xin *et al.*).[187] Several cell surface TLRs, including TLR1, TLR2, TLR4, and TLR5, both TLR adaptors MYD88 and TICAM-1, and several TLR-associated signaling components were found to be overexpressed, specifically in M4 and M5 AML when compared against their expression in other FAB subtypes. This microarray was used to support the presence of inflammatory signaling in the M4/M5 FAB subtype propagated by RIPK1 and RIPK3. However, this microarray also indicates a specific upregulation of TLRs and their signaling intermediaries.



A		B	
IRF2	MYD88	JUN	SOD2
RIPK3	TNFRSF1B	CD40	IL1B
IRF5	TLR5	TRAF1	IRAK2
TNFRSF10B	TNFRSF10	BIRC3	TICAM1
TLR2	TLR4	CYLD	CDKN1A
CSF1R	IFNGR2	IRF1	SOCS3
IFNGR1	IRF8	IL1A	TNFAIP3
TLR1	CDKN2B	TNF	PTGS2
			JUNB

Figure 5. Primary Patient Microarray Demonstrates Upregulation of TLR-Associated Transcripts. Microarray and clustering analysis performed by Jianjun Chen, Ph.D., and published in *Leukemia*.[187] It has been reproduced here with permission. Specific genetic alterations and clusters of upregulated TLR transcripts are highlighted, and TLR-associated genes in clusters A and B are highlighted in red.

Informed by this data and literature, I hypothesized that TLR signaling, through both MYD88 and TICAM-1, protects the leukemia cell by activating downstream NF-κB in certain leukemia subtypes. To determine a possible differential role of TLR signaling pathways in AML subtypes, this study examined multiple leukemia subtypes, but primarily focused on MLL-AF9 murine leukemia models and MLL-rearranged human cell lines. I divided this study into two broad aims. First, the precise contribution of MYD88 and MYD88-dependent signaling to TLR signaling was to be determined both *in vitro* and *in vivo*. Second, the contribution of TICAM-1 and TICAM-1-dependent signaling was to be determined both *in vitro* and *in vivo*. These studies utilized both pharmacological inhibition of kinases dependent on these adaptors and direct genetic deletion or knockdown of MYD88 and TICAM-1.

AIM 1: Determine the contribution of MYD88 and MYD88-dependent signaling to TLR signaling both *in vitro* and *in vivo.*

AIM 2: Determine the contribution of TICAM-1 and TICAM-1-dependent signaling to TLR signaling both *in vitro* and *in vivo.*

CHAPTER TWO

MATERIALS AND METHODS

Cell Culture

All cells are cultured at 95% humidity, 37° Celsius, and 5% carbon dioxide. 1640 Roswell Park Memorial Institute (RPMI) medium with 2.05mM glutamine with 10% heat-inactivated fetal bovine serum (FBS), 100IU penicillin, and 100µg/mL streptomycin (Corning™ CellGro™, MT30002CI) was used for *in vitro* cell culture. Human leukemia cell culture was applied as indicated by the American Type Culture Collection. For murine leukemia and hematopoietic cell culture, it was supplemented with 100 ng/mL recombinant murine stem cell factor (rmSCF), 20 ng/mL recombinant murine IL-3 (rmIL-3), 50 ng/mL recombinant murine IL-6 (rmIL-6), and 20 ng/mL recombinant murine granulocyte/macrophage colony stimulating factor (rmGM-CSF). Human 293T cells were cultured in HyClone™ Dulbecco's modified eagles medium (DMEM, catalog number: SH3002201, Fisher Scientific) supplemented with 10% FBS, 100IU penicillin, and 100µg/mL streptomycin.

Retrovirus and Lentivirus Generation and Infection

Both retrovirus and lentivirus were generated using calcium phosphate transfection using the CalPhos™ Mammalian Transfection Kit as indicated (Clontech®, catalog number 631312). For retrovirus to be used to infect cells of murine origin, Phoenix-Eco cells were transfected and

used to generate retrovirus. For generation of lentivirus or retrovirus for infection of human cell lines 293T cells were transfected. 15µg DNA of vectors bearing the desired shRNA or gene to be expressed were mixed with packaging and backbone vectors. For retroviral infections, 15µg pCL-Ampho was used as the packaging vector and 3.5µg pCMV-VSVG was used as the backbone vector. For lentiviral infections, 6.5µg pCMV-dR8.2 was used as the packaging vector, and 3.5µg pCMV-VSVG was used as the backbone vector. The DNA mixture was transfected into $3x10^6$ virus-producing 293T or Phoenix-Eco cells overnight at 37° Celsius. After sixteen hours, the original medium to which the transfection was added was removed, and 5mL DMEM with 10% FBS was added. The transfected cells were then kept at 32° Celsius for 48 hours. Media was collected at 24 hours and 48 hours after the initial media change and kept as virus-enriched media to be used for infections. Before infection, virus-enriched media was spun down at 1600RPM for 6 minutes at 32° Celsius to pellet any cells and debris. Both lentiviral and retroviral infections were performed by adding 1mL virus-enriched media to pelleted cells and mixing with 8µg polybrene per mL. The cells were centrifuged for 3-4 hours at 2000RPM at 32° Celsius. After infection, cells were plated in a fresh 12-well plate in complete culture medium.

Generation of Murine Leukemia Cell Lines

Female C57BL/6J mice bearing wildtype *Myd88*$^{+/+}$, *Myd88-loxP* (*Myd88*$^{fl/fl}$), and *Myd88* knockout (*Myd88*$^{-/-}$) were obtained from the laboratory of Katherine Knight, Ph.D., who originally acquired them from Jackson Labs. These mice were sacrificed by carbon dioxide asphyxiation, followed by cervical dislocation, as indicated by our laboratory animal protocol. Within 30 minutes of sacrifice, the long bones of the mouse hind limb, the tibia and femur, were isolated and flushed with media using a 27 gauge syringe. A 70µM filter was applied to filter the

bone marrow. Red blood cells were lysed and the remaining cell culture was selected for CD117-positive hematopoietic progenitors.

EasySep mouse CD117 positive selection kit (Stemcell Technologies) was applied to select CD117-positive cells from whole bone marrow culture. CD117 selection was individually applied to such cultures obtained from mice bearing wildtype *Myd88*$^{+/+}$, *Myd88-loxP* (*Myd88*$^{fl/fl}$), and *Myd88* knockout (*Myd88*$^{-/-}$) to isolate CD117^{+} cells bearing the same genotype. CD117^{+} cells were transformed by infection with retrovirus containing vectors bearing human cDNA MLL-AF9, MLL-ENL, or AML-ETO. Retroviral infection was performed by centrifuging cells with aliquoted virus for three hours at 2000 rotations per minute (RPM) at 22° Celsius. MLL-AF9 and MLL-ENL are expressed on the MSCV expression vector, which also has a neomycin resistance gene. Thus, they were selected on methylcellulose in the presence of 1.25mg/mL G418. AML-ETO is expressed on the Migr1-GFP vector, and cells infected with AML-ETO were purified by fluorescent-activated cell sorting (FACS). Once a purified population of leukemia cells was established, these cells are referred to as pre-leukemia cells (PLCs). To generate leukemia cells from these newly transformed cells, one million (10^6) PLCs were transplanted per female C57BL/6J mouse after lethal 9.0 Gray irradiation using the RS-2000 Biological Research Irradiator (Rad Source). In addition to leukemia cells, $2x10^5$ bone marrow cells from a syngeneic donor Transplantation was performed several hours after irradiation, to allow mice time to rest. Upon leukemia development, the circulating leukemia cells (LCs) were collected from the spleen or bone marrow and purified under culture conditions.

Derivation of *Myd88*$^{\Delta/\Delta}$ MLL-AF9 Leukemia Cells from *Myd88*$^{fl/fl}$ MLL-AF9 Leukemia Cells

To determine the effect of *Myd88* deletion on established leukemia cell lines, MLL-AF9 murine leukemia cells bearing a LoxP-flanked *Myd88* gene (*Myd88*$^{fl/fl}$) were established. Deletion of the *Myd88* gene was achieved using two approaches. First, retroviral infection of *Myd88*$^{fl/fl}$ with Cre on a Migr1-mCherry vector was performed by centrifugation (2,000RPM for 3 hours at 22° Celsius) with 8µg polybrene per mL. Cre-expressing cells were identified by detection of mCherry by flow cytometry and selectively sorted. Polymerase chain reaction (PCR) using primers obtained from Jackson Laboratory™ and a designed primer to confirm *Myd88* deletion. Alternatively, *Myd88* deletion was achieved by first expressing a fusion protein of a modified estrogen receptor (ERT2) and Cre (CreERT2). The fusion protein is excluded from the nucleus in conditions without ERT2 stimulation, so Cre can only delete *Myd88* in conditions of ERT2 stimulation. 4-hydroxytamoxifen (4OHT) is widely used to induce CreERT2 activation, and 10nM of 4OHT treatment for 16 hours was used to efficiently induce *Myd88* deletion, which was confirmed by PCR as described earlier.

Secondary Transplantation of MLL-AF9 Leukemia Cells

For secondary transplant of leukemia cells, female C57BL/6J mice were obtained from Jackson Laboratory™. These mice were subjected to single sublethal irradiation doses of 4.5 or 5.0 Gray or a lethal irradiation dose of 9.0 Gray, as indicated in each experiment. If mice were lethally irradiated, then $2x10^5$ bone marrow cells per mouse from syngeneic donor mice were transplanted as well. Following irradiation, mice were allowed to rest for no less than three hours. Murine MLL-AF9 cells were transplanted by tail vein injection. As indicated in each experiment, 10,000 to 20,000 murine MLL-AF9 leukemia cells were transplanted per mouse.

Following transplant, mouse water bottles were supplemented with 0.1mg/mL enrofloxacin (Baytril) to prevent opportunistic bacterial infections following bone marrow irradiation. Enrofloxacin stock solution was made at a concentration of 10mg/mL in autoclaved ddH_2O and diluted 1:100 when added to mouse water bottles. After day 14 following transplant, enrofloxacin treatment was stopped. Beginning at 20 days after transplant, mice were monitored every two days for leukemia development and daily once leukemia development is observed in any single mouse. Upon leukemia development, as observed by mice with immobilized hind limbs, overt anemia, and/or greatly reduced mobility and strength, mice were sacrificed by CO_2 asphyxiation, followed by cervical dislocation. Following sacrifice, total body mass, liver mass, and spleen mass were measured to assess leukemia status. If necessary to confirm leukemia status, peripheral blood was collected by cardiac puncture and assessed by peripheral blood smear and Hemavet analysis. Further, bone marrow cells were aspirated and filtered before cytospin and Wright-Giemsa staining. If the transplanted leukemia cells expressed fluorescent markers, the degree of these expression of these markers was assessed by flow cytometry. Lastly, tissues from spleen, liver, lung, and kidney were fixed in zinc formalin for hematoxylin and eosin staining by the Loyola Pathology Department.

IRAK Inhibitor Drug Treatment *in vivo*

Leukemia cells were transplanted as described for a secondary transplant. Mice were sublethally irradiated at 4.5 Gray and transplanted with 10,000 cells by tail vein injection. Mouse drinking water was supplemented with 0.1mg/mL enrofloxacin to prevent mortality from opportunistic infections that would arise due to bone marrow ablation due to irradiation. Mice were divided into 4 groups of 10. Drug vehicle was composed of a 10:90 ratio of DMSO:H_2O to facilitate drug solvency. The four treatment groups were treated as follows: (1) vehicle control; (2) 4mg/kg

IRAK1/4 inhibitor (*Sigma I5409*); (3) 20mg/kg Compound 26 IRAK4 inhibitor (*Calbiochem* 531237); (4) combination treatment with both inhibitor doses. Mice were treated by i.p. injection at 10, 12, 14, 16, and 18 days after transplantation. Following treatment, leukemia was allowed to develop. Mice were sacrificed, and leukemia was verified by bone marrow cytospin, peripheral blood smear, peripheral blood Hemavet analysis, spleen mass, liver mass, total body mass, and staining of bone marrow and peripheral blood mononuclear cells with anti-Gr1 and anti-CD11b fluorescently labelled antibodies.

Genotyping and Other Polymerase Chain Reaction Applications

DNA was harvested from pelleted cells after overnight 55° Celsius incubation with lysis buffer (10mM Tris, 100mM NaCl, 10mM EDTA, 0.5% SDS) and 0.4µg/mL Proteinase K, followed by isopropanol precipitation. Precipitated DNA was washed with 4° Celsius 70% ethanol and resuspended in 30-100µL H_2O. Polymerase chain reaction was performed using the GoTaq® PCR kit as instructed (Promega Corporation, Catalog number M3001). Primers, listed 5' to 3' below were used to differentiate between wildtype, LoxP-flanked, and deleted *Myd88* genotypes. Refer to Figure 4 for the position of these primers on the gene. PCR was applied to genomic DNA isolated by isopropanol precipitation with all three primers present. Mice and leukemia cells with *wildtype* $Myd88^{+/+}$ genotypes are expected to yield a 266 base pair product. $Myd88^{fl/fl}$ genotypes are expected to yield a 353 base pair product, and $Myd88^{-/-}$ or $Myd88^{\Delta/\Delta}$ genotypes produce a 550 base pair length product if knockout is present. Additionally, if only Forward primer 1 and the reverse, are used, then the reaction yields an 852 base pair length product, so the use of this PCR primer confirms the presence or absence of exon 3 deletion.

Forward Primer 1: TCC GAG AAG CCT TTA CAG GTG

Forward Primer 2: GTT GTG TGT GTC CGA CCG T

(oIMR9481 from Jackson Laboratory)

Reverse Primer: GTC AGA AAC AAC CAC CAC CAT GC

(oIMR9482 from Jackson Laboratory)

Immunoblotting

Lysates were prepared by first counting cells from plated suspension culture or experimental conditions using 0.4% Trypan blue (LONZA) on the TC10 automated cell counter (Bio-Rad). Each count was performed twice, on thoroughly mixed cell culture, and averaged. One million (10^6) cells were centrifuged at 1,600RPM and 4° Celsius for 6 minutes and washed twice in phosphate buffered saline (PBS). Cell pellets and lysates were kept on ice through the process. After washing, supernatant was removed by aspiration and the pellet was lysed in 300µL 1x lysis buffer (Cell Signaling Technologies). Lysis buffer was supplemented with phosphatase and protease inhibitors (PhosSTOP and cOmplete Tablets *EASYpack*, both from Roche). Following addition of lysis buffer, lysates were frozen in the -80° Celsius freezer for at least 30 minutes. Lysates were then thawed and sonicated. Sonicated lysates was centrifuged for 10 minutes at 14,000RPM at 4° Celsius. Supernatant was removed and the loading dye, with sodium dodecyl sulfate (SDS) and β-mercaptoethanol (BME) was added before boiling samples for 10 minutes at 100° Celsius. Samples were electrophoresed on 10% acrylamide Tris-HCl. PageRuler Plus prestained protein ladder was used to approximate molecular weights (Thermo Scientific #26619). Samples were transferred to nitrocellulose using the Transblot-Turbo Transfer system and the Ready-to-assemble transfer kit as indicated (Bio-Rad). Ponceau stain was applied to verify successful transfer and cut the blots. Tris-buffered saline with Tween 20 (TBS-T) was added to wash the Ponceau stain from the blot. Either 5% milk in TBS-T or 4% bovine serum

Antibody Target	Species	Clonality	Isotype	Company	Catalog Number
B-ACTIN	Human, Mouse	Monoclonal	Mouse IgG	Santa Cruz Biotechnology	C-2: sc-8432
GAPDH	Human, Mouse	Monoclonal	Mouse IgG	Sigma-Aldrich ®	G8795
IRAK4	Human, Mouse	Polyclonal	Rabbit	Cell Signaling Technology ®	4363S
MYD88 (D80F5)	Mouse, Human	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	4283S
MLKL (D2I6N)	Human	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	14993S
Mlkl (D6W1K)	Mouse	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	37705S
P38 MAPK	Human, Mouse	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	9212S
SAPK/JNK	Human, Mouse	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	9252S
Ser166-p-RIP1	Human	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	44590S
Ser172-p-TBK1/NAK (D52C2)	Human, Mouse	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	5483S
Ser345-p-Mlkl	Mouse	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	37333S
Ser358-p-MLKL	Human	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	91689S
RIP1	Human	Polyclonal	Rabbit	Cell Signaling Technology ®	4926S
Rip3 (D4G2A)	Mouse	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	95702S
RIP3 (E1Z1D)	Human	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	13526S
TBK1/NAK (D1B4)	Human, Mouse	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	3504S
Thr180/Tyr182-p-p38	Human, Mouse	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	9211
Thr183/Tyr185-p-SAPK/JNK (81E11)	Human, Mouse	Monoclonal	Rabbit IgG	Cell Signaling Technology ®	4668S
TICAM-1	Human, Mouse	Purified Polyclonal	Mouse	Biolegend ®	657102 1H4B01

Table 1. Antibodies Used for Immunoblotting.

albumin (BSA) in TBS-T was used to block the nitrocellulose blot while gently rocking it for 1 hour. Primary antibodies were added at 1:1,000 dilution in fresh blocking buffer and left overnight at 4° Celsius. Three 5-minute washes were applied while rocking the blot following primary antibody probe. Secondary antibody was applied at 1:1,250 dilution. All antibodies, unless otherwise specified, were obtained from Cell Signaling Technologies. GAPDH antibody, obtained from Sigma Aldrich, was used as a loading control and was diluted 1:5,000 and corresponding secondary was also diluted 1:5,000. Horseradish peroxidase (HRP) linked secondary rabbit or mouse antibodies allowed the visualization of protein bands by chemiluminescence using the WesternBright^{TM} Quantum detection kit (Advansta).

Colony Formation Assay

Colony forming ability of cells under various genetic and pharmacological conditions was assayed by a colony forming unit assay. Cells were mixed and counted twice. Average live cell number was averaged and used to calculate the volume for 30,000 cells. 30,000 cells were mixed by vortexing in 3mL of culture medium. 3,000 cells, or 300µL by volume, was then pipetted into 3mL of mouse (M3434) or human (H4100) methylcellulose (STEMCELL Technologies). Methylcellulose and cells were vortexed for 20-30 seconds. Methylcellulose-cell mixture was plated in three 30mm plates, with 1mL and 1,000 cells per plate. After plating, cells were allowed to grow for 7-10 days and then counted and imaged.

AnnexinV Staining and Flow Cytometry Quantification

AnnexinV staining was performed to assay cell death following inducible gene deletion or drug treatment. The BD Pharmingen APC AnnexinV Apoptosis Detection Kit was used as indicated. Treated cells were washed twice in 1% FBS in 1% PBS. Following washes, cells were resuspended in AnnexinV binding buffer (0.01 Hepes/NaOH, 0.14 M NaCl, 2.5mM $CaCl_2$) and

5µL APC-AnnexinV antibody was added. Cells were counterstained with 25µL 7-aminoactinomycin D (7AAD) to exclude dead cells. 20,000 events or more were recorded for each experiment. AnnexinV with propidium iodide (PI) or 7AAD counterstains were recorded on BD LSRFORTESSA™ or FACSCANTO II™ flow cytometers. Collected data was analyzed using FlowJo V9.9.6. All flow cytometry experiments used these cytometers and FlowJo analysis.

Cell Surface Staining and Determination of Median Fluorescent Intensity

Leukemia cells of interest were plated at 100,000 cells per mL and cultured overnight. Cultures were counts by Trypan blue staining and 150,000 cells were centrifuged and washed twice in wash buffer (1x PBS with 1% FBS). Fluorescent antibodies were diluted 1:20 and added to ~100µL wash buffer before mixing cells and antibody. If compensation controls were needed, they were stained at this time. Cells were left in the dark for 20 minutes before adding 3mL wash buffer and centrifuging to rinse any excess antibody. Cells stained with antibody were then resuspended in 150-300µL wash buffer prior to flow cytometry analysis.

Cell Cycle Analysis

Cells with >90% viability as measured by Trypan Blue counting were plated at 100,000 cells per mL and cultured overnight in complete medium. Cells were then pelleted and washed in 1x PBS. Fixation and permeabilization was performed by dropwise addition of 5mL 70% ethanol chilled at -20° Celsius. Fixed samples were kept overnight at -20° Celsius. On the day of analysis, fixed cell samples were washed in 1x PBS twice, treated with 100µg/mL RNase A for 15 minutes at 37° Celsius, and stained with 50µg/mL propidium iodide or 25µg/mL 7-aminoactinomycin D (7AAD). During data collection, singlets were gated based on the height and width ratio of the relevant fluorescent channel on linear scale, and no fewer than 25,000 events were collected.

Edu Incorporation Assay

Click-iT™ Edu Pacific Blue™ Flow Cytometry Assay Kit (Catalog number: C10418) was purchased from ThermoFisher and used as indicated. Two days before the experiment, cells were plated at 50,000 cells per mL. The viability of these cells was tested the day before the experiment and cells were replated at 100,000 cells per mL if the viability was greater than 90%. Edu incorporation was tested by adding 10μM Edu directly to culture conditions, without replating, as instructed. The cells were incubated with Edu for 30 minutes before pelleting and washing cells with 1% BSA in 1x PBS. Cells were then fixed and permeabilized and the reaction was catalyzed per the manufacturer's protocol. Edu assays were performed independently of cell cycle analyses.

Details Regarding shRNA Sequences and Vectors

Over the course of this study, shRNA was purchased from Origene, Genecopia, and Sigma-Aldrich. Their respective sequences, vectors, and selection markers are detailed in Table 2. These shRNA were expressed on lentiviral vectors by centrifugation and sorting as previously described.

Real-Time Quantitative PCR

Real-time quantitative PCR (RT-PCR) was performed to assay relative transcript levels across cell lines and treatment conditions of various targets. Cells (typically more than 10^6) were pelleted and fixed in 750μL TRI Reagent (Sigma T9424), with 20μL 5N acetic acid. Organic elements were removed by addition 100μL 1-Bromo-3-Chloropropane (Sigma B9673), mixing, 4° Celsius 12500RPM centrifugation, and careful removal of the aqueous layer. RNA was when precipitated in isopropanol and washed in 70% ethanol with DEPC-treated water. RNA pellets were dried and resuspended in DEPC water at 65° Celsius. To make cDNA, 2μg of RNA was

first treated with DNase I (1UI/µL) for 30 minutes at 37° Celsius. DNase I was inactivated by adding 25mM EDTA and heating the reaction to 65° Celsius for 10 minutes. Complementary DNA (cDNA) was generated using the High Capacity Reverse Transcription Kit (Applied Biosystems; Catalog: 4368814). The thermocycler conditions were as follows: 25° Celsius for 10 minutes, 37° Celsius for 120 minutes, and 85° Celsius for 8 seconds. The reaction was then diluted six-fold and 4µL of the diluted RT reaction was used for the PCR reaction. RT-PCR primers were obtained from IDT or Applied Biosystems and resuspended to a 20x concentration. PCR reactions were performed in x2 Taqman Fast Advanced Master Mix (Applied Biosystems, catalog: 4444557). Housekeeping β-actin transcript primers were labeled with HEX/VIC dye, and experimental transcripts were amplified with FAM-labeled probes in duplex. Each assay was performed in triplicate. Final qPCR reactions were as follows: 4µL cDNA product, 1µL housekeeping gene primer, 1µL target gene primer, 4µL water, and 10µL Fast Advanced Master Mix (Applied Biosystems, catalog: 4444557). RT-PCR reactions were performed on a Quantstudio 6 Flex machine (Applied Biosciences). Cycling conditions were: 2 minutes at 50° Celsius, 20 seconds at 95° Celsius, and 40 cycles of 1 second at 95° Celsius for and 20 seconds at 60° Celsius. Data were analyzed using ΔΔCt to determine relative transcript levels.

shRNA Target	Species	Lab Name	Vendor	Catalog Number Sequence (5'-3')	Vector (Selection)	Knock-down
IRAK1	Human	A	Sigma-Aldrich	1. TRCN0000000543 2. CCGGCCCTCCTACCTGCTTACAATTCTCG AGAATTGTAAGCAGGTAGGAGGGTTTTT	pLKO.1 (Puromycin)	Yes
IRAK1	Human	B	Sigma-Aldrich	1. TRCN0000121238 2. CCGGCATCGCCATGCAGATCTACAACTCG AGTTGTAGATCTGCATGGCGATGTTTTTG	pLKO.1 (Puromycin)	Yes
IRAK1	Human	C	Sigma-Aldrich	1. TRCN0000000544 2. CCGGGCCCGAAGAAAGTGATGAATTCTCG AGAATTCATCACTTTCTTCGGGCTTTTT	pLKO.1 (Puromycin)	No
IRAK1	Human	D	Sigma-Aldrich	1. TRCN0000121138 2. CCGGGCCACCGCAGATTATCATCAACTCG AGTTGATGATAATCTGCGGTGGCTTTTTG	pLKO.1 (Puromycin)	No
IRAK1	Human	E	Sigma-Aldrich	1. TRCN0000121239 2. CCGGGAACACGGTGTATGCTGTGAACTCG AGTTCACAGCATACACCGTGTTCTTTTTG	pLKO.1 (Puromycin)	No
Irak1	Mouse	A	Sigma-Aldrich	1. TRCN0000011877 2. CCGGCCTCAACGACTGGACATTCTTCTCGA GAAGAATGTCCAGTCGTTGAGGTTTTT	pLKO.1 (Puromycin)	Yes
Irak1	Mouse	B	Sigma-Aldrich	1. TRCN0000055088 2. CCGGCAAGCAGAGTACCTAAGGAATCTCG AGATTCCTTAGGTACTCTGCTTGTTTTTG	pLKO.1 (Puromycin)	Yes
Irak1	Mouse	C	Sigma-Aldrich	1. TRCN0000055089 2. CCGGGACACCGATACCTTCAGCTTTCTCGA GAAAGCTGAAGGTATCGGTGTCTTTTTG	pLKO.1 (Puromycin)	No
Irak1	Mouse	D	Sigma-Aldrich	1. TRCN0000271170 2. CCGGTACCGAGCAGTCATGAGAAATCTCG AGATTTCTCATGACTGCTCGGTATTTTTG	pLKO.1 (Puromycin)	Yes

Irak1	Mouse	E	Sigma-Aldrich	1. TRCN0000284313 2. CCGGTGGTGAAACAGAGCTTCTTAACTCGAGTTAAGAAGCTCTGTTTCACCATTTTTG	pLKO.1 (Puromycin)	No
Tbk1	Mouse	A	Sigma-Aldrich	1. TRCN0000353117 2. CCGGACATGACGGCGCATAAGATTTCTCGAGAAATCTTATGCGCCGTCATGTTTTTTG	pLKO.1 (Puromycin)	No
Tbk1	Mouse	B	Sigma-Aldrich	1. TRCN0000345144 2. CCGGCAGAACGCAGACTAGCTTATACTCGAGTATAAGCTAGTCTGCGTTCTGTTTTTG	pLKO.1 (Puromycin)	Yes
Tbk1	Mouse	C	Sigma-Aldrich	1. TRCN0000323444 2. CCGGCCAGAATCAGAATTTCTCATTCTCGAGAATGAGAAATTCTGATTCTGGTTTTTG	pLKO.1 (Puromycin)	Yes
Tbk1	Mouse	D	Sigma-Aldrich	1. TRCN0000345204 2. CCGGGGAACAGAACCGCGGTTTAACCTCGAGGTTAAACCGCGGTTCTGTTCCTTTTTG	pLKO.1 (Puromycin)	No
Tbk1	Mouse	E	Sigma-Aldrich	1. TRCN0000027015 2. CCGGCCAGAATCAGAATTTCTCATTCTCGAGAATGAGAAATTCTGATTCTGGTTTTT	pLKO.1 (Puromycin)	Yes
TICAM1	Human	A	Origene	1. TL308823A 2. TCATCTACTCCTTGTTCAGCTCACCTGAC	pGFP-C-shLenti (GFP)	Yes
TICAM1	Human	B	Origene	1. TL308823B 2. CAGGACGCCATAGACCACTCAGCTTTCAT	pGFP-C-shLenti (GFP)	No
TICAM1	Human	C	Origene	1. TL308823C 2. CCTCATTATCCACCACGCACAGATGGTAC	pGFP-C-shLenti (GFP)	No
TICAM1	Human	D	Origene	1. TL308823D 2. CGCCACTCCAACTTTCTGTAGAAGATACC	pGFP-C-shLenti (GFP)	No
Ticam1	Mouse	A	Origene	1. TL505757A 2. CTCAGGTTCCTGTAGGAGATGATTCTCTG	pGFP-C-shLenti (GFP)	Yes
Ticam1	Mouse	B	Origene	1. TL505757B 2. CTGGAGTCCTTGAAGATGAACACAGTAGC	pGFP-C-shLenti (GFP)	Yes
Ticam1	Mouse	C	Origene	1. TL505757C 2. TGATGACAAGACTGAGTGTTCGGAGAACC	pGFP-C-shLenti (GFP)	Yes

Ticam1	Mouse	D	Origene	1. TL505757D 2. AGCTGCACTGTCTCCAAGATGCCATCGAT	pGFP-C-shLenti (GFP)	Yes

Table 2: Details for shRNA Sequences and Vectors. This table details the shRNA vectors, sequences, respective targets, annotation, vector and mammalian selection marker of all shRNA applied in this dissertation. The "Lab Name" corresponds to the annotation used for each shRNA throughout all experiments described here. Lastly, the catalog number and respective vendors are noted.

CHAPTER THREE

RESULTS

TLR Signaling Components, including MYD88 and Downstream IRAK1 are Upregulated and Associated with Poor Patient Survival in the TCGA Database

Previously, microarray analysis was applied to 580 adult primary patient AML samples by with Jianjun Chen, Ph.D., at the University of Cincinnati (Figure 4) to determine whether TLRs and downstream mediators are expressed in acute myeloid leukemia (AML) subtypes. The microarray showed that TLR1, TLR2, TLR4, and TLR5 are overexpressed in M4 and M5 leukemia. Additionally, both MYD88 and TICAM-1 are overexpressed in M4 and M5 leukemia. To determine whether TLRs and associated signaling pathways are overexpressed in other publicly available datasets, relative transcript levels were assayed using the Oncomine™ Platform.[188] These findings were confirmed in the published Wouters Leukemia dataset using Oncomine™ (Figure 5A).[189] The Balgobind dataset showed an enrichment of TLR expression in MLL-mutated AML compared to AML in which MLL is not mutated (Figure 5B).[190] However, despite established findings of increased inflammatory signaling with advancing age, surveying Oncomine yielded no correlation between TLR signaling components with any age at diagnosis in the leukemic clone (Figure 5C).

To determine whether signaling mediators in this pathway are associated with leukemia progression and outcomes, I surveyed IRAK1, IRAK1 binding protein 1 (IRAK1-BP1), and

PELLINO-1 using the Bloodspot™ TCGA dataset browser.[191] Consistent with the hypothesis that MYD88-dependent signaling promotes non-acute promyelocytic leukemia (APL) pathogenesis, overexpression of key mediators, IRAK1 and PELLINO-1 (Figure 6A and 6C), required for MYD88-dependent signaling correlates with worse survival by Log-Rank statistical analysis. In contrast, overexpression of a negative regulator of IRAK1 signaling, IRAK1-BP1, correlates with more favorable survival outcomes (Figure 6B). Lastly the engagement of the IKK complex by IRAK1 also correlates with poor survival (Figure 6D). To determine whether expression of MYD88-independent signaling factors correlate with survival, TBK1, TRAF3 activation, and TICAM-1 were surveyed using the Bloodspot™ TCGA browser.[191] High expression of TBK1 and TRAF3 correlates with poor survival when divided along median expression (Figure 7A and 7B). Expression of TICAM-1 does not correlate with better or worse survival across the whole TCGA dataset. When the TCGA dataset is narrowed to the samples with the highest 10% or 25% of TICAM-1 expression, it correlates with favorable survival when compared against the bottom 10% or 25% of samples (Figure 7C and 7D). Lastly TICAM-1 is amplified in a small subset of AML samples. Within the TCGA dataset, TICAM-1 is amplified in 1% of samples, and within the recently published TARGET dataset, it is amplified in 3% of samples, representing the second most frequently amplified gene in the TARGET AML dataset (Figure 8A and 8B). Importantly, when *TICAM-1* expression was assayed, gain or amplification of *TICAM-1* resulted in a 0.5-1.0 log increase in expression (Figure 8C). When TICAM-1 was surveyed in other available datasets, including acute lymphoblastic leukemia, chronic lymphoblastic leukemia, myelodysplasia, and chronic myeloid leukemia, no such copy number amplification was observed.

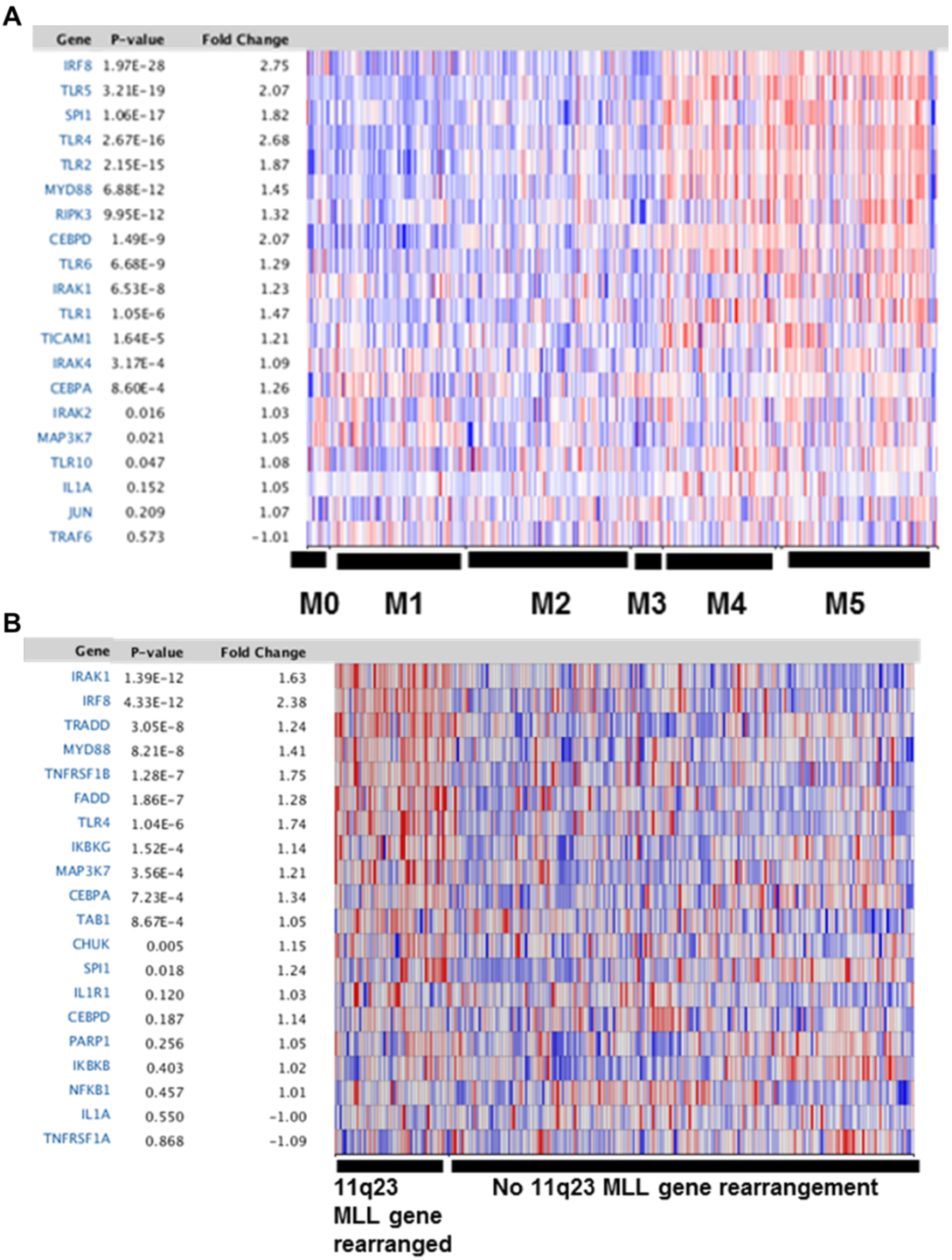
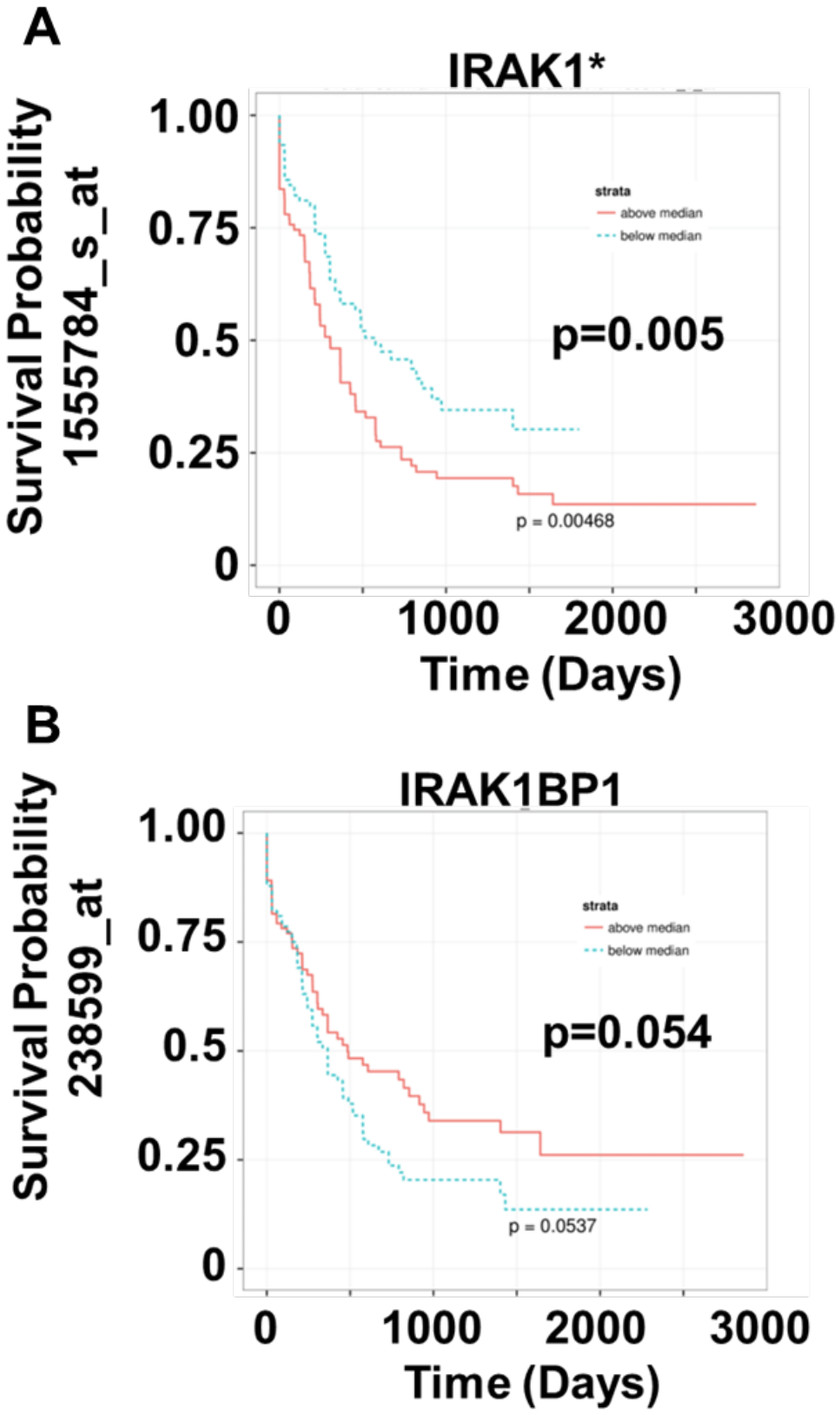


Figure 5. Toll-like Receptor Pathways are Upregulated in Myelomonocytic and Monocytic AML Subtypes and MLL-Rearranged AML. (A) Published Wouters leukemia dataset (GSE14468) reveals upregulation of TLR signaling in myelomonocytic (M4) and monocytic (M5) subtypes. P-values represent fold change of gene expression in M5 group to dataset average. (B) Published Balgobind leukemia dataset (GSE17855) shows specific enrichment of TLR signaling in MLL1-rearranged genes.



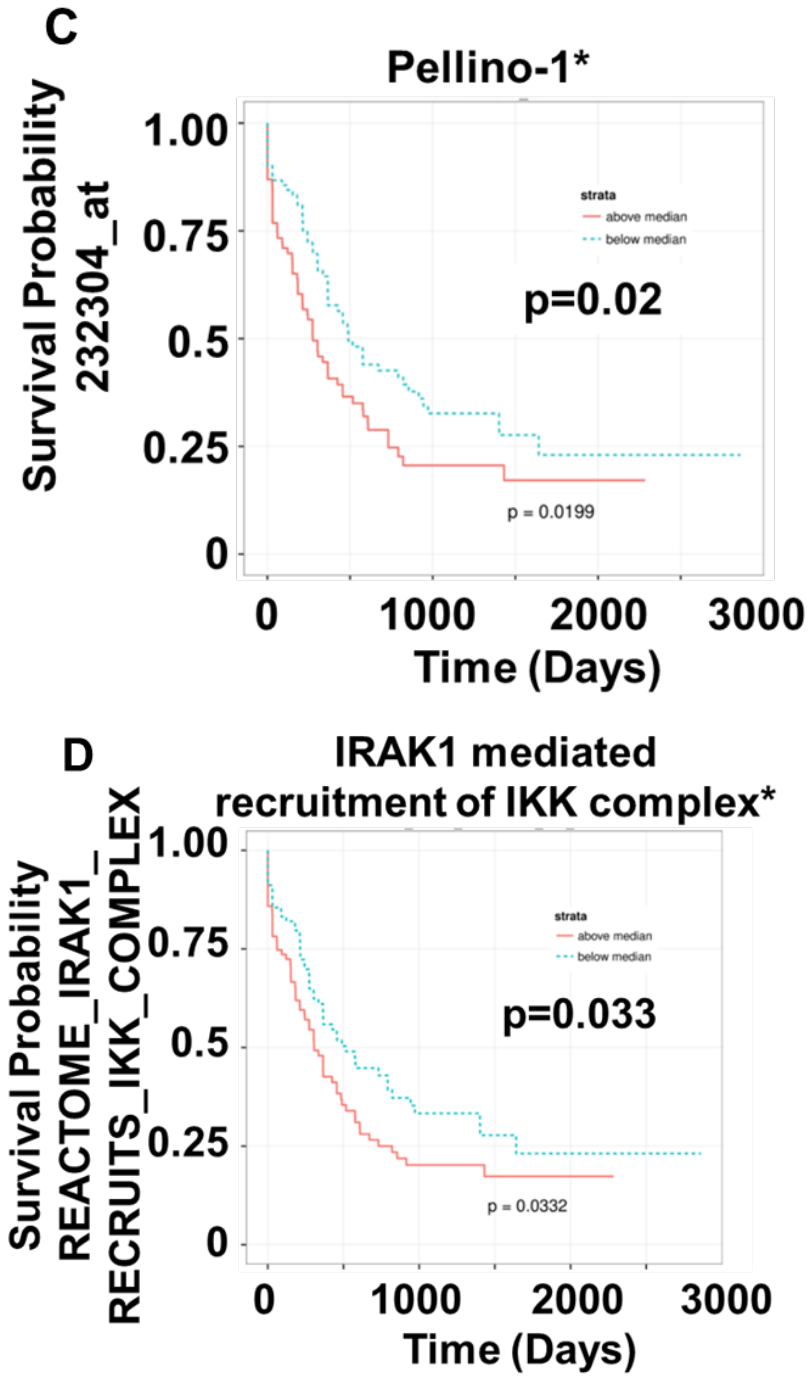
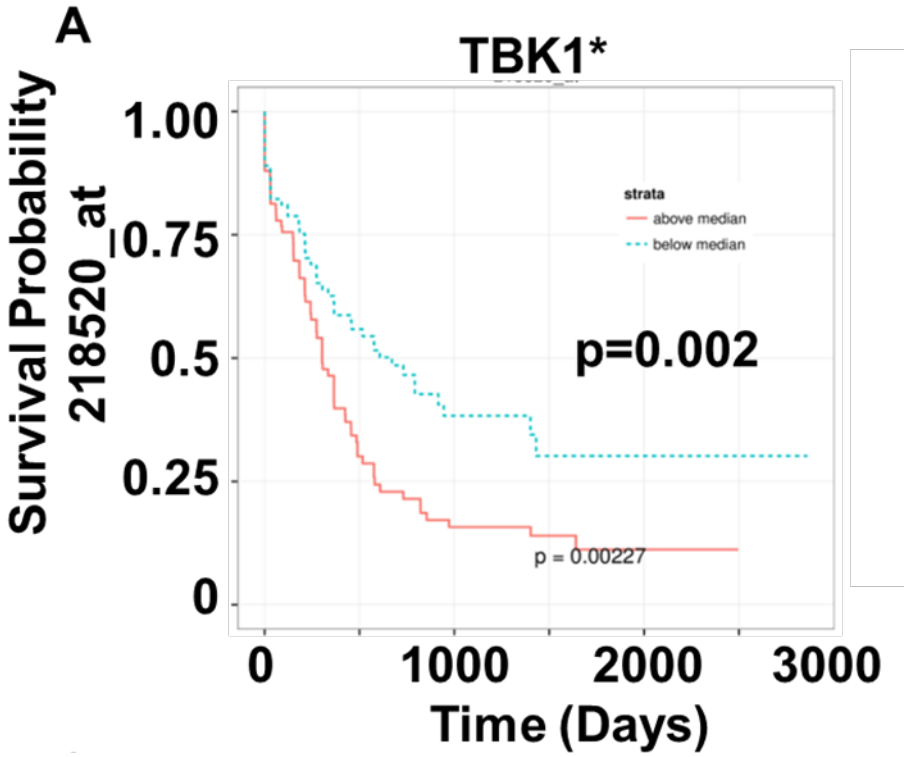
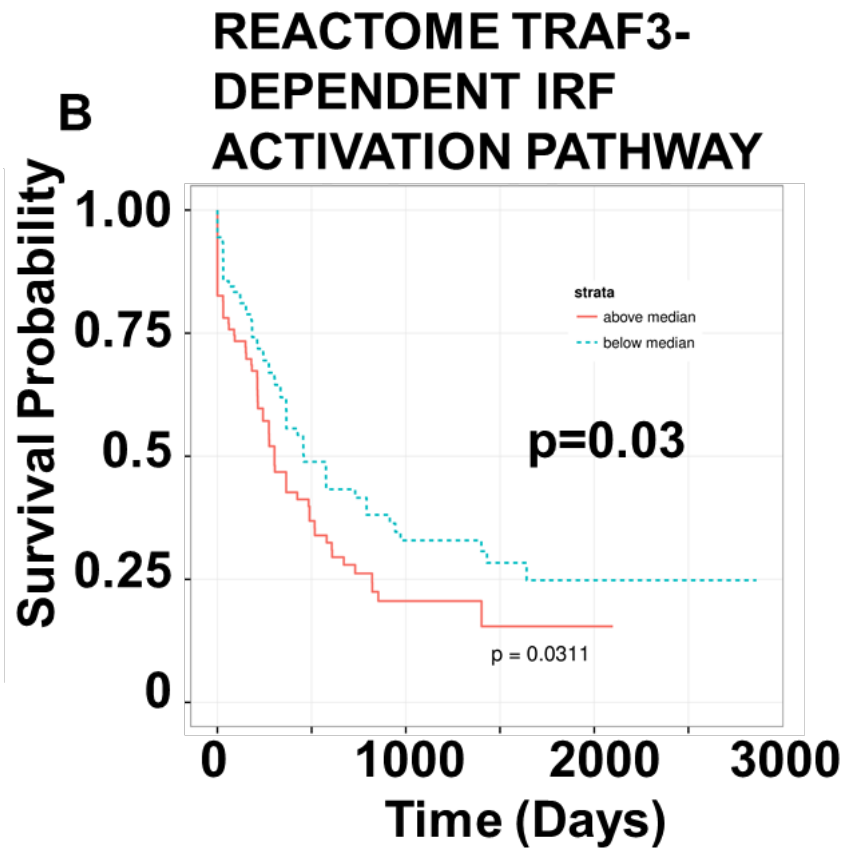


Figure 6. TCGA Dataset Shows Survival Correlation with IRAK1 and IRAK1-Associated Genes in AML. Transcript expression of IRAK1 and IRAK1-related genes across all AML samples in the TCGA dataset. Asterisks in the heading indicate a Log-rank analysis p-value<0.05. (A) Survival of TCGA AML patients is prolonged for patients with below-median expression of IRAK1 (blue, dashed), and shortened for patients with above-median expression of IRAK1 (red, solid). (B) High expression of IRAK1BP1, a negative regulator of IRAK1, is associated with favorable survival. (C and D) Pellino-1 expression and gene signature corresponding to IKK complex activity correlate with poor survival in TCGA AML samples.





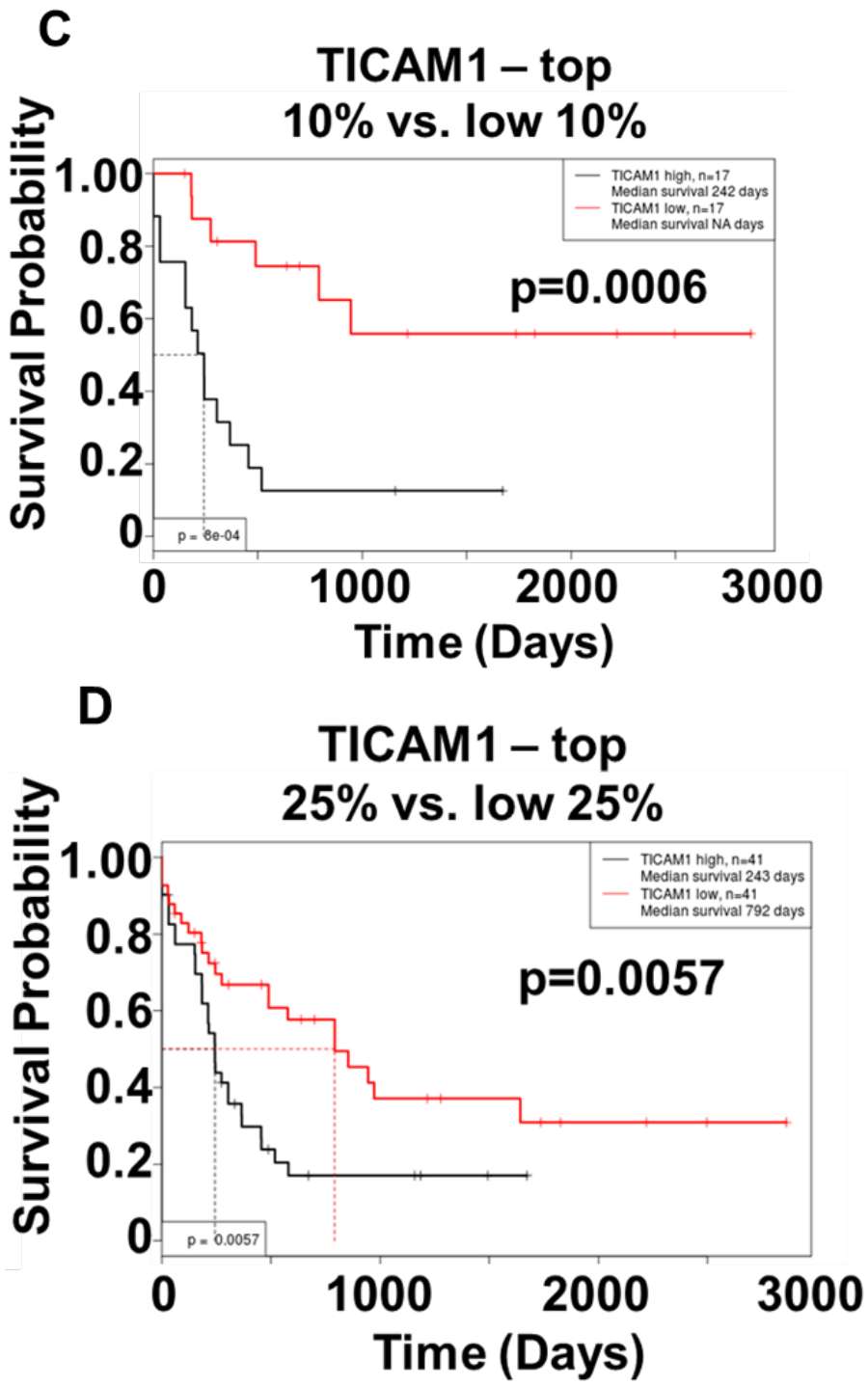


Figure 7. TCGA Survival Analysis Correlates with TICAM-1 Dependent Signaling Components. (A) TBK1 expression correlates with poor survival when compared between the upper and lower expression medians. (B) Gene signatures in the high-expressing median corresponding to TRAF3-IRF3 activation correspond with poor survival compared to those in the low expression median. (C and D) High TICAM-1 expression (black) correlates with poor survival compared to low expression (red). The survival of the highest 10% or 25% TICAM-1 expression was compared against the respective lowest 10% or 25% within the TCGA dataset.

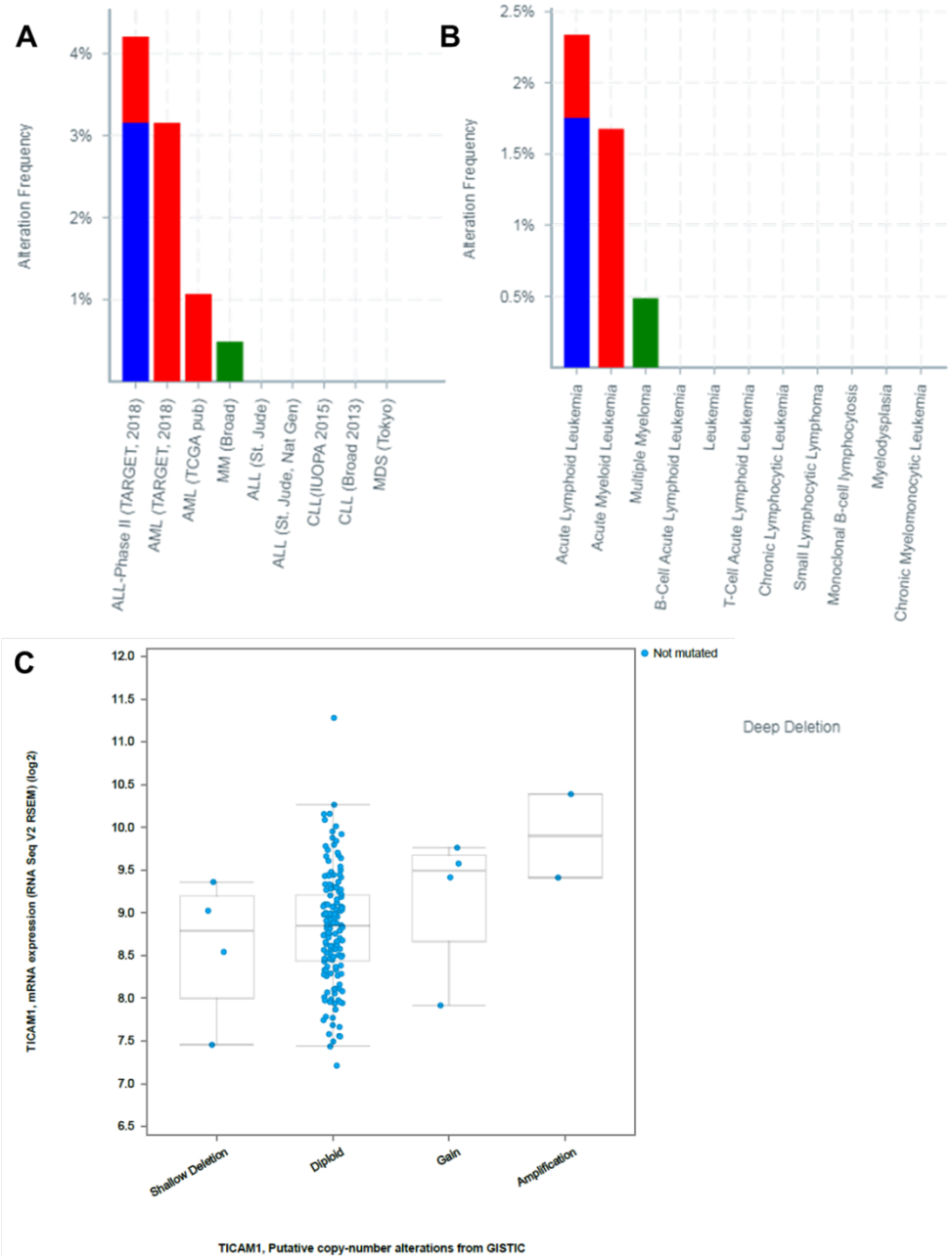


Figure 8. TICAM-1 is Recurrently Amplified in Separate AML Datasets. (A) Frequency of TICAM-1 amplification or mutation by selected study as indicated. (B) Composite frequency of TICAM-1 amplification, mutation, or deletion in leukemia types across the same study sample. (C) TCGA *TICAM-1* expression sorted by copy number amplification. Data was obtained and visualized using cBioPortal™.

Toll-like Receptors are Expressed in Human and Murine Cell Lines

Next, to determine whether TLR1, TLR2, TLR4, and TLR5 are expressed in leukemia model systems, we applied cell surface staining, using fluorescent antibodies. Consistent with transcriptional data, TLR1, TLR2, and TLR4 were detected and expressed at the cell surface for every cell line tested (Figure 9A, 9B, 9C). TLR5 is only expressed in the Mono-Mac-6 (MM6), THP1, NB4, and HL60 cells (Figure 9A). The TLR expression of murine MLL-AF9 cells mirrored that of several human cell lines in that TLR1, TLR2, and TLR4 were detected, but not TLR5 (Figure 9C). Therefore, TLR expression is conserved in M4/M5 human cell lines and murine MLL-AF9 leukemia cells.

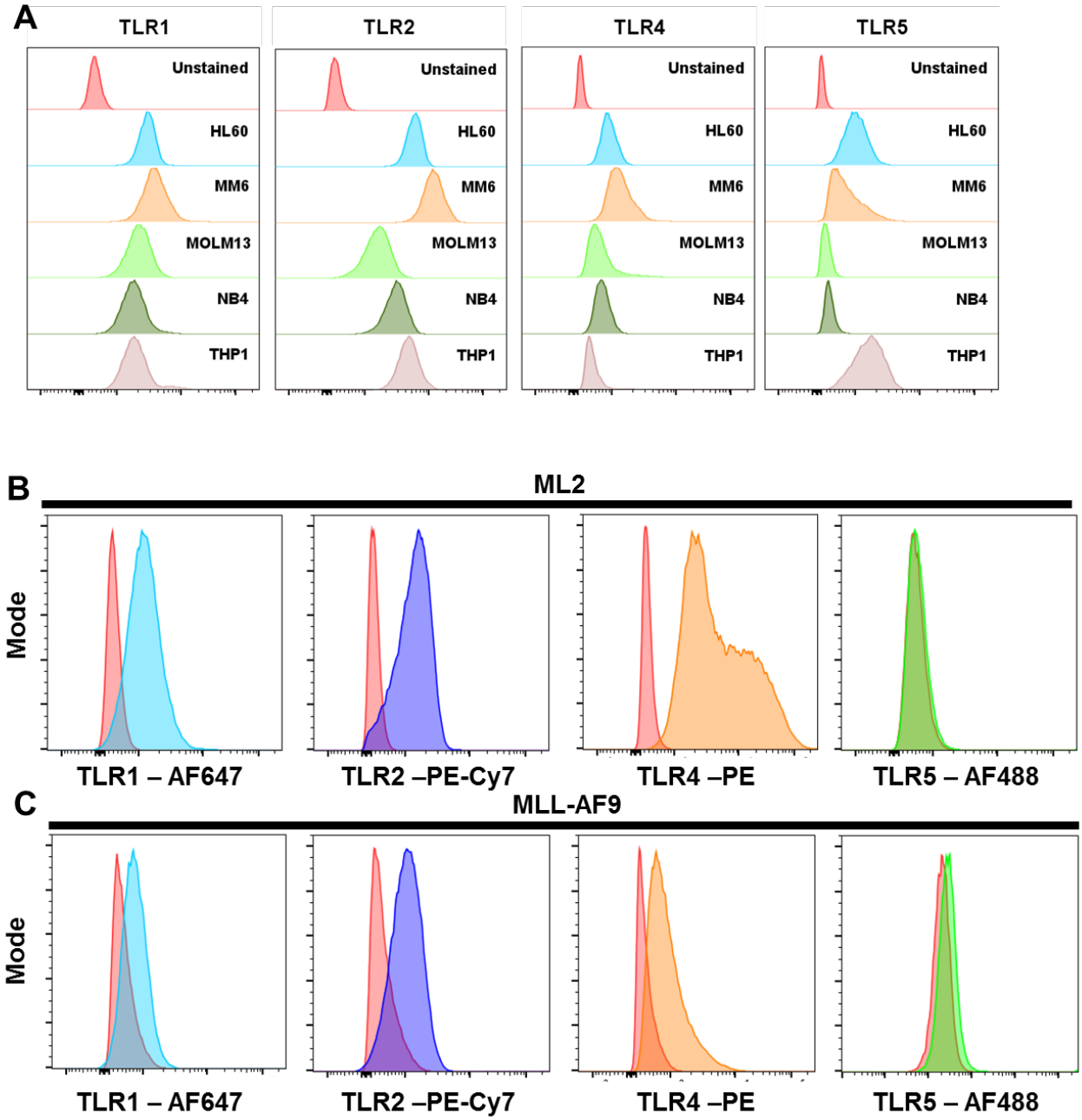


Figure 9. Cell Surface TLRs are Expressed on Human Cells and Transformed Murine Models. (A) Compared to unstained controls, TLR1, TLR2, and TLR4 are expressed on human HL60, MM6, Molm13, NB4, and THP1 cell lines. TLR5 is expressed on all cell lines except Molm13 and NB4. (B) Human ML2 cell line expresses TLR1, TLR2, and TLR4, but not TLR5. (C) Murine MLL-AF9 leukemia cells express TLR1, TLR2, and TLR4, but not TLR5.

Germline *Myd88* is Not Required for Transformation and Colony-Forming Efficiency when Transformed with Fusion Proteins Reflecting MLL-Rearrangement or Core-Binding Factor Mutations

Having shown that human and murine leukemia cell models are sensitive to IRAK inhibition, I hypothesized that leukemia cells might find a way to evade such IRAK inhibition via a novel resistance mechanism. To study the effect of complete loss of MYD88-dependent signaling in a murine model, I devised two genetic approaches. First, *Myd88*-null (*Myd88*$^{-/-}$) pre-leukemia cells were derived from *Myd88* knockout mice in parallel with leukemia cell derivation from *Myd88*-wildtype (*Myd88*$^{+/+}$) control mice (Figure 10A and 10B). Four different fusion proteins were expressed by retroviral expression to first generate pre-leukemia cells (PLCs), which were transplanted into mice and later harvested as leukemia cells (LCs) upon leukemia onset. Interestingly, neither MLL-AF9 *Myd88*$^{-/-}$ PLCs nor *Myd88*$^{-/-}$ LCs showed a statistical difference in the colony forming ability when compared to *Myd88*$^{+/+}$ cells. MLL-ENL, CBFβ-MYH11, and RUNX1-ETO PLCs were also derived and their clonogenic potential was measured by CFU assays. Like MLL-AF9 PLCs, MLL-ENL cells showed no loss of colony forming ability when derived from *Myd88*$^{-/-}$ mice and compared to *Myd88*$^{+/+}$ leukemia cells. Interestingly, both RUNX1-ETO and CBFβ-MYH11 PLCs demonstrated an increased colony number with *Myd88*$^{-/-}$ PLCs, compared to *Myd88*$^{+/+}$ PLCs (Figure 10C). Further study of *Myd88*$^{-/-}$ and *Myd88*$^{+/+}$ leukemia cells, following primary transplant of PLCs into mice, indicates that germline *Myd88*-null leukemia cells form colonies as efficiently as Myd88-expressing leukemia cells (Figure 10D). However, the remaining TLR signaling mediators are dysregulated in *Myd88*$^{-/-}$ PLCs, as Ticam-1 protein levels are dramatically induced in the *Myd88*-null PLC background (Figure 10E). Based on this data, I concluded that loss of Myd88-dependent signaling prior to

introduction of the leukemia MLL-fusion gene fails to limit leukemia cell function, as measured by CFU assays. However, it also fails to accurately represent the effect of IRAK inhibition on leukemia cells, since these *Myd88*$^{-/-}$ MLL-AF9 leukemia cells may be quite different from *Myd88*$^{+/+}$ MLL-AF9 cells.

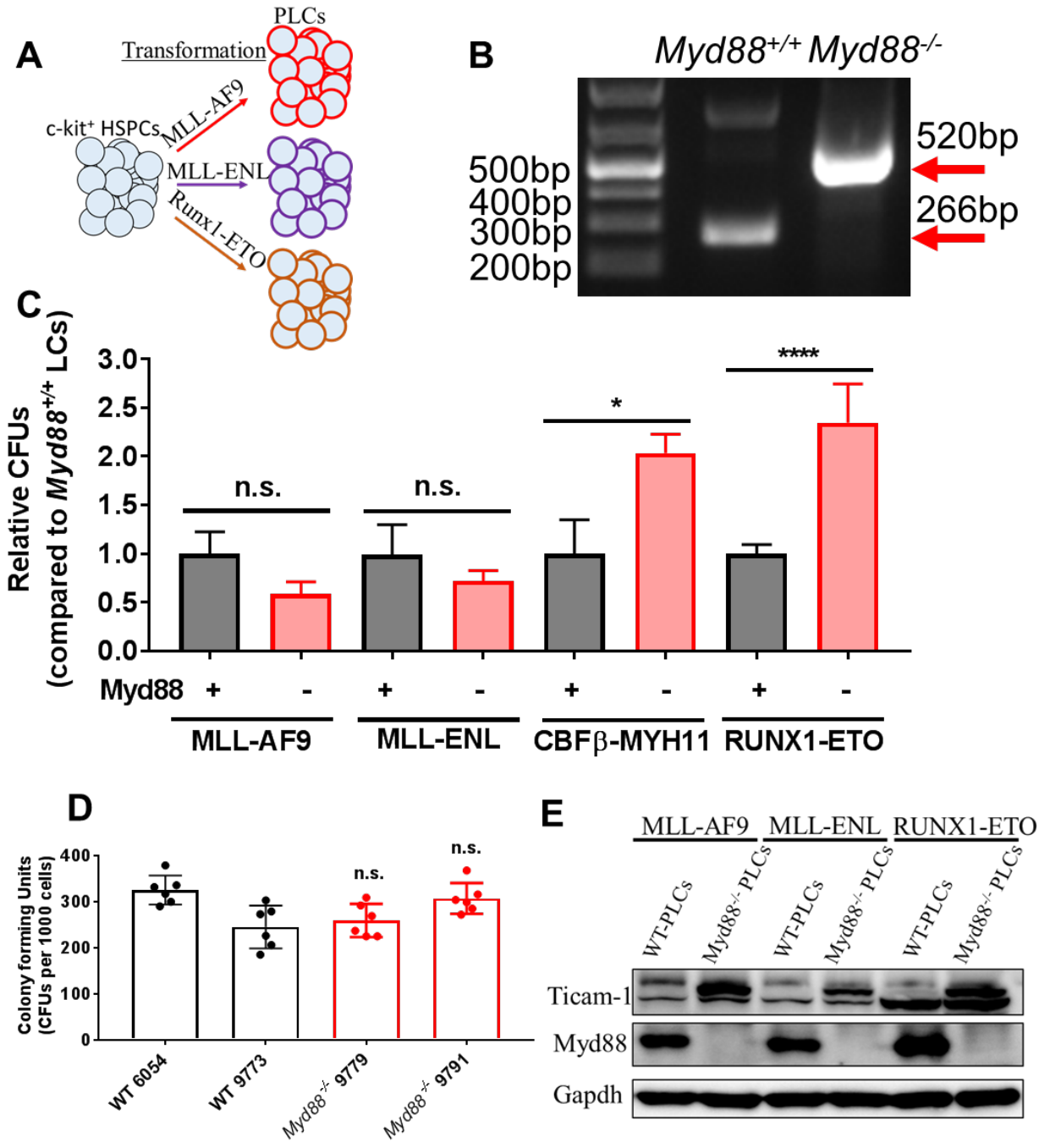


Figure 10. Myd88 is Not Required for AML Transformation and Exerts Fusion Protein-Specific Effects. (A) Experimental scheme. (B) Genotyping of mice prior to retroviral fusion protein infection. (C) Colony forming efficiency of transformed Myd88$^{-/-}$ pre-leukemia cells relative to Myd88$^{+/+}$ controls. Relative CFU efficiency was compared using a Student's t-test, where n.s. indicates $p>0.05$, * indicates $p<0.05$ and **** indicates $p<0.0001$. (D) Colony forming efficiency of leukemia cells (E) Immunoblot of MLL-rearranged and RUNX1-ETO transformations confirming Myd88 deletion and assaying Ticam-1 levels.

Cre-LoxP Inducible *Myd88* Deletion Models Disruption of Irak1/4 Signaling in MLL-AF9 Cells and Results in a Competitive Disadvantage

To accurately model the effect of chronic IRAK inhibition and lost MYD88-dependent signaling by way of *Myd88* deletion, I applied the Cre-Lox system. The Cre-Lox system utilizes recombinant genetic engineering to place conserved LoxP sequences such that they flank key genes or exons in noncoding regions. In the presence of flanking LoxP sites, expression of the bacterial Cre recombinase results in deletion of the DNA interval in between the two LoxP sites. Mice with such LoxP sites flanking exon 3 of *Myd88* (*Myd88*$^{fl/fl}$, Figure 11A) were generously provided to us for experimentation by Katherine Knight, Ph.D. and Dominick Kennedy, Ph.D. These mice bear the genotype described by and commercially available from the Jaxson Laboratory. Using these mice, *Myd88*$^{fl/fl}$ MLL-AF9 leukemia cells were derived by retroviral MLL-AF9 expression and transplantation into a primary host most as previously described (Figure 11B). Introduction of Cre-recombinase by retroviral expression was shown to efficiently and reliably delete *Myd88*, yielding *Myd88*$^{\Delta/\Delta}$ cells (Figure 11C and 11D). To determine whether short-term *Myd88* deletion alters the MLL-AF9 phenotype, Migr1-GFP empty vector controls (EV) and Migr1-GFP-Cre vectors were expressed by retrovirus in *Myd88*$^{+/+}$ controls and *Myd88*$^{fl/fl}$ MLL-AF9 leukemia cells. Based on whether the infected fraction of GFP^{+} Cre^{+} *Myd88*$^{\Delta/\Delta}$ population remained level or declined relative to levels measured at 2 days post-infection (DPI), I could infer whether *Myd88* deletion is dispensable or required for MLL-AF9 leukemia pathogenesis. Compared to control infections, the GFP^{+} Cre^{+} *Myd88*$^{\Delta/\Delta}$ population in infected *Myd88*$^{fl/fl}$ MLL-AF9 cells, demonstrated a decline, indicating that Myd88 expression and signaling are required for MLL-AF9 leukemia cell survival and a competitive growth advantage. Because prior experiments by Li *et al.*[182] have shown that joint inhibition of IL-1β

and TNFα signaling suppresses leukemia cell stemness features, I hypothesized that TNFα treatment might rescue the lost GFP$^+$ Cre$^+$ *Myd88*$^{\Delta/\Delta}$ population, and also performed the experiment in the presence of TNFα (Figure 11E). TNFα treatment partially, but not completely rescues the GFP$^+$ Cre$^+$ *Myd88*$^{\Delta/\Delta}$ population. The elevated GFP$^+$ fraction in TNFα-treated infections is lost over time and is comparable to untreated *Myd88*$^{-/-}$ MLL-AF9 leukemia cells by 8 days after infection. In conclusion, *Myd88* deletion, and consequent loss of Myd88-dependent signaling, suppresses murine MLL-AF9 leukemia cell growth. *Myd88* deletion also exerts a dominant effect over TNFα signaling such that TNFα treatment fails to rescue this phenotype. Thus, I sought to further interrogate downstream Myd88-dependent signaling and characterize the phenotype of *Myd88*-null leukemia cells.

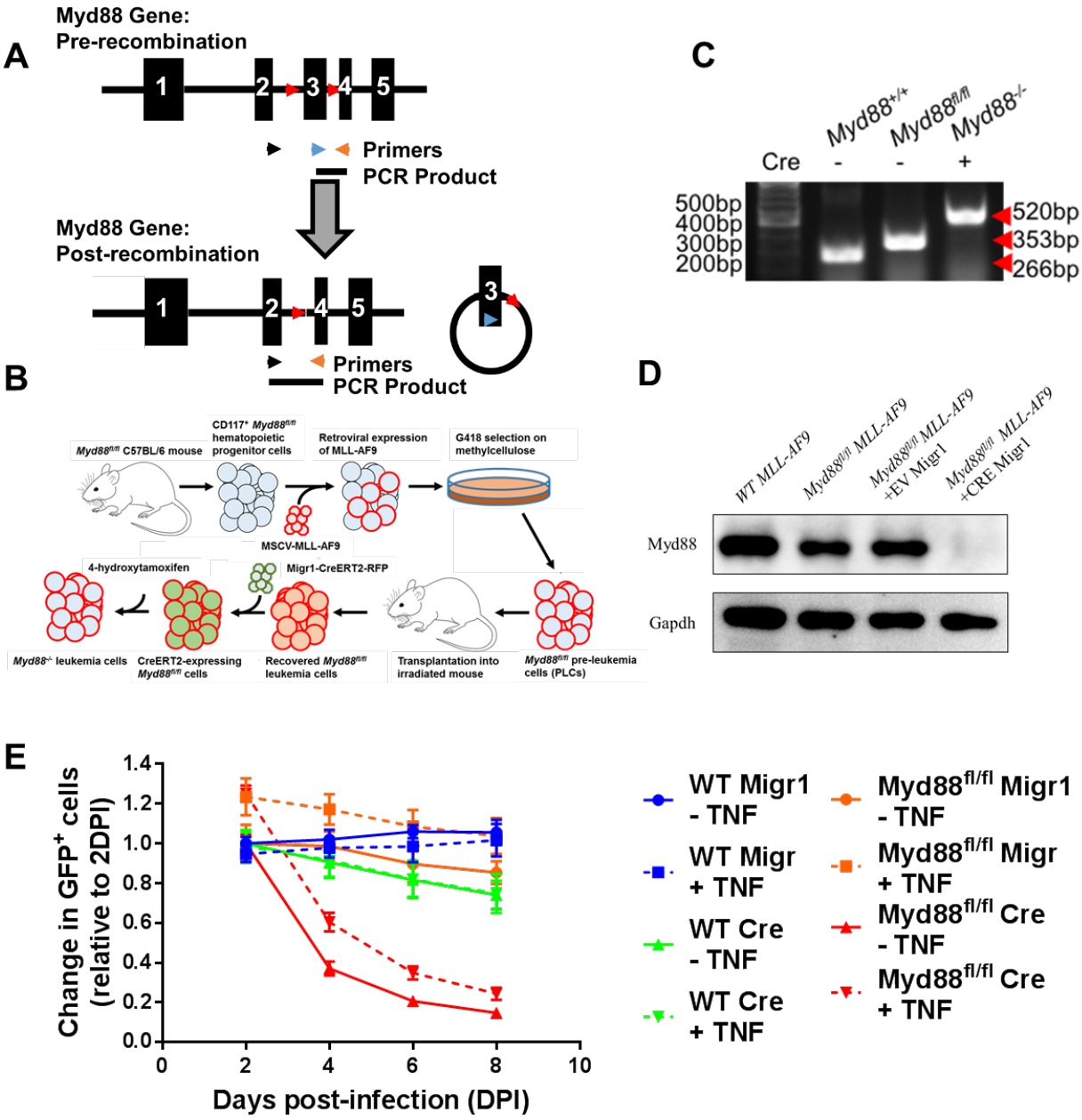


Figure 11. Inducible *Myd88* Deletion Results in Competitive Disadvantage in *in vitro* Culture. (A) Scheme for specific deletion of *Myd88* exon 3, including primer location and base pair length of murine intron and exon sequences. (B) Scheme for derivation of *Myd88*$^{fl/fl}$ leukemia cells. (C) PCR genotyping verification of efficient *Myd88* deletion after retroviral Cre recombinase expression and sorting. (D) Immunoblot of designated cells to confirm loss of Myd88 protein levels. (E) Cre recombinase or empty vector Migr1 were expressed in *Myd88*$^{+/+}$

Myd88 Deletion Fails to Reduce Myd88-Dependent Signaling to Jnk and NF-κB

Additionally, the Cre^{+} *Myd88*$^{\Delta/\Delta}$ leukemia cells were used to determine whether any significant loss of downstream signaling pathways occurred in the Cre^{+} *Myd88*$^{\Delta/\Delta}$ cell lines. Downstream Myd88 signaling is characterized by IRAK4 auto-phosphorylation in the activation domain induced by oligomerization with Myd88 assembled at the TLR or IL-1R undergoing stimulation. First, 30 minute IL-1β stimulation was used to demonstrate that Cre^{+} *Myd88*$^{\Delta/\Delta}$ cells were unable to induce IRAK4 phosphorylation (Figure 12A). Despite this, downstream Jnk and NF-κB signaling were unchanged after 30 minute stimulation at 20ng/mL or 50ng/mL (Figure 12B). Despite our initial hypothesis that MYD88-dependent signaling primarily acts through established, canonical Jnk and NF-κB signaling pathways, immunoblotting data obtained from mouse indicate that the pro-leukemic effect of MYD88 signaling occurs through a pathway independent of both NF-κB and Jnk.

Despite this, treatment of murine Cre^{+} *Myd88*$^{\Delta/\Delta}$ and *Myd88*$^{+/+}$ murine leukemia cells with high doses of TLR ligands elicit downstream activation of NF-κB and Jnk, in an Myd88-dependent manner (Figure 12C and 12D). However, Jnk activation is dependent on Myd88 expression while NF-κB is activated in the presence or absence of Myd88 when Ticam-1 independent signaling is induced. Consistent with a lack of TLR5 detected by cell surface staining (Figure 7C), bacterial flagellin failed to induce any downstream signaling. In the course of these studies, Tbk1 was found to be induced in a Tlr2-Myd88 dependent manner (Figure 12E). Tlr2-Myd88-TICAM-1-Tbk1 induction was later published in the literature using a peritoneal macrophage model.[78] These findings led me to hypothesize that chronic IL-1β treatment promotes leukemia cell growth. However, when co-cultured with recombinant murine IL-1β, murine MLL-AF9 transformed cells demonstrate no change in cell growth (Figure 13A). Further, co-culture with

LPS, results in Cre^{+} $Myd88^{\Delta/\Delta}$ leukemia cell number suppression, relative to untreated cells (Figure 13B). In conclusion, while TLR signaling may induce downstream NF-κB and Jnk activity, Myd88 is not required to maintain its expression. As in previous studies on IL-1β and TNFα, I hypothesized that the primary mechanism of pathogenesis via MYD88-dependent signaling was by activation of downstream NF-κB transcription factors and JNK kinases, and AP-1 transcription factors by extension. While high doses of LPS and PAM3CSK4 were sufficient to induce NF-κB activation by phosphorylation and JNK phosphorylation in the presence of Myd88, IL-1β stimulation failed to induce a likewise activation of these downstream signaling pathways.

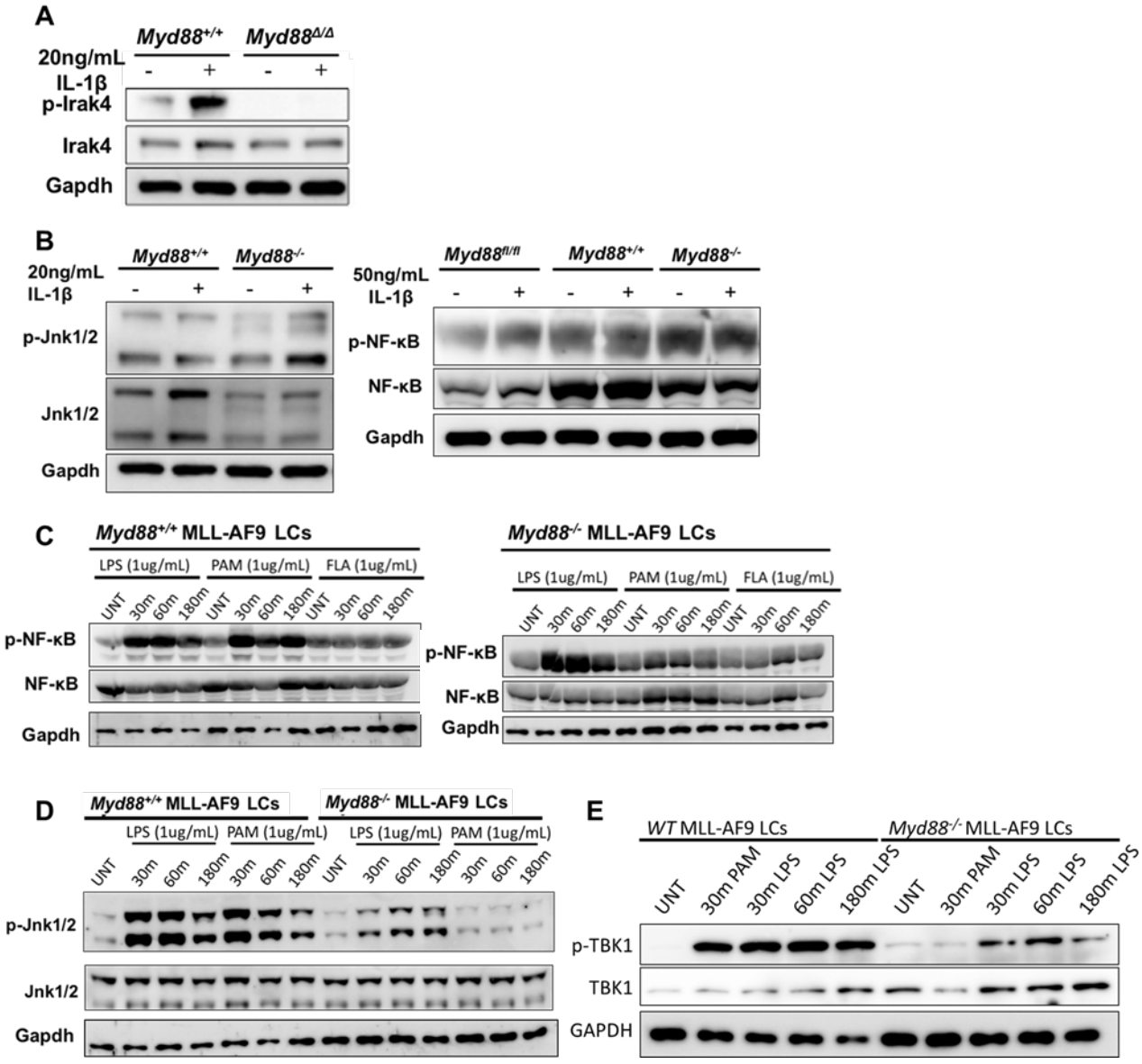


Figure 12. Downstream TLR Ligand and IL-1β Signaling is Lost in *Myd88*$^{-/-}$ MLL-AF9 Cells. (A) Immunoblot of IRAK4 phosphorylation after 30 minute 20ng/mL IL-1β treatment in *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 cells. (B) Downstream Jnk and NF-κB phosphorylation after IL-1β treatment at 20ng/mL or 50ng/mL for 30 minutes in *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 cells. (C) Immunoblot of NF-κB phosphorylation in *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 cells after treatment with LPS, PAM3CSK4, or bacterial flagellin. (D) Immunoblot of Jnk phosphorylation in *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 cells after treatment with LPS, PAM3CSK4, or bacterial flagellin. (E) Immunoblot of phosphorylated Tbk1, Tbk1, and Gadph following timed treatment of *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 cells with 1ug/mL LPS or 1ug/mL PAM3CSK4 (PAM).

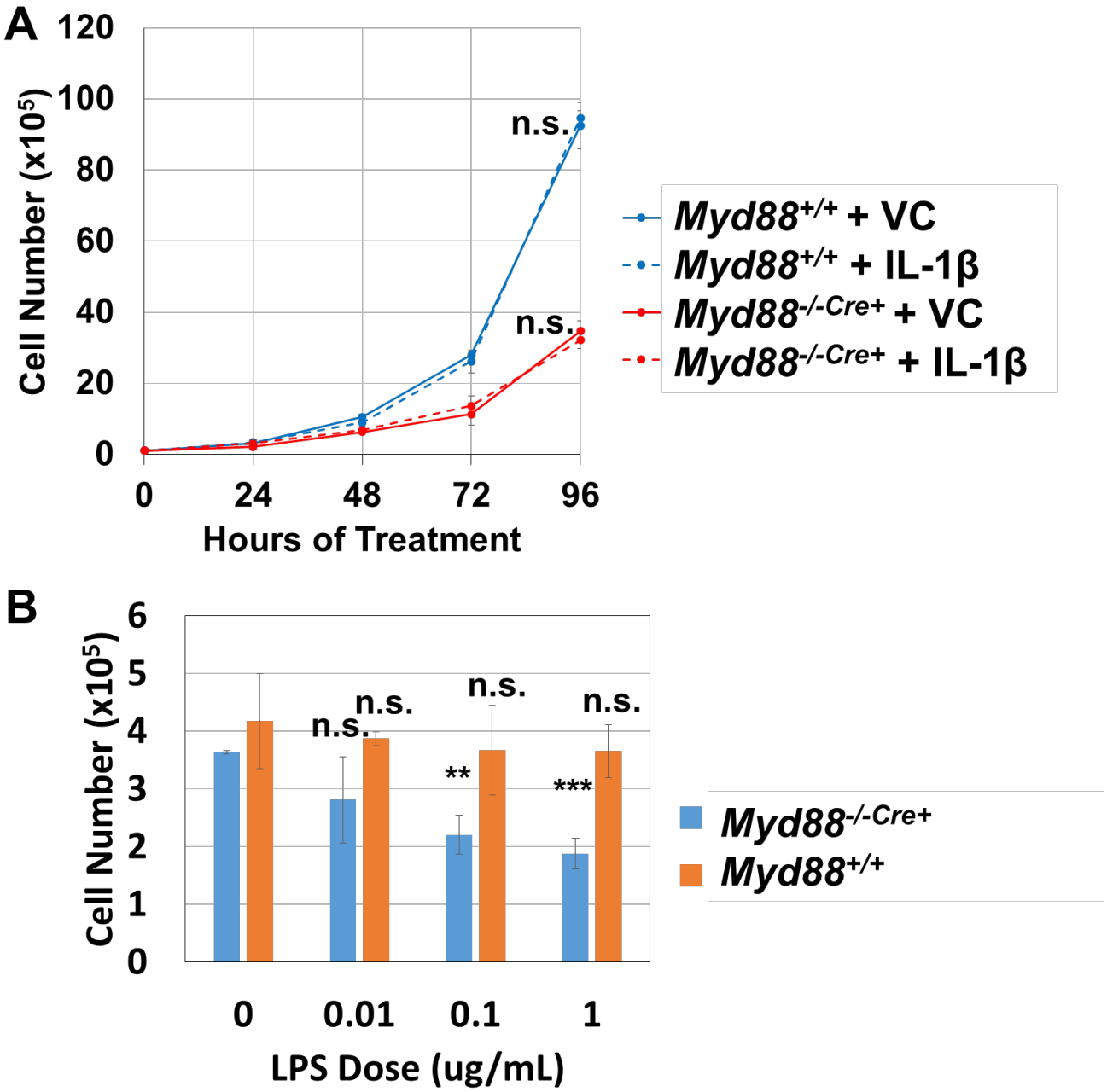


Figure 13. Chronic Stimulation with IL-1β has no Effect on Cell Growth, and LPS Specifically Suppresses *Myd88*$^{-/-Cre+}$ MLL-AF9 Cell Growth. (A) Growth curve with and without 20ng/mL IL-1β treatment in suspension culture. (B) Growth curve with and without designated doses of LPS in suspension culture. P-values are derived from Student's t-test comparison to untreated controls. N.S. indicates $p>0.05$, * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$.

Cre Toxicity Limits Colony Forming Potential

To determine whether intact genomic *Myd88* and Myd88-dependent signaling are required for colony forming ability, I likewise expressed Migr1-mCherry-Cre in *Myd88*$^{fl/fl}$ MLL-AF9 cells and control *Myd88*$^{+/+}$ MLL-AF9 cells. Within 48 hours of infection, mCherry^{+} cells were sorted by FACS and plated in M3434 methycellulose (*StemCell*) at 1000 cells per mL. Cre-expression demonstrated a clear toxicity, reducing the colonies of both the *Myd88*$^{fl/fl}$ MLL-AF9 cells and control *Myd88*$^{+/+}$ MLL-AF9 cells tenfold relative to controls expressing Migr1-EV (Figure 14A). The negative effect of Cre expression on colony formation remains when Cre-infect cells were kept in suspension culture and plated three weeks after infection (Figure 14B)

To address this issue, I used a variant of Cre recombinase with a fusion to a modified estrogen receptor, hereafter referred to as CreERT2. CreERT2 is sequestered in the cytoplasm unless ER ligands are introduced to *in vitro* culture or *in vivo* animal models, which allows for transport to the nucleus and activation of Cre. Using this approach, I hoped to reduce or eliminate any toxic side effects of Cre expression and more closely study the effect of *Myd88* deletion on leukemia cell function. To induce genomic *Myd88* deletion, I began by treating *Myd88*$^{+/+}$ controls and *Myd88*$^{fl/fl}$ MLL-AF9 leukemia cells expressing either empty vector or CreERT2 with 0.5µM 4-hydroxytamoxifen (4OHT) in methylcellulose culture to induce nuclear translocation of the CreERT2 fusion. The 0.5µM 4OHT dose yielded likewise Cre-mediated toxicity (Figure 15A). Even 40nM doses of 4OHT result in CreERT2-mediated toxicity in both methylcellulose and liquid culture (Figure 15B and 15C). Therefore, I dosed the 4OHT to the nanomolar range and found doses as low as 2.5nM 4OHT were sufficient to induce *Myd88* deletion (Figure 15D).

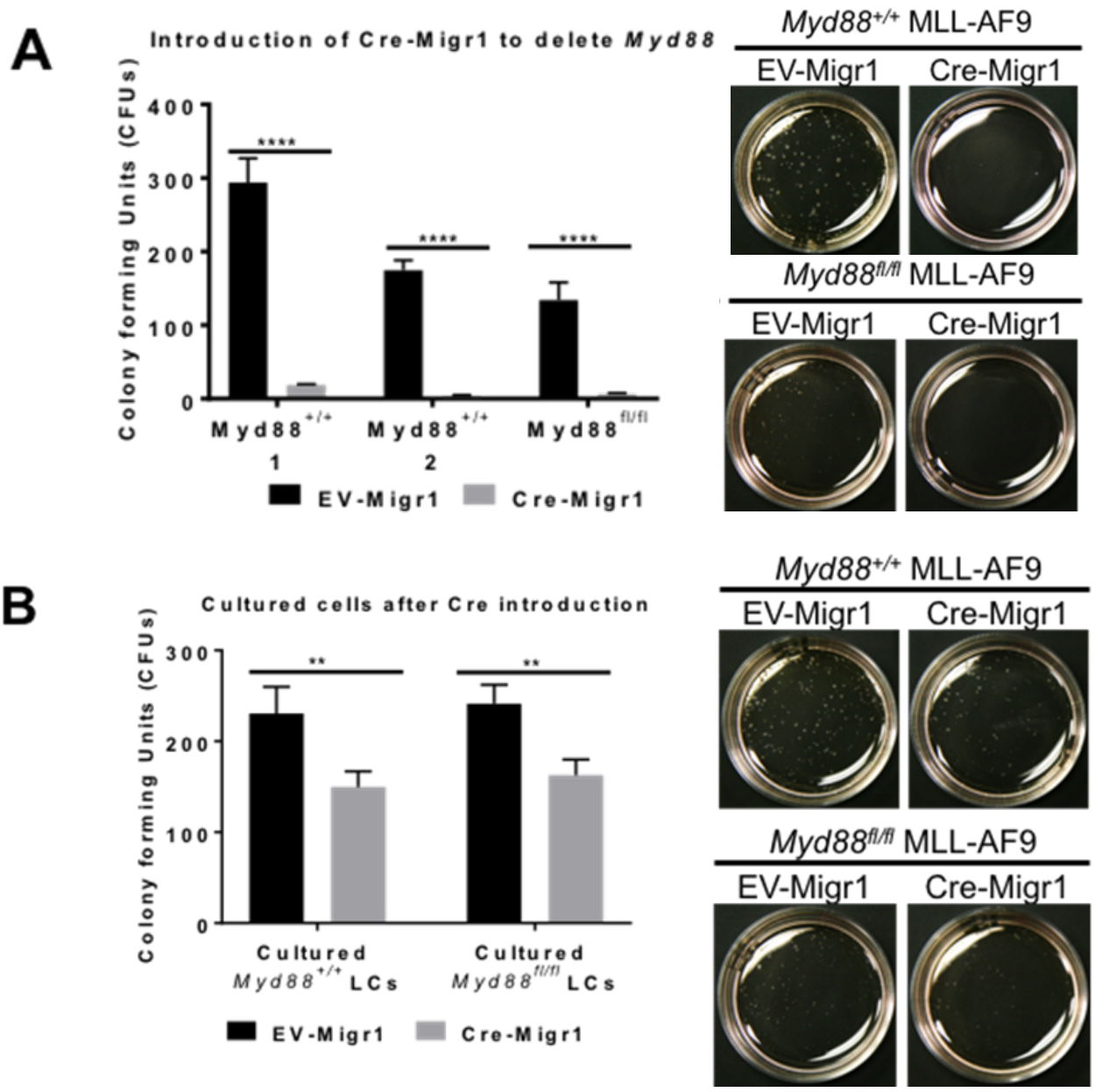


Figure 14. Cre Expression Induces Non-Specific Toxicity. (A) Colony forming efficiency of *Myd88*$^{+/+}$ and *Myd88*$^{fl/fl}$ MLL-AF9 cells with recent Cre expression. Cre recombinase was retrovirally expressed on two *Myd88*$^{+/+}$ MLL-AF9 cell lines and the *Myd88*$^{fl/fl}$ MLL-AF9 cells. 48 hours after retroviral expression, GFP^{+}-Cre expressing cells were sorted and plated on methylcellulose. (B) Colony forming efficiency of sorted Cre-expressing and empty vector-expressing cells that were maintained in culture for three weeks and then plated on methylcellulose. P-values reflect Student's t-test and indicate * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; and **** = $p<0.0001$.

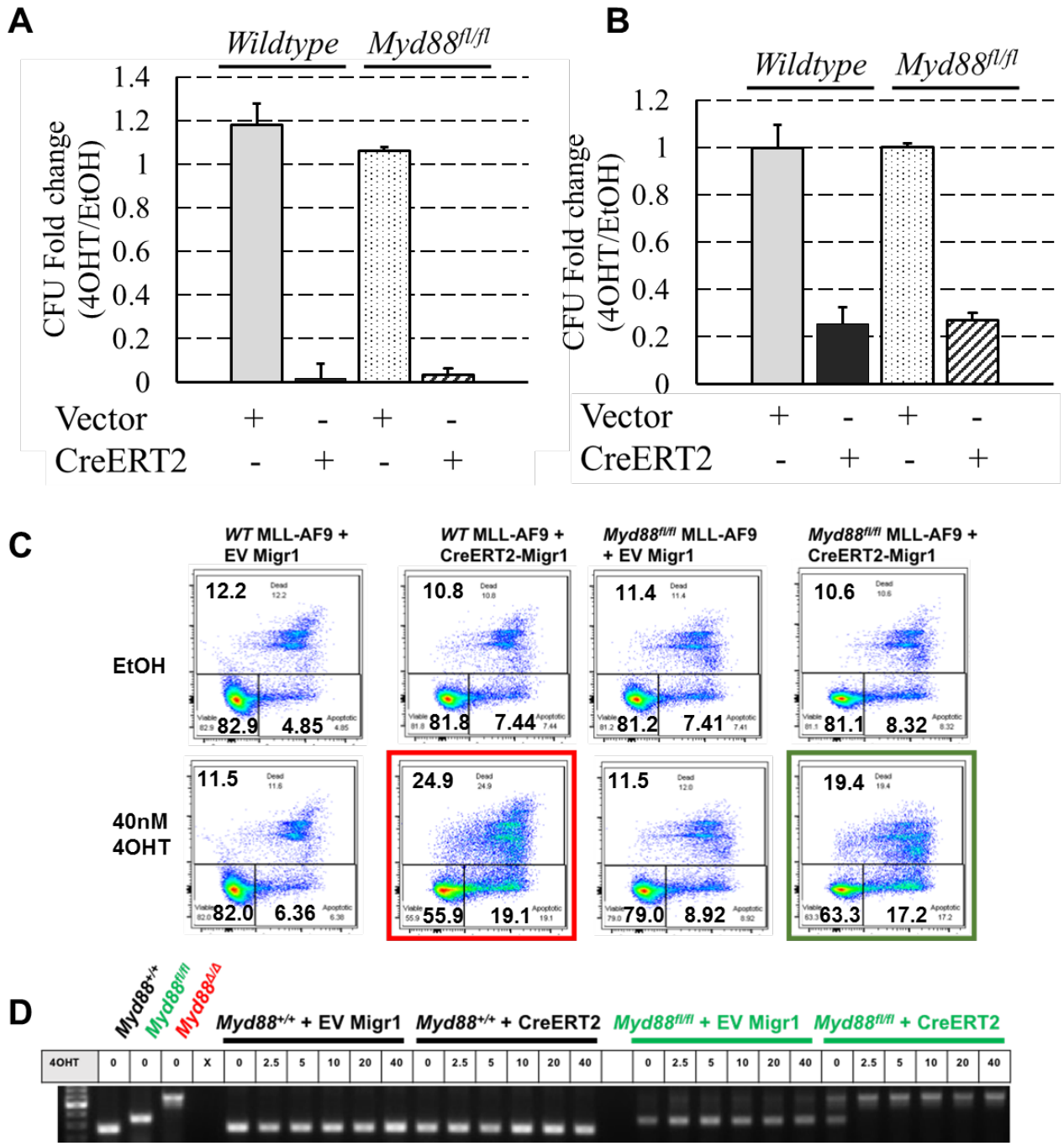


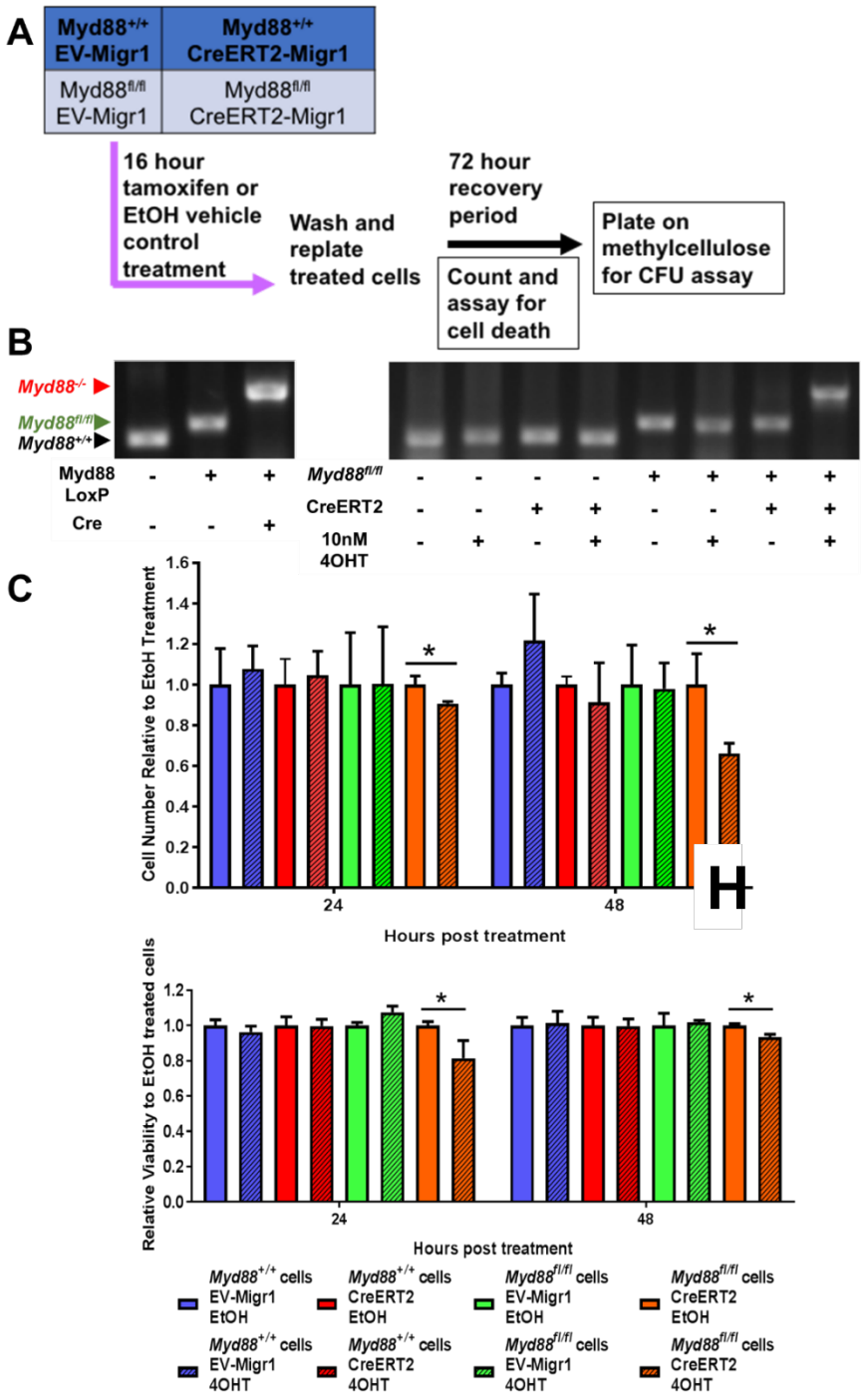
Figure 15. CreERT2 Induction Results in Highly Efficient Recombination and Toxicity at High Tamoxifen Doses. (A) CFU efficiency with induction of CreERT2 at 500nM 4OHT in methylcellulose. (B) Pretreatment of LCs with EtOH or 40nM 4OHT for 24 hours followed by CFU assay. (C) 40nM 4OHT treatment and subsequent AnnexinV/7AAD assay with EtOH vehicle control treatment, EV-Migr1, CreERT2-Migr1, *Myd88*$^{+/+}$ and *Myd88*$^{fl/fl}$. (D) PCR assay of recombination efficiency from 1nM to 40nM.

CreERT2-Mediated Myd88 Deletion Limits MLL-AF9 Leukemia Cell Proliferation and Colony Forming Ability

Thus, 10nM 4OHT was selected as a treatment dose. *Myd88*$^{+/+}$ controls and *Myd88*$^{fl/fl}$ MLL-AF9 leukemia cells expressing Migr1-EV or Migr1-CreERT2 were thus treated with 10nM 4OHT to selectively induce *Myd88* deletion. Cells were treated in suspension culture in complete media for 16 hours before washing away the 4OHT and allowing 48-hour recovery in suspension culture (Figure 16A). Using this approach, *Myd88* is efficiently deleted (Figure 16B). Within the recovery period, a slight reduction in cell number and viability was observed in cells undergoing *Myd88* deletion, but not in control groups (Figure 16C). Following the recovery period, cells were counted and plated in methylcellulose at 1,000 cells per mL. Compared to vehicle-treated controls, the *Myd88*$^{\Delta/\Delta}$ leukemia cells formed colonies at 50% lower efficiency (Figure 16G). To explain the phenotypes of growth disadvantages and reduced colony forming ability, several hypotheses were proposed. First, the deletion of *Myd88* and loss of downstream signaling led to cell death. Second, partial differentiation accounted for the loss of proliferation *in vitro*.

To test the hypothesis that *Myd88* expression is required for survival and viability, leukemia cells were assayed for cell death by AnnexinV-APC and 7AAD staining. As with the CFU assay, CreERT2^{+} *Myd88*$^{fl/fl}$ leukemia cells and controls were treated for 16 hours with 4OHT or ethanol vehicle, washed, and cultured for an additional 48 hours before staining. Surprisingly, cell viability remained comparable to vehicle control treatments (Figure 16D). In conclusion, *Myd88* expression is not required for MLL-AF9 cell viability, even shortly after *Myd88* deletion. However, *Myd88* deletion suppresses growth rate as determined by cell cycle analysis and growth curves (Figure 16E and 16F). *In vitro* treatment of *Myd88*$^{+/+}$ and *Myd88*$^{-/-CreERT2+}$ MLL-AF9 cells with daunorubicin reveals an approximately 8-fold reduction in the LC50 of *Myd88*$^{-/-}$

Creert2+ Mll-AF9 cells compared to *Myd88* expressing cells (Figure 23H)/ Therefore, *Myd88* is required for colony-forming ability, proliferation, and resistance to daunorubicin-induced cell death in MLL-AF9 cells.



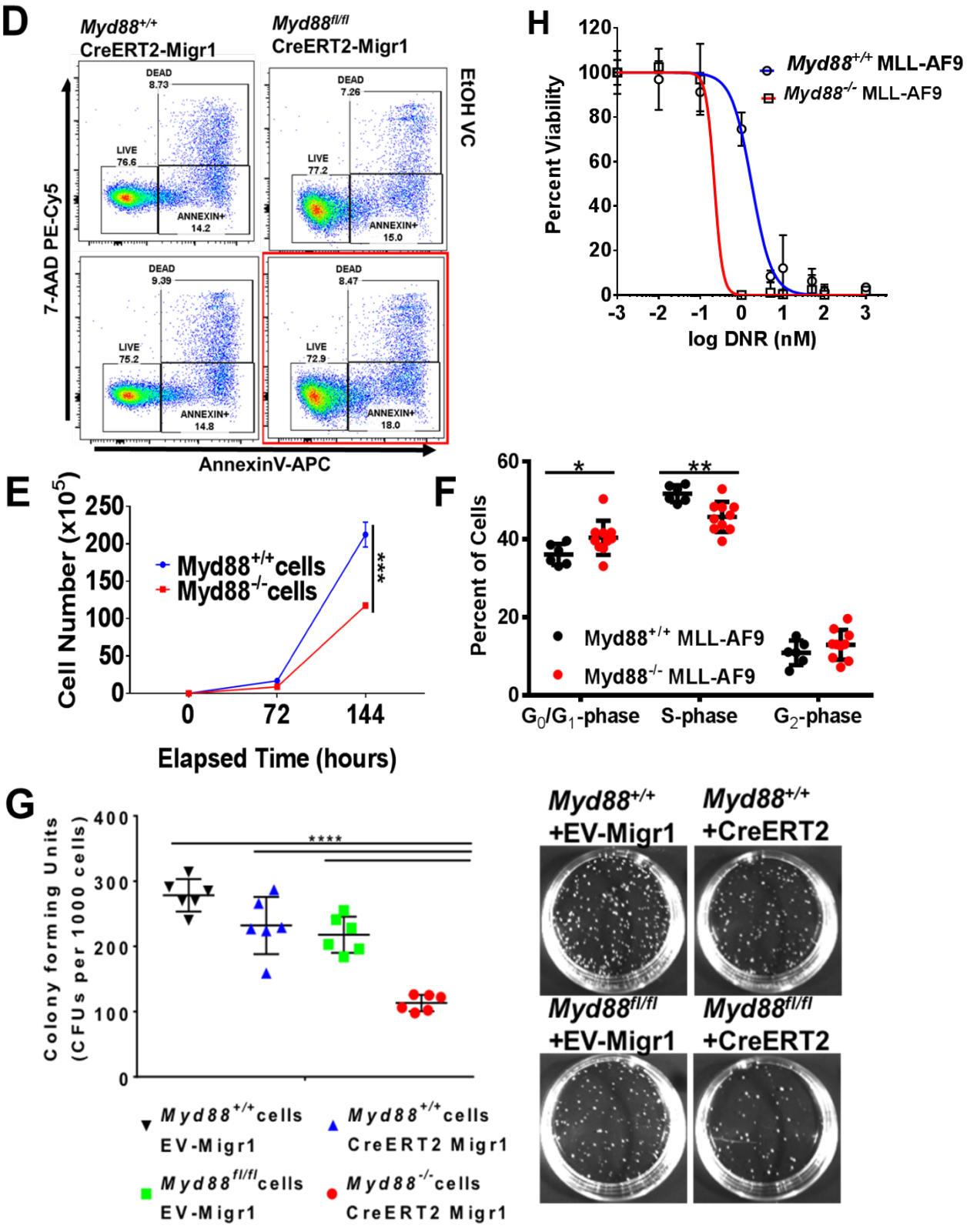


Figure 16. CreERT2-Mediated *Myd88* Deletion Reduces Colony Forming Efficiency and Proliferation.

Figure 16. CreERT2-Mediated *Myd88* Deletion Reduces Colony Forming Efficiency and Proliferation. (A) Scheme for CreERT2 induction. (B) PCR verification of *Myd88* deletion. (C) Cell counts and viability after 4OHT treatment. (D) AnnexinV/7AAD assay 48 hours after 4OHT treatment. CreERT2-activation in an $Myd88^{fl/fl}$ background is indicated by a red box. (E) Growth curve assay. (F) Cell cycle analysis after staining with 7AAD (G) Colony forming efficiency assay following 10nM 4OHT treatment. (H) MTS assay on $Myd88^{+/+}$ and $Myd88^{-/-CreERT2+}$ MLL-AF9 cells treated with increasing doses of daunorubicin. P-values reflect Student's t-test and indicate * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; and **** = $p<0.0001$.

Myd88 is Not Required for Jnk1/2, Erk1/2, Stat3α, or p38 Downstream Signaling Activation

To test whether this reduced proliferation and colony-forming phenotype occurs as a result of reduced downstream signaling, previous immunoblotting experiments were repeated on $CreERT2^{+}$ $Myd88^{\Delta/\Delta}$ leukemia cells. Of note, lysates for these experiments were collected 72 hours after 4OHT treatment. As described previously, phosphorylation of Irak4 is reduced (Figure 17A), but the phosphorylation of Jnk1/2 Erl1/2, p38α and Stat3α are unchanged (Figure 17B and 17C). Therefore, even in the event of recent *Myd88* deletion, these downstream signaling pathways are unchanged. Two lanes with lysates from *Myd88*-deleted cells are represented. The first (left) lane is from cells with recent (72 hours) *Myd88* deletion, and the second (right) lane is from cells in which *Myd88* deletion was induced 2 weeks prior to lysate collection. Therefore, Myd88 and Myd88-dependent signaling are not required to maintain the activation of these pathways in MLL-AF9 cells.

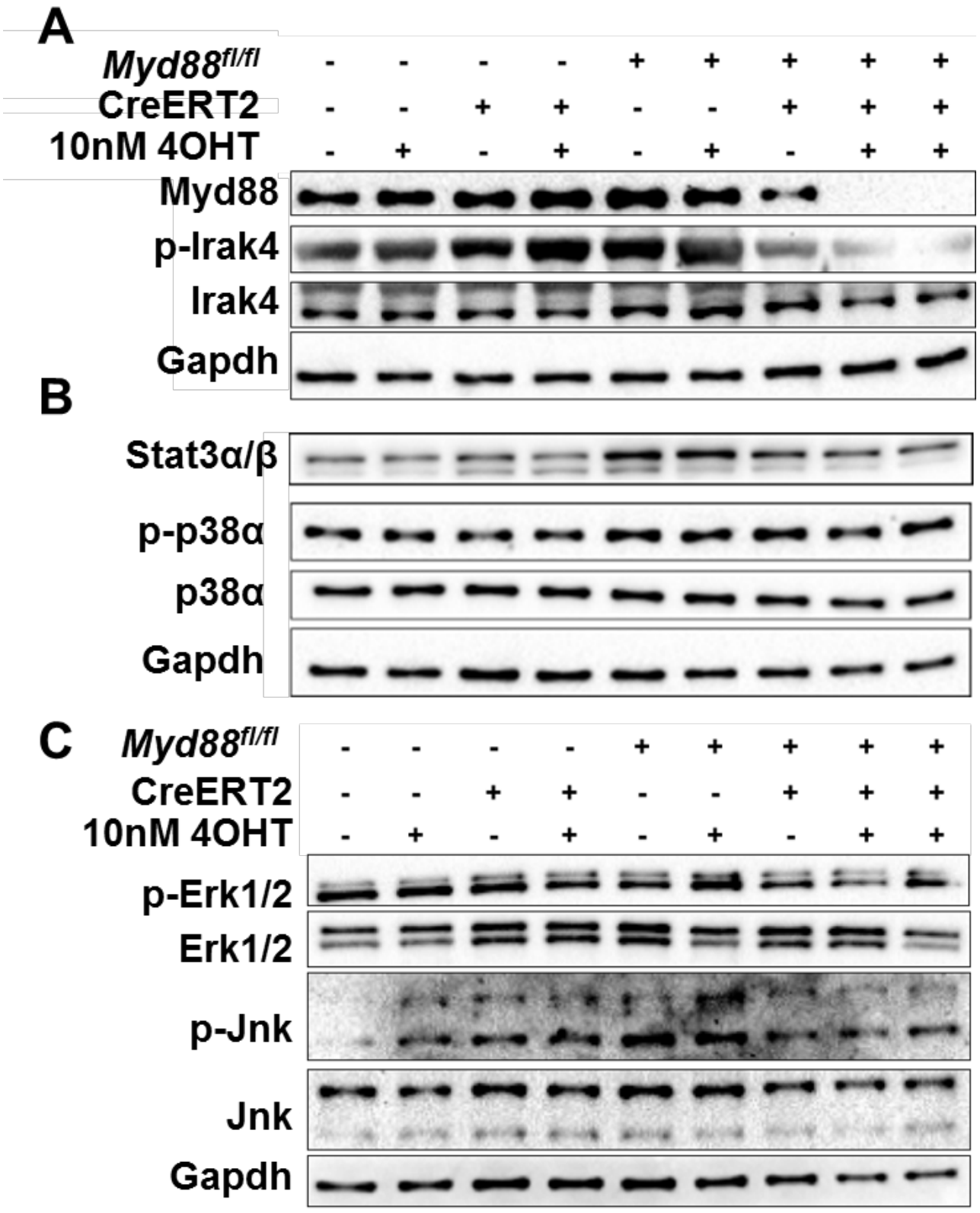


Figure 17. Myd88 and Myd88-Dependent Signaling are Not Required for JNK, p38, or ERK Activation in MLL-AF9 Cells.

Figure 17. Myd88 and Myd88-Dependent Signaling are Not Required for JNK, p38, or ERK Activation in MLL-AF9 Cells. Myd88-expressing leukemia cells and those with inducible *Myd88* deletion were assayed for downstream activity. The *Myd88*-deletion cell line on the right are cells that have *Myd88* deletion induced for 2 weeks, and the lane to its left are lysates of cells with *Myd88* deletion induced 72 hours earlier. (A) Immunoblot of Myd88, p-Irak4, Irak4, and Gapdh for MLL-AF9 cells with and without Myd88 expression. (B) Immunoblot of Stat3α/β, p-p38, p38, and Gapdh. (C) Immunoblot of p-Jnk, Jnk, p-Erk, Erk, and Gapdh.

Myd88 is Required to Maintain MLL-AF9 Leukemia Cell Surface Markers and Prevent Partial Differentiation

To test the hypothesis that Myd88 is required for maintenance of murine MLL-AF9 in a stem-like state, first, Wright-Giemsa staining was used to assess the morphologic changes in CreERT2^{+} *Myd88*$^{\Delta/\Delta}$ leukemia cells (Figure 18A) CD117 and CD11b were used as markers for leukemia cell stemness and differentiation, respectively. When stained for CD11b, CreERT2^{+} *Myd88*$^{\Delta/\Delta}$ leukemia cells show a 2.5-fold gain over *Myd88*$^{fl/fl}$ cells (Figure 18B). *Myd88*$^{\Delta/\Delta}$ leukemia cells demonstrated a partial loss of the leukemia cell stemness marker CD117 relative to *Myd88*$^{fl/fl}$ controls (Figure 18C). Together, these findings indicate that *Myd88*$^{\Delta/\Delta}$ leukemia cells are partially differentiated. To determine whether this partial differentiation phenotype is specific to inducible *Myd88* deletion or shared among MLL-AF9 *Myd88*-null cells, several previously described *Myd88*$^{-/-}$ leukemia cell lines and Cre^{+} *Myd88*$^{\Delta/\Delta}$ leukemia cells were stained for CD117 and CD11b and compared to *Myd88*$^{+/+}$ controls (Figure 18D). The mean fluorescence intensity (MFI) of CD117 showed a similar 25-50% loss in all cell lines, and the MFI of CD11b showed a 2-2.5 fold increase compared to *Myd88*$^{+/+}$ controls. This same ratio of 2.5 fold increase in CD11b and partial reduction of CD117 occurs in cells bearing germline *Myd88* deletion as well (Figure 18E). Myd88 is therefore required for maintenance of MLL-AF9 cells in an undifferentiated state, regardless of whether *Myd88* deletion occurs before or after introduction of the MLL-AF9 fusion protein.

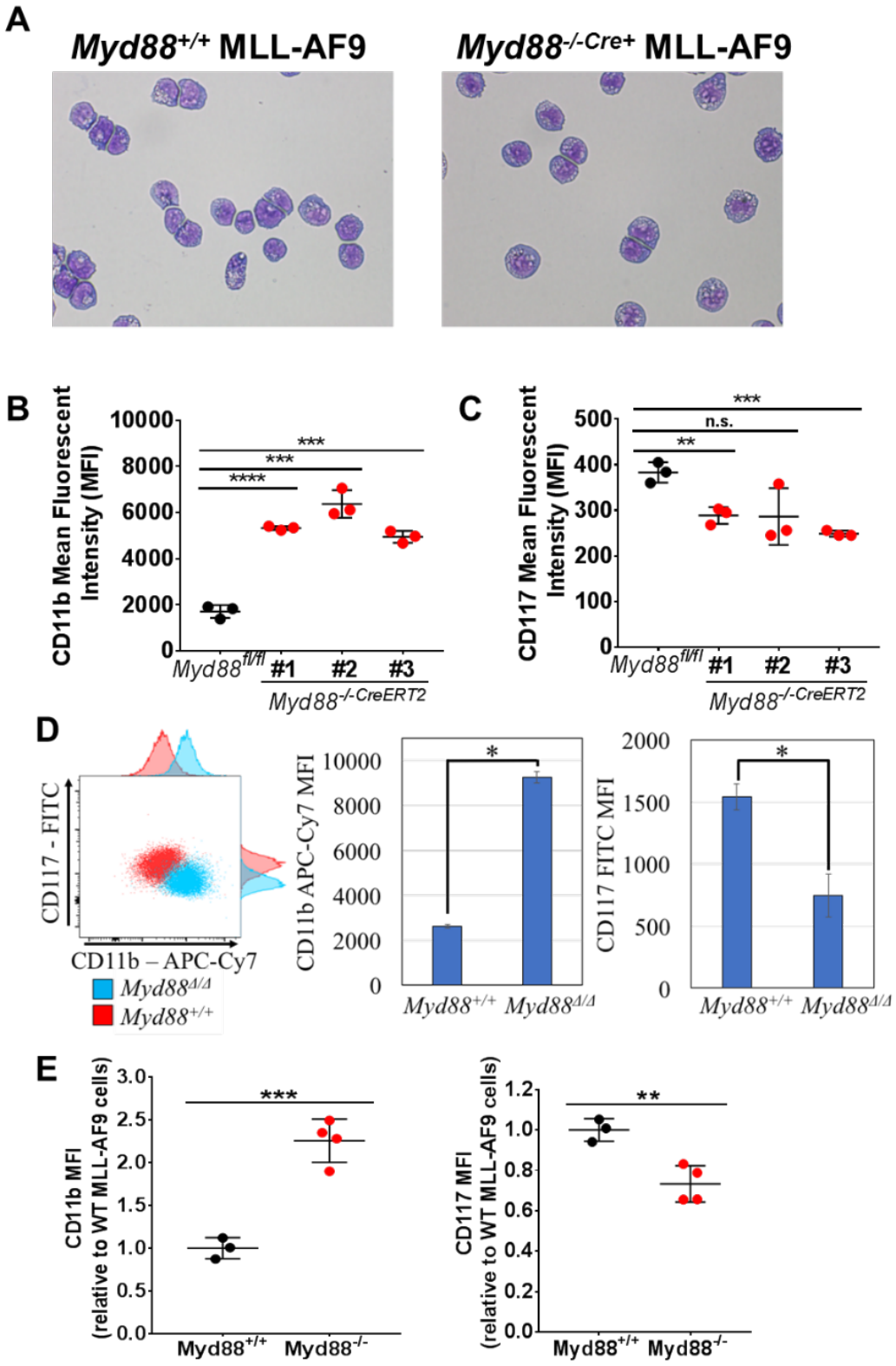


Figure 18. *Myd88* Deletion Results in Partial Differentiation of MLL-AF9 Cells. (A) Wright-Giemsa microscopy images of *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 cells. (B) CD11b median fluorescent intensity (MFI) in isogenic *Myd88*$^{fl/fl}$, *Myd88*$^{-/-}$ bulk culture, and clonal *Myd88*$^{-/-}$ MLL-

AF9 populations with CreERT2-mediated *Myd88* deletion. (C) CD117 MFI in isogenic $Myd88^{fl/fl}$, $Myd88^{-/-}$ bulk culture, and clonal $Myd88^{-/-}$ MLL-AF9 populations with CreERT2-mediated *Myd88* deletion. (D) CD117 and CD11b overlay plot for $Myd88^{-/-}$ MLL-AF9 cells expressing Cre. (E) MFI for CD117 and CD11b for MLL-AF9 cells with germline $Myd88^{-/-}$ genotype, with PCR genotyping. MFIs represent three independent experiments and were compared using a Student's t-test and indicate * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; and **** = $p<0.0001$.

Transplantation of $Myd88^{\Delta/\Delta}$ Leukemia Cells Yields Prolonged Leukemia Latency Compared to *Myd88*-Expressing Leukemia Cells

To determine whether observed partial differentiation has an effect on *in vivo* leukemogenesis, $CreERT2^{+}$ $Myd88^{\Delta/\Delta}$ MLL-AF9 leukemia cells were transplanted into irradiated mice and monitored for leukemia development. Leukemia development was confirmed by bone marrow microscopy, peripheral blood smears, and hepatomegaly and splenomegaly caused by infiltrating leukemia cells. Compared to those transplanted with $Myd88^{+/+}$ MLL-AF9 controls, mice transplanted with $CreERT2^{+}$ $Myd88^{\Delta/\Delta}$ MLL-AF9 leukemia cells develop leukemia at a median of 40 days after transplantation, 14 days after controls develop leukemia, at a median of 26 days (Figure 19). These data also correspond to the treatment of transplanted MLL-AF9 leukemia cells with high doses of IRAK1/4 inhibitors previously described *in vivo*. Therefore, the deletion of *Myd88* in the MLL-AF9 background delays leukemogenesis in an *in vivo* transplantation model. Further, intact *Myd88* is required for MLL-AF9-transformed cells to cause leukemia at a comparable time to *Myd88*-expressing cells.

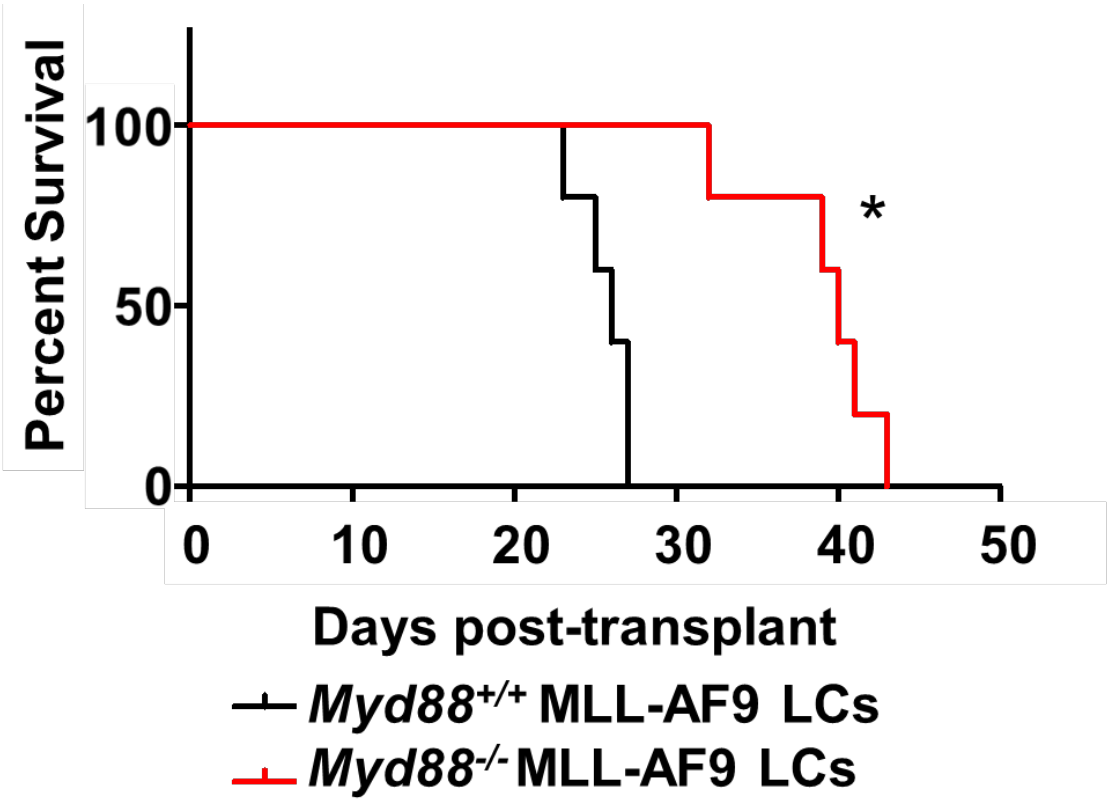
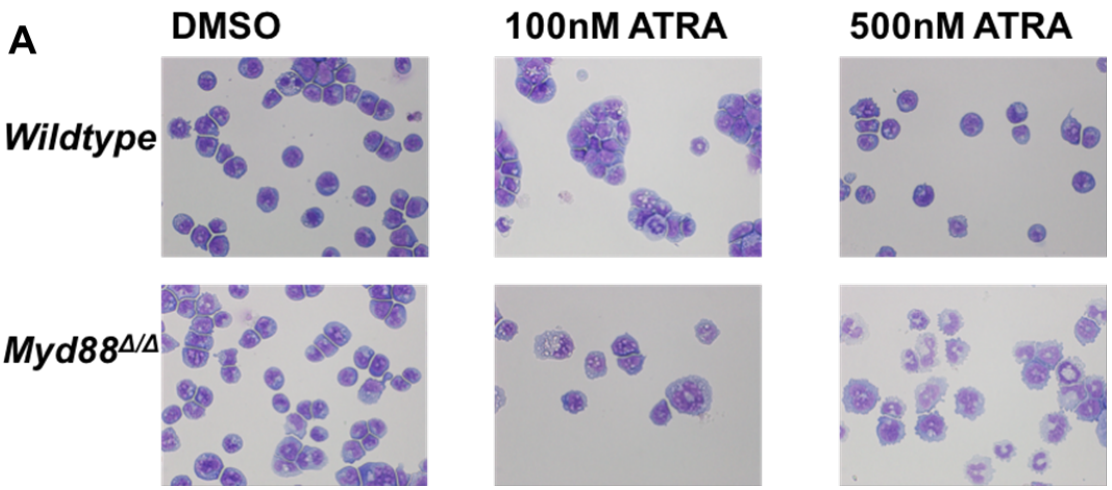
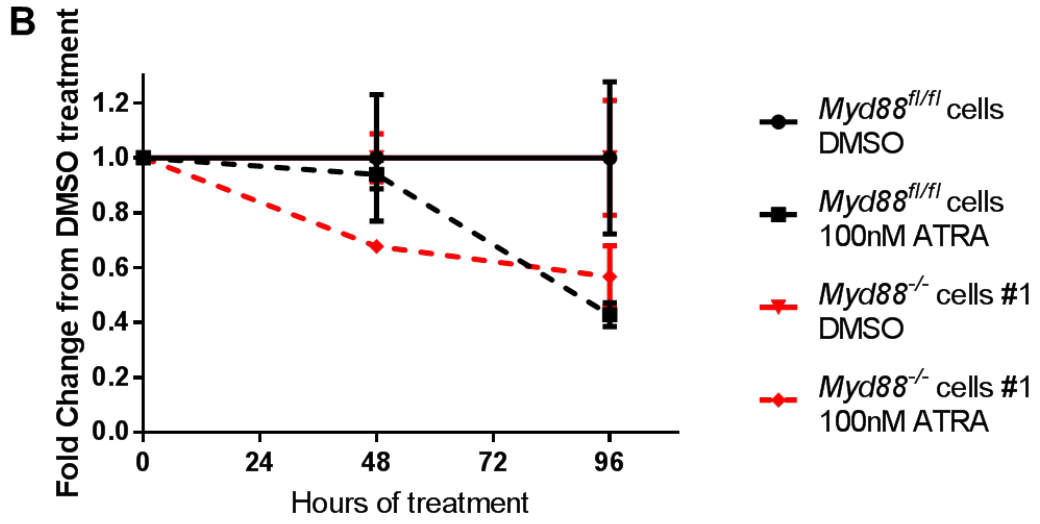


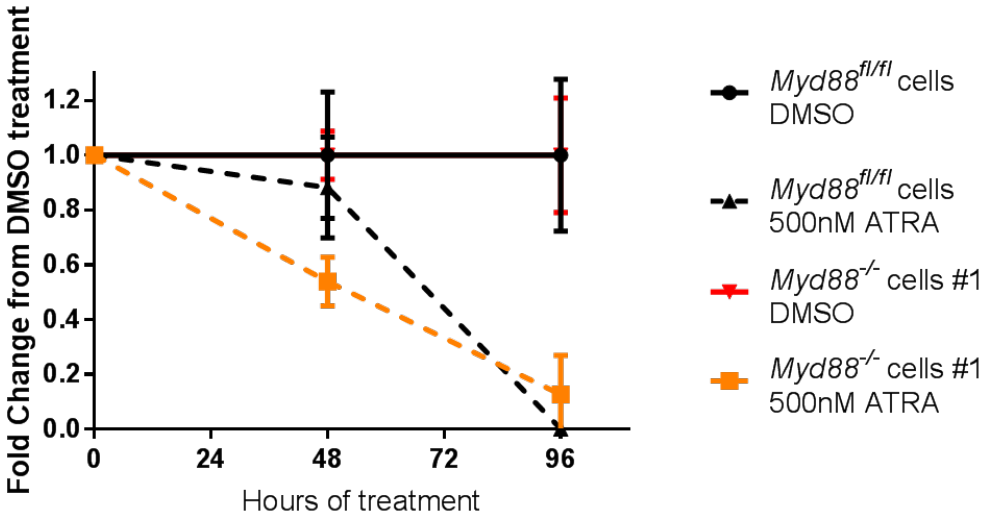
Figure 19. ***Myd88*$^{-/-}$ MLL-AF9 Leukemia Cell Transplantation Results in Delayed Leukemogenesis *in vivo*.** Survival of C57BL/6J mice transplanted with *Myd88*$^{+/+}$ or *Myd88*$^{-/-Cre+}$ MLL-AF9 leukemia cells (n=5 each). Statistical comparison reflects results of Log-rank and Mantel-Cox comparisons, with * indicating $p<0.05$.

Myd88 Deletion or IRAK1/4 Inhibitor Sensitizes MLL-AF9 Cells to ATRA

Because Myd88 is required for the maintenance of the MLL-AF9 leukemia cell surface markers associated with stem cell-like features (CD117) and the suppression of those associated with differentiation (CD11b), I hypothesized that *Myd88*$^{-/-}$ MLL-AF9 leukemia cells might be sensitive to ATRA-induced differentiation. When treated in suspension culture, a clear morphologic differentiation is induced in *Myd88*$^{-/-}$ MLL-AF9 cells compared to *Myd88*$^{+/+}$ MLL-AF9 cells (Figure 20A), despite ATRA suppressing cell growth to a similar degree in suspension culture (Figure 20B). When *Myd88*$^{-/-}$ and *Myd88*$^{+/+}$ MLL-AF9 cells were plated in methylcellulose following 5 days of treatment with 500nM ATRA, colony forming ability is suppressed (Figure 20C). Treatment with ATRA for 5 days induces CD11b, specifically in *Myd88*$^{-/-}$ MLL-AF9 cells (Figure 20D). However, CD117 levels are unchanged. *In vivo*, treatment with ATRA prolongs leukemia development in mice transplanted with *Myd88*$^{-/-}$ MLL-AF9 cells, but not in those transplanted with *Myd88*$^{+/+}$ MLL-AF9 cells (Figure 20E). These data support the conclusion that *Myd88* deletion partially sensitizes the MLL-AF9 cell to ATRA-induced differentiation. To determine if IRAK1/4 inhibitor can sensitize wildtype cells to ATRA-induced differentiation, methylcellulose was treated with DMSO, 5μM IRAK1/4 inhibitor, 100nM ATRA, or both. The treatment of *Myd88*$^{+/+}$ MLL-AF9 cells with both IRAK1/4 inhibitor and 100nM ATRA, both not either individually suppressed colony forming ability (Figure 20F). In conclusion, IRAK1/4 inhibitor treatment, while suppressing cell growth, prevents cell surface marker alterations associated with ATRA-induced differentiation in MLL-AF9 cells.







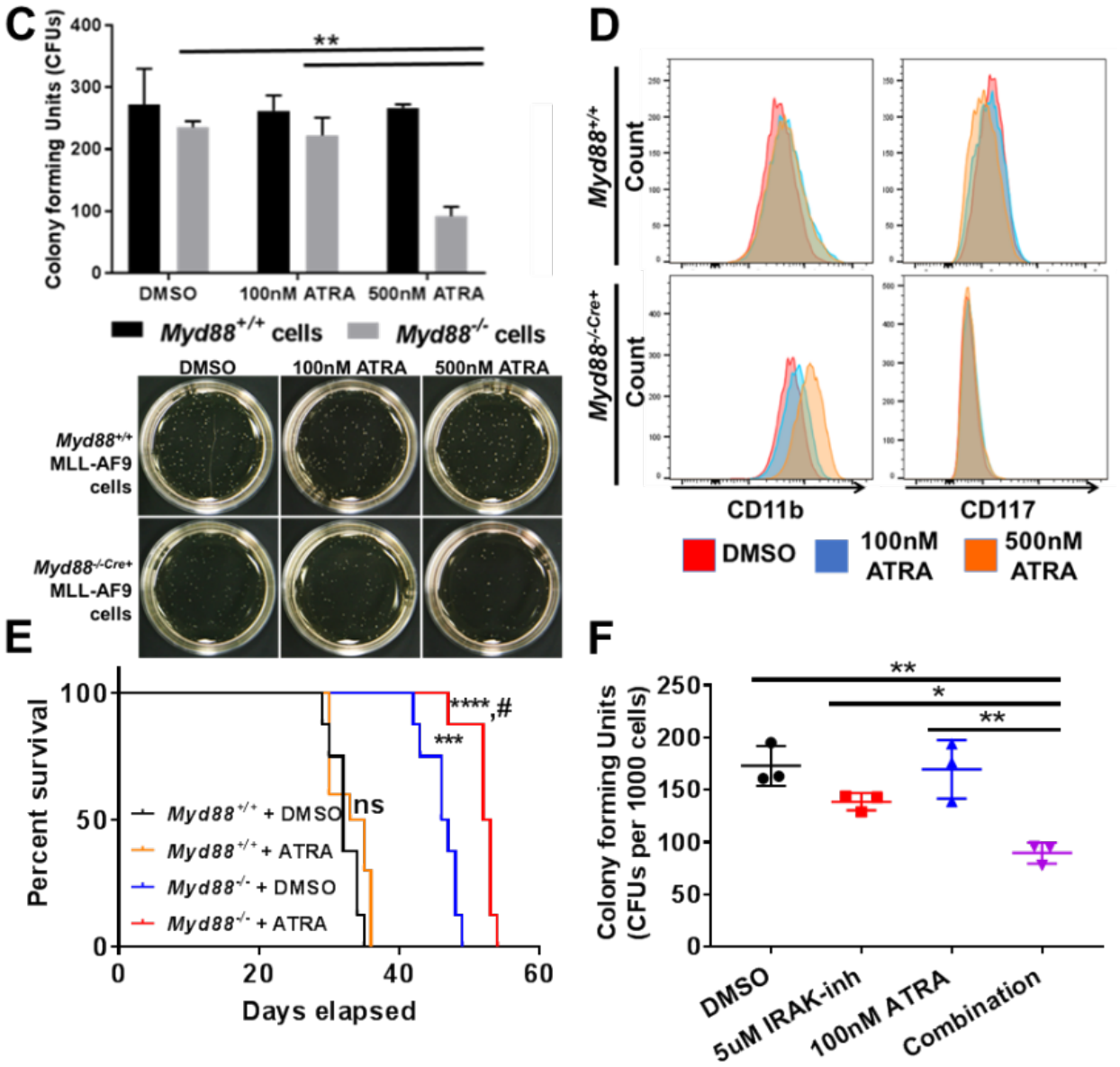


Figure 20. ATRA Treatment Augments *Myd88*$^{-/-}$ MLL-AF9 Cell Partial Differentiation and Delays Leukemia Development *in vivo*. (A) Wright-Giemsa staining of *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 cells treated with ATRA or DMSO vehicle. (B) Growth curve of *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 cells treated with ATRA or DMSO. (C) CD117 and CD11b histogram with ATRA treatment. (D) RT-PCR of *Hoxa9* with *in vitro* ATRA treatment. (E) Treatment of *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 cells with ATRA *in vivo* delays leukemia development for *Myd88*$^{-/-}$, but not *Myd88*$^{+/+}$ MLL-AF9 cells. (F) Co-treatment of MLL-AF9 cells with 5μM IRAK1/4 inhibitor, 100nM ATRA, or both. Data represent three independent experiments and were compared using a Student's t-test or ANOVA with Tukey's HSD test, with n.s. indicating $p>0.05$, * indicating $p<0.05$, ** indicating $p<0.01$, *** indicating $p<0.001$, and **** indicating $p<0.0001$.

Inhibition or Knockdown of IRAK1 in MLL-AF9 Leakemia Cells Results in Phenotype Distinct from the *Myd88* Knockout Phenotype

The correlation between *in vivo* studies of IRAK1/4 inhibitors and CreERT2^{+} *Myd88*$^{\Delta/\Delta}$ MLL-AF9 leukemia cells led to the hypothesis that Myd88-dependent signaling, as mediated by IRAK4 and IRAK1 is required to prevent the observed partial differentiation phenotype. This hypothesis is further validated by lost IRAK4 phosphorylation. To test this hypothesis, IRAK1/4 inhibitor (*Sigma*) was applied to *wildtype* MLL-AF9 cells *in vitro*, over five (Figure 21A-D) and seven days (Figure 21E-F), sufficient time periods to acquire partial differentiation. Surprisingly, CD11b and CD117 cell surface markers remained unchanged relative to DMSO-treated controls (Figure 21C-D). Because the inhibitors act on IRAK1 and IRAK4, this finding suggests that Myd88-mediated partial differentiation occurs independently of IRAK signaling. Interestingly, treatment over seven days with IRAK1/4 inhibitor induces accumulation of CD117, and loss of CD11b, indicating that treated leukemia cells gain cell surface markers associated with cell stemness and lose markers associated with differentiation. Co-treatment with ATRA abolishes this effect and the cell surface markers resemble those of cells treated with DMSO controls (Figure 21E-F).

To test this, IRAK1 was selected as a knockdown target because of its position downstream of Irak4. Knockdown to Irak1 was applied by lentiviral shRNA infection and selection (*Sigma-Aldrich*) and validated by quantitative real-time PCR (qRT-PCR) and immunoblotting (Figure 22A-B, 22G-H). Despite efficient knockdown (*Irak1*^{KD} MLL-AF9), these cells failed to recapitulate the partial differentiation findings of *Myd88*$^{\Delta/\Delta}$ MLL-AF9 leukemia cell lines (Figure 22C-D). Furthermore, *Irak1*^{KD} MLL-AF9 cells demonstrated no meaningful change in colony-forming efficiency or consistent change in proliferation (Figure 22E-F). Thus, the *Myd88*-

deletion-dependent partial differentiation occurs independently of Irak signaling, as evidenced by robust and consistent evidence from pharmacologic inhibition and shRNA genetic knockdown. Comparable to *Myd88*$^{+/+}$ cells, *Irak*^{KD} in a CreERT2^{+} *Myd88*$^{\Delta/\Delta}$ MLL-AF9 background elicits no phenotype change (Figure 22I-L). Based on these shRNA data, the *Myd88* deletion partial differentiation phenotype occurs in an Irak1-independent manner.

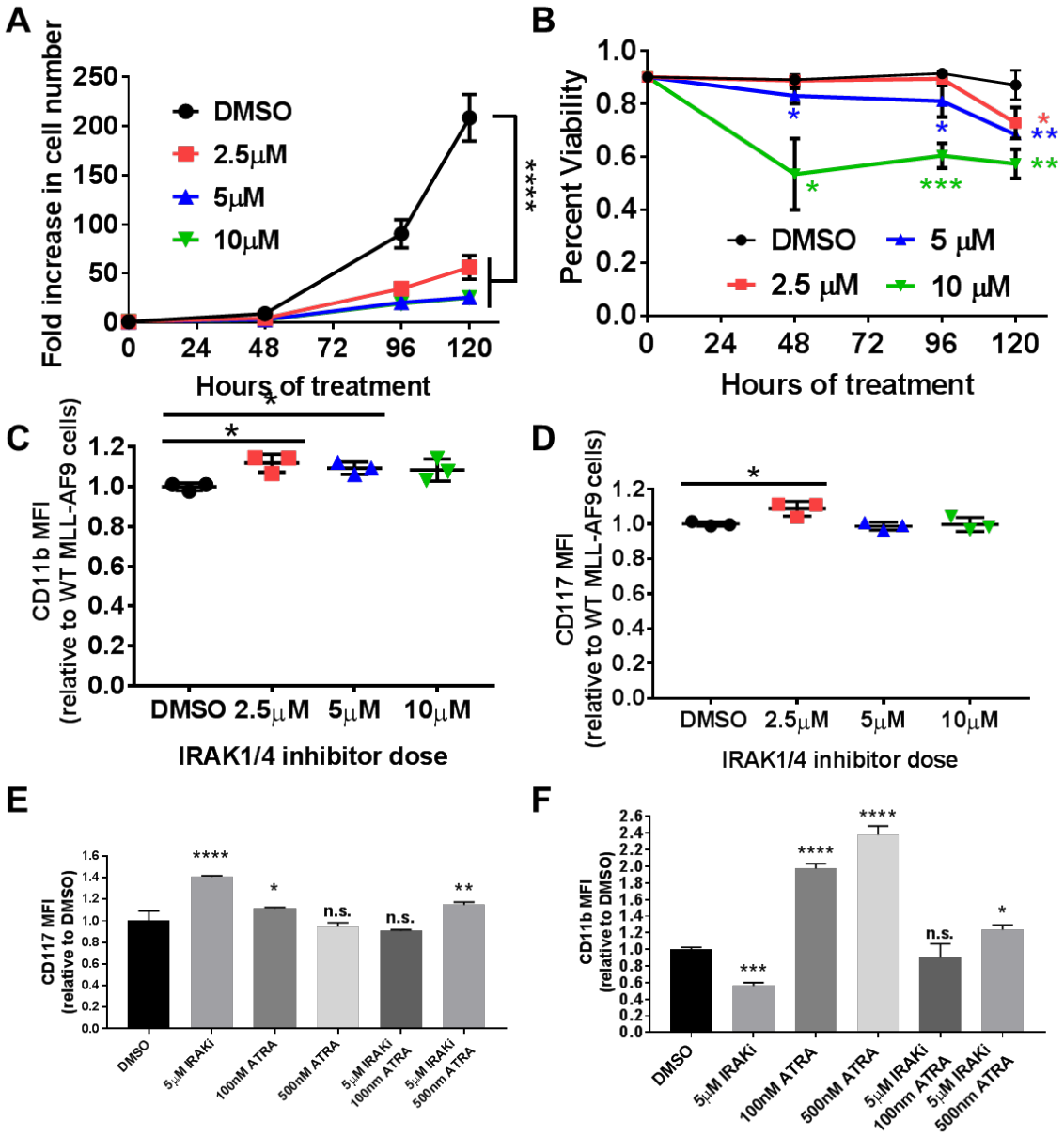


Figure 21. IRAK1/4 Inhibitor Treatment Alone or Combined with ATRA Treatment Fails to Induce Similar Partial Differentiation Phenotype. (A) Growth curve of IRAK1/4 inhibitor treatment over five days. (B) Viability of five-day IRAK1/4-inhibitor treatment. (C) CD117 MFI of MLL-AF9 cells after 5 days of IRAK1/4 inhibitor treatment. (D) CD11b MFI of MLL-AF9 cells after 5 days of IRAK1/4 inhibitor treatment. (E) CD117 MFI of MLL-AF9 cells after seven-day treatment with IRAK1/4 inhibitor, ATRA, or both. (F) CD11b MFI of MLL-AF9 cells after seven-day treatment with IRAK1/4 inhibitor, ATRA, or both. Data represent three independent experiments and were compared using a Student's t-test or ANOVA with Tukey's HSD test, with n.s. indicating $p>0.05$, * indicating $p<0.05$, ** indicating $p<0.01$, *** indicating $p<0.001$, and **** indicating $p<0.0001$.

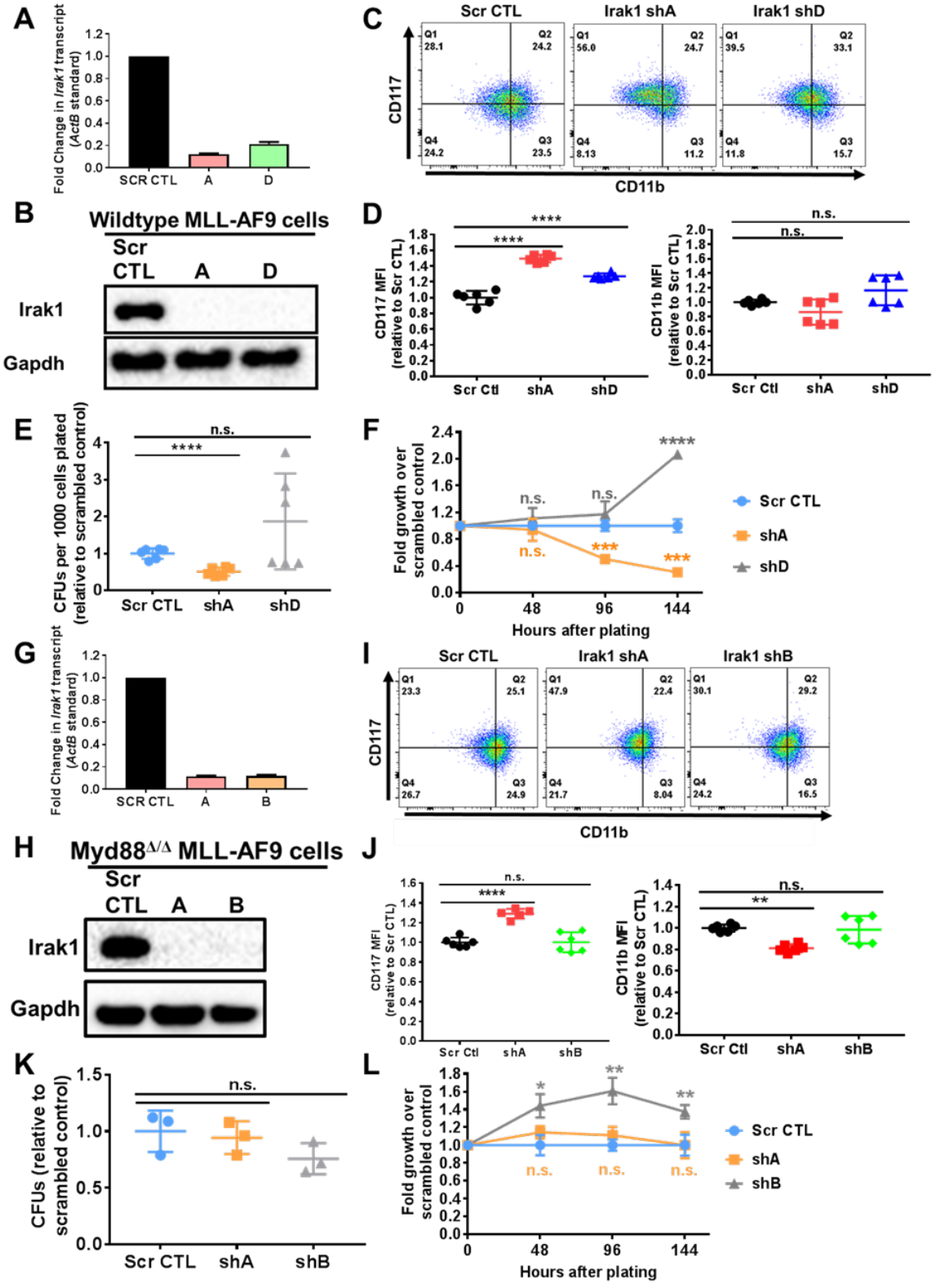


Figure 22. Irak1 is Not Required for MLL-AF9 Leukemia Undifferentiated State, Proliferation, Colony Formation, or Sensitivity to IRAK1/4 Inhibitor in *Myd88*$^{+/+}$ or *Myd88*$^{-}$

Background. (A) RT-PCR validation of *Irak1* knockdown (*Irak1*^{KD}) in *Myd88*$^{+/+}$ MLL-AF9 cells. (B) Immunoblot validation of Irak1 knockdown in *Myd88*$^{+/+}$ MLL-AF9 cells. (C) Representative plots of *Irak1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (D) CD117 and CD11b MFI of *Irak1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (E) Colony forming efficiency of *Irak1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells relative to scrambled controls. (G) Growth curve of *Irak1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells relative to scrambled controls. (G) RT-PCR validation of *Irak1*^{KD} in *Myd88*$^{-/-CreERT2}$ MLL-AF9 cells. (H) Irak1 immunoblot of *Irak1*^{KD} *Myd88*$^{-/-CreERT2}$ MLL-AF9 cells. (I) Representative plots of *Irak1*^{KD} *Myd88*$^{-/-CreERT2}$ MLL-AF9 cells. (J) CD117 and CD11b MFI of *Irak1*^{KD} *Myd88*$^{-/-CreERT2}$ MLL-AF9 cells. Data represent three independent experiments and were compared using a Student's t-test, with n.s. indicating $p>0.05$, * indicating $p<0.05$, ** indicating $p<0.01$, *** indicating $p<0.001$, and **** indicating $p<0.0001$.

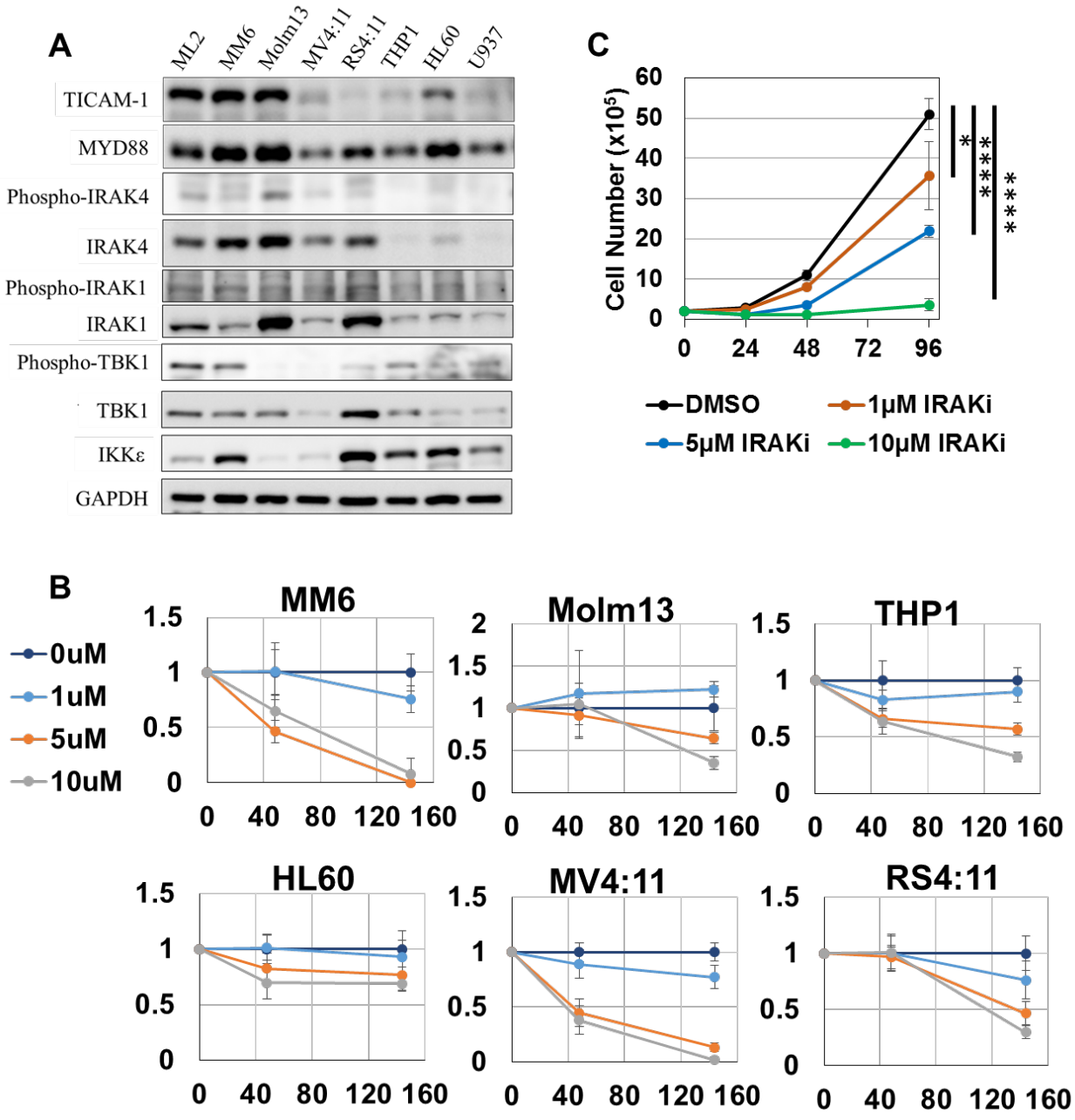
TLR Signaling is Active in Human AML Cell Lines, which Respond to IRAK1/4 Inhibitor Treatment

To demonstrate that TLR signaling is activated, human cells were assayed by immunoblotting for active phosphorylated downstream kinases. Phosphorylated IRAK4, IRAK1, and TBK1 were observed in the majority of human cell lines, indicating that, even *in vitro*, basal TLR stimulation is present in these cells (Figure 23A). Having observed overexpression in several primary patient datasets, expression of key MYD88-dependent TLR signaling molecules, and the activation of said MYD88-dependent TLR mediators, I hypothesized that MYD88-dependent signaling is required for leukemia cell pathogenesis. To test this idea, I first treated human cell lines with an IRAK1/4 inhibitor (*I5409 Sigma,* Figure 23B). A subset of human cells lines treated with IRAK1/4 inhibitor demonstrated a dose-dependent reduction in cell number over several days of treatment when assayed by growth curves. Unlike MV4:11, MM6, and THP-1, which showed both early and late dose-dependent reduction in cell number, Molm13 and RS4:11 showed only late dose-dependent responses, and HL60 showed a minimal response. Published data by Liang *et al.* indicates that this selective sensitivity is due to the presence or absence of MLL-rearrangements, whose phenotypes are augmented by degradation of MLL1 downstream of MYD88-dependent signaling.[181] Likewise, murine leukemia cell lines transformed with human

MLL-AF9 (MA9 LCs), demonstrated a dose-dependent sensitivity to IRAK1/4 inhibitors *in vitro* (Figure 23C).

Since IRAK1/4 inhibitors suppress leukemia cell growth in *in vitro* models, I hypothesized that inhibition of IRAKs might limit the colony-forming capacity as measured by methylcellulose colony forming unit assays (CFU). Plating cells on methylcellulose serves as an *in vitro* assay to approximate leukemogenic potency by measuring the efficiency of colony forming ability, out of the total number of cells plated. In the setting of leukemia cells, the CFU assay measures the ability of individual cells or a small cohort of cells to generate and propagate colonies in semi-solid medium. This quantification approximates non-adherent cell growth, proliferation, and the degree of leukemia cell differentiation or stemness. Despite other reports that IRAK1/4 inhibitors suppress colony-forming ability, IRAK1/4 inhibition of murine MLL-AF9 cells while plated in methylcellulose failed to elicit a loss of CFUs in *wildtype*, *Myd88*$^{-/-}$, and *Tnfr1/2*$^{-/-}$ MLL-AF9 backgrounds (Figure 23D). Interestingly, IRAK1/4 inhibitor is also effective in suppressing cell proliferation in *Myd88*$^{-/-}$ MLL-AF9 leukemia cells, in addition to *Myd88*$^{+/+}$ MLL-AF9 cells (Figure 23E). These findings, with those of IRAK1/4 inhibitors on *wildtype Myd88*$^{+/+}$ MLL-AF9 cells showed no change in CD117 and CD11b levels, suggest that IRAK1/4 inhibitors suppress the growth of the bulk leukemia cell population, but leave colony-forming cells and leukemia stem cells intact. Lastly, the IRAK1/4 inhibitor, when applied as a single agent, does not induce a partial differentiation phenotype in the murine MLL-AF9 model as measured by any of the applied assays. Additionally, the sensitivity of *Myd88*$^{-/-}$ MLL-AF9 to

IRAK1/4 inhibitors suggests that IRAK1/4 inhibitors have off-target effects or that Irak4 or Irak1 are activated in a *Myd88*$^{-/-}$ MLL-AF9 background.



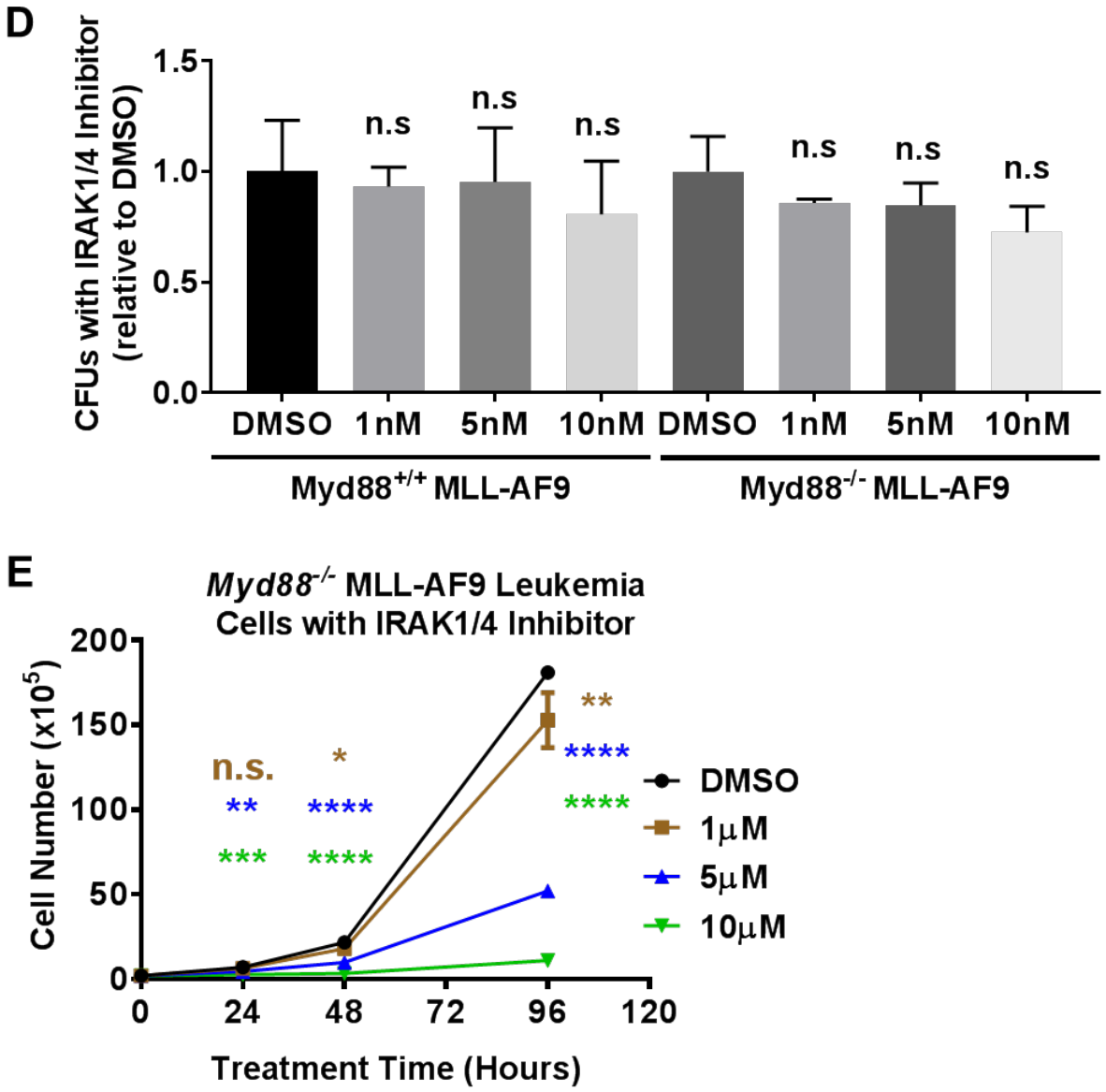


Figure 23. Human Cell Line Subsets Express Downstream TLR Signaling Components and are Sensitive to IRAK1/4 Inhibitors. (A) Immunoblot of human AML cell lines and RS4:11 B-cell acute lymphoblastic leukemia cell line demonstrate varying degrees of TLR mediator expression and activity. (B) Inhibitors of downstream IRAK1/4 kinase (IRAKi) result in a dose-dependent reduction in cell number compared to DMSO-treated controls. (C) Treatment of murine MLL-AF9 cells with IRAKi results in dose-dependent reduction of cell number. (D) Irak1/4 inhibitor treatment of both *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ MLL-AF9 leukemia cells on methylcellulose and resulting colony forming efficiency. Data represent three independent experiments with error bars as standard deviations and were compared using a Student's t-test, with n.s. indicating $p>0.05$, * indicating $p<0.05$, ** indicating $p<0.01$, *** indicating $p<0.001$, and **** indicating $p<0.0001$.

IRAK1/4 Inhibitor Treatment Delays Leukemia Development *in vivo*

To follow up with the IRAK1/4 inhibitor *in vitro* study, IRAK1/4 (*Sigma I5409*) and IRAK4 (*Calbiochem* 531237) inhibitors were applied *in vivo*. I performed the following experiment in collaboration with Kevin Liang, Andrew Volk, Ph.D., and Ali Shilatifard, Ph.D after they independently determined that MYD88-dependent signaling was required for AML pathogenesis using human leukemia cell line models.[181] To determine whether a murine model is sensitive to likewise loss of MYD88-dependent signaling, inhibitors of downstream IRAKs were applied in an *in vivo* syngeneic transplant study. Mice were divided into treatment groups of vehicle control, 4mg/kg IRAK1/4 (*Sigma*), 20mg/kg Compound 26 IRAK4 inhibitor (*Calbiochem* 531237), and combination treatment with both inhibitors. Ten mice per treatment group were sublethally irradiated (4.5Gy) and transplanted with 5,000 *wildtype* MLL-AF9 leukemia cells each. 4mg/kg IRAK1/4 (*Sigma*) and/or 20mg/kg Compound 26 IRAK4 inhibitor (*Calbiochem*) were administered by five i.p. injections at days 10, 12, 14, 16, and 18 after transplantation. Vehicle control and combination treatments were applied concurrently. When treated with IRAK1/4 inhibitor (*Sigma*) survival was prolonged by a median of 2.5 days, as determined by Log-rank Mantel-Cox statistical testing (Figure 24A). However, the alternative IRAK4 inhibitor (*Calbiochem*) treatment group failed to yield an effect, and treated mice showed no survival advantage (Figure 24B). To expand upon this *in vivo* study, we hypothesized that the dose was not sufficient to effectively inhibit IRAK1/4 *in vivo*, and repeated this experiment after increasing the dose five-fold and delaying treatment initiation. This second *in vivo* transplantation experiment was performed in collaboration with Ali Shilatifard, Ph.D. at Northwestern University by Andrew Volk, Ph.D. using murine MLL-AF9 leukemia cells that I derived and used in previous transplant studies. Treatments of 8mg/kg IRAK1/4 inhibitor and

75mg/kg IRAK4 inhibitor leukemia development significantly delayed leukemia development when mice were treated early, starting at day 10 post-transplantation (Figure 24C), or late, during 'blast phase' with treatments beginning at day 19 post-transplantation (Figure 24D).[181]

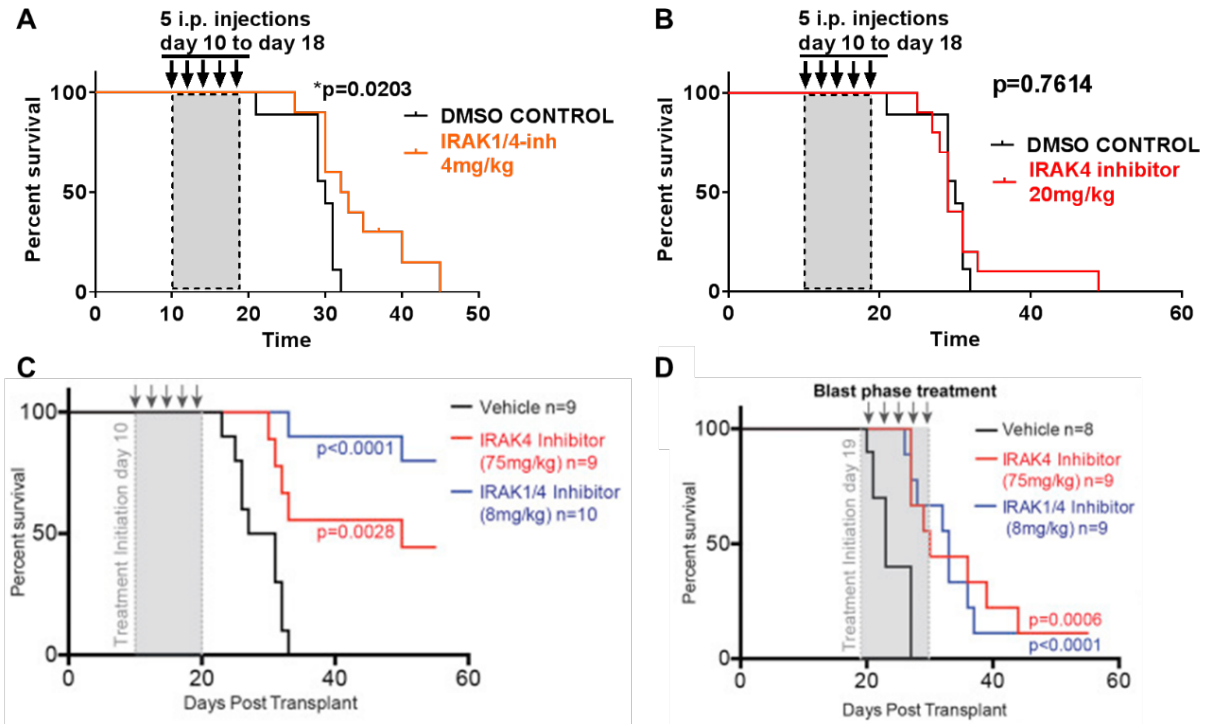


Figure 24. IRAK1/4 Inhibitor Treatment Delays Leukemia Development *in vivo*. All mice were sublethally irradiated (4.5Gy) and transplanted with $5x10^3$ MLL-AF9 leukemia cells. Treatment for panel A, B, and C reflects i.p. drug administration every 48 hours from day 10 to day 18. P-values reflect Log-rank Mantel-Cox survival analysis. (A) Survival of mice (n=10) treated with 4mg/kg IRAK1/4 inhibitor (p=0.0203). (B) Survival of mice (n=10) treated with 20mg/kg IRAK1/4 inhibitor treatment (p=0.7614). (C) Survival of mice (n=9-10) with 75mg/kg IRAK4 inhibitor (p=0.0028) or 8mg/kg IRAK1/4 inhibitor (p<0.0001). (D) Survival of mice treated with 75mg/kg IRAK4 inhibitor (p=0.0006) or 8mg/kg IRAK1/4 inhibitor (p<0.0001) beginning on day 19. Panels C and D are representative of *in vivo* transplantation study and IRAK1/4 inhibitor treatment at higher doses performed by Andrew Volk using MLL-AF9 leukemia cells that I derived. They were previously published in *Cell* by Liang *et al.* and are used here with permission.

Microarray Expression Data and TCGA Survival Correlations Implicate TICAM-1 Expression with AML Pathogenesis

The previously described primary patient microarray showed an overexpression of TICAM-1/TRIF in the acute monocytic leukemia (AMoL, M4/M5) subset. Overexpression of TLR4 but not TLR3, receptors known to induce TICAM-1/TRIF-dependent signaling, was also detected. Upon survey of the TCGA dataset, 2% of AML cases were shown to have amplification of TICAM-1/TRIF, with few other know mutations, indicating it is relatively conserved (Figure 7). Likewise, a clear survival correlation is found between low transcript levels of TICAM1/TRIF in AML. Furthermore, TICAM-1/TRIF transcripts are elevated in patients with AML relapse (Figure 6).

The study of TICAM-1/TRIF in AML was first prompted by the observation that TICAM-1/TRIF protein level is elevated in *Myd88*-null PLCs with retroviral overexpression of MLL-AF9, MLL-ENL, or AML1/RUNX1-ETO immunoblots (Figure11). This observation lead to the hypothesis that the induction of TICAM-1-TRIF functionally compensates for *Myd88* deletion, and, by extension, IRAK1/4 inhibtion. This hypothesis was later tested by Ticam-1/Trif shRNA knockdown in *Myd88*$^{-/-}$ MLL-AF9 cells. Despite efficient knockdown with all shRNAs (Figure 25A-B), the CFUs for all groups remain comparable (Figure 25C), and cell surface markers remain unchanged (Figure 25D) so Ticam-1/Trif does not act to compensate for Myd88 signaling in MLL-AF9 leukemia.

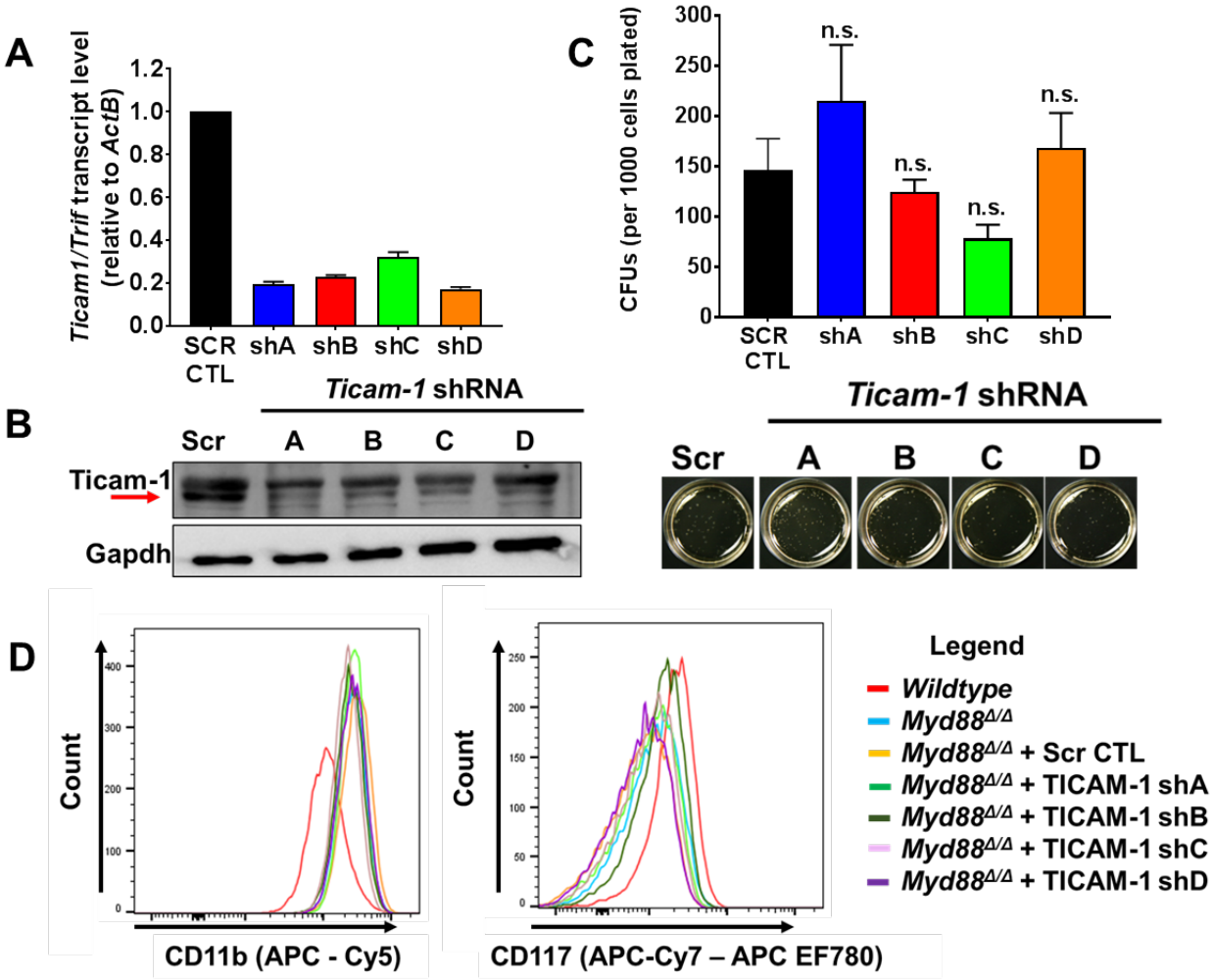


Figure 25. *Ticam-1* Knockdown Fails to Augment *Myd88*$^{-/-Cre+}$ MLL-AF9 Partial Differentiation Phenotype. (A) RT-PCR validation of *Ticam-1* knockdown (*Ticam-1*^{KD}) in *Myd88*$^{-/-Cre+}$ MLL-AF9 cells compared to scrambled controls. (B) Knockdown efficiency of *Ticam-1*^{KD} *Myd88*$^{-/-Cre+}$ MLL-AF9 cells as measured by immunoblot. (C) Colony forming unit assay of *Ticam-1*^{KD} *Myd88*$^{-/-Cre+}$ MLL-AF9 cells relative to scrambled controls. (D) Histogram comparison of scrambled control expressing to *Ticam-1*^{KD} *Myd88*$^{-/-Cre+}$ MLL-AF9 cells CFUs. Data assayed in triplicate and were compared using a Student's t-test, with n.s. indicating $p>0.05$, * indicating $p<0.05$, ** indicating $p<0.01$, *** indicating $p<0.001$, and **** indicating $p<0.0001$.

Ticam-1 is Required for Efficient MLL-AF9 Leukemia Cell Proliferation and Colony-Forming Ability

However, despite this, the primary patient and TCGA data suggest an independent role for the TLR adaptor TICAM-1/TRIF in AML. To further explore whether Ticam-1/Trif contributes to AML pathogenesis in a murine model, lentiviral Ticam-1/Trif shRNA was expressed in *wildtype* MLL-AF9 leukemia cells and confirmed by qRT-PCR and immunoblotting (Figure 26A-B). Confirmed Ticam-1/Trif knockdown led to reduction in CFUs (Figure 26C). *Ticam1*^{KD} MLL-AF9 cells demonstrate a profound growth deficiency, which is made clear by growth curves (Figure 26D), Edu incorporation (26E), and cell cycle analysis (26F).

To determine whether partial differentiation is responsible for reduced proliferation and CFUs in *Ticam1*^{KD} MLL-AF9 cells, Wright-Giemsa staining and cell surface markers were studied. Wright-Giemsa staining showed a morphological change in the leukemia cells indicative of partial differentiation (Figure 27E). Upon cell surface staining, the *Ticam1*^{KD} MLL-AF9 cells showed a 70-80% reduction in CD117 MFI (Figure 27A-B) and all knockdowns except for one showed a likewise increase in CD11b MFI (Figure 27C-D), relative to MLL-AF9 cells expressing scrambled control vectors. Interestingly, the most efficient knockdown, shTicam-1 A, shows no change in CD11b, so Ticam-1 may not be required to prevent CD11b accumulation. These phenotypes indicate that Ticam-1 is required for MLL-AF9 CD117 and CD11b cell surface marker maintenance, proliferation, and colony forming ability.

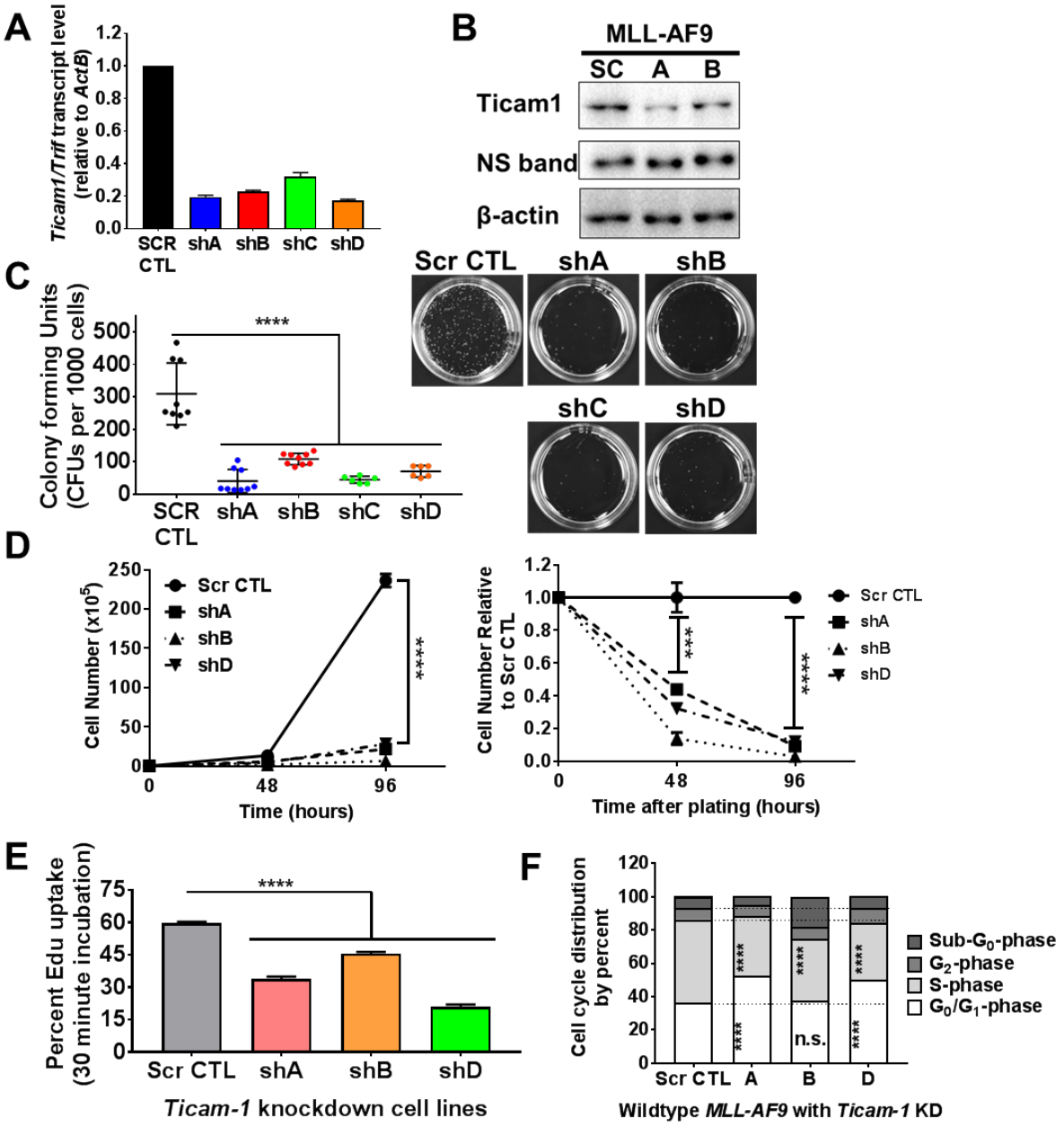


Figure 26. *Ticam-1* Knockdown Suppresses Colony Forming Efficiency and Proliferation in MLL-AF9 Cells. (A) RT-PCR validation of *Ticam-1* knockdown (*Ticam-1*^{KD}) in *Myd88*$^{+/+}$ MLL-AF9 cells compared to scrambled controls. (B) Ticam-1 immunoblot of *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (C) Colony forming efficiency in *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (D) Absolute and relative growth curve of *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (E) Edu uptake after 30 minutes for *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (F) Propidium iodide cell cycle analysis of *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. Data represent three or more independent experiments, performed in triplicate, and were compared using a Student's t-test, with n.s. indicating $p>0.05$, * indicating $p<0.05$, ** indicating $p<0.01$, *** indicating $p<0.001$, and **** indicating $p<0.0001$.

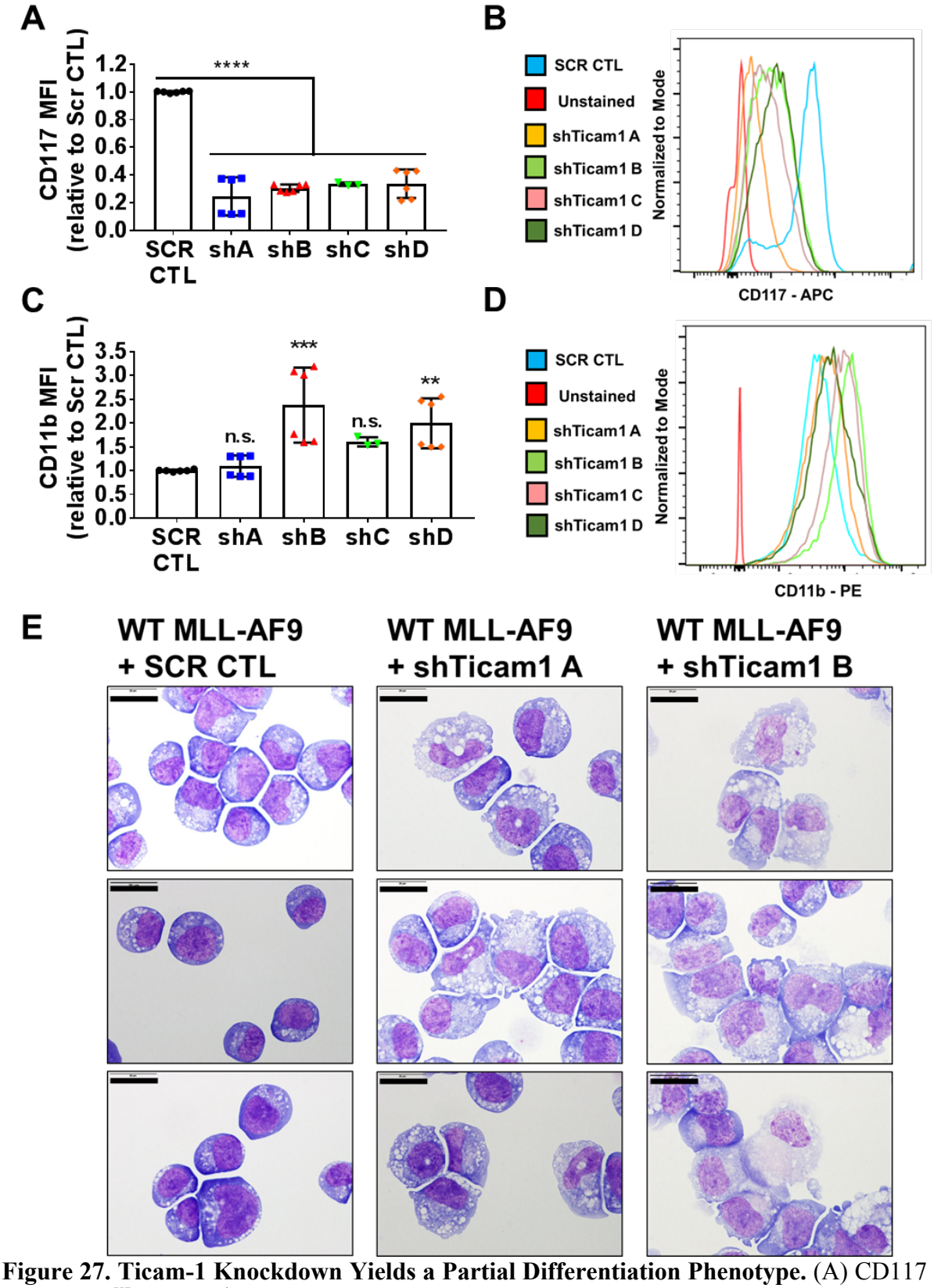


Figure 27. Ticam-1 Knockdown Yields a Partial Differentiation Phenotype. (A) CD117 MFI of *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (B) Histogram of CD117 distribution for each *Ticam-1*^{KD}

MLL-AF9 cell line. (C) CD11b of *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (D) Histogram of CD11b distribution for each *Ticam-1*^{KD} MLL-AF9 cell line. (D) Wright-Giemsa stained SCR CTL-expressing and *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells taken with 100x lens. Data represent three independent experiments, performed in triplicate, and were compared using a Student's t-test, with n.s. indicating $p>0.05$, * indicating $p<0.05$, ** indicating $p<0.01$, *** indicating $p<0.001$, and **** indicating $p<0.0001$.

Tbk1 Knockdown Does Not Result in the Ticam-1 Knockdown Phenotype

To determine how *Ticam-1* knockdown leads to a partial differentiation and suppressed proliferation phenotype, the downstream signaling pathways of Ticam-1 were interrogated. TLR signaling downstream of Ticam-1 includes activation of Tbk1 and IKKε, leading to their phosphorylation of IRFs to induce an interferon response. Both *Myd88*$^{+/+}$ and *Myd88*$^{\Delta/\Delta}$ MLL-AF9 leukemia cells show a robust sensitivity to the Tbk1 inhibitor BX795 (*Invivogen*, tlrl-bx7) in methylcellulose (Figure 28A). To determine whether this effect is specific to Tbk1 and whether the Ticam-1/Trif knockdown phenotype is due to a loss of Tbk1 activation, shRNA knockdown was applied. Genetic knockdown of Tbk1 was confirmed by qRT-PCR and immunoblotting (Figure 28B). To determine whether Tbk1 signaling contributes to the Ticam1/Trif phenotype, cell lines were assayed for CD117 and CD11b surface markers, growth curves, and cell cycle alteration. Surprisingly, no changes occurred in these assays relative to scrambled control MLL-AF9 cell lines (Figure 28C). *Tbk1*^{KD} MLL-AF9 cells were assayed by cell cycle analysis and colony forming assay, but knockdown of *Tbk1* yields no changes in these phenotypes (Figure 28D and 28E). Therefore, Tbk1 is not required for the phenotype observed with Ticam-1/Trif knockdown. Additionally, the BX795 inhibitor is likely inhibiting other targets in addition to Tbk1 to suppress colony forming ability.

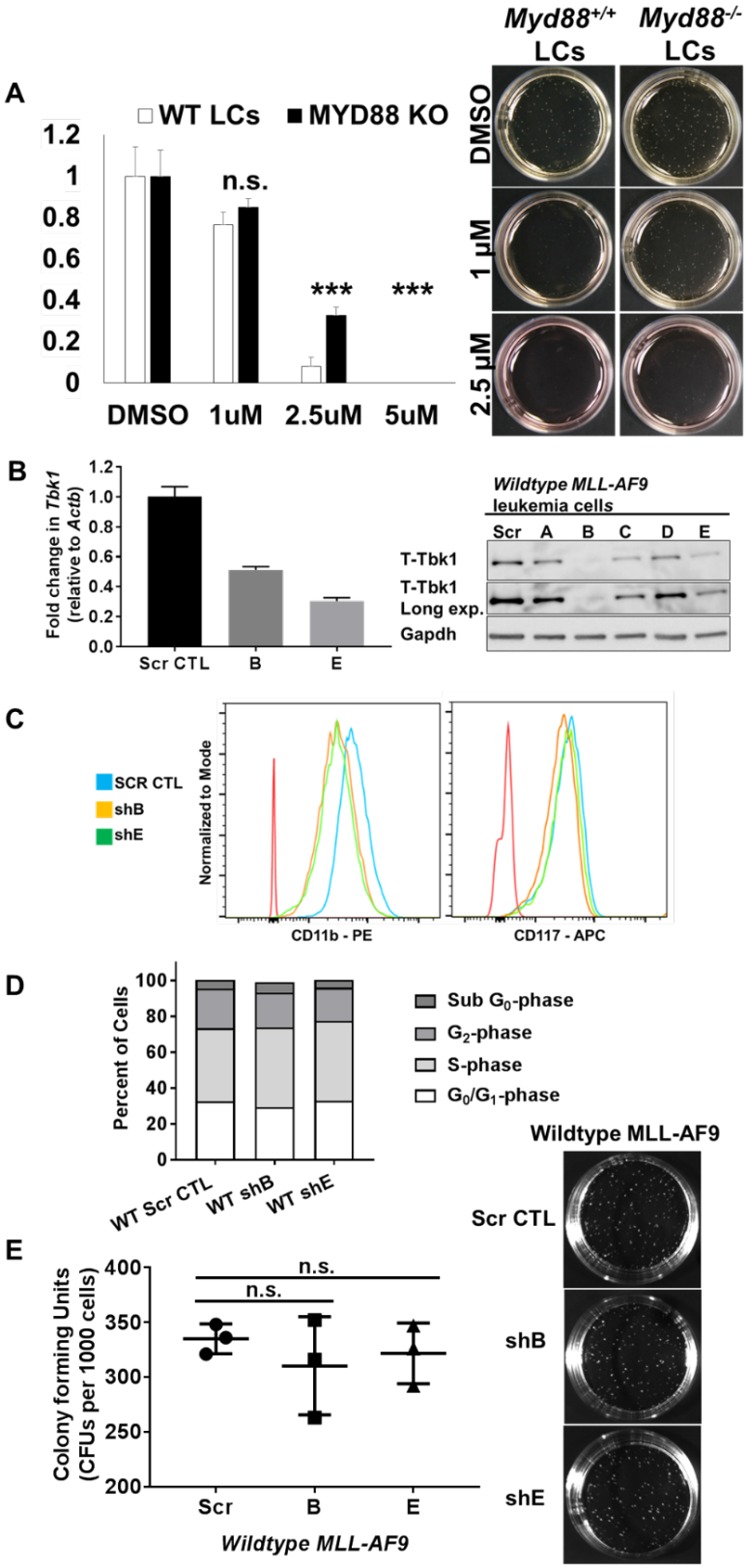


Figure 28. Tbk1 Knockdown Does Not Result in a Phenotype Comparable to Ticam-1 Knockdown. (A) Treatment of MLL-AF9 cells with *Myd88*$^{+/+}$ and *Myd88*$^{-/-}$ backgrounds with

BX795 TBK1 inhibitor. (B) RT-PCR and immunoblot validation of *Tbk1* knockdown (*Tbk1*^{KD}) in MLL-AF9 cells. (C) Colony forming efficiency of *Tbk1*^{KD} MLL-AF9 cells. (D) Cell cycle analysis of *Tbk1*^{KD} MLL-AF9 cells. (D) CD117 histogram comparison of *Tbk1*^{KD} MLL-AF9 cells to those expressing scrambled controls. (E) CD11b histogram comparison of *Tbk1*^{KD} MLL-AF9 cells to those expressing scrambled controls. Data represent three independent experiments and were compared using a Student's t-test, with n.s. indicating $p>0.05$, * indicating $p<0.05$, ** indicating $p<0.01$, *** indicating $p<0.001$, and **** indicating $p<0.0001$.

TICAM-1 Maintains RIPK1 and RIPK3 to Protect Leukemia Cell Function

Alternative downstream signaling pathways for Ticam-1/Trif were explored. Ticam-1/Trif is known to have a RHIM domain by which it interacts with the necroptotic signaling pathway components RIPK1 and RIPK3. Previous studies by our lab have implicated by RIPK1 and RIPK3 in the pathogenesis of MLL-AF9 leukemia using human and murine samples and knockout cell lines. Like Ticam-1 knockdown cells, Ripk1 and Ripk3 knockout cells demonstrate a phenotype of partial differentiation.[187] To test whether Ticam-1 phenotype is related to the Ripk1/3 knockout phenotype, murine MLL-AF9 cells with *Ticam1/Trif* knockdown were probed for phosphorylated and total Ripk3 (Figure 29A), as well as phosphorylated and total Mlkl itself (Figure 28B). In *Ticam-1*^{KD} cell lines, the knockdown of Ticam-1 is accompanied by a loss of total Ripk3, which leads to a loss of p-Mlkl. Less efficient Ticam-1 knockdown (shB) does not elicit this effect. Thus, the Ticam-1 protein is required to protect the protein stability of RIPK3 and enable pro-leukemic necroptotic signaling. Upon transplantation into mice, Ticam-1 knockdown delays leukemogenesis in a *Myd88*$^{+/+}$ MLL-AF9 background (Figure 30A, 30B). Despite this finding, leukemia development was delayed overall, and it could not be established that *Ticam-1* knockdown in addition to *Myd88* deletion further delays leukemogenesis *in vivo* (Figure 30C).

To determine whether this phenotype is reproducible in human cell lines, shRNA against TICAM-1 was expressed in all available human leukemia cell lines, including HL60, ML-2,

MM6. Molm13, MV4:11, RS4:11 (B-cell leukemia), THP1, and U937. Despite this, the only cell line in which durable knockdown at protein and transcript level was confirmed was the ML-2 cell line (Figure 31A and 31B). ML-2 is a human cell line derived from an acute myelomonocytic leukemia patient with t(6;11)(q27;q23) translocation, resulting in MLL-AF6 fusion protein, a near-tetraploid karyotype, and deletion of the wildtype *MLL1* allele.[192] When this cell line was likewise assayed for Tbk1 phosphorylation, a partial, but not complete, reduction of signal was observed (Figure 31C). When ML-2 cells bearing TICAM-1 knockdown were assayed for RIPK1, RIPK3, and MLKL, a loss of total protein was observed for both RIPK1 and RIPK3 (Figure 31D). Interestingly, *RIPK3* transcript was induced 30-fold compared to scrambled controls and inefficient knockdowns (Figure 31E). Consistent with this, the reduction in TICAM-1, RIPK1, and RIPK3, resulted in a loss of p-MLKL (Figure 31F). When assayed for colony-forming ability, these cells demonstrated a 4-fold reduction in CFU ability relative to scrambled controls (Figure 31G). Thus, when TICAM-1 knockdown is achieved in human ML-2 cells, it leads to destabilization of RIPK1 and RIPK3 protein level and disrupts their pro-leukemic signaling described in Xin *et al*.[187]

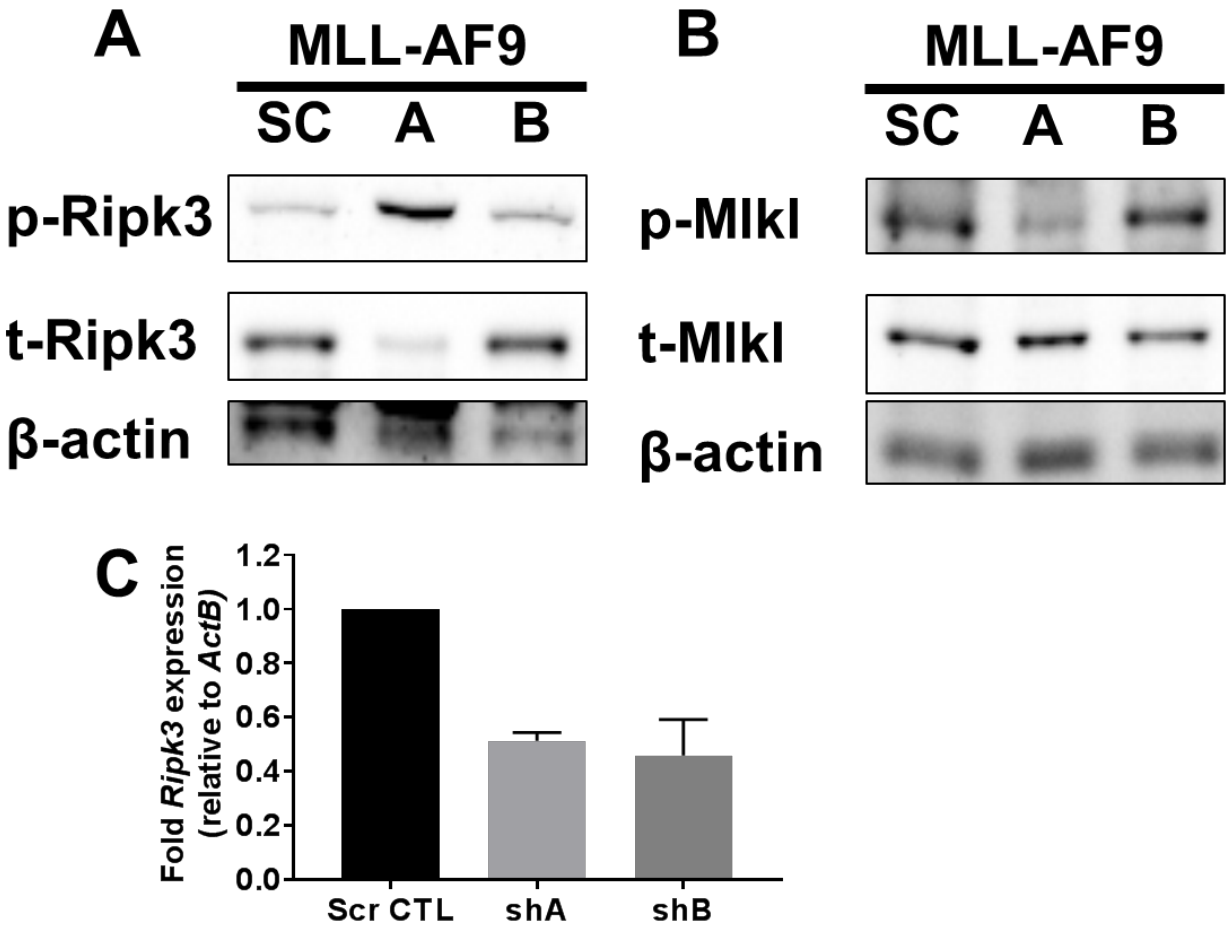


Figure 29. Ticam-1 Knockdown Efficiency Correlates with Ripk3 Protein Level and Mlkl Activity in Murine MLL-AF9 Cells. (A) Immunoblot of phosphorylated Ripk3 and total Ripk3 in *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (B) Immunoblot of phosphorylated Mlkl and total Mlkl in *Ticam-1*^{KD} *Myd88*$^{+/+}$ MLL-AF9 cells. (C) RT-PCR of *Ripk3* in murine MLL-AF9 cell lines.

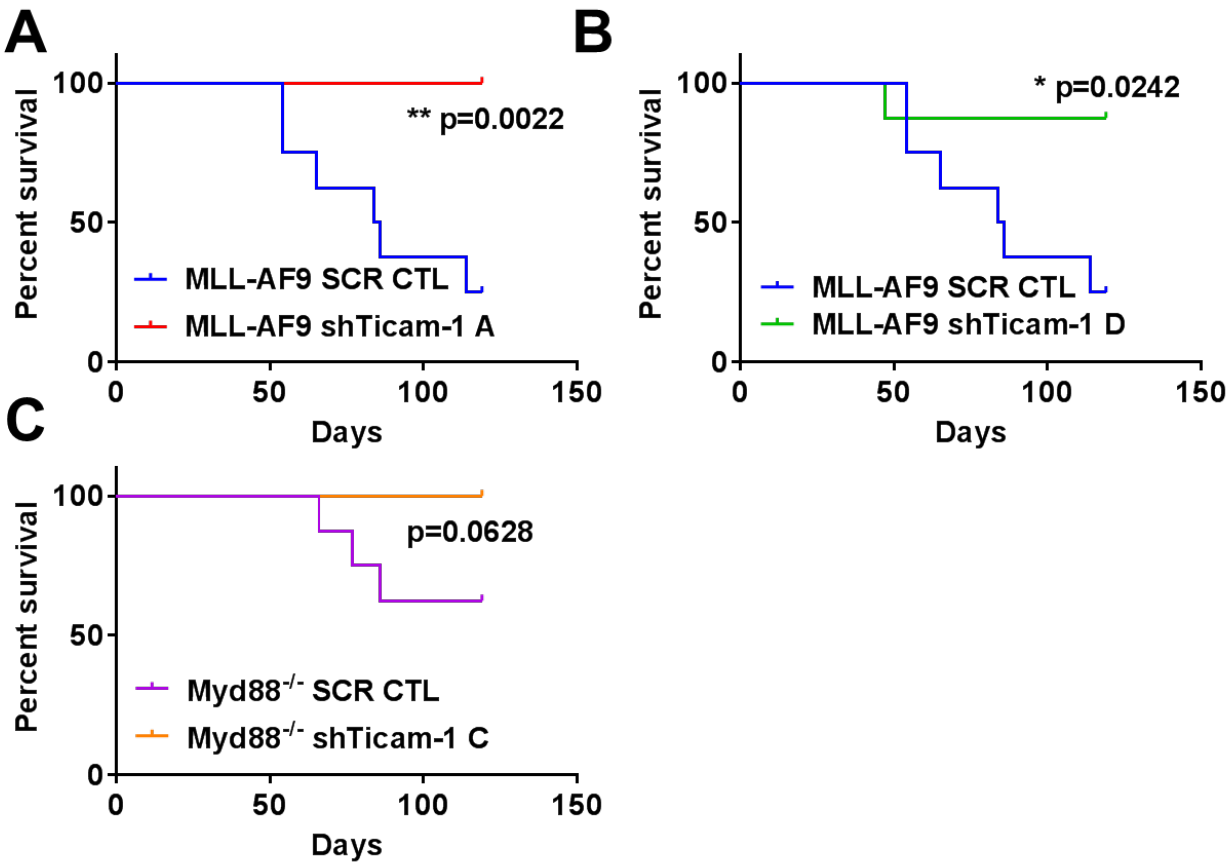
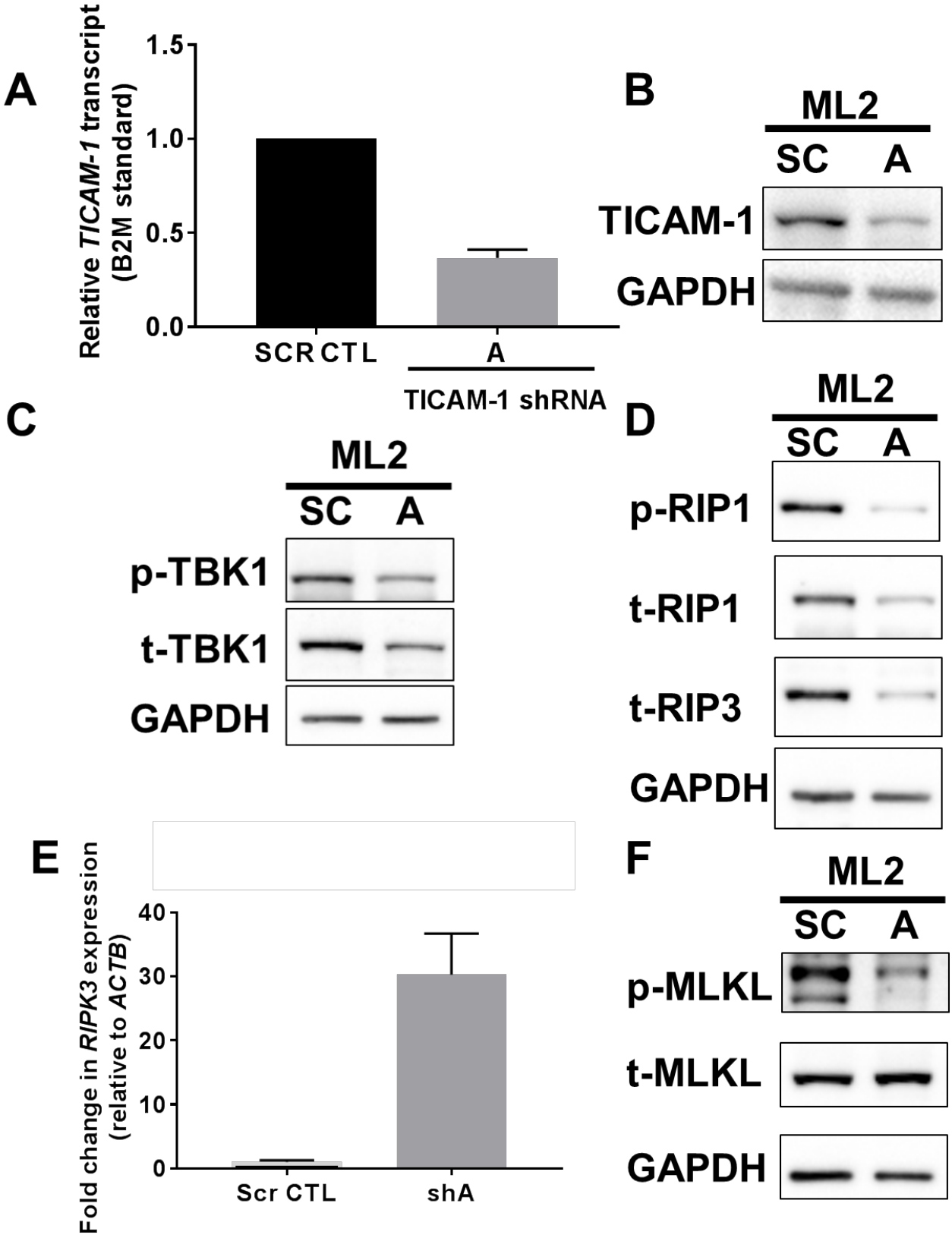


Figure 30. Ticam-1 is Required for Leukemogenesis *in vivo* in the Murine MLL-AF9 Model System. Transplantation study of mice transplanted with 10,000 MLL-AF9 cells expressing scrambled control or one of two *shTicam-1* constructs, annotated as shA (A) and shD (B) vectors (n=8 for each group). (C) Survival of mice transplanted with *Myd88*$^{-/-}$ MLL-AF9 cells expressing either scrambled control or shTicam-1 C. Difference between groups is determined using Log-rank Mantel-Cox statistical analysis, with n.s. indicating $p>0.05$, * indicating $p<0.05$, and ** indicating $p<0.01$.



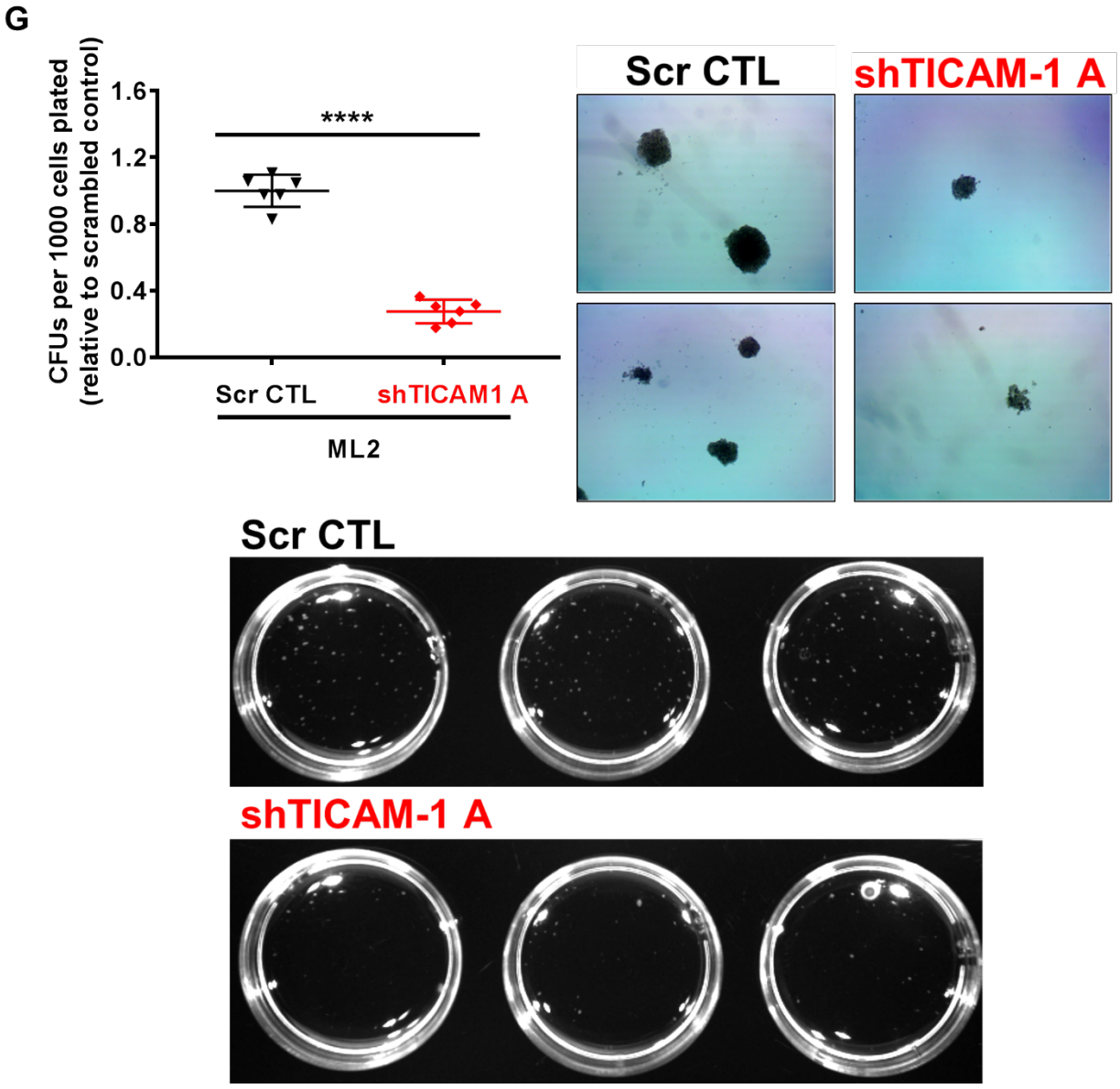


Figure 31. TICAM-1 Knockdown in Human ML-2 Cell Lines Suppresses Colony-Forming Ability and Destabilizes RIPK1 and RIPK3 Protein Level. (A) RT-PCR validation of *TICAM-1*^{KD} in ML-2 cell line. (B) Immunoblot of TICAM-1 in ML2 cell line. (C) TBK1 immunoblot of ML-2 TICAM-1 knockdown and scrambled control expressing cell lines. (D) RIPK1 and RIPK3 immunoblot of *TICAM-1*^{KD} ML-2 cells. (E) *RIPK3* transcript in scrambled control and *TICAM-1*^{KD} ML-2 cells as measured by RT-PCR. (F) MLKL immunoblot in *TICAM-1*^{KD} ML-2 cells and scrambled control. (G) Colony forming assay of scrambled control and *TICAM-1*^{KD} ML-2 cells.

CHAPTER FOUR

DISCUSSION

This dissertation sought to determine whether MYD88-dependent and TICAM-1-mediated signaling have a role in the context of AML model systems. I hypothesized that MYD88-dependent signaling and TICAM-1-dependent TLR signaling promote leukemia cell proliferation and loss of cell surface markers associated with stemness. Accordingly, I predicted that their inhibition or genetic ablation would result in increased cell death, reduced proliferation, and partial differentiation. In this study, signaling components of TLR signaling were found to be expressed in human and murine leukemia model cell lines and associated with poor patient survival. Disruption of TLR signaling by genetic knockdown or deletion of MYD88 or TICAM-1 reduced leukemia cell function in several assays. Both MYD88-dependent and TICAM-1-mediated signaling support the undifferentiated leukemia stem cell phenotype through independent mechanisms. The respective deletion and knockdown of MYD88 and TICAM-1 reduced cell stemness markers, colony forming units, proliferation, leukemogenesis *in vivo*, while increasing differentiation markers (Figure 32). These results indicate a novel function of both TLR adaptors in MLL-AF9 leukemia to protect the undifferentiated status of leukemia cells and their leukemogenic capacity.

In M4/M5 AML subtypes, overexpression of cell surface TLRs and adaptors MYD88 and TICAM-1 were shown in a large patient cohort and published datasets. Further, several TLR

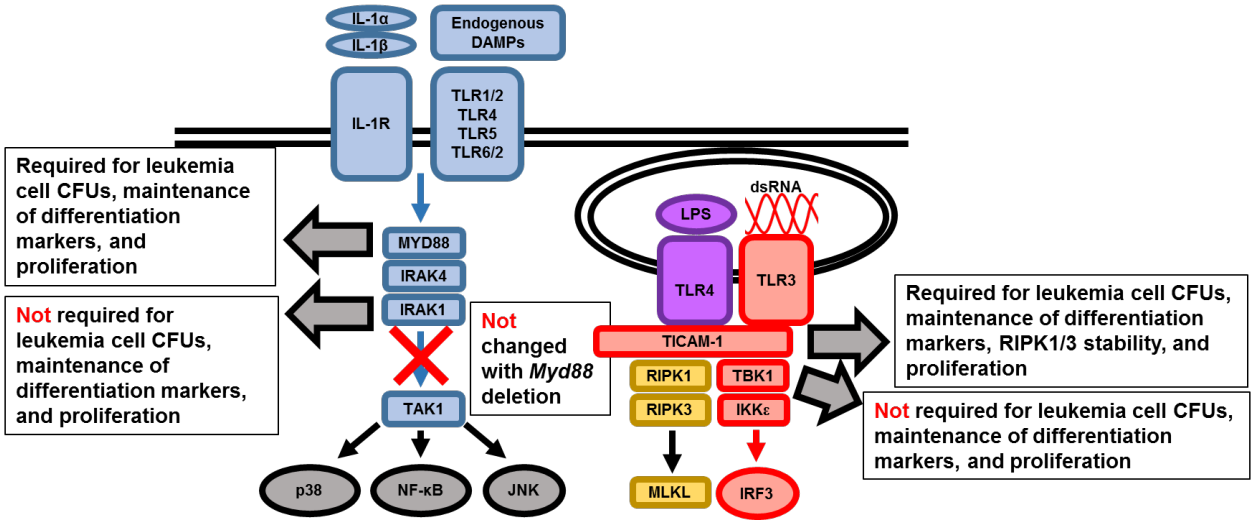


Figure 32. Toll-like Receptor Signaling in AML – Conclusions.

mediators for both MYD88 and TICAM-1-dependent signaling, including IRAK1 and TBK1, are correlated with adverse outcomes in the TCGA dataset. This led to the hypothesis that TLR signaling is activated in a subset of leukemia cells, with prognostic and therapeutic implications.

In both human and mouse cell lines, TLRs and their downstream signaling components are expressed, functionally responsive to known TLR stimuli, and phosphorylated, indicating constitutive activity. Both MYD88-dependent and MYD88-independent signaling pathways were shown to have baseline activity. With this preliminary data, the hypothesis that TLR signaling supports the leukemia cell phenotype was proposed. To test this hypothesis, both pharmacological and genetic approaches targeting critical adaptor proteins MYD88 and TICAM-1 were employed.

Much of the study on TLR signaling in AML has been performed in MLL-rearranged models or those driven by genes activated by MLL fusions.[181, 182, 185] The first approach I employed transformed *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ CD117^{+} progenitor cells. Surprisingly, following transformation, colony forming efficiency is unchanged for both MLL-AF9 and MLL-ENL constructs. When colony forming potential was tested in transformed *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$

MLL-AF9 leukemia cells, following primary transplantation, colony forming ability again remained unchanged. Therefore, Myd88 and Myd88-dependent signaling are not required for MLL-rearranged transformation and leukemogenesis in murine models.

Like other studies, these experiments primarily explored the contribution of TLR signaling to leukemia pathogenesis, using models that primarily reflect the AML myelomonocytic and monocytic subtypes in which TLR and adaptor protein expression is enhanced. However, the role of TLR signaling in AMLs in which TLR expression is relatively reduced, such as RUNX1-ETO and CBFβ-MYH11 fusions, remains unexamined. The microarray performed by Jianjun Chen, Ph.D. revealed that, in these leukemia subtypes, cell surface TLRs, adaptor proteins MYD88 and TICAM-1, and downstream mediators were downregulated, relative to average expression in the leukemia sample cohort. Interestingly, when murine progenitor cells were transformed with RUNX1-ETO expression vectors, the *Myd88*$^{-/-}$ genotype enhances CFU efficiency. These findings suggest that the inhibition or genetic deletion of TLR signaling may result in more potent forms of these AML subtypes. Therefore, MYD88-dependent TLR signaling exerts an anti-leukemic effect in these cells, but is required in other leukemia subtypes. Further study is merited to determine why RUNX1-ETO and CBFβ-MYH11 colony forming efficiency is enhanced in an *Myd88*$^{-/-}$ background. Perhaps a reduction of MYD88-dependent signaling protects RUNX1-ETO and CBFβ-MYH11 leukemic colony-forming and stem cells. Conversely, TLR stimulation in leukemia cells bearing these fusion proteins may act to attenuate these leukemia cells through an unstudied mechanism, either in a cell autonomous manner or via cytokine signals secreted in response to the TLR stimulation. However, since much of the supporting literature examined the role of TLR signaling in MLL-rearranged models, this study was planned around that leukemia cell subtype.

Rather than starting with a *Myd88*$^{-/-}$ background MLL-AF9 leukemia cell, the MLL-AF9 model was used to generate a leukemia cell line with Cre/LoxP-mediated inducible *Myd88* deletion. Using this approach, the deletion of *Myd88* could be used to mimic complete inhibition of Myd88-dependent signaling in an established leukemia cell line. With the MLL-AF9 model, CreERT2-mediated inducible deletion of *Myd88* was applied to test this hypothesis and carefully controlled to avoid Cre-mediated and CreERT2-mediated off-target toxicity. Of note, the low threshold for CreERT2-mediated toxicity and cell death underlies the need to carefully control the application of the Cre/LoxP system in model systems for leukemia and other malignancies. In this study, the expression of Cre and over-activation of CreERT2 in MLL-AF9 leukemia cells resulted in cell death, reduction in proliferative capacity, and impaired colony forming capacity. These findings reflect the hazards of permanently overexpressing targeted recombinases and nucleases, including CRISPR Cas9, in cell lines for experimentation and were consistent with published literature on Cre recombinase.[193, 194] Cre is also known to act on cryptic LoxP sites found in mammalian genomes and suppresses proliferation in several model systems.[194, 195] Activation of Cre or CreERT2, with no other treatment, causes regression of murine lymphomas *in vivo*.[196] Despite the efficiency of targeted deletion of LoxP flanked genes or sgRNA-targeted genome sequences, the expression of genomic recombinases and nucleases must be carefully controlled to account for these off-target effects. Previous studies on Cre recombinase have demonstrated a range of expression in which Cre recombinase is effective, yet exerts no detectable toxicities.[197] Application of CreERT2 or self-deleting Cre recombinase has been shown to avert genomic toxicity caused by Cre in mammalian systems.[198] These studies illustrate the need for appropriate LoxP-free controls, even in experiments in which Cre recombinase is expressed under the control of an endogenous promoter.

Inducible *Myd88* deletion and subsequent loss of downstream signaling yields a partial differentiation phenotype, as measured by colony forming ability, cell surface markers for both leukemia cell stemness (CD117) and macrophage differentiation (CD11b), proliferation, and *in vivo* transplantation. Interestingly, despite the numerous studies linking MYD88-dependent TLR signaling to downstream TAK1-dependent NF-κB, JNK/AP-1, and p38 pathways, both germline and inducible *Myd88*$^{-/-}$ leukemia cells show no change in these pathways. The unchanged activation of NF-κB may be due to dysregulation of other inflammatory mediators, such as TNFα, that converge on NF-κB activation in leukemia cells. With the autocrine production of and stimulation by these factors, leukemia cells may be able to maintain NF-κB activity even with genetic deletion of upstream TLR mediators.

These findings are consistent with those of Liang *et al.*, who, using shRNA screen and RNA-sequencing showed that a MYD88-IRAK4-UBE20 signaling axis in MLL-rearranged leukemia is required for UBE20-mediated ubiquitination of the MLL1/KMT2A C-terminal domain. This results in the degradation of MLL1/KMT2A and allowed the mutated fusion protein to dominate epigenetic loci. In their human cell line model, they noted that knockdown of any upstream TLR/IL-1R mediator failed to alter transcripts of NF-κB-regulated genes as measured by RNA sequencing.[181] While the study by Liang *et al.* links IL1R/TLR-MYD88-IRAK4 signaling to the stability of the MLL1 to tip the epigenetic balance toward MLL-fusion proteins, and used IRAK and UBE20 inhibitors to suppress leukemogenesis *in vitro* and *in vivo* to great effect, several discrepancies persist between our studies. First, Cre-mediated *Myd88* deletion, in our system, delayed MLL-AF9 leukemogenesis by two weeks, whereas application of the IRAK1/4 inhibitor delays median leukemia much greater that two weeks, beyond the course of the experiment. The

knockdown of *Irak1* in murine MLL-AF9 cells failed to elicit a comparable phenotype and failed to suppress growth or colony forming ability consistently. These findings lead to the concern that IRAK1/4 inhibitors exert unknown off-target effects because the inhibitors yield a stronger phenotype than either genetic model.

Recent studies examining S100A8 and S100A9-mediated TLR4 stimulation link the observed partial differentiation phenotype to JNK signaling, but not NF-κB signaling.[185] Of note, S100A8 and S100A9, both TLR4 ligands, elicits ligand-specific effects, with S100A8 blocking the differentiation induced by S100A9. The study described in the dissertation applied genetic deletion and knockdown of TLR adaptors to determine their net contribution to the leukemia cell phenotype. The findings presented here indicate that basal stimulation of TLR pathways protect MLL-AF9 leukemia cells in an undifferentiated state. However, downstream signaling of p38, JNK, and NF-κB in *Myd88*$^{-/-}$ MLL-AF9 leukemia cells remain unchanged. These observations merit further study into the precise role of mediators of downstream MYD88-dependent signaling, TAK1 and TRAF6, in AML and related disorders. Numerous studies have shown that TRAF6 is specifically dysregulated in MDS, but the impact of this dysregulation on TAK1 activity and that of TAK1-induced pathways also is unclear. If activation of terminal downstream signaling is conserved and activated in malignancy and MDS, then the precise pathogenic mechanism remains undetermined.

These findings are also in contrast to those of lymphoma models bearing L265P mutations.[123] Both the initial characterization of the MYD88 L265P mutation enriched in subsets of lymphomas describe its mechanism as one of spontaneous MYD88 oligomerization and constitutive activation of IRAK and BTK downstream kinases. This results primarily in a molecular signature characterized by upregulation of NF-κB-regulated genes. NF-κB is also

known to be downstream of activated MYD88-dependent signaling in preclinical models for acute T-cell lymphoblastic leukemia.[199] The decoupling of MYD88-dependent TLR activity at baseline from NF-κB activity may be specific to myeloid malignancy. The persistence of NF-κB activity in leukemia cells may also be due to the presence of other cytokines and oncogenic, active signaling that maintain NF-κB activation. Also, it may be partially explained by differential effects of TAK1 in myeloid cells compared to other tissues. While TAK1 is required for the phosphorylation of IKKα, p38, ERK, and JNK, in TLR signaling, it is known to have suppressive effects on inflammation as well. In unstimulated conditions, TAK1 suppresses inflammasome activation in myeloid cells by suppressing NLRP3 inflammasome activation and reducing basal NF-κB activity to limit downstream TNFα transcription.[46] The inflammatory modulatory effect of TAK1 extends to hepatic tissues, since hepatocyte-specific TAK1 deletion results in an inflammatory state progressing to fibrosis and hepatocellular carcinoma.[200] This progression is limited by TLR4 or MYD88 deletion. While this study demonstrates the immunomodulatory effect of TAK1, it also shows that TLR4- and MYD88-dependent signaling have pro-inflammatory and pro-tumorigenic effects in the absence of TAK1, which might indicate additional TLR-dependent downstream pathways. With respect to the malignant myeloid cell population, the specific role of TAK1 for NF-κB induction and other downstream signaling, as well as TLR-dependent, TAK1-independent pro-inflammatory pathways must be determined. Interestingly, myeloid-specific *Tak1* deletion results in spontaneous myelomonocytic leukemia development, though the status of downstream NF-κB and other signaling pathways is unclear.[201]

In all MLL-AF9 cells tested bearing *Myd88* deletion, the cell surface markers and morphologic staining indicate a partial differentiation phenotype. Therefore, Myd88 is required to maintain the MLL-AF9 leukemia cells in an undifferentiated state. Interestingly, similar cell stemness and differentiation marker levels were observed, relative to *Myd88*-expressing controls, in all *Myd88*-null cells, whether deletion is germline, Cre-mediated, or CreERT2-mediated. To determine whether downstream activity of IRAK1 and IRAK4 were required to protect the undifferentiated leukemia cell, *wildtype* leukemia cells were treated with IRAK1/4 inhibitor for five or seven days and assayed for cell surface markers. IRAK1/4 inhibitor treatment suppressed cell growth in suspension culture, but failed to induce partial differentiation cell surface phenotype. The inhibitor also failed to suppress leukemia cell colony-forming efficiency, contrasting with other studies.[181] Further, IRAK1/4 inhibitor is equally effective in $Myd88^{+/+}$ and $Myd88^{-/-}$ MLL-AF9 backgrounds, even when the $Myd88^{-/-}$ MLL-AF9 cells were later found to lack detectable Irak1 protein. These results were particularly surprising, since published data indicated that IRAK1/4 inhibitor selectively suppresses murine and human IRAK with comparable treatment doses. These findings suggest one of two conclusions, either Irak signaling is required and active in $Myd88^{-/-}$ MLL-AF9 leukemia cells, even in the absence of Myd88, or off-target effects are partially responsible for effects of IRAK1/4 inhibitors.

Unlike in $Myd88^{-/-}$ MLL-AF9 cells, efficient IRAK1 knockdown failed to induce partial differentiation in MLL-AF9 leukemia cells, indicating that IRAK1 and IRAK4 are not required for induction of partial differentiation in MLL-AF9 leukemia cells. These findings suggest either a specific role for MYD88, perhaps independent of its functions in TLR signaling, or that MYD88 is capable of inducing unknown downstream signaling to mediate this anti-differentiation phenotype independently of IRAK signaling. Recently, several novel attributes of

MYD88 have been described. The MYD88 accessory adaptor MAL is known to interact directly with TRAF6, and this interaction is required for TLR-induced NF-κB induction.[202] If IRAK1/4 are not required for TRAF6-NF-κB induction in leukemia cells, then that might explain the observed phenotype. Proteomic and RNA sequencing approaches would be best equipped to determine the MYD88 interactome and transcriptional consequences of *MYD88* deletion or mutation.

The role of TICAM-1 and TICAM-1-dependent signaling in AML and leukemia cell lines and models, outside of this study, remains unexamined. TICAM-1 is established as an inducer of IRFs and transcription of type I interferon, which has antileukemic effects.[187, 203] To determine the contribution of MYD88-independent signaling, mediated by TICAM-1, shRNA knockdown against *TICAM-1* was applied in murine models and human cell lines. The knockdown of *Ticam-1* in *wildtype* MLL-AF9 leukemia cells elicited a phenotype marked by loss of colony forming efficiency. *Ticam-1* knockdown also resulted in reduction in proliferation, as measured by growth curves, Edu uptake, and cell cycle analysis. Morphological analysis and cell surface staining revealed that this reduction in proliferative capacity of *Ticam-1*^{KD} MLL-AF9 cells is due to a partial differentiation phenotype independent from that of the *Myd88* deletion model. To determine precisely how Ticam-1 contributes to the maintenance of the undifferentiated leukemia cell phenotype, known downstream signaling pathways were surveyed.

TICAM-1 is known to induce IRFs in a TRAF3-TBK1-IKKε-dependent manner. MLL-AF9 leukemia cells responded robustly to TBK1-targeted inhibitor BX-795. To determine the precise relationship of TBK1 to this phenotype, a *Tbk1*-specific shRNA was applied in the MLL-AF9 mouse model. Knockdown of *Tbk1* failed to elicit a partial differentiation response or a reduction in proliferation. *Tbk1* knockdown also failed to reduce CFU efficiency observed with *Ticam1*

knockdown. Therefore, the *Ticam-1*-dependent partial differentiation phenotype is not mediated by Tbk1.

Ticam-1 is known to interact with Ripk1 and Ripk3 to induce necroptotic cell death in immune cells with TLR3 stimulation. Ripk3 protein level is reduced in *Ticam1*^{KD} MLL-AF9 cells despite modest reduction in *Ripk3*. Therefore, Ticam-1 is required for Ripk3 protein stability. The reduction of Ripk3 protein level yields a reduction in phosphorylated Mlkl, indicating a reduction in necroptotic signaling. *TICAM-1* knockdown in the ML2 cell line resulted in a comparable phenotype with a reduction in proliferation and colony forming efficiency. Further, *TICAM-1* knockdown resulted in a likewise reduction in RIPK1 and RIPK3 protein level, again resulting in a loss of phosphorylated MLKL. Necroptotic signaling has previously been shown to be required for leukemia cells in both murine and human models.[187] The ability of TICAM-1 to stabilize critical necroptotic kinases RIPK1 and RIPK3 is a novel relationship between these signaling pathways.

Although relatively unexamined, systemic TICAM-1 stimulation or administration of downstream interferons is known to have anti-leukemic effects as described in previous sections. Study of downstream TICAM-1 signaling found that the pro-leukemic effect of TICAM-1 occurs independently of TBK1, the downstream TLR mediator involved in IRF activation. Further study showed, in both human and murine leukemia cell models, that TICAM-1 protects RIP kinases from degradation, permitting their pro-leukemic activation of MLKL and NF-κB as previously described. Whether TICAM-1 is able to interact with RIP kinases and prevent their degradation when TICAM-1 specific TLR stimulation is induced remains to be determined. Perhaps, in addition to inducing transcription of an interferon response, recruitment of TICAM-1 to the stimulated receptor sequesters TICAM-1, allowing RIPK1/RIPK3 degradation in leukemia cells.

This phenomenon might also account for the neutral or negative phenotypic responses to TLR3 and TLR4 stimulation reported here and elsewhere. Alternatively, TNFα signaling is known to be induced in AML, and RIPK1 and TRAF E3 ligases are known to function in the TNFα receptor complex. Perhaps TICAM-1 plays an as of yet undetermined role in this receptor complex. The precise mechanism of TICAM-1 in these complexes, however, remains to be determined.

However, the findings of *TICAM-1* knockdown in the ML-2 cell line must be further discussed. ML-2 is a human AML cell line with a distinct genetic background. It has a near-tetraploid karyotype, with duplications of several chromosomes, including chromosome 11. It has two copies of the MLL-AF6 fusion protein, and no functional *MLL1* gene. Therefore, because *TICAM-1* knockdown is able to induce such a robust phenotype in the ML-2 genetic background, TICAM-1 is acting independently of MLL1 and may require MLL-rearrangement. However, whether the presence of functional MLL1 masks the effects of TICAM-1 knockdown remains undetermined. Though *TICAM-1*-targeting shRNA expression vectors were expressed in several human cells lines, the knockdown was verified at protein and transcript levels was only achieved in the ML-2 cell line. To determine whether *TICAM-1* knockdown elicits a similar phenotype in other human cell lines, future study will expand *TICAM-1* knockdown to human cell lines, with attention given to establishing *TICAM-1* knockdown in genetic backgrounds with and without MLL-rearrangements.

Likewise, the murine MLL-AF9 model, while an efficient and useful preclinical model for MLL-AF9 AML, is not without flaws. When AML arises in a patient due to a MLL translocation, one allele of both MLL and its fusion partner are consumed in the generation the genomic translocation and resulting in-frame fusion protein. The murine MLL-AF9 model

system, while bearing a close resemblance to human AML with the same translocation, does not exactly mimic human disease in a molecular and genetic sense. The introduction of human MLL-AF9 to murine hematopoietic progenitor cells is achieved by retroviral infection and selection of infected clones using neomycin resistance as a selection marker. First, in contrast to MLL-AF9 rearrangements treated in the clinic, the introduction of the MLL fusion protein in this manner conserves two allelic copies of both MLL and AF9. The occurrence of a translocation, in comparison, would leave only one functional allele of both MLL and AF9, while retaining their respective N- and C-terminals in the fusion protein. Second, the introduction of MLL-AF9 by retroviral infection yields two additional unfavorable possibilities. First, the genomic incorporation of the retroviral vector may generate additional mutations and disrupt other signaling pathways or gene expression regulatory pathways by integrating into the genome at an essential site, resulting in a MLL-AF9 leukemia model bearing additional, undetected alterations. Second, the use of retrovirus to introduce the human MLL-AF9 fusion protein introduces a risk inherent to the technique that multiple retroviral sequences, and multiple MLL-AF9 expression vectors, may integrate into the genome, resulting in an excessive overexpression of the fusion protein.

Because each of these experimental flaws introduce the possibility that the data that we have collected and presented in this study do not accurately model the pathogenesis of AML in patients, additional studies must be undertaken to clarify the roles of MYD88 and TICAM-1 in human systems and primary patient samples. First, whether TICAM-1-dependent signaling is required in other human cell lines, in addition to ML-2 must be investigated. This approach will be undertaken using either shRNA knockdown or CRISPR/Cas9 genomic deletion. Alternative shRNA sequences will be obtained, as the human shRNA obtained from Origene was only

effective in the ML-2 cell line and failed to knockdown TICAM-1 in any other cell line. Accordingly, whether TICAM-1 knockdown correlates with RIPK1 and RIPK3 protein level reduction in other human cell lines, and whether this phenotype correlates with specific human cell line genotypes, will be determined. If such a correlation is reproducible, RIPK1 and RIPK3 will be re-expressed to determine if TICAM-1-mediated protein-level stabilization is the mechanism by which RIPK1 and RIPK3 protein level is maintained, as I would hypothesize based on data presented here. Further, the precise interaction between TICAM-1 and RIP proteins will be explored to determine how TICAM-1 maintains RIP protein levels, whether it is through a transcriptional effect, direct interaction, or some other mechanism. Lastly, the mechanism of RIPK1 and RIPK3 degradation will be determined using inhibitors directed against proteasome, A20, autophagy, and other degradation processes.

If the reduction of RIPK1 and RIPK3 protein level is shown to be unrelated to the TICAM-1-mediated phenotype, then targeted disruption of the highly redundant TRAF network will be explored, first by overexpressing TICAM-1 with specific mutations in domains required for TRAF2, TRAF3, and TRAF6 interactions. If interaction with TRAFs is also not required for leukemia cell colony-forming ability, proliferation, and findings, then whole exome RNA-sequencing will be applied to identify specific transcriptional alterations between both human and murine cell lines with *TICAM-1* knockdown and their respective scrambled controls.

With respect to MYD88, future studies include generating both MYD88 and IRAK1 knockdown in several human cell lines to determine whether the phenotypes reflecting the requirement for MYD88 to prevent partial differentiation and maintain colony-forming ability and proliferation, but no change with IRAK1 knockdown or inhibition, are reproducible in human cell lines. If, as expected, MYD88, but not IRAK1, is required for leukemia cell

proliferation, colony-forming ability, and maintenance of cell surface markers, then whole-exome RNA sequencing will be applied to identify novel IRAK1-independent factors downstream of MYD88 that mediate the pro-leukemic effects of MYD88.

In conclusion, TLR signaling adaptors MYD88 and TICAM-1 are required for MLL-AF9 pathogenesis in myelomonocytic and monocytic (M4/M5) AML subtypes. IRAK1/4 and TBK1, downstream of MYD88 and TICAM-1 respectively, are both efficiently targeted by specific inhibitors, but those inhibitors and their specific knockdown fail to elicit a phenotype comparable to MYD88 or TICAM-1 knockdown. These findings indicate that alternative functions or complex partners for MYD88 and TICAM-1 facilitate pro-leukemic effects. Despite the phenotypic observations that led to this conclusion, the exact downstream mechanisms of the phenotypes derived by MYD88 and TICAM-1 deletion or knockdown remain the subject of several key questions that will hopefully be clarified by future studies.

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VITA

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