Proapoptotic Bid inhibits the Execution of Programmed Necrosis Affecting Hematopoietic and Intestinal Homeostasis

By

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To my brother, Brinson Edward Wagner, who never had the opportunity to grow into the intelligent man he would have been today. I do this in your memory and know you would be proud.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

AGM	Aorta-gonad mesonephrous
ALS	Amyotropic lateral sclerosis
AML	Acute myeloid leukemia
AMPs	Antimicrobial proteins
APAF-1	Apoptotic protease activating factor-1
APCs	Antigen presenting cells
ASXL1	Additional sex combs-like 1
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad-3 Related
ATRIP	ATR- interacting protein
BAK	BCL-2 homologous antagonist killer
BAX	BCL-2 Associated X protein
BCL-2	B cell lymphoma-2
BCL-xL	BCL-2 related gene, long isoform
BH Domain	BCL-2 homology domain
BID	BH3-interacting domain death agonist
CARD	Caspase activation and recruitment domain

Caspases	Cysteine-dependent aspartate specific proteases
CBC cells	Crypt base columnar cells
CBL	Casitas B-Lineage Lymphoma
CD	Clusters of differentiation
CDAMs	Cell death-associated molecules
CEBPα	CCAAT Enhancer-binding protein alpha
CED	Cell Death Abnormal
cFlip	Cellular FLICE-like inhibitory protein
CFU-S	Colony forming unit- Spleen
CHIP	Clonal hematopoiesis of indeterminate potential
cIAP1 & 2	Cellular inhibitor of apoptosis 1 and 2
CK1	Casein Kinase 1
CK2	Casein Kinase 2
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CYLD	Cylindromatosis
DAI	DNA-dependent activator of interferon regulatory factors
DAMPs	Damage-associated molecular patterns
DD	Death Domain

DDR	DNA Damage Response
DED	Death Effector Domain
DIK	Delta interaction protein kinase/ Rip4
DISC	Death-inducing signaling complex
DNMT3A	DNA(Cytosine-5)-Methyltransferase 3 Alpha
DRP1	Dynamin-related protein 1
dsDNA	Double-stranded DNA
DSS	Dextran Sodium Sulfate
EBI	Erythroblastic islands
EGL	Egg-laying defective
EPO	Erythropoietin
FCGBP	Fc-gamma binding protein
FSC	Forward Scatter
GATA2	GATA- binding factor 2
G-CSF	Granulocyte- Colony stimulating factor
GIP	Glucoinsulinotropic peptide
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
GMP	Granulocyte-macrophage progenitor
HMGB1	High mobility group box 1

HOIL-1	Heme-oxidized iron regulatory protein 2 ubiquitin ligase -1
HOIP	HOIL-1 interacting protein
HSC	Hematopoietic Stem Cell
HSPCs	Hematopoietic stem and progenitor cells
HSPs	Heat shock proteins
HSV-I&II	Herpes Simplex Virus Type I and I
IAPs	Inhibitors of apoptosis
IBDs	Inflammatory bowel diseases
IDH1 & 2	Isocitrate dehydrogenase 1 and 2
IFN	Interferon
IFNAR1	Interferon α/β receptor 1
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IL-1β	Interleukin-1 Beta
IL-3	Interleukin-3
IL-6	Interleukin-6
IRF	Interferon regulatory factors
ISC	Intestinal Stem cell
ISGF3	Interferon-stimulated gene factor 3 complex

ISEL	*In situ* end labeling
JAK1/2	Janus Kinase 1/ Janus Kinase 2
JNK	Jun amino-terminal kinase
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LPS	Lipopolysaccharide
LRRK	Leucine-rich repeat kinase
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia-1
MCMV	Mouse cytomegalovirus
M-CSF	Macrophage- Colony Stimulating Factor
MDS	Myelodysplastic Syndrome
MEF	Murine embryonic fibroblast
MEKK1	MAPK kinase kinase 1
MEKK2	MAPK kinase kinase 2
MEP	Megakaryocyte erythrocyte progenitor
MFN1-2	Mitofusin1 and 2
MHC I	Major histocompatibility complex class I
MLKL	Mixed lineage kinase domain-like
MOMP	Mitochondrial outer membrane permeabilization

MPN	Myeloproliferative neoplasm
MPP	Multipotent progenitor
MSU	Monosodium urate
MTCH2	Mitochondrial carrier homolog 2
MYD88	Myeloid differentiation on primary response gene 88
Nec-1	Necrostatin-1
NEMO	NF-κB essential modulator/ Inhibitor of NF-κB kinase γ
NETs	Neutrophil extracellular traps
NK cell	Natural killer cell
NLR	NOD-like receptor
NLRP3	NACHT, LRR, and PYD domains-containing protein 3
NOD	Nucleotide-binding and oligomerization domain
NOX1	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1
NPM1	Nucleophosmin
NRAS	Neuroblastoma Rat Sarcoma viral oncogene homolog
NSA	Necrosulfonamide
OMM	Outer mitochondrial membrane
PAMP	Pathogen-associated molecular patterns
PAS	Para-aortic splanchnopleura

PCD	Programmed Cell Death
PGAM5	Phosphoglycerate mutase family member 5
PINK1	PTEN-induced putative kinase 1
PKC	Protein kinase C
PKK	Protein kinase C-associated kinase/ Rip4
Poly I:C	Polyinosine:polycytidylic acid
PRR	Pattern-recognition receptor
PTEN	Phosphatase and tensin homolog
RBC	Red blood cell
RELMβ	Resistin-like molecule β
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor-interacting protein
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
Runx1	Runt-related transcription factor 1
Sca-1	Stem cell antigen 1
SCF	Stem Cell Factor
SF3B1	Splicing factor 3b subunit 1
SHARPIN	SHANK-associated RH domain-interacting protein

SLAM-HSC	Signaling lymphocyte activating molecule- hematopoietic stem cell
SMases	Sphingomyelinases
SRSF2	Serine/Arginine Rich Splicing Factor 2
SSC	Side scatter
STAT1/2	Signal transducer and activator of transcription 1 and 2
TA cells	Transit amplifying cells
TAB2 & 3	TAK-1 Binding protein 2 and 3
TAK1	Transforming Growth Factor- β Activating Kinase
TET2	Tet Methylcytosine Dioxygenase 2
TFF3	Trefoil factor 3
TGF-β	Transforming growth factor- beta
TIRAP	Toll/ interleukin-1 domain-containing adapter protein
TLR	Toll-like receptor
TP53	Tumor protein 53
TRAF	TNF Receptor-associated factor
TRAIL	TNF-Related Apoptosis Inducing Ligand
TRIF	Toll/Interleukin-1 receptor domain-containing adapter inducing interferon β
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP nick end labeling
U2AF1	U2 small nuclear RNA auxiliary factor 1

UPR	Unfolded protein response
VE-Cad	Vascular endothelium cadherin
vIRA	viral Inhibitor of Rip Activation
VV	Vaccinia Virus
XIAP	X-linked inhibitor of apoptosis

CHAPTER I

INTRODUCTION

Programmed cell death (PCD) is an important process by which developing organisms remove excess cells, form complex structures, and through which adult organisms maintain homeostasis and remove harmful or abnormal cells (*1*). Deficiencies in this process can lead to abnormalities at the cellular to whole-organism level or organism death. Two key types of PCD include Apoptosis and Necroptosis (programmed necrosis). Apoptosis is characterized morphologically by shrinkage of the cell membrane and organelles and formation of apoptotic blebs which are swiftly taken up by phagocytic cells (*2*, *3*). Conversely, necroptosis is characterized by swelling and bursting of the cell membrane and organelles and loss of plasma membrane integrity (*4*, *5*). While much is known regarding the signaling pathways involved in apoptosis execution, necroptosis is an emerging area of programmed cell death. Developing a better understanding of these pathways holds implications for understanding and treating diseases such as cancer, where targeting aberrant cell death is an essential objective.

The B cell lymphoma-2 (BCL-2) family is well known for their role in the regulation of apoptosis (*6*). However, many recent studies demonstrate that several of these proteins have alternative functions in survival, metabolism, and mitochondrial dynamics (*7–10*). In my studies I have characterized an alternative function of Bid (BH3-interacting domain death agonist), a proapoptotic, BH3 domain-only containing protein within the BCL-2 family in the inhibition of the necroptotic (programmed necrosis) pathway. This discovery was made through study of mice and cells deficient for Bid, as well as Bax and Bak to remove the apoptotic arm of Bid's function

in the hematopoietic system, and allow for characterization of Bax/Bak independent functions of Bid. These studies reveal that overwhelming necrosis of the bone marrow negatively impacts the hematopoietic system leading to bone marrow failure and premature death in these animals. Additionally, through development of cell lines from our mice attuned to undergo either apoptosis or necroptosis following stimulation, we were able to mechanistically begin to understand how Bid functions to inhibit necroptotic death. These studies revealed that Bid's presence promotes the cleavage of Rip1 through modulation of the activity of the proteases Caspase-8 and Granzyme B.

Programmed Cell Death

While there are many types of programmed cell death, there are two key types which will be the focus of my studies, apoptosis and necroptosis (programmed necrosis). Apoptosis is important in the crafting of complex structures of anatomy such as digits (*1*). Similarly, necroptosis is key in development as deficiency for receptor-interacting kinase 3 (Rip3) rescues the embryonic lethality of Caspase-8 deficiency in mice (*11*). While the outcome of both apoptosis and necroptosis is cell death, the morphological characteristics, signaling pathways, execution, and consequences of execution differ significantly between these two paths to death. Additionally, while apoptosis and necroptosis can be stimulated downstream of death receptors such as Tumor necrosis factor receptor 1 and 2 (TNFR1 and 2), and a number of the proteins involved in each are similar, the downstream mediators of apoptotic or necroptotic signaling differ (*12*). Apoptotic execution is dependent upon this upstream pathway to signal activation of cysteine-dependent aspartate-specific proteases (Caspases), whereas necrosis is dependent upon

activation of the RIP kinases 1 and 3, as well as mixed lineage kinase domain-like (MLKL) (*5*, *13*). Lastly, the consequences of apoptotic versus necroptotic death are drastically different and as a result can have distinct effects on the surrounding cells in the organism. Because apoptotic cells form small blebs that bud off from the main cell body, and then are immediately taken up by phagocytes, there is typically no immune response. Conversely, necroptotic death leads to loss of membrane integrity and/or bursting of the cell causing leakage of damage-associated molecular patterns (DAMPs) in the extracellular space (*14*). This in turn promotes inflammation and an innate immune response (*15*).

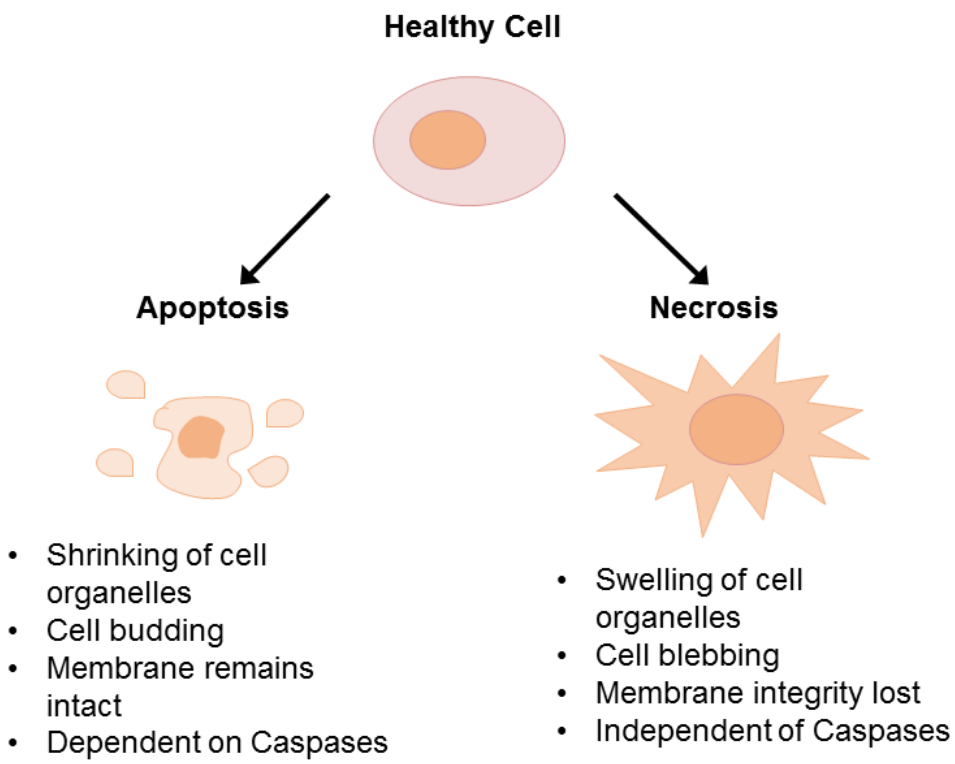


Figure 1.1 Morphological characteristics of apoptosis versus necrosis
Death by apoptosis versus necrosis is morphologically distinct and requires distinctly different proteins. Apoptosis is characterized by an immunologically silent death in which the cells shrink and bud off into small buds which are rapidly phagocytosed. Conversely, necrosis is characterized by the swelling of the cell, leading to the loss of plasma membrane integrity. While apoptosis requires Caspases for its execution, necrosis is independent of Caspases and instead requires the activity of Rip kinase 1 and 3.

Programmed cell death in human disease

Aberrant cell death has devastating effects on an organism, demonstrating the importance of programmed cell death in maintaining homeostasis. A number of human diseases are characterized by lack of, or too much programmed cell death (apoptosis and/or necroptosis). One of the best known instances in human disease where lack of programmed cell death is recognized as being paramount is in the development of cancer (*16*). Evasion of programmed cell death is a contributing factor to the development of many neoplasms. Other settings where lack of or altered programmed cell death promotes disease is in the development of autoimmunity, where immune cells that are reactive to "self" are not properly eliminated. Diseases that manifest as a result of this include Diabetes mellitus, Lupus erythematosus, and Rheumatoid arthritis (*17–20*). Another setting in which lack of programmed cell death promotes a disease state is in the clearance of viral infection. In some viral life cycles, death of cells infected with virus blocks the completion of the viral propagation cycle blocking further infection. As such, prevention of both apoptotic and necroptotic death are demonstrated to be important in the viral defense against the host (*21*, *22*). An example of this is in infection with the vaccinia virus in mice, which contains the viral protein B13R. B13R inhibits apoptosis, which in turn promotes RIP3-mediated necrosis, aiding in the clearance of infection (*23*, *24*). Similarly, in infection with the Influenza A virus *in vitro* both apoptotic and necroptotic death are important in blocking further infection (*25*). Conversely, infection with the T1L strain of Reovirus appears to require apoptosis during its life cycle to promote viral growth and aid in infection and pathogenesis *in vivo* (*26*, *27*). However, more recent studies demonstrate that another Reovirus strain, T3D, induces necroptosis at a different stage in the viral life cycle which also promotes virulence, suggesting that programmed cell death is often, but not always key in viral clearance (*28*). These examples reiterate the

importance of programmed cell death execution in maintenance of tissue homeostasis, proper immune regulation, and in viral clearance.

There are also several settings in which too much cell death promotes a number of human diseases. Programmed cell death following ischemia/ reperfusion injury can lead to apoptotic and/or necroptotic death of tissue. Studies with human tissue and mouse models of myocardial infarction demonstrate a role for both apoptosis and necroptosis in damage and remodeling of cardiac tissue (*29*, *30*). Similarly, following stroke, both apoptotic and necroptotic death are implicated in the death of neurons (*31*–*33*). Importantly, while studies on both mouse and human tissues following ischemic/reperfusion to study apoptosis activation have been utilized, only mouse models have been utilized in the study of necroptosis following ischemia/reperfusion injury, suggesting our understanding of this may be incomplete (*34*). In neurodegenerative disease, death of neurons or other neural cell types leads to progressive loss cognitive and motor capabilities and eventually death (*35*, *36*). Both apoptosis and necroptosis are implicated in these events. In Alzheimer's disease Caspase activation has been implicated in the death of neurons along with the formation of plaques of Amyloid-β and neurofibrillar tangles of the protein tau (*37*). Additionally, more recent preliminary studies *in vitro* and *in vivo* suggest a role for necroptosis in neuronal death in a mouse model of Alzheimer's Disease (*38*, *39*). In Parkinson's Disease, *in vitro* studies demonstrate that overexpression of α- synuclein, the major component of Lewy Bodies found in Parkinson's Disease, promoted death in dopamine neurons (*40*). Similarly, in Huntington's Disease neurons die as a result of accumulation of mutant Huntingtin protein. Preliminary studies in mice *in vivo* and *in vitro*, as well as in human *in vitro* studies demonstrate that both apoptotic and necroptotic death lead to loss of neurons (*41*, *42*). Lastly, recently multiple sclerosis (MS) was shown to be associated with necroptosis of

oligodendrocytes, the myelin-producing cells of the nervous system. Through study of mouse models of MS as well as human samples with MS lesions, the authors demonstrate that necroptosis execution may play a role in this process through use of the necroptosis inhibitor, 7N-1. Additionally through proteomic studies, the authors find that a number proteins found to aggregate in both Alzheimer's and Parkinson's disease are also upregulated in human MS patient samples (*43*). This same group also recently discovered that necroptosis mediated by Rip1, Rip3, and MLKL plays a role in axonal degeneration, contributing to the pathogenesis of amyotropic lateral sclerosis (ALS) (*44*). While apoptotic death has been implicated in the pathogenesis of many diseases for many years, more recent studies implicate necroptotic death as well. As such, a better understanding of necroptotic signaling and its role in human disease *in vivo* present a new avenue of therapeutic targets to pursue in the treatment of human disease (*45*).

Table 1.1 Human diseases associated with abnormal programmed cell death

Disease/ Pathology	Death Dysregulation	Type of PCD	Result of Dysregulation	Reference
Cancer	Loss	Apoptosis	Overgrowth of cells due to apoptosis resistance	(*16*)
Diabetes Mellitus	Loss	Apoptosis	Apoptosis of β-Cells in the pancreas as a result of autoimmunity due to failure of removal of cells recognizing "self"	(*17*)
Lupus Erythematosus	Loss	Apoptosis	Fas Resistance of "self" recognizing lymphoid cells to PCD	(*18*, *19*)
Rheumatoid Arthritis	Loss	Apoptosis	Resistance of macrophages to apoptosis	(*20*)
Clearance of viral infection	Loss	Apoptosis/ Necroptosis	Continued increase of viral infection	(*21–25*)
Myocardial Infarction	Too much	Apoptosis/ Necroptosis	Death of cardiac tissue after ischemia/reperfusion injury	(*29*, *30*)
Stroke	Too much	Apoptosis/ Necroptosis	Death of neural tissue after ischemia/reperfusion injury	(*31*, *33*, *34*, *46*)
Alzheimer's Disease	Too much	Apoptosis/ Necroptosis	Death of neurons due to accumulation of Amyloid-β plaques and neurofibrillar tangles of the tau protein	(*37–39*)
Huntington's Disease	Too much	Apoptosis/ Necroptosis	Accumulation of Huntingtin protein in neurons causing PCD	(*41*, *42*)
Parkinson's Disease	Too much	Apoptosis	Death of dopamine neurons due to accumulation of α-synuclein, the principle component of Lewy Bodies	(*40*)
Multiple Sclerosis	Too much	Necroptosis	Necroptotic cell death of oligodendrocytes leading to loss of myelination	(*43*)
Amyotropic Lateral Sclerosis	Too much	Necroptosis	Degeneration of axonal portion of neurons due to necroptotic death	(*44*)

Apoptosis

Apoptosis is a programmed cell death process that is highly conserved among multi-cellular organisms, and is extremely important in the crafting of complex structures within different tissues and organs during development (*1*). Apoptosis also maintains homeostasis and balance of cell numbers within tissues. Lastly, apoptosis regulates the removal of damaged, defective (e.g. transformed), or excess cells (e.g. hematopoietic cells generated during an immune response which are no longer required).

Apoptotic signaling is carried out through two distinct pathways; the extrinsic and intrinsic apoptotic pathways (*47*, *48*). Activation of the extrinsic pathway occurs when death receptors such as TNFR (tumor necrosis factor receptor), TNF-related apoptosis inducing ligand (TRAIL) Receptor, and FAS (Apoptosis antigen 1 (APO-1), tumor necrosis factor superfamily member 6 (TNFSF6)) are activated through the binding of their cognate ligands, TNFα, TRAIL, and Fas ligand, respectively (*49*, *50*). Upon ligand binding, these receptors trimerize, bringing together their death domain-containing cytoplasmic tails, and with adapter proteins form the Death-inducing signaling complex (DISC) (*51*). Adapter proteins such as Fas-associated death domain protein (FADD) and TNF-related associated death domain protein (TRADD) contain death domains (DD), and allow for association with the cytoplasmic regions of the trimerized death receptors (*52*, *53*). The DISC serves to transduce the death signal into the cell, and promotes the direct activation of initiator cysteine-dependent aspartate-specific proteases (Caspases) -8 and -10 through engagement of Death effector domains (DED) present in these proteins (*54*–*56*).

In extrinsic apoptosis activation, initiator Caspases-8 and -10 promote the direct activation of executioner Caspases, which include Caspase-3 and Caspase-7 (*48*). These

proteases initiate a Caspase cascade that promotes the breakdown of cellular contents. The intrinsic pathway of apoptosis functions as an amplification loop of apoptosis activation, through an increase in Caspase activation. The intrinsic pathway is activated as a result of an intracellular death stimulus, which may result from irreparable DNA damage, or as a result of activation through the extrinsic apoptotic pathway, through cleavage of the BCL-2 family member Bid by Caspase-8. This cleavage activates Bid, causes its translocation to the mitochondrion where it promotes activation of the Bax and Bak, proapoptotic BCL-2 family members. Bax and Bak form pores within the OMM causing MOMP and allowing the release of several factors from the intermembrane space (IMS), including high-temperature-requirement-protein A2 (HTR2A/OMI), and Second mitochondrial-derived activator of Caspases/ direct inhibitor of apoptosis-binding protein with low pI (SMAC/Diablo), Cytochrome C, Apoptosis-Inducing Factor (AIF), and Endonuclease G (*57*). SMAC/Diablo and HTR2A/OMI block the activity of X-linked inhibitor of apoptosis (XIAP), an E3 ligase which promotes the degradation of Caspases, through directly binding to them (*58–61*). Cytochrome C release promotes apoptosis activation through formation of a complex with Apoptotic protease activating factor-1 (APAF-1), known as the apoptosome, which recruits and activates Caspase-9 (*62, 63*). Caspase-9 activates Caspase-3 and Caspase-7, promoting the Caspase cascade, which leads to the digestion of cellular contents to promote enzymatic activation and to cause protein cleavage (*64*). DNA fragmentation additionally occurs through activation of Caspase activated DNase (CAD) (*65*). Following MOMP and Caspase activation, mitochondrial membrane potential is lost, rendering cells unable to make adenosine triphosphate (ATP), the necessary energy required for survival. Cells additionally expose phosphatidylserine on the outer plasma membrane, a phospholipid

primarily present on the inner leaflet of the plasma membrane, which serves as an “eat me” signal for phagocytic cells to engulf an apoptotic cell (*66*).

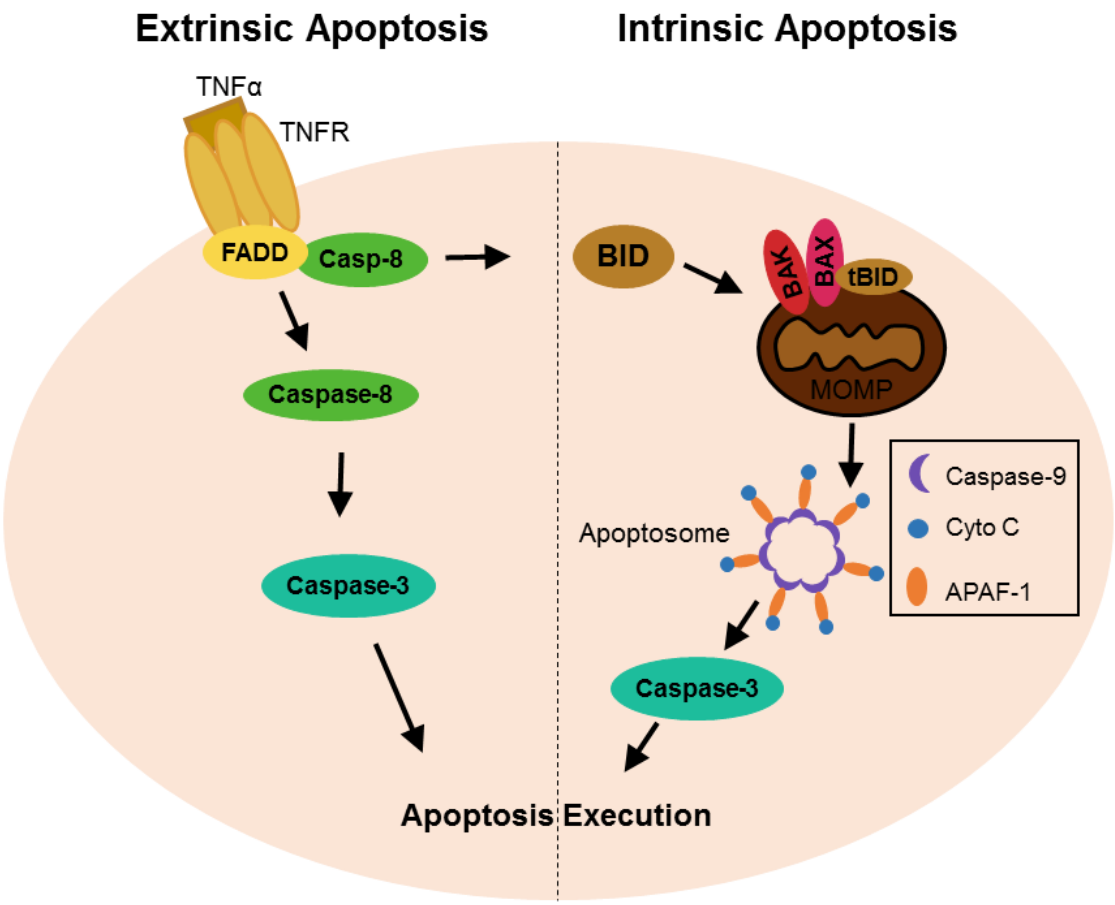


Figure 1.2 Extrinsic versus intrinsic apoptosis and reliance on each pathway in Type I and Type II cells

There are two pathways to apoptotic cell death, extrinsic and intrinsic apoptosis. Extrinsic apoptosis is activated downstream of death receptor signaling. Activation of Caspase-8 promotes the cleavage and activation of Caspase-3, activating the apoptotic program. Cells reliant on extrinsic apoptosis activation to execute cell death are known as Type I cells. Intrinsic apoptosis is activated through the cleavage of Bid by Caspase-8. As a BH3-Only activator, Bid translocates to the mitochondrion where it activates Bax and Bak, promoting their activation and oligomerization. Pore formation leads to the release of several factors, including Cytochrome C. Cytochrome C associates with APAF-1 and Pro-Caspase-9 to promote activation of Caspase-9. Caspase-9 activates Caspase-3 activation the apoptotic program, executing apoptosis. Cell reliant on intrinsic apoptosis to execute cell death are termed Type II cells.

Type I and Type II cells

Early studies of death receptors revealed the intrinsic and extrinsic apoptotic pathways, and determined that different cell types rely more on one pathway versus the other following death receptor activation to execute apoptosis. The reliance of a cell on extrinsic versus intrinsic apoptosis to die classifies cells as being either Type I or Type II, respectively (*67*). The key difference between these types of death is direct activation of downstream caspases through Caspase-8 (extrinsic) versus a mitochondrial amplification loop that promotes increased activation of executioner caspases and Caspase-8 through Caspase-9 activation. Through examination of the type of Caspase activation downstream of Fas receptor activation and of kinetics of Caspase activation in relation to mitochondrial permeabilization and apoptosis execution it was determined that 1) Caspase-8 activation differed when it occurred within the DISC versus after mitochondrial permeabilization and 2) Caspase-8 activation primarily occurs in the DISC in Type I cells, whereas it primarily occurs downstream of the mitochondrion in Type II cells (*48*). Later studies demonstrate that minimal activation of Caspase-8 in Type II cells was transmitted to the mitochondrion through cleavage and translocation of the BCL-2 family member, Bid. The reliance of Type II cells on mitochondrial amplification of the apoptotic signal versus the independence of Type I cells makes apoptosis execution inhibitable at this level of signaling. Anti-apoptotic BCL-2 and BCL-X_L, members of the BCL-2 family, promote this inhibition highlighting the importance of these proteins in apoptosis regulation.

Caspases

Caspases are cysteine proteases that cleave peptide bonds specifically after aspartic acid residues, serving to either activate or inactivate proteins (*68*). For example, Caspase-1, the first

member of this family of cysteine proteases discovered, was originally demonstrated to cleave Pro interleukin-1β (proIL-1β) (the immature form of IL-1β), generating the mature cytokine that promotes an inflammatory response (*69*, *70*). Since this discovery there have been a total of 10 caspases identified in the *Mus Musculus* species (Caspase 1-3, 6-9, and Caspase-11, 12, and 14) and 12 caspases identified in humans (Caspase 1-10, and Caspase-12 and -14). These proteins have various roles in inflammatory and apoptotic signaling pathways, as well as keratinocyte differentiation and hematopoiesis (*71*, *72*). There are three subclasses of caspases, which are delineated based upon the structure and function of the caspase in each pathway. The first subclass includes Caspases that are important for the activation of inflammatory cytokines such as IL-1β and IL-18 (*73–75*) . These caspases function to promote the cleavage of substrates that incite inflammation, namely intracellular cytokines. Caspase-1, Caspase-4 (human only), Caspase-5 (human only), Caspase-11 (mouse only), and Caspase-12 are members of this group of caspases, which primarily function as initiators of inflammatory responses. The second subclass of caspases is classified as apoptotic effectors and function to promote the execution of the apoptotic program through cleavage of crucial substrates for apoptotic execution. Caspases that are members of this subclass include Caspase-3, Caspase-6, and Caspase-7. These caspases become activated by initiator caspases, which make up the third subclass of caspases. Initiator caspases include Caspase-8, -9, and -10, which serve as initiators of the caspase cascade in the extrinsic and intrinsic apoptotic pathways. These caspases become activated through extracellular and intracellular signals which promote apoptotic signaling and allow for the execution of the apoptotic program.

Apoptotic caspases begin as inactive zymogens and become activated through a systematic dimerization and/or cleavage process, which can be autocatalytic or be mediated by

other caspases (*71*). The general structure of caspases includes a prodomain, which may contain either a Death-effector domain (DED) or a caspase activation and recruitment domain (CARD)), along with a large subunit, connected through a linker region to the small subunit (*76*). Initiator caspases involved in apoptosis exist in the cytosol as inactive monomers and become activated through interaction with the DISC (Caspase-8, -10) or through association with the proteins APAF-1 and cytochrome C (Caspase-9). This activation was described as the "induced proximity" model in which caspases dimerize without cleavage (*77*). However, activation of Caspase-8 and Caspase-10 requires cleavage (*78*). Caspase interaction within the DISC occurs through binding of Caspase-8 zymogens to FADD through DEDs. This docking of Caspase-8 serves as an activation platform whereby Caspase-8 can form dimers with either another Caspase-8 molecule or with a cellular FLICE-like inhibitory protein (cFlip) molecule, a protein similar to Caspase-8, which lacks catalytic cleavage activity. cFlip exists in two main isoforms ($cFlip_S$ and $cFlip_L$) which have differential effects on Caspase-8 activity. Binding of $cFlip_S$ blocks Caspase-8 apoptotic activity, blocking apoptotic activation. Conversely, binding of $cFlip_L$ allows Caspase-8 activation, but alters its activity (*79*). This Caspase-8: $cFlip_L$ heterodimer displays differential substrate specificity than Caspase-8 homodimers, a point that will be important in later discussions of Caspase-8's role in necroptotic signaling (Chapter III) (*80*). Binding of another Caspase-8 molecule allows for homodimerization of these molecules, and is the first step in activation. Next, an initial autocatalytic cleavage occurs in the linker region. In Caspase-8 during apoptosis this cleavage occurs at Asparagine 374 or 384. This is followed by cleavage between the prodomain and large subunit at Asparagine 210, 216, or 223 (*56*, *77*, *81*). Following cleavage, the molecule is fully activated and can then cleave other substrates such as Bid or Rip1. Activation of Caspase-9 does not require an autocatalytic cleavage event, but does

require association with the apoptosome, a heptameric molecule that forms through association of APAF-1 and cytochrome C. There is controversy within the field regarding whether activation of Caspase-9 occurs as a dimer or monomer, however it has been clearly established that this activation occurs through a single interaction of CARDs present in APAF-1 and Caspase-9 (*82*, *83*). More recently the CARD has been purported to play a key role in activation through three distinct interactions, with CARD domains from two APAF-1 molecules interacting with a single Caspase-9 CARD (*84*). Lastly, executioner caspases exist in the cytosol as inactive dimers, held in check due to steric hindrance from the interdomain linker (*85*). While there are three identified executioner caspases (Caspase-3, -6, and -7) many studies on this class of caspases have focused on activation mechanism of Caspases-7. Studies of Caspase-3 suggest a similar method of activation to Caspase-7 through highly conserved residues (*85*). Caspase-6 is implicated to have a role in not only apoptosis, but also in alternative functions such as degeneration of axons (*86*). Studies of Caspase-7 indicate that cleavage of the interdomain linker allows for a conformational change and stabilization of the active site loop, and full activation (*87*, *88*). Cleavage of this domain by initiator caspases allows for full activation, activation of the caspase cascade, and execution of apoptosis.

While the execution of apoptosis by caspases is an important process in the maintenance of tissue homeostasis, this process, like all biological processes requires regulation through inhibition. There are three types of inhibitors of caspases: viral inhibitors, cellular-derived inhibitors, and chemical inhibitors. The one known viral inhibitor in mammals is a protein known as CrmA and is derived from the cowpox virus (*89*, *90*). In infected organisms it inhibits the activity of Caspase-1 and Caspase-8. Additionally, there is a single confirmed cellular-derived inhibitor in mammals known as X-linked apoptosis inhibiting protein (XIAP) (*91*). XIAP

contains three baculoviral IAP repeat (BIR) domains, which aid in interaction with Caspases. XIAP blocks the catalytic activity of Caspase-3 and Caspase-7 through interaction with the linker region between BIR1 and BIR2 in XIAP with the active site in these caspases (*92*). XIAP also blocks the activity of Caspase-9 through interaction of BIR3 with the amino terminal after processing (*93*). While another set of proteins, the inhibitors of apoptosis (IAPs) known as cellular IAP 1 and 2 (cIAPs), were initially purported to have a role in apoptosis inhibition through direct inhibition of catalytic caspase activity, later studies determined that interaction with caspases did not modulate caspase activity (*94*). Chemical inhibitors of Caspases are synthesized molecules that specifically bind to the active site in Caspases, that may also contain modifications allowing for irreversible or reversible binding. These inhibitors are generated to either inhibit all caspases (pan-caspase inhibitors) or specific to certain caspases (e.g. Z-DEVD-FMK which inhibits Caspase-3).

Caspases also have alternative roles in cell survival and proliferation, as well as inhibition of other programmed cell death pathways. For example, Caspase-8 in addition to promoting apoptotic death is also important for survival, as Caspase-8 deficiency leads to embryonic lethality in mice (*95*). Additionally, further studies implicate a role for Caspase-8 in the inhibition of programmed necrosis (also referred to as necroptosis) through cleavage of the Receptor-interacting protein (RIP) kinases 1 and 3 (*96*, *97*). Our studies (discussed in Chapters II and III) with mice and cells deficient for BCL-2 family members Bax, Bak, and Bid suggest a role for Bid in the mediation of Caspase-8 activity. Caspase-8 promotes the cleavage of Bid to promote intrinsic apoptosis execution. The studies detailed later will explore an alternative interaction between Caspase-8 and Bid in the setting of necroptotic death and how this affects the stability of Rip1.

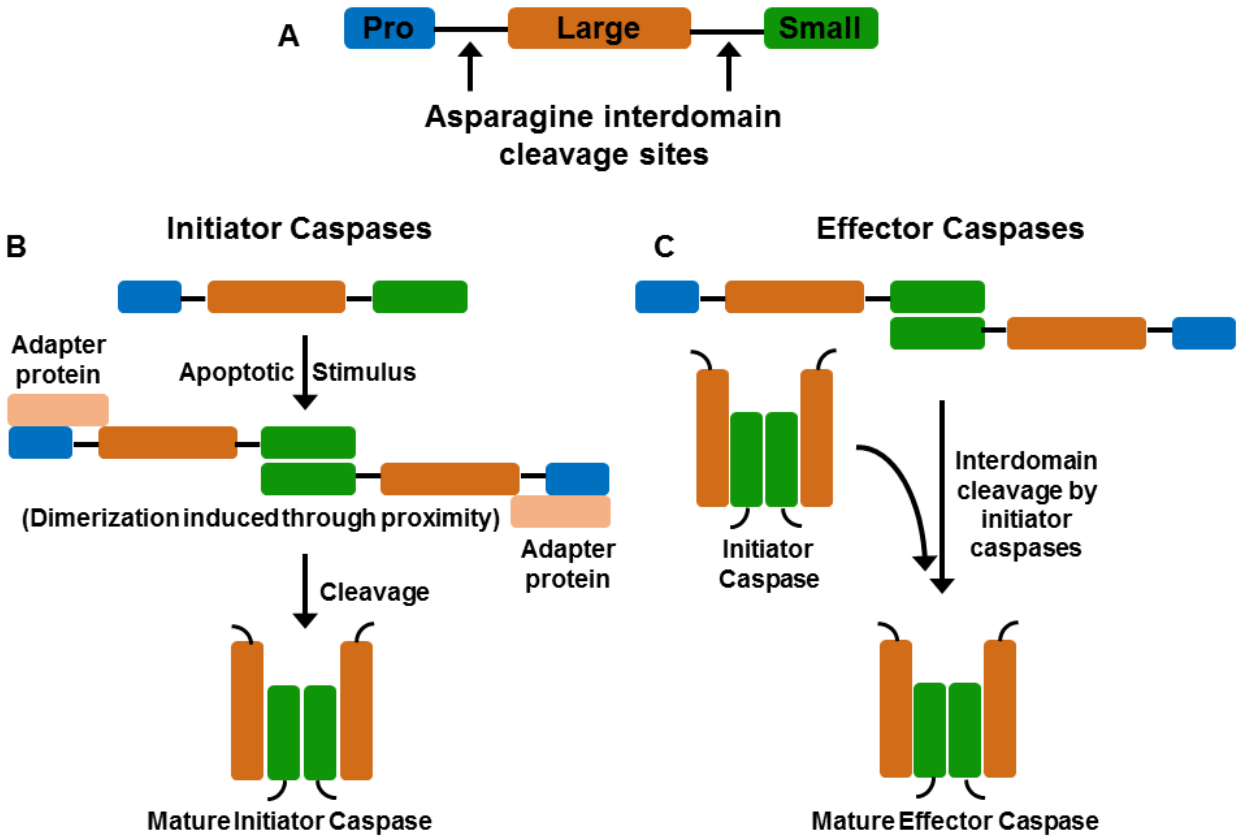


Figure 1.3 Structure of Caspases
A) Caspases contain three main domains: The prodomain which may contain death effector domains (DED) or death domains (DD), the large subunit, and the small subunit. Between the prodomain and the large subunit is the prodomain linker region. The linkage region between the large and small subunit is the interdomain linker region. Asparagine within these regions serve as sites of cleavage. B) Initiator caspases exist as monomers in healthy cells. Upon receipt of an apoptotic stimulus, these caspases are recruited to activation platforms through their Death effector or Caspase activation and recruitment domains (DED or CARD). At these sites Caspases undergo induced dimerization through proximity, promoting autocatalytic activity. An autocatalytic cleavage leads to the formation of the mature initiator caspase. C) Effector caspases exist as dimers within healthy cells and are maintained in an inactive state by the intact interdomain linkage. Activated initiator caspases cleave this region promoting activation of effector caspases. Adapted from (*76*).

The BCL-2 Family and Apoptosis Regulation

Early studies of the development of nematode *Caenorhabditis elegans* demonstrated that a specific number of cells were always eliminated during development of the organism (*98*). Mutagenesis screens led to the determination that programmed cell death is very important in the proper development of these animals. Utilizing these screens, egg-laying defective mutants (EGL) were identified and characterized, which led to the identification of EGL-1, an ortholog of BH3-only members of the BCL-2 family (*99*). Further mutagenesis screens resulted in the identification of cell death abnormal (CED) mutants. Initially *CED-3* (corresponding to a caspase) and *CED-4* (corresponding to APAF-1) mutants were identified due to a lack of death of specific cells identified to undergo programmed cell death in wild type animals (*100*). Further studies led to the discovery *CED-9* gain-of-function mutants (ortholog of *BCL-2*), which were able to rescue the defects in *EGL-1* mutants, and also could dominantly block cell death in cells which typically died in wild type animals (*101*).

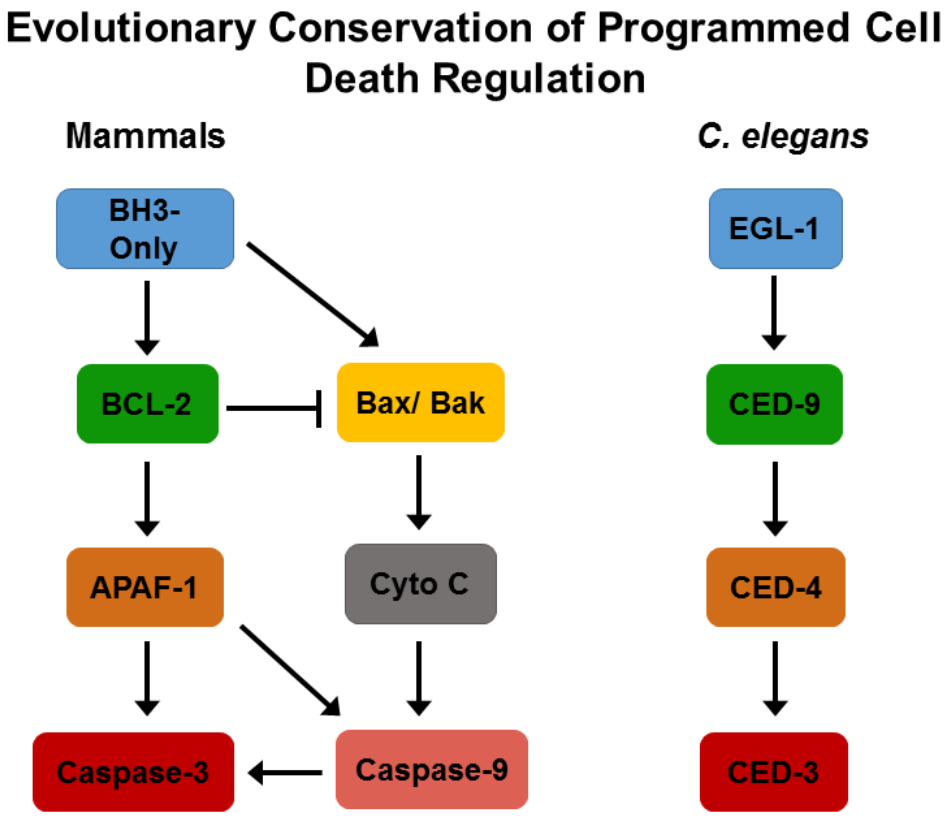


Figure 1.4 Conservation between Mammalian BCL-2 Family and regulators of cell death in *C. elegans*

Programmed cell death in *C. elegans* like mammalian organisms is regulated by a family of proteins. The BH3-Only protein EGL-1 activates apoptotic death through interaction with CED-9, similar to interaction of BH3-only proteins with antiapoptotic proteins such as BCL-2. Mammalian and *C. elegans* signaling diverge in the transduction of the apoptotic death in that the CED-9 homolog (BCL-2) is able to directly bind CED-4 (homolog of Apaf-1) to inhibit apoptosis. In the absence of this interaction, CED-4 can bind CED-3 promoting its activation and execution of apoptosis. Thus *C. elegans* do not require MOMP to promote apoptosis. This is in contrast to mammalian signaling in which BH3-only proteins can additionally activate execution of apoptosis through Bax and Bak, which allows for MOMP and promotes Caspase activation. Adapted from (*102*).

Around the same time the founding member of the BCL-2 family of proteins, which are orthologs to several proteins identified in *C. elegans* (as described above), was identified. B cell lymphoma-2 (BCL-2), which was first identified when the breakpoint of the chromosomal translocation between chromosomes 14 and 18 t(14:18), was cloned. This translocation was found to result in the fusion of the *BCL-2* gene to the immunoglobulin locus in human follicular B cell lymphomas and B cell leukemias (*103*, *104*). Further investigation revealed the exact location of the break, that it caused BCL-2 overexpression, and that it was similar in most patients that developed follicular lymphoma, suggesting it was a result of defective class-switching in B cells (*105*). Soon after it was discovered that c-MYC cooperates with BCL-2 to protect hematopoietic progenitors from cytokine withdrawal-induced cell death through introduction of a human BCL-2 transgene (overexpressiong BCL-2) into bone marrow from Eμ-Myc mice (*106*). The lab of Stanley Korsmeyer developed a transgenic mouse with an artificial chromosome containing the t(14:18) mutation. These studies caused overexpression of BCL-2 and confirmed that it promotes increased survival in B cells (*107*). The increased survival led to accumulation of mutations within cells in the follicles of the spleen that over time had the capability to transform to lymphoma (*107*, *108*). These studies established BCL-2 as a part of a novel class of proteins. The overexpression identified BCL-2 as a new type oncogene at the time, one playing a role in cell death (*109*) (as evasion of cell death is a defining feature of cancer found with the discoveries of both BCL-2 and TP53 (*110*)).

Members of the BCL-2 family function as regulators of intrinsic apoptosis, regulating execution of mitochondrial outer membrane permeabilization (MOMP), the “point of no return” in apoptosis execution. These proteins share conserved domains of homology known as the BCL-2 Homology (BH) domains, of which there are four. These domains are made up of α

helices which are key structures in BCL-2 family members, and also promote their function through interaction with each other and membranes. All four BH domains are present in the founding member BCL-2, and each member contains homology to at least one BH domain. BH domains allow the BCL-2 family to interact with each other and subsequently for exertion of their functions. Proteins within the BCL-2 family are subdivided into three subgroups based upon the presence of these domains and their function. These include the multi-domain antiapoptotic proteins, the multi-domain proapoptotic effector proteins, and the BH3-only sensitizer and direct activator proteins. Like most biological pathways, the BCL-2 family is regulated through several post-translational modifications (phosphorylation, cleavage, ubiquitylation, and fatty acid addition), and through subcellular localization (*6*).

The BH3-only subgroup of the BCL-2 family contains members with homology to only the BH3 domain (e.g. Bid, Bim, Bad, Noxa, and Puma). Several of these proteins also contain a transmembrane domain which allows for membrane interaction. BH3-only proteins serve as the first layer of regulation in intrinsic apoptotic activation, sensing apoptotic stimuli through a variety of cellular stresses and transducing the cellular response through post-translational modification. These proteins are further divided into two groups whose function is mutually exclusive and is to either sensitize proapoptotic multi-domain members (through quenching of antiapoptotic multi-domain members) to activate apoptosis, or to promote the activation of apoptosis directly through activation of the multi-domain proapoptotic members (*111*). The BH3 domain within these proteins is key in the interaction with other members of the BCL-2 family to carry out functions in the promotion of apoptosis (*112*). While the levels of each of these proteins is important in determining the outcome of apoptotic activation in a cell, other factors, such as the stimulus and method of activation, are important in their activation and the final

outcome. For instance, Bid is activated through cleavage by Caspase-8 following death receptor activation which promotes translocation to the mitochondrion and Cytochrome C release (*113, 114*). PUMA and Noxa are directly transcriptionally upregulated by p53 following DNA damage (*115–117*). Conversely, Bad is phosphorylated which promotes binding to other non-BCL-2 family proteins, and blocks its proapoptotic activity (*118, 119*). Following their activation, the BH3-only proteins promote multi-domain proapoptotic protein activation and inhibit antiapoptotic proteins. These proteins serve distinct roles in sensing different death stimuli and transmitting the signal downstream to promote apoptosis.

The multi-domain proapoptotic proteins, which include Bax, Bak, and Bok, contain BH domains 1-3, as well as a transmembrane domain. These proteins interact with the OMM and upon activation disrupt the OMM through pore formation. As such, these proteins serve as the guards of OMM integrity in the face of BH3-only protein activation and anti-apoptotic protein inhibition. Loss of Bax and Bak inhibits cytochrome C release, inhibiting intrinsic apoptosis (*120, 121*). Previous studies demonstrate that Bax and Bak are activated through direct and indirect methods leading to conformational changes which allow for OMM localization as well as homo- and heteroligomerization to form pores in the mitochondrion (*122–124*). While Bax is localized to the cytosol, and translocates to the mitochondrion upon activation by Bid (*125*), Bak is constitutively localized to the OMM. This oligomerization event forms a pore in the OMM and allows for the release of cytochrome C, and apoptosis activation. The outcome of interaction of members of this subgroup with BH3-only and multi-domain antiapoptotic proteins at the OMM, determines if apoptosis proceeds (activation) or is halted (inhibition).

Members of the multi-domain antiapoptotic group (e.g. MCL-1, BCL-X_L) contain all homology domains present in BCL-2 as well. These proteins block apoptosis by inhibiting the

activation of multi-domain and BH3-only proapoptotic proteins. Inhibition occurs through sequestration, which provides a second opposing layer of regulation in apoptosis activation. This event is described as “mutual sequestration” and results in two effects: first antiapoptotic proteins binding to BH3-only proteins prevents BH3-only proteins from activating Bax and Bak; second binding of BH3-only proteins to antiapoptotic proteins blocks antiapoptotic proteins from hindering the activation of Bax and Bak. This in turn leaves other BH3-only proteins free to bind and activate Bax and Bak. As a result, the relative stoichiometry and interaction of antiapoptotic proteins to multi-domain and BH3-only proapoptotic molecules plays some role in the outcome of apoptotic signaling (*126*). Another factor that plays a role in this outcome is the subcellular localization of these proteins. While antiapoptotic proteins are primarily found in the OMM they may also be present in the inner mitochondrial membrane (IMM), cytosol, or at the endoplasmic reticulum (*127*, *128*). Depending upon their location these proteins can exhibit differential functions involved in apoptosis or alternative roles within the cell.

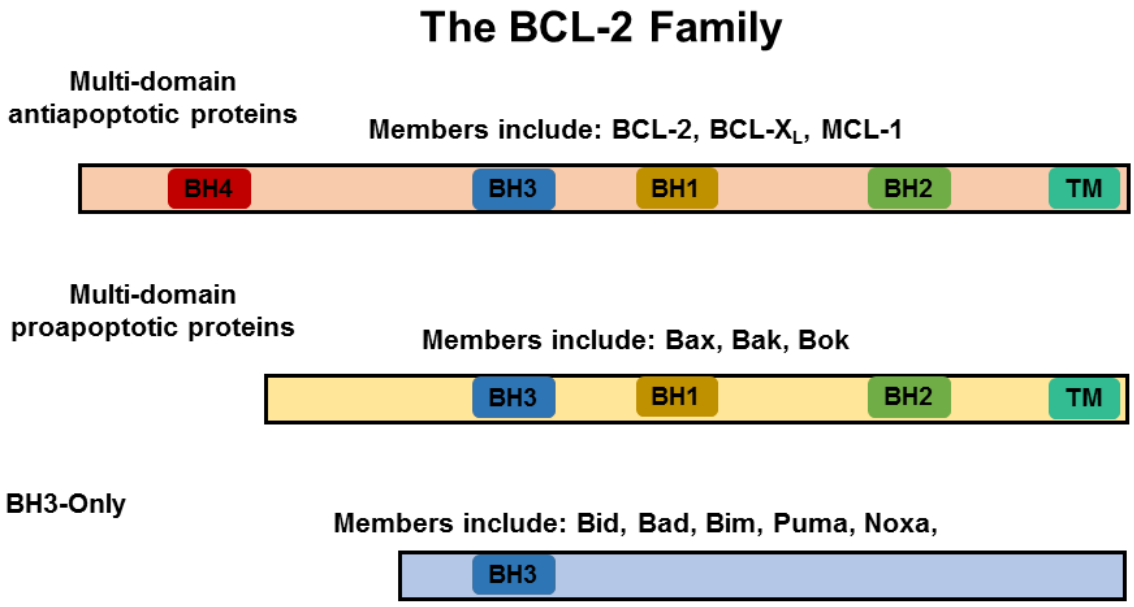


Figure 1.5 Members of the BCL-2 family have distinct functions in the regulation of apoptosis

The BCL-2 Family is made up of proteins sharing domains of homology known as BCL-2 homology domains (BH domains) found within the founding member BCL-2. There are four BH domains, and the presence or absence of these domains as well as function categorize these proteins into subgroups. There are three subgroups within this family; the multi-domain antiapoptotic proteins, the multi-domain proapoptotic proteins, and the BH3-Only proteins. The multi-domain antiapoptotic proteins serve as inhibitors of apoptosis and function at the mitochondrion to inhibit the actions of multi-domain proapoptotic and BH3-Only proteins. Multi-domain antiapoptotic proteins contain all BH domains as well as a transmembrane domain aiding in association with membranes. The multi-domain proapoptotic proteins function to execute apoptosis through formation of pores in the OMM. These proteins contain BH domains 1-3 and also contain a transmembrane domain aiding in their association with membranes. BH3-Only proteins serve as sensitizers and activators of apoptosis through displacement of multi-domain proapoptotic proteins from multi-domain antiapoptotic proteins (removing inhibition) and through direct activation of multi-domain proapoptotic proteins, respectively. Adapted from (*6*).

BCL-2 family members interact with each other through α helical BH domains. Previous studies demonstrate that the BH1, BH2, and BH3 domains present in antiapoptotic proteins form a hydrophobic groove that is capable of binding the BH3 α helix present in proapoptotic proteins (*6*). However, while members of each subgroup contain the same BH domains, members display different specificity in their binding to each other. This in turn affects apoptotic activation downstream of different stimuli and/or in different cell types. These differences in specificity are particularly important in the activation of Bax and Bak by BH3-only proteins which led to the classification of these proteins as sensitizers and activators of apoptosis (*111*, *129*). Activator BH3-only proteins (e.g. Bid, Bim, and Puma) are able to directly promote the activation of Bax and Bak (*122*, *130–133*). Conversely sensitizer BH3-only proteins Bad and Noxa are unable to promote the direct activation of Bax and Bak (*111*, *134*). These proteins instead inhibit antiapoptotic proteins through direct interaction, which in turn lowers the threshold of BH3-only protein needed for activation of Bax and Bak. Additionally, BH3-only proteins demonstrate specificity in binding to other BCL-2 family members based upon the structure of their BH3 domain. For instance, while Bad is capable of binding BCL-2 and BCL-X_L, Noxa is only able to bind MCL-1 (*130*, *134*).

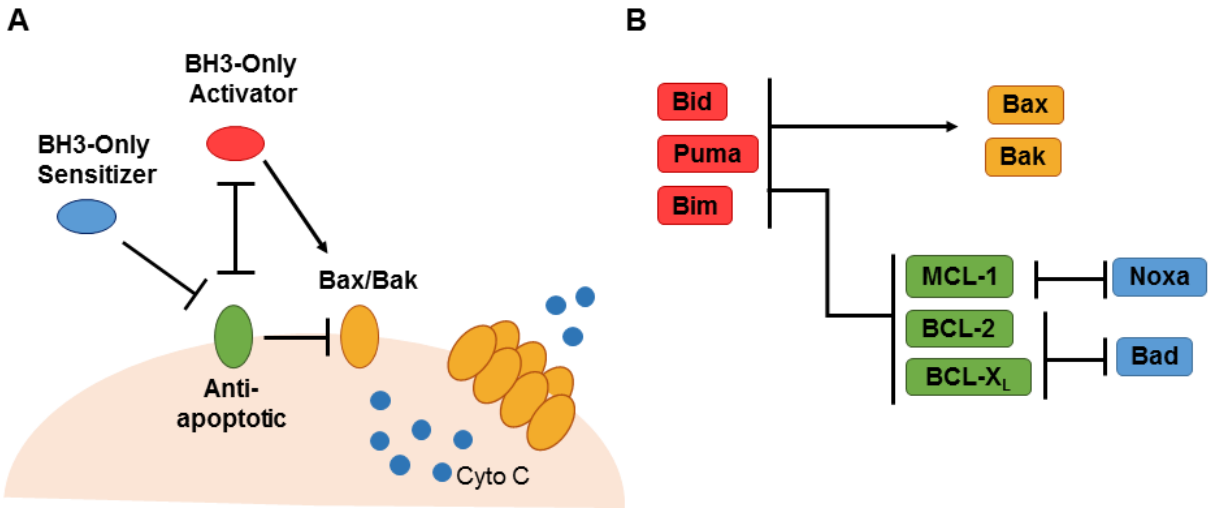


Figure 1.6 Apoptotic regulation by BH3-only sensitizers and activators

BH3-Only sensitizers and activators function in a mutually exclusive manner to regulate intrinsic apoptosis. A) BH3-only proteins are classified based on their ability to interact with different classes of BCL-2 family members. Activator BH3-only proteins can interact with proapoptotic Bax and Bak to promote apoptosis. Sensitizer BH3-only proteins interact exclusively with the antiapoptotic proteins, and can aid in apoptosis activation through release of Bax and Bak from antiapoptotic protein inhibition. Interaction between antiapoptotic proteins and Bax/Bak results in "mutual sequestration", a phenomenon in which the binding of these proteins to each other inhibits binding to other BCL-2 family proteins. This results in an apoptotic threshold that must be overcome to activate apoptosis. As such, this threshold is quite sensitive to the stoichiometry of BCL-2 family members at the mitochondrion. B) Activator BH3-only proteins are capable of interaction Bax and Bak. Sensitizer BH3-only proteins are limited to binding certain antiapoptotic proteins. While Bad is capable of binding both BCL-2 and BCL-X_L, Noxa is only capable of binding MCL-1. Adapted from (*135*).

In addition to their canonical functions in MOMP in apoptosis, mounting evidence demonstrates that members of the BCL-2 family also have alternative functions in many important cellular functions (*136*). For example, there are many examples of BCL-2 family members playing roles in mitochondrial fusion/fission, metabolism, and respiration. These include Bax and Bak, which are implicated in the fusion of mitochondria in homeostatic settings through formation of a complex with mitofusin-2 (MFN2) (*137*, *138*), and Bax which aids in fission along with dynamin-related protein 1 (DRP1) in the setting of cell death (*139*). Bax and Bak also localize to the endoplasmic reticulum (ER) where they can promote apoptosis following calcium depletion through activation of Caspase-12 (*140*). An alternative isoform of MCL-1 present in the IMS is also implicated in the maintenance of IMM cristae structure, regulation of membrane potential, mitochondrial fusion, and ATP production (*141*). Additionally BCL-X_L aids in the efficiency of respiration through the interaction with mitochondrial F_1F_0 ATP synthase aiding in the stabilization of mitochondrial membrane potential (*142–144*). The BCL-2 family additionally has many prosurvival roles at the cellular level. For example, BAD promotes survival prior to apoptotic activation through formation of a mitochondrial complex with glucokinase (hexokinase IV) and other factors that aid in glycolysis (*145–147*). Additionally, NOXA promotes survival through promotion of glucose metabolism through the pentose phosphate pathway (*148*). Additionally, Bax, BCL-2, and BCL-X_L are implicated in calcium homeostasis through localization to the ER and release of calcium to the mitochondria independent of their apoptotic functions (*136*). BCL-2 family antiapoptotic members BCL-2, MCL-1, and BCL-X_L also inhibit autophagy through interaction with the BH3-only protein Beclin. Lastly, previous studies, and studies completed in the Zinkel lab demonstrate a role for Bid in the DNA damage response (DDR). These studies demonstrate that Bid is phosphorylated

by Ataxia Telangiectasia Mutated (ATM) and ATM and Rad-3 related (ATR) and is a part of the ATR-directed DNA damage sensing complex, which also contains ATR-interacting protein (ATRIP) (*8*, *9*, *149*, *150*). Bid is also implicated in the regulation of mitochondrial ROS downstream of ATM, which is important in the maintenance of hematopoietic stem cell quiescence. This role is mediated through cooperation with mitochondrial carrier homolog 2 (MTCH2) which resides at the mitochondrion (*151*). This alternative function of Bid is also mediated through phosphorylation (*152*). Bid was also implicated in the inflammatory and innate immune response following nucleotide-binding and oligomerization domain (NOD) receptor signaling (*153*).

Table 1.2 Alternative function for the BCL-2 Family

Member	Alternative Function	Reference
Bax	Mitochondrial fusion/ fission, calcium homeostasis at the ER	(*136–139*)
Bak	Mitochondrial fusion	(*138*)
MCL-1	Maintenance of mitochondrial cristae structure and fusion, ATP production	(*141*)
BCL-X_L	Maintains efficiency of respiration, calcium homeostasis at the ER	(*136*, *142–144*)
Bad	Aids in glucose metabolism promoting survival	(*145–147*)
Noxa	Promotes glucose metabolism through the pentose phosphate pathway	(*148*)
BCL-2	Calcium homeostasis at the ER	(*136*)
Bid	Aids in the DNA damage response, inflammatory, and innate immune signaling	(*8*, *9*, *149*, *150*, *153*, *154*)

BH3-only protein Bid

The BH3-only protein Bid (BH3-interacting domain death agonist) was discovered in 1996 as a protein that was key in the mitochondrial activation of apoptosis through interaction with Bax (*155*). These studies revealed that competition between the interaction of the BH3 domain of Bid and the BH1 domain of Bax or BCL-2 determines the outcome following stimulation. Formation of Bid-Bax heterodimers allows for apoptosis execution, while Bid-BCL-2 heterodimers inhibits this process. Further study revealed that Bid is cleaved by the cysteine-protease Caspase-8 at aspartic acid 59 to promote apoptosis, translocates to the mitochondria, activates Bax, and promotes Cytochrome C release (*113*, *114*, *133*, *156*). Cleavage of Bid by Caspase-8 was further determined to be important in the activation of apoptosis at the organismal level through examination of mice after tail-vein injection of Fas ligand to promote activation of the Fas receptor and to promote apoptotic death of the liver. These studies found that mice deficient for Bid were protected from fulminant liver apoptosis as compared to wildtype animals (*157*), demonstrating the key role of Bid in apoptosis activation.

The solution structure of human and mouse Bid was determined in the same year and provided some insight into how Bid mediates apoptosis activation (*158*, *159*). These studies revealed that contrary to other BH3-only proteins, Bid is very structured, and maintains that structure even after cleavage by Caspase-8 (*160*). Further structural studies demonstrate that Bid's structure is more similar to the multi-domain anti- and pro-apoptotic proteins (Bax in particular), fitting with its strong ability to promote apoptosis (*161*). The NMR structure of Bid reveals that is composed of 8 α helices, with two hydrophobic helices making up a core surrounded by 6 amphipathic helices and an unstructured loop. The Caspase-8 cleavage site of Bid is contained in the unstructured loop of Bid. The resulting cleavage allows for the dissociation of α helices 1 and 2 from α helix 3, exposing the BH3 domain. OMM interaction is facilitated by a protein known as (MTCH2) which is present in the OMM. MTCH2 facilitates the targeting of cleaved Bid to the mitochondrion, and promotes unfolding of Bid which is critical for its insertion into the OMM (*162*, *163*).

While Bid is best-known for it role in activation of apoptosis following cleavage by Caspase-8, several studies implicate its cleavage by other proteases. For example, Bid can be cleaved by activated Caspase-3 and is believed to be a part of a positive feedback loop that promotes propagation of the apoptotic signal (*164*). Additionally Calpains were demonstrated to promote cleavage of Bid *in vivo* in the setting of ischemia/reperfusion injury and *in vitro* following Cisplatin treatment (*165*, *166*). Cathepsins, proteases found in lysosomes and activated by low pH can also promote cleavage of Bid and promote apoptosis following damage of the lysosome (*167–169*). Lastly Granzyme B, a protease present in cytotoxic T cells and other immune cell types, is demonstrated to promote rapid apoptosis through the release of cytolytic granules into cells. Bid was determined to be the factor mediating this rapid response through

cleavage by Granzyme B (*170*, *171*). My studies presented in Appendix B also implicate an alternative interaction of Granzyme B and Bid that is important in the inhibition of necroptotic signaling.

Bid has multiple alternative functions promoting survival and modulating the innate immune response. These alternative functions are mediated through another post-translational modification of Bid, phosphorylation. Phosphorylation of mouse Bid at serine 61 (S61) and serine 64 (S64) by Casein Kinase I II (CK1 and CK2) blocks cleavage by Caspase-8 and blocks apoptotic activation (*172*). Bid is phosphorylated at S61, S64 and S78 by Ataxia Telangiectasia Mutated (ATM) and ATM and Rad-3 related (ATR) kinases in response to DNA Damage (*7*, *9*, *150*, *173*) aiding in efficient activation of the DNA damage response. Additionally, phosphorylation of human Bid at S64, S65, and S76 as well as the aforementioned sites in mouse Bid is important in activating innate immunity and the inflammatory response downstream of NOD signaling (*10*). Phosphorylation in the unstructured loop aids in the binding of NOD1 and NOD2. These findings demonstrate an important role in phosphorylation in alternative functions for Bid.

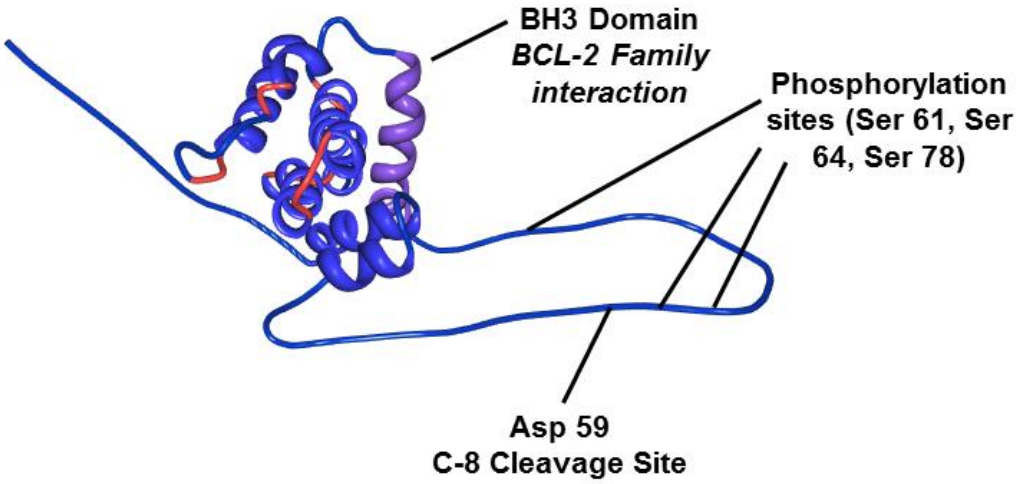


Figure 1.7 NMR Solution Structure of Mouse Bid
The NMR solution structure of Bid revealed that it was composed of 8 α helices and an unstructured loop between helix 2 and 3 (Protein data bank entry:1DDB) (*158*, *159*). Upon death receptor activation Bid is cleaved at Aspartic Acid 59 by Caspase-8 and translocates the mitochondrial membrane where it promotes the activation of Bax and Bak and apoptosis. Phosphorylation of Bid promotes alternative functions (e.g. Phosphorylation by ATM/ATR and participation in the DNA damage response). In our studies of Bid's role in necroptosis we hypothesize that Caspase-8's specificity for Bid is altered in necroptosis inhibition.

Necroptosis

The study of necroptosis (also known as programmed necrosis) is an emerging area of the programmed cell death field, which was only recognized in the last 16 years. Previously, necrosis was purported to be an unprogrammed, default cell death executed in settings where an overwhelming cell death stimulus occurred (e.g. freeze-thaw cycles, ischemia/reperfusion injury, or oxidative stress) (*174*). As a result, necrosis was largely disregarded in the study of programmed cell death within the field. However, the recognition that stimulation with TNFα could promote both an apoptotic and necrotic death in different cell types implicated that cell death programs might be an intrinsic, programmed process (*12*). Studies of TNF signaling continued, with the proteins involved in the upstream signaling processes of TNFR and FasR rapidly being elucidated, but the main outcomes recognized being apoptosis and NF-κB mobilizing cytokine signaling (*53*). However, in 2000 studies with mouse and human T cells revealed that Receptor-interacting protein 1 (Rip1) was required for the execution of necroptotic death if Caspase activation was inhibited, providing evidence that necrosis was likely a programmed process, and that the Rip kinases were involved in the signaling of this pathway (*175*). Previous studies implicated necrosis as an outcome of TNF signaling as well as a role for Rip1 in apoptosis, but the finding from Jurg Tschopp's group was the first to implicate the involvement of a signaling protein in this process (*176*, *177*). The term necroptosis was coined with development of Necrostatin-1 (Nec-1) which was first determined to block necrosis in ischemic brain injury through an unbiased screen of small molecules (*46*). Later studies identified Rip1 as a target of Nec-1 through inhibition of the kinase activation of Rip1 (*178*). Since that time our understanding of this signaling process has steadily increased through study of necroptotic signaling downstream of death receptors (e.g. TNFR1, TNFR2, FAS), and pattern

recognition receptor (PRR) signaling. However, many key questions still exist such as; What other upstream signaling pathways can feed into the downstream necroptotic pathway? What are the distinct complexes (and members within them) that lead to necroptotic activation? And importantly, my data will address: What causes a cell to signal toward an apoptotic versus necroptotic death?

Necroptosis Regulation

Signaling through death receptors utilizes the same proteins upstream of either an apoptotic or necroptotic outcome. As such, necroptotic signaling downstream of death receptors begins in the same manner as described above with the activation of death receptors through trimerization and conformational change of the cytoplasmic domains. The cytoplasmic domains then associate with adapter proteins forming the DISC or Complex I (*51*). Three possible outcomes exist through modulation of stability of key proteins, post-translational modifications, and Caspase activation; Apoptosis activation, necroptosis activation, and activation of NF-κB and mitogen-activated protein kinase (MAPK)/ Jun amino kinase (JNK) signaling. The activation of Caspases-8/-10 leads to apoptosis activation through cleavage of executioner Caspases and apoptotic substrates such as BH3-only Bid. Additionally, Caspase-8 promotes the cleavage of several other proteins, including Rip1, which in turn blocks NF-κB, MAPK-JNK, and necroptosis activation. Within Complex I, Caspase-8 promotes the cleavage of cylindromatosis (CYLD) a deubiquitylating enzyme that removes ubiquitylation from Rip1 (*179–183*). Necroptosis proceeds in cases where Rip1 and Rip3 are activated through phosphorylation. However, the Rip kinases can also be inactivated through cleavage and degradation (leading to

an apoptosis outcome if Caspases are activated) or ubiquitylated (Rip1 only, promoting NF-κB and MAPK-JNK signaling activation).

Ubiquitylation of these proteins is carried out by cIAP1 and cIAP2, E3 ubiquitin ligases which are recruited to Complex I and stabilized by TNFR-associated factor 2 (TRAF2) which allows for the formation of linear ubiquitin chain assembly complex (LUBAC) (*184*, *185*). Formation of LUBAC is facilitated by SHANK-associated RH domain-interacting protein (SHARPIN), Heme-oxidized iron regulatory protein 2 ubiquitin ligase-1 (HOIL-1), and HOIL-1 interacting protein (HOIP) (*186*, *187*). This ubiquitylation of Rip1 through Lysine 63 on ubiquitin (K63-linkage) allows it to serve as a docking site for the proteins Transforming growth factor-β activated kinase 1 (TAK1) and TAK1-binding protein 2 and 3 (TAB2 and TAB3) promoting TAK1 activation (*188*, *189*). NF-κB signaling is promoted through the recruitment of the IKK complex (Inhibitor of NF-κB kinase γ/ NF-κB essential modulator (NEMO), Iκα kinase (IKKα), Iκβ kinase (IKKβ) to complex I. Phosphorylation of IKKβ by TAK1 activates the IKK complex, and in turn allows it to phosphorylate IκBα (*190*). Phosphorylation of IκBα promotes its ubiquitylation and degradation, releasing NF-κB. MAPK-JNK signaling proceeds through the phosphorylation of MAPKs by TAK1 which stimulates the activation of JNK signaling (*190*) A20, a protein with both deubiquitylation and E3 ligase domains, has a controversial role in this process. While initial studies implicated A20 in the inhibition of NF-κB through removal of K63-linked ubiquitin and replacing it with K48-linked (degradative) ubiquitin, more recent studies implicate a role for A20 in the stabilization of the LUBAC assembly, blocking its removal by deubiquitylating enzymes and cell death (*191*, *192*).

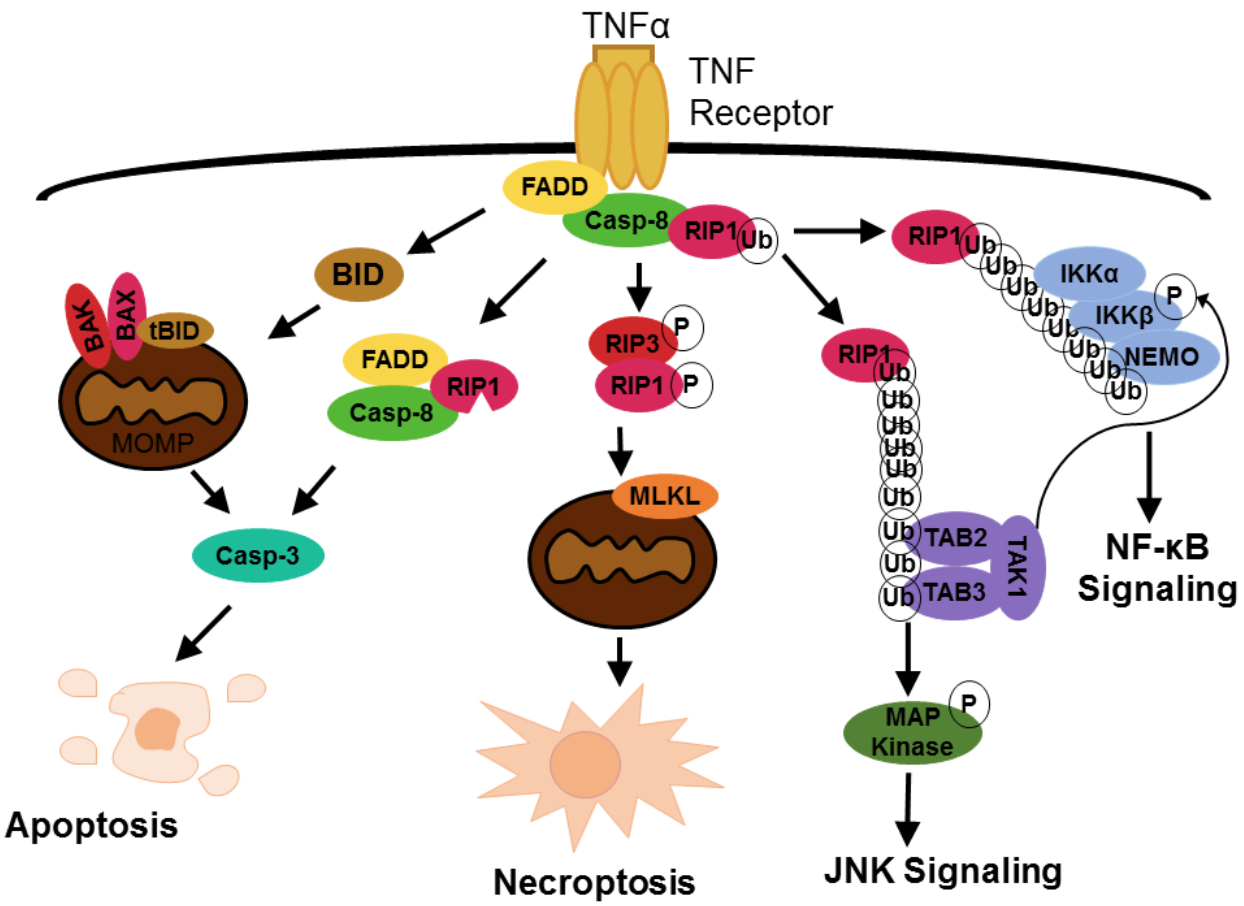


Figure 1.8 Outcomes of death receptor signaling
Activation of death receptor signaling can have two main outcomes, signaling to promotes death, survival, or stress signaling pathway activation. Death signaling can result in apoptotic or necroptotic signaling, depending upon the activation of Caspase-8/10. Additionally, survival signaling through NF-κB occurs with the addition of linear ubiquitin by LUBAC, which serves as a scaffold for binding of NEMO. Similarly stress signaling through MAPK-JNK is facilitated by phosphorylation of the MKK6 by TAK1, which is activated through ubiquitylation, that promotes phosphorylation of JNK and activation of JNK signaling. Adapted from (*190*).

Signaling downstream of death receptors proceeds with the formation of Complex II containing Rip1, Rip3, FADD, and/or TRADD, and Caspase-8, which are capable of binding to each other through DEDs and DDs (*193*). This complex can promote both apoptosis and necroptosis. As discussed above, apoptosis occurs in the presence of Caspase-8/10 activation, in this instance, this complex is proapoptotic and is termed Complex IIA. Necroptosis proceeds in cases where Rip1 is deubiquitylated effectively allowing its translocation to the cytosol and recruitment to this complex. Necroptosis also proceeds in cases where Caspases cannot be activated to promote Rip kinase cleavage. Within this complex Rip1 and Rip3 become activated through either an autophosphorylation event or cross phosphorylation of the kinases on each other and is known as Complex IIB (*24*, *194*). In human Rip, phosphorylation occurs on Serine 161 and 166 in Rip1, and Serine 199 in Rip3 (*195*, *196*). In mouse Rip this event occurs on Rip1 at Serine 161 and in Rip3 at Serine 204. Necroptosis inactivation by Caspase-8 occurs through the cleavage of both Rip1 and Rip3 within this complex, inhibiting their phosphorylation (*96*, *197*). Importantly, formation of active Caspase-8/c-Flip$_L$ heterodimers (as opposed to active Caspase-8 homodimers) also promotes the cleavage of Rip1, but has decreased specificity for cleavage of Bid, which will become an important point in our later studies of necroptotic signaling (*80*, *198*). Following phosphorylation of Rip3, mixed-lineage kinase domain like (MLKL) is recruited to and phosphorylated by Rip3 at Threonine 357/ Serine 358 in human MLKL and at Serine 345 in mouse MLKL (*199–201*). This phosphorylation event releases a "latch" on a four helix bundle within MLKL that allows for its oligomerization and translocation to membranes containing phosphatidylinositol phosphates and cardiolipin (e.g. plasma and intracellular membranes). MLKL oligomers are capable of binding to these lipids, disrupting membrane integrity and promoting execution of necroptosis (*199*, *202–204*). This leads to loss of

membrane integrity in the plasma and organelle membranes, often causing bursting of cells, and release of alarmins, molecules found within the cell released after execution of programmed cell death (*14*).

In the context of the studies presented here we hoped to answer a key unknown in the field of programmed cell death: How is the decision made to undergo either an apoptotic or necroptotic cell death downstream of death receptor signaling? Several proteins (including Caspase-8 and $cFlip_L$) are purported to be key in this decision, however incomplete studies of distinct complex formation cloud the understanding of the mechanistic regulation of this decision. Our studies suggest that Bid cooperates with Caspase-8 and $cFlip_L$ as well as Rip1 to determine the path to death. Additionally, several groups believe that Rip3, but not Rip1 is required for necroptosis activation and execution. Our studies, as well as studies from other groups including Junying Yuan and Michelle Kelliher suggest that Rip1 is also important in necroptosis activation (*43, 44, 205*). Overall, our studies will provide insight into understanding this key unknown, with the hope that this may provide not only better understanding of necroptotic signaling, but also targeting of this pathway in human disease.

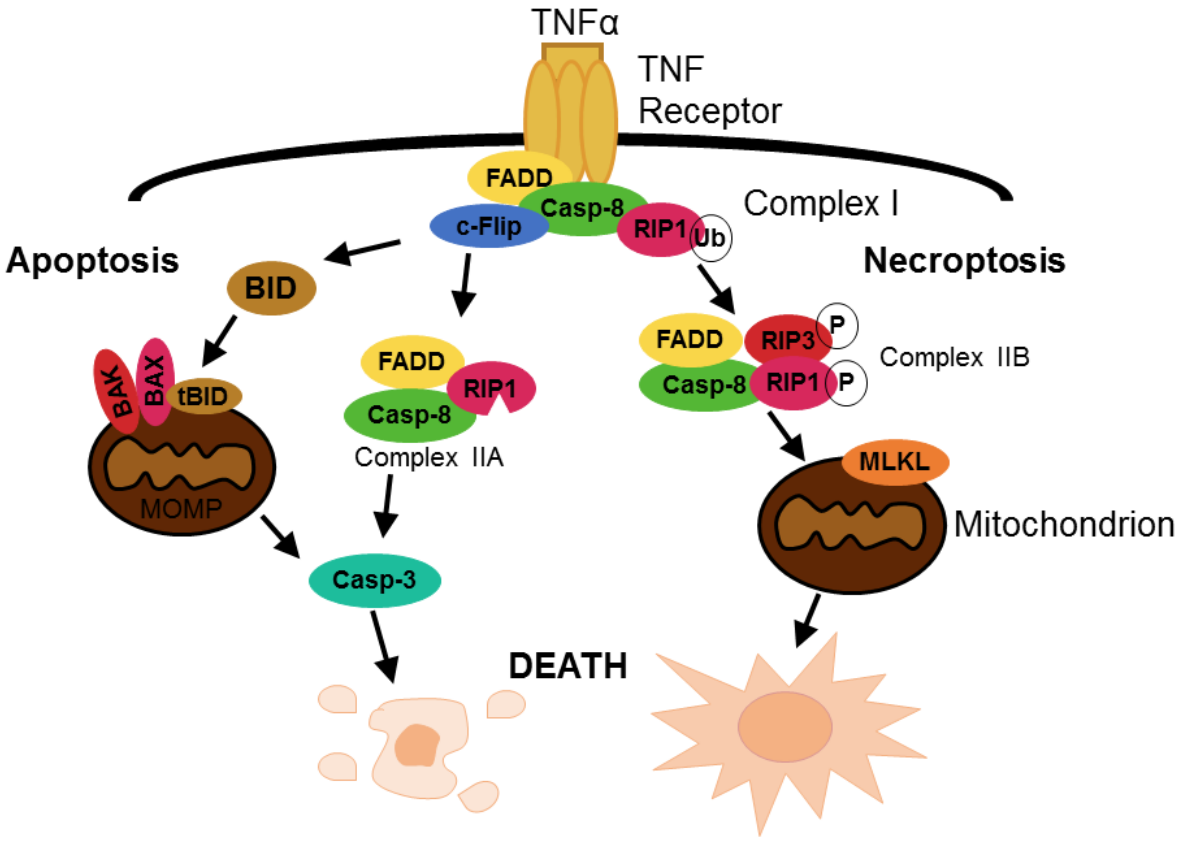


Figure 1.9 Downstream death receptor signaling toward apoptosis or necroptosis
Death receptor signaling can lead to two potential outcomes of death; apoptosis or necroptosis. Apoptosis proceeds in cases where Caspase-8/10 becomes activated and can cleave BID, Caspase-3, Rip1 and other substrates. Cleavage of Rip1 inhibits promotion of necroptosis. Necroptosis proceeds in cases where ubiquitylation of Rip1 is effectively removed or when Caspase-8/10 is unable to be activated. In these settings Rip1 forms Complex IIB with Rip3, Caspase-8, and FADD and is able to be activated through phosphorylation. Phosphorylation of Rip3 occurs as well, allowing it to activate MLKL and execute necroptosis.

Necroptosis Execution

Additionally, a protein known as phosphoglycerate mutase family member 5 (PGAM5), a phosphatase that localizes to the mitochondrion, has also been implicated in necroptosis execution through the activation of DRP1 (*206*). However, the involvement of PGAM5 in necroptosis is controversial, as more recent studies suggest that PGAM5 functions instead to inhibit necroptosis. One *in vivo* study of *PGAM -/-* mice demonstrates increased necroptosis and inflammation, manifest by increased infarct size after ischemia/reperfusion injury. This study suggests that PGAM5 is important in promoting mitophagy, a process that removes defective mitochondria and prevents necroptosis. PGAM5 mediates this process through binding with Phosphatase and Tensin (PTEN)- induced putative kinase 1 (PINK1) to promote mitophagy. In the absence of PGAM5 mitophagy does not occur, leading to accumulation of defective mitochondria, increased ROS production, and increased necroptosis (*207*). Another study implicates decreased size and survival in PGAM5 deficient mice. *In vitro* studies of cells from these animals demonstrate increased necroptosis but decreased IL-1β production, mediated through decreased NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome activation which is necessary for the production of mature IL-1β (*208*). These studies suggest that PGAM5 functions to instead inhibit necroptosis through regulation of mitochondrial turnover, however further studies are necessary to fully understand the role of this mitochondrial protein in necroptosis in relation to normal cell homeostasis.

Production of reactive oxygen species (ROS) is also implicated in necroptosis execution, although it is not required in some instances (*194*, *209*). The first instance of this was reported downstream of TNFR signaling, which demonstrated that downstream ROS production could be inhibited by antioxidants and causes ultrastructural damage to mitochondria (*210*). ROS is

believed to be generated from two main structures, the mitochondrial respiratory chain and plasma membranes. Respiratory bursts as a result of increased glycolysis, glutaminolysis, and glycogenolysis are believed to promote the production of ammonia and ROS as well as leading to mitochondrial uncoupling which is toxic to the cell (*211, 212*). The enzyme NADPH oxidase 1 (NOX1) is believed to promote ROS production from plasma membranes following stimulation of TNFR through recruitment by Rip1 (*213*). Additionally, increased production of ceramide through the hydrolysis of sphingomyelin by sphingomyelinases (SMAses) is believed to promote ROS production as well as lipid peroxidation (*214, 215*). Lipid peroxidation promotes breakdown of lipid structure through removal of electrons forming radicals (*216*). The production of reactive nitrogen species (RNS) is also implicated to play a role in necroptosis execution (*217, 218*). Studies of RNS suggests that they promote oxidation and peroxidation of proteins and lipids, destroying these structures and promoting death (*219*). While there is still much to be learned about the involvement of ROS and RNS in the execution of necroptosis, the evidence available thus far suggests they can be important in programmed cell death.

Necroptosis activation following other stimuli

Much of the early studies regarding necroptosis describe our understanding following death receptor signaling following TNFR or FasR activation. However, more recently necroptosis stimulation downstream of pattern-recognition receptors (PRR) family members, which are associated with innate immunity, has been demonstrated (*220*). For example, the Toll-like receptors (TLRs) can promote necroptotic death. Stimulation of necroptosis is often mediated through the RHIM domain, and interaction between Rip1 and Rip3 or Rip3 with a RHIM-containing adapter protein. TLR signaling, which is activated in response to binding of

pathogen-associated molecular patterns (PAMPs) (e.g. lipopolysaccharide (LPS) or Polyinosine:polycytidylic acid (Poly I:C)), signals through Toll/Interleukin-1 receptor domain-containing adapter inducing interferon β (TRIF), myeloid differentiation on gene 88 (Myd88), or Toll-interleukin-1 domain containing adapter protein (TIRAP)(*190*). TRIF binds to Rip3 through the RHIM and promotes necroptotic execution through MLKL activation (*221*, *222*). These studies implicate this signaling in response to activation of TLR2, TLR3, TLR4, TLR5, and TLR9. Additionally, DNA-dependent activator of interferon (IFN) regulatory factors (DAI) can directly sense foreign DNA within the cytoplasm, and bind to Rip3 to stimulate necroptosis, as well as NF-κB and interferon type 1 signaling responses mediated through interferon regulatory factor (IRFs) (*223–225*). Additionally, activation of Interferon signaling through Interferon α/β Receptor (IFNAR) by IFNα or IFNβ stimulates Janus Kinase 1 (JAK1) which promotes the formation of the Interferon-stimulated gene factor 3 (ISGF3) complex containing Signal transducer and activator of transcription 1 and 2 (STAT1, STAT2) and IRF9. This complex promotes transcription-dependent necroptosis activation through activation of Rip1 and Rip3 (*226*). Necroptotic signaling through other receptors provides an explanation for the activation of necroptotic death from diverse stimuli, and also aids in understanding how crosstalk between receptor signaling determines the final outcome of the signal.

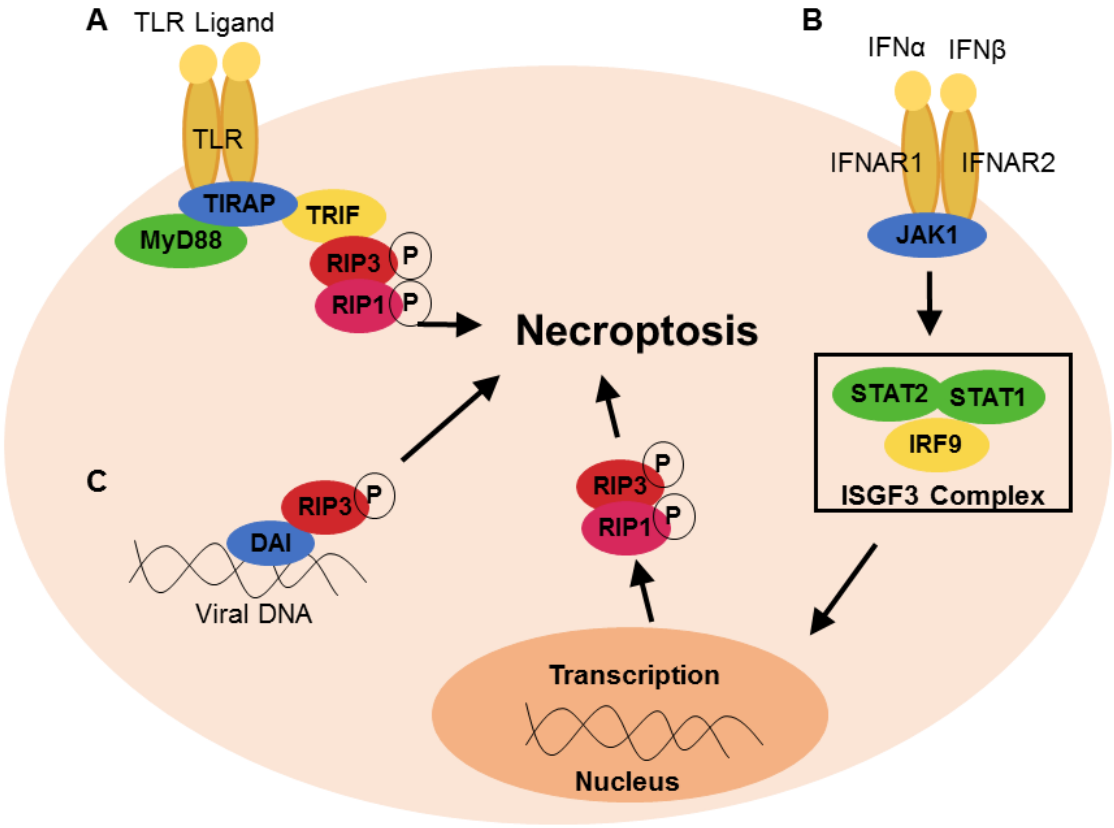


Figure 1.10 Alternative stimuli for necroptosis
In addition to being stimulated through death receptor signaling, necroptosis can also be stimulated through Toll-like receptors (TLRs) and Interferon α/β Receptor 1(IFNAR). A) Stimulation through TLRs can occur through several adapter proteins including MyD88, TIRAP, and TRIF. TRIF is capable of binding Rip1 and Rip3 through the RHIM domain to promote their activation and necroptosis signaling. B) Signaling through IFNAR promotes the activation of JAK1. JAK1 signaling leads to formation of the Interferon-stimulated gene factor 3 complex containing STAT1, STAT2, and IRF9. This complex activates transcription and promotes the activation of Rip1 and Rip3, leading to necroptosis. C) Lastly, binding of DNA-dependent activator of IFN regulatory factors (DAI) can directly bind viral DNA, and in turn complex with Rip3 through its RHIM domain to promote its activation and necroptosis execution. Adapted from (*15*).

Inhibitors of necroptosis

Since the discovery that necrosis is a programmed process mediated by the activation of Rip kinase 1 and 3, and MLKL, many inhibitors that block necroptosis at specific levels of signaling have been identified. The first inhibitor of necroptosis was discovered before the identification of necrosis as a programmed process. The laboratory of Junying Yuan initially identified Necrostatin-1 (Nec-1) as an inhibitor of ischemic/reperfusion injury in the brain and cardiac tissue (*32*, *227*). Later, Nec-1 was identified as a direct inhibitor of the kinase activity of Rip1 (*178*). While Nec-1 held much promise for therapeutic potential of pathological necrosis in disease, it was later demonstrated that Nec-1 has off target effects on another kinase and poor stability *in vivo* (*228*). Later Yuan's group identified a more specific Rip1 inhibitor with implications for blocking death in neurologic disease. 7N-1 blocked the death of myelin-producing cells known as oligodendrocytes which are lost in multiple sclerosis, implicating a role for necroptosis in oligodendrocyte death (*43*). 7N-1 also inhibits phosphorylation of Rip1 blocking necroptosis and can function to inhibit both human and mouse Rip1. Additionally, GlaxoSmithKline (GSK) developed specific inhibitors of Rip1 and Rip3. The GSK'963 Rip1 inhibitor demonstrates promise in specificity and stability, inhibiting necroptosis *in vitro* in mouse and human cells following pharmacologic necroptotic stimuli as well as viral and bacterial infection, and *in vivo* in a TNF- induced septic shock model (*229–231*). GSK Rip3 inhibitors showed initial promise in necroptotic inhibition following TLR and TNF signaling, but later proved to be toxic due to the induction of apoptosis at higher concentrations (*221*, *232*). Necrosulfonamide (NSA) was identified as an inhibitor of Rip3-mediated necroptosis through a chemical screen of compounds that could block necroptosis following a known chemical stimulus. The authors reporting this story determined that this inhibition occurs downstream of

Rip1 and Rip3 at the mitochondrion, and thus identified MLKL as a target of NSA in the inhibition of necroptosis. This compound binds directly to MLKL blocking the downstream execution of necroptosis through a specific cysteine residue in that is not conserved between mice and humans in MLKL (*233*). As such, this compound is only effective on human MLKL. In addition to pharmacologic inhibitors of the Rip kinases, viruses have evolved proteins which are also capable of inhibition of necroptosis. One such instance is in the infection with murine cytomegalovirus. Certain strains of this virus encode a protein known as m45/ viral inhibitor of Rip activation (vIRA) containing a RHIM domain that blocks the activation of necroptosis through interaction with Rip1 or Rip3, inhibiting their ability to bind together and become activated (*234, 235*). Additionally, Herpes Simplex virus type I and II (HSV-I and HSV-II) have evolved proteins which are a part of the R1 large subunit of ribonucleotide reductase (ICP6 and ICP10, respectively) that are capable of blocking both Caspase-8 mediated apoptosis and Rip kinase mediated necroptosis (*231*). ICP6 and ICP10 mediate this function through binding to Caspase-8's death domain as well as the RHIM domain within the Rip kinases in murine cells, to promote necessary interactions for apoptosis and necroptosis activation. However, this role is controversial in human cells as *in vitro* studies suggest this protein inhibits necroptosis (*236*).

Table 1.3 Inhibitors of necroptosis

Name	Source	Target	Specificity	Reference
Necrostatin-1	Chemically produced small-molecule	Activating phosphorylation site in Rip1	Human and Mouse Rip1	(*46*, *178*, *227*)
7N-1 (Nec-1s)	Chemically produced small-molecule	Activating phosphorylation site in Rip1	Human and Mouse Rip1	(*43*)
GSK '963	Chemically produced small-molecule	Blocks kinase activity of Rip1	Human and Mouse Rip1	(*229–231*)
GSK '843 and '872	Chemically produced small-molecule	Blocks kinase activity of Rip3	Human and Mouse Rip3	(*237*)
Necrosulfonamide	Chemically produced small-molecule	Blocks activation of MLKL	Human MLKL only	(*233*)
vIRA	Protein found in Murine Cytomegalovirus	Rip1 and Rip3 RHIM domain	Mouse Rip1 and Rip3	(*234*, *235*)
ICP6/ICP10	Herpes Simplex Virus-I, -II	Rip1 and Rip3 RHIM domain	Mouse Rip1 and Rip3	(*231*, *236*)

Rip Kinases

The Rip kinases are a family of proteins demonstrated to interact with receptors to transduce signals in response to external stimuli promoting cellular cytokine signaling, death and survival pathways, and keratinocyte differentiation (*238*). There are seven Rip kinases (Rip1-Rip7) identified, with four having demonstrated definitive roles in cell signaling identified thus far (*239*). While all members of this family contain a conserved kinase domain, each protein has unique domains, which have been demonstrated to or likely play an important role in the function of each member. The intermediate domain present in Rip1, Rip2, Rip4, and Rip5, allows for interaction with TRAFs 1,2, and 3 which are important for transduction of death receptor signals inside the cell (*240, 241*). The Rip homotypic interaction motif (RHIM) allows interaction between Rip1 and Rip3, the only members containing this motif, along with other proteins such as TRIF (*221, 238*). The death domain is present only in Rip1, and is key in its

interaction with other death domain containing proteins such as FADD and TRADD, transducers downstream of death receptor signaling. Rip2 contains a CARD which allows for interaction with Caspase-1 to promote inflammatory and survival signaling (*242*, *243*). Rip4- Rip7 contain Ankyrin domains which are demonstrated to be important for protein-protein interactions (*244*). Rip6 and Rip7 (also known as Leucine-rich repeat kinase 1 and 2 (LRRK1 and 2)) contain leucine-rich repeats, which are also key in protein-protein interactions (*245*). While the domains present in the members of the Rip kinase family are diverse, the known functions of these proteins cluster in similar facets of cell biology.

Rip kinase 1 was originally identified as a protein that interacted with the intracellular domain of the Fas receptor and TNFR1 (*246*). Later, Rip1 was also implicated in NF-κB signaling downstream of TNFR activation and study of this protein focused exclusively on this function (*247*). This was due to the finding that loss of Rip1 led to early postnatal death due to lack of NF-κB and MAPK mediated survival signaling, overwhelming inflammation of the dermis, and cell death in the intestine (*247*). Similarly, acute global deletion of Rip1 in adult mice leads to rapid cell death due to increased apoptosis in the intestinal and hematopoietic cell lineages (*248*). While these data to suggest that Rip1 is required for activation of this signaling pathway is plentiful, some controversy regarding this exists after *in vitro* studies with *Rip1-/-* mouse embryonic fibroblasts (MEFs) were found to maintain the ability to activate NF-κB signaling (*249*). Several years later, with the finding that Rip1 was required for necrosis activation downstream of TNF signaling and that it could be inhibited, the term programmed necrosis was coined and studies continued to understand how Rip1 modulated necrotic signaling (*175*). Rip1 was also demonstrated to functionally complement the embryonic lethality of loss of FADD in mouse embryos, suggesting that necroptosis and/or survival signaling is important in

development as well (*250*). As discussed above, previous structural studies revealed that Rip1 contains a death domain, which was key in its interaction with other adapter proteins involved in death receptor signaling, and that this death domain alone could stimulate apoptosis (*246*, *247*). Additionally studies revealed this domain is additionally important in survival signaling through activation of MAPK kinase kinase 1 and 2 (MEKK1 and MEKK2) to stimulate NF-κB signaling, as well as Focal adhesion kinase (FAK), which suppresses Rip1 activation of apoptosis (*251*–*253*). While the kinase domain of Rip1 is important in mediating its necroptotic function, it is not necessary for embryonic development as mice with a Rip1 knock-in of a mutation to render the kinase domain inactive (henceforth referred to as kinase dead) survive into adulthood (*254*). However, as expected these animals and cells demonstrate deficiencies in the activation of necroptosis in response to known necrotic stimuli, demonstrating the importance of Rip1 kinase activation in the activation of necroptosis. While Rip1 has recently been implicated in only being important in survival and apoptotic signaling (*255*), (with Rip3 being touted as the key activator of necroptosis) our and others studies with mice and hematopoietic cells suggest that modulation of the stability of Rip1 is also very important in necroptotic activation. As mentioned previously, in addition to its pronecrotic activity Rip1 is also implicated in the activation of apoptosis. This was first discovered with the finding that Caspase-8 mediated cleavage of Rip1 promoted death *in vitro* (*97*). Later, it was found that removal of ubiquitylation on Rip1 was an important step in this process, as this blocked NF-κB survival signaling (*256*). Further studies seeking to understand Rip1 activation of apoptosis identified that formation of the ripoptosome in the absence cIAPs regulated the outcome of TNF signaling, depending upon the presence of c-Flip isoforms (*198*, *257*). This process is inhibited by $cFlip_L$ but promoted by $cFlip_s$ through modulation of Caspase-8 activity. Additionally, Rip1 is implicated in the activation of

inflammasomes downstream of TLR signaling in a FADD and Caspase-8 dependent manner (*258*). Lastly, Rip1 is also implicated in the production of TNFα following caspase inhibition through activation of Jun amino-terminal kinase (JNK) signaling. This production is believed to promote autocrine TNF signaling in certain contexts that promotes necroptotic death in response to certain stimuli (*259*).

Rip kinase 2 was discovered in 1998 by several groups and identified to be important in NF-κB, MAP kinase signaling, and alteration of apoptosis (*243, 260, 261*). Further study demonstrates that the kinase activity of Rip2 is important in maintaining its stability as well as MAPK signaling, but not NF-κB signaling (*262, 263*). NF-κB and MAPK signaling through Rip2 is mediated through interaction with NOD1 and NOD2, which mediate the mucosal innate immune response (*264*). Rip2 is recruited to NODs following their activation and oligomerization and is ubiquitylated (*265, 266*). This ubiquitylation serves as a docking site for adapter proteins important in activation of NF-κB and MAPK signaling, similar to activation of these pathways following Rip1 ubiquitylation downstream of death receptor signaling (*267*). Additionally, the BH3-only BCL-2 family member Bid is implicated to associate with Rip2 in the setting of NOD signaling, playing an important role in activating the innate immune response in intestinal mucosa (*10*). Accordingly, earlier studies implicated that loss-of-function mutations in NOD2 increases susceptibility to the inflammatory bowel disease (IBD), Crohn's Disease (*268–270*). Additionally gain-of-function NOD2 mutations lead to sarcoidosis and Blau Syndrome, inflammatory diseases affecting numerous areas of the body (*271, 272*).

Rip kinase 3 was identified though the screening of a human fetal brain cDNA library for proteins with homology to Rip1 and Rip2 (*273*). These initial studies implicated Rip3 overexpression in apoptosis activation, through binding to Rip1 and partial blockage of its NF-

κB signaling capability. This role of Rip3 persisted within the field until it was discovered that Rip3 forms a complex with Rip1 through RHIM domain interaction, and this in turn promotes necroptosis through its phosphorylation (*24*, *96*, *194*, *274*). Mice deficient for Rip3 survive into adulthood, and demonstrate lack of necroptotic death in response to necrotic stimuli. Additionally, loss of Rip3 rescues the embryonic lethality of Caspase-8 deficiency, suggesting a role for necroptosis in embryonic development (*11*). These findings solidified the role of Rip3 in necroptotic signaling.

The role of Rip3 kinase activity in embryonic development was determined through the development and characterization of kinase dead knock-in mice. These animals die at E11.5 due to apoptosis of the vasculature in the yolk sac (*275*). However, crossing these animals to Caspase-8 deficient mice rescued viability, demonstrating that both apoptosis and necroptosis activation are important in embryonic development. Rip3 is also important in necroptotic execution through activation of MLKL, and correspondingly Rip3 deficiency has important implications in settings where necroptotic death is necessary. For example, studies with models of viral infection demonstrate that necroptotic death becomes an important pathway to viral clearance in the setting of apoptosis inhibition by viruses. The Vaccinia virus (VV) has evolved a number of proteins geared toward the inhibition of apoptosis through Caspase inhibition (*276*). However, necroptosis execution through Rip3 activation is a key factor in clearance of this virus in mice (*24*, *277*). Conversely, murine cytomegalovirus (MCMV) evolved to produce proteins that inhibit both apoptosis and necroptosis through inhibition of Bax and Bak and through inhibition of Rip1 association with Rip3, respectively (*278*, *279*). These findings demonstrated that necroptosis is also key in mediating host defense, and has implications for treatment of viral infection.

In addition to its role in necroptosis, Rip3 is also implicated in apoptosis activation. Several *in vitro* studies implicate that Rip3 promotes apoptosis activation following TNFR stimulation through activation of Caspase-8 in contexts where cIAP1 and 2 are depleted, when TAK1 is inhibited, or in MLKL deficiency (*280, 281*). In the setting of viral infection (Influenza A) Rip3 is demonstrated to activate not only necroptosis through MLKL but also apoptosis in a Rip1 independent, but FADD dependent manner (*25*). Additionally, Rip3 is implicated in the activation of the NLRP3 inflammasome through activation of Caspase-1 (*281*). This process is inhibited by Caspase-8, and can occur with or without Rip3 kinase activity (*258*, *282*).

Rip4 was originally identified through a yeast two hybrid screen of a human keratinocyte cell line to identify proteins interacting with Protein kinase C (PKC)-δ (Delta) isoform, and was originally named Delta protein interacting kinase (DIK) (*283*). A year later, a mouse ortholog was identified through its interaction with PKC-β isoform, and was named protein kinase C-associated kinase (PKK) (*284*). Further investigation revealed that these proteins contained a kinase and intermediate domain similar to previously identified Rip kinases as well as ankyrin repeat domains. As such, these proteins were renamed as human (DIK) and mouse (PKK) Rip4 (*285*, *286*). Overexpression studies of Rip4 *in vitro* revealed increases in NF-κB and JNK signaling. Signaling through NF-κB can occur independent of or require phosphorylation by MEKK2 and 3 depending on the stimulus (*287*). Examination of NF-κB signaling revealed that the IKKα and IKKβ subunits of the IKK complex were required for this signaling. Additionally, Rip4 was found to bind to several members of the TRAF family, and that dominant negative versions of TRAF1, TRAF3, and TRAF6 (lacking the ability to bind to elements necessary for this signaling pathway) blocked NF-κB signaling. Lastly, cleavage of Rip4 by Caspases inhibits NF-κB activation (*286*). Deletion of Rip4 in mice leads to lethality at birth, due to lack of

differentiation of epithelial cells into keratinocytes leading to fusion of the airway (*285*). This phenotype is quite similar to the phenotype seen in IKKα knockout animals, suggesting that both proteins may be part of a similar signaling pathway that is important in keratinocyte differentiation (*288*).

Sugen kinase 288 has high sequence homology and structure to Rip4 as well as ankyrin repeats, which lead to classification of this protein as a member of the Rip kinase family, and naming as Rip5 (*238*). Thus far, a function for Rip5 has not been discovered, but its overexpression leads to programmed cell death that causes DNA fragmentation (*289*).

Similar to Rip5, the function of Rip6 and Rip7 are not known, however, these proteins are referred to as such due to their structural homology to Rip kinases (*238*). These proteins contain unique domains not present in other members of this family such as leucine-rich repeats (LRR) (both), C-terminal of ras of complex proteins (ROC) domain (COR) (Rip6), and WD40 repeats (Rip7). The most prominent feature is the LRR repeat, and as such these proteins are also known as Leucine-rich repeat kinase 1 (LRRK1) (Rip6) and LRRK2 (Rip7). In seeking to understand their function, it was discovered that mutations in Rip7 as opposed to Rip6 are more neuronally toxic *in vitro* (*290*). Correspondingly, mutations in Rip7 are demonstrated to play a role in the pathogenesis of autosomal dominant and sporadic mutations in Parkinson's Disease (*291*).

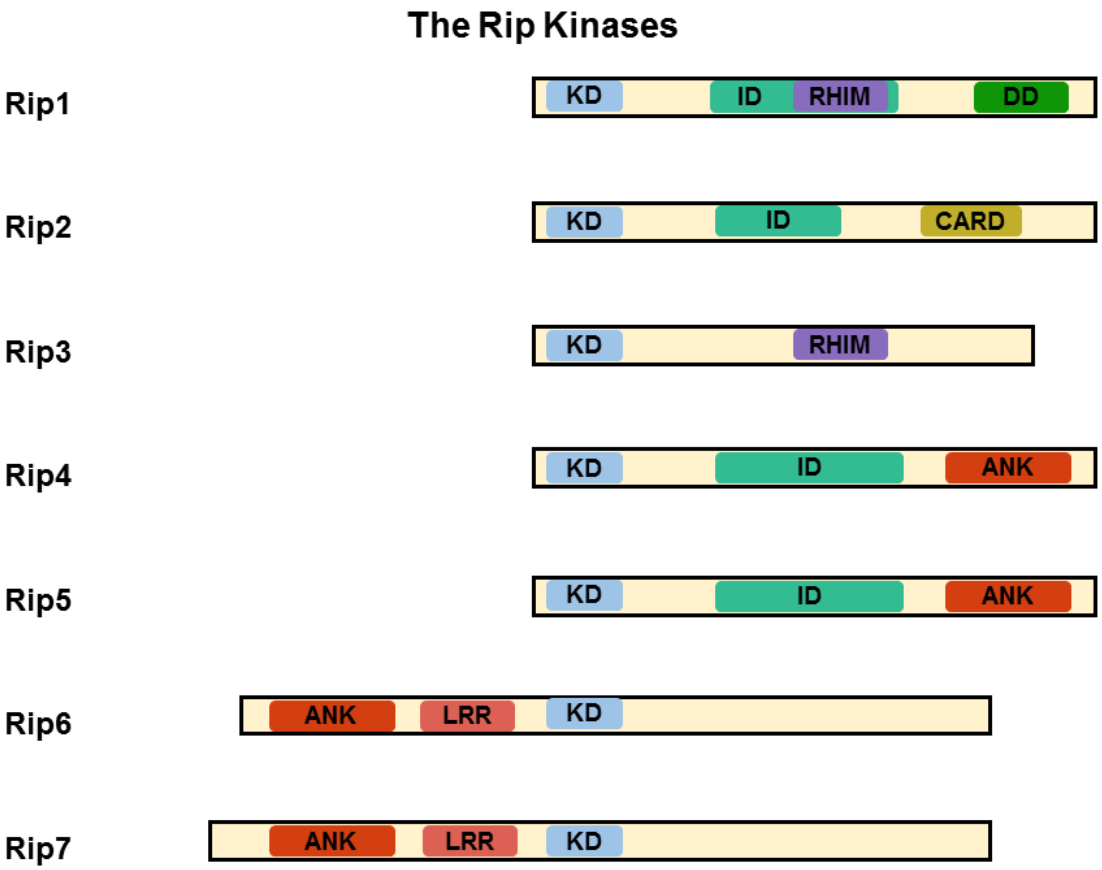


Figure 1.11 Structure of the Rip Kinases
The Rip kinases are a family of proteins implicated in cell signaling. Of the 7 members, 4 have defined roles (Rip1-4), and the remaining are purported to be involved in cell death (Rip5) and in the pathology of Parkinson's Disease (Rip6 and Rip7). Adapted from (*239*).
ID= Intermediate Domain, KD= Kinase Domain, RHIM= Rip homotypic interaction motif
ANK= Ankyrin Repeats, DD= Death Domain, CARD= Caspase activation and recruitment domain, LRR= Leucine Rich Repeats

Inflammatory and innate immune activation following necroptosis

Execution of necroptosis leads to the release of a number of DAMPs which can be categorized as pathogen-associated molecular patterns (PAMPs) and cell death-associated molecules (CDAMs) (also known as alarmins). Release and detection of these molecules promotes the activation of inflammatory and innate immune responses, with these molecules serving as adjuvants functioning with cytokines to promote signaling (*292*). This belief is as a result of the danger model, a model of how the immune system distinguishes between the presence of a molecule as being harmful or innocuous, as opposed to simply recognizing "self" versus "non-self" (*293*). DAMPs serve as the triggers which suggest the presence of danger/damage hence their characterization as such in this context.

One of the most prominent CDAMs includes a protein that associates with the chromatin known as high mobility group box 1 (HMGB1). This protein is released into the extracellular space from the nucleus following necroptotic death, promoting inflammation (*294*). Another set of CDAMs includes Heat shock proteins (HSPs), which are demonstrated to be released specifically from necrotic, but not apoptotic cells (*295*). Release of HSPs gp96, 90, and 70 promotes cytokine release from macrophages and activation of antigen presenting cells (APCs). Additionally, the release of uric acid into the sodium-rich extracellular space is believed to cause the formation of monosodium urate (MSU) crystals, which stimulates dendritic cell maturation (*296*). Another CDAM is the release of ATP, which is capable of signaling through P2 receptors leading to innate and adaptive immune signaling (*297*).

DNA can serve as either a CDAM or a PAMP depending upon its source. Double-stranded genomic DNA (dsDNA) from the dead cell itself can be sensed by the adapter DAI, which in turn can promote inflammatory and death signaling as well as the maturation of APCs

(*298*). DNA from a viral or bacterial source serves as a PAMP, and signals through the TLRs through the adapter Myeloid differentiation primary response gene 88 (MYD88) which again can promote cytokine release, death, and innate immune signaling (*299*). Similarly, the detection of foreign RNA from a viral or bacterial source stimulates signaling through TLRs through the adapter MYD88 to promote a similar outcome (*300*). IL1-α is a DAMP released from necroptotic cells that is capable of inducing inflammation through activation of macrophages and promotion of cytokine secretion (*14*). While many DAMPs have been identified thus far, it is believed that many more have yet to be defined.

Other types of programmed cell death

While my studies primarily focus on the crossroads of programmed cell death pathways in apoptosis and necroptosis, there are several identified programmed cell death pathways within the literature. For example, ferroptosis is a programmed cell death requiring iron uptake that is executed by ROS, and morphologically characterized by shrinkage of the mitochondria, loss of cristae, and ATP depletion (*301*). It was originally identified in tumor cells as a different type of cell death downstream of Ras targeted treatments in Ras-mutated cell lines (erastin) (*302*, *303*). This death is morphologically distinct from apoptosis and necroptosis, and additionally does not require any of the proteases implicated in cell death (Caspases, Cathepsins, or Calpains), nor autophagic, or lysosomal function (*301*). This ROS-mediated death is executed through lipid peroxidation and believed to be promoted through the NOX enzyme family, which produces superoxides. This death can be inhibited by a compound the discovering authors identified called ferrostatin-1, through iron chelation, or blocking iron uptake.

Another type of programmed cell death is termed pyroptosis and is associated with Caspase-1 and Caspase-7 activation, and was originally identified as a specific type of death in macrophages after infection with *Salmonella Typhimurium* (*304*). Continued study revealed that this death occurred downstream of infection with several types of bacteria including *Bacillus Anthracis* and *Listeria Monocytogenes* (*305*, *306*). This death displays morphological features of both apoptosis and necroptosis, including DNA fragmentation and loss of plasma membrane integrity (*307*). Activation of inflammasomes downstream of NLR signaling through either Apoptosis-associated speck-like (ASC) or Absent in melanoma 2 (AIM2) adapter proteins promotes the activation of Caspase-1 (*308*, *309*). Caspase-1 activation also occurs through the formation of the pyroptosome made up of ASC dimers (*310*). This activation leads to the production of IL-1β and IL-18, which promotes inflammatory signaling. In certain but not all instances, this promotes the activation of Caspase-7 which in turn promotes programmed cell death (*311*).

Autosis is a programmed cell death process caused by overactive autophagy, which is promoted *in vitro* through overexpression of Beclin-1 (a protein associated with autophagy) and *in vivo* in a model of hypoxia-ischemia in the rat cerebrum (*312*). This cell death is characterized by severe vacuolization, accumulation of autophagosome/ autolysosomes, nuclear shrinkage with perinuclear ballooning, and plasma membrane rupture that leads to extrusion of cellular contents. Autosis is inhibited by the cardiac glycoside digoxin, which blocks the function of the Na^+K^+ATPase pump present at the plasma membrane. Autosis is unaffected by ROS, inhibition of the Rip kinases, and deletion of Bax and Bak, suggesting it is executed through a signaling pathway independent of apoptosis and necroptosis (*313*). While these types of cell death are new

with well-defined morphologies and requirements, other types of cell death are still under consideration in terms of their consideration as a true programmed cell death process.

Autophagic cell death is defined as a rare cell death directly caused by autophagy due to massive cytoplasmic vacuolization due to accumulation of autophagosomes (*314*). The controversy surrounding classifying this process as an actual programmed cell death arises in data suggesting that autophagy is actually a mode of clearance for cells that are already targeted to die by a programmed cell death process. For example, in a model of mouse development, embryoid bodies lacking genes important in autophagy were unable to clear dead cells, and embryos similarly shows defects in the clearance of dead cells within tissue (*315*). Additionally, while this death is inhibited by suppression of autophagy, studies reveal difficulty in determining what qualifies as a true inhibition (e.g. inhibition of plasma membrane disruption in the presence of DNA fragmentation), making it difficult to understand what role autophagy plays in the cell's demise (*316*). Lastly, this death so far has only been observed *in vitro* (but not *in vivo*) in mammalian systems or in other non-mammalian systems, leaving in question the actual applicability of this process in humans.

Another programmed cell death known as Netosis is characterized by caspase-inhibition, activation of NOX enzymes, and release of neutrophil extracellular traps (NETs). This programmed cell death is characteristic of eosinophils and neutrophils, but the release of NETs is a process that can occur in the absence of cell death. NETs are comprised of chromatin, histones, and antimicrobial granules and are released from viable cells in response to physiological stimuli such as IL-8 and LPS as part of host defense (*317*, *318*). However, NET release and cell death occurs in response to treatment with Phorbol-12-myristate-13-acetate (a non-physiological stimulus) (*319*, *320*). This death is characterized by ROS production, although it is not required

in this process. Correspondingly, this death is inhibited with NOX family of enzymes inhibitors (*317*, *321*). Another process of netosis is the formation of NETs and involves a process known as histone-citrunillation, in which positively charged arginine side chains are made into polar uncharged molecules (*322*). The controversy in classifying this process as a programmed cell is that not all cells dying with these features release NETs, and not all cells releasing NETs die. While there are several types of programmed cell death programs and several emerging programmed cell death programs, much study is still needed to fully understand a number of these new processes and to understand their applicability to human health.

Hematopoiesis in the Mouse

Mouse hematopoietic development

Hematopoiesis is an important biological process that ensures the production of new blood cells and maintenance of the hematopoietic system. Multi-cellular organisms rely on hematopoiesis for the oxygenation of tissues, defense against outside pathogens, and maintaining hemostasis at external sites of injury. Hematopoietic cells are characterized into a hierarchy based upon their ability to self-renew and differentiate into more mature cell types (potency). Hematopoietic stem cells (HSCs) sit at the top of this hierarchy with the ability to both self-renew (ability to divide repeatedly to produce another stem cell) and to develop multiple cell types (multipotent) (*323*). Next in the hematopoietic hierarchy after HSCs are progenitor cells which can self-renew, but primarily differentiate into fewer cell types. Lastly, terminally differentiated cells are capable of proliferation (limited divisions only producing another

terminally differentiated cell), but do not self-renew or differentiate. In our studies of mice with overwhelming necrosis of the bone marrow we explore how this context affects stem and progenitor populations, and also how this affects hematopoietic homeostasis.

Hematopoiesis begins early during embryogenesis, and is characterized by two hematopoietic bursts. The first burst, the primitive wave, produces temporary cells which are not found in the adult organism (*324–326*). This wave begins in the yolk sac within mesodermal cell masses also known as "blood islands" at E7.5 (*327*). These blood islands produce the first red blood cells (RBCs) known as primitive RBCs, which function to oxygenate the rapidly growing embryo (*328*). These cells are characterized by increased size (as compared to adult RBCs), the presence of nuclei, and the presence of fetal hemoglobin (*329–331*). The first macrophage progenitors are also formed, followed by an early erythroid-myeloid progenitor (*332*, *333*). Cells from the macrophage progenitor population eventually migrate to the brain to become microglia (specialized macrophages present in the brain) and to the epidermis where they become Langerhans cells (specialized macrophages present in the skin) (*334*, *335*).

The blood islands within the yolk sac fuse forming vasculature of this structure and allow the primitive RBCs to circulate. Because the cells forming the primitive RBCs and the vasculature (endothelium) appear at approximately the same time and from the same spatial area, it is hypothesized that they derive from one cell type, known as the hemangioblast (*336*). This phenomenon was first described in the 1800s, and was later examined *in vitro* in mouse embryonic stem cells and human pluripotent cells (*337*, *338*). While this process has not been observed *in vivo*, there are multiple studies in the literature supporting this phenomenon.

The second burst of hematopoiesis is known as the definitive wave, during which cells that will be present in the adult are formed. Studies with several species of organisms (including

chick, quail, and frogs) demonstrates that these cells are derived exclusively from the embryo, and not the yolk sac (*325, 326, 339*). *In vitro* studies were performed to examine the intricacies of which portion of the developing embryo had multipotent hematopoietic potential, as well as reconstitution capability. Culture of cells from the para-aortic splanchnopleura (PAS) (region surrounding the dorsal aorta) demonstrates that these cells have multipotent and lymphoid capability (*340, 341*). Additionally, cells from the aorta-gonad mesonephrous (AGM), which derives from the PAS, also demonstrate multipotency through the presence of colony forming unit-spleen (CFU-S) cells which are capable of forming multipotent hematopoietic cells within the spleens of irradiated mice (*342*). From here, these cells move to the fetal liver, the next site of hematopoiesis.

Next, hematopoietic cells seed into the fetal liver, the site of hematopoiesis within the fetus until just after birth (*343, 344*). The fetal liver is capable of producing not only blood, but also macrophages and other myeloid cells (*345*). Development of mature RBCs occurs in erythroblastic islands (EBIs) (*346*). These terminally differentiated myeloid cells are produced in a hierarchical fashion with CMPs giving rise to these cells, similar to adult hematopoiesis (*345*). Cells are believed to seed from the AGM directly to the fetal liver as well as the bone marrow, however is it also suggested that cells may seed from the fetal liver and then to the bone marrow (*341, 347, 348*). Cells from the AGM also migrate to the thymus and spleen (*328*).

In the adult organism, all hematopoietic cells arise from HSCs which are primarily present in the bone marrow. These cells differentiate and mature within the bone marrow, or within peripheral hematopoietic organs (thymus, spleen, and lymph nodes) (*349–351*). Hematopoietic cells differentiate and commit to a specific lineage and proliferate in response to cues from cytokines. In response to exposure to certain cues, HSCs can self-renew or

differentiate into the multipotent progenitor (MPP), which can further differentiate into the common myeloid progenitor (CMP) or the common lymphoid progenitor (CLP). The CMP differentiates into the megakaryocyte-erythrocyte progenitor (MEP) or the granulocyte-macrophage progenitor (GMP). MEPs produce RBCs and megakaryocytes, the producers of platelets. The GMP produces granulocytes and macrophages. These cells are important in a number of roles including tissue oxygenation, hemostasis, and the innate immune response. The CLP produces B and T cells, as well as natural killer (NK) cells, which are important in launching the adaptive immune response, and the maintenance of immunity (*351*).

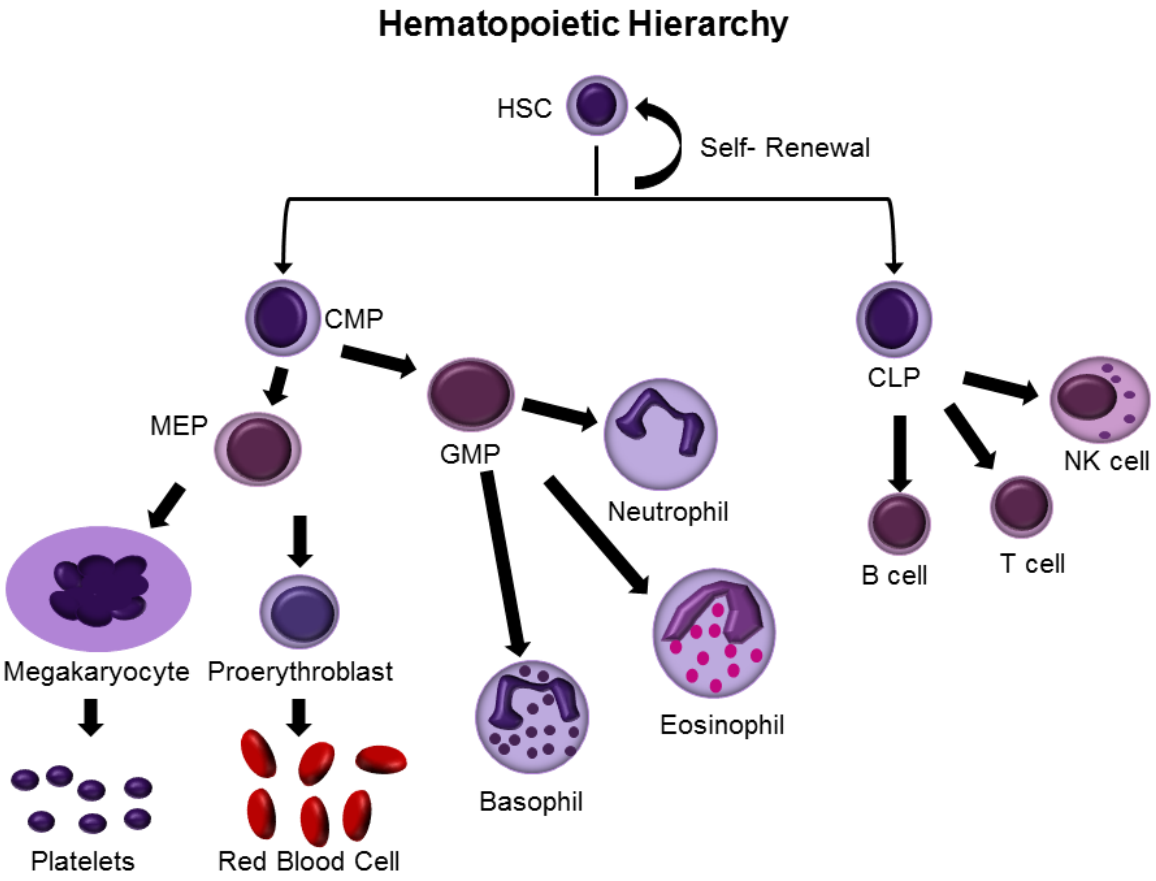


Figure 1.12 The Hematopoietic Hierarchy
The hematopoietic hierarchy demonstrates the classification of hematopoietic cells by their capability to self-renew, as well as their ability to differentiate into different cell types. At the top of the hierarchy is the HSC which is capable of differentiating into all cell types as well as self-renew. Underneath the HSC are the progenitors, which can be subdivided into the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP) which give rise to myeloid and lymphoid cells, respectively. The CMP gives rise to all cells of the myeloid lineage including the platelets and RBCs through the Megakaryocyte erythrocyte progenitor (MEP), as well as basophils, eosinophils, and neutrophils through the Granulocyte macrophage progenitor (GMP). The CLP gives rise to lymphoid cells including B and T cells, as well as natural killer cells. In our studies, the myeloid arm of hematopoiesis is affected with decreases in myeloid progenitor populations. Adapted from (*352*).

Hematopoietic stem cells

Hematopoietic stem cells (HSCs) are essential to the maintenance of the hematopoietic system and were first discovered as cells that were able to form colonies from the spleen of irradiated animals (*353*). Hematopoietic cells derived from the yolk sac do not contribute to the adult hematopoietic system, however there is data to suggest that fetal HSCs from other sources do contribute to adult HSC populations. The discovery that adult HSCs do not derive from yolk sac hematopoiesis was made during studies to examine the origin of the HSC. These studies demonstrated that cells from the AGM had long-term reconstitution ability suggesting that this population of cells contained stem cells (*354*). Additionally, utilization of fate-mapping studies to examine expression of proteins expressed exclusively in early HSCs determined that the definitive wave during which these cells were produced was relatively short. These studies demonstrated that vascular endothelium Cadherin (VE-Cad) and Runt-related transcription factor 1 (Runx1) turned on from E8.5-E9.5, consistent with a burst of cell production characterizing the definitive wave (*355*, *356*). These immature HSCs were later demonstrated to be present in a VE-Cad^{+} CD41^{+} population of cells. Later designation of the pre-HSC (the next step in HSC maturation) was identified to be distinguishable through examination of CD45 positivity. Pre-HSCs were characterized as being Type I (VE-Cad^{+} CD41^{+} CD45^{-}) and then maturing into Type II (VE-Cad^{+} CD41^{+} CD45^{+}) through acquisition of CD 45 positivity (*357*). A similar study later identified that the acquisition of CD45 positivity as well as markers for the major histocompatibility complex class I (MHC class 1) was also important in the maturation of HSCs (*358*). This process is hypothesized to occur in the fetal liver, and to contribute to the development of the adult hematopoietic system (*359*). While it is understood that HSCs are

present in the fetal liver and beyond in hematopoietic development, it is not clear from where and when HSCs present in the adult bone marrow originate.

Previous studies imply that in the adult organism, many HSCs remain primarily in a quiescent (G_0) state, with many HSCs entering into the cell cycle only in response to insults or cytokine cues (*352*). These studies utilized BrdU or tritiated Thymidine to understand how HSCs proliferate in how HSCs proliferate (*360*, *361*). However, use of these labels present a number of limitations including loss of label overtime due to slow cycling and BrdU labeling that is not specific to HSCs (*362*). More recently, several studies demonstrate that at some point within a matter of months, all HSCs divide but do so quite slowly (*363*–*365*). These studies utilized mice ubiquitously expressing fusion proteins of Histone 2B and GFP following doxycycline treatment to fate-map HSCs over time. Examination of these mice over time after doxycycline pulse revealed that HSCs are maintained in this state through localization to microenvironments within the bone marrow known as niches (*365*). These niches are important in the maintenance and self-renewal of HSCs through HSC interaction with stromal cells and exposure to cytokines which maintain their quiescent/ slow proliferating state (*352*). In response to hematopoietic injury or insult, an increased number of HSCs cycle and self-renew as well as differentiate to replace lost cells. HSC proliferation maintains hematopoiesis through the generation of progenitors which rapidly proliferate and differentiate to replace lost terminally differentiated cells. Several studies suggest that amongst the HSC population, there are further distinguishing factors including reconstitution ability and proliferation state (entrance into cell cycle), which further distinguish functionality of this population (*364*, *365*). As such, HSCs are characterized into units of function that are heterogeneous, and are capable of short-term reconstitution and while other HSCs capable of maintaining hematopoiesis long-term. One of the key methods in which this is

tested is through hematopoietic cell or HSC transplantation and serial transplantation (*366*). However, there are some discrepancies regarding how these assays are completed, the potential heterogeneity of hematopoietic populations, as well as what this type of assay truly tests (*367*). As such, transplant studies utilized in the measurement of true HSC activity are questioned in terms of their ability to understand how HSCs behave under homeostatic conditions. Several recent studies are attempting to understand how HSCs function under homeostatic conditions, and to understand how HSC function over time versus single snapshot measurements of quiescence (*368*). These studies suggest that despite the identification of a relatively pure HSC population utilizing CD and SLAM markers, there is still heterogeneity within this population.

Identification of hematopoietic cells by flow cytometry using immunophenotyping

Different types of hematopoietic cells are identified through expression of surface markers known as clusters of differentiation (CD). These markers identify the immunophenotype of hematopoietic (and many other types) of cells (*369*). Utilizing flow cytometry, and antibodies conjugated to fluorescent probes, hematopoietic populations are readily identified using established CD markers for various cell types. While terminally differentiated cells express 1-2 of these markers (indicating terminal differentiation), less terminally differentiated cells are typically identified by expression (or lack there of) of three or more markers (*370*). Pioneering studies from the lab of Irving Weissman elucidated the markers to identify HSC and progenitor populations, revolutionizing the study of these populations in mice with implications for treatment of human disease (*371*). In the studies presented here, cells of the myeloid lineage, including progenitors, as well as HSCs will be the focus of our findings.

Identification of HSCs and myeloid progenitors

Hematopoietic stem and progenitor cells (HSPCs) are identified through their phenotypic expression (or lack there of) of CD markers on their surface. As discussed above, HSCs and progenitors are the least mature cells of the hematopoietic system, and as a result are negative for markers of terminally differentiated cells including: (B cells (B220/CD45R), T cells (CD3), Erythroid cells (Ter119), and granulocytic cells (Ly6G), and a marker for less terminally differentiated lymphocytes (C127/IL-7Rα). Cells negative for these markers are termed "lineage negative" (Lin^-). Identification of an HSPC enriched population is achieved through examination of Lin^- cells and examination of positivity for c-Kit, a cytokine tyrosine receptor, and stem cell antigen 1 (Sca-1), also designated as the LSK population (*372–374*). The myeloid progenitor-enriched population (defined as such since CLPs are depleted through use of IL-7Rα as a marker for differentiated cells) is only positive for c-Kit and is designated as Lin^- $Sca\text{-}1^-$ $c\text{-}Kit^+$. This myeloid population can be further examined to identify the CMP, GMP, and MEP populations through examination of CD34 and FcγIIR/CD16/CD32. These populations are identified as follows: CMP: $CD34^+$ $Fc\gamma IIR^-$, GMP: $CD34^+$ $Fc\gamma IIR^+$, and MEP: $CD34^-$ $Fc\gamma IIR^-$ (*375*). The HSC-enriched population is identified by positivity for both markers and is designated as Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$. The HSC-enriched population can be further purified through the examination of the status of CD135/Fetal liver kinase 2 (flk2)/ FMS-like tyrosine kinase3 (flt3) (*376*). Cells negative or low for this marker are termed Long term-HSC (LT-HSC), where as cells that are intermediately positive are classified as short-term HSCs (ST-HSC), and cells that are highly positive are MPPs. The LT-HSC population can be further purified through examination of the status of two signaling lymphocyte activation molecule (SLAM) markers, CD48 and CD150. Cells that are negative for CD48, but positive for CD150 represent the most pure HSC

population identified in mice and are known as the SLAM-HSC population (*377*). In my studies, examination of the myeloid progenitor enriched and SLAM-HSC populations revealed defects in hematopoietic homeostasis as a result of overwhelming necrosis in the bone marrow of *VavBaxBakBid* TKO mice (see Chapter II).

Table 1.4 Table of Clusters of Differentiation and other markers to identify hematopoietic cells

Cell Type	Marker(s)	Function/ Description
SLAM-HSCs	Lin^- $Sca\text{-}1^+$ (Ly6A/E) c-Kit^+ (CD117) $Flt3^{Low}$ (CD135/Flk2) $CD150^+$	Repopulation of the hematopoietic system
Long Term-HSC	Lin^- $Sca\text{-}1^+$ c-Kit^+ $Flt3^{Low}$	Long-term repopulation of the hematopoietic system
Short Term-HSC	Lin^- $Sca\text{-}1^+$ c-Kit^+ $Flt3^{Int}$	Short-term repopulation of the hematopoietic system
Multipotent Progenitor	Lin^- $Sca\text{-}1^+$ c-Kit^+ $Flt3^{Hi}$	Progenitor capable of differentiating into terminally-restricted progenitors
CLP	Lin^- c-Kit^+ $CD34^+$ IL-$7R^+$	Lymphoid restricted progenitor capable of differentiating into mature lymphoid cells
B cells	$CD45R^+$ (B220)	Part of adaptive immune response, production of antibodies
T cells	$CD3^+$	Part of adaptive immune response, targeting cells for death and activation of other immune cells
CMP	Lin^- c-Kit^+ $CD34^+$ $Fc\gamma IIR^-$ (CD16/32)	Myeloid restricted progenitor capable of differentiating into mature myeloid cells
GMP	Lin^- c-Kit^+ $CD34^+$ $Fc\gamma IIR^+$	Progenitor capable of differentiation into monocytes, granulocytes, and macrophages
MEP	Lin^- c-Kit^+ $CD34^-$ $Fc\gamma IIR^+$	Progenitor capable of differentiation into megakaryocytes and reticulocytes
Monocytes	$CD11b^+$ (MAC) $Gr\text{-}1^+$ (Ly6G/C)	Precursor for macrophages capable of phagocytosing antigens
Macrophages	$CD11b^+$	Innate immune cells that monitor tissues for antigen and phagocytose antigens to present to other immune cells
Neutrophils	$Gr\text{-}1^+$	Innate immune cells capable of phagocytosing antigen or releasing granules capable of killing cells and bacteria
Pro-Erythrocyte	$CD71^+$ $Ter119^-$	RBC precursor capable of differentiating into a mature RBC
Basophilic Erythroblast (Ery A)	$Ter119^{Hi}$ $CD71^+$ $FSC^{Int\text{-}Hi}$	RBC precursor developing after the Pro-erythrocyte
Polychromatic Erythroblast (Ery B)	$Ter119^{Hi}$ $CD71^+$ FSC^{Lo}	RBC precursor developing after the Basophilic erythroblast
Orthochromatic Erythroblast (Ery C)	$Ter119^{Hi}$ $CD71^-$ FSC^{Lo}	RBC precursor developing after the polychromatic erythroblast
Erythroid cells	$Ter119^+$	General marker for precursors/progenitors of RBCs
Megakaryocytes	$CD41^+$	General marker for megakaryocytes producers of platelets

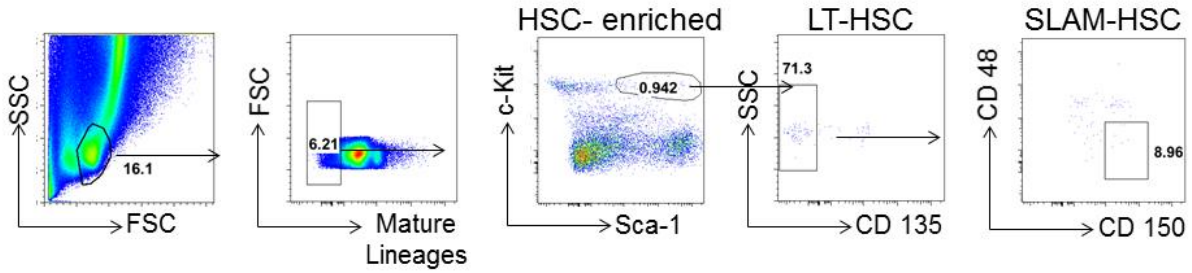


Figure 1.13 Flow schematic for Identification of hematopoietic stem cells
Immunophenotyping by flow cytometry allows for the characterization of the bone marrow to look for expression (or absence of expression) of surface markers which allows for the determination of what types of cells are present in the bone marrow. The forward scatter (FSC) and side scatter (SSC) of the cells allows for examination of their size and complexity, respectively. A gate is placed on the smaller, less complex cells which are typically less differentiated. The next gate is place on cells that are negative for markers expressed on terminally differentiated cells (e.g. Granulocytes, lymphocytes, megakaryocytes, etc.). From this population cells that are positive for both c-Kit and Sca-1 are gated. This subset is the stem cell-enriched population. From the stem cell-enriched gate cells that are negative for CD135/Flt3 are gated on. This population is known as the long-term HSC (LT-HSC). Lastly, from the LT-HSC gate the CD48- CD150+ subset of cells are gated on. This population is known as the signaling lymphocyte activation molecule (SLAM-HSC) and currently the purest mouse HSC population identified in the literature. Adapted from (*364*, *378*).

Cytokine signaling in hematopoietic cells

Cytokines are small proteins secreted by various hematopoietic cells as well as other cell types that direct hematopoietic cells to proliferate, survive, differentiate, die, mature, and activate (*379*). These functions are exerted through two types of action to promote a response, through lineage restriction and through action on multiple lineages at once. However, while a single cytokine is often enough to stimulate action, certain cell types require stimulation with multiple cytokines to promote a response (e.g. HSCs) (*380*). Additionally, cytokines may be present in both secreted and membrane-bound forms, aiding in their ability to act on specific cell types (*381*). While initial studies revealed cytokines are important for normal maintenance and regulation of hematopoiesis, other cytokines have been identified in the promotion of inflammation, inhibition of inflammation, and immune responses. Studies discussed within this dissertation will focus on inflammatory cytokines.

Cytokines were first identified as factors that could stimulate colony production *in vitro* (*382*). The first cytokine discovered, erythropoietin (EPO) a stimulator of RBC production, was found in a patient with aplastic anemia and later demonstrated to promote RBC production directly once it was purified (*383*). Key studies also demonstrated that both granulocyte-macrophage colony stimulating factor (GM-CSF) and Granulocyte- Colony-stimulating factor (G-CSF) were important in maintaining viability of neutrophils and eosinophils (*384*). Systematic studies of cell cycle and signaling also revealed that colony-stimulating cytokines also promote proliferation (*385*). Several studies also demonstrated how increased cytokine stimulation promoted further increases in proliferation, differentiation, or further cytokine release, or that artificial or genetic loss of the cytokine led to dramatic decreases in action clearly demonstrated their importance in hematopoiesis. For example, studies with G-CSF, which

promotes the production of neutrophils, demonstrated that injecting mice with G-CSF promoted overwhelming production of neutrophils, and this was exacerbated with removal of the spleen, a key site for extramedullary myelopoiesis in settings of hematopoietic stress (*386*). Additionally, the ability of a cytokine to promote multiple actions became more clear with the discovery of cytokine receptors and understanding of the signaling following cytokine stimulation (*387*). These studies also delved into demonstration of the ability of cytokines to promote differentiation and maturation of hematopoietic cells. For GM-CSF, this was demonstrated through expression of the receptor for GM-CSF and subsequent stimulation with GM-CSF led to differentiation down the myeloid lineage, demonstrating the importance of cytokines in promoting differentiation (*387*). Cytokines, though small proteins are important in regulating hematopoietic cells to maintain homeostasis and respond to insults.

Inflammatory cytokines promote inflammation and in turn stimulate the activation of innate and adaptive immune responses. The action of these cytokines are important in initiating the front line of host defense in a setting of infection. Four of the most important inflammatory cytokines include TNFα, IL-6, IL-1β, and the interferon (α and γ). Our studies focus on the role of inflammatory signaling in hematopoietic cell death and bone marrow failure. These studies suggest the inflammatory cytokine, TNFα, plays a role in this process. TNFα was originally identified as a protein with the ability to induce necrosis of tumor cells from both mice and humans (*388, 389*). It is expressed by a wide variety of cells including macrophages, T cells, fibroblasts, and natural killer cells (NK cells) (*390*). TNFα functions to promote inflammation through activation of NF-κB signaling in target cells which stimulates the production of many other cytokines including GM-CSF and M-CSF (*391*). TNFα is known to cause systemic

inflammation and its overproduction is implicated in a number of inflammatory diseases including those discussed in this manuscript, MDS and inflammatory bowel diseases (*392*, *393*).

IL-6 was first discovered in the supernatant of T cells, and was later shown to be important in T cell activation (*394*). It was additionally implicated in the activation of antibody production in B cells, following stimulation with bacteria. In addition to lymphocytes, IL-6 acts on many other cell types and is also crucial in the activation of myelopoiesis during infection stimulating the acute innate immune response (*395*). IL-1β is a potent inducer of fever in organisms which led to its discovery in 1977 (*396*). It is primarily produced by monocytes, macrophages, NK cells, and B cells (*397*). IL-1β is highly inflammatory and is not secreted in the absence of stimulation in normal conditions. Detection of an antigen by TLRs stimulates IL-1β production and promotes an inflammatory response. Interferons were first identified as agents that could block the growth of the influenza virus in chick embryos (*398*). There are two types of interferons, Type I (α) and Type II (γ). IFNα is expressed by lymphocytes and promotes the expression of IL-1β following bacterial infection through gene expression (*399*). IFNα is also implicated in anti-inflammatory responses, and as such its action is context-dependent (*400*). IFNγ production is the quintessential macrophage activation factor and is produced by NK cells, lymphocytes, and antigen presenting cells (macrophages/dendritic cells) (APCs) (*401*). It attracts leukocytes to the site of infection, and directs the proliferation, maturation, and differentiation of several cell types (*402*). Inflammatory cytokines are important factors in the clearance of infection, but can also be pathogenic if they promote too much inflammatory signaling.

More recently, the involvement of inflammatory cytokines in HSPC development and populations has become a topic of great interest. Recent studies suggest that inflammatory cytokine signaling is required for the proper development of HSPC populations in mice and

zebrafish (*403*). Discovery of an inflammatory cytokine profile in this population prompted investigation of transcriptional activation of this cytokine expression. Speck and colleagues found that knockdown of IRF2, a negative modulator of interferon transcriptional activation lead to increases in the expression of interferon targets, and increases in HSPC production in development (*403*). Additional studies implicate inflammatory cytokines in increases in proliferation states, and suggest that TNFα aids in HSC development, and suggests that cytokine-producing myeloid cells are required for proper HSC development (*404*). While these studies have been performed in zebrafish, their insight provide potential therapeutic value for human disease (*405*).

Table 1.5 Abbreviated list of cytokines important in hematopoietic regulation and inflammation

Name	**Year Discovered**	**Function**	**Reference**
EPO	1977	Promotes production of RBCs	(*383, 406*)
GM-CSF	1984	Stimulates the production of granulocytes and maturation of macrophages	(*407, 408*)
G-CSF	1985	Stimulates production of and survival of neutrophils	(*409, 410*)
M-CSF	1979	Stimulates the production of macrophages	(*411, 412*)
IL-3	1986	Required for survival of myeloid cells, potentiates growth, involved in inflammatory signaling	(*406, 413*)
SCF	1990	Ligand for c-Kit, promotes proliferation	(*406, 414*)
TNFα	1985	Promotes cell death, survival and stress signaling, stimulates the production of other cytokines	(*388, 389, 391*)
IL-6	1990	Promotes activation of lymphocytes and stimulate production of other cytokines regulating inflammatory response	(*394, 395, 415, 416*)
IFNγ	1957	Key activator of macrophages	(*401, 402, 417*)
IFNα/β	1957	Stimulates production of other inflammatory cytokines/ anti-inflammatory in some contexts	(*399, 418*)
IL-1β	1977	Potent activator of inflammation	(*396, 397*)

Hematopoietic homeostasis

Following the completion of hematopoietic development, birth, and maturation of the organism, the hematopoietic system must still be maintained to function normally and respond to insults over the lifetime of the organism. As such, hematopoiesis is intricately controlled by proliferation and programmed cell death, and is extremely responsive to insults (e.g. blood loss, infection). In response to any perturbations, more terminally differentiated cells are removed, and as a result the HSCs and progenitors proliferate and differentiate to replace lost cells. As such, HSCs and progenitor populations are the most important cells in the maintenance of hematopoiesis. Often, issues with hematopoiesis arise from defects within these populations, leading to a failure to maintain terminally differentiated populations (*371*).

Additionally, the hematopoietic system must constantly address maintenance of the hematopoietic system to keep the number of cells within each population of the hierarchy constant. This is due to the constant turnover of terminally differentiated cells such as platelets (7-10 days), neutrophils (6-8 hours), RBCs (120 days), and T cells (70-90 days) (*419–422*). Again proliferation of hematopoietic stem and progenitor populations, as well as programmed cell death to remove excess, damaged, and autoreactive cells is very important to maintain hematopoiesis in a healthy state.

Early studies of understanding the role of HSPCs in hematopoietic homeostasis focused on methods to intervene in the ablative properties of irradiation. Irradiation greatly affects the bone marrow, leading to death of cells shortly after exposure, and death of the organism in high doses (*423*). Later studies determined that delivery of adult bone marrow could rescue this lethality through reconstitution of the hematopoietic system with the injected cells (*424*). This finding suggested that certain cells within the bone marrow had the capability to carry out

hematopoiesis long-term, and as such, studies with limiting dilution assays of bone marrow were carried out to understand how many cells were needed to reconstitute the hematopoietic system (*425*). Additionally, in studying these transplanted mice, it was noted that they developed nodules on the spleen soon after transplantation. These nodules contained clones of transplanted cells, suggested they derived from transplanted cells. Colony-forming assays were utilized to test the capability of these cells to proliferate and produce mature blood cells which at the time were called colony-forming units of the spleen (CFU-S) (*366*). Additionally, the finding that cells within these colonies had the capability to produce nodules in secondarily irradiated hosts suggested that daughter cells derived from these assays were capable of again developing clones (*426*). This finding introduced the concept of "self-renewal", which is a defining feature of HSCs (*427*). Continued study of these cells suggested that they are typically quiescent in the setting of the bone marrow, which made them resistant to cytotoxic treatments (e.g. irradiation) that target dividing cells (*428*). Lastly, while initial CFU-S assays demonstrated that myeloid cells typically arose from nodules, it was also determined that these cells had lymphoid potential, demonstrating that some cells within these nodules had the capability to generate all mature cell types (*429*). Together, these studies implicated the existence of HSCs, capable of generating all mature hematopoietic cell types and of long-term repopulating capability. Progenitors were later identified through *in vitro* assays which revealed that these populations were distinct from the CFU-S, and served as the intermediate step between HSCs and mature cells types (*430*, *431*).

HSC proliferation is an important aspect of maintenance of hematopoietic homeostasis in the setting of injury or insult, however how HSCs become activated in order to maintain homeostasis was initially not well understood. Typically, HSCs are thought to be in a quiescent state, however in the setting of transplantation (an insult), when host cells are lost and

transplanted cells are injected to reconstitute the hematopoietic system, it was found that self-renewal and proliferation of the injected HSCs was necessary for reconstitution to occur (*432*). Alternatively, it was hypothesized that a process known as "Clonal succession" occurred in which new HSCs are activated when active HSCs became exhausted. This was examined through the retroviral-mediated transfer of genes into hematopoietic cells as a marker, and then examining the fate of these cells over time (*433*). More recently, with the development of better labeling strategies which includes the use of conditional expression of fusion proteins (see hematopoietic stem cells), the LT-HSC, the most immature cell within the hematopoietic system, is thought to have 20% of its population slowly proliferating on a daily basis (*365*). These cells undergo what is termed a "Perfect self-renewal" in which the number of divisions resulting in self-renewal and differentiation are equal, and the number of cells required to maintain this population is minimal (*434*). Cells differentiating from the LT-HSC become ST-HSCs, which undergo "Near self-renewal", as the number of differentiating divisions is slightly greater than self-renewing divisions. These cells feed downstream into MPP and committed lymphoid and myeloid populations.

Correspondingly, as a balance to proliferation, programmed cell death is also required in the maintenance of the HSC population and in turn its homeostasis. Studies with hematopoietic cells and mice overexpressing BCL-2 first implicated this through demonstration that blocking apoptosis increases HSC number and their repopulating capability (*435*). Additionally BCL-2 overexpression decreased the sensitivity of the hematopoietic system to irradiation (*436*). Further studies demonstrate that more HSCs are produced than are needed at any given time likely to support death or egress of HSCs from the bone marrow (*437*). Studies showing that HSC number is tightly controlled, that studies with BCL-2 overexpression increases this number, and that

apoptotic cells are present within an isolated fraction of these cells, demonstrate that apoptosis is an important factor in the control of this population (*364*, *435*).

Maintenance of the progenitor populations is additionally key in hematopoietic homeostasis. MPPs, the next most mature population after ST-HSCs, maintain downstream production of committed progenitors through maintaining the ability to self-renew and proliferate (*434*). In contrast to MPPs, lineage committed progenitors (CMPs and CLPs) constantly proliferate and differentiate in order to produce terminally differentiated cells (*438*). This "transit" population has far greater divisions producing differentiated cells than divisions promoting self-renewal (*434*). As a result of this increased differentiation, many cells are required to maintain this population, and as such the input into its development is much larger and faster than it is for HSCs. Production of CMPs and CLPs varies quite substantially, with CMP production outweighing CLP production by several fold, likely as a result of the fact that lymphoid cell turnover much slower than myeloid cells. While, cell death is not characteristic of this population, aberrant cell death within committed progenitor populations can lead to bone marrow failure disorders, which particularly affect myeloid populations (discussed in greater detail in Hematopoietic Disease) (*439*, *440*).

Stress and emergency hematopoiesis

Settings of stress hematopoiesis are activated in response to moderate insults such as local tissue infection or inflammation and promote activation of similar response pathways as seen in emergency hematopoiesis, but to a lesser extent (*441*, *442*). For example, in the setting of local infection innate immune cells present in tissues before insult perform their antimicrobial tasks and additionally secrete cytokines and chemokines. This in turn promotes increases in

innate immune cell production locally through cytokine release and HSPC differentiation and recruits more immune cells (including lymphocytes) (*443*). Once the infection or inflammatory insult is cleared, excess cells are removed through programmed cell death. Additionally in the setting of repeated RBC loss, HSC proliferation and self-renewal increases within the bone marrow and spleen as well as the number of erythroid progenitors (Ter119+) (*444*).

Emergency hematopoiesis is characterized by insults that affect systemic hematopoiesis. In the setting of systemic infection with bacterial or viral pathogens, this threat is sensed by not only terminally differentiated cells, but also progenitors and HSCs (*445–448*). Initial studies of this process suggest that the constant egress of HSPCs from the bone marrow to peripheral tissues aids in surveillance for infections and in the production of innate immune cells in host defense (*449*). These infections are primarily sensed through TLRs and promote cytokine release. Mature cells release Granulocyte-Colony Stimulating Factor (G-CSF), Interleukin-3 (IL-3), IL-6, and Flt3 stimulating increased production of granulocytes, macrophages, and other myeloid cells (*450–452*). Rapid depletion of neutrophils induces a response known as "Emergency Granulopoiesis" in which neutrophils are generated *de novo* by G-CSF leading to release of mature and immature neutrophils (*453*). This process is believed to be essential for survival of systemic infection as defects in this process quite often lead to decreases in survival (*454*).

HSCs sense infections through TLRs and as a result can secrete similar cytokines secreted by mature cells including IL-6, however this process is thought to be negligible in the overall response (*455*). Release of inflammatory cytokines such as IFNα and IFNγ during infection also affect HSCs (*445*). IFNα stimulates increased HSPC production through increased Signal transducer and activator of transcription 1 (STAT1) signaling (*418*). However the amount

of this cytokine is important in its activity as too little limits repopulation capability, while too much is detrimental and causes HSC exhaustion (*364*, *456*). IFNγ affects HSPC function through stimulation of HSC activity and the accumulation of a c-Kit Hi progenitor population (*457*, *458*). Artificial settings in which these cells types are ablated such as with irradiation or treatment with chemotherapeutics likely produce similar response as discussed above, but additional studies are needed to understand these settings which are termed "reactive granulopoiesis" (*443*).

While programmed cell death is important in the maintenance of homeostatic hematopoiesis, it can also be important or detrimental to the activation of emergency hematopoiesis (*459*). Execution of pyroptotic and necroptotic death promotes the release of cytokines directly and indirectly through release of DAMPs and through their secretion from inflammatory innate immune cells, respectively. For example IL-1β, which promotes pyroptotic death, promotes production of IL-6 and G-CSF, key cytokines in emergency granulopoiesis (*460*). Similarly release of IL-1α from necroptotic or pyroptototic cells can promote release of these same cytokines to promote emergency granulopoiesis (*299*, *461*). Pyroptosis and necroptosis also block emergency hematopoiesis through indirect blockade of HSC activation. This is mediated through IFNα, which inhibits HSC proliferation and differentiation in settings of chronic exposure (*418*). Additionally, increased IFNα signaling is also purported to increase Rip3-mediated necroptosis, and Caspase-11 mediated pyroptosis through TLR and inflammasome activation in mature populations (*226*, *462*). Additionally, is it also hypothesized that pyroptosis and necroptosis may directly impact emergency hematopoiesis through the death of HSCs. This is mediated through TNFR-dependent Rip3-dependent necroptosis as well as Caspase-1 and Caspase-11 dependent pyroptosis (*248*, *463*).

Hematopoietic Disease

Perturbations in the hematopoietic system typically promote a response that causes proliferation and differentiation of the HSPCs and death of terminally differentiated cells. However, in settings in which these cells do not respond properly, or are unable to respond hematologic disease arises. In the aging hematopoietic system, disease can arise as a result of increased DNA damage, development of a myeloid bias in hematopoiesis, or changes in epigenetic regulation leading to changes in gene expression. While mounting evidence suggests that these defects in HSCs occur with increased age, there is some controversy regarding whether these effects are specific to aged HSCs or are characteristics of HSCs which are not functioning properly (*464*). Further functional and genomic studies of aged and young HSCs are needed to clarify this point.

In the aging hematopoietic system HSCs begin to accumulate DNA damage due to increased ROS and telomere shortening (*465–467*). This accumulation of damage is likely due to poor DNA repair mechanisms present in aging HSCs (*468*, *469*). In support of this, a comprehensive study in mice suggests that the number of times HSCs divide is directly related to this increased DNA damage, suggesting entry into the cell cycle increases the potential for DNA damage (*470*). This damage often affects HSC capability to self-renew and in turn reconstitution ability. Studies examining transplant of HSCs from younger and older individuals suggests that HSCs from older individuals have less reconstitution ability that is associated with decreased survival of recipients (*471*).

DNA damage can additionally affect the potential of HSCs, biasing HSCs toward a myeloid potential (*438*, *472*, *473*). This bias is demonstrated to occur not due to changes in HSC activity, but instead as a result of a clonal expansion of a HSC with an inherent myeloid

potential, and a decrease in HSCs with lymphoid potential. This in turn leads to a decrease in immune competence with age (*472*). Another study demonstrated that with age HSCs had increased expression of genes associated with myeloid potential, but decreases in genes associated with gene potential (*474*). These data taken together along with the fact that myeloid cells are produced more often than lymphoid cells in normal hematopoietic homeostasis, likely leads to the increased occurrence of malignancy in myeloid lineage in older individuals (*475, 476*).

Aging in HSCs also affects gene expression through epigenetic regulation, increasing expression of genes involved in stress responses and inflammation (*477*). Expansion of these initial studies has also revealed decreases in multiple genes including those expressed in DNA repair and DNA replication, and differentiation. Through pathway analysis they identified that the increases in the transforming growth factor -Beta (TGF-β) pathway, a key pathway in the activation of proliferation and differentiation in HSCs, also occurs in aged HSCs (*478*). The authors found that these changes were mediated by alterations in epigenetic marks with increases in activating acetylation marks on genes involved in HSC identity and self-renewal, and overall decreases in inactivating methylation marks. The authors also found that expression of the enzymes important in adding/removing these marks were altered with a decrease in DNA methyltransferases and histone deacetylases suggesting that overall gene regulation was perturbed in ages HSCs. Taken together, these data suggest that overall gene expression is altered in aging HSCs and that it is likely changes in epigenetics that contribute to the characteristic changes in of older HSCs.

Clonal hematopoiesis

Damage to HSCs may also confer increased ability to survive over other HSCs, leading to increased representation of these HSCs and its progeny in the hematopoietic population, a state known as clonal hematopoiesis (*472*). Clonal hematopoiesis primarily affects older individuals (*479*). This process is characterized by increased presence of the same "clone" of hematopoietic cells within the hematopoietic system containing certain acquired somatic mutations. Current studies focus on examination of the genomes and exomes of hematopoietic cells within the peripheral blood for mutations (*479*, *480*). While many somatic mutations are present in individuals with clonal hematopoiesis, three genes are commonly mutated at a higher variant allele frequency, DNA (Cytosine-5)-Methyltransferase 3 Alpha (DNMT3A), Additional Sex Comb-like 1 (ASXL1), and Tet Methylcytosine Dioxygenase 2 (TET2) (*479*). These genes are associated with epigenetic control of gene expression in myeloid malignancies (*481*).

The clinical significance of clonal hematopoiesis is not well understood. However, it is hypothesized that individuals with clonal hematopoiesis at an older chronological age are nearly 13 times more likely to progress to hematologic malignancies such as myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) (*480*, *482*). This process is hypothesized to primarily occur as a result of the increased likelihood that additional "driver mutations" will develop in these clonal cells (*483*, *484*). Driver mutations are causally linked to malignancy development and drive the development of cancer. Several studies of populations of individuals demonstrate that mutations often found in individuals with AML and MDS often have mutations in the same genes commonly mutated in clonal hematopoiesis discussed above (*485–487*). The presence of these mutations and others in HSPCs that are still able to function normally are referred to as preleukemic HSPCs (*488*). Mutations in DNMT3A, ASXL1, and TET2 are considered to be

preleukemic due to their association with malignancy. However, few people with clonal hematopoiesis due to these mutations develop these hematologic malignancies, a distinction called clonal hematopoiesis of indeterminate potential (CHIP) (*489*). Some individuals additionally develop these malignancies but lack driver mutations, suggesting another random mutation likely stochastically occurs in order for malignancy to arise (*464*). This process of transformation is not well understood, as well as how other factors including the variant allele frequency of specific mutations and structural chromosomal mutations (e.g. inversion or translocation) affect the likelihood of transformation.

Myelodysplastic Syndrome

MDS is a believed to be a group of chronic hematologic diseases characterized by cytopenias in at least one lineages, presence of dysplasia, and hypocellular bone marrow. The presence of hypocellular bone marrow is attributed to increased programmed cell death. For over 20 years, studies of the type and cause of programmed cell death in MDS has concluded that apoptosis was the culprit of increased death within the bone marrow. However, the majority of these studies were completed during a time before another prominent type of cell death, necroptosis, was recognized in 2000. As such, while the findings presented during that time were sufficient to suggest an apoptotic death, reexamination of this data suggests that an apoptotic or necroptotic death could be occurring. Initial studies utilized a new technique at the time termed *in situ* end labeling (ISEL), which measured ends of nicked DNA. While DNA cleavage was purported to only occur in apoptosis, data also suggest that cleavage of DNA (although in a less ordered fashion) also occurs in necrosis (*490*, *491*). These initial studies found that ISEL positivity was more prominent in the three lineages most affected by programmed cell death in

MDS samples and that this correlated with increased levels of TNFα (*492–494*). Later studies additionally demonstrate increased TNFR1 and FasR in MDS patient samples, suggesting increased death receptor signaling, which can have an outcome of both apoptotic and necroptotic death (*495*). With increased understanding of the apoptotic pathway, these studies next moved to understand the role of activation Caspase-3 in this process, and through *in vitro* studies found that cleaved Caspase-3, Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL), and Annexin V positivity were increased in MDS samples (*496*, *497*). However, these studies were completed on cultured MDS samples, which likely have decreased viability as a result of being outside of their natural niche. Additionally, as previously mentioned, increased DNA presence of nicked ends (TUNEL) is not necessarily indicative of apoptosis.

Current treatments for MDS include chemotherapeutics, demethylating agents, transfusions, and allogeneic bone marrow transplantation. While these treatments often palliate patient symptoms, many patients have no response or lack a sustained response to these interventions. The only potential cure for MDS is an allogeneic transplant, which often is difficult to receive as it requires the bone marrow donor to be closely related. As such, more therapeutics are needed to aid in the treatment of this disease.

MDS is hypothesized to be propagated by the accumulation of mutations within the HSC and progenitor populations, quite often within the same HSPCs which in turn propagate clonal hematopoiesis (*489*, *498*). MDS is characterized by structural (Cytogenetic abnormalities) and/ or molecular (gene) mutations. Structural mutations often involve deletion of chromosomal arms, the most common of which is deletion 5q, (a loss of the long arm on chromosome 5) but may also include deletions of chromosome 7 and 20 (*464*, *482*, *499*). Molecular mutations are much more numerous and typically occur in not only DNMT3a, ASXL1, and TET2, but also Runt-

related transcription factor 1 (RUNX1), Splicing factor 3b Subunit 1 (SF3B1), Serine/Arginine Rich Splicing Factor 2 (SRSF2), U2 Small Nuclear RNA Auxiliary Factor 1 (U2AF1), Tumor protein 53 (TP53), Enhancer of Zeste Homolog 2 (EZH2), GATA-binding factor 2 (GATA2), Janus Kinase 2 (JAK2), Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS), and Casitas B-Lineage Lymphoma (CBL) (*482*, *500*) .

MDS is currently classified according to recommendations from the World Health Organization (WHO) which considers morphologic, cytogenetic, hematologic, and molecular genetic findings (*501*). This group now utilizes a designation of MDS-qualifying description (e.g. Single lineage dysplasia, excess blasts). This replaces designations such as Refractory Anemia with Ringed Sideroblasts (RARS) or Refractory Anemia with Excess Blasts (RAEB-1) with MDS-RS and MDS- with excess blasts, respectively. This new designation was made because cytopenias are an essential quality of MDS and do not require designation, and understanding dysplasia and percent of blasts present is more pertinent in disease classification and treatment development.

Table 1.6 Comparison of old and new WHO classifications of MDS. Adapted from (*501*, *502*).

WHO Previous Classification (2008)	**WHO New Classification (2016)**
Refractory Anemia (RA)	MDS
Refractory anemia with ring sideroblasts (RARS)	MDS with ring sideroblasts
Refractory cytopenia with unilineage dysplasia	MDS with single lineage dysplasia
Refractory cytopenia with multilineage cytopenia (RCMD)	MDS with multilineage dysplasia
Refractory anemia with excess blasts-1 (less than 10% blasts) (RAEB-1)	MDS with excess blasts (% blasts)
Refractory anemia with excess blasts-2 (10-20% blasts) (RAEB-2)	MDS with excess blasts (% blasts)
Myelodysplastic Syndrome with del (5q)	MDS with del (5q)

My studies demonstrate that inflammatory cytokine signaling is increased in the bone marrow, and that death seems to particularly affect myeloid progenitor populations. Additionally, my own studies suggest that Annexin V, a protein that binds to phosphatidylserine, binds to both apoptotic and necroptotic cells, again suggesting this is not a marker specific to apoptosis. Studies presented within Chapter II implicate programmed necrotic death in the death of bone marrow in not only our mouse model of MDS, but also in samples of MDS. These findings suggest that further study of this process is needed to clarify the type of programmed death occurring in MDS and also provide a potential new therapeutic target for the treatment of MDS, blockage of programmed cell death. My studies contribute to this exploration through demonstration that necroptosis is a prominent source of death in both a mouse model of MDS and in MDS patient samples.

Acute Myeloid Leukemia

Acute myeloid leukemia is a heterogeneous collection of diseases characterized by cytogenetic and molecular abnormalities that leads to overproduction of non-functional myeloid blasts which impedes normal hematopoiesis (*503*). This overproduction can occur within any cell of the myeloid lineage. AML is the most common of the acute leukemias and most often develops *de novo,* as a new malignancy (*504*). However AML can also occur after cytotoxic therapies, which is known as therapy-related AML (t-AML) or as a result of transformation from MDS (*505, 506*). This process occurs through "clonal evolution" the development of a new mutation within an existing clone that confers increased survival and allows for cancer development (*507*). AML primarily affects older individuals, and the risk for development increases with age. AML may additionally affect children.

AML is diagnosed through examination of cytogenetics, peripheral blood counts, examination of the bone marrow for blasts, immunophenotyping, and examination of malignant cells for molecular mutations (*508*). Treatment consists of induction therapies with chemotherapy and in cases where a complete remission is achieved, patients often undergo consolidation therapy which may include further cytotoxic treatment or allogeneic hematopoietic cell transplant. While AML is quite treatable in younger patients (younger than 60), it becomes more difficult to treat in older patients as they are more likely to be resistant to standard treatments, suffer from therapy-related mortality, or to have a relapse of their disease (*503*, *508*).

AML is characterized by several types of mutations, and similar to MDS these mutations tend to be structural cytogenetic or molecular genetic issues. As in clonal hematopoiesis and MDS, many of the same genes are mutated in AML. However, many of these genes are unique to AML, and are recognized driver mutations that develop as a result of clonal evolution. In addition to DNMT3A, RUNX1, ASXL1, TET2, and TP53, mutations in FLT3, Nucleophosmin (NPM1), CCAAT Enhancer-binding protein alpha (CEBPα), Kit (gene encoding c-Kit), Neuroblastoma Rat Sarcoma viral oncogene homolog (NRAS), and Isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) are also common (*501*, *503*). As with MDS, presence or absence of these mutations often predicts prognosis and additionally aids in classification of AML. Additionally, it was recently demonstrated that clonal evolution also comes into play with the relapse of AML in patients after achieving complete remission (*509*).

As a heterogeneous disease characterized by many structural and molecular mutations, AML is classified into a number of categories based upon specific genetic abnormalities as well as dysplasias (a rare occurrence), as well as the particular lineage of cell affected, and lastly myeloid sarcoma, an isolated myeloid extramedullary tumor (also rare) (*501*). The great number

of structural and molecular mutations present in AML leads to a single classification category focused on recurrent mutations that commonly occur in AML 11 subtypes focus on chromosomal translocations, inversions, and deletions, t-AML related to cytotoxic therapies, as well as commonly mutated genes (*510*). AML is also classified according to the presence of myelodysplasia, as the association of these dysplasias with previously diagnosed MDS or myeloproliferative neoplasms (MPNs) has prognostic relevance for AML. Lastly, AML- not otherwise specified (NOS) covers AML with blasts at varying stages of differentiation, and blasts that tend to be rarer (e.g. acute basophilic leukemia). Classifications of AML utilizing these criteria aid in diagnosis and additionally aid in determination of treatment regimens.

Table 1.7 WHO Classification of AML. Adapted from (*501*).

Classification (# of subtypes)	**Description**
AML with recurrent genetic abnormalities (11)	AML with structural and molecular mutations
AML with myelodysplasia-related changes	AML present in patients with previous diagnosis of myedysplasia
Therapy-related AML	Patient diagnosed with AML after cytotoxic therapy for a primary neoplasm
AML, Not otherwise specified (9)	AML characterized by more mature blasts (e.g. megakaryoblast) or immature blasts
Myeloid sarcoma	Solid tumor composed of myeloid blasts outside of the bone marrow

The Intestinal System

Intestinal development in the mouse

The development and maintenance of the gastrointestinal system is crucial for the survival of an organism as it is key in breaking down and taking up nutrients necessary for the maintenance, growth, and restoration of the body. This complex system consists of the mouth, esophagus, stomach, cecum, small intestine, large intestine (i.e. colon), rectum, and anus. Development of the gastrointestinal system begins very early in fetal development and completes by two weeks postnatally. This process is characterized by constant proliferation and differentiation. Following gastrulation, the endodermal cells accumulate to form the hindgut and foregut. Through a folding wave these two sets of cells meet to form the primitive gut tube which includes the foregut, midgut, and hindgut (*511*). The foregut gives rise to esophagus, thyroid, trachea, lungs, stomach, liver, biliary system, and pancreas. The midgut gives rise to the small intestine and the hindgut gives rise to the colon. As the fetus continues to develop, the gut tube continues to grow through elongation and increasing girth. The once monolayer epithelium becomes pseudostratified and develops polarity (apical and basolateral poles) (*512*). Additionally, expansion of cells within the submucosa and layer of muscle expand to aid in expansion of the epithelial layer. In late development, the rostral to caudal wave promotes reorganization of the epithelium. The pseudostratified epithelial cells within the mid and hindgut become simple polarized columnar epithelium. Intraepithelial cavities form at the basolateral membrane of the pseudostratified epithelium and grow larger forming a secondary lumen that eventually fuses with the primary lumen (*513*). Around the same time, the mesenchyme invaginates into the epithelium to form *de novo* villi. These initial villi split to form more villi, a

process that is believed to be control by tension from the underlying muscle layer (*514*). This process similarly occurs in the colon, however the villi that form here are transient and are replaced by crypts as development continues. The colon additionally forms a multilayer epithelium that develops secondary lumina that fuse with the primary lumen as intervillous regions (*513*). By late gestation the small intestine and colon are composed of villi and intervillous regions. During the first two weeks of life the intervillous regions become crypts of Lieberkühn and the gastrointestinal system matures (*514, 515*). After maturation, further growth of the intestine occurs through fission of crypts throughout the lifetime of the organism (*516*).

Adult intestinal system

The mature gastrointestinal system plays two key roles in helping an organism to survive; breaking down and absorbing nutrients obtained from foods and maintaining a tight barrier to keep potential pathogens (e.g. microbes present on ingested food or commensals present within the intestinal tract) from infiltrating the body. There are four main layers of the intestine. The inner mucosa is the home of the intestinal epithelium which serves as a barrier from the intestinal lumen. The submucosa is a supportive layer of the mucosa and also contains the enteric neurons. The muscle layer contains several layers of muscle which are important for the movement of chyme (intestinal contents) through the intestine (*517*). The outer layer, the serosa, aids as a visceral membrane that covers the intestine and anchors it in place within the peritoneum (*518*). Within the intestinal epithelium there are four main types of terminally differentiated cell types that carry out these functions; enterocytes (colonocytes within the colon), goblet cells, enteroendocrine cells, and Paneth cells. Enterocytes make up the majority of the epithelial barrier within the intestinal barrier easily comprising 80% or more of the cells covering villi (*519*).

These highly polarized cells are responsible for the uptake of nutrients present in the digesting food moving through the small intestine and also secrete hydrolyzing enzymes that aid in digestion (*520*). These cells additionally participate in host defense against potential antigens through secretion of antimicrobial proteins (AMPs), expression of PRRs that allow for innate immune signaling, and through presentation of antigens to immune cells (*519, 521, 522*). Goblet cells are localized to both crypts and villi and secrete mucus within the lumen providing an extra barrier against potential antigens. Goblet cells primarily secrete mucin 2, a primary component of mucus, as well as trefoil factor 3 (TFF3), FC-gamma binding protein (FCGBP), and resistin-like molecule β (RELMβ). TFF3 and FCGBP promote the cross-linking of mucins which is important in maintaining the stability of the mucus layer protecting the epithelium (*523*). While the role of RELMβ is not completely understood, it is known that its expression promotes increased mucin 2 expression and secretion, and it is induced when goblet cells are exposed to commensal bacteria, as well as within mouse models of helminth infection and inflammatory bowel disease (IBD) (*523*). These cells are also demonstrated to play a role in host defense through the presentation of antigens to dendritic cells (*524*). Enteroendocrine cells are hormone secreting cells that sense the luminal environment for nutrient uptake and monitor energy status and in turn secrete hormones in response to these stimuli. This is important in regulation of the enteric nervous system, facilitating communication between different portions of the gastrointestinal system as well as with other organs important for nutrition such as the pancreas (*525*). There are 10 distinct populations of enteroendocrine cells each secreting different types of hormones in response to various stimuli. For example, detection of energy sources such as glucose leads to the secretion of glucoinsulinotropic peptide (GIP) from enteroendocrine cells which targets β cells within the pancreas to produce insulin and promote glucose uptake (*525*).

Paneth cells serve as the primary host defense proteins within the intestine and are localized to the bottom of crypts alternating positions with intestinal stem cells (ISCs). These cells secrete AMPs such as lysozyme, cryptdins, and cathelicidins, which disrupt bacterial membranes (*526*). Paneth cells also secrete necessary factors for maintenance and survival of ISCs (*520*). Paneth cells are also implicated in the management of inflammation and intestinal homeostasis, as loss of these cells leads to increased inflammation in the intestine of mice and development of a phenotype similar to the IBD Crohn's disease (*527*). Three other less common types of cells (cup cells, tuft cells, and microfold cells) exist within the intestinal system, however their function is less well understood. More recently, microfold cells, which are localized to Peyer's patches in the small intestine, were demonstrated to sense antigens within the lumen and present these antigens to adaptive immune cells (*528*). Additionally, tuft cells which are chemosensory sensing epithelial cells, were shown to accumulate at site of infection with protozoa and helminth within the intestine (*529*).

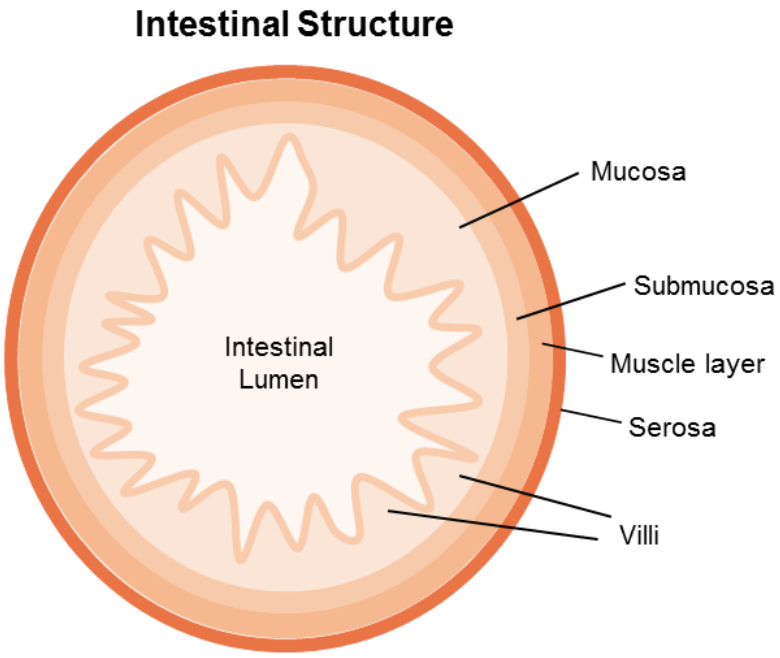


Figure 1.14 Structure of the Intestine
The intestine is an important site of nutrient absorption, and also serves to aid in the movement of intestinal contents throughout the gastrointestinal system. The intestinal mucosa is part of the intestinal epithelium which is important for nutrient absorption and serves as a barrier between the intestinal lumen and the gastrointestinal system. The submucosa serves as a supportive layer of the mucosa and also contains the enteric nerves. The muscle layers contain several layers of muscle which are important for the movement of the intestinal contents through the gastrointestinal system. Lastly, the serosa is an outer layer serving as a visceral membrane covering the intestine. This membrane also serves as an anchor for the intestine within the peritoneum. Adapted from (*517*).

Maintenance and homeostasis of the intestine

The intestinal lumen is an extremely harsh environment due to the high pH of the lumen, constant mechanical motion, and the presence of enzymes designed to aid in the breakdown and digestion of the contents moving through it. As a result, the turnover of the epithelial cells within the intestinal lumen is quite high with most lineages renewing every 3-5 days and as many as 300 million cells being lost daily through anoikis (*530*). This great loss creates a need to replace cells quickly. Intestinal stem cells (ISCs) replace these lost cells by replenishing terminally differentiated populations through proliferation and self-renewal. Replicating ISCs differentiate into transit amplifying cells (TAs) an intermediate compartment within the crypt (*531*). Next, TAs divide 3-4 times, traveling up the crypt as they divide, before differentiating into all terminally differentiated epithelial cells with the exception of Paneth cells. Once this lining of cells reaches the base of the villus, proliferation ceases (*530*). Paneth cells renew every 3-6 weeks and differentiate from dedicated progenitor cells which reside in the TA cell portion of the crypt. Once these cells differentiate they migrate downward toward the crypt bottom where they are interspersed between ISCs (*532*).

Maintenance of the intestinal epithelium relies on ISCs which are capable of generating all intestinal epithelial cells. Current studies have led to the development of a two stem cell zone model which posits the existence of at least two stem cell populations; the crypt base columnar (CBC) stem cells and the +4 stem cells which are present 4 cell diameters up from to the crypt base. This model arises from several studies suggesting that cells residing within the crypts proliferate and self-renew rapidly and function as short-term ISCs while +4 stem cells are more quiescent and function as long-term ISCs (*530*). While the identity of CBC stem cells is well-established with the identification of Leucine-rich G protein-coupled receptor 5 (Lgr5) as a

marker, a +4 stem cell has not been firmly established (*531*). Several proteins are purported to be markers of the +4 stem cell population, including B-cell specific Moloney murine leukemia virus integrating site 1 (BMI1), Hop homeobox (HOPX), Leucine-rich Repeats and Immunoglobulin like domains 1 (Lrig1), and Telomerase reverse transcriptase (TERT) (*533–537*). However, while initial reporter and fate-mapping studies in mice suggested these proteins as markers of this population, further studies have fallen short in reliably marking this population, were not reliably being expressed in exclusively multipotent cells, or were expressed in highly proliferative cells (*536, 538, 539*). The fate of ISCs after differentiation into TAs and terminally differentiated cells is well understood, however many more studies are needed to understand stem cell biology within the intestine.

The adult ISC population arises from early ISCs that are present during initial crypt development. These early ISCs rapidly expand through several self-renewing cycles, and then switch to a differentiating state to establish the adult stem cell pool (*540*). These cells further undergo a symmetric division that can result in either long-lived ISCs or TA cells. Through a stochastic event each crypt has a single remaining ISC, a process termed “neutral drift” (*530*). Overtime, each crypt becomes clonal, as this single long-term ISC remains after the cells dividing and differentiating are lost through normal cell turnover. The remaining ISC gives rise to all cells within the crypt, creating a clonal population.

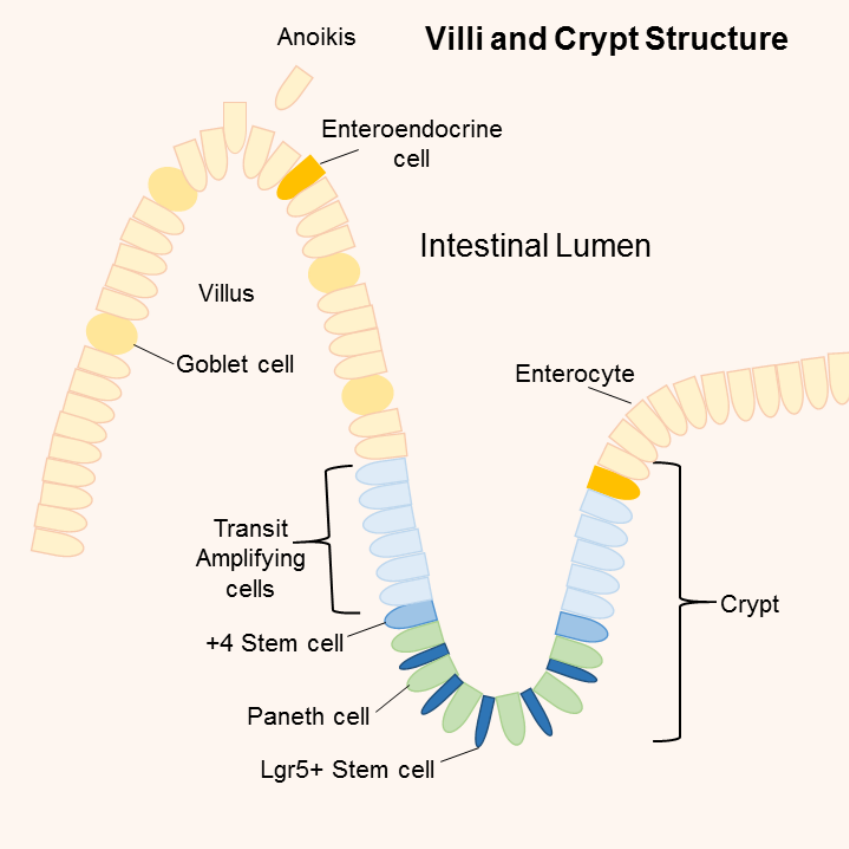


Figure 1.15 Crypt and villi structure
The epithelial layer of the small intestine is composed of villi and crypts which absorb nutrients and are the sites of intestinal cell generation, respectively. These structures provide increased surface area, increasing exposure of the contents of the intestinal lumen to chyme. Enterocytes are the principle absorptive cells. Goblet cells secrete protective mucus that coats all surfaces of the intestinal lumen and serve as the first line of defense against foreign antigens. Entero-endocrine cells secrete hormones related to the presence or absences of certain nutrients and monitor energy status. Paneth cells serve as intestinal host defense cells, secreting antimicrobial peptides, and are also implicated in control of inflammation in the intestine. Transit amplifying cells serve as the precursors of each of these terminally differentiated cell types except Paneth cells. The cells of the intestine derive from intestinal stems cells (ISCs). The current model of ISC maintenance purports the existence of two types of ISCs; Lrg5+ and +4 ISCs. Lgr5+ cells are positive for this marker and reside at the bottom of crypts in alternating position between Paneth cells. Lgr5+ cells are believed to be short term ISCs that are highly proliferative. The +4 ISC is located at position 4 within the crypt, however, there is controversy on which markers it expresses. The +4 ISCs are believed to be a more quiescent population that is long lived. The colon is made up of these same cells populations, but with enterocytes instead being termed colonocytes. Additionally, the colon primarily contains crypts, and villi are absent. Adapted from (*530*).

Inflammatory Bowel Diseases

Inflammatory Bowel Diseases (IBDs) are pathological states affecting the intestines characterized by chronic inflammation due to defective inflammatory signaling and is often driven by cytokine signaling (*521*). There are two major types of IBDs, Crohn's Disease (CD) and Ulcerative Colitis (UC) (*541*). Other types include microscopic colitis, Behcet's Disease, and indeterminate colitis, which are much less common (*541*, *542*). While an exact etiology of IBDs is not known, it is believed to be caused by a combination of potential genetic susceptibility, issues with maintenance of epithelial cell barrier function, innate immune signaling, the status of the microbiota, and diet (*543–545*). IBDs typically affect individuals between the ages of 15-30, but it is also diagnosed in children and is often more severe than adolescent and adult cases. Mouse models of IBDs suggest a major role for perturbed immune signaling and barrier function, as well as the microbiota, in the development of IBDs and as such are topics are of active research to develop and discover therapeutic targets (*546*). One of the new methods that is being utilized is the identification of biomarkers which can aid in the classification of IBDs and distinguish between CD and UC (*547*).

Crohn's disease and ulcerative colitis, while being characterized by inflammation are two different diseases. CD can affect any tissue of the gastrointestinal system, but primarily affects the intestines, and is characterized by patchy presence of inflamed sites within healthy tissue. UC exclusively affects the colon and typically affects the distal portion and the rectum in continuous regions. Symptoms of both diseases include similar features; diarrhea, chronic abdominal pain, weight loss, and often a family history of IBD issues, as nearly 20% of patients have a family history of IBD (*544*, *545*, *548*). Patients may also display extraintestinal diseases such as osteoporosis, uveitis (inflammation of the uvea in the eye), or erythema nodsum (formation of

painful nodules under the skin) (*544*, *545*). Diagnosis of both diseases follows the same process which includes endoscopic and colonoscopic examination and biopsies, examination of tissue histology, magnetic resonance enterography, and blood serum examination and fecal examination for markers of inflammation such as C-reactive protein and calprotectin(*544*, *545*). Additionally, while CD has clear mutations associated with its occurrence, UC does not (*544*, *545*).

Classification of inflammatory bowel diseases, particularly CD and UC, is based upon the age of onset, location within the gastrointestinal system, activity of the disease, and growth (*544*, *545*). In the treatment of IBDs, clinicians also measure the activity of the disease according to a score known as the Crohn's Disease Activity Index in CD and the Truelove and Witts Severity Index for UC in adults (*545*, *549*). Activity is characterized as being mild, moderate, or severe and give implication for the effectiveness of patient treatment.

There are a number of treatments utilized in patients with IBDs which target hormone, immune, or inflammatory/cytokine signaling (*546*). Steroids are a common treatment for IBDs and include corticosteroids and adrenocorticotropic hormones (*550*). These steroid often cause a complete remission in patients within a year. Immunosuppressants such as mercaptopurine and azathioprine are utilized in patients if steroids are ineffective, or for patients that are steroid-dependent (*551*). Cyclosporine is another immunosuppressant that is specifically utilized in severe cases of UC that are steroid-refractory (*552*). Methotrexate is an anti-inflammatory drug utilized in patients with CD that are steroid-dependent. It is also used to maintain steroid-free remission, or to maintain remission (*553*, *554*). Lastly, more recently, anti-TNFα therapy is demonstrated to be extremely effective in some patients with IBDs. As a pleiotropic cytokine the mechanism of action is not well understood, but may provide clues to understanding the

pathogenesis of IBDs (*555*). When drug therapies are ineffective, or if inflammation in the affected intestine is severe, patients may also receive surgery to remove affected portions of tissue or to bypass affected regions (*556*, *557*). While many patients are successfully treated with these therapies, many are not, while others fail to have a sustained response. As such, the need for better understanding of IBDs and for more treatment options is needed in order to provide better treatment options for patients, and to prevent the progression of this disease which can lead to formation of fistulas, strictures, or intestinal malignancy (*544*, *545*).

CHAPTER II

LOSS OF BID-REGULATED NECROSIS INHIBITION LEADS TO MYELODYSPLASIA AND BONE MARROW FAILURE SIMILAR TO THE HUMAN DISEASE MYELODYSPLASTIC SYNDROME

Introduction

Programmed cell death (PCD) is an essential process, required to craft distinct structures in early embryonic development (*1*), and to maintain homeostasis in dynamic systems such as hematopoiesis in adult organisms (*102*). The two main forms of PCD, apoptosis and necroptosis, result in markedly different outcomes with important implications for the cellular microenvironment: apoptotic cells are removed with minimal inflammation, whereas necroptotic cells release DAMPs which incite inflammation (*15*). In settings such as hematopoiesis, where cells are primed to respond to cytokine-directed environmental cues in order to maintain homeostasis, biasing cell death fate to necroptosis could seed chronic inflammation. Inflammation-driven cytokine production has the potential to alter the microenvironment to impact response to infections, myelosuppression from toxins (e.g chemotherapy), and transformation to leukemia. The potential impact of cell death fate on hematopoietic homeostasis is substantial and poorly understood.

The Bcl-2 (B-cell lymphoma) family of proteins are situated at this central decision point of cell death fate, functioning downstream of death receptor signaling yet before activation of

executioner Caspases and cell death. While their central role in apoptotic cell death is well understood, the mechanistic link between the Bcl-2 family and necroptotic cell death has not been described. In particular, the BH3-only family member Bid, acts as a sensor and amplifier of signaling through death receptors, serving to activate mitochondrial outer-membrane permeabilization (MOMP) and initiate intrinsic apoptosis following interaction with and cleavage by Caspase-8 (*113, 114*). Importantly, Bid has been shown to function in a pro-survival role in which it acts to restrain cell death execution in certain contexts (*7*, *8*, *173*, *558*). We propose that this pro-survival function of Bid extends to its role in actively restraining necroptosis, mediated through its modulation of Caspase-8 activity (discussed in Chapter III). Thus Bid acts at the central decision point between apoptotic and necroptotic cell death commitment.

In order to test whether Bid may influence cell death fate, we generated two mouse models. First, we conditionally deleted *Bax* using VavCre, and crossed this to a mouse model with germ line deletion of *Bak* in order to create hematopoietic-specific *BaxBak* double knockout (DKO) mice. This cross results in the loss of intrinsic apoptotic execution in hematopoiesis, however importantly leaves the upstream signaling pathway of interest intact. To specifically assess the role of *Bid*, we crossed these mice with *Bid-/-* mice to create *BaxBakBid* triple knockout (TKO) mice (*121*, *157*). These models allow us to determine *BaxBak* independent roles of *Bid* at the central decision node upstream of the mitochondria yet still downstream of the receptors and immediate DISC complexes (*47*, *51*).

We confirm that deletion of *BaxBak* completely blocks apoptotic cell death, but that this deficiency is not sufficient to initiate necroptotic cell death. Further deletion of Bid, however, leads to robust activation of necroptosis and specifically early death due to bone marrow failure

(BMF), manifest by a disproportionate anemia and thrombocytopenia. TKO mice display highly increased numbers of necrotic bone marrow cells, as assessed by Transmission Electron Microscopy (TEM). In addition, they display a dramatic depletion of progenitor cells accompanied by expansion of hematopoietic stem cell populations. Increased necroptotic death of bone marrow thus has a disproportionate effect on an early hematopoietic progenitor cell that give rise to erythrocytes and megakaryocytes. We have thus created genetic mouse models in which hematopoietic cells are protected from PCD (*BaxBak* DKO), and show that by removing Bid, a third pro-apoptotic Bcl-2 family member (*BaxBakBid* TKO), we switch cell death fate to necroptosis from apoptosis (wild type) under homeostatic conditions in mouse bone marrow.

To further examine the function of hematopoietic stem and progenitor cells in our mouse models, we performed competitive reconstitution studies in which TKO or DKO whole bone marrow is transplanted in a 1:1 ratio with wild type bone marrow into lethally irradiated wild type (*Bid +/+)* recipient mice. Mice transplanted with TKO as well as mice transplanted with DKO bone marrow initially display increased competitive reconstitution compared to the wild type control, suggesting that early reconstitution requires restraint of BaxBak dependent apoptosis. Over time however, mice transplanted with TKO bone marrow develop progressive anemia and thrombocytopenia leading to early mortality despite the continued presence of wild type bone marrow. This suggests that the presence of necroptotic hematopoietic cells can impair hematopoietic function of normal cells.

Evaluation of hematopoietic stem and progenitor compartments in the transplant setting reveals significant differences between the impact of DKO versus. TKO bone marrow on homeostasis in this compartment. Whereas DKO stem and progenitor cell numbers are decreased relative to wild type, TKO cell numbers are preserved or even increased. This increased number

of TKO stem and progenitor cells is due to a disproportionate increase in LSK and SLAM-HSC cell numbers relative to wild type. In a secondary reconstitution experiment, TKO but not DKO bone marrow fails to reconstitute in a secondary transplant, demonstrating that increased necroptosis impacts hematopoietic stem cell repopulating ability.

Necroptotic cell death elicits an inflammatory response that can impact hematopoietic differentiation and stem cell function. We note increased TNFα production in tissues of mice transplanted with TKO bone marrow, and increased TNFα production in response to LPS stimulation in TKO bone marrow. Importantly, treatment of TKO mice with the decoy TNFR, Enbrel, can partially rescue progenitor cells as well as TKO anemia and thrombocytopenia, further demonstrating that the preferential progenitor cell death and resultant cytopenias observed are driven at least in part by increased inflammatory cytokine production. Notably, we also demonstrate increased necroptotic signaling in the human bone marrow failure disorder, Myelodysplastic Syndrome (MDS), demonstrating the impact of necroptosis signaling on normal bone marrow function is relevant to human disease.

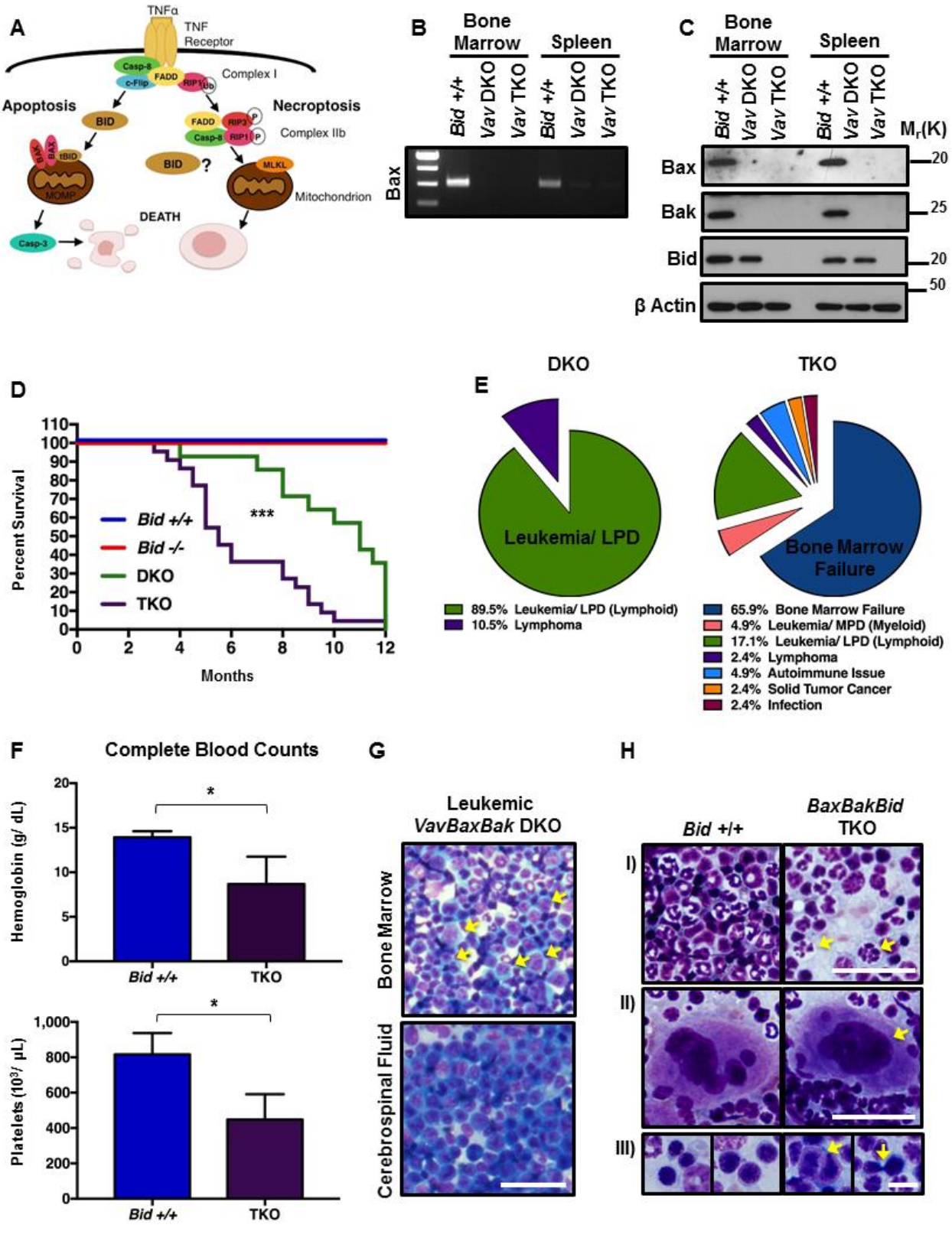
Results

VavBaxBakBid TKO mice die of bone marrow failure (BMF)

As described above, our mouse models enable us to study apoptosis inhibition at the central decision node upstream of the mitochondria yet still downstream of the receptors and immediate DISC complexes (*47, 51*) (Figure 2.1A). VavCre efficiently deletes Bax in the bone marrow and spleen of DKO and TKO mice (Figure 2.2A). Consequently, there is no detectable mRNA by RT-PCR (Figure 2.1B) or protein by Western blot (Figure 2.1C). Phenotypes present

in *VavCre TKO* mice distinct from *VavCre DKO* mice indicate *BaxBak*- independent functions of *Bid,* allowing us to interrogate Bid-dependent upstream signaling events driving commitment to apoptosis versus necroptosis in hematopoiesis.

As shown previously, *BaxBak* DKO mice die predominantly (89%) of lymphoid leukemia or myeloproliferative disease (MPD) that can be transferred to recipient mice, consistent with loss of mitochondrial –mediated death (Figure 2.1D, E, and G) (*120*, *559*). In contrast, mice harboring loss of *Bid* in addition to *Bax/Bak* (TKO mice) display decreased survival relative to *Bid*+/+ (WT), *Bid-/-* (*Bid* KO), and *BaxBak* DKO mice (Figure 2.1D), with death predominantly due to overwhelming cell death in the bone marrow that results in bone marrow failure (BMF), in 66% of TKO mice. TKO mice manifested classical signs of BMF, including decreased hemoglobin concentrations (red blood cell (RBC) numbers) and platelet numbers (Figure 2.1F). Furthermore, TKO bone marrow demonstrates extensive myeloid dysplasia (abnormal differentiation and development) (Figure 2.1H): I) neutrophils (hyper-segmentation), II) megakaryocytes (hypo-lobulation), and III) erythroid precursors (binucleation and intrachromosomal bridging) (*560*). Transformation to leukemia/MPD only occurs in 22% of TKO mice. This marked difference in hematopoietic phenotype with additional Bid deletion establishes that Bid can regulate hematopoietic homeostasis independent of its Bax/Bak activator role.


A
TNFα
TNF Receptor
Complex I
Casp-8
c-Flip
FADD
RIP1
Ub
Apoptosis
BID
tBID
BAK
BAX
MOMP
Necroptosis
FADD
RIP3
Casp-8
RIP1
P
Complex IIb
BID
?
MLKL
Mitochondrion
DEATH
Casp-3
B
Bone Marrow
Spleen
Bid +/+
Vav DKO
Vav TKO
Bid +/+
Vav DKO
Vav TKO
Bax
C
Bone Marrow
Spleen
Bid +/+
Vav DKO
Vav TKO
Bid +/+
Vav DKO
Vav TKO
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Bax
Bak
Bid
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25
20
50
D
Percent Survival
Months
Bid +/+
Bid -/-
DKO
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65.9% Bone Marrow Failure
4.9% Leukemia/ MPD (Myeloid)
17.1% Leukemia/ LPD (Lymphoid)
2.4% Lymphoma
4.9% Autoimmune Issue
2.4% Solid Tumor Cancer
2.4% Infection
F
Complete Blood Counts
Hemoglobin (g/ dL)
Bid +/+
TKO
*
Platelets (10³/ µL)
Bid +/+
TKO
*
G
Leukemic VavBaxBak DKO
Bone Marrow
Cerebrospinal Fluid
H
Bid +/+
BaxBakBid TKO
I)
II)
III)

Figure 2.1 *VavBaxBakBid* TKO mice die of Bone Marrow Failure (BMF)
A) Schematic and rationale for development of the *BaxBakBid* KO (TKO) mouse. (B) Examination of the deletion of Bax in bone marrow and spleen as determined by RT-PCR. (C) Immunoblot examining expression of Bax, Bak, and Bid, in *Bid* +/+, *VavBaxBak* DKO, and *VavBaxBakBid* TKO mice. (D) Survival curves of *Bid* +/+, *Bid* -/-, DKO, and TKO mice. Statistics demonstrate differences between DKO and TKO animals. ***= P< 0.001 *Bid*+/+ n=4 *Bid*-/- n=4 DKO n=14 TKO n=22. (E) Cause of death in DKO and TKO mice was determined based on findings upon necropsy in DKO versus TKO mice. (F) Hemoglobin (a measure of red blood cell (RBC) numbers) and platelet counts from complete blood counts. Data are representative of mean +/- SEM from three and four *Bid*+/+ and TKO mice, respectively. *= P< 0.05 (G) Examination of bone marrow and brain fluid from *Bid* +/+ mice transplanted with leukemic *VavBaxBak* DKO bone marrow after sub-lethal irradiation. Scale bar denotes 50 microns. (H) Cytospins from the bone marrow of *Bid* +/+ and TKO mice denoting I) neutrophils, II) megakaryocytes, and III) erythroid precursors. Scale bars in I) and II) denote 50 microns and in III) denotes 10 microns.

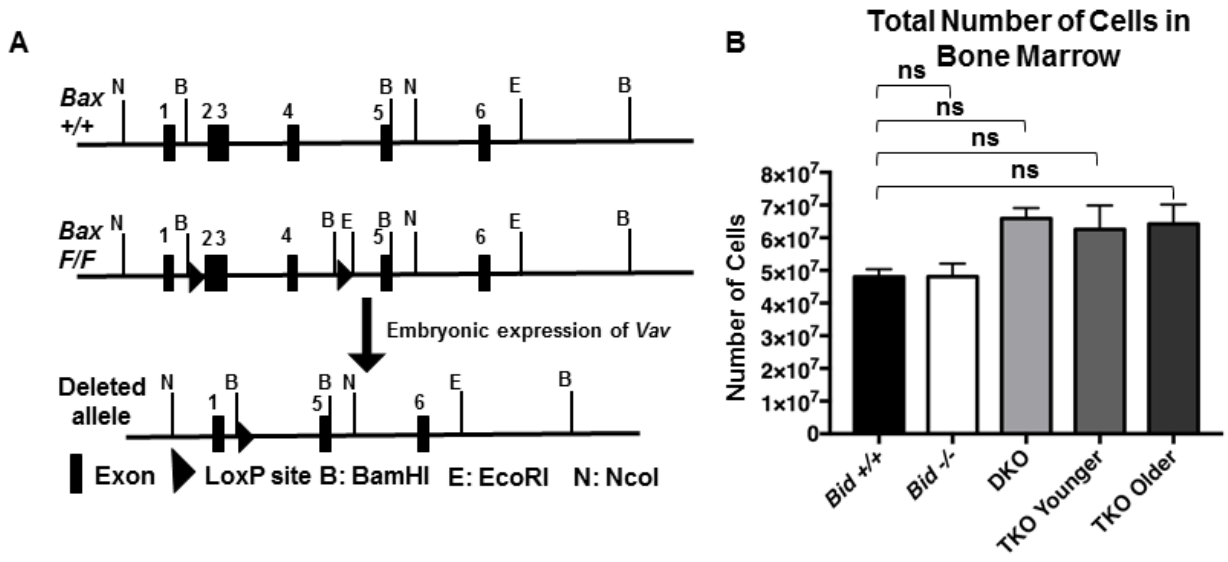


Figure 2.2 *VavBaxBakBid* TKO Bax allele organization and total bone marrow count
A) Schematic of the Bax allele in *VavBaxBak* and *VavBaxBakBid* mice. B) Total cell number within the bone marrow (before sickness) in *Bid* +/+, *Bid* -/-, DKO, TKO Younger, and TKO Older mice. *Bid* +/+ n=5, *Bid* -/- n=5, DKO n=6, TKO Younger n=5, TKO Older n=9

TKO bone marrow dies by necroptosis

The presence of markedly decreased cells in TKO blood despite preserved bone marrow cellularity (Figure 2.2B) strongly suggests that there is increased cell death in the bone marrow. To determine how the additional loss of Bid impacts bone marrow cell death in TKO mice, we performed transmission electron microscopy (TEM) to examine cellular morphology, a defining feature of both apoptosis and necroptosis. Image comparison of *Bid*+/+ and TKO cells reveal morphologies characteristic of apoptosis (e.g. pyknotic nuclei, cell membrane and organelle shrinkage) and necroptosis (e.g. membrane integrity loss, cell membrane and organelle swelling), respectively (Figure 2.3A). Image quantification reveals necroptotic morphology in nearly 25% of cells in TKO bone marrow versus 7% in *Bid*+/+ and 10% in DKO bone marrow (Figure 2.3B). Necroptotic PCD signaling is executed by Rip1 and Rip3 (*561*). Accordingly, Rip1 levels are increased by immunofluorescence in TKO but not *Bid*+/+, *Bid*-/-, or DKO bone marrow (Figure 2.3C). No significant cleaved Caspase-3 was observed in DKO or TKO bone marrow (Figure 2.3D and E), inconsistent with apoptosis as the primary cell death mechanism in this setting. Immunofluorescence data further supports necroptotic PCD in TKO bone marrow, establishing that while loss of *BaxBak* prevents MOMP and apoptosis in the bone marrow, the additional loss of *Bid* induces necroptotic cell death, suggesting that Bid may function as a brake on necroptotic death in the bone marrow.

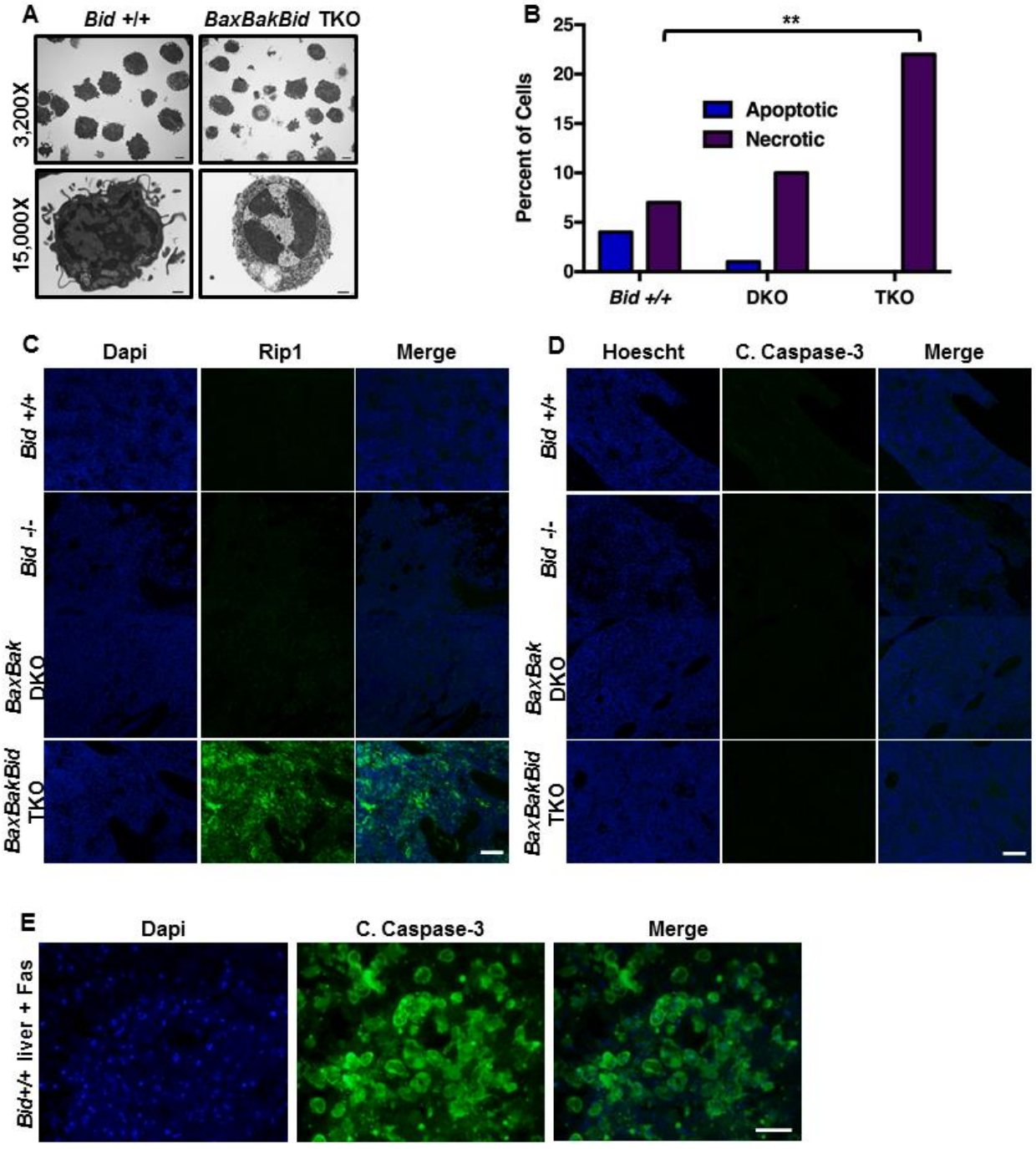

A
Bid +/+
BaxBakBid TKO
3,200X
15,000X
B
**
Percent of Cells
25
20
15
10
5
0
Apoptotic
Necrotic
Bid +/+
DKO
TKO
C
Dapi
Rip1
Merge
Bid +/+
Bid -/-
BaxBak DKO
BaxBakBid TKO
D
Hoescht
C. Caspase-3
Merge
Bid +/+
Bid -/-
BaxBak DKO
BaxBakBid TKO
E
Dapi
C. Caspase-3
Merge
Bid+/+ liver + Fas

Figure 2.3 *VavBaxBakBid* TKO bone marrow dies by necrosis

A) *Bid* +/+, DKO, and TKO bone marrow was prepared for transmission electron microscopy. Representative transmission electron microscopy images from *Bid* +/+ and TKO mice. (Upper: Scale bar indicates 2 microns, Lower: Scale Bar indicates 500 nanometers). **= P< 0.01 B) 100 cells with a nucleus from lower magnification images (Upper) were scored as being either apoptotic, necroptotic, or live based on cell and organelle morphology. Quantification of transmission electron microscopy of *Bid* +/+, DKO, and TKO bone marrow. C) Fluorescent immunohistochemistry was performed on paraffin-embedded bone marrow sections from *Bid* +/+, *Bid* -/-, DKO, and TKO mice were for Rip1. Experiment was performed three independent times. Scale bar indicates 50 microns. D) Fluorescent immunohistochemistry was performed as above for cleaved Caspase-3. Scale bar indicates 100 microns. E) Fluorescent immunohistochemistry on *Bid* +/+ liver after tail vein injection with Fas ligand as a positive control for cleaved Caspase-3 staining.

Unrestrained bone marrow necrosis disrupts hematopoietic homeostasis

Hematopoiesis is a highly dynamic biological process that is tightly regulated by proliferation and death. In response to stress, terminally differentiated cells are removed through PCD and normally quiescent hematopoietic stem cells (HSCs) proliferate and differentiate, expanding the progenitor cell pools, which then rapidly proliferate to restore hematopoiesis (*364*). Programmed cell death serves to reset homeostasis once the stress is resolved.

Long-term hematopoietic homeostasis therefore depends on proper regulation of programmed cell death. We asked whether the mechanism of cell death (apoptosis versus necroptosis) may also impact long-term hematopoietic homeostasis, as necroptotic cells create an inflammatory microenvironment by releasing DAMPs and cytokines such as TNFα and IL-1β into the extracellular space (*562*). Inflammatory cytokines have been shown to impair HSC function (*445*), suggesting the possibility that bone marrow necroptosis may impair HSC function and hematopoietic homeostasis.

To evaluate the effect of increased necroptosis on hematopoietic homeostasis in our TKO mice, we examined hematopoietic stem and progenitor cells (HSPCs). LSK (Lin^- $Sca1^+$c-Kit^+**)** cell populations were expanded in TKO but not *Bid*+/+, *Bid* -/-, or DKO mice (Figure 2.4A). The signaling lymphocyte activating molecule-HSC (SLAM-HSC, LSK $Flt3^{Lo}CD48^-CD150^+$) population (*377*), more highly enriched for HSCs, continues to expand in TKO but not DKO mice with age. Accordingly, TKO but not DKO SLAM-HSCs displayed increased BrdU incorporation, consistent with an appropriate response to bone marrow stress (Figure 2.4B). LT-HSC (Lin^- $Sca1^+$c-Kit^+CD135^{Lo}) populations were not significantly changed between genotypes (Figure 2.5A). The above results are consistent with an appropriate SLAM-HSC response to bone marrow stress.

Given the apparent bone marrow stress noted in TKO SLAM-HSCs, we anticipated that progenitor populations would be similarly expanded with increased BrdU incorporation. In contrast, we found that both TKO and DKO myeloid progenitor (Lin^- $Sca1^-$c-Kit^+**)** populations are decreased (Figure 2.4C) and display significantly decreased BrdU incorporation as compared to *Bid*+/+ mice (Figure 2.4D). The expanded SLAM-HSCs with decreased progenitors in TKO mice are consistent with increased sensitivity of the progenitor population to cell death with compensatory HSC proliferation. Importantly, DKO mice do not display increased SLAM-HSC proliferation despite decreased progenitor cell proliferation (Figure 2.4B and D), suggesting a distinct defect in hematopoietic homeostasis in DKO versus TKO mice.

Consistent with the increased programmed cell death noted in myeloid cells, TKO but not *Bid*+/+, *Bid*-/-, or DKO mice display splenomegaly with increased $Ter119^+$ cells (erythroid), indicative of extramedullary hematopoiesis that is progressive with age (Figure 2.5B and C). Notably B cell and monocyte populations are not significantly different between genotypes at necropsy, but TKO T cell populations are expanded (Figure 2.5D). Examination of erythroid progenitor populations including the Pro-erythrocyte and erythroblast populations before phenotype manifestation and at death reveal significant decreases in the basophilic erythroblast populations in both DKO and TKO mice (Appendix A).

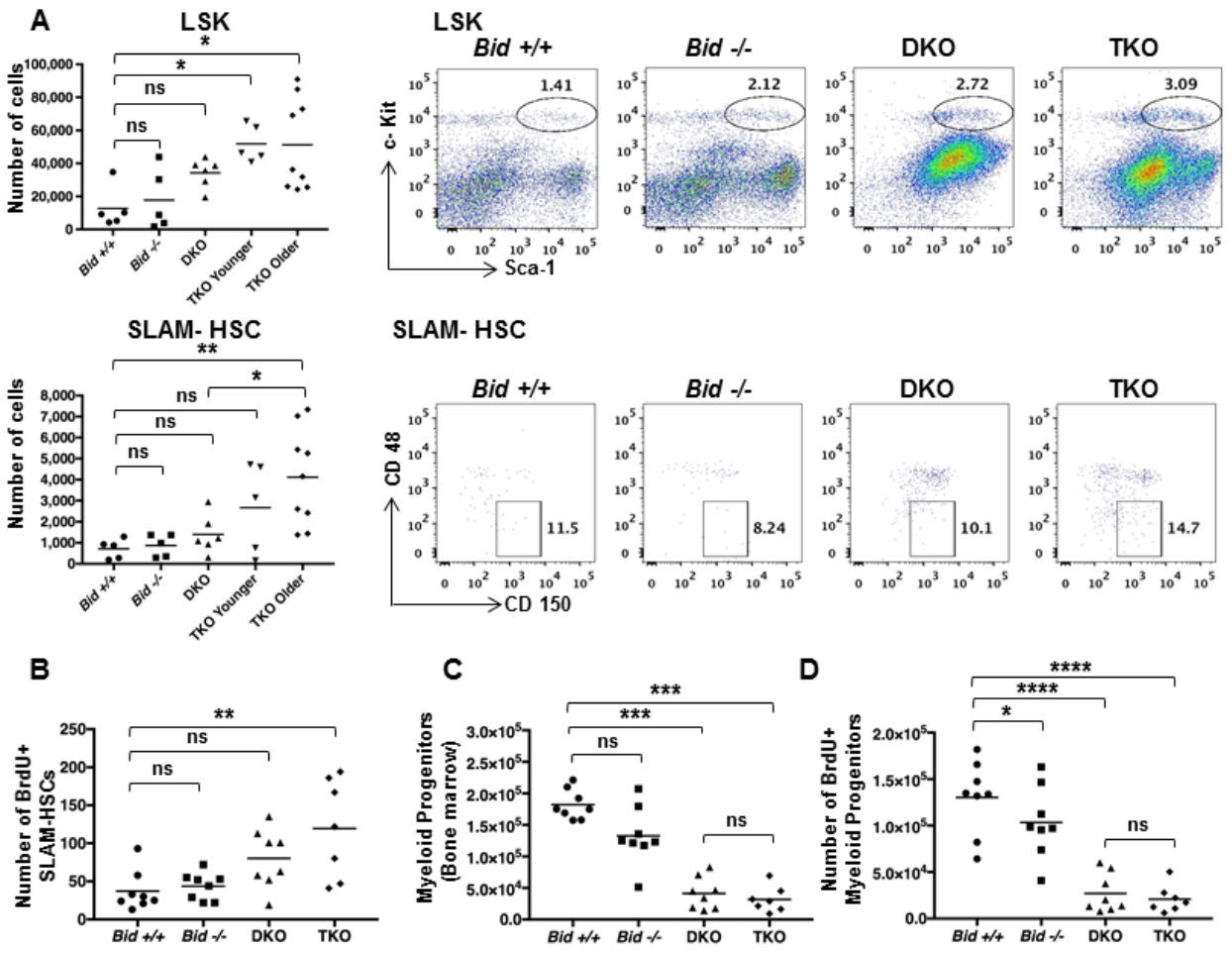


Figure 2.4 *VavBaxBak* DKO and *VavBaxBakBid* TKO bone marrow displays altered hematopoietic homeostasis

A) Immunophenotyping analysis by flow cytometry of bone marrow to examine LSK (Lineage$^-$, Sca-1$^+$, c-Kit$^+$) and SLAM-HSC (Signaling lymphocyte activating molecule- hematopoietic stem cell) populations. Mice were examined before onset of sickness. Younger mice were 11-15 weeks old and older mice were 15-20 weeks of age. *Bid* +/+ n=5, *Bid* -/- n=5, DKO n=6, TKO Younger n=5, TKO Older n=9 B) Examination of the number of BrdU+ SLAM-HSCs in bone marrow as determined from BrdU assay in mice were injected with a total of 4mg of BrdU in three doses over the course of 36 hours. Bone marrow was collected, depleted for terminal lineages, and then stained for flow cytometry. All remaining cells were analyzed by flow cytometry. Mice were 18-20 weeks of age. C) Numbers of myeloid progenitors (Lineage$^-$, Sca-1$^-$, c-Kit$^+$) as in (B). Mice were 18-20 weeks of age. D) Examination of the number of BrdU+ myeloid progenitors. BrdU positivity in the myeloid progenitor population was examined in mice treated as above. For (A) *Bid*+/+ n=5, *Bid* -/- n=5, DKO n=6, TKO Younger n=5, TKO Older= 9. For (B), (C), and (D) *Bid*+/+ n=8, *Bid* -/- n=7, DKO n=8, TKO n=7.
Ns= not significant (p>0.05) *= P<0.05 **=P<0.01 ***=P<0.001 ****=P<0.0001

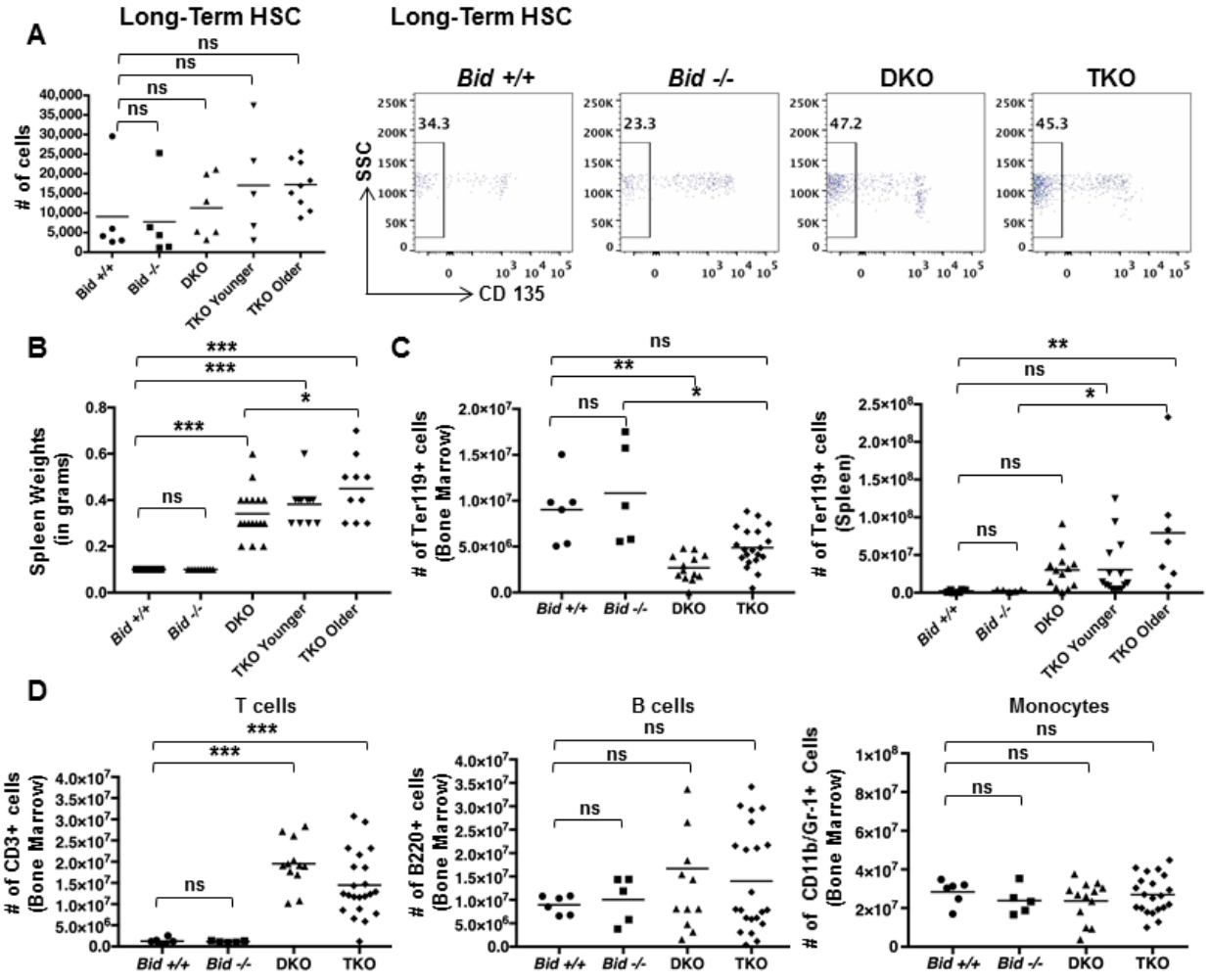


Figure 2.5 Unrestrained bone marrow necrosis does not significantly impact LT-HSC, B cell, or monocytic populations

A) Examination of the LT-HSC population in *Bid* +/+, *Bid* -/-, DKO and TKO mice before sickness (11-20 weeks old). Younger TKO mice are aged 11-15 weeks, and Older TKO mice are 15-20 weeks old. *Bid*+/+ n=5, *Bid*-/- n=5 DKO n= 6 TKO Younger n=5, TKO Older n=10 B) Spleen weights of *Bid* +/+, *Bid* -/-, DKO and TKO mice before sickness as in (A) *Bid*+/+ n=11, *Bid*-/- n=9 DKO n= 15 TKO Younger n=10, TKO Older n=10. C) Examination of Ter119+ cells in the bone marrow and spleen of mice at death (At time of phenotype manifestation in DKO and TKO mice) In examination of spleen, TKO younger mice are 12 months old or younger, and TKO older mice are 12 months or older in age. *Bid*+/+ n=6, *Bid*-/- n=5 DKO n= 13 TKO n=22 D) Examination of T, B, and myeloid cells in the bone marrow of mice at death (At time of phenotype manifestation in DKO and TKO mice). Populations examined by flow cytometry for CD3 (T cells), B220 (B cells), and CD11b and Gr-1 double positivity (Myeloid cells). *Bid*+/+ n=6, *Bid*-/- n=5 DKO n= 13 TKO n=22

TKO cells outcompete Bid+/+ cells, but fail to maintain hematopoiesis in competitive repopulation experiments

The BMF noted in TKO mice strongly indicates abnormal hematopoietic stem cell function. To stringently evaluate TKO HSPC function, we required test (TKO) bone marrow to compete with normal bone marrow to repopulate a lethally irradiated congenic mouse (competitive repopulation). Accordingly, we injected a 1:1 ratio of TKO (Ly45.2+) to wild type (*Bid +/+)* (Ly45.1+) bone marrow into lethally irradiated wild type (*Bid +/+)* (Ly5.1+) mice, and evaluated peripheral blood for Ly45.2+ and Ly45.1+ mononuclear cells. Two additional cohorts of mice were examined in which a 1:1 ratio of wild type (Ly5.2+) to wild type (Ly5.1+) or a 1:1 ratio of DKO (Ly5.2+) to wild type (Ly5.1+) marrow was transplanted into lethally irradiated wild type (Ly5.1+) mice. Surprisingly, both DKO and TKO bone marrow displayed increased repopulating ability relative to *Bid+/+* marrow (Figure 2.6A). However, peripheral blood counts reflected the presence of bone marrow stress that was more severe in TKO mice, with decreasing RBC counts (anemia) and platelets (thrombocytopenia) and increasing platelet size (Mean platelet volume) (Figure 2.6B, C, and D).

Hematopoietic stem and progenitor compartments reflect a distinct phenotype between DKO and TKO HSPCs

To further explore how altered cell death mechanism impacts non-cell autonomous interactions in the HSPC and progenitor compartment, we evaluated progenitor, LSK, and SLAM HSC populations post 20 weeks in primary competitive repopulation experiments. Similar to untransplanted mice, DKO and TKO-transplanted progenitor cell numbers were similar, and markedly decreased relative to *Bid +/+* (wild type) transplanted mice. Strikingly,

whereas DKO and wild type LSK cell numbers are similar, TKO bone marrow displays a substantial increase in LSK (~7x) and SLAM HSC (~2x) numbers. This increase in TKO LSK and SLAM HSCs results in an expansion in the HSC population overall, despite a relative decrease in progenitor cells- consistent with stress hematopoiesis. In contrast, DKO HSPCs are ~2 fold decreased relative to wild type HSPCs, consistent with bone marrow crowding due to increased mature cells that did not die (Figure 2.6E). Beginning at 20 weeks, mice transplanted with both DKO and TKO bone marrow but not *Bid*+/+ bone marrow alone, began to die (Figure 2.6F). In addition, TKO but not DKO-transplanted mice displayed evidence of BMF (Appendix B, A and B), increased bone marrow debris. Examination of blood and bone marrow from mice transplanted with TKO bone marrow reveals dysplasia and bone marrow cell death similar to that observed in TKO mice. At the time of death, TKO cells represented 80-90% of the bone marrow. Despite the presence of 10-20% wild type bone (*Bid +/+)* marrow, hematopoiesis was not maintained, suggesting a cell-extrinsic effect of TKO bone marrow on wild type HSPCs.

Secondary transplantation reveals defective TKO HSPC repopulating ability

To further evaluate HSPC function, and to compare DKO and TKO bone marrow reserve, we performed a secondary transplant. DKO bone marrow continues to out-compete wild type bone marrow even in secondary transplant conditions, indicating continued HSPC self-renewal capacity. In contrast, TKO bone marrow displays strikingly decreased competitive repopulating ability, consistent with decreased HSPC self-renewal capacity (exhaustion) in secondary transplant conditions (Figure 2.6G). We thus demonstrate that increased necroptosis impairs long-term HSPC function.

Mice transplanted with TKO bone marrow display increased TNFα production

As increased necroptosis promotes an innate immune response, we evaluated our transplanted mice for evidence of increased inflammation and inflammatory cytokine production. Notably, mice transplanted with TKO but not wild type (*Bid* +/+) bone marrow displayed marked inflammation in the lungs, kidney, and liver upon necropsy. Immunofluorescence revealed increased TNFα expression in lungs from mice transplanted with TKO but not wild type or DKO bone marrow (Figure 2.6H), suggesting that transplantation of TKO but not wild type cells promotes inflammatory cytokine production. Accordingly, TKO but not wild type or DKO bone marrow displayed significantly increased TNFα expression on the lower side scatter population but not the middle side scatter population, as measured by intracellular flow cytometry, following LPS stimulation (200 ng/mL) (Figure 2.6I and Appendix B, C.). The above results are consistent with inflammation induced by dying TKO cells leading to increased TNFα, which kills wild type and TKO HSPCs, producing BMF.

Treatment with TNF decoy receptor (Enbrel) restores HSPCs and improves cytopenias in TKO mice

Given the marked increase in TNFα observed in TKO transplanted mice as well as TKO bone marrow, we sought to determine whether inhibiting TNFα could improve TKO cytopenias. We treated a cohort of *Bid*+/+, DKO and TKO mice with Enbrel to inhibit TNFα. Enbrel treatment increased the number of myeloid progenitor cells and $BrdU^+$ myeloid progenitor cells such that were not significantly different from *Bid* +/+ myeloid progenitor cell and $BrdU^+$ myeloid progenitor cell numbers (Figure 2.6J). In addition to rescuing progenitor cell numbers and proliferation, Enbrel treatment also improved peripheral cytopenias in TKO mice. Both RBC

and platelet counts in Enbrel-treated TKO mice (Fig. 2.6K) increased, further demonstrating that increased TNFα elicited by necroptosis impairs hematopoiesis. Significantly, TNFα inhibition can improve bone marrow function to improve peripheral blood counts.

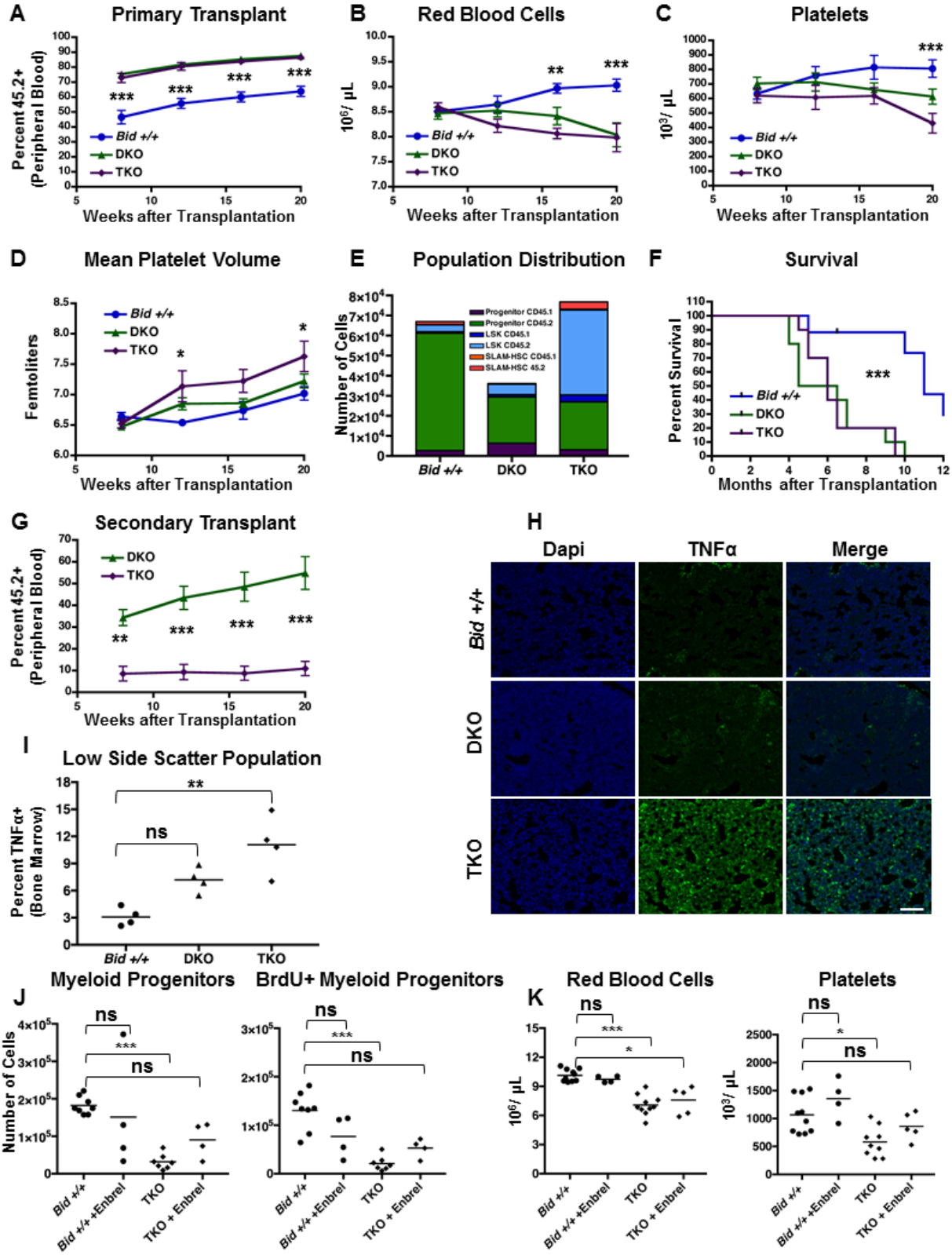

A
Primary Transplant
Percent 45.2+ (Peripheral Blood)
Weeks after Transplantation
Bid +/+
DKO
TKO
B
Red Blood Cells
10^6/ µL
C
Platelets
10^3/ µL
D
Mean Platelet Volume
Femtoliters
E
Population Distribution
Number of Cells
Progenitor CD45.1
Progenitor CD45.2
LSK CD45.1
LSK CD45.2
SLAM-HSC CD45.1
SLAM-HSC 45.2
F
Survival
Percent Survival
Months after Transplantation
G
Secondary Transplant
H
Dapi
TNFα
Merge
I
Low Side Scatter Population
Percent TNFα+ (Bone Marrow)
ns
J
Myeloid Progenitors
BrdU+ Myeloid Progenitors
Bid +/+ +Enbrel
TKO + Enbrel
K
Red Blood Cells
Platelets

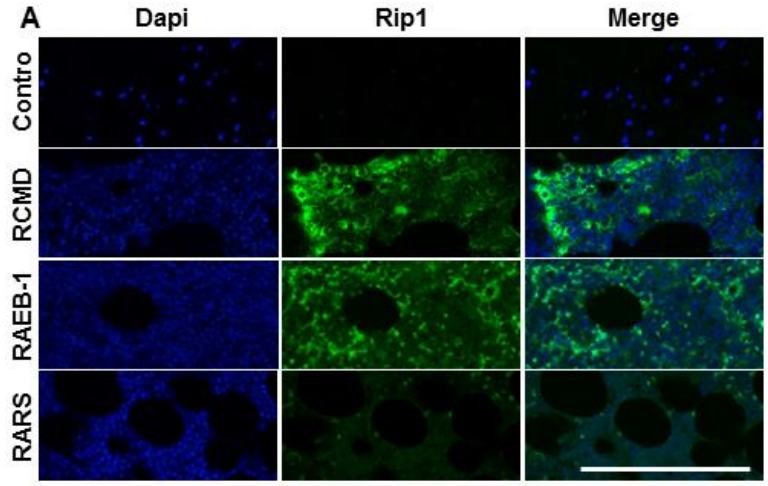
Figure 2.6 *VavBaxBakBid* TKO bone marrow outcompetes *Bid* +/+ bone marrow but fails to maintain hematopoiesis in competitive reconstitution experiments
A) Percent CD45.2+ cells in *Bid* +/+, DKO, and TKO transplant mice at 8,12,16, and 20 weeks after transplantation. Mice were transplanted with experimental and control bone marrow at a 1:1 ratio. *Bid* +/+ n=7 DKO n=7 TKO n=6. Statistics demonstrate differences between *Bid* +/+ and TKO animals. B) Examination of red blood cell counts in transplanted *Bid* +/+, DKO, and TKO mice at 8, 12, 16, and 20 weeks after transplantation. *Bid* +/+ n=5 DKO n=8 TKO n=8. Statistics demonstrate differences between *Bid* +/+ and TKO animals. C) Examination of platelet counts in transplanted *Bid* +/+, DKO, and TKO mice at 8, 12, 16, and 20 weeks after transplantation. *Bid* +/+ n=5 DKO n=8 TKO n=8. Statistics demonstrate differences between *Bid* +/+ and TKO animals. D) Examination of mean platelet volume in transplanted *Bid* +/+, DKO, and TKO mice at 8, 12, 16, and 20 weeks post transplantation. *Bid* +/+ n=5 DKO n=8 TKO n=8. Statistics demonstrate differences between *Bid* +/+ and TKO animals. E) Examination of the distribution of myeloid progenitor, LSK, and SLAM-HSC populations in *Bid* +/+, DKO, and TKO transplanted mice. *Bid* +/+ n=5 DKO n=7 TKO n=6. F) Survival of *Bid* +/+, DKO, and TKO transplanted mice. *Bid* +/+ n=10 DKO n=10 TKO n=10. Statistics demonstrate differences between *Bid* +/+ and TKO animals. G) Secondary transplantation of DKO and TKO bone marrow completed through transplantation of bone marrow from primary transplant in a 1:1 ratio with *Bid* +/+ to stringently test HSC function. DKO n=8 TKO n=7. H) Fluorescent immunohistochemistry of TNFα expression in lung of *Bid* +/+, DKO, and TKO mice. Scale bar indicates 50 microns. Experiment was completed two independent times. I) Examination of TNFα expression in the low side scatter population of bone marrow of *Bid* +/+ and TKO mice following 5 hours of *in vitro* stimulation with 200ng/mL LPS. Data are represented as mean +/- SEM. Experiment was completed four independent times. J) Examination of myeloid progenitor populations and BrdU incorporation within this population in *Bid* +/+ and TKO mice after treatment with Enbrel, a TNF inhibitor. K) Examination of red blood cell and platelet counts in *Bid* +/+ and TKO mice. *Bid* +/+ n=4 TKO n=4
ns= $P>0.05$ *= $P<0.05$ **=$P<0.01$ ***=$P<0.001$ ****=$P<0.0001$

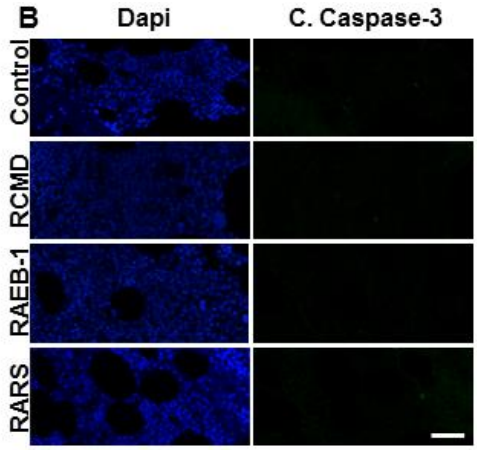
The human disease MDS demonstrates increased Rip1 and Phospho-MLKL expression, consistent with increased necroptotic signaling

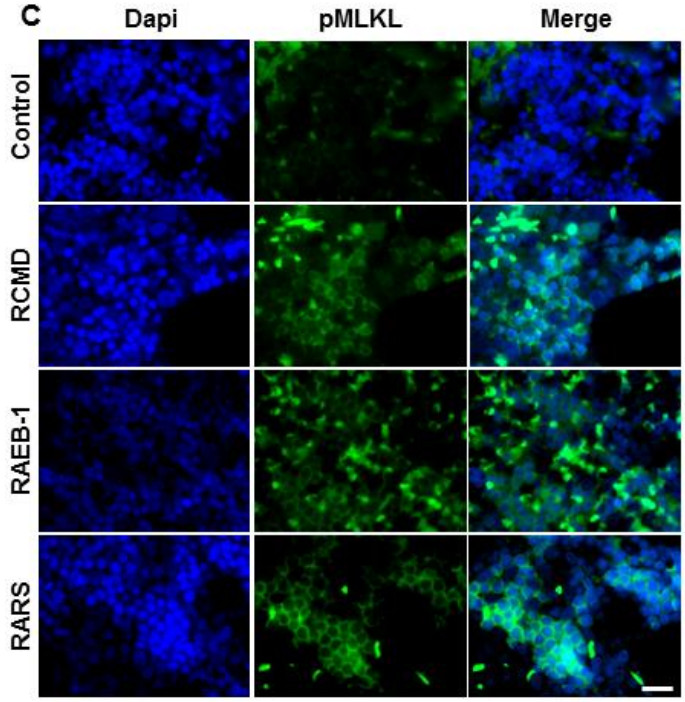
We demonstrated above that increased necroptosis in mouse bone marrow results in BMF with a cellular bone marrow, prominent dysplasia, and a small frequency of transformation to leukemia, phenocopying the human BMF disorder, Myelodysplastic Syndrome (MDS). Increased cell death in MDS bone marrow has been attributed to apoptosis. However, review of the published data in light of current knowledge reveals that early studies measured cell death using techniques that do not distinguish between apoptotic and necroptotic cells (*563*): increased *in situ* end labeling, increased TUNEL staining, or increased DNA laddering on gels (*392*, *492*, *493*, *564*). Increased Caspase-3 activity was seen in cultured MDS bone marrow (*496*), but in only 10% of MDS samples when measured directly *ex vivo* (*565*). Thus evidence to date does not distinguish between apoptotic and necroptotic cell death in MDS bone marrow *in vivo*.

As the salient features of MDS are recapitulated in our Vav TKO mice, we investigated necroptosis and apoptosis in MDS patient bone marrow samples. Rip1 staining revealed increased expression in all samples of RCMD, and 50% of RAEB-1 and -2 subtypes of MDS in our 22-patient cohort (Figure 2.8A) consistent with increased necroptosis signaling in MDS bone marrow. Conversely, staining for cleaved Caspase-3 reveals modest staining in only a few samples, including controls (Figure 2.8B), inconsistent with significant apoptosis in our cohort of patients. We further stained samples from our MDS patient cohort with anti-phospho MLKL. We find increased phospho-MLKL staining in several subtypes of MDS patient samples, corresponding to those in which we find increased RIP1 kinase staining (Figure 2.8C and D). Furthermore, we observed an inverse correlation between Rip1 and Bid expression in MDS but not control patient samples: we observed a decrease in Bid expression, but an increase in Rip1

expression in MDS (Figure 2.8E). This correlation corresponds to the pattern of expression seen in myeloid progenitor cells (MPCs) in mechanistic studies (Chapter III). While this study does not rule out a role for apoptosis in a subset of MDS patients, our study clearly implicates necroptosis signaling in MDS.







D Percent of samples positive for markers versus total number of samples

Marker	Control	RCMD	RAEB-1/2	RARS
Rip1	1/4	6/6	3/7	1/1
pMLKL	1/4	5/5	5/7	1/1

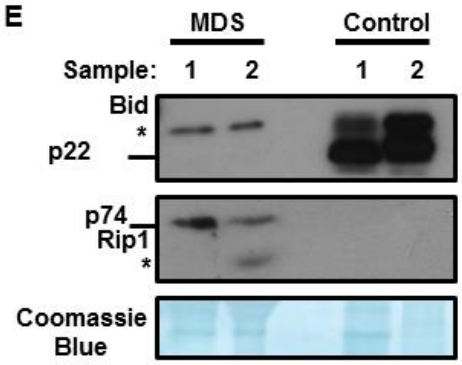


Figure 2.7 The human disease MDS demonstrates increased Rip1 and phosphor-MLKL expression, consistent with increased necroptotic signaling
(A) Rip1 staining on paraffin-embedded human bone marrow aspirate with Dapi as a nuclear stain. Scale bar indicates 100 microns. Experiment was performed three independent times. (B) Caspase-3 staining on paraffin-embedded human bone marrow aspirate with Dapi as a nuclear stain. Experiment was performed three independent times. Scale bar indicates 50 microns. (C) Phospho-MLKL staining on paraffin-embedded human bone marrow aspirate with Dapi as a nuclear stain. Experiment was performed two independent times. Scale bar indicates 50 microns. (D) Table demonstrating positivity of human samples for Rip1 and Phosph-MLKL by subtype. E) Lysate from control and MDS patient bone marrow examining Rip1 and Bid levels by immunoblot. Demonstrates a similar relationship between these proteins, with increased *Rip1* expression and decreased *Bid* expression in MDS samples as seen in TKO myeloid progenitor cell lines. Experiments in A-E were performed by Qiong Shi.

Discussion

Using our mouse models in which we altered programmed cell death fate in hematopoiesis, we demonstrate that increased necroptotic cell death in the bone marrow, as demonstrated in our TKO mice, leads to bone marrow failure with prominent dysplasia, and a hyper/normocellular bone marrow. We further show that this bone marrow failure is driven by loss of the progenitor cell populations: SLAM-HSCs show expansion over time, consistent with a stress response. Competitive repopulation experiments demonstrate that necroptotic cells can cause bone marrow failure, even in the presence of normal hematopoietic cells, suggesting that necroptotic cell death results in a cell extrinsic impairment of both normal and mutant hematopoietic stem cells. We postulate that this cell extrinsic effect is mediated by the release of DAMPS, which promotes the release of TNFα, and show increased TNFα in organs of TKO transplanted mice as well as increased TNFα production by TKO bone marrow. Hematopoiesis is a dynamic system that responds to environmental cues transmitted through cytokines such as TNFα, to promote proliferation and PCD function to remove damaged cells and to reset homeostasis. Our data demonstrate that, in addition to the degree of cell death, the mechanism by which cells die can have a dramatic impact on bone marrow homeostasis, and that skewing death to necroptosis results in BMF.

Studies focused on BaxBak DKO MEFs and cardiac myocytes have implicated Bax and Bak in necroptosis execution as *BaxBak* DKO cells are protected from necroptotic stimuli (*566*). Our studies in hematopoietic cells are in agreement with the finding that *BaxBak* DKO cells do not undergo necroptosis. However, we clearly demonstrate that necroptosis can be executed in the absence of Bax and Bak both *in vitro* as well as *in vivo* in hematopoietic cells following the

additional loss of Bid. Further studies will be required to determine the molecular mechanism of necroptosis execution in hematopoietic cells versus fibroblasts and cardiac myocytes, and to clarify the requirement for Bax and Bak.

The bone marrow failure phenotype of our TKO mice phenocopies the human BMF disorder, MDS, and we demonstrate increased necroptosis signaling in primary MDS bone marrow. Our results thus shed light on how increased programmed necrotic cell death can amplify bone marrow cell death and lead to BMF. The ability to restore hematopoietic progenitor function by inhibiting the inflammatory cytokine TNFα raises the possibility that inhibiting inflammatory cytokines (TNFα or other inflammatory cytokines) may provide therapeutic benefit in BMF disorders such as MDS. Clinical trials targeting TNF in MDS suggest that TNF signaling can drive cytopenias in this disease (*567*). However the disappointing results of the randomized phase 2 trial suggest that targeting TNFα at the receptor level is not sufficient (*568*). Additional studies will be required to determine whether combining necroptosis and inflammatory cytokine inhibition can provide additional benefit.

Two recent studies demonstrate BMF due to HSC dysfunction in mice harboring a kinase-inactive form of Rip1 (*248*), and impaired engraftment of fetal liver hematopoietic cells from *Rip1 -/-* mice (*569*) demonstrating that signaling through Rip1 kinase is required for proper HSC function. However, the loss of stem cell function in these studies precluded further evaluation of the impact of Rip1 kinase on hematopoiesis as no blood cell development beyond HSCs ensues. In contrast, our study designed to interrogate increased necroptosis signaling, demonstrates an expanded HSC population with increased repopulating ability, further highlighting the importance of Rip1 kinase signaling in HSC function. As our system allows differentiation beyond the HSC, we show that in contrast to HSCs that appear to require RIP1

activity for proper function, myeloid progenitors are sensitive to increased Rip1 signaling and undergo necroptosis that results in bone marrow failure through both cell intrinsic as well as cell extrinsic mechanisms. Our study has important implications for settings in which an insult such as infection or chemotherapy is delivered to the entire bone marrow and induces necroptotic cell death. The ability to intervene to inhibit necroptosis in these settings may provide a mechanism to ameliorate myelosuppression.

In summary, we have developed a novel set of mouse models tuned to undergo apoptosis (wild type) or necroptosis (TKO), to explore the impact of necroptotic PCD on hematopoiesis. Although the impact of necroptosis signaling on early embryonic development has been carefully dissected using genetic mouse models, the role of necroptosis in dynamic systems such as hematopoiesis under homeostatic conditions has not been determined. Our mouse models provide insights into how increased necroptosis impacts hematopoietic homeostasis and HSC function leading to BMF. We further demonstrate how aberrant bone marrow necroptosis contributes to bone marrow failure disorders such as MDS. Substantial data have established the presence of increased cell death and increased inflammation in MDS bone marrow. We now demonstrate increased necroptosis in MDS bone marrow, and elucidate how aberrantly increased bone marrow necroptosis may contribute to the pathogenesis of bone marrow failure disorders such as MDS.

CHAPTER III

BID MODULATES CASPASE-8 ACTIVITY TOWARDS RIP1

Introduction

The two main forms of PCD, apoptosis and necroptosis, result in markedly different cellular outcomes as a result of similar methods of upstream stimulation. The upstream molecular signaling machinery through death receptors such as TNF (Tumor necrosis factor receptor), FAS, and DR4/5 (Death receptor 4/5) is shared among apoptosis and necroptosis, but diverges prior to activation of effector Caspases or Rip kinases culminating in apoptotic or necroptotic death, respectively. Seminal studies of genetic mouse models further demonstrate that the upstream activators of apoptosis, FADD and Caspase-8, act as key inhibitors of necroptotic cell death during embryonic development. The embryonic lethality of *Caspase 8-/-*, and *FADD -/-* mice can be rescued by additional loss of the necroptosis effectors Rip1/Rip3. (*230*, *569–574*). These data strongly suggest that the molecular interactions that commits a cell to necroptosis through Rip1 kinase activity or to apoptosis through Caspase-8 activity lie downstream of receptor-ligand interactions, yet must take place before the preferential activation of either effector Caspases or Rip kinases.

The Bcl-2 family of proteins are key regulators of apoptosis, required for execution of through the intrinsic mitochondrial apoptotic pathway. Despite their central role in cell death signaling, the mechanistic link between the Bcl-2 family and necroptotic cell death has not been

described. The BH3-only family member Bid, acts as a sensor and amplifier of apoptotic signaling through death receptors, serving to activate mitochondrial outer membrane permeabilization (MOMP) and initiate intrinsic apoptosis following interaction with and cleavage by Caspase-8 (*113*, *114*). Furthermore, Bid has been shown to play a cell death or a survival role depending on the context, suggesting the possibility that it can act as a brake on cell death (*8, 9, 149, 558*). Bid thus is situated at the nexus between apoptosis and necroptosis commitment, well positioned to serve as a mediator between these two pathways.

Our previous studies (See Chapter II) with mice deficient for the upstream regulators of apoptosis activation (*VavBaxBak* DKO) as well as mice deficient for these proteins plus *Bid* (*VavBaxBakBid* TKO) suggests that loss of Bid in the setting of loss of apoptosis execution promotes increased necroptotic signaling. In this work we show that the Bcl-2 protein Bid, traditionally associated with apoptosis execution, plays a previously unrecognized regulatory role early in cellular commitment to necroptosis. We found that Bax/Bak deletion completely blocks apoptotic cell death upon treatment of hematopoietic progenitor cells with TNFα but this knockout is not sufficient to shift the balance toward necroptotic cell death. Further deletion of Bid, however, leads to robust activation of necroptosis. The presence of Bid correlates with Rip1 degradation and apoptosis execution through a non-canonical role to modulate Caspase-8 activity. This occurs through an IETD-inhibitable activity mediated by Caspase-8 in myeloid progenitor cells. We thus show that Bid, a Bcl-2 protein typically linked to MOMP and apoptosis plays a central role in apoptotic versus necroptotic cell death outcome by pushing the “brake” on necroptosis.

As necroptosis causes inflammation that alters hematopoietic differentiation, and stem cell function, and causes additional tissue damage that can drive bone marrow failure,

understanding the key signaling components that determine whether a cell will die by apoptosis or necroptosis provides a first step towards therapeutic intervention in diseases such as Myelodysplastic Syndrome (MDS), driven by dysregulated cell death.

Results

TKO myeloid progenitors die by necroptosis

In our previous studies with mice deficient for *Bax*, *Bak*, and *Bid* we found that the myeloid progenitor populations were particularly affected as a result of unrestrained necrosis of the bone marrow. Our studies suggest that this population is particularly susceptible to TNFR signaling, and likely as a result undergoes increased necroptosis as a result. To interrogate cell death signaling in these hematopoietic progenitor cells, we generated *Hox11* immortalized myeloid progenitor cells (MPCs), from the bone marrow of *Bid* +/+, *Bid* -/-, *MxBaxBak* DKO, and *MxBaxBakBid* TKO mice, as previously discussed (*8*). We then treated these MPCs with TNFα plus Actinomycin D (TNFα/ActD) to activate TNFR signaling and block survival signaling, and utilized Annexin V/ PI staining by flow cytometry to examine viability. As expected for Type II cells, *Bid-/-* and DKO cells exhibited less death in response to TNFα/ActD (*133*). Notably, DKO MPCs do not undergo necroptosis following TNFα/ActD, suggesting that inhibiting apoptosis is insufficient to elicit necroptotic cell death. TKO and *Bid*+/+ cells display similar death kinetics (Figure 3.1A, I), and *Bid*+/+ but not TKO MPCs displayed increased cleaved Caspase-3 (Figure 3.1A, II). Additionally, TKO MPCs that are positive for Annexin V following TNFα/ActD exhibit increased size (Figure 3.2A), consistent with necroptotic cell

death. TEM of MPCs treated with TNFα/ActD reveals predominantly apoptotic morphology in *Bid*+/+ cells, whereas TKO cells display overwhelmingly necroptotic cell morphology (Figure 3.1B). Untreated MPCs show minimal cell death (Figure 3.2B). Our MPCs therefore behave in a manner consistent with death receptor signaling: *Bid*+/+ (wild type) MPCs undergo apoptosis in response to TNFα/ActD; removal of *Bax* and *Bak* prevents cell death; and removal of *Bid* in addition to *Bax* and *Bak* results in necroptotic cell death in response to TNFα/ActD, consistent with a role for Bid's inhibition of necrosis.

TKO MPCs display increased necroptotic signaling

The above studies suggest that TKO bone marrow and MPCs do not die by apoptosis based on absence of activated Caspase-3. This instead suggests the possibility that TKO MPCs may die by necroptosis. To establish if TKO MPCs also display increased necroptosis signaling, we examined kinetics of Rip1 phosphorylation after LPS and TNFα stimulation. Phosphorylation of Rip1 has been shown to stabilize its association with a pro-necroptotic complex, and activate necroptotic kinase activity (*24*). TKO but not *Bid*+/+, *Bid*-/-, or DKO MPCs displayed constitutive and increased kinetics of Rip1 phosphorylation in response to LPS or TNFα, manifested by a phosphatase-sensitive shifted band (*575*) (Figure 3.1C and Figure 3.2C and D). Interestingly, Bid levels were inversely correlated with Rip1 phosphorylation: DKO MPCs have more *Bid* than *Bid*+/+ cells (Figure 3.2E), and no detectable phospho-Rip1. TKO MPCs (lacking Bid) display constitutive Rip1 phosphorylation. We further examined necroptotic execution by probing MLKL trimerization (*203*). TKO MPCs display increased MLKL trimerization with or without LPS stimulation, while *Bid*+/+ and DKO MPCs display minimal trimerization further supporting constitutive necroptosis signal execution in TKO MPCs (Figure 3.1D). These results

suggest that loss of *Bid* in addition to *Bax* and *Bak* is sufficient to stimulate necroptotic signal execution, consistent with release of a Bid-directed brake on necroptosis.

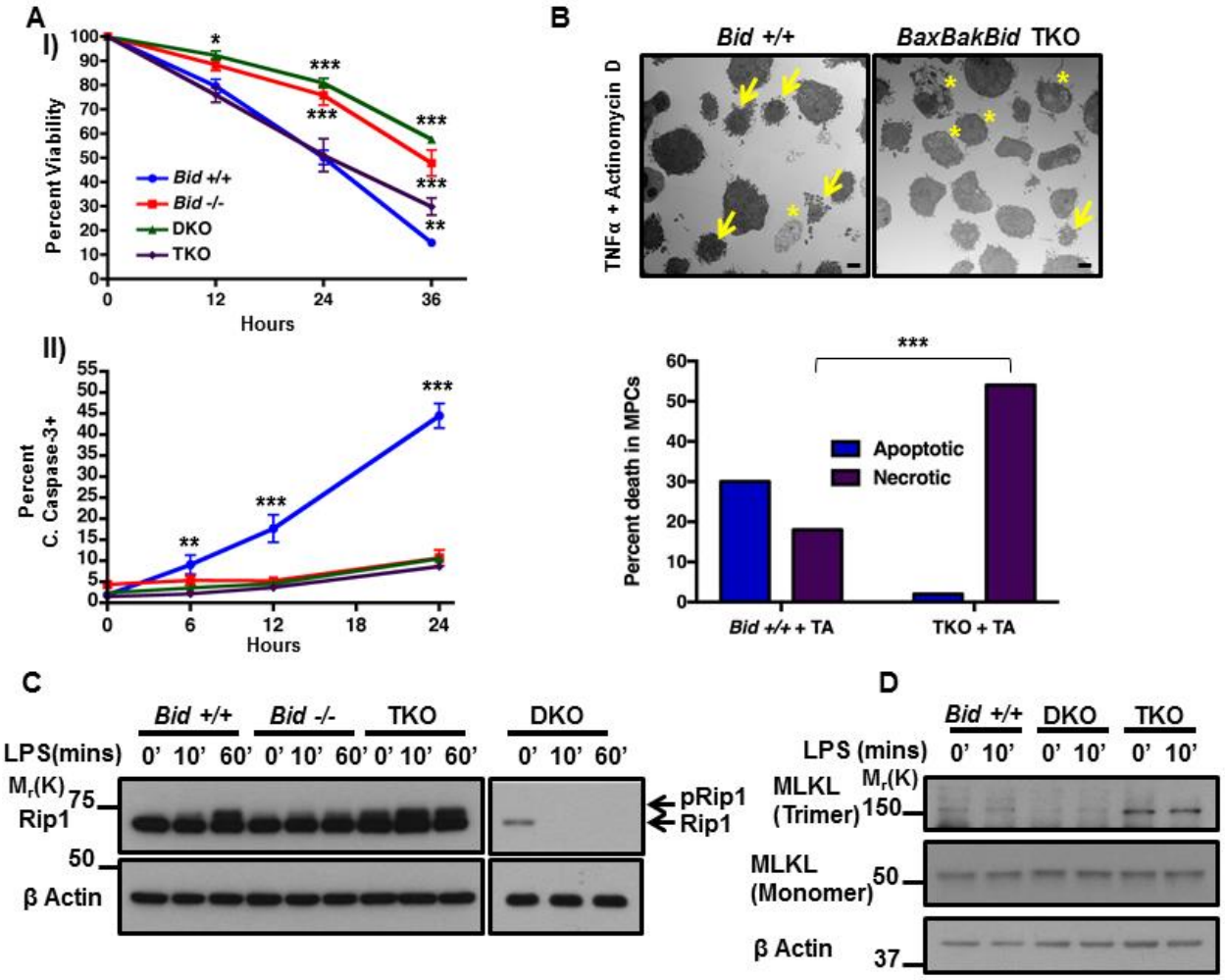
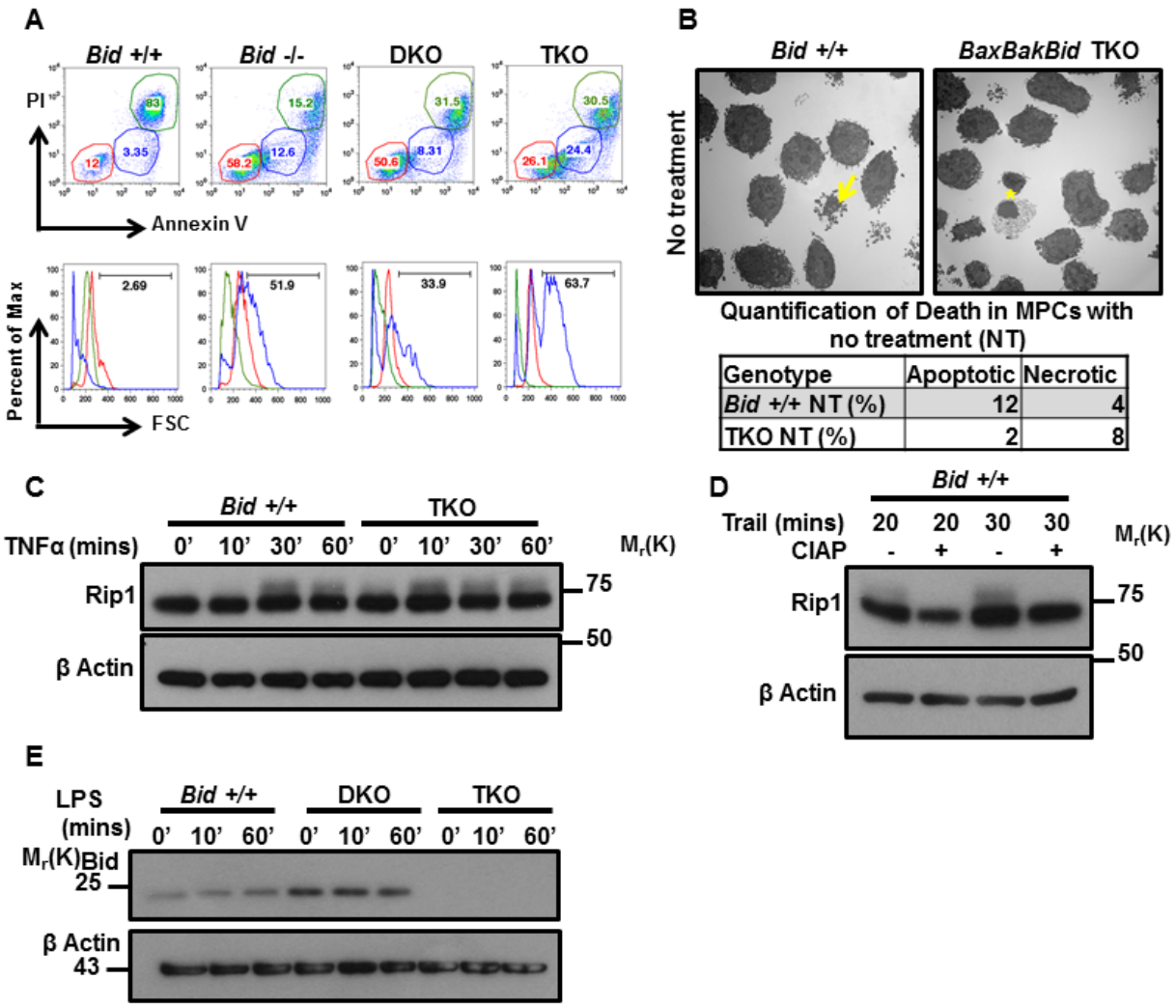


Figure 3.1 *MxBaxBakBid* TKO myeloid progenitor cells (MPCs) die by necroptosis
A) Examination of death in myeloid progenitor cells (MPCs). I) MPCs were treated with 25ng/mL TNFα + 50ng/mL Actinomycin D. Viability was determined by Annexin V/ PI staining. II) MPCs treated with TNFα/ ActD were stained for cleaved Caspase-3 by intracellular flow. Experiment was performed three independent times. Data are represented as mean +/- SEM. Statistics indicate differences between *Bid* +/+ versus *Bid* -/-, DKO, and TKO. B) *Bid* +/+ and TKO MPCs treated with TNFα + Actinomycin D were examined by transmission electron microscopy (TEM). 50 cells with a nucleus were examined and characterized as being apoptotic, necrotic, or live. Arrows indicate apoptotic cells, and asterisks indicate necrotic cells. Scale bar indicates 2 microns. Graph with death quantification below. ***= P< 0.001 **=P< 0.01. C) MPCs were stimulated with 250 ng/mL LPS and status of Rip1 was examined. Experiment was performed four times. D) Examination of trimerization of MLKL in *Bid* +/+, DKO, and TKO MPCs following stimulation with LPS as in (C). Experiment was performed three times. Experiments in C) and D) were performed by Qiong Shi.



Genotype	Apoptotic	Necrotic
Bid +/+ NT (%)	12	4
TKO NT (%)	2	8

Figure 3.2 *MxBaxBakBid* TKO can also be stimulated by TNFα and increase in size following stimulation

A) Examination of Size of *Bid* +/+, *Bid* -/-, DKO, and TKO myeloid progenitor cells (MPCs) in response to stimulation with TNFα + Actinomycin D by utilizing forward scatter measurements from flow cytometry. Experiment was performed three independent times. B) TEM of *Bid* +/+ and TKO MPCs without stimulation with quantification. 50 cells with a nucleus were scored as being live, apoptotic, or necroptotic. Scale bar indicates 2 microns. C) Examination of Rip1 phosphorylation by immunoblot following stimulation of *Bid* +/+, *Bid* -/-, and TKO MPCs with TNFα. Experiment was performed two independent times. D) Examination of Rip1 state by immunoblot after phosphatase pre-treatment (CIAP) of lysates after trail stimulation. Experiment was performed two independent times. E) Bid expression in MPCs after stimulation with LPS. Experiment was performed two independent times. Experiments in C), D), and E) were performed by Qiong Shi.

Bid inhibits association of Rip1 with Complex IIB

Rip1 recruitment to a complex with FADD (Complex IIB), is associated with Rip1 kinase activity and this association is correlated with necroptosis execution (*24*). We next examined the effect of Bid on Rip1 recruitment to Complex IIB (FADD: Rip1: Caspase-8: $cFlip_L$) through FADD immunoprecipitation (IP) following TNFα or LPS stimulus (*24*, *576*, *577*). IP for FADD following LPS or TNFα stimulation of TKO MPCs, revealed a marked increase in association of Rip1, $cFlip_L$, and Caspase-8 with Complex IIB relative to *Bid*+/+ MPCs (Figure 3.3A, B, and C and Figure 3.4A, B, and C) suggesting increased necroptotic signaling in TKO MPCs. FADD IP of *VavBaxBak* DKO and *VavBaxBakBid* TKO MPC lysates yielded similar results (Figure 3.4D), establishing that the observed signaling is not a reflection of the Cre utilized to delete Bax. Retroviral reintroduction of Bid into *MxTKO* MPCs completely abrogated Rip1 presence in this complex (Figure 3.3D and Figure 3.4E), demonstrating that the association of Rip1 in Complex IIB was inhibited by Bid.

Bid regulates Rip1 stability through modulation of Caspase-8 activity

Our previous studies with MPCs reveal that the levels of Rip1 vary between genotypes (Figure 3.1C and 3.3D). In particular we note that Rip1 levels in DKO MPCs, which express increased levels of Bid (Figure 3.2D), are markedly decreased relative to TKO MPCs (Figure 3.1C). Importantly, reintroduction of Bid into TKO MPCs by retroviral transduction results in decreased Rip1 levels (Figure 3.3D), demonstrating that the decreased Rip1 observed in DKO MPCs is due to the presence of Bid.

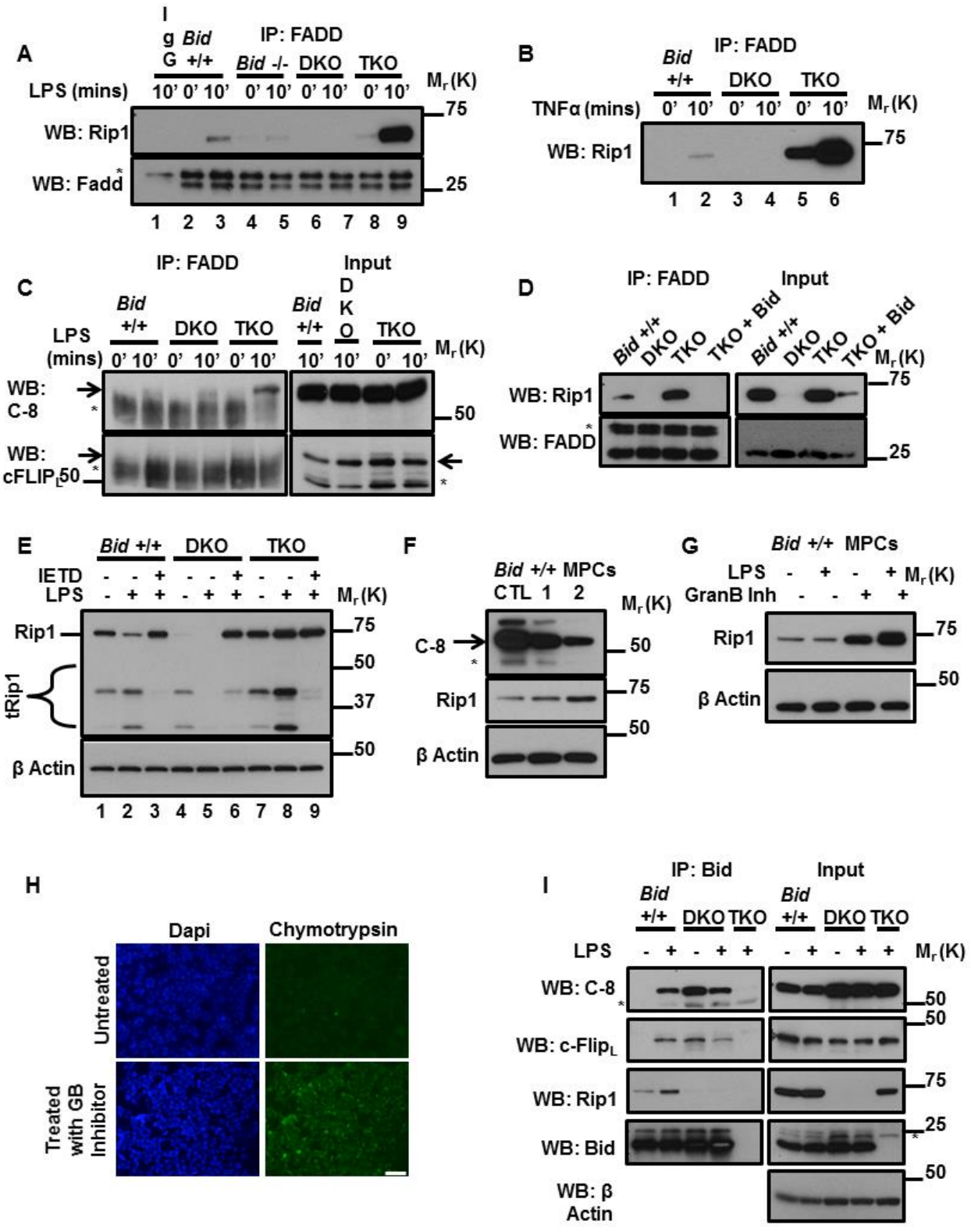
Rip1 can be ubiquitylated or cleaved by proteases such as Caspase-8 (*97*), and Cathepsins (*578*) to promote its degradation. Treatment with MG132 (proteosome inhibitor) or

Z-VAD-FMK (pan-caspase inhibitor) did not recover full length Rip1 in *Bid*+/+, DKO, or TKO MPCs (Figure 3.4F). Pretreatment with IETD (Caspase-8 inhibitor) following LPS stimulation completely recovered full length Rip1 in DKO MPCs and decreased truncated Rip1 in *Bid*+/+ and TKO MPCs, indicating that an IETD-inhibitable enzyme cleaves Rip1 (Figure 3.3F). Similar recovery of full-length levels of another Caspase-8 substrate, Cylindromatosis (CYLD), was also observed (Figure 3.4G). Additionally, Rip1 presence was recovered in Complex IIB in *VavBaxBak* DKO MPCs pretreated with IETD followed by FADD IP (Figure 3.4D). Furthermore, deletion of Caspase-8 using CRISPR/CAS9 with 2 independent gRNAs, results in an increase in Rip1 levels in *Bid* +/+ MPCs (Figure 3.3F) that is proportional to the degree of Caspase-8 knockdown achieved. While the above results are consistent with a role for Caspase-8 in mediating Rip 1 levels, the inability to restore RIP1 levels with ZVAD is inconsistent with a singular role for Caspase-8 in modulating of Rip1 stability, suggesting another protease may be involved in this process.

Another protease containing an IETD active site is Granzyme B. Our studies with a protein inhibitor of Granzyme B suggest that it may also play a role in the degradation of Rip1 (Figure 3.3G). Permeabilization of MPCs with digitonin and addition of this protein to the culture medium, followed by treatment with LPS rescued full length levels of Rip1 in *Bid* +/+ MPCs. Presence of the Granzyme B inhibitor in MPCs was confirmed by immunofluorescence for chymotrypsin, the backbone of the inhibitor (Figure Previous studies suggest that Granzyme B may play a role in the restraint HSC function, as Granzyme B -/- HSPCs display increased competitive repopulating ability mediated through NF-κB signaling (*579*). However, whether Granzyme B exerts its HSPC effect through RIP1 kinase will require further investigation.

Bid forms an intermediate complex with Rip1, Caspase-8, and cFlip$_L$

We next sought to understand where in the necroptotic pathway Bid functioned to toggle the switch between apoptosis and necroptosis. Because we saw dramatic changes in Rip1 in Complex IIB, we first examined this complex to determine if Bid could function within this complex to block Rip1's association. However, upon multiple examinations of this complex, we were unable to detect the presence of Bid in this complex (Figure 3.4H). To probe Bid's role in necroptosis directly, we next performed an IP for Bid with and without LPS stimulation. As shown in Figure 3.3I, we found an association of Bid with Rip1, Caspase-8, and c-Flip$_L$ with stimulation, suggesting the possibility that this complex could be involved in Rip1 degradation. Therefore, our work suggests that Bid plays a central role in PCD outcome through modulation of an IETD- inhibitable activity, and can serve as an alternate switch that determines whether a hematopoietic cell will execute apoptosis or necroptosis. However further study is needed to understand the interaction between these proteins and the activity of this complex.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Ig G
Bid +/+
IP: FADD
Bid -/-
DKO
TKO
LPS (mins)
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0' 10'
0' 10'
0' 10'
Mr(K)
WB: Rip1
WB: Fadd
75
25
1 2 3 4 5 6 7 8 9
B
IP: FADD
Bid +/+
DKO
TKO
TNFα (mins)
0' 10'
0' 10'
0' 10'
Mr(K)
WB: Rip1
75
1 2 3 4 5 6
C
IP: FADD
Input
Bid +/+
DKO
TKO
LPS (mins)
0' 10'
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Mr(K)
WB: C-8
WB: cFLIPL
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D
IP: FADD
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Bid +/+
DKO
TKO
TKO + Bid
Mr(K)
WB: Rip1
WB: FADD
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25
E
Bid +/+
DKO
TKO
IETD
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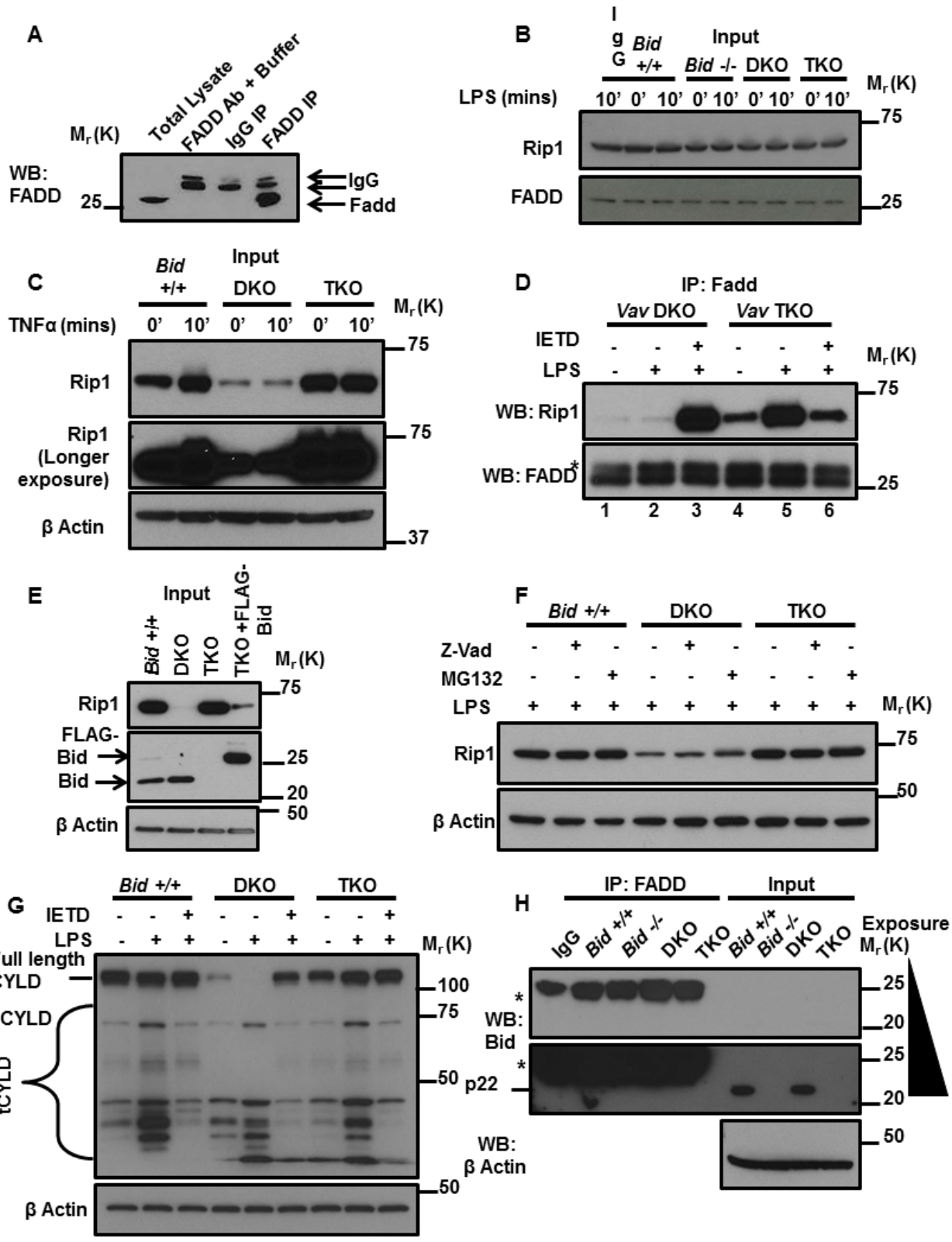
Rip1
tRip1
β Actin
75
50
37
50
1 2 3 4 5 6 7 8 9
F
Bid +/+ MPCs
CTL 1 2
Mr(K)
C-8
Rip1
β Actin
50
75
50
G
Bid +/+ MPCs
LPS
GranB Inh
Mr(K)
Rip1
β Actin
75
50
H
Dapi
Chymotrypsin
Untreated
Treated with GB Inhibitor
I
IP: Bid
Input
Bid +/+
DKO
TKO
LPS
Mr(K)
WB: C-8
WB: c-FlipL
WB: Rip1
WB: Bid
WB: β Actin
50
50
75
25
50

Figure 3.3 Bid inhibits association of Rip1 with Complex IIB through modulation of Caspase-8 activity towards Rip1 in *MxBaxBakBid* MPCs
A) Immunoprecipitation (IP) for FADD following stimulation with 250 ng/mL LPS for 10 minutes, and immunoblotted for Rip1 and FADD. *Indicates light chain IgG. Experiment was performed four independent times. B) IP for FADD as in (A) with MPCs stimulated with 10ng/mL TNFα for 10 minutes. Experiment was performed three independent times. C) Repeat of IP for FADD as in (A) with MPCs stimulated with 250ng/mL LPS to examine the status of Caspase-8 and $CFlip_L$. Experiment was performed two independent times. D) IP for FADD as in (A) with TKO MPCs that had *Bid* reintroduced. Experiment was performed two independent times. All samples were stimulated with LPS as in (A). *Indicates light chain IgG. E) Examination of Rip1 in *Bid*+/+, DKO, and TKO MPCs by immunoblot following stimulation with LPS (as in A) and pre-treatment with an IETD inhibitor to Caspase-8 (20μM). Experiment was performed three independent times. F) Examination of Rip1 levels by immunoblot after deletion of Caspase-8 utilizing the Crispr-Cas9 system in *Bid* +/+ cells. Experiment was performed two independent times. G) Examination of Rip1 following addition of Granzyme B to *Bid* +/+ MPCs. Experiment was performed two independent times. H) Immunofluorescence for chymotrypsin (the backbone of the Granzyme B inhibitor) to examine the presence of the Granzyme B inhibitor in MPCs. I) IP for Bid following LPS stimulation and immunoblot for Caspase-8, Rip1, and $cFlip_L$. MPCs were untreated or stimulated with 250ng/mL LPS. * indicates IgG. Experiment was performed three independent times. Experiments in A-I performed by Qiong Shi.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Total Lysate
FADD Ab + Buffer
IgG IP
FADD IP
Mr(K)
WB: FADD
25
IgG
Fadd
B
Ig G
Bid +/+
Input
Bid -/-
DKO
TKO
LPS (mins)
10' 0' 10' 0' 10' 0' 10' 0' 10'
Mr(K)
Rip1
FADD
75
25
C
Bid +/+
Input
DKO
TKO
TNFα (mins)
0' 10' 0' 10' 0' 10'
Mr(K)
Rip1
Rip1 (Longer exposure)
β Actin
75
75
37
D
IP: Fadd
Vav DKO
Vav TKO
IETD
LPS
Mr(K)
WB: Rip1
WB: FADD*
75
25
1 2 3 4 5 6
E
Input
Bid +/+
DKO
TKO
TKO +FLAG-Bid
Mr(K)
Rip1
FLAG-Bid
Bid
β Actin
75
25
20
50
F
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TKO
Z-Vad
MG132
LPS
Mr(K)
Rip1
β Actin
75
50
G
Bid +/+
DKO
TKO
IETD
LPS
Mr(K)
Full length CYLD
CYLD
tCYLD
β Actin
100
75
50
50
H
IP: FADD
Input
IgG
Bid +/+
Bid -/-
DKO
TKO
Bid +/+
Bid -/-
DKO
TKO
Exposure
Mr(K)
WB: Bid
p22
WB: β Actin
25
20
25
20
50

Figure 3.4 Bid inhibits association of Rip1 with Complex IIB in *VavBaxBakBid* MPCs
B) Input from IP for FADD before and after LPS (250ng/mL) stimulation in *Bid* +/+, *Bid* -/-, DKO, and TKO MPCs. WB for FADD demonstrates equal loading. Experiment was performed four independent times. C) Input from IP of FADD from MPCs stimulated with 10ng/mL TNFα immunoblot for Actin demonstrates equal loading. *denotes IgG. Experiment was performed three independent times. D) IP of FADD from MPCs developed from *VavBaxBakBid* TKO mice with and without Caspase-8 inhibitor (IETD, 20μM). MPCs were pre-treated with IETD and/or stimulated with LPS (250 ng/mL). *denotes IgG. Experiment was performed two independent times. E) Examination of Bid expression by immunoblot in MPCs after retroviral reintroduction of Bid into TKO MPCs and LPS stimulation to demonstrate Bid overexpression after retroviral reintroduction. Experiment was performed two independent times. F) Effect of MG132 (proteosome inhibitor) and Z-Vad-FMK (Pan-caspase inhibitor) on Rip1 levels; MPCs were stimulated with LPS and/ or pre-treated with MG132 or Z-Vad-FMK and Rip1 was examined by immunoblot. Experiment was performed three independent times. G) Effect of IETD on CYLD; MPCs were pre-treated with IETD (20μM) and/or stimulated with LPS (250ng/mL) and CYLD was examined by immunoblot. Experiment was performed two independent times. (H) Immunoblot for Bid following IP for FADD in *Bid* +/+, *Bid* -/-, DKO, and TKO MPCs demonstrating Bid is not found in Complex II. * Indicates IgG. Experiment was performed twice, independently. Experiments in A-H were performed by Qiong Shi.

Discussion

A key unknown in the field of cell death is understanding what determines an outcome of apoptotic versus necroptotic death. The Rip kinases are important in the activation of necroptosis, whereas the Caspases are important in the activation of apoptosis. Additionally, multiple lines of evidence demonstrate that both Rip1 and Caspase-8 are important factors in upstream signaling pathways. Our studies in the context of hematopoiesis indicate that Rip1 and Caspase-8, in the presence or absence of Bid in the context of Bax/Bak deletion, determines the downstream cell death outcome. This occurs through the formation of a complex with Rip1, Caspase-8, $cFlip_L$, and Bid that likely promotes the degradation of Rip1. In the absence of Bax, Bak, and Bid Rip1 levels are increased, and necroptosis is enhanced. Our data demonstrates that, in addition to blocking apoptosis, necroptosis in hematopoiesis must be activated by releasing the constraint imposed by Bid on Caspase-8-mediated Rip1 degradation.

Consistent with previous studies, we show that cells in which both Bax and Bak are deleted are resistant to apoptotic stimuli, and also do not die by necroptosis. This indicates that it not sufficient to block apoptosis to promote necroptosis. We find that Rip1 levels in BaxBak DKO MPCs (which also have increased Bid levels) are markedly diminished, due to Caspase-8 activity. Rip1 levels are completely restored upon additional loss of Bid, demonstrating that in this setting, Bid functions to modulate Rip1 levels through Caspase-8 (Figure 3.3E). Collaboration with a mathematical modeling group, suggests that this occurs through the modification of Bid. Leveraging cell lines from our mouse models that are tuned to undergo apoptosis or necroptosis, we demonstrate that in addition to activating apoptosis, Bid restrains necroptosis through a Bid:Caspase-8 axis, that cleaves and inactivates Rip1.

CHAPTER IV

ROLE OF BID IN INTESTINAL HOMEOSTASIS AND INFLAMMATION

Introduction

Multicellular organisms remove damaged or superfluous cells through a highly regulated cellular process known as programmed cell death. There are two main forms of programmed cell death, apoptosis and necroptosis. Apoptosis, a highly regulated process, was for many years purported to be the only type of programmed cell death. Conversely, programmed necrosis (necroptosis), previously thought to be an unregulated death pathway, was recently found to be highly regulated. Necroptosis results in swelling of the cell and organelles, and is mediated by RIP kinases. While most of the activators and transducers of apoptosis have been identified, the necroptotic pathway is not well understood, but genetic evidence implicates upstream apoptotic proteins in necroptosis inhibition.

In the setting of intestinal homeostasis and disease, programmed cell death balances with proliferation to maintain the integrity of the intestinal barrier and to limit inflammation (*580*). This process is intricately controlled, with deviations leading to pathologies such as malignancy or inflammatory bowel diseases (IBDs) (*581*). While apoptosis is a normal feature of cells within the crypt and anoikis of cells at the tips of villi, too much apoptotic death is detrimental and has been implicated in inflammation and pathology of IBDs (*582–584*). Similarly, necroptosis recently was implicated in maintaining intestinal homeostasis through inhibition of programmed cell death. Intestinal epithelial cell-specific deletion of Caspase-8 and FADD promotes increased

inflammation and necrosis of the intestinal epithelium in mice (*585*, *586*). Additionally, necrosis is also implicated in the pathogenesis of IBDs, as it was reported in Crohn's Disease with necrotic cells being visualized in portions of involved and uninvolved tissue (*587*, *588*). The increase in death in this setting is likely mediated through increased inflammatory signaling, as increases in inflammatory cytokines, including TNFα, is a common finding in patients with IBD (*393*). These findings demonstrate the importance of programmed cell death in the intestine.

The BCL-2 family has been demonstrated to regulate apoptotic cell death with the capability to promote or inhibit apoptosis execution. We recently identified a role for the BH3-only member Bid in the inhibition of programmed necrosis in hematopoiesis (Chapters II and III). Because programmed cell death is also an important factor in maintaining intestinal epithelial homeostasis, and is likely a factor in the IBD pathological state, we wanted to understand what role Bid might also play in programmed cell death in the intestine independent of its apoptotic function.

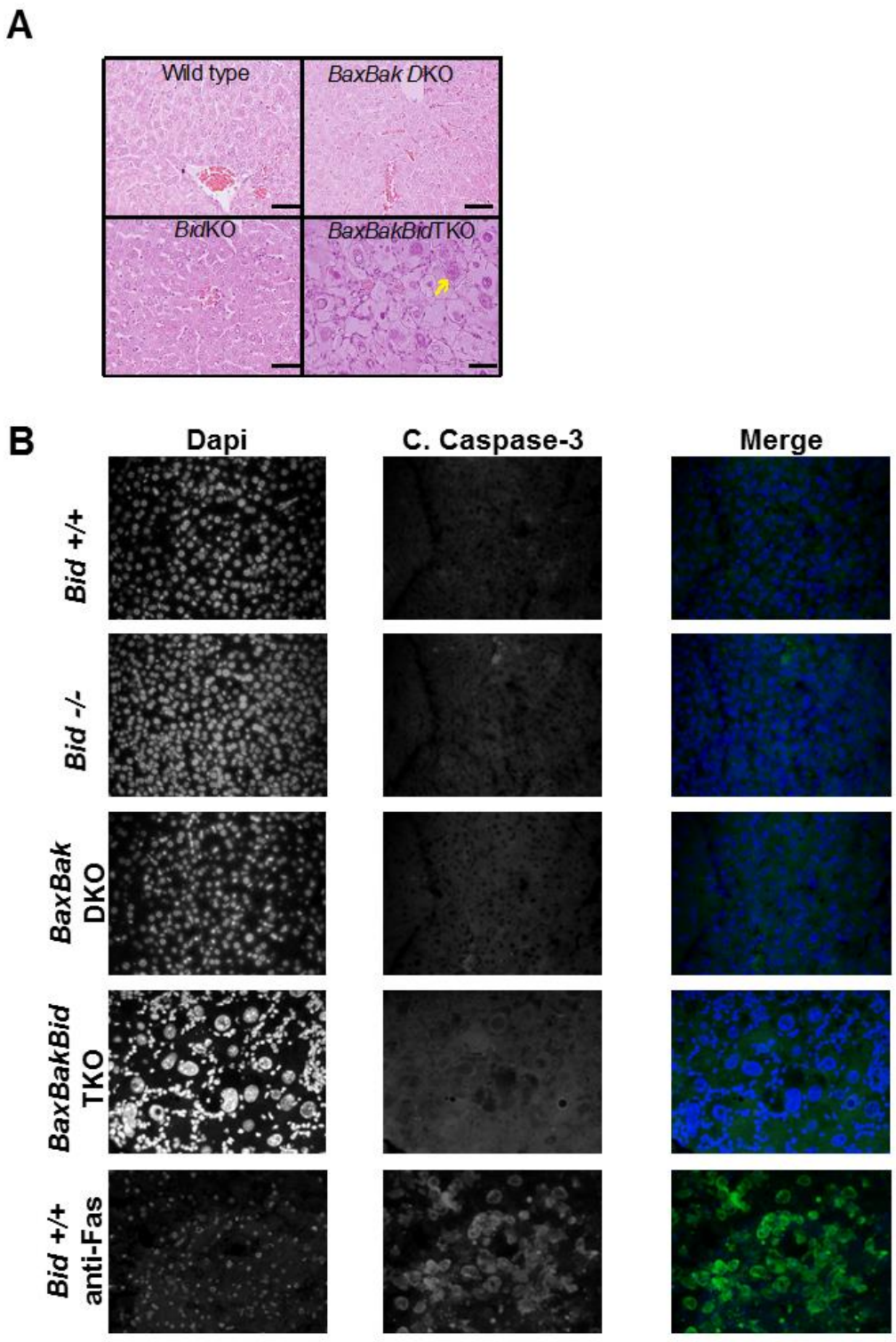
To interrogate this role for Bid, we generated a mouse model with a triple knockout (TKO) of the apoptotic proteins Bax, Bak, and Bid. In knocking out all three proteins we removed not only Bid, but also the apoptotic branch of Bid's function (Bax and Bak). To compare and contrast the impact of loss of apoptosis (*MxBaxBak* DKO mice) versus increased necrosis (*MxBaxBakBid* TKO mice) in intestinal epithelial injury, we subjected our mice to DSS colonic injury model for 6 days. We hypothesized that mice with unrestrained necrosis (*BaxBakBid* TKO) would have increased epithelial injury as compared to *Bid* +/+ (wild type). We saw differences between *Bid* +/+ and *Bid* -/- versus DKO and TKO colons, suggesting increased inflammation in DKO and TKO colons. Further study is needed to understand the role of inflammatory signaling in settings of lack of apoptosis and increased necroptosis.

Results

MxBaxBakBid TKO mice display fulminant liver necrosis

We began our studies with the development of *MxBaxBakBid* TKO mice to understand what function Bid might play intestinal homeostasis. We utilized *Mx1Cre* to induce the deletion of *Bax* in the setting of a *Bid* and *Bak* germline deletion. *Mx1Cre* is induced through injection with Poly Inosinic: Cytidylic (Poly (I;C), a dsRNA mimic that promotes a type I interferon response inducing Cre expression (*589*). Mice were given three doses every other day to induce Cre recombination. Following induction with Poly I;C a small percentage of TKO mice became moribund after a single injection. Upon necropsy we noted that these mice seemed to have livers and kidneys devoid of blood, as well as hemolysis of the peripheral blood. This effect did not occur in *MxBaxBak* DKO, *Bid* +/+, or *Bid* -/- mice when injected with Poly (I;C). We examined paraffin-embedded sections of liver, and found that the hepatocytes from TKO mice were increased in size, with some having a swollen appearance, along with increased size of the nuclei (Figure 4.1A). The morphology and size of hepatocytes of *Bid* +/+, or *Bid* -/-, and *MxBaxBak* DKO were normal, even with (Poly I;C) injection, suggesting that this effect was not due solely to the induction of an interferon response. Next, we wanted to examine the type of death occurring in the liver of TKO mice. Utilizing fluorescent immunohistochemistry, we examined the status of cleaved Caspase-3 in paraffin-embedded liver sections from our mice. We observed no significant staining for cleaved Caspase-3 in liver sections from *Bid* +/+, or *Bid* -/-, DKO, or TKO mice as compared to a positive control for cleaved Caspase-3, a *Bid* +/+ mouse tail-vein injected with Fas ligand, which stimulates liver apoptosis (Figure 4.1B). From this finding and

previous studies, we hypothesized that Bid was functioning to inhibit necrotic death, a process that often leads to increased inflammatory signaling. We next wanted to understand what role Bid might play in inflammatory diseases/ conditions in which necrosis and inflammation cause pathology. As discussed previously, necrosis and inflammation are both involved in the pathology of IBDs, and as such we subjected our mice to a model of colitis to understand what role Bid might play in this setting.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Wild type
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BidKO
BaxBakBidTKO
B
Dapi
C. Caspase-3
Merge
Bid +/+
Bid -/-
BaxBak DKO
BaxBakBid TKO
Bid +/+ anti-Fas

Figure 4.1 *MxBaxBakBid* TKO mice display fulminant liver necrosis
A) We utilized *Mx1Cre* to promote the deletion of Bax in the setting of a Bid and Bak germline deletion to generate TKO mice (or Bak deletion for *MxBaxBak* mice). Liver sections from TKO mice demonstrate the classic feature of necrosis including loss of cell membrane integrity and swelling of nuclei. B) Fluorescent immunohistochemistry on sections of liver for Cleaved Caspase-3 to examine if dying cells in the TKO liver were undergoing apoptosis. Animals tail-vein injected with Fas ligand as a positive control.

TKO mice have increased inflammation and damage in response to DSS model of colonic injury

To examine the role of programmed cell death in the setting of inhibited apoptosis and uninhibited necrosis on inflammatory signaling in the intestine, we subjected our *Bid +/+*, *Bid -/-*, DKO, and TKO mice to the acute Dextran Sodium Sulfate (DSS) colitis model (*590*). Treatment with DSS is toxic to intestinal epithelial cells and disrupts the intestinal epithelial barrier in the colon promoting inflammation through innate immune signaling. Mice were treated with H_2O or 4% DSS for 6 days and weighed each day. On day 6, mice were sacrificed, and tissues prepared for histologic analysis. Hematoxylin and Eosin stained sections of colon were scored for pathologic criteria including inflammation, percent involvement of inflammation, distortion of intestinal epithelium, crypt damage, and percent involvement of crypt damage. Each of these criteria may be scored from 0 to 4, and the total from all criteria is added together as a pathological score. While TKO mice treated with DSS have a pathological score that trends higher than *Bid +/+*, or *Bid -/-*, and DKO mice treated with DSS, this finding was not statistically different from the other genotypes (Figure 4.2A). Surprisingly, examination of weight loss in mice treated with DSS revealed less weight loss in TKO mice which was significantly different from *Bid -/-* and DKO mice (Figure 4.2B). The colons were further examined on Day 5 of the DSS treatment by endoscopy. Interestingly, upon examination of colons by endoscopy, both DKO and TKO mice appeared to have the greatest pathology with increased inflammation and an ulceration in the colon of the TKO (Figure 4.2C). These findings suggest that loss of Bid and its apoptotic arm of function may potentiate inflammation in the acute DSS model.

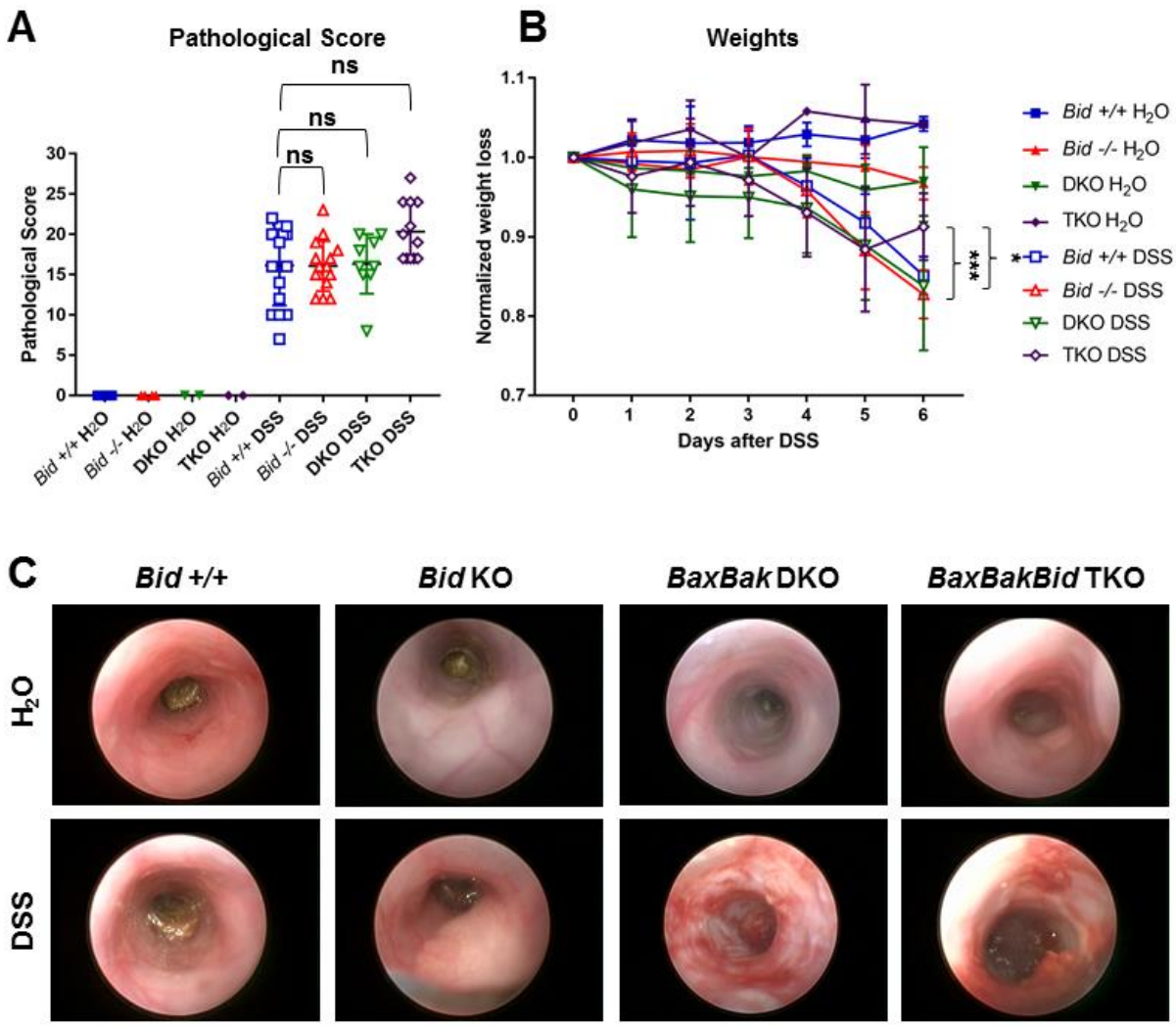


Figure 4.2 *MxBaxBakBid* TKO mice demonstrate increased inflammation and damage in the colon in response to intestinal injury

A. Pathological scoring evaluating the degree of inflammation, distortion of the intestinal epithelium, crypt damage, and percent involvement of damage and inflammation in *Bid* +/+, *Bid* -/-, DKO, and TKO mice treated with H2O or DSS. B) Normalized weights of mice each day during H2O or DSS treatment in each genotype. C) Endoscopy images from mice treated with H2O and DSS on day 5 of DSS treatment. Pathological scoring in (A) completed by Mary Kay Washington. Endoscopy in (C) performed by Amber Bradley.

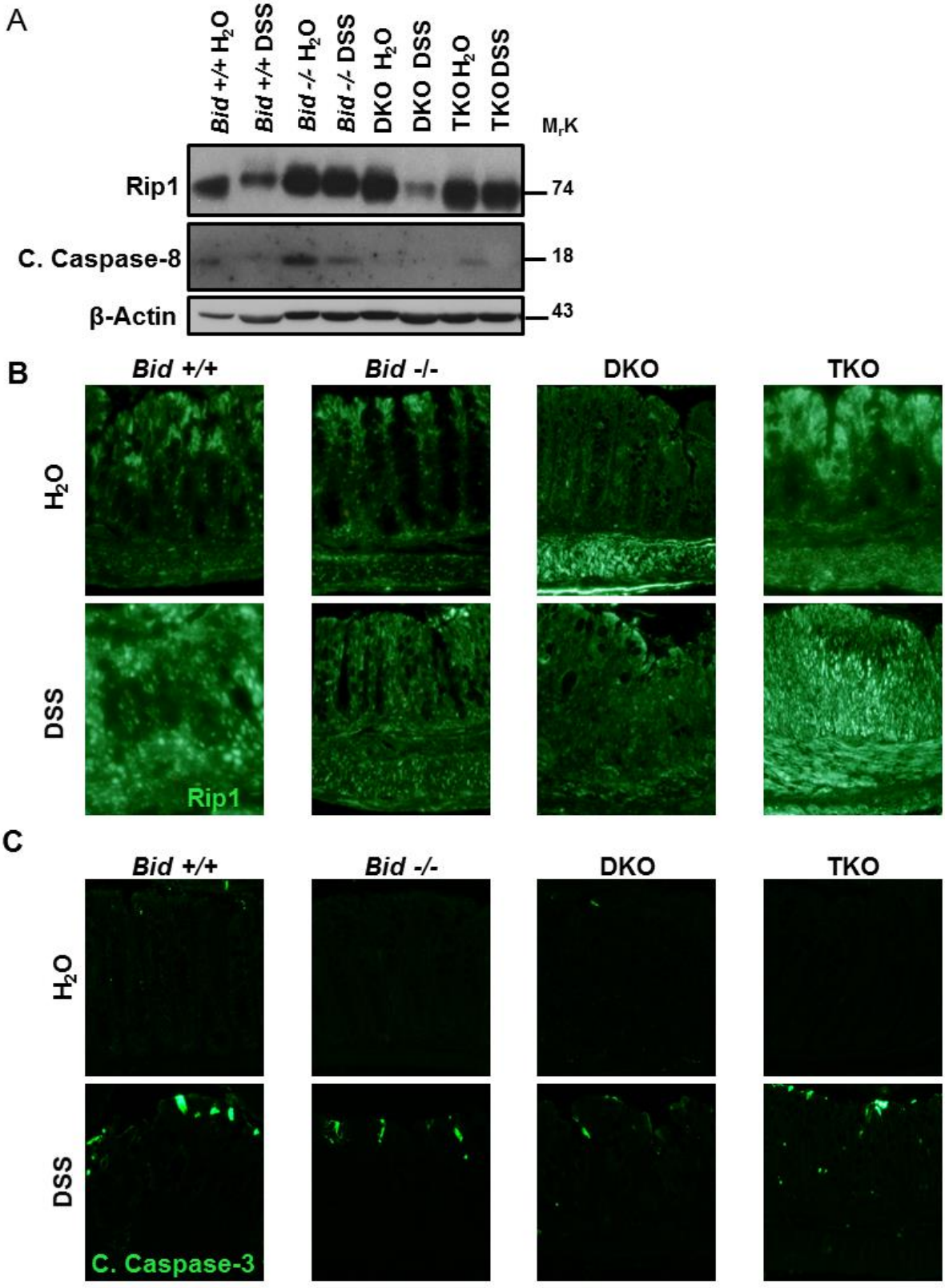
TKO mice treated with DSS have increased Rip1 expression in the colon following DSS

The toxicity of DSS to the intestinal epithelial cells promotes disruption of the intestinal epithelium, likely through programmed cell death. As discussed previously, there is a delicate balance between cell production and death in the intestine, and pathologies arise in settings in which this balance is perturbed. Additionally, previous studies implicate increased apoptosis and necroptosis in intestinal pathologies such as IBDs. We next wanted to explore if cell death was occurring in the intestine of *Bid* +/+, or *Bid* -/-, DKO and TKO mice, and to determine if this death was occurring through apoptosis or necroptosis. To explore this, we first examined whole cell lysate from colons of *Bid* +/+, or *Bid* -/-, DKO and TKO mice treated with H_2O or DSS. We examined the status of Rip1 to determine levels of expression, as well as differences in mobility which would be indicative of phosphorylation, demonstrating activation of Rip1. Examination of Rip1 revealed increased expression and some lower mobility species in *Bid*+/+, *Bid* -/-, and TKO colons with H_2O and/or DSS treatment. This finding correlates with the previous observation of an inverse relationship between *Bid* and *Rip1* expression, as previously seen in MPCs (Figure 3.3C). While the *Bid* +/+ colon demonstrates about half the level of Rip1 expression with H_2O treatment, it interestingly is diminished and is of a lower mobility with DSS treatment. Interestingly, there is also dramatic loss of full length Rip1 in DKO colons treated with DSS, similar to the trend we note in our DKO MPCs following stimulation with LPS or TNFα. This finding suggests that any death occurring in DKO is likely not occurring through necroptosis (Figure 4.3A). While there are differences in Rip1 expression between our different genotypes of mice and with DSS treatment, further study is needed to interpret these differences.

Our western blot only contains lysate from a single mouse, however variability between mice, as well as random sampling of colons taken from mice at sacrifice could cause sampling error. We additionally examined the status of cleaved Caspase-8, a marker of apoptosis activation. While there is only a modest activation in *Bid* +/+ colon, *Bid* -/- colon demonstrates robust cleaved Caspase-8 expression that decreases with DSS treatment, suggesting apoptotic signaling occurs in homeostatic conditions (H_2O), but that with DSS treatment any death within the colon does not occur through apoptosis. While examination of Rip1 and Caspase-8 by Western blot was informative regarding levels of expression of these proteins, further study is needed to understand the effect of DSS on the mobility/modifications within the colon.

While examination of whole cell lysate provided some understanding of the overall status of cell death in our different genotypes of mice, it did not provide information regarding where within the colon this expression was occurring. Inflammation from DSS treatment primarily affects the intestinal epithelium, however examination of lysate from the whole colon includes this layer as well as the underlying submucosa, muscle layer, and serosa. To understand what type of programmed cell death was occurring specifically within certain portions of the colon we examined paraffin-embedded sections of colon for Rip1 and cleaved Caspase-3, a more definitive marker of apoptosis. Examination of Rip1 in colons treated with H_2O or DSS revealed a strong increase in Rip1 expression in the colon of TKO mice following DSS suggesting death of the epithelium by necroptosis. TKO mice also demonstrate increased Rip1 on the tips of the villi as compared to *Bid* +/+, *Bid* -/-, and DKO mice with H_2O treatment, suggesting increased necrotic signaling in the TKO colon even without stimulation. While the *Bid* +/+ colon has increased Rip1 expression as well with DSS, the extent of this increase is not quite to the level seen in TKO. Interestingly, *Bid* -/- had only modest increases in Rip1 expression with DSS

within the villi, while the DKO colon demonstrates minimal staining in the villi (Figure 4.3B). Next we examined apoptosis signaling through cleaved Caspase-3 staining. The positivity for cleaved Caspase-3 was modest and uniform between genotypes with both H_2O and DSS treatment, at the tips of the villi within the colon, suggesting death by anoikis. The minimal positivity also suggests that minimal apoptotic death is occurring within the intestinal epithelium (Figure 4.3C). Increased Rip1 expression in TKO but not DKO colons with a corresponding lack in increase of cleaved Caspase-3 suggests that death in the TKO but not DKO colons is mediated by programmed necrotic death.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Bid +/+ DSS
Bid -/- H2O
Bid -/- DSS
DKO H2O
DKO DSS
TKO H2O
TKO DSS
MrK
Rip1
74
C. Caspase-8
18
β-Actin
43
B
Bid +/+
Bid -/-
DKO
TKO
H2O
DSS
Rip1
C
Bid +/+
Bid -/-
DKO
TKO
H2O
DSS
C. Caspase-3

Figure 4.3 *MxBaxBakBid* TKO mice have increased Rip1 expression in the colon following DSS treatment

A) Western blot examining expression of Rip1 as a marker of programmed necrosis and cleaved Caspase-8 as a marker of apoptosis activation. B) Examination of Rip1 expression in mice treated with H_2O and DSS by fluorescent immunohistochemistry. C) Examination of cleaved Caspase-3 as a marker of apoptosis activation in mice treated with H_2O and DSS by fluorescent immunohistochemistry. Experiments in A-C were performed by Qiong Shi.

Cytokine signaling following DSS stimulation is similar between DKO and TKO mice

The differences between Rip1 staining between DK0 and TKO colons after DSS, despite the presence of inflammation in both suggested that inflammatory and innate immune signaling pathways could differ between these two genotypes. These pathways are mediated by the release of cytokines which promote responses from epithelial and hematopoietic cells to attract immune cells, promote proliferation, differentiation, and programmed cell death. We examined the status of cytokine signaling within the colons of to *Bid* +/+, *Bid* -/-, DKO, and TKO mice following H_2O and DSS through examination of lysate from these tissues. Utilizing a Luminex multiplex ELISA assay, the expression of several cytokines was examined. Comparison between genotypes and treatments reveals a similar pattern of cytokine expression between *Bid* +/+ and *Bid* -/- colon, as well as similarities in DKO and TKO colons (Figure 4.4A). Interestingly, while DKO and TKO colons have increases in similar cytokines, the TKO colons demonstrate slightly increased expression as compared to DKO colons (more intense red), suggesting loss of Bid on top of Bax and Bak further potentiates cytokine signaling. Examination of graphs of cytokines with substantial increases following DSS treatment reveals significant increases in TNFα and G-CSF in TKO colons treated with DSS, suggesting increased inflammatory and innate immune signaling occurs in TKO colons following acute DSS injury (Figure 4.4B).

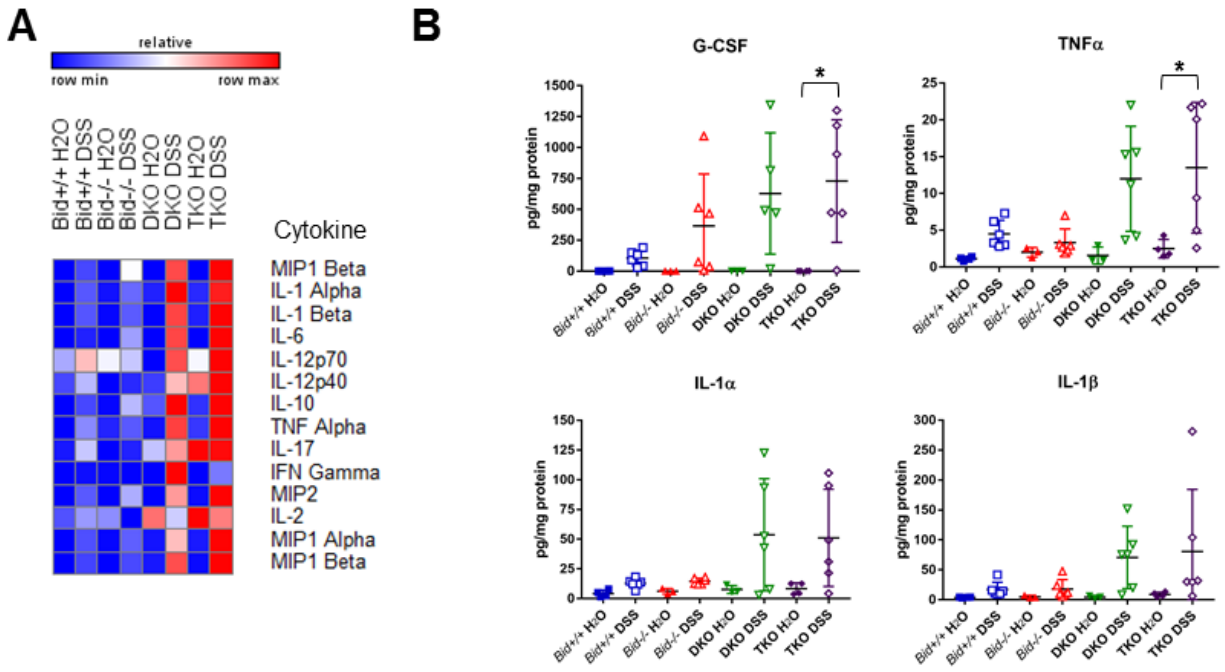


Figure 4.4 *MxBaxBakBid* TKO colons treated with DSS have increased inflammatory cytokine expression

Examination of protein from colons of mice treated with H_2O and DSS for expression of inflammatory and innate immune signaling cytokines. A) Heatmap of cytokine expression for inflammatory and innate immunity signaling cytokines. B) Graphs of cytokines with greatest change in expression in colons of mice treated with H_2O and DSS. Experiments in (A) and (B) were performed in collaboration with the lab of Keith Wilson and with the help of Qiong Shi.

Rip1 expression is decreased in transformed samples of IBD

As discussed previously, programmed cell death has been implicated in the pathology of IBDs. While the role of increased apoptosis is established in this process, the studies on the role of necroptosis are preliminary, with few studies investigating the role of necroptosis in this disease and more recent studies completed in mice (*583*, *585*, *586*). As such, we wanted to understand if necroptotic signaling might be increased in samples of involved tissue from Crohn's Disease (CD) and Ulcerative Colitis (UC). To do this, we acquired two tissue microarrays (TMAs) with samples of Control intestine, CD involved and uninvolved tissue, CD tumor, UC involved and uninvolved tissue, and UC tumor. We stained these microarrays for Rip1 and quantified expression through scoring for positivity. Representative images demonstrate increases in Rip1 staining in both Control and involved samples of CD and UC, but a striking lack of positivity in tumor samples (Figure 4.5A). Scoring of UC samples revealed significantly decreased Rip1 levels in uninvolved and tumor samples as compared to involved samples. Interestingly, in Crohn's Disease, both the involved and uninvolved samples had increased Rip1 levels as compared to tumor samples. These data suggest that the transformation of IBDs to a malignant state decreased Rip1-mediated necrotic signaling. Further study to understand the activation of necroptotic signaling is needed to better understand the role of Rip1-necroptotic signaling in IBDs.

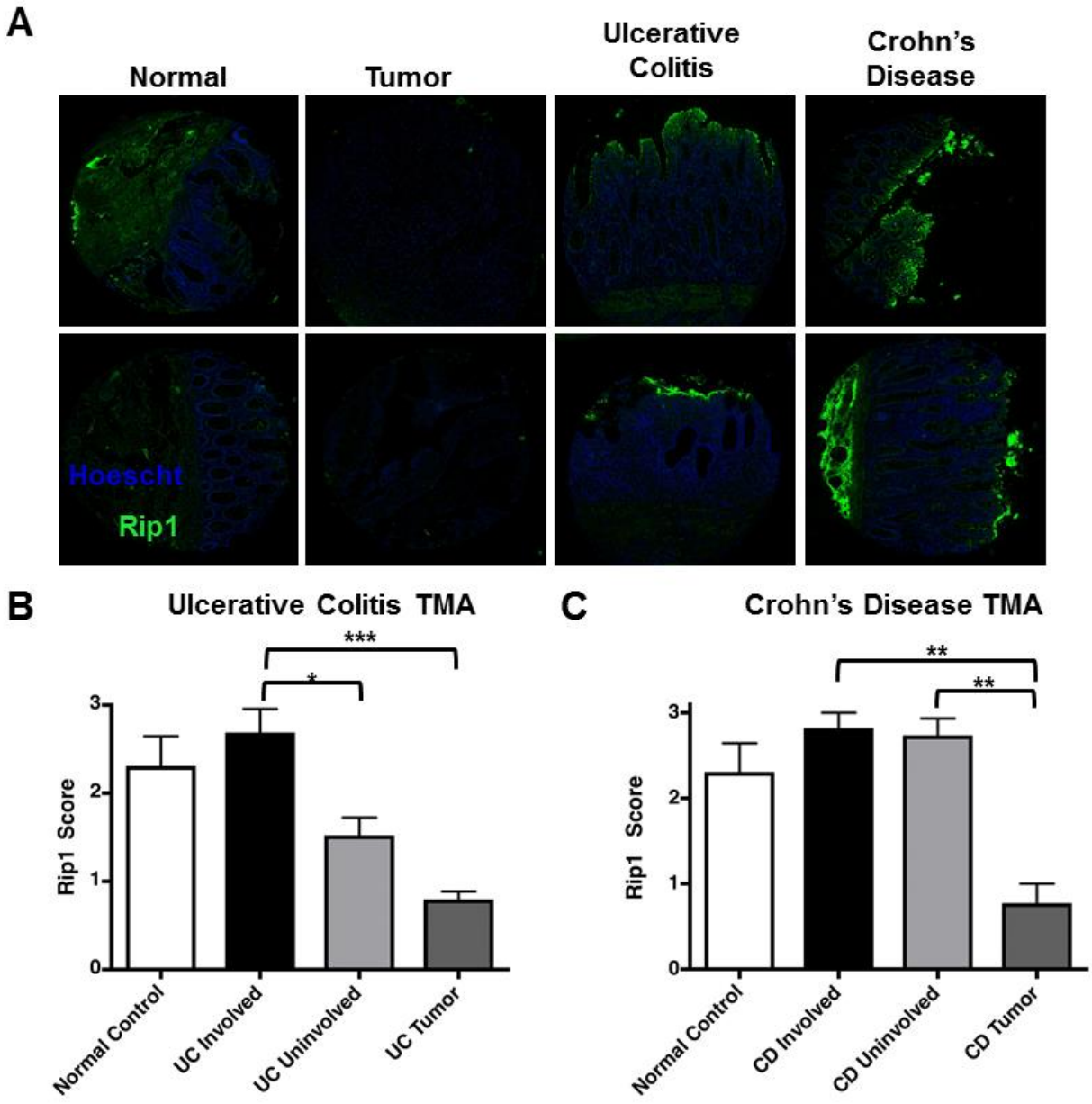


Figure 4.5 Rip1 levels are decreased in tumor samples of IBDs
A) Representative samples of tissue from a tissue microarray (TMA) of IBD samples. B) Rip1 score of UC samples of from two TMA arrays. C) Rip1 score of Crohn's Disease samples from two microarrays. Experiments in A-C were performed in collaboration with the laboratory of Chris Williams. Staining in (A) was performed by Qiong Shi.

Discussion

Through my studies with mice utilizing the acute DSS injury model I sought to understand if Bid-mediated programmed necroptotic death might play a role in inflammatory processes within the intestine. These studies suggest that inhibition of apoptosis (*MxBaxBak)* and loss of three proapoptotic proteins (*MxBaxBakBid)* within the colon causes increased pathology within the colon. My studies suggest this is mediated by increased inflammation through increased cytokine expression, but may be due to other factors such as programmed cell death. Increases in Rip1 expression in the colons of TKO mice as compared to DKO suggests that necroptotic death may be a factor in this process. Both necroptotic and increased apoptotic death can promote increased inflammation in the intestine (*583*, *588*). Activation of similar cytokines to varying extents in DKO and TKO colons suggest that inflammatory signaling could be the result of activation of different upstream pathways. While our studies do not definitively delineate a role for Bid-mediated programmed cell death in intestinal inflammation, they do suggest that modulation of programmed cell death affects intestinal inflammation. Further study is needed to clarify what role Bid might play in this process.

Inflammatory bowel diseases are mediated by increased inflammatory signaling within the intestine. Several studies implicate increased cytokine signaling in patients with IBDs including through increased expression of TNFα, IL-6, and IL-1β. Signaling through TNFα can promote both an apoptotic and necroptotic death. Additionally, both apoptotic and necroptotic death is implicated in the pathology of IBDs suggesting that understanding how programmed cell death functions in IBD pathology may be a plausible target in treatment of this disease. Our studies with IBD samples suggest that necroptotic signaling may play a role in involved tissues in Crohn's Disease and Ulcerative Colitis that is lost with transformation to malignant state.

However, further study to understand if this is an activation of Rip1 and if necroptotic signaling is occurring is needed to understand that status of necrotic signaling in these samples.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Summary of findings

Loss of Bid removes restraint of programmed necrosis perturbing hematopoietic homeostasis

The canonical role of Bid as a potent activator of apoptosis is well-established in the field of apoptosis, however my studies suggest that Bid also functions to inhibit the activation of necroptosis. This finding of an alternative role for Bid is not unprecedented, as previous studies from our lab and others suggest alternative functions for Bid in the DNA damage response and in innate immune signaling (*7*, *9*, *10*, *150*, *173*). Through investigation of *VavBaxBakBid* mice, which removed Bid as well as its apoptotic arm of function, I found that Bid restrains necroptosis in hematopoiesis leading to a bone marrow failure phenotype and decreased survival in mice. This phenotype is distinct from *VavBaxBak* mice, which have hematopoietic cells that lack the ability to undergo apoptosis and as a result develop lymphoid leukemia and lymphoproliferative disease. This previously unknown function implicates a novel role for Bid in determining the cell death fate following stimulation of death and Toll-like receptors.

Characterization of Vav TKO mice through complete blood counts, pathological characterization, immunophenotyping, and competitive bone marrow transplantation revealed their phenotype was very similar to the human disease myelodysplastic syndrome (MDS). Complete blood counts revealed decreases in red blood cell/ hemoglobin and platelet counts suggesting deficiencies in hematopoiesis. Additionally, examination of the bone marrow revealed increased necrosis in the bone marrow of TKO mice through examination of Rip1

expression and morphological characteristics through transmission election microscopy. This creates a hostile environment with the release of DAMPs and inflammatory cytokines within the bone marrow milieu. TKO mice additionally demonstrate dysplasia within several myeloid cell types similar to those seen in MDS, which include hypersegmentation of neutrophils, and hypolobulation of megakaryocytes suggesting this environment additionally perturbs differentiation. I further examined the hematopoietic system through characterization of hematopoiesis by immunophenotyping by flow cytometry. Examination of terminally differentiated cell types revealed minimal perturbation, as only T cell populations were significantly increased in DKO and TKO mice, whereas B cell, monocyte, and erythroid populations were not significantly different. However, examination of less mature populations revealed stark differences between TKO mice and *Bid* +/+, *Bid* -/-, and DKO mice. The hematopoietic system is highly responsive to insults, and as a result the HSC and progenitor populations are perturbed in TKO mice. The hematopoietic stem cell (HSC) populations were significantly increased in TKO mice as a result of compensatory proliferation as expected due to increased necrosis of the bone marrow. Surprisingly, the myeloid progenitor populations were significantly decreased in both DKO and TKO populations, however lack of a corresponding increase in the HSC population in DKO mice suggests a different hematopoietic defect. The decrease in TKO myeloid progenitor populations suggests that this population is particularly susceptible to the hostile environment created by the increased necrosis of the bone marrow. Reconstitution assays revealed that the presence of TKO cells in a *Bid* +/+ mouse promotes increased inflammation particularly in the lungs mediated by TNFα. My studies further revealed that TKO cells produced increased TNFα following necrotic stimulation, suggesting TKO cells have increased signaling through this pathway.

I utilized competitive transplant assays to examine the ability of TKO cells to function and reconstitute the hematopoietic system in competition with *Bid* +/+ cells. Because TKO mice die of bone marrow failure without any stimulation, I hypothesized that TKO cells would fail to compete against *Bid* +/+ cells within a *Bid* +/+ mouse. Surprisingly, TKO cells outcompeted these cells and continued to do so throughout the course of the experiment for 5 months. Immediately after this time period, these mice began to die of bone marrow failure phenotype which also demonstrated increased inflammation. This result was surprising at the time, but was consistent with the original TKO phenotype which promotes increased TNFα signaling. Immunophenotyping of transplanted mice revealed similar perturbations seen in the bone marrow of TKO mice. HSC populations were increased within the transplanted TKO population, and progenitor populations were decreased as compared to *Bid* +/+ derived populations. Additionally, a secondary transplantation reveals that TKO HSCs have decreased function, as TKO bone marrow fails to outcompete *Bid* +/+ cells a second time, while DKO bone marrow continues to outcompete *Bid* +/+ bone marrow. From these findings we hypothesized that 1) myeloid progenitors are susceptible to increased TNFα signaling in the bone marrow, 2) that increased necrosis in the bone marrow propagates a feed-forward TNFα signaling increase, as a result of increased TNFα production by TKO cells, and 3) TKO mice likely die of bone marrow failure resulting from stem cell exhaustion due to increased cycling in order to replace cells lost by necrosis, and that this is distinct from the defect in hematopoiesis in DKO mice.

The phenotype of TKO mice was very similar to the human disease MDS, in particular the refractory cytopenias with multilineage dysplasia (RCMD) subtype. MDS is characterized by unrestrained programmed cell death in the bone marrow leading ineffective hematopoiesis and potential transformation to acute myeloid leukemia. For more than two decades, the death in

MDS has been implicated as an apoptotic death. However, my studies very clearly implicate necrotic death in the bone marrow failure phenotype of TKO mice. In examination of the type of cell death occurring in MDS patient samples, I found significant expression of Rip1 and phospho-MLKL in several subtypes of MDS samples, particularly the RCMD subtype. Every sample of RCMD within our cohort stained positively for both Rip1 and phospho-MLKL, suggesting that these proteins could serve as markers for these subtypes in diagnosis. Conversely, staining for cleaved Caspase-3 was minimal, with one to two positive cells in few samples. These findings suggest that there is increased necroptotic signaling in MDS samples, as opposed to apoptotic signaling. One limitation of this finding is that I have only examined expression of Rip1 instead of phospho-Rip1, the activated form. While the degree of Rip1 staining suggests that necroptotic death is occurring, this staining must be repeated with a phospho-Rip1 antibody to definitively show this.

In summary, my studies demonstrate the effect of unrestrained necrosis on hematopoietic homeostasis, a context previously uncharacterized within the literature. I show that loss of Bid in addition to Bax and Bak (TKO mice), but not Bax and Bak alone (DKO mice), promotes unrestrained necrosis of the bone marrow in mice. My studies implicate a role for Bid independent of Bax and Bak in the restraint of programmed necrosis. This finding places Bid at the crossroads of apoptosis and necroptosis execution, much like other apoptotic proteins such as Caspase-8 demonstrated to play a role in necroptosis inhibition (*78*). It additionally is the first implication of a member of the BCL-2 family in the regulation of necroptotic death, a novel noncanonical function distinct from their canonical role in apoptotic death. My studies also implicate necroptotic death in the pathology of MDS, which has not previously been explored in this disease. These studies provide the foundation for the use of TKO mice as a mouse model of

MDS to better understand it's pathology, potentially identify novel markers of certain MDS subtypes, and also new potential therapeutic targets of MDS in the targeting of necroptotic death.

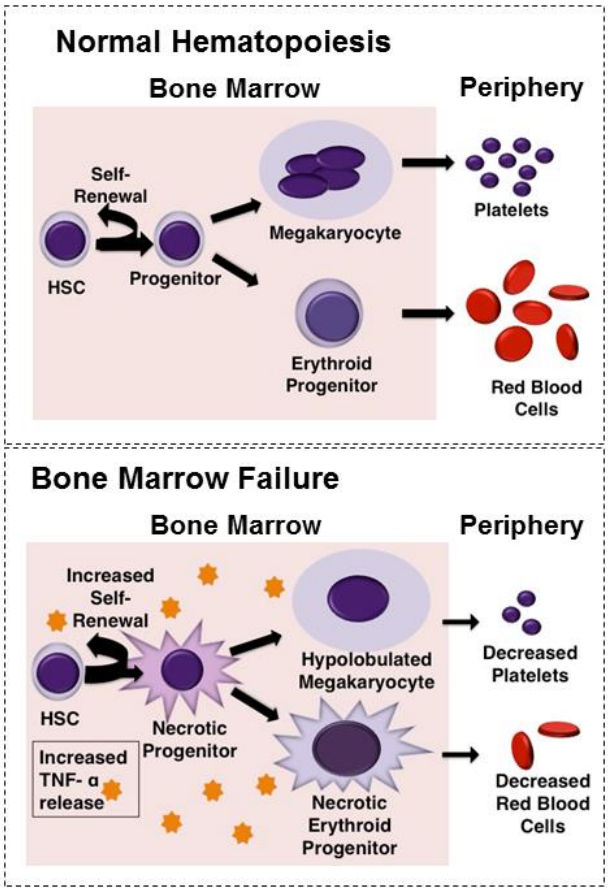


Figure 5.1 Loss of Bax, Bak, and Bid promotes bone marrow failure in mice as a result of increased TNFα expression that promotes necrosis of the bone marrow
Studies of *VavBaxBakBid* TKO mice reveal bone marrow failure as result of increased necrosis of the bone marrow. Increased TNFα expression perturbs normal hematopoiesis causing necrotic death of myeloid progenitor populations and dysplasia, leading to low peripheral counts.

Bid modulates Caspase-8 activity towards Rip1

In finding a role for Bid at the physiological level in restraint of necrosis my next set of studies sought to understand the role of Bid in necroptosis at the molecular level. To understand this, I developed myeloid progenitor cell lines from the bone marrow of our mice. These myeloid progenitor cells (MPCs) served as a medium to understand necroptotic signaling following death receptor and Toll-like receptor signaling. From these studies, we find a role for Bid in the restraint of necroptotic signaling through modulation of an IETD-inhibitable activity towards Rip1. While we favor this activity to be mediated by Caspase-8, we also have data to suggest that Granzyme B is involved in this process. Our mechanistic studies, informed by mathematical modeling studies (data not shown) suggests that Bid performs this function in an intermediate complex between complex I and II. Our studies shed light on where in the necroptotic pathway Bid functions, however, more questions remain, including which proteins Bid directly interacts with? How does Bid's presence modulate Caspase-8 and/or Granzyme B activity? Is there a modification of Bid (e.g. phosphorylation) that inhibits its cleavage by Caspase-8 to inhibit apoptosis?

Critical to the completion of these studies, I generated *MxBaxBak* DKO and *MxBaxBakBid* TKO MPCs from DKO and TKO bone marrow. Along with *Bid* +/+, *Bid*-/- MPCs, I demonstrated that deficiency for proapoptotic proteins in these Type II cells yields in the expected pattern of death through examination of their ability to die in response to TNFα to stimulate the TNFR plus Actinomycin D to block survival signaling (TNF/ActD). (Loss of Bid or Bax and Bak yields protection from death, whereas cells wildtype for Bid or deficient for Bax, Bak, and Bid have increased death) These studies solidified that these cells could serve as a tool to understand primarily apoptotic (*Bid* +/+), inhibition of apoptotic (DKO), and necroptotic

(TKO) signaling. We next evaluated necroptotic signaling in each of these cells lines, and found that signaling through the necroptotic pathway was increased with or without stimulation, suggesting loss of Bid and its apoptotic arm of function removes restraint on necroptotic signaling. Throughout these studies, we noticed that the levels of Rip1 varied dramatically between genotypes, with DKO MPCs having significantly less full length Rip1 as compared to TKO MPCs, which had more than *Bid* +/+ MPCs. We hypothesized that this might be due to degradation of Rip1 mediated by Caspases (or other proteases such as Granzyme B or Cathepsins) and/or the ubiquitin/proteasome system. We ruled out the role of Cathepsins, the ubiquitin/proteasome pathway, and other Caspases (through treatment with a pan Caspase inhibitor) in the degradation of Rip1. Our studies instead revealed a role for Caspase-8 and/ or Granzyme B in this process, suggesting that the presence or absence of Bid potentiates the degradation of Rip1.

These studies were followed up with investigation into the location of Bid's function within the necroptotic pathway. To examine this, we performed an immunoprecipitation (IP) for FADD, as this is demonstrated to allow for exclusive examination of Complex II downstream of death receptor/TLR signaling. Our studies revealed that in the absence of Bid and its apoptotic arm of function, Rip1 presence was increased within Complex II, suggesting increased necrotic signaling was occurring. This was strikingly different from the result in DKO MPCs, which demonstrated little to no presence of Rip1 in this complex. While other expected members were also present within this complex, including Caspase-8 and c-Flip, Bid was not. This suggested that Bid may instead function more upstream in the necroptotic pathway before Complex II, as Rip1 levels were greatly increased within Complex II in TKO MPCs. We next performed an IP for Bid, and found that Bid formed a complex containing Bid, Caspase-8, c-$Flip_L$, and Rip1.

Collaboration with the Dr. Carlos Lopez (VUMC) aided in modeling this process, and suggested that this complex was intermediate between Complex I and Complex II.

My studies provide a solid foundation for Bid's function in the necroptotic arm of signaling downstream of death and TLR receptors, however several questions remain. For instance, how is it that Bid modulates the activity of Caspase-8 such that it is more specific toward Rip1? Previous studies from the lab of Guy Salvesen suggest that Caspase-8 can form homodimers with itself or heterodimers with $cFlip_L$. Additionally, these studies suggest that the substrate specificity differs between homodimers and heterodimers, with heterodimers having decreased specificity toward the canonical apoptotic substrate Bid (*80*). Further understanding of the interaction between these proteins, in particular between Bid, Rip1, and Caspase-8 will likely provide some insight into how Caspase-8's substrate specificity is affected within a heterodimer with $cFlip_L$. Another question of interest is if there is a post-translational modification of Bid that potentially inhibits its processing by Caspase-8, such as a phosphorylation event (*172*). Previous studies also suggest that Bid is phosphorylated by ATM in the DNA damage response, and that this phosphorylation event is required for its role in this process (*7, 9, 150, 173*). While my initial studies did not reveal phosphorylated Bid following IP, there are further methods that can be utilized to interrogate a modification of Bid in necroptotic signaling.

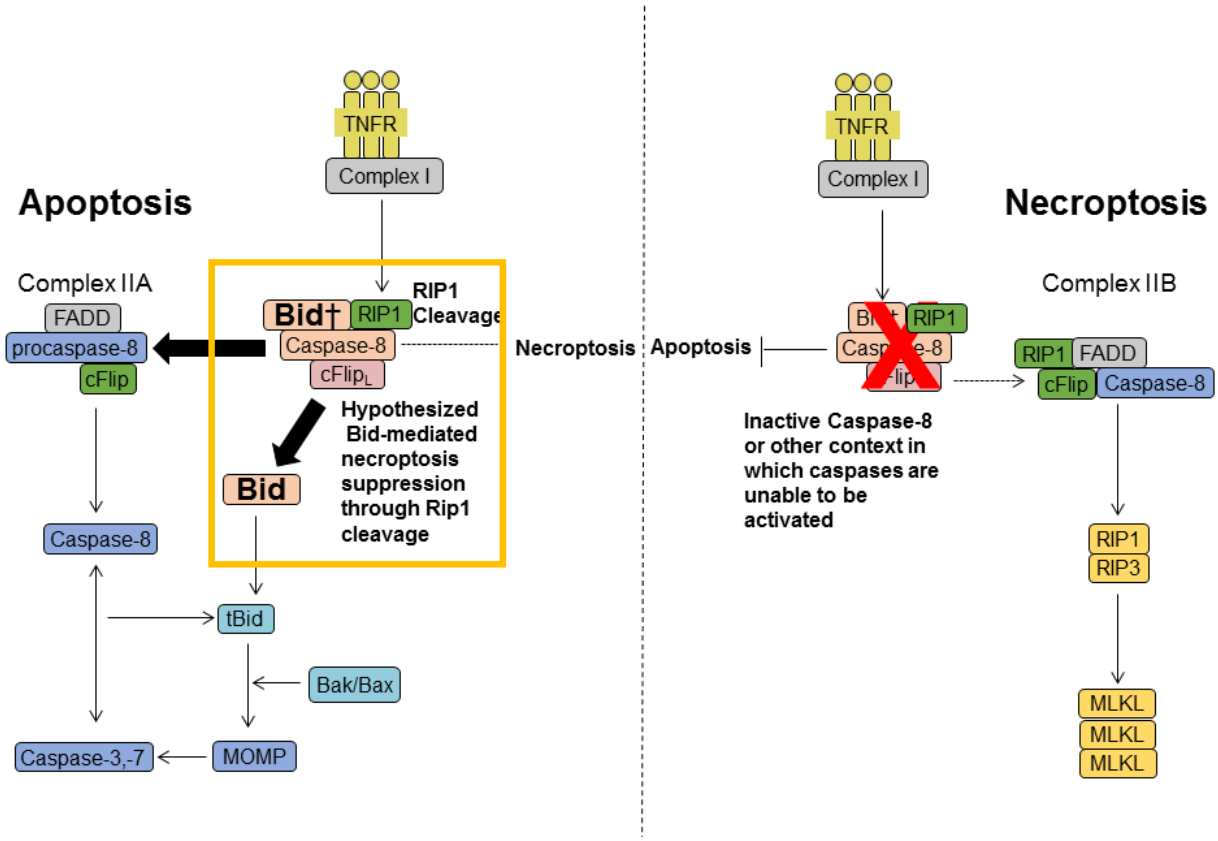


Figure 5.2 Proposed role of Bid in necroptotic signaling downstream of death receptors
Death receptor signaling can result in two types of programmed cell death, apoptosis and necroptosis. The role of the BCL-2 family member Bid, in the promotion of apoptosis is well-established, however my studies suggest a novel role for Bid in the inhibition of necroptosis as well. My studies suggest this inhibition of necroptosis is mediated through cleavage of Rip1 in an intermediate complex with Caspase-8, cFlip$_L$, and Bid. While further study is needed to understand how this complex forms, I hypothesize that key interactions, as well as an alternative state of Bid (e.g. phosphorylation) are important in the formation and catalytic activity of this complex.

Potential role for Bid in intestinal inflammation

In my initial studies with *MxBaxBakBid* TKO mice, I found that loss of these three proteins promotes necrosis of the liver, often leading to lethality. With this finding, I hypothesized that loss of these three proapoptotic proteins may play a role in necrotic death which frequently causes inflammation. However, while necrotic death is known to promote inflammation regardless of cell type, increased or excessive apoptotic death is also purported to promote increased inflammation in the setting of the intestine (*580*). In order to explore how apoptotic or necroptotic death might promote inflammation of the intestine, I wanted to subject these mice to an inflammatory stimulus within the intestine to better understand how loss of these proteins potentiates necrotic signaling. To do this, I utilized the acute DSS colitis model to explore how loss Bid and its apoptotic arm of function might promote necrosis and inflammation in the intestine.

My studies primarily revealed increased inflammation in both DKO and TKO colons following DSS, suggesting that loss of these proapoptotic proteins promotes increased inflammation in response to DSS. While pathological scores were not different amongst all genotypes of mice tested (*Bid* +/+, *Bid* -/-, DKO, and TKO), endoscopy reveals increased inflammation in DKO and TKO colons. However, the inflammation occurring in DKO and TKO colons appeared to be visually different, in a manner that was not captured by the pathological scoring method. To attempt to further understand the role of inflammatory signaling, we performed a Luminex ELISA on protein extracts from colon sections to examine the levels of several cytokines. This assay revealed similar levels of increase in inflammatory cytokines, with the TKO having slightly higher levels of cytokine expression as compared to DKO. This finding may be due to sampling error as section of colon were taken randomly at sacrifice, and

inflammation within the colon after DSS is patchy. However, examination of proteins involved in different death signaling pathways revealed increases in Rip1 in TKO but not DKO colons. This suggests that epithelial cells in DKO and TKO colons are executing different pathways to death.

My studies provide evidence that loss of Bax and Bak, or Bax, Bak, and Bid promote increased inflammation in response to intestinal epithelial cell cytotoxicity. While these studies provide initial evidence that loss of apoptosis or loss of necroptotic restraint can promote increased inflammation, there are limitations to my studies: 1) A small limitation is that we are interested in understanding inflammation in intestinal epithelial cells, but have utilized mice that have a germline deletion of Bak and Bid, but not Bax. We have utilized *MxCre* to promote deletion of Bax deletion in any hematopoietic cells present in the colon, and should also promote deletion of intestinal epithelial cells. However, this Cre can be inefficient, although we anticipated that the primary inflammatory effect would be mediated by hematopoietic cells. 2) Additionally, while we hypothesize the death in the epithelial cells of the colons of DKO and TKO mice die differently, we have not specifically identified how these cells die. Staining for markers of apoptosis (cleaved Caspase-3) and necroptosis (Phosphorylated Rip1) along with a nuclear stain and/or electron microscopy studies are needed to definitively classify death in the colons of these mice. While these studies hint at a role for the BCL-2 family in intestinal inflammation and a role for necroptosis in IBDs, further studies are needed to understand the full extent of involvement.

In summary, my studies demonstrate a novel role for Bid in the inhibition of necroptosis in hematopoiesis. I established this role for loss of Bid and its apoptotic arm of function in the perturbation of hematopoietic homeostasis through characterization of a mouse deficient for *Bax*,

Bak, and *Bid* developed within our laboratory. From my studies, I determined that increased TNFα signaling promotes inflammatory signaling that specifically affects the HSPC compartment and eventually leads to bone marrow failure due to overwhelming necrosis of the bone marrow, with a particular effect on the myeloid progenitor and HSC populations. These perturbations of homeostasis within the bone marrow manifest in the animal through decreased peripheral RBC and platelet counts, as well as dysplasia in multiple myeloid lineages. Additionally, studies of TKO mice reveal that their phenotype of bone marrow failure is very similar to the human disease MDS. My findings suggest that TKO mice could be utilized as a mouse model of MDS in order to provide insights into this disease including methods of diagnosis, through examination for positivity of Rip1 and/ or phospho-MLKL, and new therapeutic targets, such as targeting cell death in the treatment of this disease. Additionally, this finding adds another novel, alternative function for Bid, reinforcing the idea that members of the BCL-2 family participate in other cellular functions aside from apoptotic programmed cell death. Together these insights contribute knowledge to the field of programmed cell death within the emerging field of programmed necrosis, with potential implications for human disease.

Future Directions

Exploring the role of Bid and programmed necrosis in hematopoiesis

My studies provide a solid role for Bid in the inhibition of necroptosis and explores how increased necrosis in the hematopoietic system perturbs hematopoietic homeostasis. However, several questions remain in understanding the mechanism of how increased necrosis actually affects hematopoiesis. I preliminarily explore this through examination of TNFα expression in

the bone marrow of TKO and TKO transplanted mice and through treatment of mice with an inhibitor for TNF signaling, Enbrel. Although I have not included these studies within this dissertation, I have also examined lysate from whole bone marrow treated with LPS by Luminex ELISA, as well as LSK and myeloid progenitor populations for cytokine expression by flow cytometry (Data not shown). These studies reveal increases in TNFα, IL-6, and IL-1β. Utilization of pharmacologic inhibitors for other inflammatory cytokines (Such as IL-6 and IL-1β) or inhibitors of necroptosis (Necrostatin-1 or 7N-1) would provide further insight into the role of the role of inflammatory signaling and necroptotic signaling in the bone marrow failure of TKO mice. This in turn could provide insight into potential therapeutics for MDS, as initial studies with an inhibitor of TNF did not reveal promising responses (*568*).

Another method to explore the role of necroptosis in the TKO phenotype is to inhibit necroptotic signaling through crossing *VavBaxBakBid* mice to mice with knockins of the kinase dead (KD) version of Rip1 or Rip3 to evaluate the role of Rip kinase mediated necrotic signaling. Our hypothesis would be that a Rip1-KD knock-in mouse would be optimal to block the bone marrow failure phenotype, as our mechanistic studies suggest that the phenotype is mediated through Rip1. However, because Rip1 is also implicated in inflammatory signaling, and Rip3 in apoptotic signaling, this cross may produce alternative results.

Necroptotic signaling in MDS

The bone marrow death in MDS is purported to be apoptotic in nature. However, the data presented in the literature examines samples in less than optimal conditions or utilized methods of death detection which do not distinguish between apoptosis or necroptosis (*491*, *493*, *496*, *497*). Conversely, my initial studies with samples of MDS suggest that this death is instead

occurring through necroptosis. One limitation of this conclusion is that we have examined these samples for expression of all Rip1 species, but not for phospho-Rip1 specifically. In order to firmly establish a role for necroptosis in MDS it would be necessary to firmly establish increased necroptotic signaling (increased Rip1 phosphorylation) as well as to examine the morphology of MDS cells through transmission electron microscopy. Although this was previously explored, I think it would be useful to continue to evaluate the status of cleaved Caspase-3 in MDS patients to establish that apoptosis, while potentially present in MDS samples, is not the primary method of programmed cell death in the pathology of this disease. Another limitation is that we examined a small cohort of 26 samples, with only a single sample of two of the subtypes. In order to establish that necroptosis is the means by which death occurs in MDS overall, it will be important to examine a much larger cohort of MDS samples with representation of all subtypes to evaluate the role of necroptosis in pathology of certain subtypes.

Understanding if necroptosis plays a key role in the pathology of the MDS provides great potential to target this programmed cell death pathway in treatment of this disease. About one-third of patients with MDS will transform to an acute myeloid leukemia, and will typically be less responsive to treatment. I hypothesize that blocking necroptotic death could potentially stop the cycle of death in the bone marrow, in turn blocking the self-renewal and proliferation of HSPC populations, and preventing the production of new mutations. Additionally, because there is only one curative therapy for MDS that is often difficult to receive (Allogeneic bone marrow transplantation), there is a great need for new therapeutics for the treatment of this disease. Thus, targeting programmed cell death could be of great therapeutic value in MDS treatment.

How do Bid, Rip1, and Caspase-8 interact to inhibit necroptotic signaling?

Utilizing cell lines developed from the bone marrow of *Bid* +/+, *Bid* -/-, DKO, and TKO mice, we found that 1) loss of Bid, Bax, and Bak in MPCs leads to increased presence of Rip1 overall and within the pronecrotic complex IIB, 2) that TKO MPCs additionally have increased downstream necrotic signaling, 3) that loss of Bax and Bak alone leads to decreased Rip1 expression and increased Bid expression, and 4) that Bid forms a complex with Rip1, Caspase-8, and $cFlip_L$ that promotes the cleavage and degradation of Rip1, inhibiting necroptosis. Each of these proteins have existing interactions with each other, however it not clear how Bid and Rip1, Rip1 and Caspase-8, and Caspase-8 and Bid (in the setting of necroptosis inhibition) interact, and in what state these proteins are in, to promote an outcome of Rip1 degradation, and maintenance of Bid in the setting of Caspase-8 activity. While the Caspase-8:$cFlip_L$ is already established, the interactions between the remaining members of this complex is not known, although one could speculate that a novel interaction between Bid and Rip1 is a key factor in the organization of this complex. Another factor that should also be explored is the presence of other proteins within this complex. I hypothesize that this complex is intermediate between complex I present at the membrane, and complex II within the cytosol. There are many proteins present in complex I that may also be present in this intermediate complex with Bid which we did not explore, such as cIAPs and TRAFs that could potentiate the outcome of Rip1 stability. Thus this possibility should be explored to ensure a clear picture of the mechanism and the proteins involved in this process.

Understanding how Bid potentiates Caspase-8 activity toward Rip1

My studies suggest that Bid may modulate the catalytic activity of Caspase-8 and/ or Granzyme B in a manner that increases protease activity towards Rip1. One way to understand this is to understand how these proteins interact, and what other proteins might be involved. Additionally, examination of the catalytic activity of this complex in the presence of known involved proteins *in* vitro would be critical to evaluate the catalytic activity of Caspase-8. A common method utilized to understand the proteolytic activity of Caspases is to perform an assay utilizing purified proteins included activated versions of the Caspases of interest (*591*). Utilizing this method, and armed with the understanding of the state of each member of the complex (e.g. post-translational modification or activation) would aid in understanding how protease activity is modulated toward Rip1. Based upon data from our studies, as well as through collaboration with our mathematical modeling colleagues, we found that Bid is likely in an alternative state within this complex. Previous studies would suggest that this could be a phosphorylation event, however further study of Bid within this complex is needed to understand what, if any modifications occur and if this modification of Bid is required in the activation of this complex. Another state that is likely required in this complex is the formation of a Caspase-8:cFlip$_L$ heterodimer, a catalytic complex demonstrated to have altered substrate specificity (*80*). In the setting of these modifications, the catalytic activity of this complex can be evaluated through an *in vitro* assay utilizing purified proteins (*592*). However, examination in this manner has limitations as Caspases molecules would be activated in an artificial manner, and the use of purified proteins, versus examination within cell lysate (which is possible, but more difficult) may not provide the full story regarding how activity is altered within this complex. However,

despite these limitations, this type of experiment could provide great insights so that we can begin to better understand the novel role of Bid in this pathway.

Exploring the role of Bid and the BCL-2 family in intestinal homeostasis and IBDs

My studies with *MxBaxBakBid* mice with the acute DSS injury model were less clear in understanding what role loss of Bid and its apoptotic arm of function might play in intestinal homeostasis and injury. While these studies were inconclusive in demonstrating that loss of Bid, Bax, or Bak potentiates the response to colonic injury, they did provide some clues as to how loss of apoptosis (DKO mice) versus loss of restraint of necroptosis (TKO mice) may affect the consequences of colonic injury. For instance, DKO and TKO mice, while exhibiting a similar cytokine profile, displays differences in the pattern of expression of markers for apoptosis and necroptosis. These differences suggest altered consequences of colonic injury in these different genotypes of mice. However, in order to parse these differences out several limitations of my initial studies must be addressed.

My studies utilize *MxCre* to promote the deletion of Bax in DKO and TKO mice. *MxCre* promotes the deletion of Bax in hematopoietic cells, including the in the spleen and the bone marrow, as well as the liver, kidney, and expression in the epithelium of the intestines (*593, 594*). However, it is possible that Cre recombination is incomplete in all tissues, as efficiency is not always 100%. It would be useful to explore the degree and location of Bax deletion in the intestinal epithelium to ensure that Bax is deleted. Alternatively, future experiments could utilize *VillinCre* or *VillinCreERT2* ,expressed within the intestinal epithelium within the small intestine and colon, to promote the deletion of Bax embryonically, or conditionally in mature animals with tamoxifen treatment, respectively (*595, 596*). However, because it is unclear whether increased

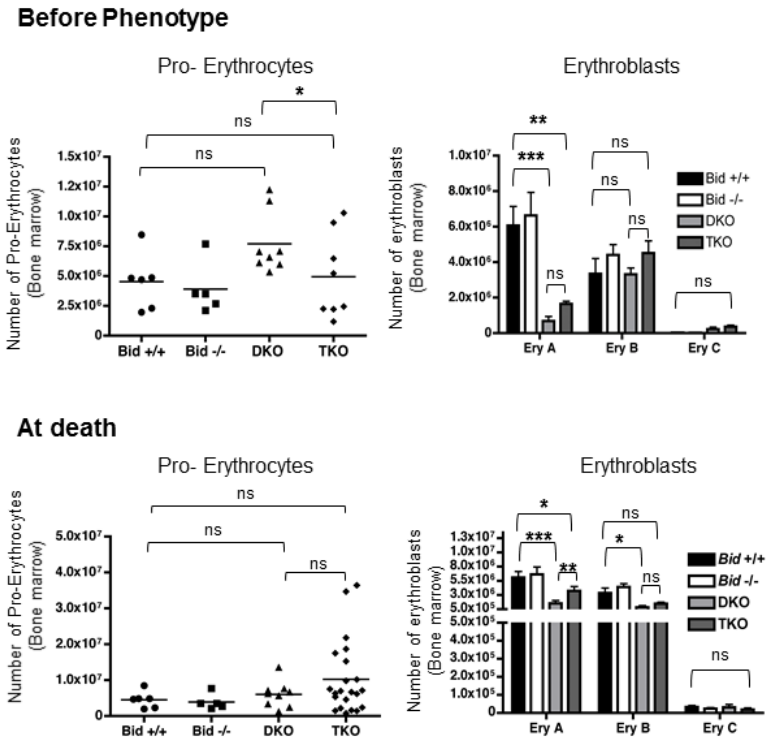
inflammation in this model is caused by intestinal epithelial cell death or hematopoietic inflammatory cells, it is unclear how significant use of an alternative Cre recombinase would be.

Another key limitation of these studies, is that it is difficult to make a true conclusion regarding cell death in the setting of DSS injury and IBDs. While I have performed immunohistochemistry to examine if death in these samples was occurring through apoptosis or necroptosis, it is difficult to determine how these cells are dying due to our examination of all species of Rip1. To truly understand the role of necroptosis in this process it is imperative to evaluate if inhibition of necroptosis can block the inflammatory phenotype in TKO mice. Understanding the status of necroptotic signaling in IBDs could provide insight into the pathology of these diseases, and potentially provide a therapeutic target in the blockage of cell death occurring as a result of inflammation.

In my dissertation, I have completed studies to understand a novel role for Bid in the inhibition of programmed necrosis in the setting of hematopoiesis and intestinal homeostasis and inflammation. My studies in hematopoiesis reveal that increased necroptosis perturbs hematopoietic homeostasis leading to bone marrow failure in mice that is similar to a human disease, MDS. While my studies in the intestine were less conclusive, they do suggest that the BCL-2 family and/or programmed cell death may affect intestinal response to insult and maintenance of homeostasis. Further studies are needed to better understand these possibilities. While my studies have focused on the hematopoietic and gastrointestinal systems and how necroptosis dysregulation might be involved in related human diseases, there are a number of other settings in which necroptotic death and inflammation lead to human pathologies. My studies demonstrate how alterations in necroptotic regulation can contribute to the pathophysiology of disease and provide insight into how these diseases can be therapeutically

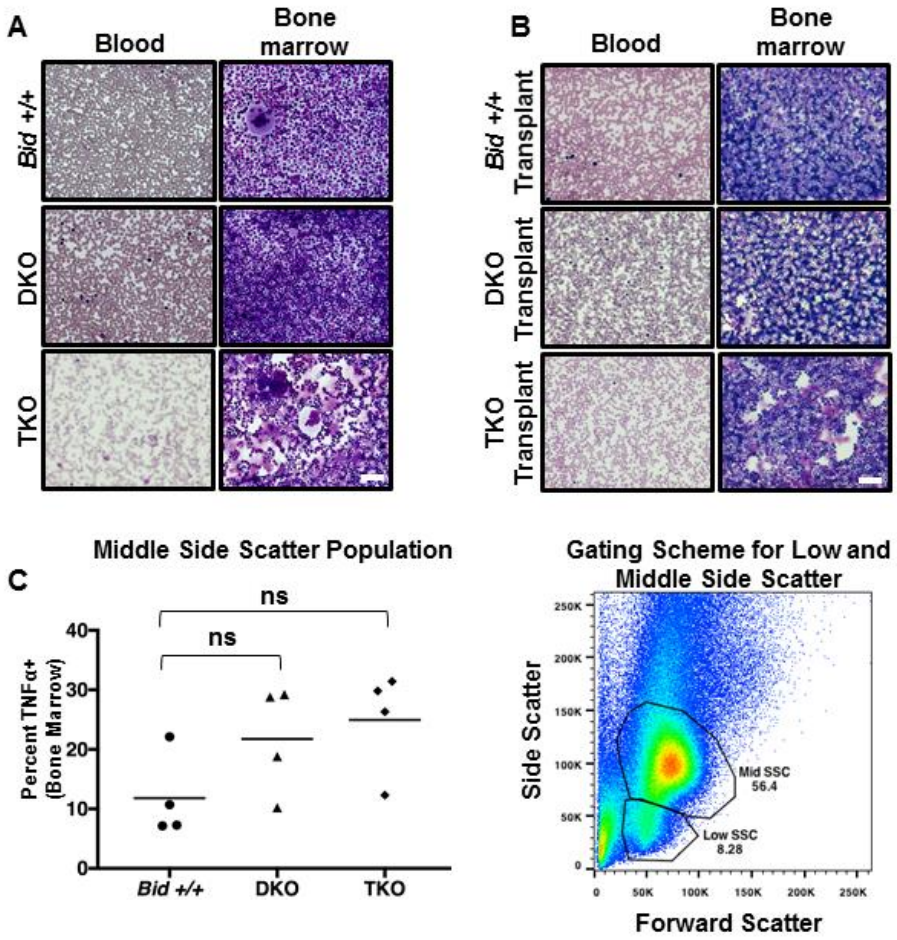
targeted. Additionally, these studies also provide novel insight into the role of the BCL-2 family in necroptotic regulation, a previously unrecognized role.

APPENDIX



Appendix A. Examination of erythroid cell development in *Bid*+/+, *Bid*-/-, DKO, and TKO mice

Because TKO mice have drastic decreases in RBCs, we hypothesized that erythroid development would be greatly affected. However, in addition to loss of myeloid progenitor cell populations, both DKO and TKO mice demonstrate decreases in the erythroblast A population (basophilic erythroblast) before phenotype manifestation, and also at time of sacrifice after phenotype manifestation. Interestingly, Pro-erythrocyte populations differ between DKO and TKO before phenotype manifestation, but are no longer significant at the time of phenotype manifestation.



Appendix B. TKO transplanted mice display similar dysplasia to *VavBaxBakBid* mice and *VavBaxBakBid* bone marrow does not display significant differences in TNFα positivity
A) Blood and bone marrow smears from *Bid* +/+, DKO, and TKO mice. B) Blood and bone marrow smears from *Bid* +/+, DKO, and TKO transplant mice. Scale bar indicates 50 microns. C) Examination of TNFα in the middle side scatter population in bone marrow from *Bid* +/+, DKO, and TKO mice. *Bid* +/+ n=4, DKO n= 4, TKO n=4.

Materials and Methods

Mice

VavCre BaxBakBid and *MxCre BaxBakBid* null mice were developed in our laboratory by crossing *VavCre+* or *MxCre+* mice with *Bax F/F Bak -/-* mice then subsequently crossing these Cre+ *Bax F/F Bak -/- Bid* mice to *Bid -/-* mice. *VavCre* and *MxCre BaxBak* null mice were generated through crosses of *Bax F/F Bak -/-* mice with *VavCre+* mice. Mice were backcrossed 8 generations with C57/BL6 mice from Jackson Laboratories. *MxCre BaxBakBid* mice were induced to delete *Bax* with three injections with Poly I;C (GE Healthcare; 27-4732-01) at a dosage of 200 mg/kg. Blood was collected in microcontainers with EDTA (365973; BD Pharmingen). Complete blood counts completed using a Hemavet 950. Cytospins were completed using a Shandon Cytospin 4 and stained using a Protocol HEMA 3 Stat pack (Thermo Fisher Scientific; 123-869). For Enbrel experiments: Mice were treated with two doses of Enbrel (eternacept) one week apart at a dose of 50mg/kg. Complete blood counts and analysis were performed one week after last dose of Enbrel was given. The Vanderbilt University Institutional Animal Care and Use Committee approved all experiments.

Myeloid progenitor cell lines

Myeloid progenitor cells (MPCs) were generated as previously described (Zinkel, *et al.*, 2003). Briefly, whole bone marrow was removed from sex-matched 6 to 8 week old mice and depleted of mature populations utilizing biotinylated antibodies from BD Pharmingen for B220 (553086), Ly6G and C (553125), CD127 (555288), Ter119 (553672), and CD3 (553060). The cells were then *Hox11*-immortalized by growing on irradiated MEFs expressing *Hox11*. Cells were grown in IMDM (Iscove's modified Dulbecco Medium) supplemented with 20% Calf-serum, WEHI-

conditioned medium as a source of IL-3, 100U/mL penicillin/streptomycin, 2mM Glutamine, and 0.1mM β-mercaptoethanol (IMDM 20 WEHI). Media was further supplemented with G-CSF, GM-CSF, SCF, and IL-3. Cells were cultured at 37°C in 5% CO_2. After immortalization cells were grown in IMDM 20 WEHI.

Cell Death Analysis

For Annexin V/ PI staining, MPCs were collected at designated time points after treatment with 25 ng/ mL TNFα (T7539; Sigma) and 50 ng/mL Actinomycin D (A9415; Sigma). Cell viability was determined using Annexin V (1001; Biovision) and Propidium Iodide (Sigma) staining by flow cytometry. Briefly cells were stained in Annexin V staining buffer (10x Staining Buffer: 0.1M HEPES, pH 7.4; 1.4M NaCl; 25mM $CaCl_2$, Diluted to 1x before use) + Annexin V (500x, Diluted to 1x in Staining Buffer) and PI was added at 50 μg/mL just before analysis. Samples were run on a BD FACScalibur with CellQuest software and analyzed using FlowJo software (TreeStar). For cleaved Caspase-3 staining, MPCs were treated as in death assays, collected at designated time points and fixed, permeabilized, and stained per manufacturer's instructions (BD Biosciences). Cells were then stained with rabbit polyclonal Cleaved Caspase-3 antibody (Asp175) (9661; Cell Signaling), followed by anti-Rabbit Alexa Fluor 488 (A11008; Invitrogen) Samples were run and analyzed as above.

For electron microscopy, whole bone marrow or MPC samples were washed in a 0.1M cacodylate buffer (pH 7.4) then fixed with 2.5% glutaraldehyde + 0.1M cacodylate Buffer (pH 7.4) solution for 1 hour then overnight at 4° C. Samples were post-fixed in 1% osmium tetraoxide and washed 3 times with 0.1 M cacodylate buffer. The samples were dehydrated through a graded ethanol series followed by incubation in 100% ethanol and propylene oxide

(PO) with 2 exchanges of pure PO. Samples were embedded in epoxy resin and polymerized at 60°C for 48 hours. For each sample 70-80nm ultra-thin sections were cut and mounted on 300-mesh copper grids. Sections were stained at room temperature with 2% uranyl acetate and lead citrate. Samples were imaged on a Phillips T-12 TEM utilizing Tecnai interface software and an AMT 2k X 2k CCD camera to capture images. 50-100 cells were scored as being as alive, apoptotic or necroptotic based upon presence of morphological features representative of each condition. Cells were only scored if the nucleus could be visualized in the image.

Hematopoietic Characterization

For examination of hematopoietic stem and progenitor cell populations, analysis was performed on whole bone marrow. A power analysis was utilized to calculate the number of animals needed to analyze from each genotype. Cell suspensions were obtained by flushing the femurs and tibia with media. Erythrocytes were lysed using erythrocyte lysis buffer (100μM Tris pH 8, 157mM NH_4CL, + H_2O) and then samples stained with biotinylated antibodies obtained from BD Pharmingen (as above in generation of MPC lines) and fluorescent-conjugated antibodies from eBioscience CD117 (17-1171), Sca-1 (25-5981), Flt3 (15-1351), CD48 (11-0481), Streptavidin eFluor 450 (48-4317), and CD150 (12-1501) to analyze HSC populations. Samples were run on a BD LSRII flow cytometer with FACSDiva software and analyzed using FlowJo software (Tree Star). For examination of mature populations, single-cell suspensions were obtained from bone marrow as above and from spleen through dissociation through a filter. Cells were stained with antibodies from BD Pharmingen for CD3 (553062), B220 (553090), CD11b (553311), and Gr-1 (553127). Samples were run on a BD FACScalibur and analyzed using FlowJo software (Tree Star). For intracellular analysis of TNFα, single-cell suspensions of bone marrow were obtained

as above. Cells were cultured overnight in IMDM 20 WEHI. Cells were replated the next day with fresh media and treated with 200ng/ mL lipopolysaccharide (L4391; Sigma) and Golgi Plug per manufacturer's instructions (BD Biosciences) or Golgi plug alone for five hours. Cells were then fixed, permeabilized, and stained with TNFα according to the manufacturer's instructions (554419; BD Pharmingen). Samples were then run and analyzed as above. Numbers of cells were calculated through back-calculation of percentages from FlowJo analysis to the total number of cells counted when isolated.

Competitive Reconstitution Assay

Whole bone marrow (femur and tibia) from CD45.2 *Bid* +/+ and *BaxBakBid* TKO, and CD45.1 *Bid* +/+ mice was isolated and erythrocytes were lysed as above. Bone marrow was mixed in a 1:1 ratio with *Bid* +/+ CD45.1 bone marrow: CD45.2 experimental bone marrow then injected retro-orbitally into lethally irradiated CD45.1 *Bid* +/+ mice. Irradiation was performed in two doses five hours apart for a lethal 9 gy dose. Approximately 1 million cells were injected. Reconstitution was examined every 4 weeks beginning at 8 weeks post-transplantation. This was examined through retro-orbital bleeding of mice and separation of nucleated cells from red blood cells using lymphocyte separation medium. Nucleated cells were stained with antibodies for CD45.1 (553776) and CD45.2 (553772) from BD Pharmingen and analyzed as above. Upon sacrifice organs were placed in 10% formalin and were embedded in paraffin wax. Sections of lung tissue were utilized for TNFα staining.

Secondary transplantation was performed through collection of bone marrow from primary transplanted animals as above. Percent 45.2 positivity was determined and then number of cells needed to have 1:1 ratio with *Bid* +/+ 45.1 positive cells was calculated.

For secondary transplantation of leukemic*VavBaxBak* DKO bone marrow, bone marrow was collected from *VavBaxBak* DKO mice displaying the leukemia phenotype. *Bid* +/+ mice were sublethally irradiated at a dose of 5gy. Mice were retro-orbitally injected with 5 million bone marrow cells.

In Vivo BrdU Assay

A power analysis was utilized to determine the number of animals needed to analyze from each genotype. Mice were injected three times with BrdU over the course of 36 hours (once every 12 hours) with a total of 4mg of BrdU (550891; BD Pharmingen) injected. Whole bone marrow was isolated from mice four hours after the last injection and then lineage-depleted using antibodies for CD3, B220, Ly-6G, CD127, and Ter119 from BD Pharmingen (as mentioned above) with DynaBeads (11035; Life Technologies). All remaining cells were stained for stem and progenitor surface stains from eBioscience (CD117, Sca-1, Flt3, and CD150 as described above) and from BioLegend (CD48; 103432). Cells were lastly treated with DNase type I (Sigma; Cat: D5025) for 1 hour at 37°C and then stained for BrdU from BD Pharmingen (Cat: 556028). All remaining cells were run on a LSRII flow cytometer and analyzed using FlowJo software (Tree Star).

Fluorescent Immunohistochemistry

For mouse bone marrow analysis paraffin-embedded samples of whole tibia were fixed in formalin solution (10% formaldehyde) and then decalcified before embedding. Embedded sections were stained with either Rip1 (H-207)(sc-7886; Santa Cruz Biotechnologies, no antigen retrieval required) or Cleaved Caspase-3 (9661; Cell signaling). Signal was amplified utilizing Fluorescein-conjugated Tyramide Sample amplification kit (SAT701001EA; Perkin Elmer), an

Avidin/Biotin blocking kit (SP-2001; Vector laboratories), and a biotinylated anti-Rabbit antibody (BA-1000; Vector Laboratories). Samples were mounted with DAPI containing mounting buffer (P36941; Life Technologies) or stained with Hoescht (H21492; Life technologies) and mounted (P10144, life technologies). For TNFα staining in lungs from transplant mice, samples were fixed (as above) and embedded, then stained as above with TNFα (ab9739; Abcam). For human bone marrow aspirate, paraffin-embedded samples of human bone marrow aspirate were stained as above as well as with phospho MLKL (S358) (Ab187091;Abcam). Samples were imaged on a Nikon AZ100 microscope and images were captured using a Nikon DS-Ri1 color camera or on a Zeiss Axioplan microscope using a Hamamatsu ORCA-ER monochrome digital camera.

Immunoblot and Immunoprecipitations

For analysis of whole cell lysate, cells were lysed utilizing Lysis Buffer (25mM HEPES pH 7.5, 250mM Sodium Chloride, 2mM EDTA, 0.5% NP-40, 10% Glycerol, 1X Complete Mini Protease Inhibitor, EDTA free (Roche), 10mM β Glycerophosphate, 0.1 mM Sodium Orthovanidate, 10mM Sodium Fluoride, 1mM Sodium Pyrophosphate). Samples were denatured by boiling in Laemmli Buffer (containing β-mercaptoethanol), and then run utilizing SDS-PAGE. For MLKL trimerization, cells were lysed as above and lysates were prepared under non-denaturing conditions. Stimulation was completed with LPS (L4391; Sigma) and treatment with Z-IETD-FMK (550380; BD Pharmingen), Z-VAD-FMK (sc-311561; Santa Cruz Biotechnology), and MG132 (BML-PI102; Enzo Biologicals). Samples were immunoblotted with antibodies listed below.

For immunoprecipitation, cells were lysed utilizing lysis buffer (as above) and then IP was performed utilizing sepharose beads (17-0618-02; GE Healthcare) with α-FADD (M-19) (sc-6036; Santa Cruz Biotechnology) or Biotinylated α-Bid (AF860; R&D systems) with Streptavidin agarose beads were used (6923; Novagen) and then lysates were run by SDS-PAGE, transferred and immunobloted with antibodies specified below.

Immunoblotting was performed for Bid (developed by S. Korsmeyer laboratory), Caspase-8 (4927; Cell Signaling), c-FLIP$_L$ (H-150) (sc-8346; Santa Cruz Biotechnology), CYLD (GTX100228; GeneTex), FADD (S-18) (sc-6035; Santa Cruz Biotechnology), Rip1 (610459; BD Pharmingen), MLKL (LS-C323026; LifeSpan Biosciences), c-Flip (ADI-AAP-440-E; Enzo life sciences), and β-Actin (A5441; Sigma)

Retroviral Transduction

Retroviral supernatants were generated through transient transfection of 293T cells using Fugene (11814443001; Roche) to introduce a FLAG-HA-tagged *Bid* +/+ expression vector with a retroviral packaging vector. Bid was introduced into *BaxBakBid* KO MPCs by infection and was over-expressed.

Reverse- Transcription PCR to examine Bax deletion

RNA isolation was completed utilizing Trizol reagent (15596; Invitrogen) from single-cell suspensions of bone marrow and spleen prepared as above. Isolation was completed according to manufacturer's instructions. cDNA was prepared using GoScript Reverse Transcription System (A5000; Promega). PCR was performed utilizing primers Bax Forward (ACAGATCATGAAGACAGGGG) and Bax Reverse (CAAAGTAGAAGAGGGCAACC).

Treatment with Granzyme B Inhibitor

Cells were plated with fresh media on the previous day. The first day, cells were permeabilized with 7.5μg/μL Digitonin (dissolved in DMSO) for five minutes. Cells were then treated with 2μg of the Granzyme B inhibitor for 90 minutes in the culture medium. The cells were then placed in fresh media, and allowed to recover overnight (approximately 18 hours). Cells were then treated with LPS for 30 minutes. Afterward lysate was collected as above.

Treatment with DSS and pathological scoring

MxBaxBakBid and *MxBaxBak* mice were treated with three doses of 200μg/ ml of Poly(I:C) every other day for 6 days. Mice were left for two weeks after the last injection to allow for activation of Cre recombination and Bax deletion. Mice were then treated with a 4% DSS solution (14489;Affymetrix) in water for 6 days. Mice were weighed each day. On day 5, endoscopy was performed on a single mouse per genotype. Mice were sacrificed on day 6 and colons were collected for fixation. H&E stained sections were utilized for pathological scoring and which was performed by Dr. Kay Washington (VUMC).

Statistical Analysis

All analyses were completed using either GraphPad Prizm (GraphPad Software) or with the help of the Vanderbilt University Medical Center (VUMC) Center for Quantitative Sciences (Heidi Chen). Analysis of survival curve was determined through Kaplan-Meier log-rank test between all genotypes and DKO versus TKO to examine difference between all genotypes and specifically DKO and TKO. Analysis of cell numbers from mouse experiments (Lymphocyte,

progenitor, and stem cell populations) was analyzed by one-way analysis of variance to examine differences between genotypes. Examination of percent 45.2 reconstitution in competitive reconstitution assay (Primary and Secondary) was determined by two-way analysis of variance to examine differences in 45.2 reconstitution between genotypes at different timepoints after transplantation. Comparison of necrotic death by transmission electronic microscopy in bone marrow and MPCs was determined utilizing a chi-squared test on the proportions of the type of cell death. Analysis of intracellular cleaved Caspase-3 was completed utilizing a two-way analysis of variance to compare differences between genotypes at different timepoints post-stimulation. Analysis of Enbrel *in vivo* BrdU assay and complete blood counts completed through one-way ANOVA.

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