The Roles of the Bcl-2 Family Proteins in T Lymphocyte Development and Homeostasis

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Immunology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Throughout their development in the thymus and during their maintenance and the immunological response in the periphery, T cells rely on the regulation of classical apoptotic pathways to promote cell survival or death. Several proteins of the Bcl-2 family have been shown to be critical in thymocyte and T cell survival and consequently, in T cell function. Among these proteins, the antiapoptotic proteins Bcl-2 and Mcl-1 are critical for promoting T cell survival at multiple stages of the T cell “life cycle.” While these proteins have been reported to interact with several of the proapoptotic members of the Bcl-2 family, the specific interactions by which Mcl-1 in particular promotes T cell survival in vivo were not well understood. Further, how different stimuli (for example, cytokine signaling and T cell activation) modulate the specific functions of Mcl-1 had also not been thoroughly explored.

We utilized mouse models to dissect the roles of Mcl-1 at multiple stages of T cell development and function. We utilized conditional knockout and double knockout strategies to build genetic pathways for Mcl-1 activity during thymocyte development and in peripheral T cells under a variety of conditions. In the thymus, the major role of Mcl-1 is to inhibit the activity of proapoptotic Bak because the loss of Bak, but not the loss of proapoptotic Bax or Bim, rescued the survival of Mcl-1-deficient thymocytes at both the double negative and single positive stages. Further, we concluded that this role
is not shared with Bcl-2 because overexpression of Bcl-2 did not rescue DN or SP survival.

In peripheral T cells, the loss of Bak rescued T cell survival in the presence of IL-7, but not during conditions of cytokine withdrawal. Interestingly, the overexpression of Bcl-2 or the loss of Bim partially rescued the survival of T cells during cytokine withdrawal, indicating that Mcl-1 has dual roles in T cells: cytokine-dependent and cytokine independent. Additionally, we found that cytokines of the common gamma chain family have different effects on the activity of Mcl-1 due to the differential regulation of other proteins of the Bcl-2 family, most notably Bim.

Finally, we utilized a Bcl-2 reporter mouse model to examine the role of Bcl-2 in the establishment of CD8+ T cell memory to infection. Although it is known that Bcl-2 is dynamically regulated in response to activation, the importance of this regulation in the establishment of T cell memory is not yet clear. We show that a subset of effector T cells within a previously defined memory precursor population retained high Bcl-2 expression at the peak of the immune response. Using adoptive transfer of sorted effector T cells, we provide preliminary evidence that the cells with memory potential lie within a strict range of Bcl-2 expression. These studies indicate that the regulation of Bcl-2 is likely critical in establishing T cell memory and provide a platform for the future study of the factors that influence T cell memory.
Dedication

This is dedicated to my parents, for instilling in me a love of exploration. To Erica, for sharing it with me (not to mention, you know, like, clothes). And to Jason, with whom I will explore the future.
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List of Abbreviations

4OHT — 4-hydroxytamoxifen

AIF — apoptosis inducing factor

APAF-1 — apoptotic protease activating factor 1

APC — allophycocyanin

APC/Cy7 — allophycocyanin-Cy7

BAC — bacterial artificial chromosome

Bad — Bcl-2-antagonist of cell death

Bak — Bcl-2-antagonist/killer

Bax — Bcl-2-associated X protein

Bcl-2 — B cell lymphoma 2

BH — Bcl-2 homology

Bid — BH3-interacting-domain death agonist

Bim — Bcl-2 interacting mediator of cell death

BSA — bovine serum albumin

CAD — caspase-activated DNase

cFLIP — cellular FLICE-like inhibitory protein

CFU — colony-forming units

DD — death domain
DED — death effector domain
DNA — deoxyribonucleic acid
DN — double negative
DP — double positive
DTT — dithiothreitol
ER — estrogen receptor
ERK — extracellular signal-related kinase
ES (cell) — embryonic stem (cell)
EtOH — ethanol
FACS — fluorescence-activated cell sorting
FADD — Fas-associated protein with death domain
FasL — Fas ligand
FBS — fetal bovine serum
FITC — fluorescein
FKHRL1 — forkhead transcription factor like 1
FSC — forward scatter
HRP — horseradish peroxidase
HSC — hematopoietic stem cell
IAP — inhibitor of apoptosis
IgG — immunoglobulin G
IL — interleukin

IL-(2,4,7,15)R — interleukin-(2,4,7,15) receptor

ISP — immature single positive

KLRG1 — killer cell lectin-like receptor subfamily G 1

LAT — linker of activated T cells

Lck — lymphocyte-specific protein tyrosine kinase

LCMV — lymphocytic choriomeningitis virus

LM-OVA — Listeria monocytogenes-ovalbumin

MAPK — mitogen-activated protein kinase

Mcl-1 — myeloid cell leukemia 1

MEF — mouse embryonic fibroblast

MFI — mean fluorescence intensity

MHC — major histocompatibility complex

MPEC — memory precursor effector cell

mRNA — messenger ribonucleic acid

mTOR — mammalian target of rapamycin

NF-κB — nuclear factor-κB

NK (cell) — natural killer (cell)

OVA — ovalbumin

PAGE — poly-acrylamide gel electrophoresis
SSC — side scatter

STAT6 — signal transducer and activator of transcription 6

tBid — truncated Bid

TCR — T cell receptor

TNF — tumor necrosis factor

TRADD — tumor necrosis factor receptor type 1-associated protein with death domain

TRAIL — TNF-related apoptosis-inducing ligand

UV — ultraviolet

VDAC — voltage-dependent anion channel

XIAP — X-linked inhibitor of apoptosis

YFP — yellow fluorescent protein
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1. Introduction

1.1 Cell death in T lymphocytes

As a major component of the adaptive immune response, T lymphocytes make up a dynamic population in which cell death must be carefully regulated. During their development and normal function, T cells undergo several checkpoints and utilize several mechanisms to regulate whether the cells live or die. Although several proteins and pathways have been connected with the regulation of cell death in T cells, our understanding of this regulation in different in vivo contexts is still incomplete. In this chapter, we will discuss the critical points at which T cell survival is regulated and briefly discuss some of the known mechanisms that control this regulation.

1.1.1 Thymic selection and cell death during T cell development

In order to allow the recognition of a variety of foreign antigens, the cells of the adaptive immune system, namely T and B cells, undergo a unique process in which different gene segments are rearranged in a combinatorial manner to create a diverse pool of T and B cell receptors, each of which is genomically encoded in an individual cell [Reviewed in (Nikolich-Zugich et al., 2004)]. In addition to the combinatorial rearrangement of the gene segments, imprecise joining and nucleotide addition lead to an increased diversity of receptor sequences. These methods of receptor diversification have clear benefits: they allow for reactivity to a wide variety of antigens using relatively little genomic “space” (compared to the millions of genes that would be necessary if
each receptor was encoded individually (Arstila et al., 1999; Casrouge et al., 2000), and they allow the cells to respond to novel antigens for which there may be no evolutionary pressure. Further, it has been suggested that receptor diversity contributes to functional responses (Turner et al., 2009).

In spite of the many benefits, diversification also occurs at a cost. Because imprecise joining and N-nucleotide addition leads to a largely random number of nucleotides between the different segments, only a predicted 1/3 of the receptors formed during this process would be in-frame with the rest of the gene, and additional factors may further reduce the number of functional receptors. Additionally, the random nature of the recombination process generates receptors that are reactive with the organism’s own antigens (self-antigens), which could lead to aberrant activation and autoimmunity. Because of these issues, T and B cells undergo several checkpoints during (and after) their development to ensure the expression of functional, non-self-reactive receptors. Cells that do not pass such checkpoints can be redirected to alternative fates, such as anergy and receptor editing, but most often are eliminated by apoptosis.

1.1.1 β-selection

For conventional αβ T cells, several “selection” events occur during development in the thymus. During thymic development, bone marrow-derived early thymic progenitors seed the thymus and progress first through four CD4CD8 double negative (DN) stages, which are distinguished by the expression of CD44 and CD25. Then, cells
continue to the CD4\(^+\)CD8\(^+\) double positive (DP) stage before maturing into CD4\(^+\)CD8\(^-\) or CD4\(^-\)CD8\(^+\) single positive (SP) cells. The rearrangement of the T cell receptor (TCR) \(\beta\) chain occurs first and begins in the DN stage. At the DN3 stage, the newly rearranged TCR\(\beta\) subunit pairs with the pre-TCR\(\alpha\) chain to form the pre-TCR. Pre-TCR signaling through Lck, SLP76/LAT, and Rho, amongst other signaling molecules, is required for the proliferation, differentiation, and survival of thymocytes past this checkpoint, ensuring the rearrangement of a functional TCR\(\beta\) allele [Reviewed in (Michie and Zuniga-Pflucker, 2002)].

Several studies have linked p53, a tumor suppressor that can induce cell cycle arrest and apoptosis, with the \(\beta\)-selection checkpoint and have implicated Rho signaling in the suppression of p53-mediated apoptosis (Michie and Zuniga-Pflucker, 2002). Pre-TCR activation of the transcription factor NF-\(\kappa\)B has also been linked to cell survival (Voll et al., 2000). One potential target of NF-\(\kappa\)B is the antiapoptotic Bcl-2 family protein A1, which has been shown to be upregulated in response to pre-TCR signaling (Mandal et al., 2005). Interestingly, it is thought that Bcl-2 does not have a dominant role in promoting survival at this checkpoint because it is downregulated at the later DN stages in which \(\beta\)-selection occurs (Michie and Zuniga-Pflucker, 2002; Voll et al., 2000).

### 1.1.1.2 Positive and negative selection

After TCR\(\alpha\) rearrangement, DP thymocytes undergo a second selection step, termed positive selection, to ensure that a complete TCR (consisting of a rearranged \(\alpha\)
and β chain) is expressed and capable of reacting to antigen presented on major histocompatibility complex (MHC). For positive selection, the cell requires a weak signal from self-peptide presented on MHC. Cells that do not pass positive selection are thought to undergo death by neglect, meaning that they do not receive the necessary signals to survive. Both Bcl-2 and CD127, the interleukin 7 receptor (IL-7R) α subunit, have been connected with positive selection because they are downregulated upon the cell’s transition to the DP stage but are upregulated again in post-selection DP thymocytes (Linette et al., 1994; Mazzucchelli and Durum, 2007; Moore et al., 1994; Sudo et al., 1993). However, overexpression of neither CD127 nor Bcl-2 greatly affected thymic selection, indicating that other signals may be needed to rescue DP cells from apoptosis (Linette et al., 1994; Munitic et al., 2004; Sentman et al., 1991; Yu et al., 2006).

While positive selection requires a low-affinity reactivity to self-peptide, if the signal is too strong, the cell undergoes negative selection. Several studies have addressed the differences in negatively selecting and positively selecting signals (Minter and Osborne, 2003). The proapoptotic protein Bim has been shown to mediate negative selection of thymocytes, and deficiency in Bim leads to autoimmunity, emphasizing the importance of this process (Bouillet et al., 1999; Bouillet et al., 2002).

1.1.2 T lymphocyte homeostasis

In order to maintain a population of T cells that can respond to a variety of antigens, the immune system requires that the body retain both antigen-inexperienced
“naïve” and antigen-experienced “memory” T cells over time. However, not surprisingly, these two populations have different requirements for survival [Reviewed in (Jameson, 2002; Khaled and Durum, 2002; Surh and Sprent, 2008)]. Naïve T cells balance thymic output with the retention and attrition of cells in the periphery by relying on specific signals for survival. First, naïve T cells require a low-level signal from self-peptide/MHC. Second, naïve T cells require a signal from a pro-survival cytokine; specifically, IL-7 has been shown to be the critical cytokine for naïve T cell survival in vivo (Jameson, 2002; Khaled and Durum, 2002; Surh and Sprent, 2008). Naïve CD8⁺, but not CD4⁺ T cells have been shown to also utilize IL-15 (Berard et al., 2003).

Because memory T cells have previously encountered their cognate antigen (presumably an invading pathogen), it is in the organism’s best interest to maintain these cells for defense against future encounters with the same pathogen. Thus, memory cells are maintained in a separate niche and do not require the same signals as naïve cells. Specifically, memory T cells do not require signal from self-peptide/MHC and are less reliant on IL-7. However, the maintenance of memory must be balanced with the maintenance of naïve cells, and thus the size of the memory cell niche is regulated. Like with naïve cells, this can be achieved through responsiveness to cytokines, although memory cells can utilize IL-15 in addition to IL-7 for survival (Jameson, 2002; Khaled and Durum, 2002; Surh and Sprent, 2008).
1.1.3 The regulation of survival during the immune response and contraction

In addition to the homeostatic mechanisms that regulate naïve and memory T cell survival under steady-state conditions, the survival of T cells is closely regulated during an active immune response. It is estimated that the antigen-specific T cell population expands up to or even over 1,000-fold upon activation by the cognate antigen, but most of these cells will die during the contraction period (Blattman et al., 2002; Murali-Krishna et al., 1998). Upon activation, T cells downregulate the IL-7R and upregulate the high-affinity IL-2 receptor, CD25 (Ma et al., 2006; Schluns et al., 2000). While naïve cells (with the exception of regulatory T cells) do not typically rely on IL-2, IL-2 promotes the survival of the activated T cells and the expansion of effector T cell populations (Ma et al., 2006; Vella et al., 1998).

After the effector phase, the responding T cell population undergoes a contraction period in which 90-95% of the cells die. A number of factors, including cytokines and inflammation have been shown to influence contraction, but this phase appears to be largely pre-programmed because it can occur regardless of the persistence of antigen [Reviewed in (Hand and Kaech, 2009)]. The proapoptotic protein Bim, either alone or in conjunction with Fas, has been shown to be important for facilitating T cell contraction after activation (Hildeman et al., 2002; Hughes et al., 2008; Willis and Adams, 2005; Wojciechowski et al., 2006).
1.2 Apoptosis

1.2.1 Morphologic and physiologic characteristics of apoptosis

Apoptosis is the major form of cell death in most metazoan organisms (Danial and Korsmeyer, 2004). Also known as type I programmed cell death, apoptosis occurs through a series of morphological and molecular changes [Reviewed in (Elmore, 2007)]. Morphologically, apoptosis can be observed through decreased cell size and nuclear condensation. Later morphologic characteristics are plasma membrane blebbing and the budding of apoptotic bodies, which contain densely packed cellular material, off of the dying cell.

In general, apoptosis is thought to be immunologically silent: The phagocytic cells (usually macrophages) that take up the apoptotic vesicles are not activated, inducing no inflammation (Elliott and Ravichandran, ; Elmore, 2007; Savill and Fadok, 2000). In contrast, when cells die by necrosis, which is characterized by cell swelling and a loss of organelle and membrane integrity, a number of intracellular components are released into the surrounding environment and have proinflammatory effects. Because cells inevitably die during the lifespan of an organism, the containment of inflammatory mediators and self-proteins within apoptotic vesicles is a method of preventing unnecessary inflammation and autoimmunity (Elliott and Ravichandran).
1.2.2 Molecular mechanisms of apoptosis

One of the molecular hallmarks of apoptosis is the activation of a group of cysteine proteases known as caspases, which cleave at aspartic acid residues [Reviewed in (Danial and Korsmeyer, 2004; Elmore, 2007)]. Caspases can be divided into two groups: initiator caspases and effector caspases. While initiator caspases, such as caspase 9 and caspase 8, are responsible for propagating the apoptotic signal, effector caspases, such as caspases 3, 6, and 7, are responsible for cleaving essential intracellular proteins, leading to the morphologic changes discussed above and ultimately cell death. Initiator caspases are activated through the formation of dimers and the autocatalytic cleavage of the “pro” form of the enzyme. For example, pro-caspase 9 forms a complex known as the apoptosome with the proteins apoptotic protease activating factor 1 (APAF-1) and cytochrome c, which leads to the cleavage and activation of caspase 9. To continue the apoptotic cascade, active caspase 9 then cleaves the effector caspase caspase 3 to the active form.

In addition to the degradation of essential proteins, caspase activation contributes to DNA fragmentation and chromatin condensation by activation of the nuclease caspase-activated DNase (CAD) (Sakahira et al., 1998). A caspase-independent contributor to the DNA fragmentation process is apoptosis-inducing factor (AIF), which is released from the mitochondria later in apoptosis (Danial and Korsmeyer, 2004; Elmore, 2007). Another class of conserved proteins involved in the regulation of
apoptosis are the inhibitors of apoptosis (IAPs), such as XIAP. These proteins contain a motif that binds to the active site and inhibits the activity of caspases. However, the IAP proteins themselves can be inhibited by other apoptotic proteins, such as SMAC/DIABLO, that promote the release of caspases from the IAPs. Thus, a variety of proteins regulate the activity of caspases and contribute to the regulation of apoptosis (Danial and Korsmeyer, 2004; Elmore, 2007).

### 1.2.3 The intrinsic pathway of apoptosis

While the caspase-dependent and caspase-independent events described above are the hallmark of apoptotic cell death, these processes are initiated by—and regulated by—multiple upstream mechanisms. Two major pathways for the initiation of apoptosis have been described: the intrinsic (or mitochondrial) pathway and the extrinsic pathway. The intrinsic pathway of apoptosis is initiated in response to cellular stress signals. Stress signals, such as DNA damage, the unfolded protein response, and growth factor withdrawal, trigger the activation of proapoptotic signals, specifically the activation of proapoptotic Bcl-2 family proteins (Strasser, 2005). For example, the tumor suppressor p53, which is activated in response to DNA damage among other cellular stresses, induces the expression of the proapoptotic proteins Noxa and Puma (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). As another example, growth factor withdrawal can lead to the activation of the proapoptotic protein Bad, which in the presence of growth factors is phosphorylated by Akt and is sequestered in the cytosol in
complex with 14-3-3 proteins (Datta et al., 1997; Datta et al., 2000). The activation of these and other proapoptotic proteins of the Bcl-2 family (discussed further in the next section) ultimately leads to the depolarization of the mitochondria and the release of mitochondrial contents, specifically cytochrome c, into the cytosol via a mechanism that involves the proteins Bak and Bax (Danial and Korsmeyer, 2004; Strasser, 2005). As discussed above, cytochrome c is a critical component of the apoptosome, and its release facilitates the activation of caspase 9 and downstream caspases.

The intrinsic pathway of apoptosis is regulated at many levels. The Bcl-2 family (discussed further in Section 1.3) is responsible for regulating most of the upstream signals and for delivering (or preventing the delivery of) the proapoptotic signal to the mitochondria. This regulation is achieved through the regulation of both the expression and the activity of the Bcl-2 family proteins (Strasser, 2005). While Bak and/or Bax, members of the Bcl-2 family, are required for apoptosis to proceed through the intrinsic pathway (Wei et al., 2001), the activity of Bak and Bax is also regulated by non-Bcl-2 family proteins. Separate from its role as a transcription factor, p53 has been shown to induce mitochondrial permeabilization by Bax by binding to Bcl-xL (Chipuk et al., 2004), and Bak has been shown to be regulated by the mitochondrial protein VDAC2 (Cheng et al., 2003).
1.2.4 The extrinsic pathway of apoptosis

In addition to intracellular stress, cells can also be signaled to die by environmental signals, both soluble and cell-bound factors, through a process known as the extrinsic pathway of apoptosis. In this pathway, signals such as FasL, tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL) bind to specific receptors on the cell membrane to trigger an apoptotic cascade [Reviewed in (Danial and Korsmeyer, 2004; Opferman and Korsmeyer, 2003; Zhang et al., 2005)]. In the Fas/FasL pathway, FasL binds to the receptor Fas (CD95) on the surface of the responding cell. The intracellular portion of Fas contains two death domains (DDs). These domains recruit and activate the protein FADD, which contains both DDs and a death effector domain (DED). FADD in turn recruits pro-caspase 8, which also contains a DED, leading to the homodimerization and cleavage of pro-caspase 8 into its active form. Then, caspase 8 feeds into the downstream pathway by cleaving and activating the effector caspases 3 and 7. The molecular events following TNF and TRAIL signaling are similar to those that follow FasL signaling. In the TNF pathway, an additional adaptor protein, TRADD functions as an intermediate between the receptor and FADD.

The extrinsic pathway is also regulated at many levels. In addition to the availability of extracellular apoptotic signals, the regulation of receptor expression levels also serves as a means of regulation. Additionally, the protein cFLIP (also known as CASH, MRIT, I-FLICE, CLARP, usurpin, Casper, and FLAME-1), which has structural
homology to caspase 8 in that it contains two DED domains, prevents apoptosis through the extrinsic pathway (Goltsev et al., 1997; Han et al., 1997; Hu et al., 1997; Inohara et al., 1997; Irmler et al., 1997; Rasper et al., 1998; Shu et al., 1997; Srinivasula et al., 1997). Differential splicing of cFLIP mRNA results in three major isoforms in human and two in mouse (Budd et al., 2006; Golks et al., 2005; Thome and Tschopp, 2001; Ueffing et al., 2008). While all three isoforms contain two DED domains, the longer form of cFLIP, known as cFLIPL, also contains a catalytically inactive caspase-like domain. The shorter forms of cFLIP, known as cFLIPs and cFLIPr, contain no caspase-like region. All of the forms of cFLIP are thought to have a “dominant negative-like” effect on caspase 8 activation in that they bind to caspase 8 through the DED domains, but caspase 8 cannot be cleaved to its active form due to the lack or inactivity of the caspase-like domain on cFLIP (Budd et al., 2006; Thome and Tschopp, 2001). It is possible that cFLIPL has additional mechanisms of action because it allows partial cleavage of pro-caspase 8—in particular, a role in T cell signaling has been suggested—but the functional differences between the different isoforms are still not well described (Boatright et al., 2004; Budd et al., 2006; Chang et al., 2002; Dohrman et al., 2005a; Dohrman et al., 2005b; Micheau et al., 2002). Downstream of caspase 8 activation, the extrinsic pathway converges with the intrinsic pathway in the activation of effector caspases, and this pathway can also be inhibited by the IAPs.
1.2.4 Crosstalk between apoptotic and other cell survival and cell death pathways

While the intrinsic and extrinsic pathways of apoptosis converge downstream at the level of effector caspase activation, there are other points at which these two pathways connect. The best-described crosstalk mechanism is via the protein Bid. Bid is a proapoptotic protein of the Bcl-2 family that is processed into a truncated form, tBid in response to apoptotic signals (Li et al., 1998; Wang et al., 1996). tBid is a potent inducer of apoptosis and has been shown to directly activate Bak and Bax (Kuwana et al., 2002; Wei et al., 2000). It has been shown that caspase 8 can cleave Bid into tBid, thus allowing the initiation of the mitochondrial pathway downstream of death receptor signaling (Li et al., 1998).

Cell survival is intimately linked with the maintenance and regulation of metabolic and catabolic processes. Thus, it is not surprising that metabolic pathways in the cell are also molecularly linked to apoptotic pathways. In T cells, the metabolic regulators mammalian target of rapamycin (mTOR) and Akt have been shown to be important regulators of T cell survival as well as regulators of the Bcl-2 family proteins (Plas et al., 2002; Powell and Delgoffe).

Autophagy is a conserved catabolic process by which the cytoplasmic contents of a cell are recycled, particularly during times of starvation (Klionsky and Emr, 2000; Lum et al., 2005). In addition, autophagy has also been proposed to be a mechanism of programmed cell death, “type II programmed cell death” (Baehrecke, 2005; Yu et al.,
Treatment with the autophagy inhibitor 3-methlyadenine or silencing of autophagy proteins was shown to inhibit cell death following etoposide or staurosporine treatment in Bak−/−Bax+ embryonic fibroblasts (Shimizu et al., 2004). Further, knockdown of autophagy genes has been shown to inhibit cell death in response to the caspase inhibitor zVAD (Yu et al., 2004a). However, because these examples involved the manipulation of apoptotic pathways, it is not yet clear whether autophagic cell death is a relevant pathway in vivo. These data do highlight, however, that there is likely crosstalk between autophagic and apoptotic pathways.

Autophagy proteins have been shown to interact with proteins of both the intrinsic and the extrinsic pathways of apoptosis. The autophagy protein Beclin1 was first identified as a protein that binds Bcl-2, and both Bcl-2 and Bcl-xL have been shown to bind to and inhibit Beclin1 (Liang et al., 1998; Pattingre et al., 2005). Atg5, another autophagy protein, has been shown to interact with FADD, indicating a possible mechanism of crosstalk with the extrinsic pathway (Pyo et al., 2005).

1.3 The Bcl-2 family

1.3.1 Overview of the Bcl-2 family

1.3.1.1 Identification and basic structure of Bcl-2

Bcl-2 was originally identified as a critically deregulated gene in follicular lymphomas (Tsujimoto et al., 1984). It was found to be the mammalian counterpart to C. elegans CED9 and to have antiapoptotic function (Hengartner and Horvitz, 1994;
Hockenbery et al., 1990; Vaux et al., 1992). The first indication that Bcl-2 might represent a larger family of proteins was the subsequent discovery of Mcl-1 as a protein that is expressed in differentiating myeloid leukemia cells and that has homology to Bcl-2 (Kozopas et al., 1993). A few different transcripts of Bcl-2, which consist of either two or three exons, have been described, and the Bcl-2 gene spans nearly 200 kB due to a large intron between the exons (Negrini et al., 1987). The protein consists of a long C-terminal tail that contains four regions known as the Bcl-2 homology (BH) domains (Figure 1). By definition, one or more these domains is conserved amongst Bcl-2 family members. Additionally, the BH domains function in protein-protein interactions between Bcl-2 family proteins. Bcl-2 also contains a transmembrane region, and has been shown to associate with the outer mitochondrial membrane (Hockenbery et al., 1990).

1.3.1.2 Classification and function of Bcl-2 family members

Members of the Bcl-2 family are defined by sharing at least one of the BH domains with Bcl-2. The members of the Bcl-2 family can be classified based on both the presence of different BH domains and by their function. Proteins such as Mcl-1, A1, Bcl-xl, Bak, and Bax share multiple BH domains, while another subset of the Bcl-2 family, known as the BH3-only proteins, share only the BH3 region (Figure 1). The BH3-only proteins, such as Bad, Bim, Bid, Noxa, and Puma, are primarily proapoptotic. As discussed in Section 1.2.3, cell stress signals lead to the activation of BH3-only proteins by a number of different mechanisms.
The “multidomain” proteins of the Bcl-2 family can be either antiapoptotic (Bcl-2, Mcl-1, A1, Bcl-w, and Bcl-xL) or proapoptotic (Bak and Bax). Of the antiapoptotic proteins, Bcl-2, Mcl-1, and Bcl-xL are the most widely expressed and studied, and as such (and because of their dominant expression in T cells), these three antiapoptotic proteins will be the focus throughout most of this dissertation. Bak and Bax, which contain BH1-3 regions, are critical for initiating mitochondrial disfunction, the release of cytochrome c, and activation of downstream molecules such as APAF-1 and effector caspases (Wei et al., 2001). Although the exact method of activation of Bak and Bax are still unclear, apoptotic stimuli lead to conformational changes in both proteins as observed by exposure of new epitopes at the N-terminus, and this facilitates the formation of homooligomeric pores on the mitochondrial membrane, causing depolarization and release of cytochrome c (Griffiths et al., 1999; Hsu and Youle, 1997; Hsu et al., 1997; Kuwana et al., 2005; Kuwana et al., 2002).
1.3.1.3 Interactions between Bcl-2 family members

One of the primary ways in which Bcl-2 family proteins are known to function is through interactions with other Bcl-2 family members. These interactions are largely through the BH domains, and a number of specific protein-protein interactions have been demonstrated within the Bcl-2 family both in vitro and in vivo. The BH3 region of the BH3-only proteins has been shown to be important for interactions with Bcl-2, Bcl-xL, and/or Mcl-1 (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). Similarly,
mutational analyses showed that the BH3 regions of Bax and Bak are critical for binding to Bcl-2, Bcl-xL, and/or Mcl-1 (Chittenden et al., 1995; Willis et al., 2005; Zha et al., 1996). Within the antiapoptotic proteins, the BH1, BH2, and BH3 regions are all important for these interactions (Sedlak et al., 1995; Yin et al., 1994).

The crystal and solution structures of Bcl-xL and Bcl-2 elucidated that the BH1, BH2, and BH3 regions form a hydrophobic cleft that is the binding site for an amphipathic helix within the BH3 region of proapoptotic proteins such as Bak and Bim (Liu et al., 2003; Muchmore et al., 1996; Petros et al., 2001; Sattler et al., 1997). The solution structure of Mcl-1 also demonstrated this groove, but it differed from that of Bcl-2 and Bcl-xL by the presence of positively charged residues and a more “open” conformation (Day et al., 2005). Interestingly, a similar groove is formed by the BH1, BH2, and BH3 regions of Bax, but it is blocked by an alpha helix of Bax itself when Bax is in its monomeric form (Liu et al., 2003; Suzuki et al., 2000).

Although all Bcl-2 family members contain a BH3 region, some proteins interact with only select other Bcl-2 proteins. While earlier overexpression studies indicated that Noxa can bind to Bcl-2 and Bcl-xL (Oda et al., 2000), it was later shown to preferentially bind Mcl-1 and A1 of the antiapoptotic proteins (Chen et al., 2005; Kuwana et al., 2005). Similarly, the BH3-only protein Bad has been shown to interact via its BH3 domain with Bcl-2 and Bcl-xL, but not Mcl-1 (Chen et al., 2005). While Bax has been shown to bind the
antiapoptotic proteins Bcl-2, Bcl-xL, Mcl-1, and A1, Bak is specific for only Mcl-1 and Bcl-xL. (Sedlak et al., 1995; Willis et al., 2005)

1.3.1.4 Localization of Bcl-2 family members

As might be predicted by the presence of the transmembrane domain and their roles in regulating the mitochondrial pathway of apoptosis, most of the multi-domain Bcl-2 family members have been shown to associate with the mitochondria. Bcl-2, Mcl-1, and Bcl-xL localize predominantly to the outer mitochondrial membrane but have also been detected in other membrane fractions (Hockenbery et al., 1990; Hsu et al., 1997; Wolter et al., 1997; Yang et al., 1995). Both Bak and Bax have been shown to localize to the mitochondria during active apoptosis, but they are found in different locations at a basal state. Interestingly, Bak is constitutively located at the mitochondria, while Bax is primarily located in the cytosol until the onset of apoptosis (Hsu et al., 1997; Wolter et al., 1997). The translocation of Bax to the mitochondria corresponds with the conformational shift to the form associated with active apoptosis.

Only some of the BH3-only proteins contain transmembrane regions, while others can be presumed to be either cytosolic or associated with membranous structures through protein-protein interactions. Bim has been shown to associate with the microtubule dynein-motor complex, and the release of Bim from the microtubules has been associated with apoptosis (Puthalakath et al., 1999). However, it has also been reported that Bim can be found constitutively at the mitochondria (Zhu et al., 2004). The
BH3-only proteins Bid, Puma, and Noxa have been shown to localize to the mitochondria (Nakano and Vousden, 2001; Oda et al., 2000; Wei et al., 2000; Yu et al., 2001), although Bid has also been reported in the cytosol (Wang et al., 1996). For Noxa, mitochondrial localization is dependent on the BH3 region (Oda et al., 2000), while BH3 mutants of Puma and Bid have been reported to still have mitochondrial localization (Nakano and Vousden, 2001; Wei et al., 2000; Yu et al., 2001). Bad has been shown to associate with Bcl-xL at the mitochondria in its unphosphorylated form but is released from Bcl-xL and sequestered by 14-3-3 proteins in the cytosol upon phosphorylation of key serine residues by prosurvival kinases (Datta et al., 2000).

1.3.2 Models of how the Bcl-2 family regulates apoptosis

The individual Bcl-2 family members are regulated on several levels, including regulation of transcription and protein expression, phosphorylation, and protein degradation, but the interactions between the proapoptotic and antiapoptotic proteins are ultimately what determine whether or not the cell undergoes apoptosis (Oltval et al., 1993; Strasser, 2005). It is known that Bak and Bax are required for the downstream mitochondrial events of the intrinsic pathway of apoptosis (Wei et al., 2001). However, although multiple interactions have been demonstrated between the different Bcl-2 family members, precisely how the Bcl-2 family regulates apoptosis upstream of Bak/Bax activation has been debated.
1.3.2.1 The direct activation model

Two opposing, but not necessarily exclusive, models have emerged for the mechanism of the antiapoptotic molecules Bcl-2, Mcl-1, and Bcl-xL. The “direct activation model” suggests that the antiapoptotic proteins function to inhibit the activity of BH3-only activator proteins (Figure 2) (Kim et al., 2006; Kuwana et al., 2005; Letai et al., 2002). By this model, certain BH3-only molecules, namely Bim, Bid, and possibly Puma, directly activate Bak and Bax, and binding of the antiapoptotic proteins to these BH3-only activators inhibits apoptotic pathways (Kim et al., 2006; Kuwana et al., 2005; Letai et al., 2002). In contrast, “sensitizer” or “inactivator” BH3-only molecules do not bind Bak or Bax but rather affect the availability of the antiapoptotic proteins to bind their BH3-only activator targets.

**Figure 2: Direct activation model for the Bcl-2 family proteins**

Bak and Bax (rectangles) are directly activated by certain BH3-only proteins termed “direct activators” (Bim, Bid, and Puma; large pentagons). The
antiapoptotic proteins (Mcl-1, Bcl-xl, and Bcl-2; diamonds) function by inhibiting
direct activators. “Sensitizer” BH3-only proteins (such as Noxa and Bad; small
pentagons) inhibit one or more of the antiapoptotic proteins.

In support of this model, several studies have shown the ability of truncated Bid
(tBid), Bim, or Puma to induce oligomerization of Bax and Bak, to form pores in isolated
mitochondrial membranes, and to cause cytochrome c release (Cheng et al., 2001; Kim et
al., 2006; Kuwana et al., 2002; Letai et al., 2002; Wei et al., 2000). To rule out the effects of
other mitochondrial proteins, Bim and tBid, but not Puma, were also shown to directly
activate Bax to form pores in liposomes (Kuwana et al., 2005). Stable tBid-Bcl-2
complexes have been found in the mitochondria, and saturation of Bcl-2 with excess tBid
correlated with apoptosis, implying that Bcl-2 sequesters tBid from activating Bak/Bax
(Cheng et al., 2001). Further, mutant forms of Bak and Bax that could not bind Bcl-2,
Bcl-xl, or Mcl-1 could still be inhibited by these antiapoptotic proteins, indicating that
inhibition of Bak and Bax by the antiapoptotic proteins is indirect (Kim et al., 2006).
Finally, a recent study showed that the Bim+/Bid+/Puma+/ mouse, in which all of the
proposed “direct activators” were knocked out, had a phenotype similar to that of the
Bak/Bax knockout, indicating that these proteins are required for apoptosis through the
Bak/Bax pathway (Ren et al., 2010).

1.3.2.2 The indirect activation model

Under the “indirect activation” or “Bak/Bax sequestration” model, the
antiapoptotic proteins bind Bak and Bax directly to prevent their oligomerization and/or
activation (Figure 3) (Willis et al., 2007). By this model, all BH3-only proteins function upstream to affect the ability of antiapoptotic proteins to bind Bak/Bax, and the distinction between “activators” and “sensitizers” is really in the differential abilities of the BH3-only proteins to bind the antiapoptotic proteins (Chen et al., 2005; Willis et al., 2005; Willis et al., 2007).

**Bak/Bax Sequestration**

Figure 3: Indirect activation model for the Bcl-2 family proteins

Bak and Bax (rectangles) are sequestered by the antiapoptotic proteins (diamonds). Bax can be inhibited by all three proteins shown, while Bak is bound by Mcl-1 and Bcl-xL, but not Bcl-2. The BH3-only proteins (pentagons) promote apoptosis by interfering with the ability of the antiapoptotic proteins to sequester Bak/Bax. Their potency in inducing apoptosis (indicated by size) is related to their ability to broadly neutralize the antiapoptotic proteins.

In support of a Bak/Bax sequestration role for the antiapoptotic proteins, Bcl-xL can be immunoprecipitated with Bak in healthy cells and this interaction was diminished upon treatment with apoptotic stimuli (Griffiths et al., 1999). In contrast to earlier studies, one group found that Bim and Bid peptides had only weak binding to
active Bax and did not bind inactive Bax in solution competition assays (Willis et al., 2007). Additionally, a mutant form of Bid that could bind the antiapoptotic proteins but not Bax still induced apoptosis in MEFs (Willis et al., 2007). Co-expression of the BH3-only proteins Noxa and Bad, both of which would be considered “sensitizer” BH3-only proteins by the direct activation model, leads to the induction of apoptosis at a level equivalent to that induced by expression of Bim (Chen et al., 2005). It was proposed the concurrent inactivation of Mcl-1 and Bcl-xL/Bcl-2 by either the Noxa/Bad combination or by Bim leads to apoptosis and that the ability of different BH3-only proteins to induce apoptosis is related to their ability to broadly inhibit the anti-apoptotic proteins (as demonstrated in Figure 3; more potent/less selective BH3-only proteins are displayed larger) (Chen et al., 2005). Another study showed that apoptosis occurs at wild type levels in Bim/Bid double knockout cells in response to ultraviolet (UV) irradiation, etoposide, or expression of Noxa plus the Bad BH3-mimetic ABT-737, and the same was found when Puma was additionally knocked down, indicating that none of the “direct activators” were required for apoptosis in this system (Willis et al., 2007). Finally, Mcl-1 and Bcl-xL but not Bcl-2 have been shown to bind Bak, and loss of Bcl-xL rendered cells sensitive to killing by Noxa (inhibition of Mcl-1), while Bcl-2 appeared to have no effect on sensitivity, indicating that cells undergo apoptosis when neither Mcl-1 or Bcl-xL are available to inhibit Bak (Willis et al., 2005).
One limitation of the studies leading to the two models above is that the specific interactions between the Bcl-2 family members have been largely defined using BH3-peptides and were often performed by overexpressing proteins in cell lines (Chen et al., 2005; Willis et al., 2005). Because the balance of Bcl-2 family proteins is critical in both models and may be different in different cell types, further studies are needed to examine the in vivo consequences of the interactions between Bcl-2 family members. Further, much of the “evidence” for either model relies to some extent on interpretation. For example, while the phenotype of the Bim/Bid/Puma triple knockout mouse (Ren et al., 2010) indicates that these proteins are critical for the efficient induction of apoptosis (strong evidence for their roles as direct activators), it does not formally rule out that they do so by inhibiting the antiapoptotic proteins (indirect activation). Finally, it is possible that both mechanisms are utilized by the Bcl-2 family to regulate apoptosis. One study using knock-in mice expressing mutant forms of Bim showed that both the ability of Bim to bind the antiapoptotic proteins (indirect activation) and its ability to bind Bax (direct activation) are important for its full apoptotic effects (Merino et al., 2009). Therefore, it is important to study the context-specific, in vivo roles of the Bcl-2 family proteins to obtain a full understanding of how they function in a physiological context.

### 1.3.3 Knockout mouse models of the Bcl-2 family

While several in vitro studies have demonstrated the molecular features and functions of the Bcl-2 family proteins, knockout mouse models have allowed the study
of the physiologic roles of many of the proteins of this family. In this section, we will review the data from the available knockout mouse models of both the antiapoptotic and the proapoptotic proteins of the Bcl-2 family.

1.3.3.1 The antiapoptotic Bcl-2 family proteins

Knockout of the Bcl-2, Mcl-1, and Bcl-x genes, the three best-studied of the antiapoptotic Bcl-2 family members, has proven to be lethal, although the specific phenotype and the timing of the lethality is different for each of these genes. Knockout of the Mcl-1 gene results in peri-implantation lethality at approximately the blastomere stage (Rinkenberger et al., 2000). Bcl-x knockout embryos die around embryonic day 13, and the major defects are apoptosis of the developing neurons in the brain and spinal cord and apoptosis of hematopoietic cells in the liver (Motoyama et al., 1995). In contrast to the embryonic phenotypes of the Mcl-1 and Bcl-x gene knockouts, Bcl-2 knockout animals are born, but succumb to death after just a few weeks largely due to renal failure resulting from polycystic kidney disease (Nakayama et al., 1994; Veis et al., 1993b). Further, these mice exhibit loss of the lymphoid compartment over time and hypopigmentation of the hair beginning at the second follicle cycle (Nakayama et al., 1994; Veis et al., 1993b). Germline knockout of A1, another antiapoptotic protein of the Bcl-2 family, has not been possible due to the existence of multiple isoforms at different chromosomal loci (Hatakeyama et al., 1998). Knockout of the A1a isoform alone results in accelerated apoptosis of neutrophils and decreased inflammatory responses to
Toxoplasma gondii infection but yields no obvious developmental abnormalities (Hamasaki et al., 1998; Orlofsky et al., 2002).

In addition to conventional knockout models, conditional and inducible deletion of the different Bcl-2 family members, particularly of the antiapoptotic genes for which the conventional knockouts are lethal, has allowed researchers to better study the roles of these proteins in specific cell types. For example, Mcl-1 has been shown to be important for the survival of T cells (discussed in Section 1.4), B cells, hematopoietic stem cells (HSCs), and neutrophils (Dzhagalov et al., 2008; Dzhagalov et al., 2007; Opferman et al., 2005; Opferman et al., 2003; Vikstrom et al.).

1.3.3.2 Bak and Bax

Although Bak and Bax are critical for apoptosis proceeding through the intrinsic pathway (Wei et al., 2001), knockout models indicated that these two proteins are largely redundant. Indeed, in the definitive study by Wei et al. that concluded the role of these proteins, both Bak and Bax had to be eliminated to have an effect on apoptosis (Wei et al., 2001). The Bak<sup>−/−</sup> animals have no reported phenotype, and the only defect reported in Bax<sup>−/−</sup> animals is male sterility due to a defect in spermatogenesis (Knudson et al., 1995; Lindsten et al., 2000). A minor lymphoplasia in Bax<sup>−/−</sup> animals was reported by some groups, but not others (Dunkle et al., 2010; Knudson et al., 1995; Rathmell et al., 2002). In contrast, the Bak/Bax double knockout mice have several physiologic
abnormalities, including interdigital webbing of the feet, neurological defects, splenomegaly, and lymphadenopathy (Lindsten et al., 2000; Rathmell et al., 2002).

1.3.3.3 The BH3-only proteins

In contrast to the multiple defects observed upon loss of proapoptotic Bak and Bax, the phenotypes of the knockout mice for the other family of proapoptotic proteins, the BH3-only proteins, are much less severe (Strasser, 2005). The most profound BH3-only knockout phenotype is that of Bim: Bim<sup>+</sup> animals have a perturbed hematopoietic system and exhibit autoimmune kidney disease (Bouillet et al., 1999). Puma and Noxa knockout mice have no apparent developmental phenotype, normal body and organ weight, and a normal hematopoietic system (Jeffers et al., 2003; Villunger et al., 2003).

1.4 The Bcl-2 family in T lymphocytes

1.4.1 Expression patterns of the antiapoptotic Bcl-2 family proteins in T lymphocytes

In order to understand the function of the antiapoptotic proteins of the Bcl-2 family in T lymphocytes, we must first consider the expression pattern of these proteins throughout the T cell lineage. Bcl-2, Bcl-xl, Mcl-1, and A1 are all expressed in T lymphocytes at some point in their lineage, but over the “lifespan” of the T cell, the expression patterns of the different antiapoptotic Bcl-2 family members differ. Bcl-2 is expressed in DN thymocytes, downregulated at the DP stage, and upregulated following positive selection to be expressed in SP thymocytes (Gratiot-Deans et al., 1993; Gratiot-Deans et al., 1994; Veis et al., 1993a). In peripheral T cells, Bcl-2 is highly
expressed in naïve and memory populations, but has been shown to have lower expression in effector T cells (Grayson et al., 2001; Grayson et al., 2000). Interestingly, Bcl-xL, which is the predominant splice product of the Bcl-x gene in the T cell lineage (Ma et al., 1995), exhibits a nearly inverse pattern of expression to Bcl-2. Bcl-xL is highly expressed in DP thymocytes, but not the other thymocyte subpopulations, and is upregulated in activated/effector T cells (Boise et al., 1995; Grillot et al., 1995; Ma et al., 1995).

The expression of Mcl-1 is somewhat less dynamic because it is expressed at all of the major stages of thymocyte development and in peripheral T cells (Dzhagalov et al., 2008; Opferman et al., 2003). However, Mcl-1 levels have been shown to be upregulated in response to TCR signaling and stimulation by the cytokines IL-7 and IL-15 (Dzhagalov et al., 2008; Opferman et al., 2003).

The A1b and A1d isoforms are the predominant isoforms of A1 in lymphocytes, while A1a is highly expressed in neutrophils (Hatakeyama et al., 1998). A1 gene and protein expression has been detected in thymocytes and peripheral T cells, most highly in DP thymocytes (Tomayko et al., 1999). A1 (predominantly the A1d isoform) is expressed in response to pre-TCR signaling; expression is low/undetectable in DN3 cells and high in DN4 and DP cells (Mandal et al., 2005). In peripheral T cells, A1 is expressed in an NF-κB- and Ca2+-dependent manner in response to TCR, but not cytokine, signaling (Gonzalez et al., 2003; Verschelde et al., 2003).
1.4.2 Roles for the proapoptotic Bcl-2 family members in T lymphocytes

1.4.2.1 Bak and Bax

As discussed in Section 1.3.3, Bak/Bax double-knockout mice exhibit splenomegaly and lymphadenopathy (Lindsten et al., 2000; Rathmell et al., 2002). Closer examination of the T cell phenotype demonstrated that thymic development was perturbed and that T cells were resistant to apoptosis under a variety of conditions (Lindsten et al., 2000; Rathmell et al., 2002). Thus, the intrinsic pathway of apoptosis through Bak and Bax is clearly important for T cell development and homeostasis.

1.4.2.2 Bim

The knockout mouse for Bim has one of the most severe phenotypes of the BH3-only knockout models, exhibiting autoimmune kidney disease and an increased number of hematopoietic cells (Bouillet et al., 1999). The T cell phenotype of these mice includes an increased number of thymocytes and T cells, a skewing of the thymic profile as indicated by an expansion of the SP (particularly CD4+) populations, and an increased resistance of T cells to cytokine withdrawal and other apoptotic stimuli (Bouillet et al., 1999). The thymic phenotype and the observed autoimmunity are thought to be due to impaired negative selection because Bim−/− thymocytes are resistant to TCR-induced death and are not deleted when crossed onto a transgenic background expressing an autoreactive TCR (Bouillet et al., 2002; Villunger et al., 2004). In addition, Bim, either alone or in concert with Fas, has been shown to have a role in promoting the apoptosis
of peripheral T cells after activation (Hildeman et al., 2002; Hughes et al., 2008; Pellegrini et al., 2003; Weant et al., 2008).

1.4.3 Roles for the antiapoptotic Bcl-2 family members in thymocytes and peripheral T cells

1.4.3.1 Bcl-2

Bcl-2 is required for the proper development and maintenance of T lymphocytes. As discussed, Bcl-2−/− mice are born and initially appear normal but develop a lethal phenotype typically within a few weeks of birth (Nakayama et al., 1994; Veis et al., 1993b). Interestingly, the fate of thymocytes and peripheral T cells in these mice follows a similar pattern. At birth, Bcl-2−/− mice have similar numbers and percentages of thymocyte subsets and T cells in the spleen compared to wild type mice (Nakayama et al., 1994; Veis et al., 1993b). However, by approximately 3-4 weeks of age, the numbers of both thymocytes and peripheral T cells are dramatically reduced, indicating a failure in both the generation and maintenance of T cells (Nakayama et al., 1994; Veis et al., 1993b). The same result was observed when comparing congenically marked Bcl-2−/− with wild type cells in chimeric animals (Nakayama et al., 1993). While Nakayama et al. reported that the CD4/CD8 profile of the depleted Bcl-2−/− thymus was relatively normal (Nakayama et al., 1994), Veis et al. observed a skewing toward the DN compartment (Veis et al., 1993b). Both sets of authors agreed, however, that there was a likely survival defect in an early progenitor population in the thymus.
One explanation as to why Bcl-2\(^{-}\) thymocytes and T cells are initially normal but develop a survival defect after a few weeks is that neonatal T cells are derived from progenitor cells from the fetal liver, whereas after birth, bone marrow-derived progenitor cells seed the thymus and give rise to T cells. To address this issue, one study compared the ability of bone marrow-derived and fetal liver-derived Bcl-2\(^{-}\) HSCs to reconstitute the lymphoid compartment of irradiated mice (Matsuzaki et al., 1997). In this study, Matsuzaki et al. found that Bcl-2\(^{-}\) HSCs derived from the adult bone marrow did not give rise to T cells in irradiated recipients. Although DP thymocytes could be observed early after reconstitution, they were lost at later timepoints. In contrast, Bcl-2\(^{-}\) fetal liver-derived HSCs initially repopulated the thymus and splenic T cell compartments to a similar degree as wild type cells. However, like the Bcl-2\(^{-}\) mice, both thymocytes and peripheral T cells were lost at later timepoints. Importantly, in both models, the persistence of the HSCs themselves was not affected, indicating that Bcl-2 is not required for the survival of HSCs. This study showed that there is a fundamental difference in the survival requirements of bone marrow-derived and fetal liver-derived thymic progenitors and T cells that could explain the age-related defect of Bcl-2\(^{-}\) mice. Taken together, the results suggest that in addition to being required for peripheral T cell survival, Bcl-2 is critical for the survival of an early (DN) thymic progenitor cell population.
1.4.3.2 Bcl-xL

Early studies of the role of Bcl-xL in T cells utilized a system in which Bcl-xL−/−
embryonic stem (ES) cells were injected into RAG2−/− blastocysts to yield chimeric mice in
which the only thymocytes capable of maturation (due to the ability to rearrange the
TCR genes) were the Bcl-xL−/− thymocytes (Ma et al., 1995; Motoyama et al., 1995). These
mice displayed reduced percentages and numbers of SP thymocytes and T cells in the
spleen compared to wild type/RAG2−/− chimeras, but it was clear that mature cells still
developed and were maintained without Bcl-xL (Ma et al., 1995; Motoyama et al., 1995).
Further, mature SP and peripheral T cells displayed normal cell death in vitro, but
Bcl-xL−/− DP cells displayed enhanced cell death under a variety of in vitro conditions,
including culture in medium alone and treatment with the steroid dexamethasone, an
anti-CD3 antibody, and γ-irradiation (Ma et al., 1995; Motoyama et al., 1995).

When Bcl-x was knocked out specifically in the T cell lineage using a loxP/Cre
system, a similar phenotype was observed in the thymus and spleen: the mice had lower
overall numbers of thymocytes and T cells in the spleen, and DP thymocytes exhibited a
survival defect upon in vitro culture (Zhang and He, 2005). Peripheral Bcl-x-deficient
T cells did not have a survival defect upon in vitro activation, and their primary and
memory responses to in vivo infection with Listeria monocytogenes expressing the
ovalbumin (OVA) protein was normal (Zhang and He, 2005). Together, these data
indicate that Bcl-xL is largely dispensable for the development, maintenance, and function of T lymphocytes.

In spite of the mild phenotype of the Bcl-xL-deficient T cells, it is worth noting that Bcl-xL does appear to have some role in DP cell survival. Bcl-xL expression is highest in DP cells, and multiple groups have demonstrated an in vitro survival defect of Bcl-xL-deficient DP thymocytes (Ma et al., 1995; Motoyama et al., 1995; Zhang and He, 2005). Further, because DP cells make up a vast majority of thymocytes, the decreased numbers of thymocytes observed in Bcl-xL−/− mice could be attributed to an in vivo loss of DP thymocytes. Interestingly, in a double knockout model of Bcl-x and Mcl-1, the mice exhibited a severe decrease of total thymocyte and DP cell numbers compared to those of the wild type and single knockout animals, indicating that Bcl-xL and Mcl-1 likely cooperate to promote DP cell survival in vivo (Dzhagalov et al., 2008).

1.4.3.3 A1

Although germline targeting of A1 has not been possible, overexpression and siRNA-mediated knockdown experiments suggested that A1 promotes survival of DN thymocytes after signaling through the pre-TCR (Mandal et al., 2005). Overexpression of A1a, which is typically expressed more highly in neutrophils than in T and B lymphocytes (Hatakeyama et al., 1998), has also been shown to increase the survival of T cells under a variety of stimuli, including activation (Gonzalez et al., 2003).
1.4.3.4 Mcl-1

The use of conditional knockout mice has demonstrated the importance of Mcl-1 in T cells. When Mcl-1 is deleted in DN cells, the mice exhibit an approximately 90% reduction in thymocyte number and a severe block at the DN stage (Dzhagalov et al., 2008; Opferman et al., 2003). Using Cre-mediated deletion of Mcl-1 at the DN-DP transition, it was shown that Mcl-1 alone is not critical for DP survival, but the mice do exhibit a block in SP thymocyte maturation leading to an absence of mature SP thymocytes and peripheral T cells (Dzhagalov et al., 2008). However, as discussed, the loss of Mcl-1 and Bcl-x in DP thymocytes leads to a severe loss of DP cells, indicating that Mcl-1 does indeed have a critical, but non-exclusive, role in DP cell survival (Dzhagalov et al., 2008). In addition to its roles in thymocytes, Mcl-1 has been shown to be important to the survival of resting and activated peripheral T cells using drug-inducible knockout models both in vivo and in vitro (Dzhagalov et al., 2008; Opferman et al., 2003).

1.5 Overview of results

An interesting observation from the knockout and expression data discussed above was that Mcl-1 and Bcl-2, while often co-expressed, are individually required for thymocyte and T cell survival. This suggested that these two proteins have molecularly distinct roles in T cells. However, although several proteins have been shown to interact with Bcl-2 and/or Mcl-1, which of the previously described interactions is most relevant
in vivo was also not known. To address these issues, we took a genetic approach, using conditional Mcl-1 knockout mouse strains in combination with the genetic manipulation of other Bcl-2 family members. In short, we observed that Mcl-1 and Bcl-2 do indeed have molecularly distinct roles in thymocytes, with the major role of Mcl-1 being to inhibit Bak. Further, we found that while this role is also important in peripheral T cells, Mcl-1 has both cytokine-dependent and cytokine-independent roles in T cells, some of which are shared with Bcl-2. These studies not only allowed us to determine the molecular function of Mcl-1 in thymocytes and T cells but also have implications for the current models of how the Bcl-2 family regulates apoptotic pathways and the roles of cytokines. In the final section, we present data that the gradient expression of Bcl-2 in effector T cells has important implications in the formation of T cell memory to infection, providing new evidence for the role of this antiapoptotic protein in T cell function.
2. Materials and Methods

2.1 Mouse models

To examine the roles of the Bcl-2 family in vivo and in vitro, a number of genetically modified mice were used. Because knockout of the \textit{Mcl-1} gene is peri-implantation lethal (Rinkenberger et al., 2000), in order to perform loss-of-function studies for \textit{Mcl-1} in primary T cells, we used mice that were homozygous for a “floxed” allele of the \textit{Mcl-1} gene (\textit{Mcl-1}\textsuperscript{fl/fl} mice) so that \textit{Mcl-1} could be deleted upon expression of Cre recombinase (Dzhagalov et al., 2007). To examine the effect of ablation of \textit{Mcl-1} in thymocytes, we utilized mice that express Cre under the control of the proximal \textit{Lck} gene regulatory elements (LckCre mice), in which Cre expression is turned on in the DN2-DN3 stages, and mice that express Cre under the control of the \textit{CD4} gene regulatory elements (CD4Cre mice), which express Cre beginning at the late DN to early DP stage (Lee et al., 2001). To examine the effect of deletion of \textit{Mcl-1} in peripheral T cells, we utilized the drug-inducible estrogen receptor-Cre fusion protein (ERCre). This fusion protein is expressed by gene knock-in at the \textit{Rosa26} locus, and in the cells expressing this protein, Cre remains inactive until the addition of the drug tamoxifen or its active metabolite 4-hydroxytamoxifen (4OHT) (Shapiro-Shelef et al., 2005).

In addition to the conditional/inducible deletion of \textit{Mcl-1}, we utilized previously established models in which other Bcl-2 family members had been genetically modified. These models included Bim\textsuperscript{+/−} (Bouillet et al., 1999), Bak\textsuperscript{+/−} (Lindsten et al., 2000), and Bax\textsuperscript{+/−}
(Knudson et al., 1995) mice as well as mice that overexpress human Bcl-2 from a transgene using the H-2K\textsuperscript{b} promoter [Bcl-2\textsuperscript{tg} mice; (Domen et al., 1998)]. For the examination of the role of Bcl-2 in T cell memory, we used Bcl-2\textsuperscript{YFP} reporter mice generated in our laboratory, which express yellow fluorescent protein (YFP) from the Bcl-2 locus on a bacterial artificial chromosome (BAC) transgene (unpublished data from Ivan Dzhagalov). Bcl-2\textsuperscript{YFP} mice had been backcrossed to the C57BL/6 (CD45.1 or CD45.2) background for at least 15 generations. These mice were further crossed to OT1 mice, which transgenically express a TCR that is specific for OVA peptide presented on MHC class I. The OT1, Bcl-2 family knockout and Cre-expressing mouse strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) or Taconic (Hudson, NY, USA). Mice were maintained under specific pathogen-free conditions, and all animal work was performed under protocols approved by the Duke University Animal Institutional Care and Use Committee.

2.2 Experimental methods for Chapter 3

The methods below have been adapted from published material (Dunkle et al., 2010).

2.2.1 Cell counts of thymus and spleen

Mice were analyzed between 4-8 weeks of age. For each experiment, littermate controls were used or age-matched controls substituted as necessary. To obtain thymic and spleen cellularity, the thymus and spleen were harvested into FACS buffer
(phosphate-buffed saline (PBS) containing 2% fetal bovine serum (FBS) and 0.2% NaN₃) and kept on ice. Single-cell suspensions were made by smashing organs with a plunger and passing cells through a nylon mesh. Red blood cells (RBCs) were lysed using ACK buffer. Cells were enumerated on a hemocytometer in 0.1% trypan blue.

### 2.2.2 Flow cytometry to identify cell populations in the thymus and spleen

Before staining, Fc receptors were blocked by a 10-15 minute incubation with 24G2 supernatant. Cells were stained for 20-30 minutes with antibodies to various surface markers (CD4, CD8, CD69, Qa2, TCRβ, B220, CD44, and CD25) directly conjugated to the fluorochromes FITC, PE, PE/Cy5, PE/Cy7, APC, or APC/Cy7. All antibodies were obtained from eBioscience (San Diego, CA, USA) or Biolegend (San Diego, CA, USA) and were used at a concentration of 0.125-0.5 µl per 10⁶ cells in 100 µl. For analysis, cells were washed, resuspended in FACS buffer containing 2 µg/ml propidium iodide (PI) for exclusion of dead cells, and run on a BD FACSCanto™ cytometer (BD Biosciences, San Jose, CA, USA). All analyses were performed using FlowJo software (Tree Star, Inc. Ashland, OR, USA).

### 2.2.3 Intracellular staining for Mcl-1

Cells were fixed after surface staining in 2% paraformaldehyde (PFA) for 20-60 minutes, washed, and stored in FACS buffer at 4°C. Fixed cells were permeabilized for 20 minutes in FACS buffer with 0.1% saponin, 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA), and 10% 24G2 supernatant. Cells were stained
with rabbit polyclonal anti-Mcl-1 (Rockland, Inc. Gilbertsville, PA, USA) or 0.1 µg rabbit IgG (Rockland) at 0.1 µg/10⁶ cells in 100 µl for 30-60 minutes in the same buffer. Cells were washed with 0.1% saponin in FACS buffer and stained with 1 µl/10⁶ cells FITC-donkey-anti-rabbit (Jackson Immunoresearch) for 30 min in 0.1% saponin. Cells were run immediately after washing on a BD FACSCanto™ cytometer.

2.2.4 Statistical analyses

Data were further analyzed and graphed using Prism (Graphpad Software, San Diego, CA, USA). Statistical significance was defined by p values less than 0.05 as calculated by an unpaired two-tailed t test.

2.3 Experimental methods for Chapter 4

The methods below have been adapted from a manuscript under consideration for publication at the time this dissertation was written (Dunkle et al., submitted).

2.3.1 Antibodies, cytokines, and other reagents

The antibodies used for sorting and surface staining for flow cytometry (against CD4, CD8, CD44, and TCRβ) and the anti-CD28 antibody were obtained from Biolegend (San Diego, CA, USA). The 2C11 anti-CD3 monoclonal antibody was previously purified from ascites and was stored at -80°C then 4°C before use. Rabbit anti-Bak, -Bax, -Bim and -β-tubulin as well as the horseradish peroxidase (HRP)-conjugated anti-rabbit antibody were from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-Mcl-1 was from Rockland Immunochemicals (Gilbertsville, PA, USA). The whole rabbit IgG
isotype control and PE-anti-rabbit IgG were from Jackson Immunoresearch (West Grove, PA, USA). The PE-conjugated Bcl-2 staining kit was obtained from BD Biosciences (San Jose, CA, USA). Murine IL-7, IL-4, and IL-15 were purchased from Peprotech (Rocky Hill, NJ, USA) and were dissolved according to the manufacturer’s instructions. Cytokines were then diluted to 1,000x stocks in medium (RPMI) and stored at -80°C until use. Recombinant hIL-2 was obtained from the Duke University pharmacy and was diluted to a stock concentration of 10^5 units (U)/ml in medium and stored at 4°C. 4OHT was obtained from Sigma (St. Louis, MO, USA) and was dissolved in 100% ethanol to a stock concentration of 500 µM. PI was obtained from Sigma and was dissolved in PBS to a 500x stock concentration of 1 mg/ml. All media and related solutions were from Invitrogen (Carlsbad, CA, USA).

### 2.3.2 Total splenocyte cultures and deletion of Mcl-1

Spleens were smashed and filtered through a nylon mesh to make a single cell suspension in PBS. RBCs were lysed using ACK buffer for 1-2 min at room temperature, and the remaining cells were washed, counted, and resuspended in complete RPMI (+10% FBS and 1x non-essential amino acids, L-glutamine, and penicillin/streptomycin) to a concentration of 2-4 x 10^6 cells/ml. Although the cell concentrations used varied slightly between different experiments, the cell concentrations of the different samples were kept constant within each experiment. One-hundred microliters of cells was plated in triplicate onto 96-well Corning Costar plates with an equal volume of complete RPMI
containing 4OHT or ethanol and cytokines as appropriate (final cell concentration was 1-2 x 10^6 cells/ml). 4OHT was used at a final concentration of 100 nM, and the ethanol vehicle control was prepared by adding an equivalent volume of 100% ethanol to the control medium. The final concentration of IL-7 was 1 ng/ml, the concentration of hIL-2 was 100 U/ml, and the concentrations of IL-15 and IL-4 were both 10 ng/ml. For the activation of T cells, the wells were pre-coated with anti-CD3 (5 µg/ml) and anti-CD28 (1 µg/ml) in PBS for several hours at room temperature and were rinsed with PBS immediately before use. Cells were cultured in a 5% CO_2, 37°C humidified atmosphere for one, three, or five days before analysis. Unless otherwise indicated, the data shown are from a three-day culture of the cells.

2.3.3 Sorting of CD44<low> and CD44<high> cells

For experiments using sorted T cells, splenocytes as well as mesenteric and inguinal lymph nodes were collected, and RBCs were lysed as described above. Cells were resuspended in 2% FBS in PBS for T cell enrichment prior to sorting. T cells were enriched using the EasySep T Cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada) following the manufacturer’s instructions. Cells were surface-stained and sorted into CD44<high> and CD44<low> populations on a FACSDiva (BD Biosciences, San Jose, CA, USA) or MoFlo (Beckman Coulter, Miami, FL, USA) cell sorter. To increase the plating density of sorted cells, CD4<sup>+</sup> and CD8<sup>+</sup> cells were cultured together within the
CD44\textsuperscript{high} and CD44\textsuperscript{low} populations. Cells were cultured in triplicate as described above except with cell concentrations of 0.1-1 x 10\textsuperscript{6} cells/ml.

**2.3.4 Flow cytometry and measurement of cell survival**

Cells were transferred into a U-bottom 96-well plate, centrifuged, and resuspended in 25% 24G2 hybridoma supernatant (Fc block) in 2% FBS. After a 10-15 minute blocking step, cells were stained with antibodies to surface molecules for 15-30 minutes. Cells were washed with 2% FBS and resuspended in 2% FBS containing PI at 2 \( \mu \text{g/ml} \). Cells were kept on ice throughout staining and until analysis on a FACSCanto cytometer (BD Biosciences). Flow cytometry data were analyzed on FlowJo software (Tree Star, Inc., Ashland, OR, USA). Events were first gated on live and dead cells (and “debris” was removed) based on forward and side scatter (FSC and SSC). Then CD4\textsuperscript{+}TCR\textsuperscript{β+} and CD8\textsuperscript{+}TCR\textsuperscript{β+} populations were gated (in some cases with the addition of the marker CD44), and the percentage of cells in each population that was negative for PI staining was determined.

**2.3.5 Intracellular staining for Mcl-1, Bcl-2, and Bim**

T cells were enriched as described above using the Easy Sep T Cell Enrichment Kit following the manufacturer’s instructions. Cells were plated in a 96-well plate at a density of 1-2 x 10\textsuperscript{6} cells/ml in the presence of IL-2 (100 U/ml), IL-4 (10 ng/ml), IL-7 (1 ng/ml), or IL-15 (10 ng/ml) or without cytokine. After three days in culture, cells were surface-stained as described above, fixed in 1% PFA on ice for 20 min, and stored at 4°C.
overnight. Cells were permeabilized with 0.1% saponin in 2% FBS. For the Mcl-1 and Bim stains, the permeabilization buffer contained 5% donkey serum and 10% 24G2 supernatant. After permeabilization, cells were centrifuged and resuspended in 90 µl of the same buffer. PE-anti-Bcl-2 and the corresponding isotype control were added at 10 µl/stain as per the manufacturer’s recommendation. Stock solutions of anti-Mcl-1 (0.1 mg/ml in 50% glycerol in PBS) and anti-Bim (0.234 mg/ml in 50% glycerol) were diluted so as to add 10 µl of the diluted antibody solution to yield a final dilution of 1:100 (Mcl-1) or 1:200 (Bim). For the isotype controls, stock solutions of rabbit IgG were prepared to equivalent concentrations in 50% glycerol in PBS and were diluted and added to the appropriate wells accordingly. For each condition, one well was stained with each isotype control, and duplicate wells were used for each of the specific antibody stains. After primary antibody staining, cells were washed in 0.1% saponin two times, and cells for the Mcl-1 and Bim stains as well as the isotype controls were incubated in PE-anti-rabbit IgG (1 µl/well) in 0.1% saponin. Cells were washed two times and were analyzed immediately using a FACSCanto cytometer. For subsequent analysis, only those cells in the “live” gate by FSC and SSC and the CD44low gate were used.

### 2.3.5 Western blotting

Enriched T cells were prepared as described and were plated onto 6-well plates at a density of 2 x 10^6 cells/ml (6 ml, 12 x 10^6 cells per well) in the presence or absence of
cytokines. After one day (22 h), the cells were collected into 15-ml conical tubes, washed with PBS, and resuspended in 150 µl of SDS sample buffer (50 mM Tris HCl, pH 6.8 and 2% SDS) containing 20 mM freshly added dithiothreitol (DTT). Cells were lysed on ice for 30 minutes then 30 µl of 6x sample buffer containing bromophenol blue and glycerol (at final concentrations of 0.1% and 10%, respectively) was added, and samples were boiled for 5 min. Protein lysate aliquots were stored at -80°C until use. For “fresh” T cells, an equal number of cells was lysed immediately after T cell enrichment, and the sample was prepared and stored as described. Protein samples were subjected to SDS-PAGE and were transferred to a polyvinylidene fluoride (PVDF) membrane (ImmobilonP; Millipore, Billerica, MA, USA). To allow simultaneous staining of Bak, Bax, or Bim and the loading control (β-tubulin), the membranes were cut at the 37 kDa marker, and the top half was used for β-tubulin, while the bottom half was used for the Bcl-2 family protein. The membranes were blocked with 5% milk in PBS-T (PBS containing 0.2% Tween-20) at room temperature with gentle shaking for 45 min followed by incubation in primary antibodies (all used at 1:1,000 in 3% BSA in PBS-T) for 1-1.5 h also at room temperature with gentle shaking. After washing in PBS-T (3 x 10 min), the HRP-conjugated anti-rabbit secondary antibody was added (1:5,000 in 3% BSA in PBS-T), and the membranes were gently shaken at room temperature for one hour. The membranes were washed (3 x 10 min in PBS-T) and incubated in the
SuperSignal West Pico Substrate (Pierce/Thermo Scientific, Rockford, IL, USA) for 5 min before the signal was detected by exposure to film.

2.3.6 Image analysis for Western blotting

To estimate the relative intensities of the Bak, Bax, and Bim bands corrected by the β-tubulin loading control bands, the gel analysis tools of the ImageJ software (http://rsbweb.nih.gov/ij/) were used. For each blot, a histogram was plotted for the signal in each lane, and the peak corresponding to the band was defined by drawing a line across the bottom of the peak based on the signal in the background region. The area under the peak was calculated relative to Lane 1 (fresh T cells, arbitrarily set to 1). To correct for slight differences in protein loading, the relative intensity of Bak, Bax, or Bim was divided by the relative intensity of the corresponding β-tubulin band to yield a loading-corrected estimated intensity of Bak, Bax, or Bim relative to Lane 1.

2.3.6 Statistical analyses

Additional data analysis and the graphing of data were performed using Prism (GraphPad Software, Inc., La Jolla, CA, USA). The mean percent survival of triplicate wells in each condition was determined for each experiment. The figures from representative individual experiments depict the mean percent survival plus the standard deviation (SD) of triplicate wells. To determine the Mcl-1-dependent survival ratio, the mean survival of the wells cultured in 4OHT was divided by the mean survival of the corresponding wells in ethanol control medium (labeled EtOH in figures). The SD
of the 4OHT:ETOH ratio for individual experiments with triplicate wells was calculated using the equation \( SD_{\text{ratio}} = \sqrt{[SD_{\text{EtOH}}]^2 + [SD_{\text{4OHT}}]^2} \). To combine multiple experiments, the 4OHT:EtOH survival ratios of the individual experiments were averaged, and the mean plus standard error of the mean (SEM) are depicted in the figures. To assess statistical significance, an unpaired Student’s t test was used, and p values less than 0.05 were considered to be significant. For some figures (as indicated in the legends), a “graded” indication of significance is depicted using the following symbols: *, \( p < 0.05 \); **, \( p < 0.01 \); and ***, \( p < 0.001 \).

2.4 Experimental methods for Chapter 5

2.4.1 Infection of Bcl-2\(^{\text{YFP}}\) mice

Mice were infected by tail vein injection with \( 10^4 \) colony-forming units (CFU) of \( Listeria\ monocytogenes \) expressing OVA (LM-OVA) resuspended in PBS. For analysis of Bcl-2 expression during the T cell response, Bcl-2\(^{\text{YFP}}\) mice were infected directly. For sorting experiments, 50,000-100,000 Bcl-2\(^{\text{YFP}}\) cells on the OT1 TCR transgenic background (OT1\(^{\text{YFP}}\)) were transferred by intraperitoneal injection into congenically marked recipients one day before infection. Briefly, spleens from OT1\(^{\text{YFP}}\) mice were removed and made into a single cell suspension. RBCs were lysed by incubation in ACK buffer for 1-2 min at room temperature. Cells were resuspended in 2% FBS in PBS for CD8\(^+\) T cell enrichment prior to sorting. CD8\(^+\) T cells were enriched using the EasySep CD8\(^+\) T Cell
Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada) following the manufacturer’s instructions. Cells were resuspended in PBS for injection.

2.4.2 Analysis of effector T cell populations

Seven or eight days after infection (the “peak” of the immune response), spleens were removed and prepared to a single-cell suspension in 2% FBS in PBS as above. Cells were incubated with 24G2 supernatant for 10 min on ice then with free mouse IgG1 (BD Biosciences) for 10 min. Then, a pre-prepared mixture of OVA peptide (American Peptide Company; Sunnyvale, CA) bound to DimerX (an H-2Kb-IgG1 fusion protein; BD Biosciences; bound to OVA overnight in PBS at 37°C) with secondary antibody (PE-anti-mouse IgG1; BD Biosciences) was added to the cells along with antibodies to surface molecules. Staining was allowed to proceed for 30-90 minutes, and cells were analyzed on a FACSCanto cytometer (BD Biosciences).

2.4.3 Sorting and adoptive transfer of Bcl-2YFP populations

Day 7 or 8 splenocytes were prepared and enriched for CD8+ T cells as described above. Typically, 3-4 mice were pooled for each experiment. Enriched CD8+ cells were stained with antibodies to CD8, CD44, the congenic marker CD45.1/CD45.2, the OT1 TCR subunit Vα2, CD127, and KLRG1 (all from Biolegend) for at least 20 minutes. Cells were sorted on a FACSDiva sorter (BD Biosciences). After sorting, cells were washed once with PBS and resuspended in PBS for intravenous injection into the tail vein of naïve, congenically marked recipient mice. Approximately 10,000 cells from each
population were transferred (see figure legends for the cell number transferred for each experiment). In some experiments, $5 \times 10^6$ unsorted, enriched CD8$^+$ cells were transferred as a positive control.

**2.4.4 Memory analysis of recipient mice**

After transfer of OT1$^{YFP}$ cells, at least five weeks were allowed to pass before secondary challenge of the recipient mice. A secondary LM-OVA infection and T cell analysis were performed as described above with the exception that the analysis was performed on day 4 or 5. Splenic cellularity was determined by counting cells using a Countess cell counter (Invitrogen). For the flow cytometric analysis, 50,000 total events were collected to assess the percentage of CD8$^+$ cells in the spleen (only live cells were included based on PI exclusion). Then, a high number of live CD8$^+$ cells was collected to determine the frequency of the relatively rare donor cells. The total number of OT1$^{YFP}$ cells per spleen was calculated based on the observed frequencies and the total cell number. The percent recovery or fold expansion of the donor cells was calculated by dividing the calculated number of OT1$^{YFP}$ cells in the spleen by the number of cells transferred. For mice that received unsorted CD8$^+$ cells, the number of OT1$^{YFP}$ cells transferred was determined based on the percentages observed during sorting and was used as the denominator to calculate recovery/expansion.
3. Mcl-1 promotes survival of thymocytes by inhibition of Bak in a pathway separate from Bcl-2

The results in this section have been previously published, and the corresponding text and figures have been modified from the original publication (Dunkle et al., 2010).

3.1 Introduction

The antiapoptotic proteins Bcl-2, Mcl-1, and Bcl-xL are all expressed in thymocytes, but in distinct patterns and potentially for distinct purposes. Mcl-1 is expressed in all thymocyte subsets (Dzhagalov et al., 2008; Opferman et al., 2003), while Bcl-2 is expressed in DN and SP stages but is down-regulated in DP until positive selection (Gratiot-Deans et al., 1993; Gratiot-Deans et al., 1994; Veis et al., 1993a). Conversely, Bcl-xL, the predominant splice product of the Bcl-x gene in thymocytes, exhibits high expression at DP but low expression at DN and SP (Grillot et al., 1995; Ma et al., 1995).

Knockout and conditional knockout mouse models have suggested that the anti-apoptotic proteins of the Bcl-2 family may have distinct roles in thymocytes and T cells because the loss of each of these proteins exhibits a distinct phenotype. Bcl-2−/− thymocyte development is normal at early post-natal timepoints, but T cells in both the periphery and thymus are lost by four weeks of age, likely due to differences in Bcl-2-dependency between fetal liver- and bone marrow-derived hematopoietic progenitors (Matsuzaki et
al., 1997; Nakayama et al., 1994; Nakayama et al., 1993; Veis et al., 1993b). Although Bcl-x\textsuperscript{-/-} DP thymocytes have reduced survival under some conditions, Bcl-x\textsubscript{i} is not required for T cell development, effector function, or memory (Ma et al., 1995; Motoyama et al., 1995; Zhang and He, 2005). Mcl-1 is required for thymocyte progression past the DN stage and is also required for SP cell maturation and for the survival of naïve and activated peripheral T cells (Dzhagalov et al., 2008; Opferman et al., 2003).

Given that Mcl-1 and Bcl-2 are both expressed in DN and SP thymocytes (Dzhagalov et al., 2008; Gratiot-Deans et al., 1993; Gratiot-Deans et al., 1994; Veis et al., 1993a), it was intriguing that loss of Mcl-1 alone prohibited survival past these stages (Dzhagalov et al., 2008; Opferman et al., 2003). Similarly, post-natal Bcl-2\textsuperscript{-/-} thymocytes (after 3-4 weeks of age) display a similar survival defect and block in development at the DN stage (Matsuzaki et al., 1997; Nakayama et al., 1994; Nakayama et al., 1993; Veis et al., 1993b). Thus, both Bcl-2 and Mcl-1 are singly required for survival of thymocytes past the DN stage and perhaps also at the SP stage and in peripheral T cells. This raised the question of whether Bcl-2 and Mcl-1 have distinct molecular roles in thymocytes. In order to assess this, we generated genetic models to dissect the mechanisms of Mcl-1 activity. These studies yielded insight into the mechanisms of thymocyte survival and also have implications for understanding how the different types of Bcl-2 family members regulate apoptosis in vivo.
3.2 Results

3.2.1 Overexpression of Bcl-2 does not rescue survival of Mcl-1-deficient DN or SP thymocytes

Previous observations from knockout mouse models implied that Mcl-1 and Bcl-2 have distinct roles at the DN and SP stages of thymocyte development. Although both Mcl-1 and Bcl-2 are antiapoptotic proteins, these distinct roles could be the result of interactions with different proapoptotic proteins. However, an alternative explanation for the parallel phenotypes of the Mcl-1- and Bcl-2-deficient models is that Mcl-1 and Bcl-2 have overlapping roles, but the combined level of these two proteins is critical in regulating cell survival. This model would suggest that in Mcl-1-deficient thymocytes, the endogenous levels of Bcl-2 are insufficient to promote cell survival. To investigate these possibilities, we crossed mice that have floxed alleles of Mcl-1 and express Cre recombinase in the thymocyte lineage (Mcl-1<sup>f/f</sup>LckCre or Mcl-1<sup>f/f</sup>CD4Cre) to mice that over-express Bcl-2 as a transgene under the H-2K<sup>b</sup> promoter, which yields high expression in all hematopoietic cells (Domen et al., 1998). Flow cytometry confirmed expression of the Bcl-2 transgene in DN, DP, and SP thymocytes in our system (data not shown).

Consistent with previous experiments (Dzhagalov et al., 2008), Mcl-1<sup>f/f</sup>LckCre mice exhibited a dramatic reduction (~90%) in the total thymocyte number due to a block at DN, specifically an accumulation at the CD44<sup>+</sup>CD25<sup>+</sup> DN2 and CD44<sup>-</sup>CD25<sup>+</sup> DN3 stages (Figure 4). A similarly profound loss in thymocyte number was observed in
Mcl-1<sup>fl</sup>LckCreBcl-2<sup>tg</sup> mice compared to both Bcl-2<sup>tg</sup> and wild type controls (Figure 4A). In spite of the increased thymic cellularity in the Bcl-2<sup>tg</sup> control mice, the total cell numbers in Mcl-1<sup>fl</sup>LckCreBcl-2<sup>tg</sup> mice were not significantly different from those of Mcl-1<sup>fl</sup>LckCre mice (Figure 4A). As previously observed, the thymic profile of Mcl-1<sup>fl</sup>LckCre mice showed a skewing toward the DN compartment at the expense of DP cells (Figure 4B, upper panel). Although the Bcl-2 transgene alone increased the DN percentage, the block at DN was also observable in Mcl-1<sup>fl</sup>LckCreBcl-2<sup>tg</sup> mice (Figure 4B, upper panel). Additionally, the block at the DN2-DN3 stages was still observed in the Mcl-1<sup>fl</sup>LckCreBcl-2<sup>tg</sup> mice (Figure 4B).

![Figure 4: Overexpression of Bcl-2 does not rescue thymocyte number or profile in Mcl-1<sup>fl</sup>LckCre mice](image)

A. Total thymocyte number in control (ctrl), Mcl-1<sup>fl</sup>LckCre, Bcl-2<sup>tg</sup>, and Mcl-1<sup>fl</sup>LckCreBcl-2<sup>tg</sup> mice. Control is Mcl-1<sup>fl</sup> or Mcl-1<sup>+/+</sup> without LckCre. The mean value is indicated by a line. Mcl-1<sup>fl</sup>LckCre numbers were significantly different from the Cre-negative control on both the wild type Bcl-2 and the Bcl-2<sup>tg</sup> background; ns signifies that the difference between Mcl-1<sup>fl</sup>LckCreBcl-2<sup>tg</sup> and Mcl-1<sup>fl</sup>LckCre was not statistically significant (p>0.05). B. Representative flow cytometry plots of control, Mcl-1<sup>fl</sup>LckCre, Bcl-2<sup>tg</sup>, and Mcl-1<sup>fl</sup>LckCreBcl-2<sup>tg</sup> mice. Top panel: CD4 vs. CD8 staining of the thymus. Numbers represent the percent of total thymocytes in each quadrant. Lower panel: CD44 and CD25 expression.
in DN cells (DN1 is top left; DN2-4 proceed clockwise). Numbers represent the percentage of DN cells in each quadrant. All mice were 5-8 weeks of age, and the data represent two separate experiments (n = 3-4).

Because different thymic subsets may have different apoptotic mechanisms, we examined whether Bcl-2 overexpression could rescue the survival of Mcl-1-deficient SP thymocytes and peripheral T cells. We previously showed that Mcl-1<sup>f/f</sup>CD4Cre mice have normal cellularity in the thymus and spleen, but a marked reduction in mature SP cells as defined by expression of TCRβ and the markers Qa2 and CD69 (mature SP cells are TCRβ<sup>-</sup>Qa2<sup>-</sup>CD69<sup>lo</sup>) as well as both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the periphery (Dzhagalov et al., 2008). To determine whether these defects could be rescued by overexpression of Bcl-2, we examined Mcl-1<sup>f/f</sup>CD4CreBcl<sup>-2%</sup> mice. Representative profiles from the spleen (Figure 5A) and thymus (Figure 5B-D) of these mice along with wild type, Mcl-1<sup>f/f</sup>CD4Cre, and Bcl-2<sup>%</sup> controls are shown in Figure 5. Mcl-1<sup>f/f</sup>CD4CreBcl-2% mice exhibited a clear loss of T cells in the spleen, although the percentage was consistently higher than that of Mcl-1<sup>f/f</sup>CD4Cre mice (Figure 5A). Like Mcl-1<sup>f/f</sup>CD4Cre mice, the thymic profile of Mcl-1<sup>f/f</sup>CD4CreBcl-2% mice showed a reduction in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> SP cells (Figure 5B) and in the percentage of TCRβ<sup>+</sup> cells within the SP compartments (Figure 5C [CD4<sup>+</sup>SP] and 5D [CD8<sup>+</sup>SP]). However, within the CD4<sup>+</sup>TCRβ<sup>+</sup> and CD8<sup>+</sup>TCRβ<sup>+</sup> SP compartments, Mcl-1<sup>f/f</sup>CD4CreBcl-2% mice had higher proportions in the mature gate than Mcl-1<sup>f/f</sup>CD4Cre mice (Figure 5C and 5D).
Figure 5: Overexpression of Bcl-2 does not overtly rescue the spleen or SP thymocyte phenotype in Mcl-1<sup>f/f</sup>CD4Cre mice

Representative flow cytometry plots of the spleen (A), thymus (B), CD4<sup>+</sup> SP thymocytes (C) and CD8<sup>+</sup> SP thymocytes (D) from control (Mcl-1<sup>f/f</sup> or Mcl-1<sup>1/2</sup>), Mcl-1<sup>1/2</sup>CD4Cre, Bcl-2<sup>+</sup> (Mcl-1<sup>f/f</sup> or Mcl-1<sup>1/2</sup>), and Mcl-1<sup>1/2</sup>CD4CreBcl-2<sup>tg</sup> mice. Qa2 vs. CD69 SP plots were pre-gated on TCRβ<sup>+</sup> cells. Numbers represent the percent of total splenocytes/thymocytes (A, B) or of pre-gated populations (C, D) within the gate.

The numbers of mature CD4<sup>+</sup> and CD8<sup>+</sup> SP cells in Mcl-1<sup>1/2</sup>CD4CreBcl-2<sup>tg</sup> mice were increased over those of the Mcl-1<sup>f/f</sup>CD4Cre mice (Figure 6A). The same was true of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen (Figure 6B). However, consistent with previous
observations of the Bcl-2\(^{tg}\) mice (Domen et al., 1998), we observed elevated total cell
numbers in both the thymus and the spleen in the Bcl-2\(^{tg}\) mice, and the total CD4\(^{+}\) and
CD8\(^{+}\) cell numbers in both the thymus and the spleen of Mcl-1\(^{f/f}\)/CD4CreBcl-2\(^{tg}\) mice were
significantly reduced compared to Bcl-2\(^{tg}\) controls (Figure 6). Therefore, it was unclear
whether the increased numbers of mature SP and splenic T cells were the result of a
partial rescue of the survival Mcl-1-deficient cells by Bcl-2 (the survival of a proportion
of cells that would typically die upon loss of Mcl-1) or of a non-specific increase in cell
survival in the remaining cells after Mcl-1 deletion (the expansion of the few cells that
escape death).

To better compare the relative survival of different populations of cells with and
without Mcl-1 and excess Bcl-2, we compared the percentages of mature SP cells in the
thymus and T cells in the spleen over several mice to determine whether the
developmental blocks observed in the Mcl-1\(^{f/f}\)/CD4CreBcl-2\(^{tg}\) mouse were of a similar
magnitude to those observed in the Mcl-1\(^{f/f}\)/CD4Cre mouse. For CD4\(^{+}\) SP cells, there was
an observable increase in the mean percentage of mature SP cells in the thymus of
Mcl-1\(^{f/f}\)/CD4CreBcl-2\(^{tg}\) mice over that of Mcl-1\(^{f/f}\)/CD4Cre mice. However, the percentage
was significantly lower than that of the Bcl-2\(^{tg}\) control mice, which itself was increased
over wild type (Figure 6C, left). For CD8\(^{+}\) SP cells, the percentage of mature cells in the
thymus of Mcl-1\(^{f/f}\)/CD4CreBcl-2\(^{tg}\) mice was not significantly different from that of
Mcl-1\(^{f/f}\)/CD4Cre mice (Figure 6C, right). Similar results were observed for CD4\(^{+}\) and CD8\(^{+}\)
T cells in the spleen (Figure 6D). These data show that while over-expression of Bcl-2 lends a modest improvement to survival of Mcl-1-deficient mature SP thymocytes and peripheral T cells, particularly CD4+ cells, there is still a significant impairment in the survival of these cells because the transgene was unable to rescue the cell percentages to wild type or Bcl-2+ control levels, indicating a developmental block even in the presence of excess Bcl-2.

Figure 6: Overexpression of Bcl-2 does not greatly rescue mature SP thymocytes or peripheral T cells in Mcl-1−CD4Cre mice

A, B. Total number of mature (TCRβ+Qa2+CD69lo) CD4+ and CD8+ SP cells in the thymus (A) as well as CD4+ and CD8+ T cells (TCRβ+) in the spleen (B) of control, Mcl-1−CD4Cre, Bcl-2−/−, and Mcl-1−CD4CreBcl-2−/− mice. C, D. Percent mature (TCRβ+Qa2+CD69lo) CD4+ and CD8+ SP cells of the total thymus (C) and percent CD4+ and CD8+ T cells (TCRβ+) of the total spleen (D) of control, Mcl-1−CD4Cre, Bcl-2−/−, and Mcl-1−CD4CreBcl-2−/− mice. “CD4Cre” represents Mcl-1−CD4Cre. All mice were 5-8 weeks of age, and the data represent five separate experiments (n = 5-8; Qa2 vs. CD69 not available for all experiments). For all graphs, the line represents the mean total/percentage. The p value is illustrated as *p<0.05, **p<0.01, ***p<0.001, or not significant, “ns”. Mcl-1−CD4Cre numbers and percentages were significantly different from the Cre-negative control for all measures.
3.2.2 Loss of Bak but not Bax partially rescues the survival and development of Mcl-1-deficient thymocytes at the DN stage

One explanation for the non-redundancy of Mcl-1 and Bcl-2 is that these proteins have differential abilities to bind proapoptotic proteins. For example, the BH3-only protein Noxa binds Mcl-1 and A1 but not Bcl-2 or Bcl-xL (Chen et al., 2005; Kuwana et al., 2005). Intriguingly, Bak has been shown to bind Mcl-1 at relatively high affinity, Bcl-xL at a lower affinity, and to not bind Bcl-2 to a detectable level (Willis et al., 2005). However, a different group showed that when Bak protein as opposed to Bak BH3 peptide was used, Bcl-2 could bind Bak with similar or better affinity than Bcl-xL, although this was somewhat dependent on the variant of Bcl-2 (Dai et al., 2009). Thus, the in vivo specificity of Bak for Mcl-1 is unclear. To determine whether the death of Mcl-1-deficient thymocytes was occurring preferentially through Bak, we crossed Mcl-1<sup>f/f</sup>LckCre mice to Bak<sup>−/−</sup> and Bax<sup>−/−</sup> mice. While some groups but not others have observed a mild expansion in the total cellularity of the Bax<sup>−/−</sup> thymus (Knudson et al., 1995; Rathmell et al., 2002), we observed no differences in total cellularity between wild type and Bak or Bax single knockouts (data not shown), so all Cre-negative genotypes are represented as controls in our experiments.

The total thymocyte numbers in Mcl-1<sup>f/f</sup>LckCreBak<sup>−/−</sup> mice were significantly increased over those of Mcl-1<sup>f/f</sup>LckCreBak<sup>+/−</sup> mice, although the numbers did not reach wild type control levels (Figure 7A). Mcl-1<sup>f/f</sup>LckCreBak<sup>−/−</sup> mice were identical to age-matched Mcl-1<sup>f/f</sup>LckCre mice in these experiments (data not shown). In contrast, the
thymic cellularity of Mcl-1^{f/f}LckCreBax^{-/} mice was no different from that of Mcl-1^{f/f}LckCre mice (Figure 7B). To determine which stages of thymocyte development were rescued, we compared the cell numbers within the different thymic subsets. Mcl-1^{f/f}LckCreBak^{-/} mice had equivalent DN thymocyte numbers to control mice (Figure 7C, upper row), but in subsequent developmental subsets, the cellularity in Mcl-1^{f/f}LckCreBak^{-/} mice did not reach that of the controls. However, there was a significant increase in Mcl-1^{f/f}LckCreBak^{-/} cell numbers over those of the Mcl-1^{f/f}LckCreBax^{-/} mice in all subsets except for CD8^{+} SP, indicating some persistence of rescued cells into later stages (Figure 7C, upper row). Consistent with the lack of rescue of the total cellularity by the Bax^{-/} background, the cell numbers in Mcl-1^{f/f}LckCreBax^{-/} mice were not different from those in Mcl-1^{f/f}LckCre mice for any thymocyte subset (Figure 7C, lower row).

Figure 7: Bak^{−/} but not Bax^{−/} rescues thymocyte numbers in Mcl-1^{f/f}LckCre mice

A, B. Total thymic cellularity of control, Mcl-1^{f/f}LckCreBak^{+/−}, and Mcl-1^{f/f}LckCreBak^{-/} mice (A) or control, Mcl-1^{f/f}LckCre, Mcl-1^{f/f}LckCreBax^{+/−}, and Mcl-1^{f/f}LckCreBax^{-/} mice (B). Control is Mcl-1^{f/f} or Mcl-1^{f/+} with Bak^{+/−}, Bak^{−/−}, or Bak^{−/} (A) or with Bax^{+/−}, Bax^{−/−}, or Bax^{−/−} (B). C. Total numbers of thymic subsets: DN, immature single positive (ISP = CD4^{−}/CD8^{−}/TCRβ^{+}), DP, CD4^{+} SP, CD8^{+} SP
(CD4-CD8-TCRβ}). All mice were 4-7 weeks old, and the data represent five separate experiments each for Bak⁺/- and Bax⁺/- [n = 8-11 (Bak) and 3-10 (Bax)].

The flow cytometry profile of the Mcl-1+/LckCreBak⁺/- thymus revealed an increased percentage of cells in the DP gate and a decreased percentage of cells in the DN gate compared to the Mcl-1+/LckCreBak⁺/- profile (Figure 8A). Consistent with a partial rescue, the Mcl-1+/LckCreBak⁺/- thymus had a higher percentage of CD4⁺CD25⁺DN4 cells than the Mcl-1+/LckCreBak⁺/- thymus, but the elevated percentage at the DN3 stage was not completely relieved (Figure 8A). The Mcl-1+/LckCreBax⁺/- thymus showed no relief of the DN block as observable by the percentage of DN and DP cells and by the DN profile (Figure 8B). In fact, in most experiments, the Mcl-1+/LckCreBax⁺/- thymus contained higher percentages in the DN gate than the Mcl-1+/LckCreBax⁺/- thymus, even though the Bax⁺/- alone has no such increase (Rathmell et al., 2002).

![Flow Cytometry Plots](image)

**Figure 8:** Bak⁺/- but not Bax⁺/- rescues the developmental block in the Mcl-1+/LckCre thymus

A, B. Representative flow cytometry plots of control, Mcl-1+/LckCreBak⁺/-, Mcl-1+/LckCreBak⁺/- (A) and Mcl-1+/LckCreBax⁺/- (B) mice. All mice were 4-7
weeks old, and the data are representative of five separate experiments each for Bak\(^{-/}\) and Bax\(^{-/}\). The numbers indicate the percentage of total thymocytes (top row) or DN thymocytes (bottom row) within the quadrant.

Finally, we examined Mcl-1 protein levels in the different thymocyte subsets to confirm that the rescued Mcl-1\(^{+/}\)LckCreBak\(^{-/}\) thymocytes did not retain Mcl-1 expression. We have previously shown that we are able to specifically detect Mcl-1 protein by flow cytometry (Dzhagalov et al., 2008). Due to differences in background (isotype control) fluorescence levels between thymic subsets, relative Mcl-1 levels are displayed as a ratio of the mean fluorescence intensity (MFI) of the anti-Mcl-1 stain to that of the isotype control for each subset. Mcl-1\(^{+/}\)LckCreBak\(^{-/}\) thymocytes displayed wild type levels of Mcl-1, likely because the surviving thymocytes have escaped deletion of Mcl-1 (Figure 9A). This is consistent with previous results demonstrated by Western blot or PCR in Mcl-1\(^{+/}\)LckCre mice (Dzhagalov et al., 2008; Opferman et al., 2003). Mcl-1\(^{+/}\)LckCreBak\(^{-/}\) thymocytes had a detectable and statistically significant decrease in the Mcl-1 expression ratio in all of the major thymic subsets, indicating that these cells have survived without Mcl-1 and represent a true rescue (Figure 9A). No such decrease was detectable in Mcl-1\(^{+/}\)LckCreBax\(^{-/}\) thymocytes, indicating that most of the cells in these mice, like those in the Mcl-1\(^{+/}\)LckCre mice, are those that have escaped deletion (Figure 9B).
3.2.3 Loss of Bak but not Bax rescues progression to the mature SP stage and T cell numbers in the periphery

Given the rescue of Mcl-1-deficient DN cells by the Bak\textsuperscript{+/} background and that we continued to detect increased cell numbers in later subsets, we sought to determine whether the loss of Bak could rescue the survival of Mcl-1-deficient mature SP cells. As observed previously, the thymus and the spleen from Mcl-1\textsuperscript{+/}CD4Cre mice had normal total cellularity, and this was unaltered by deletion of Bak or Bax (Figure 10A). However, the percentages of mature SP cells in the thymus and T cells in the spleen were significantly reduced in the Mcl-1\textsuperscript{+/}CD4Cre mice [(Dzhagalov et al., 2008) and Figure 10B]. Flow cytometry analysis demonstrated that the percentage of T cells in the Mcl-1\textsuperscript{+/}CD4CreBak\textsuperscript{+} spleen returned to near control levels, and a partial increase over

![Figure 9: Bak\textsuperscript{+/}Mcl-1\textsuperscript{+}/LckCre thymocytes survive in the absence of Mcl-1 protein](image)
Mcl-1<sup>−/−</sup>CD4Cre was observed in mice heterozygous for Bak (Figure 10B). We observed a return of Qa2<sup>+</sup>CD69<sup>lo</sup> cells in the CD4<sup>+</sup> and CD8<sup>+</sup> SP compartments in both Mcl-1<sup>−/−</sup>CD4CreBak<sup>+/−</sup> and Mcl-1<sup>−/−</sup>CD4CreBak<sup>−/−</sup> mice (Figure 10B). Similar to what was observed with the Bcl-2 transgene, Mcl-1<sup>−/−</sup>CD4CreBax<sup>−/−</sup> mice exhibited a modest improvement over Mcl-1<sup>−/−</sup>CD4Cre in the percentage of T cells in the spleen and in the percentage of Qa2<sup>−</sup>CD69<sup>lo</sup> cells within the already reduced SP compartments (Figure 10C). However, this did not match even the Bak<sup>−/−</sup> in the extent of the rescue.

**Figure 10: Bak<sup>−/−</sup> but not Bax<sup>−/−</sup> rescues survival of SP thymocytes and peripheral T cells in Mcl-1<sup>−/−</sup>CD4Cre mice**

A. Total cell numbers in the thymus and spleen of Mcl-1<sup>−/−</sup>CD4Cre mice crossed to the Bak<sup>−/−</sup> and Bax<sup>−/−</sup> backgrounds. Bars are the mean + SD. Control (Ctrl) mice are Mcl-1<sup>−/−</sup>, Mcl-1<sup>−/−</sup>Bak<sup>−/−</sup>, and Mcl-1<sup>−/−</sup>Bax<sup>−/−</sup>, or Mcl-1<sup>−/−</sup> Mcl-1<sup>−/−</sup>Bak<sup>−/−</sup>, and Mcl-1<sup>−/−</sup>Bax<sup>−/−</sup>. B. Representative flow cytometry plots for control, Mcl-1<sup>−/−</sup>CD4Cre, Mcl-1<sup>−/−</sup>CD4CreBak<sup>−/−</sup>, and Mcl-1<sup>−/−</sup>CD4CreBax<sup>−/−</sup> mice (C) as well as Mcl-1<sup>−/−</sup>CD4CreBak<sup>−/−</sup> mice (C). The upper row shows the percentage of T cells (TCRβ<sup>+</sup>) and B cells (B220<sup>+</sup>) in the spleen. The middle and bottom rows show the percentage of Qa2<sup>−</sup>CD69<sup>lo</sup> mature cells within the CD4<sup>+</sup> TCRβ<sup>+</sup> SP compartment (middle) and the CD8<sup>+</sup> TCRβ<sup>+</sup> SP compartment (bottom) in the thymus.

In addition to the rescue of the relative percentage of cells in each population, the Bak<sup>−/−</sup> background rescued Mcl-1<sup>−/−</sup>CD4Cre mature CD4<sup>+</sup> and CD8<sup>+</sup> SP cell numbers to
wild type levels, and a dose effect was observed in Bak\(^{-/-}\) mice (Figure 11A). The same results were observed in the spleen, although the Bak\(^{-/-}\) effect was not statistically significant in CD8\(^+\) T cells (Figure 11B). Although there was a statistically significant increase in the number of mature CD8\(^+\) SP and CD4\(^+\) and CD8\(^+\) T cells in the Mcl-1\(^{-/-}\)CD4CreBax\(^{-/-}\) mice, the absolute difference in cell numbers was minor, and the numbers from the Mcl-1\(^{-/-}\)CD4CreBax\(^{-/-}\) mice were significantly different from those of the Mcl-1-sufficient controls for all subsets (Figure 11C and 11D).

Figure 11: Gene dose-dependent rescue of Mcl-1-deficient SP thymocytes and peripheral T cells by loss of Bak but not Bax

A, B. Total cell numbers of mature TCR\(\beta\)Qa2\(^-\)CD69\(^-\) SP cells in thymus (A) and CD4\(^+\) and CD8\(^+\) T cells in spleen (B) of Mcl-1\(^{-/-}\)CD4CreBak mice. C, D. Total cell numbers of mature TCR\(\beta\)Qa2\(^-\)CD69\(^-\) SP cells in thymus (C) and CD4\(^+\) and CD8\(^+\) T cells in spleen (D) of Mcl-1\(^{-/-}\)CD4CreBax mice. Mice were 5-7 weeks of age, and the data represent seven (Bak) or four (Bax) separate experiments \([n = 5-13 \text{ (Bak)} \text{ or } 5-9 \text{ (Bax) per group}]\). The mean is indicated by a line, and significance is displayed as follows: *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\), and ns = not significant (\(p\geq0.05\)).
3.2.4 Loss of Bim does not rescue the survival or development of Mcl-1-deficient SP thymocytes

One of the proposed roles of the anti-apoptotic proteins is to inhibit BH3-only proteins that are capable of directly activating Bak and Bax, such as Bim (Cheng et al., 2001; Letai et al., 2002). Bim is known to be critical for preventing autoimmunity due to its roles in thymocyte negative selection and the death of activated T cells (Bouillet et al., 1999; Bouillet et al., 2002; Hildeman et al., 2002). Moreover, it has been shown that loss of Bim can rescue the survival and phenotypic defects of Bcl-2−/− mice, including thymocyte development (Bouillet et al., 2001; Wojciechowski et al., 2007). To determine whether Bim is important for the apoptosis of Mcl-1-deficient thymocytes, we crossed Mcl-1floCD4Cre mice to Bim−/− mice. Whereas there was no difference between Bim+/+ and Bim−/− mice by our measures, the Bim−/− and Mcl-1floCD4CreBim−/− mice had higher total cell numbers in the spleen and thymus (Figure 12A). The flow cytometry profiles of the spleen and thymus showed that Bim−/− control mice had normal T cell percentages in the spleen but elevated percentages of Qa2+CD69lo mature cells within the SP compartments (Figure 12B, compare with Figures 5 and 10). However, Mcl-1floCD4CreBim−/− mice showed a similar percentage of mature SP cells and peripheral T cells to Mcl-1floCD4Cre mice (Figure 12B, compare with Figures 5 and 10). In spite of the increased total cellularity, Mcl-1floCD4CreBim−/− mice displayed comparable cell numbers to Mcl-1floCD4CreBim+ mice in the mature SP (Figure 12C) and spleen T cell (Figure 12D).
compartments. Thus, Bim does not appear to have a downstream role in promoting the death of Mcl-1-deficient thymocytes.

Figure 12: Bim<sup>+</sup> does not rescue the survival of Mcl-1<sup>f/f</sup>CD4Cre SP thymocytes

A. Total cell number in the thymus and spleen of Mcl-1<sup>f/f</sup>CD4Cre mice crossed to the Bim<sup>+</sup> background. Bars are mean ± SD. Cre-negative controls were Mcl-1<sup>+/+</sup>, Mcl-1<sup>-/-</sup>, or Mcl-1<sup>f/f</sup> and were Bim<sup>+</sup> (Bim<sup>+/+</sup> or Bim<sup>-/-</sup>) or Bim<sup>-/-</sup> as indicated. All Cre<sup>+</sup> mice were Mcl-1<sup>f/f</sup> and Bim<sup>+</sup> (Bim<sup>+/+</sup> or Bim<sup>-/-</sup>) or Bim<sup>-/-</sup> as indicated. B. Representative flow cytometry plots of Bim<sup>+</sup> control and Mcl-1<sup>f/f</sup>CD4CreBim<sup>+</sup> mice. The left side shows the percentage of T cells (TCRβ+) and B cells (B220+) in the spleen. The right side shows the percentage of Qa2<sup>-</sup>CD69<sup>lo</sup> mature cells within the CD4<sup>+</sup>TCRβ<sup>+</sup> SP compartment (middle) or the CD8<sup>+</sup>TCRβ<sup>+</sup> SP compartment (far right). C, D. The numbers of mature TCRβ<sup>+</sup>Qa2<sup>-</sup>CD69<sup>lo</sup> SP cells in the thymus (C) and CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen (D) of Mcl-1<sup>f/f</sup>CD4CreBim mice. Mice were 4-8 weeks of age, and the data represent four separate experiments (n = 4-7). Significance is displayed as follows: *p<0.05, **p<0.01, ***p<0.001, and ns = not significant (p≥0.05).
3.3 Summary and discussion

3.3.1 Inhibition of Bak is the critical role for Mcl-1 in thymocytes

By generating genetic models, we showed that Mcl-1 and Bcl-2 have separate molecular roles at two critical points in thymic development, the DN stage and the SP stage. Over-expression of Bcl-2 did not rescue the survival of Mcl-1-deficient thymocytes at either the DN or the SP stage. Additionally, while it has been shown that the Bim<sup>−/−</sup> background rescues Bcl-2-deficient thymocytes (Wojciechowski et al., 2007), crossing to the Bim<sup>+/−</sup> background did not rescue the survival or developmental block of Mcl-1-deficient thymocytes. Thus, Bim does not appear to be the downstream effector of cell death in the absence of Mcl-1. Intriguingly, the Bak<sup>−/−</sup> but not Bax<sup>−/−</sup> background rescued Mcl-1-deficient thymocytes at both the DN and SP stages, yielding evidence that the specific inhibition of Bak may be the major role of Mcl-1 in thymocytes.

3.3.2 Specific roles for Mcl-1 in different cell types

It was interesting that we observed a more complete rescue of Mcl-1-deficient thymocytes by the Bak<sup>−/−</sup> background in SP cells than in DN cells, indicating that even between different thymic subsets, the dominant activity of Mcl-1 is different. Similarly, the minor rescue observed in Bcl-2<sup>−/−</sup> and Bax<sup>−/−</sup> SP thymocytes was more evident in CD4<sup>+</sup> cells than CD8<sup>+</sup> cells, indicating that CD4<sup>+</sup> and CD8<sup>+</sup> cells may have a different balance between Mcl-1-specific and Mcl-1/Bcl-2-shared pathways. A Mcl-1 hypomorphic mouse model showed a defect that was more severe in CD8<sup>+</sup> than CD4<sup>+</sup> T cells (Yang et al.,
2009), correlating well with our data that CD8\(^+\) cell survival was not improved by the Bax\(^{-/+}\) or Bcl-2\(^{-/-}\) background. These data suggest that CD8\(^+\) T cells are more exclusively dependent on Mcl-1 than CD4\(^+\) T cells.

In addition to T cells, Mcl-1 is required for neutrophil survival (Dzhagalov et al., 2007). Interestingly, unlike our observations in thymocytes, the authors of a recent study into the role of Mcl-1 in neutrophils and macrophages stated that the Bak/Bax double knockout rescues Mcl-1-deficient neutrophil survival, but neither the Bak nor Bax knockout alone rescued neutrophil survival (Steimer et al., 2009). Mcl-1-deficient neutrophils were not rescued by knocking out Bim, but the Bim\(^{-/+}\) background rescued activated Mcl-1-deficient macrophages in this study (Steimer et al., 2009). Thus, there does appear to be cell-type differences in terms of the specific role of Mcl-1, even subtly between thymocyte populations.
4. Cytokine-dependent and cytokine-independent roles for Mcl-1 in peripheral T cells

At the time of writing, portions of the text and figures from this section were under consideration for publication (Dunkle et al., submitted).

4.1 Introduction

The data in Chapter 3 indicated that the inhibition of Bak is a critical role for Mcl-1 in thymocytes (Dunkle et al., 2010). However, as discussed, our data and the data of others suggest that the dominant function of Mcl-1 may be different in different cell types and/or under different conditions (Dunkle et al., 2010; Steimer et al., 2009; Yang et al., 2009). Therefore, we wanted to determine the mechanism(s) by which Mcl-1 promotes the survival of peripheral T cells. Because most of the genes expressed in peripheral T cells are also expressed during thymic development or in other mature cell types, a peripheral T cell conditional knockout model of comparable efficiency and specificity to the LckCre and CD4Cre models used in thymocytes is not currently available. Using drug-inducible deletion of Mcl-1, however, Opferman et al. and our laboratory showed that peripheral T cells died upon loss of Mcl-1 in vivo and in a variety of conditions in vitro (Dzhagalov et al., 2008; Opferman et al., 2003).

Multiple factors are known to influence the survival of peripheral T cells. Antigen-inexperienced, or naïve, T cells require homeostatic signals, typically a low-level signal from self-peptide-MHC complexes and the cytokine IL-7, in order to be
maintained (Jameson, 2002; Surh and Sprent, 2008). Upon encountering their cognate antigen presented on MHC in the presence of co-stimulatory signals, T cells proliferate and differentiate into effector populations before “contracting” to form a population of antigen-experienced, or memory, T cells that are retained over time. Unlike naïve cells, memory cells do not require signal from self-peptide-MHC and can better utilize IL-15 for survival (Jameson, 2002; Surh and Sprent, 2008).

IL-7 is member of a family of cytokines, which includes IL-2, IL-15, IL-4, IL-9, and IL-21, that is defined by sharing the common gamma chain (γc) receptor subunit. The receptors for these cytokines are composed of the γc subunit paired with one or two other receptor subunit(s), some shared and some unique. Each of these cytokines has a distinct biological role, although several, particularly IL-7 and IL-15, promote T cell survival (Rochman et al., 2009). Studies from mice, primary cells, and cytokine-dependent cell lines have implicated the Bcl-2 family, specifically the Bcl-2/Bim/Bax axis, downstream of cytokine signaling (Khaled and Durum, 2002), but the role of Mcl-1 in the cytokine-induced survival pathway is less well-described.

The mechanism(s) by which Mcl-1 promotes the survival of peripheral T cells and the effect, if any, of trophic cytokines on these mechanisms are not yet understood. We utilized genetic models to investigate the mechanisms by which Mcl-1 promotes the survival of peripheral T lymphocytes. In the studies below, we examine the survival of Mcl-1-deficient cells on the Bak−/−, Bax−/−, Bim−/−, and Bcl-2-overexpressing backgrounds in
both the presence and absence of IL-7. Further, we examine the effects of other γc-sharing cytokines on the Mcl-1-dependency of T cells and show that these cytokines modulate Bcl-2 family protein expression, particularly that of Bim, to influence the role of Mcl-1. The data indicate that several factors in the in vivo milieu can regulate the requirement and specific roles for Mcl-1.

4.2 Results

4.2.1 Mcl-1 is required for both CD4⁺ and CD8⁺, naïve and memory T cells in a variety of conditions

The expression of the estrogen receptor-Cre fusion protein (ERCre) allows the deletion of floxed genes upon addition of the drug tamoxifen or its active metabolite 4-hydroxytamoxifen (4OHT) (Shapiro-Shelef et al., 2005). We have shown that the deletion of Mcl-1 in T cells from Mcl-1-floxed ERCre-expressing mice (Mcl-1floxed ERCre mice) caused the death of T cells in different ex vivo conditions (Dzhagalov et al., 2008).

To expand on this observation, we used this system to examine the survival of CD4⁺ and CD8⁺ T cell subsets upon deletion of Mcl-1. Similar to previous results (Dzhagalov et al., 2008), we observed that both CD4⁺ and CD8⁺ Mcl-1floxed ERCre T cells had significantly reduced cell survival upon deletion of Mcl-1 in a three-day culture of total splenocytes in medium alone, in medium supplemented with IL-7, and in the presence of activating antibodies against CD3 and CD28 (Figure 13A).

Because the baseline percentage of live cells in wild type T cells varied between the different conditions, we calculated the ratio of survival in 4OHT to survival in the
ethanol vehicle control (EtOH) for each genotype to better compare the extent to which survival was diminished upon loss of Mcl-1. The magnitude of the reduction in survival upon loss of Mcl-1 can be considered a measure of the dependency on Mcl-1. As expected, the 4OHT:EtOH survival ratio was approximately 1 in the ERCre-negative samples (Figure 13B). Interestingly, the reduction in survival without Mcl-1 was more severe in the cells cultured in medium alone than in those cultured with IL-7, indicating that IL-7 may have a protective effect and modulate the extent to which the cell depends on Mcl-1 (Figure 13B).

**Figure 13: Mcl-1 is required for peripheral T cell survival under different conditions**

A. Survival of CD4+ and CD8+ Mcl-1fl/ERCre and Mcl-1fl control T cells after a three-day culture of total splenocytes in medium alone, medium with 1 ng/ml
IL-7, or with plate-bound anti-CD3 and anti-CD28. The percentage of live cells was assessed by PI exclusion. White bars represent cells cultured in the ethanol vehicle control (EtOH), and black bars represent cells cultured in 4-hydroxytamoxifen (4OHT). The data represent the mean survival + SEM of 12-15 experiments, each performed in triplicate. n.s. signifies that the p value is not significant (p≥0.05) between the samples indicated. **p<0.01 and ***p<0.001 vs. 4OHT-treated Mcl-1<sup>f/f</sup> control. B) Ratio of survival in 4OHT to survival in EtOH of splenocytes cultured for three days in medium alone, in medium + IL-7, and with anti-CD3 and anti-CD28. The ratios calculated from triplicate wells were determined for each experiment, and the bars represent the mean of the ratios + the SEM of 12-15 experiments. ***p<0.001 vs. Mcl-1<sup>f/f</sup> control in each condition.

We also examined whether naïve and memory populations differed in their dependence on Mcl-1. When the cells were gated based on CD44 expression (Figure 14A), we observed some differences in the percent survival between CD44<sup>high</sup> memory-phenotype and CD44<sup>low</sup> naïve cells (Figure 14B). However, the magnitude of the reduction in survival upon loss of Mcl-1 was similar between CD44<sup>high</sup> and CD44<sup>low</sup> cells (Figure 14B). Because the activation of T cells induces surface expression of CD44, to compare the survival of naïve and memory T cells upon activation, T cells were sorted based on CD44 expression levels. The survival ratio of CD44-sorted cells in medium alone and in IL-7 confirmed that the decrease in survival upon loss of Mcl-1 was similar between naïve and memory cells (Figure 14C). Further, we observed that both CD44<sup>high</sup> and CD44<sup>low</sup> cells required Mcl-1 for survival upon activation by anti-CD3 and anti-CD28 (Figure 14C).
A. Gating of CD44<sup>high</sup> and CD44<sup>low</sup> cells from total splenocyte cultures. Representative flow cytometry plots and histograms are shown. Cells were pregated on both live and dead cell populations by FSC and SSC. B. Percent surviving cells by PI exclusion in total splenocyte cultures gated on CD44<sup>high</sup> and CD44<sup>low</sup> populations. Bars are the mean ± SD of triplicate wells. C. Ratio of survival in 4OHT to survival in EtOH of sorted naïve (CD44<sup>lo</sup>) and memory (CD44<sup>hi</sup>) cells. Bars are the mean ratios ± SEM of 3-5 experiments. *p<0.05; ns signifies p≥0.05 between the indicated pairs.

**4.2.2 Loss of Bak but not Bax rescues the survival of Mcl-1-deficient T cells in the presence of IL-7, but neither rescues Mcl-1-dependent survival of T cells during cytokine withdrawal.**

Previously, we showed that loss of Bak rescued the survival of Mcl-1-deficient thymocytes, and we also observed a rescue of cell numbers in the periphery of
Bak\(^{-/-}\)/Mcl-1\(^{f/f}\)CD4Cre mice [Chapter 3; (Dunkle et al., 2010)]. To more directly assess whether the inhibition of Bak by Mcl-1 occurs in peripheral T cells, we compared the survival of Mcl-1\(^{f/f}\)ERCre T cells with that of Bak\(^{-/-}\)/Mcl-1\(^{f/f}\)ERCre T cells with and without 4OHT. Somewhat surprisingly, the Bak\(^{-/-}\)/Mcl-1\(^{f/f}\)ERCre T cells did not have improved survival over Mcl-1\(^{f/f}\)ERCre T cells when cultured in medium alone as measured by the percentage of live cells or by the 4OHT:EtOH survival ratio (Figure 15A and 15C). In contrast, the survival Mcl-1\(^{f/f}\)ERCre T cells on a Bax\(^{-/-}\) background was slightly improved over that of the Bax\(^{+/+}\) controls, although the difference in the ratio was not statistically significant for CD8\(^{+}\) T cells (Figure 15B and 15C). The Bax\(^{-/-}\) genotype has been shown to rescue the survival of neonatal IL-7R\(^{-/-}\) thymocytes in vivo and adult thymocytes upon IL-7 withdrawal in vitro (Khaled et al., 2002), indicating a role for Bax in cytokine-withdrawal induced cell death.

Figure 15: Bak\(^{-/-}\) does not rescue the survival of Mcl-1-deficient T cells cultured in RPMI, but Bax\(^{+/+}\) slightly improves survival
To better mimic in vivo homeostatic conditions, we examined the survival of Bak\(^{+/+}\) and Bax\(^{+/+}\) T cells in medium supplemented with IL-7. In IL-7, Bak\(^{+/+}\)Mcl\(^{-/-}\)ERCre T cells treated with 4OHT had comparable survival to wild type, Bak\(^{+/+}\) (ERCre\(^{+/+}\)), and ethanol-treated Bak\(^{+/+}\)Mcl\(^{-/-}\)ERCre control cells (Figure 16A and 16C). In contrast, the Bax\(^{+/+}\) background did not rescue the survival of Mcl-1-deleted T cells in IL-7 (Figure 16B and 16C).

**Figure 16: Bak\(^{+/+}\) but not Bax\(^{+/+}\) rescues survival of Mcl-1-deficient T cells cultured with IL-7**

A, B. Percent surviving Bak\(^{+/+}\) (A) and Bax\(^{+/+}\) (B) Mcl-1\(^{-/-}\) and Mcl-1\(^{+/+}\)ERCre cells by PI exclusion after three days in culture in medium alone. Bars are the mean + SD of triplicate wells. Data are representative of three independent experiments each for Bak\(^{+/+}\) and Bax\(^{+/+}\). *p<0.05 vs. EtOH control within each genotype. C. Survival ratios of Bak\(^{+/+}\) and Bax\(^{+/+}\) cells upon three-day culture in medium alone (RPMI). Bars are the mean + SEM of three experiments. *p<0.05 between the ERCre\(^{+/+}\) samples. †p<0.05 between ERCre\(^{+/+}\) and ERCre\(^{+/+}\) samples within the Bak\(^{+/+}\) or Bax\(^{+/+}\) genotype. ns indicates that p≥0.05 between the indicated samples (designated by lower case letters).
each genotype. C. Survival ratios of Bak\(^{+}\) and Bax\(^{-}\) cells upon three-day culture in IL-7. Bars are the mean + SEM of three experiments. *p<0.05 between the ERCre\(^{+}\) samples. †p<0.05 between ERCre\(^{+}\) and ERCre\(^{-}\) samples within the Bax\(^{-}\) genotype. ns indicates that p\(\geq\)0.05 between the indicated samples (designated by lower case letters).

To address the possibility that the loss of Bak altered the kinetics of survival, but not the ultimate fate of Mcl-1-deleted cells, we assessed the survival of Bak- and/or Mcl-1-deficient T cells in culture with IL-7 over a five-day period. The Bak\(^{-}\) background rescued the survival of Mcl-1-deficient cells for five days when a single dose of IL-7 was added at the beginning of the culture (Figure 17).

Figure 17: Bak\(^{-}\) rescues survival over time of Mcl-1-deficient T cells cultured in IL-7

Timecourse of survival of Mcl-1\(^{+/+}\), Mcl-1\(^{-/-}\), Mcl-1\(^{-/-}\)ERCre, Bak\(^{-/-}\)Mcl-1\(^{+/+}\), and Bak\(^{-/-}\)Mcl-1\(^{-/-}\)ERCre T cells in IL-7. The mean 4OHT:EtOH survival ratios from two experiments (triplicate wells for each) are plotted vs. time.

4.2.3 Overexpression of Bcl-2 rescues cytokine dependency of T cells but not requirement for Mcl-1

IL-7 has been shown to positively regulate not only the levels of Mcl-1, but also Bcl-2 levels, and Bax and Bim have also been implicated in cytokine withdrawal-induced cell death (Bouillet et al., 1999; Dijkers et al., 2000; Khaled et al., 2002). In previous
studies, overexpression of Bcl-2 largely rescued thymic development and T cell survival in IL-7R<sup>−/−</sup> mice (Akashi et al., 1997; Maraskovsky et al., 1997), but Bcl-2 overexpression failed to rescue Mcl-1-deficient thymocyte development [Chapter 3; (Dunkle et al., 2010)]. To determine whether overexpression of Bcl-2 modulates the survival of Mcl-1-deficient peripheral T cells, we used mice that express a Bcl-2 transgene using the MHC class I promoter [Bcl-2<sup>tg</sup> mice; (Domen et al., 1998)]. As expected, the overexpression of Bcl-2 rescued the survival of (Mcl-1-sufficient) T cells cultured in medium alone to the levels observed with IL-7 (Figure 18). We observed a very modest improvement in the survival of Bax<sup>−/−</sup>, but not Bak<sup>−/−</sup>, cells over wild type cells in medium alone, but the mild extent of this improvement indicated that the apoptotic pathway initiated by cytokine withdrawal largely occurs through either Bak or Bax (Figure 18). Bim<sup>−/−</sup> cells were also resistant to cytokine withdrawal-induced cell death [Figure 18 and (Bouillet et al., 1999)].

![Figure 18: Bcl-2<sup>tg</sup>, Bim<sup>−/−</sup>, and to a small extent Bax<sup>−/−</sup> T cells are resistant to cytokine withdrawal-induced cell death](image)

Percent survival by PI exclusion of wild type (WT), Bak<sup>−/−</sup>, Bax<sup>−/−</sup>, Bcl-2<sup>tg</sup>, and Bim<sup>−/−</sup> CD<sup>4+</sup> T cells in culture of total splenocytes with or without IL-7 for three days. CD<sup>8+</sup> T cells yielded similar results (not shown). *p<0.05, **p<0.01, and ***p<0.001 vs. WT control. Items not marked with an asterisk are not significantly different from the WT control value (p≥0.05).
Upon deletion of Mcl-1, Mcl-1\(^{\text{f/f}}\)ERCre cells on the Bcl-2\(^{-}\) background showed improved survival over Mcl-1\(^{\text{f/f}}\)ERCre cells (with endogenous Bcl-2) as measured by the percentage of live cells and by the 4OHT:EtOH survival ratio (Figure 19A and 19B). However, overexpression of Bcl-2 did not fully rescue the survival of Bcl-2\(^{\text{tg}}\)Mcl-1\(^{\text{f/f}}\)ERCre cells in 4-OHT to ethanol control (as measured by actual percent survival) or wild type (by survival ratio) levels (Figure 19A and 19B). When the cells were supplemented with IL-7, conditions in which endogenous Bcl-2 levels are naturally higher, the survival of Bcl-2\(^{\text{tg}}\)Mcl-1\(^{\text{f/f}}\)ERCre cells was no different from that of Mcl-1\(^{\text{f/f}}\)ERCre cells (Figure 19C and 19D). Thus, the withdrawal of T cells from cytokines initiates an apoptotic pathway that can be inhibited both by Mcl-1 and Bcl-2.
Figure 19: Overexpression of Bcl-2 partially rescues survival of Mcl-1 deficient T cells during cytokine withdrawal but not in IL-7

A, C. Percent surviving cells by PI exclusion after three-day culture of total splenocytes without (A) and with (C) IL-7. Cells overexpressing Bcl-2 (Bcl-2tg) and cells expressing only endogenous Bcl-2 (wt Bcl-2) are shown. Bars are the mean ± SD of triplicate wells from a single experiment. Data are representative of four independent experiments. *p<0.05 vs. EtOH control within each genotype.

B, D. Survival ratios of wt Bcl-2 and Bcl-2tg cells upon three-day culture in medium alone (B) or in IL-7 (D). Bars are the mean ± SEM of 2-4 independent experiments. *p<0.05 between the ERCre+ samples (wt Bcl-2 vs. Bcl-2tg); †p<0.05 between ERCre+ and ERCre- samples within the Bcl-2tg genotype; ns indicates that p≥0.05 between the indicated samples (designated by lower case letters).

Because a complete rescue by Bcl-2 overexpression was not observed during cytokine withdrawal, it is likely that Mcl-1 also has a separate cytoprotective role(s) from Bcl-2. This was further indicated by the fact that overexpression of Bcl-2 did not stabilize
the survival of Mcl-1-deficient T cells in cytokine withdrawal over time (Figure 20A). The Bcl-2\textsuperscript{b} background also did not alter the kinetics of Mcl-1 deletion-induced death in IL-7 (Figure 20B).

![Figure 20: "Rescued" Bcl-2\textsuperscript{b}Mcl-1\textsuperscript{f/f}ERCre T cells continue to die over time](image)

A. B. Timecourse of survival of wt Bcl-2 or Bcl-2\textsuperscript{b} and Mcl-1\textsuperscript{f/f} or Mcl-1\textsuperscript{f/f}ERCre T cells in RPMI alone (A) or with IL-7 (B). The mean of the 4OHT:EtOH survival ratios from 1-3 experiments is plotted vs. time.

4.2.4 Loss of Bim partially rescues cytokine dependency of T cells but not the requirement for Mcl-1

The BH3-only protein Bim has been shown to be induced by cytokine withdrawal and to modulate the cytokine dependency of T cells (Bouillet et al., 1999;
Dijkers et al., 2000). We similarly observed that Bim$^{-}$ cells survived better than wild type cells, although not as well as Bcl-2$^{+}$ cells, under conditions of cytokine withdrawal (Figure 18). Bim$^{-}$/Mcl-1$^{f/f}$ERCre T cells, particularly CD8$^{+}$ cells, had improved survival compared to Mcl-1$^{f/f}$ERCre cells when cultured in medium alone (Figure 21A and 21B). Like the Bcl-2$^{+}$ background, the Bim$^{-}$ background did not improve the survival of Mcl-1-deficient T cells in IL-7 (Figure 21C and 21D). These results indicate that the inhibition of Bim is not a major role of Mcl-1 in T cells with sufficient IL-7, but Bim likely contributes to the accelerated death of Mcl-1-deficient T cells during cytokine withdrawal.
Figure 21: Bim\(^{-/-}\) partially rescues survival of Mcl-1-deficient T cells during cytokine withdrawal but not in IL-7

A, B. Percent surviving cells (A) and ratio of survival in 4OHT:EtOH (B) of control and Bim\(^{-/-}\), Mcl-1\(^{1ff}\) and Mcl-1\(^{1ff}/\text{ERC}r\text{e}\) cells cultured in medium alone. C, D. Percent surviving cells (C) and ratio of survival in 4OHT:EtOH (D) of control and Bim\(^{-/-}\), Mcl-1\(^{1ff}\) and Mcl-1\(^{1ff}/\text{ERC}r\text{e}\) cells cultured in IL-7. The data in A and C are the mean + SD of triplicate wells from a single experiment and are representative of two independent experiments. *p<0.05 vs. EtOH control within each genotype. Data in B and D are the mean + SD\(_{\text{ratio}}\) (see Materials and Methods) of the ratios from one representative of two experiments. *p<0.05 between the ERC\text{e}+ samples (wt Bim vs. Bim\(^{-/-}\)). *p\text{a} the Bim\(^{-/-}\) survival is significantly lower. †p<0.05 between ERC\text{e}+ and ERC\text{e}neg samples within the Bim\(^{-/-}\) genotype. ns indicates that p\geq0.05 between the indicated samples (designated by lower case letters).
4.2.5 Different common gamma chain cytokines differentially regulate the dominant role of Mcl-1

Because IL-7 appeared to modulate the role of Mcl-1 in T cells, we examined how other cytokines of the common gamma chain family, namely IL-4 and IL-15, affected T cell dependence on Mcl-1. Naïve and memory T cells are known to respond differently to certain cytokines, so sorted CD44\textsuperscript{high} (effector/memory phenotype) and CD44\textsuperscript{low} (naïve) T cells were used. Although IL-2 promotes the survival of activated T cells, resting (non-T\textsubscript{reg}) T cells do not express the high affinity IL-2 receptor subunit CD25, and we did not observe a survival response to IL-2 in our system (data not shown). Consistent with previously published results (Rathmell et al., 2001; Vella et al., 1997), IL-4 improved the survival of CD44\textsuperscript{low} (Figure 22A) and CD44\textsuperscript{high} (Figure 22B) T cells over that of cells cultured in medium alone. Upon deletion of Mcl-1, the survival of T cells in IL-4 was significantly diminished (Figure 22A and 22B).
Figure 22: IL-4, IL-7, and IL-15 promote T cell survival, but Mcl-1 is still required

A, B. Percent surviving cells after three-day culture of sorted CD44^{low} (A) and CD44^{high} (B) cells in IL-4 (10 ng/ml), IL-7 (1 ng/ml), or IL-15 (10 ng/ml). Data are the mean ± SD of triplicate wells from a single experiment and are representative of 2–5 independent experiments. *p<0.05 vs. EtOH control within each genotype. *^a^ the survival in 4OHT was significantly higher.

We also tested the ability of IL-15 to modulate T cell dependence on Mcl-1. IL-15 has been previously shown to upregulate Mcl-1 in T cells and to promote T cell survival (Opferman et al., 2003; Rochman et al., 2009). As expected, IL-15 promoted the survival of CD44^{high} cells (Figure 22B). However, CD44^{high} cells cultured in IL-15 exhibited reduced survival upon deletion of Mcl-1 (Figure 22B). IL-15 also promoted the survival of CD44^{low}CD8^{+} T cells to a similar extent as IL-7 (Figure 22A). CD44^{low}CD4^{+} T cells were
less responsive to IL-15, with a percent survival less than half that of CD8+ cells or CD4+ cells with IL-7 (Figure 22A).

The magnitude of the drop in survival upon loss of Mcl-1 (measured by the 4OHT:EtOH survival ratio) was greater in IL-4 than in IL-7, indicating that the cells cultured in IL-4 were more sensitive to the loss of Mcl-1 (Figure 23). The difference in the survival ratios between cells cultured in RPMI alone and cells in IL-4 was not statistically significant. In cells cultured in IL-15, the sensitivity to the loss of Mcl-1 correlated with the overall survival response to IL-15: CD44lowCD4+ T cells, which did not respond to IL-15, were more sensitive to the loss of Mcl-1 than the IL-15-responsive CD44lowCD8+ and CD44high T cells (Figure 23).

![Figure 23: Differential effects of IL-4, IL-7, and IL-15 in T cell dependence on Mcl-1](image)

The 4OHT:EtOH survival ratios of sorted CD44low (top) and CD44high (bottom) Mcl-1\textsuperscript{f/f}ERCre cells after three-day culture in the presence of different cytokines.
Data are mean ratios + SEM of 3-5 experiments. The statistical significance using survival in RPMI or survival in IL-7 as a comparison is indicated as follows: *p<0.05 vs. ratio in RPMI; †p<0.05 vs. ratio in IL-7. The absence of either of these symbols indicates that the difference is not statistically significant (p≥0.05)

Consistent with the fact that CD44<sup>low</sup>CD4<sup>+</sup> T cells did not respond to IL-15, while CD44<sup>high</sup> and CD44<sup>low</sup>CD8<sup>+</sup> T cells did, the loss of Bak completely rescued the survival in IL-15 of CD44<sup>high</sup>CD4<sup>+</sup>, CD44<sup>high</sup>CD8<sup>+</sup>, and CD44<sup>low</sup>CD8<sup>+</sup> T cells, but not CD44<sup>low</sup>CD4<sup>+</sup> T cells (Figure 24). Thus, although some cells respond better to IL-15 than others, IL-15 has IL-7-like effects on the 4OHT:EtOH survival ratio in responsive cells. In contrast, the Bak<sup>−/−</sup> background did not completely rescue the survival of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells in culture with IL-4 (Figure 24). This is consistent with the fact that the T cells in IL-4 were more similar to cells cultured in RPMI than in IL-7 in their dependence on Mcl-1.

Figure 24: Bak<sup>−/−</sup> rescues memory and CD8<sup>+</sup> naive Mcl-1-deficient T cells in IL-15, but not cells in IL-4

Survival of sorted CD44<sup>low</sup> and CD44<sup>high</sup> Bak<sup>−/−</sup>Mcl-1<sup>−/−</sup>ERCre cells after three-day culture in different cytokines. Bars are the mean + SD of triplicate wells. White bars represent cells treated with vehicle control (EtOH), and black bars were treated with 4OHT
4.2.6 Cytokines of the common gamma chain family differentially regulate protein expression of Bcl-2 family members

To determine why T cells cultured in IL-4, IL-7, and IL-15, all of which promoted survival, had differing levels of dependence on Mcl-1, we examined the protein expression of select Bcl-2 family proteins in side-by-side cultures with different γc cytokines. By intracellular staining, IL-4, IL-7, and IL-15 induced higher expression of Mcl-1 and Bcl-2 than IL-2 or RPMI alone on day three (Figure 25). Surprisingly, the protein level of Bim was also upregulated in the survival cytokines, but this upregulation was most pronounced in IL-4 (Figure 25). The upregulation of Bim by IL-4 (but not IL-7 or IL-15) could also be observed on day one by intracellular staining (data not shown) and to a modest extent (increase of ~20%) by Western blotting (Figure 26). At this timepoint, Bim expression was highest in RPMI (cytokine withdrawal) and lowest in IL-15. IL-15 has been shown to negatively regulate Bim expression in natural killer (NK) cells (Huntington et al., 2007).

Figure 25: Intracellular levels of Bcl-2, Mcl-1, and Bim in response to γc cytokines
Histograms of intracellular staining for Bcl-2, Mcl-1, and Bim on day 3. Cell populations were first gated on live cells by FSC and SSC then on the CD44\textsuperscript{low} population within the CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell gates. The shaded histogram is a representative isotype control (consistent for all conditions). Data are representative of 1-2 experiments.

We also examined the expression of Bak and Bax on day one of culture by Western blotting. Day one was selected because of the low cell survival by day three in some conditions. Bak protein levels were slightly higher in the pro-survival cytokines but were equivalent between these cytokines. Bax exhibited the highest expression in IL-4, indicating another mechanism that could explain the increased sensitivity of T cells in IL-4 to the loss of Mcl-1.

![Western blot of Bak, Bax, and Bim protein expression](image)

**Figure 26: Western blot of Bak, Bax, and Bim in response to γc cytokines**

Western blot of Bak, Bax, and Bim protein expression in T cells cultured with cytokines for 22 h. The corresponding loading control (β-tubulin) for each blot is shown. The estimated relative intensities of Bak, Bax, and Bim, normalized to
loading controls, were calculated as described in the Materials and Methods and are shown underneath each blot. Lane 1 (“Fresh” T cells) was arbitrarily defined as 1, and the number indicates the intensity relative to Lane 1. For Bim, the combined intensity for the Bim\_L and Bim\_EL isoforms was used.

### 4.2.7 Role of Mcl-1 in activated T cells

Finally, we examined the mechanism(s) by which Mcl-1 promotes the survival of activated cells. Mcl-1 is required for activated T cell survival [Figure 13 and (Dzhagalov et al., 2008)]. Because of the number of signaling pathways that are activated in response to activation, including some that alter cytokine and cytokine receptor expression, it was possible that the activation of T cells might alter the mechanism by which Mcl-1 promotes survival in activated T cells. Using the same double knockout and overexpression models above, we observed that the Bak\_f background, but not the Bax\_f or Bcl-2\_f backgrounds, yielded a significantly improved 4OHT:EtOH survival ratio compared to the single knockout of Mcl-1 (Figure 27). Further, the ratio in the Bak\_f/Mcl-1\_f\_ERCre cells was close to 1 and was not significantly different than that of the ERCre-negative control, indicating that knockout of Bak completely rescues the death due to loss of Mcl-1 in activated T cells (Fig. 27). Given that the Bak\_f background rescues Mcl-1-deficient T cell survival in the IL-7 culture system, this result is not surprising; activated cells upregulate cytokine production and thus likely produce sufficient cytokines to drive the cells toward the Mcl-1/Bak pathway of survival. However, more studies would be needed to separate the effects due to cytokine production and the effects due to other activation-induced signals.
Figure 27: Bak\(^{-/+}\) rescues the survival of activated Mcl-1-deficient T cells

The 4OHT:EtOH survival ratios of Mcl-1\(^{ff}\) and Mcl-1\(^{ff}\)ERCre cells crossed onto the Bak\(^{-/+}\) (top), Bax\(^{-/-}\) (middle), and Bcl-2\(^{tg}\) (bottom) backgrounds three days after activation using anti-CD3 and anti-CD28. CD4\(^{+}\) and CD8\(^{+}\) cells are shown. Bars are the mean + SEM of at least three separate experiments. Statistical significance is indicated as follows: *p<0.05 between Mcl-1-deleted samples [Mcl-1\(^{ff}\)ERCre (wild type for Bak/Bax/Bcl-2) vs. Mcl-1\(^{ff}\)ERCreBak\(^{-/-}\)/Bax\(^{-/-}\)/Bcl-2\(^{tg}\) cells]; #p<0.05 between Mcl-1\(^{ff}\) (Mcl-1-sufficient) and Mcl-1\(^{ff}\)ERCre (Mcl-1-deficient) cells on the Bak\(^{-/-}\)/Bax\(^{-/-}\)/Bcl-2\(^{tg}\) backgrounds; and ns signifies that there is no significant difference (p≥0.05) between the indicated samples (pairs indicated by lower case letters).

### 4.3 Summary and discussion

#### 4.3.1 Changing roles for the Bcl-2 family members in different contexts

Data from our previous studies and those of others indicated that Mcl-1 is critical for the survival of peripheral T lymphocytes. We observed that T cells in culture with the trophic cytokine IL-7 required Mcl-1 but that IL-7 altered the magnitude of the
reduction in survival upon loss of Mcl-1. This indicated that Mcl-1 has roles in multiple pathways: cytokine-dependent and cytokine-independent. When the cells were cultured without added IL-7, the Bak<sup>−/−</sup> background, which rescues Mcl-1-deficient thymocyte development [Chapter 3 and (Dunkle et al., 2010)], was insufficient to rescue Mcl-1-deficient peripheral T cells. When IL-7 was added to the culture, the Bak<sup>−/−</sup> background rescued the survival of Mcl-1-deficient cells. In contrast, the Bcl-2<sup>tg</sup> background did not rescue the survival of Mcl-1-deficient cells in IL-7 but partially rescued the cells under conditions of cytokine withdrawal. Thus, Mcl-1 and Bcl-2 share a role in protecting cells from death under conditions in which cytokines are scarce or absent. However, with sufficient IL-7, the critical role for Mcl-1 in peripheral T cells is to inhibit Bak, a role that Mcl-1 does not share with Bcl-2 (Willis et al., 2005).

The loss of Bim also provided a partial rescue of the death of Mcl-1-deficient cells without, but not with, IL-7. The lack of rescue in IL-7 could be because there are relatively low levels of Bim in this condition and/or because the endogenous levels of Bcl-2 maintained by IL-7 are sufficient to suppress Bim activity. Protein expression analysis indicated that both Bcl-2 and Bim were higher after three days in culture with IL-7 compared to cells cultured in medium alone, lending strength to the latter interpretation. However, it is possible that the increased levels of Bim in IL-7 are not of the active protein.
Because the rescue of death with the Bim<sup>+</sup> genotype in cytokine withdrawal conditions did not quite equal that with the Bcl-2<sup>+</sup>, it is possible that other BH3-only proteins are also inhibited by Bcl-2 and Mcl-1. The fact that even the Bcl-2<sup>+</sup>, which rescued the survival of wild type cells without cytokines to the levels observed with IL-7, did not fully rescue the survival of Mcl-1-deficient cells in cytokine withdrawal conditions implies that Mcl-1 has a separate, specific role in promoting T cell survival during cytokine withdrawal. Even though the Bak<sup>+</sup> genotype provided no rescue under these conditions, this role could still be the inhibition of Bak. The activation of Bim and perhaps other BH3-only proteins, which could induce apoptosis through Bax, could mask any potential survival effect of the Bak<sup>+</sup>. To address this possibility, Bak and Bim would need to be simultaneously neutralized, perhaps even in combination with other BH3-only proteins. Alternatively, the BH3-only protein Noxa has been shown to specifically bind Mcl-1 and not Bcl-2. Both Noxa and Bim have been shown to be upregulated in response to IL-15 withdrawal in NK cells (Huntington et al., 2007).

4.3.2 Differential roles of γc cytokines in T cell survival

We compared the ability of γc cytokines to modulate the roles of Mcl-1. IL-15 had IL-7-like effects on memory phenotype T cells and naïve CD8<sup>+</sup> T cells in terms of the survival of wild type cells, the reduction in survival upon loss of Mcl-1, and the rescue of this reduced survival by the loss of Bak. Naïve CD4<sup>+</sup> T cells, which did not respond well to IL-15, exhibited a similar trend to cells cultured without cytokine in both the
reduction in survival upon loss of Mcl-1 and in the resistance to rescue by the Bak\(^{−/−}\) background. Interestingly, IL-4 promoted the survival of T cells but unlike IL-7, did not alter the dependence on Mcl-1 compared to cells cultured without cytokine. T cells in IL-4 were not rescued by crossing to a Bak\(^{−/−}\) background.

Protein expression analysis showed that while IL-4 upregulated Mcl-1 and Bcl-2 to a similar level as IL-7 and IL-15 (and Bak was relatively constant in these conditions), IL-4 induced higher levels of proapoptotic Bax and Bim. This could explain why the T cells in IL-4 were more sensitive to the loss of Mcl-1. All of the cytokines of the \(\gamma c\) family signal through the JAK-STAT pathway and activate JAK1 and JAK3 through the \(\gamma c\) subunit (Rochman et al., 2009). While the IL-7 and IL-15 receptors signal primarily through STAT5, the IL-4 receptor signals through STAT6 (Rochman et al., 2009). STAT6 has been shown to be dispensable for the pro-survival effect of IL-4 (Vella et al., 1997) but remains a candidate for the upregulation of the proapoptotic molecules observed here. Alternatively, IL-7 and IL-15 could repress Bim activation via a pathway that is absent downstream of IL-4 signaling. The PI3K/Akt pathway, which is activated in response to \(\gamma c\) cytokine signaling, been implicated in regulating Bim protein levels (Dijkers et al., 2000; Huntington et al., 2007; Jiang et al., 2005; Khaled and Durum, 2002; Obexer et al., 2007), and it would be worthwhile to examine any differences in the magnitude or duration of this signal in response to the different cytokines. Regardless of
the mechanism, the upregulation of Bim is a novel function of IL-4, the biological reason for which is unknown.

Our data indicate that IL-7, IL-15, and IL-4, which all promote T cell survival, differentially regulate the dominant role of Mcl-1 and the expression of Bcl-2 family proteins in T cells. The data here highlight the complex pathways that regulate survival in primary T cells, particularly with regards to the role of Mcl-1 and the different functions of the γc cytokines.
5. Regulation of Bcl-2 in the generation of CD8$^+$ T cell memory

The data in this section are the results of pilot experiments from a study ongoing at the time of writing.

5.1 Introduction

During an acute response to infection, antigen-specific T cells undergo preprogrammed expansion and contraction phrases that ultimately lead to the formation of a stable memory population. However, what allows the survival of the memory cells while the other ~90% of effector T cells die is still unclear. A number of factors, including signal strength, inflammation, and cytokines, have been proposed to contribute to the formation of T cell memory [Reviewed in (Hand and Kaech, 2009)]. Similarly, a number of surface molecules have been proposed to have functional and/or identifying roles in the cells fated to become memory.

Expression of the IL-7Rα subunit (CD127) has been shown to mark effector cells fated to become memory cells, which is consistent with the evidence that IL-7 is important for memory cell survival (Kaech et al., 2003; Schluns et al., 2000). However, some evidence has cast doubt on the true importance of CD127 and IL-7 signaling in memory formation because cells in an IL-7$^+$ environment exhibit fairly normal CD127 modulation and establishment of memory (Klonowski et al., 2006). Other studies showed that CD127 expression alone is not sufficient for the establishment of T cell
memory, calling CD127 expression a “permissive” rather than an “instructive” feature (Hand et al., 2007; Haring et al., 2008). More recently, CD127 has been used with another surface marker, KLRG1, to distinguish memory precursor effector cells (MPECs) from short-lived effector cells (SLECs) at the peak of the immune response (Joshi et al., 2007).

Because of the important roles of the antiapoptotic proteins of the Bcl-2 family in T cell survival, these proteins may regulate the formation of T cell memory. Both Bcl-2 and Mcl-1 are expressed in memory T lymphocytes, whereas Bcl-xL is upregulated in effector cells (Boise et al., 1995; Dzhagalov et al., 2008; Grayson et al., 2001; Grayson et al., 2000; Opferman et al., 2003). Using a conditional knockout model, Bcl-xL was shown to be dispensable for T cell memory (Zhang and He, 2005). The roles of Mcl-1 and Bcl-2 in memory formation have been more difficult to assess because of the importance of these proteins in thymocyte and naïve T cell survival. An examination of Bim+/−/Bcl-2−/− cells (which partially escape Bcl-2−/−-induced death in the thymus and periphery due to the loss of one allele of Bim) showed that Bcl-2 was not required for memory to lymphocytic choriomeningitis virus (LCMV) infection (Wojciechowski et al., 2007). However, it is possible that the loss of one allele of Bim and/or the lymphopenic environment affected the results of these experiments and the requirement for Bcl-2 in this system. Therefore, there is still some question as to the role of Bcl-2 in the formation of memory.
5.2 Results

5.2.1 Bcl-2<sup>YFP</sup> reporter mouse

In order to more closely examine the regulation and importance of Bcl-2 during an immune response, our lab generated a reporter mouse in which the gene encoding yellow fluorescent protein (YFP) was inserted into the Bcl-2 locus on a BAC transgene (Bcl-2<sup>YFP</sup> mice; unpublished data from Ivan Dzhagalov and You-Wen He). This allowed the expression of YFP to parallel that of Bcl-2 without interfering with the endogenous expression of Bcl-2 (unpublished data from I.D., Y.W.H.). YFP expression was shown to mirror known patterns of Bcl-2 expression in the thymus and in the peripheral lymphoid system and was dynamically regulated during an immune response (unpublished data from I.D., Y.W.H.).

5.2.2 Analysis of YFP expression compared to other activation and memory markers during Listeria monocytogenes infection

To examine the regulation of Bcl-2 during an immune response to infection, we utilized an infection model in which Bcl-2<sup>YFP</sup> mice were infected with a sublethal dose of Listeria monocytogenes expressing OVA (LM-OVA). These mice produce a dominant CD8<sup>+</sup> T cell response to the OVA peptide OVA<sub>257-264</sub>. CD8<sup>+</sup> T cells specific for this peptide can be identified by the ability to bind to OVA<sub>257-264</sub> presented on a commercially available MHC fusion protein in which the MHC class I protein H-2K<sup>b</sup> is attached to the Fc region of mouse IgG1 (DimerX), allowing flow cytometric detection upon incubation with a fluorescently labeled secondary antibody. Consistent with previous Bcl-2 expression
data, seven days after infection, we observed that most activated OVA-specific CD8+ cells downregulated YFP compared to naïve cells (Figure 28 and unpublished data from I.D., Y.W.H.). However, a small population of cells remained YFP\textsuperscript{hi} at this timepoint. In addition, this pattern mimics that of CD127, but we observed both CD127\textsuperscript{hi}Bcl-2\textsuperscript{hi} and CD127\textsuperscript{lo}Bcl-2\textsuperscript{lo} populations (Figure 28 and unpublished data from I.D., Y.W.H.).

**Figure 28: A subset of effector T cells remains Bcl-2\textsuperscript{hi} after LM-OVA infection**

Bcl-2\textsuperscript{YFP} mice were infected with a sublethal dose (10\textsuperscript{4} CFU) of LM-OVA and CD8+ T cells from the spleen were examined seven days later. OVA-specific cells were gated based on upregulation of CD44 (an activation/memory marker) and binding to the DimerX-OVA peptide complex. Bcl-2 (YFP) expression was compared with CD127 expression. Because the CD127 antibody yields low-level staining, cells that are low for this marker are often observed on or below the axis. Both the dot plot (middle) and the contour plot (right) are shown for this reason.

In addition to CD127, we also examined the relationship between Bcl-2 expression and other markers associated with memory. We examined the expression of KLRG1, which is reported to be expressed on terminally differentiated SLECs but not on MPECs (Joshi et al., 2007). Indeed, a majority OVA-specific effector T cells were KLRG1\textsuperscript{YFP\textsuperscript{lo}}, but a subset of KLRG1\textsuperscript{lo} cells was observed (Figure 29). A small percentage
of the population also remained YFP$^{hi}$, and the YFP$^{hi}$ cells were more enriched for the KLRG1$^-$ fraction than YFP$^{lo}$ cells (Figure 29B). Gr1 has been associated with the formation of memory (Walunas et al., 1995), but we did not observe a definable pattern with regards to Gr1 and Bcl-2 expression (Figure 29). Another example of CD127 staining is also shown in Figure 29 to demonstrate that there is some variation in terms of the size of the CD127- and/or Bcl-2-high populations, likely due to mouse-to-mouse variation as well as the low-level signals of the DimerX-OVA and CD127 stains resulting in differences between experiments in how the gates are drawn.
Figure 29: Comparison of Bcl-2 expression with other markers associated with memory

Mice were infected with $10^4$ CFU LM-OVA (or left uninfected), and CD8 cells were examined on day 7 of infection. A. Flow cytometry plots of Bcl-2 vs. KLRG1, Gr1, and CD127 (IL-7Ra) of uninfected CD8+ cells (left), naïve (CD44+) CD8+ cells from an infected mouse (middle), and effector CD8+Dimer/OVA+ T cells. B. A histogram comparing the expression of KLRG1, Gr1, and CD127 in Bcl-2 hi (line) and Bcl-2 lo (shaded) OVA-specific effector cells.

Finally, we utilized the co-staining of KLRG1 and CD127 to look at Bcl-2 expression within the previously described MPEC and SLEC populations. Similar to what was observed when examining CD127 alone vs. Bcl-2, while the Bcl-2 level of the
total MPEC population was higher than the SLEC population, there appeared to be a range of expression within the MPEC population wherein some cells expressed higher Bcl-2 than others (Figure 30).

Figure 30: Bcl-2 expression in memory precursor and short-lived effector cell populations

Splenic CD8+ T cells were examined seven days after infection with 10^4 CFU LM-OVA. CD44^hi, OVA-specific (DimerX-OVA^+ ) cells were gated and split into short-lived effector cell (SLEC; CD127^hiKLRG1^+ ) and memory precursor effector cell (MPEC; CD127^loKLRG1^− ) populations. The YFP expression of these two populations was compared (shown on the right vs. CD127). For MPECs, high and low Bcl-2-expressing populations were observed. The numbers represent the percentage of the parent population within each gate.

5.2.3 Sorting of effector T cells based on Bcl-2, CD127, and KLRG1 expression

It was intriguing that a subset of the CD127^hi/MPEC population retained high Bcl-2 expression, while another subset was low for Bcl-2. To determine whether or not
there is a difference in the potential of these populations to form the memory population, we sorted effector Bcl-2YFP T cells based on Bcl-2 expression and other memory markers and transferred these cells to naïve animals. At least five weeks were allowed to pass then recipient mice were given a secondary challenge of LM-OVA to assess the establishment of memory.

Our initial attempts to transfer sorted effector Bcl-2YFP cells failed, likely for one or both of the following reasons. First, the cell number transferred may not have been sufficient. Even pooling cells from multiple mice for sorting, the yield of some of the smaller populations only allowed a transfer in the range of 1,000 cells per recipient. Given that the sorting and ex vivo handling of the cells likely decreases the ability of the cells to engraft and survive, the number of transferred cells required to observe a memory response is likely much higher than the number of endogenous memory cells that would be required to observe a response of similar magnitude (i.e. the transferred cells die non-specifically before the establishment of memory). Second, in order to separate effector T cells from the non-specific memory population, which is also CD44hi, we used DimerX-OVA binding to identify cells. However, it is possible that this binding could either stimulate the TCR, providing a signal that could lead to activation-induced death, or block the TCR from receiving signals that might promote survival after transfer. For both of these reasons, we utilized a TCR transgenic model that not only would facilitate the collection of higher numbers of cells, but would also allow the use of
congenic markers to identify the cells to avoid using the TCR-binding peptide/MHC complex.

The Bcl-2<sup>YFP</sup> mice were crossed onto the OT1 TCR transgenic background (OT1<sup>YFP</sup>), in which nearly all T cells are CD8<sup>+</sup> and specific for the OVA peptide. To avoid any complications from directly infecting the OT1<sup>YFP</sup> mice (due to the overwhelming number of antigen-specific cells and the lack of CD4<sup>+</sup> cells), 50,000-100,000 OT1<sup>YFP</sup> CD8<sup>+</sup> T cells were transferred to a wild type recipient mouse one day before infection. This number of cells, while supraphysiologic, has been used in many similar studies, and in our Bcl-2<sup>YFP</sup> system, the phenotype of the responding CD8<sup>+</sup> T cells was similar to that observed in the endogenous responders when Bcl-2<sup>YFP</sup> mice were infected directly (data not shown). The use of the congenic markers CD45.1 and CD45.2 allowed the identification of OT1<sup>YFP</sup> cells both in the initial sorting and upon final analysis after secondary challenge.

In a first set of experiments, we separated effector OT1<sup>YFP</sup> cells first into SLEC and MPEC populations (Figure 31; left). Then, we selected Bcl-2<sup>hi</sup> and Bcl-2<sup>lo</sup> populations from within the MPEC population. The SLEC population was uniformly Bcl-2<sup>lo</sup>. After sorting, ~10,000 cells from each population were transferred to recipient animals, and memory was assessed on day 5 after secondary challenge (performed at least five weeks after transfer). Five million unsorted CD8<sup>+</sup> cells were transferred into separate mice as a positive control. Although the number of donor cells recovered upon secondary
challenge varied between experiments and individuals, we could detect an expansion of the memory population (as measured by the calculated number of OT1<sup>YFP</sup> cells recovered/the number transferred) in the mice that received unsorted CD8<sup>+</sup> cells (Figure 31; right). Consistent with published results, we did not detect an appreciable expansion of donor cells upon secondary challenge in mice transferred with the SLEC population. Somewhat confusingly, within the MPEC population, different experiments yielded different results. When we assigned the Bcl-2<sup>hi</sup> and Bcl-2<sup>lo</sup> gates based on what appeared to be a distinct break in YFP expression, the Bcl-2<sup>lo</sup> population was better at conferring memory (Figure 31, top). However, when we defined these populations based on the arbitrary distinction of the highest ~33% and the lowest ~33%, the high population appeared to be better (Figure 31, bottom).

Figure 31: Efficiency of different OT1<sup>YFP</sup> populations in establishing memory
One day before infection, 100,000 CD45.2^+OT1^YFP^CD8^+ T cells were transferred to recipient (CD45.1) mice that were then infected with 10^4 CFU LM-OVA. After seven days, cells were sorted by gating CD44^hi^OT1^YFP^ cells (OT1^YFP^ cells identified by CD45.2^+ with or without the addition of the OT1 TCR marker, Vα2^+^). SLEC and MPEC populations were then gated based on CD127 and KLRG1 expression, and the MPEC population was further sorted based on Bcl-2 expression. The numbers on the FACS plots indicate the percentage of the parent population within each gate. A total of 12,000 (top) or 8,000 (bottom) sorted or 5 × 10^6 unsorted cells were transferred to recipient (CD45.1) mice, and secondary challenge was performed more than five weeks later. Five days after secondary challenge, the spleens of recipient mice were analyzed, and the total number of OT1^YFP^ cells recovered per spleen was calculated based on the percentage of CD45.2^+Vα2^+^ cells detected. This was normalized to the number of cells transferred to assess the expansion (or percent recovery) of the cells at the memory timepoint. For the unsorted cells, the number of OT1 cells transferred was calculated based on the percentage of the total CD8^+^ population.

5.2.4 Intermediate-high Bcl-2 expression corresponds to the ability to confer memory in recipient mice

One potential reason that the experiments above yielded different results is that the arbitrary selection of the “high” and “low” samples based on Bcl-2 expression led to different percentages within each gate between the two experiments. When the high gate was more strict (Figure 31; top row), the high population did not confer memory. In contrast, when the high gate was expanded and the low gate more restricted (Figure 31; bottom row), the high, but not the low, cells conferred memory to the recipient. Thus, we adopted a strategy to sort roughly equal-sized populations of cells representing the full range of Bcl-2 expression. We first gated CD8^+^CD44^hi^OT1^YFP^ cells. Then, we gated four populations based on Bcl-2 expression (populations I-IV), each consisting of approximately 15-20% of the total (Figure 32).
Figure 32: Sorting of OT1<sup>YFP</sup> cells into equal-sized populations based on Bcl-2 expression

Responding day 8 OT1<sup>YFP</sup> cells from the spleens of recipient mice (transferred with 50,000 OT1<sup>YFP</sup> cells on day -1) were sorted into four populations (labeled with Roman numerals) based on YFP expression (left). While not used as markers for sorting, the expression patterns of CD127 and KLRG1 were determined for each population (right). The CD127<sup>hi</sup>KLRG1<sup>-</sup> MPEC population is gated in red. The numbers represent the percentage of the parent population that falls within the MPEC gate. A total of 10,000 sorted cells were transferred per mouse.

Consistent with previous observations that the MPEC population expresses higher Bcl-2 than SLECs, the Bcl-2<sup>hi</sup> population (I) was most enriched for MPECs, and the percentage of MPEC cells decreased with Bcl-2 levels with the exception of the Bcl-2<sup>lo</sup> cells (IV) (Figure 32). A post-sort analysis (not shown) indicated that population IV contained a higher percentage of contaminating host cells, which would appear in the MPEC gate due to the similarities of the MPEC markers with resting T cell markers, than the other three populations. Given the likelihood that differences in MPEC percentages might confound the interpretation of the results from this experiment, a parallel set of experiments was performed in which only MPEC cells were included, and this population was divided into equal parts based on Bcl-2 expression (Figure 33A). Post-
sort analysis confirmed that all of the sorted MPEC populations were >96% pure for OT1\textsuperscript{YFP} cells and demonstrated the difference in the YFP mean fluorescence intensity (MFI) between the populations (Figure 33B). Interestingly, the CD127 MFI increased along with YFP expression, but the dynamic range of CD127 was much smaller than that of YFP expression (Figure 33B). Whether this is because of a difference in the actual range of expression levels or rather in the limitations of the detection of the signal is not clear.
A. Sorting of OT1YFP cells on day 7 of infection with LM-OVA. Mice were transferred with 50,000 OT1YFP cells on day -1 and infected with 10^4 LM-OVA on day 0. CD8+ T cells were enriched from the spleens, and cells were gated as shown. Cells were first gated on the CD8^+CD45.1^+ population then CD44^hiVα2+. Memory precursors were selected as CD127^hiKLRG1^+, and this population was split into four populations based on Bcl-2-YFP expression (indicated by Roman numerals). The numbers shown indicate the percentage of the parent population within the gate. B. Post-sort analysis of populations V-VIII as described in A. To
check purity, the percentage of cells within the CD8^+CD45.1^+ gate was examined (top row). All populations were >96% OT1^YFP. We also examined the fluorescence intensities of YFP (middle) and CD127 (bottom). The number shown is the mean fluorescence intensity (MFI) of the population.

Memory analysis of mice that received populations I-IV (effector OT1^YFP cells) as shown in Figure 32 demonstrated that as the levels of Bcl-2 decreased, so did the memory potential (Figure 34A). Population I (Bcl-2^{hi}) yielded the greatest recovery of cells followed by population II (Bcl-2^{med-hi}), population III (Bcl-2^{med-lo}), then population IV (Bcl-2^{lo}). In a second experiment, in which memory recovery was not as good overall, we only detected donor cells in the mice that received population I (Bcl-2^{hi}) (Figure 34B).

**Figure 34: Recovery of memory OT1^YFP cells after secondary challenge of mice transferred with effector CD8^+ T cell populations based on Bcl-2 expression**

Mice were transferred with 10,000 (A) or 50,000 (B) cells of the indicated population at least five weeks before secondary challenge with 10^4 CFU LM-OVA (see also Figure 32 – sort for result A). Five (A) or four (B) days after secondary challenge, the spleen was analyzed by flow cytometry to detect donor cells. The number of OT1^YFP cells per spleen was calculated, and the mean ± SD of the mice within each group (four mice per group) is shown.
While these data indicate that higher levels of Bcl-2 do indeed correspond to increased memory potential, it remained possible that the differences between populations I-IV in establishing memory were due to the differences in MPEC percentages between these populations, not Bcl-2 expression per se. Upon analysis of the mice that were transferred with gradient populations of MPEC-gated OT1<sup>YFP</sup> cells (as shown in Figure 33), we observed that even within the MPEC population, the different populations based on Bcl-2 expression had different abilities to confer memory. In fact, it was not the MPEC-Bcl-2<sup>hi</sup> population (V), but the MPEC-Bcl-2<sup>med-hi</sup> population (VI), that yielded the highest recovery of memory cells (Figure 35). Together, these results indicate that the cells with the greatest memory potential can be defined within a fairly narrow range of Bcl-2 expression.

**Figure 35:** Recovery of memory OT1<sup>YFP</sup> cells after secondary challenge of recipients transferred with memory precursor effector CD8<sup>+</sup> T cell populations based on Bcl-2 expression
Mice were transferred with 10,000 cells of the indicated population at least five weeks before secondary challenge with $10^4$ CFU LM-OVA (see also Figure 33 – sorting strategy). Five days after secondary challenge, the spleen was analyzed by flow cytometry to detect donor cells. The number of OT1<sup>YFP</sup> cells per spleen was calculated, and the mean $\pm$ SD of the mice within each group (four mice per group) is shown.

5.3 Summary and discussion

The experiments in this section provide intriguing evidence that the dynamic regulation of Bcl-2 during the effector phase of the immune response could be important for establishing T cell memory. Using the OT1<sup>YFP</sup> LM-OVA infection system, we found that a subset of cells retains high Bcl-2 expression at the peak of the immune response and that this population is contained within the CD127<sup>hi</sup>KLRG1<sup>-</sup> population. Using adoptive transfer, we confirmed the observations of other groups that the CD127<sup>hi</sup>KLRG1<sup>-</sup> population is indeed enriched for memory precursors. Our preliminary results (Figures 31 and 35) indicate that within this population, a smaller population of effector cells, which can be identified based on Bcl-2 expression levels, has true memory potential. Intriguingly, based on the different results obtained with different gating strategies, it appears that the MPECs that express intermediate-high, but not the highest, levels of Bcl-2 may be the best at establishing memory.

While initially the results above seem to contrast the results of the experiments in which effector T cells (not gated on MPECs) were transferred (Figures 32 and 34), we must consider the fact that the higher Bcl-2 population (population I) in these experiments was more enriched for MPECs. Because the peak/curve of YFP expression
in MPECs is shifted to the right compared to total effector cells, the population I gate used for the transfer of effector OT1\textsuperscript{YFP} cells likely encompasses a higher percentage of the MPEC curve (corresponding to population V and at least part of population VI). This observation is also consistent with the results presented in Figure 31, in which MPECs were only split into two populations with varying strictness in the gates between the two experiments. Together, all of the experiments confirm that relatively high expression of Bcl-2 enhances the memory potential of the population. However, it appears that those cells with the highest expression of Bcl-2 may be excluded from this trend. This phenomenon and potential explanations will be discussed further in the Future Directions (Section 6.5).
6. Discussion, remaining questions, and future directions

6.1 Model of the roles of the antiapoptotic Bcl-2 family proteins in thymocytes

Previous models have suggested that Mcl-1 may inhibit apoptosis either through the inhibition of proapoptotic BH3-only proteins, specifically the “direct activators,” and/or through the inhibition of Bak and/or Bax (see Figures 2 and 3, Chapter 1). The data presented in Chapter 3 allow us to build an in vivo pathway of the specific roles of Mcl-1 in thymocytes (Figure 36). Our data support previous biochemical data showing specificity at the level of the Mcl-1-Bak interaction (Willis et al., 2005). Together, these results are supportive of a model in which Mcl-1 directly sequesters Bak in thymocytes. We showed that loss of the direct activator Bim does not rescue Mcl-1-deficient thymocytes. Although this does not rule out that other BH3-only “activators” are not downstream of Mcl-1, Bid and Puma, the only other BH3-only proteins with reported direct activator functions, have both been shown to activate Bax as well as Bak (Kim et al., 2006; Kuwana et al., 2002), and our data show that Mcl-1-deficient thymocytes die by a Bak-specific mechanism. The release of BH3-only proteins upon deletion of Mcl-1 may still contribute to the death of thymocytes, particularly in DN cells, because rescue by Bak<sup>+</sup> was incomplete at this stage. It remains possible that one of the BH3-only proteins could preferentially activate Bak over Bax, either directly or indirectly, by a mechanism that is yet to be appreciated. However, the robust rescue by the Bak<sup>+</sup> background, even
in DN cells, suggests that the major role of Mcl-1 in thymocytes is to directly antagonize Bak.

**Figure 36: Model of the roles of the antiapoptotic Bcl-2 proteins at different thymic stages**

The antiapoptotic proteins are displayed as diamonds. Those in grey are not highly expressed at the given stage. BH3-only proteins are demonstrated by pentagons. In DN thymocytes, an unknown BH3-only protein is shown because the Bak\(^{-}\) background does not fully rescue the Mcl-1-deficient phenotype (see discussion). Although not displayed for simplicity, upstream BH3-only proteins are important in affecting the ability of the antiapoptotic proteins to perform the functions displayed. In SP thymocytes, the bold line indicates the dominant pathway, whereas the dashed line indicates a minor pathway.

The role of Mcl-1 appears to be slightly different in each thymic subset. In DN cells, Mcl-1 sequesters Bak, and loss of Mcl-1 leads to Bak activation (Figure 36, left). Because the rescue by the Bak\(^{-}\) background was only partial at this stage, it is likely that Mcl-1 also inhibits other apoptotic signals that are less selective for Bak, such as BH3-only proteins that directly activate Bak/Bax (as shown in Figure 36). Although our data do not directly address the role of Bcl-2, it has been shown that the Bim\(^{-}\) background rescues Bcl-2\(^{-}\) thymic development (Bouillet et al., 2001; Wojciechowski et al., 2007), placing Bim genetically downstream of Bcl-2 and suggesting that the major
role of Bcl-2 in thymocytes is to inhibit the activation of Bax and/or Bak by Bim (Bax activation displayed in Figure 36). It is likely that Bim functions to activate Bax both directly and indirectly because mutant forms of Bim that cannot bind Bax have been shown to partially alleviate the Bcl-2−/− phenotype but did not entirely recapitulate the rescue observed with the Bim−/− (Merino et al., 2009). We observed that the Bim−/− background did not rescue Mcl-1+/−LckCre DN thymocytes (data not shown), so Bim alone is unlikely responsible for the incomplete rescue of Mcl-1-deficient DN thymocytes in the Bak−/− mice.

In SP cells, the direct inhibition of Bak appears to be a more dominant role of Mcl-1 due to the extent of rescue by the deletion of Bak, even at the heterozygous level (Figure 36, right). The Bcl-2+/− and Bax−/− backgrounds yielded a slight improvement in CD4+ SP thymocyte survival, so Mcl-1 likely also plays a minor cooperative role with Bcl-2 in inhibiting Bax. Because this was not observed on the Bim−/− background, the direct inhibition Bax by Mcl-1 could be responsible (as shown in Figure 36), although it is also possible that Mcl-1 inhibits one of the other activator BH3-only proteins for this effect.

Because Mcl-1+/−CD4Cre thymocytes have no observed defect at the DP stage, we could not use the “rescue” models to assess the specific role of Mcl-1 in DP cells. However, based on published data and the results above, we can extrapolate our DN and SP cell models to predict the role(s) of Mcl-1 at the DP stage. In a previous study, we
observed that DP cells were lost when both Mcl-1 and Bcl-xl were deleted, but not when either was deleted alone, indicating overlapping functions for these two proteins at the DP stage (Dzhagalov et al., 2008). Willis et al. showed biochemically that both Mcl-1 and Bcl-xl can bind to Bak (Willis et al., 2005). Thus, it is likely that in DP cells, both Mcl-1 and Bcl-xl function to inhibit Bak (Figure 36, middle). It is unclear whether Mcl-1 and Bcl-xl also inhibit Bax in DP thymocytes or whether Bax is inactive at this stage. It is worth noting that Bcl-xl expression is highest at the DP stage and relatively low in the DN and SP stages (Grillot et al., 1995; Ma et al., 1995), and thus, unlike Bcl-2, it is possible that overexpression of Bcl-xl in DN and SP thymocytes would rescue the survival of Mcl-1-deficient thymocytes at these stages.

### 6.2 Model of the major roles of Mcl-1 and Bcl-2 in T cells

Building on the model above, the data from Chapter 4 allow us to determine the primary roles of Mcl-1 in peripheral T cells (Figure 37). In the presence of IL-7, T cells require Mcl-1 to inhibit Bak. Bcl-2, which is induced by IL-7, likely fulfills a similar role as in thymocytes to inhibit Bax and/or Bim. Again, the Bim<sup>-/-</sup> background has been shown to rescue the development and survival of naïve mature Bcl-2<sup>-/-</sup> T cells, and IL-7, IL-15, and IL-4 could not rescue survival in Bim<sup>-/-</sup>Bcl-2<sup>-/-</sup> T cells (Wojciechowski et al., 2007). Because the Bim<sup>-/-</sup> and Bax<sup>-/-</sup> backgrounds did not rescue the survival of Mcl-1-deficient cells in IL-7, either Mcl-1 does not regulate these proteins in the presence of IL-7 or the amount of Bcl-2 present is enough to compensate for the loss of regulation.
upon deletion of Mcl-1. In memory T cells and CD8+ naïve cells, but not naïve CD4+ T cells, it appears that IL-15 has the same role as IL-7 in this model. In contrast, IL-4 alters the balance between the Bak-mediated pathway and the Bim/Bax-mediated pathway of cell death by upregulating Bim.

Under conditions of cytokine withdrawal, it is clear that Mcl-1 is important to inhibit the proapoptotic effects of Bim and Bax because knockout of these proteins improves the survival of Mcl-1-deficient T cells under this condition (Figure 37). Further, it appears that this is a role that Mcl-1 shares with Bcl-2 but that limited amounts of Bcl-2 are available during cytokine withdrawal, as evidenced by the partial rescue by the Bcl-2\(^{tg}\). Thus, the loss of cytokine signaling shifts the dominant role of Mcl-1 to include the Bim/Bax pathway. However, because the Bcl-2\(^{tg}\), which completely rescues wild type cells from cytokine withdrawal-induced death, only partially rescued Mcl-1-deficient cells, it is clear that Mcl-1 also has a separate role from Bcl-2 even during cytokine withdrawal. As discussed in Section 4.3.1, this could still be the inhibition of Bak, or it could be the neutralization of another BH3-only protein with preferential binding to Mcl-1.
Left (with cytokines): IL-7, IL-4, and IL-15 promote Mcl-1 and Bcl-2 expression. The primary role of Mcl-1 is to inhibit Bak. Meanwhile Bax remains inactive either through inhibition by Bcl-2 (perhaps through the inhibition of Bim) or through the lack of activating factors retaining Bax in the cytosol (see Section 6.3). Bim is kept relatively inactive but is induced on the protein level by IL-4. Right (cytokine withdrawal): Mcl-1 and Bcl-2 expression is lower, while the activity of Bim and perhaps other BH3-only proteins is enhanced. The dominant function of both Mcl-1 and Bcl-2 is to inhibit Bim, but Mcl-1 may still have a role in inhibiting Bak.

6.3 Non-redundancy of Bak and Bax and implications on the Bcl-2 family models of action

Our data have highlighted that the antiapoptotic proteins of the Bcl-2 family have both shared and unique roles. In addition, the data contribute new insight into how the proapoptotic members of the Bcl-2 family, particularly Bak and Bax, may be differentially regulated. The rescue of Mcl-1 deficient thymocytes and T cells by the Bak knockout was initially somewhat surprising considering that Bak and Bax are presumed to be largely redundant (Lindsten et al., 2000). Neither Bak nor Bax single knockout mice exhibit a significant phenotype, while the double knockout mouse has profound defects in several systems, including thymocytes (Knudson et al., 1995; Lindsten et al., 2000;
Rathmell et al., 2002). Typically, both Bak and Bax need to be neutralized in order to observe an effect on apoptosis. In our experiments, however, loss of Bak alone rescued Mcl-1-deficient thymocytes and T cells, while loss of Bax only had a marked impact during cytokine withdrawal.

There is some evidence that Bak and Bax, while functionally similar, could be regulated differently. An obvious example is of course the fact that Bak can be inhibited by Mcl-1 but not Bcl-2, while Bax can be inhibited by both [(Willis et al., 2005) and Chapters 3, 4]. In addition to the Bcl-2 family members, other proteins may also affect the activity of Bak and/or Bax. For example, the mitochondrial protein VDAC2 has been shown to bind monomeric Bak in the mitochondrial membrane and to contribute to the susceptibility of cells to apoptosis through Bak (Cheng et al., 2003; Ren et al., 2009). Another important consideration is the intracellular localization of Bak and Bax. Under resting conditions, Bak is localized on the mitochondrial membrane, while Bax can be found in the cytosol until the induction of apoptosis, when it relocates to the mitochondrial membrane, coincident with a conformational shift to the active form and multimerization (Griffiths et al., 1999; Hsu et al., 1997; Wolter et al., 1997).

On a conceptual level, the difference in localization could allow for Bak and Bax to be subject to different regulatory mechanisms. From our data, we could hypothesize that Bak, poised within the mitochondrial membrane, requires constant inhibition by Mcl-1. Meanwhile, Bax, inactive in the cytosol, might require activation by a BH3-only
protein, such as Bim, before it translocates to the mitochondria. Mcl-1 and Bcl-2 could inhibit the activation of Bax either through the inhibition of Bim or by directly inhibiting Bax as a second level of regulation once it reaches the mitochondria. Some evidence exists that the antiapoptotic proteins may indeed be more effective at antagonizing activated Bax. One group showed that while Bax does not pull down Bcl-xl in non-apoptotic cells, Bcl-xl does associate with the activated/N-terminus-exposed form of Bax (Hsu and Youle, 1997). Similarly, Bcl-2 was shown to undergo a structural change in response to apoptotic signals to a form that can bind the membrane-targeted form of Bax, and the ratio of this Bcl-2 conformation to Bax in the mitochondrial membrane correlated with the ability of Bcl-2 to protect the mitochondria from cytochrome c release (Dlugosz et al., 2006). Interestingly, one study identified a novel binding site for Bim on Bax that could potentially be involved in the activation of Bax, but whether similar interactions exist with the other putative direct activators and/or Bak is yet to be determined (Czabotar et al., 2009; Gavathiotis et al., 2008).

Some important questions remain regarding the differential regulation of Bak and Bax. One critical question is how Bak is released from Mcl-1 under apoptotic conditions. One mechanism could be the targeting of Mcl-1 for protein degradation: UV irradiation has been shown to lead to proteosome-mediated degradation of Mcl-1 and apoptosis that occurs through Bak preferentially over Bax in mouse embryonic fibroblasts (MEFs) (Nijhawan et al., 2003; Willis et al., 2005). Similarly, adenovirus E1A
protein has been shown to destabilize Mcl-1 protein (in addition to downregulating Mcl-1 mRNA) and disrupt the Mcl-1/Bak interaction, leading to apoptosis (Cuconati et al., 2003). Alternatively, BH3-only proteins could bind to Mcl-1 or Bak to displace these proteins from one another. Interestingly, Noxa, at least at high levels, has been shown to displace Mcl-1 from Bak and also to contribute to the degradation of Mcl-1 protein (Willis et al., 2005). Another issue that is worth noting is that many of the studies that contributed to the current models of how the Bcl-2 family functions did not distinguish between Bak and Bax. In fact, many focused solely on Bax. Therefore, it would be worth revisiting some of the experiments of the earlier studies to examine whether Bak performs similarly to Bax in these experimental models.

### 6.4 Context-specific roles for Bcl-2 family proteins

One of the predominant themes of the studies here is that the dominant roles of Mcl-1 and the antiapoptotic proteins of the Bcl-2 family are context-dependent. This is evidenced by the different roles of these proteins within different thymocyte/T cell subsets, between different cell types, and between cells responding to different stimuli. In Chapter 3 and as depicted in Figure 36, we showed that the inhibition of Bak is a dominant role of Mcl-1 in both DN and SP cells. However, the difference in the extent of rescue by the Bak<sup>−/−</sup> background and the modest effect of the Bcl-2<sup>−/−</sup> and the Bax<sup>−/−</sup> background in SP thymocytes, but not DN thymocytes, indicate that DN and SP cells have other factors that modulate the dominance of the Mcl-1/Bak pathway. The slight
rescue of Mcl-1-deficient CD4+ SP thymocytes and T cells by the Bcl-2s and Bax+/ backgrounds and the data from a hypomorphic mouse model of Mcl-1 (Yang et al., 2009) showed a potential difference between CD4+ and CD8+ cells in their dependence on Mcl-1. Using a similar double-knockout strategy to ours, Steimer et al. showed that neither the Bak nor Bax knockout alone rescued the survival of Mcl-1-deficient neutrophils, indicating that neutrophils and T cells both rely on Mcl-1, but via different mechanisms (Steimer et al., 2009).

The reasons for the differences in the roles of Mcl-1 between different cell types are not yet clear. However, because the balance between antiapoptotic and proapoptotic factors are what ultimately determine cell fate, a likely contributor would be differences in expression and activity of other Bcl-2 family members between cell types. Upstream BH3-only proteins inhibit the antiapoptotic proteins and combine to sensitize the cell towards certain stimuli. For example, while neither Noxa nor Bad alone causes apoptosis in certain experimental systems, the co-expression of these proteins or their BH3-regions led to cell death (Chen et al., 2005; Willis et al., 2005). Bad has been shown to bind Bcl-2 and Bcl-xL, but not Mcl-1, directly, while Noxa binds Mcl-1 and A1, but not Bcl-xL or Bcl-2 (Chen et al., 2005). Thus, Bad could indirectly influence the role of Mcl-1 in that the more Bad activity there is in a cell, the less Bcl-2 and Bcl-xL are able to perform shared roles with Mcl-1, and the more susceptible the cell becomes to loss of Mcl-1 protein or function. Additionally, the activity of Noxa, which inhibits Mcl-1, is
likely to have a large role in determining the availability of Mcl-1 to perform its specific roles as well as its shared roles with Bcl-2.

As important as it is to identify the distinct balance of Bcl-2 family proteins that promotes survival in individual cell types, it is equally as important to understand the factors that modulate this balance. In Chapter 4, we showed how γc-sharing cytokines differentially regulate the dominant role of Mcl-1. The evidence suggests that this modulation is indeed through the differential expression of proapoptotic Bcl-2 family members, but the precise signaling events that lead to these differences in expression are not yet clear. Akt, which is activated in response to γc cytokine signaling, has been shown to phosphorylate and inactivate the forkhead transcription factor FKHRL1 (also known as Foxo3a), which in turn has been shown to regulate both Bim and Noxa expression (Brunet et al., 1999; Dijkers et al., 2000; Huntington et al., 2007; Obexer et al., 2007). In addition to regulation at the transcriptional level, IL-15 has been shown to phosphorylate Bim through the MAPK/ERK pathway in NK cells, targeting it for proteosome degradation (Huntington et al., 2007). A thorough analysis of how these and other signaling pathways affect BH3-only protein expression in T cells downstream of different cytokines, particularly focusing on the differences between IL-4 and IL-7, will provide greater insight into how these cytokines modulate the role of Mcl-1.

As the biochemical pathways are further defined, it will be important to consider that each cell type and condition may have a different balance of Bcl-2 family members.
While the work presented here dissects the influence of different factors, particularly the γc family cytokines, on the roles of Mcl-1 in T cells, under certain in vivo circumstances, many of these factors may be present simultaneously. Thus, it will be important to continue studying the dynamics of the expression and interactions of the different Bcl-2 family members in different cell types and microenvironments. Given that this family of proteins is often disregulated in human cancers and has been identified as a target for tumor therapy, a thorough understanding of the different apoptotic pathways and the factors that influence the balance within these pathways is essential for future translational applications.

6.5 Remaining questions/future directions for the role of Bcl-2 in CD8⁺ T cell memory

The experiments presented in Chapter 5 demonstrated that effector CD8⁺ T cells that retain relatively high levels of Bcl-2 during the response to *Listeria monocytogenes* are the cells that survive to become memory cells. Further, while these cells appeared to be included within the previously described CD127⁺KLRG1⁻ memory precursor population, the true memory precursors were actually a subset of the CD127⁺KLRG1⁻ cells that could be defined within a fairly narrow range of Bcl-2 expression. The identification of this range of Bcl-2 expression that correlates with memory potential indicates that Bcl-2 likely has an important function in the formation of T cell memory. However, further studies are needed to determine whether Bcl-2 expression alone is the critical factor that allows some cells but not others, even within the CD127⁺KLRG1⁻
MPEC population, to become memory cells or whether high Bcl-2 expression is indicative of, and perhaps even secondary to, some larger developmental program of the pre-memory subset.

One way to address this question would be to manipulate Bcl-2 expression in the effector T cells, for example by crossing the OT1^YFP cells onto the Bcl-2^tg background used in our earlier studies (which overexpresses Bcl-2 in all T cells). Overexpression of CD127, which would lead to enhanced Bcl-2 expression, has been shown to be insufficient for rescuing effector T cells from contraction, although constitutive activation of STAT5 via retroviral transduction did inhibit contraction of LCMV-specific cells in one study (Hand et al., 2010; Hand et al., 2007; Haring et al., 2008). The critical question is whether changing the expression level of Bcl-2 alone would shift or expand the memory potential from one OT1^YFP subpopulation to another, particularly within the CD127^hiKLRG1^− population. Because the Bcl-2^tg system does not utilize the endogenous Bcl-2 regulatory elements, expression of the transgene would likely not affect YFP expression (although this must be confirmed), thus leaving the populations observed in our studies intact. The overexpression of Bcl-2 may also address the question of whether expression of Bcl-2 can be “too high” as suggested by our observation that the highest expressors of Bcl-2 within the CD127^hiKLRG1^− MPEC population appeared less fit to become memory cells. Like many prosurvival proteins, Bcl-2 has been shown to have a negative effect on cell

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proliferation and thus at high levels could potentially negatively regulate memory cell homeostasis or expansion.

Another explanation for the decreased potential of the Bcl-2\textsuperscript{highest} MPEC cells is that these cells might represent cells that have been activated recently or incompletely. Because naïve cells are CD127\textsuperscript{hi}KLRG1 Bcl-2\textsuperscript{hi} and differ from the MPEC Bcl-2\textsuperscript{hi} cells only by the absence of CD44, it is possible that the Bcl-2\textsuperscript{hi} MPEC cells are newly activated cells that have only completed part of the program toward an effector phenotype. As such, these cells might represent a mixed population of cells that will develop a terminal effector phenotype and cells that will develop memory potential. Under this scenario, the reason that we did not observe a secondary response in the mice that received these cells could be that the frequency of cells fated to become memory was too low or that the transfer of the cells into naïve mice at the early post-activation timepoint interfered with the developmental program toward the memory fate. Alternatively, the Bcl-2\textsuperscript{highest} cells could be naïve or memory-phenotype cells that were insufficiently activated and thus express some markers of activation (CD44) but do not represent true effector cells. It would be worthwhile to perform a more in depth analysis of the timing of the changes in YFP expression compared to other activation markers (both those already discussed and additional markers such as CD25 and CD69) as well as to track the changes in the expression patterns of these markers and Bcl-2 after transfer of the different populations.
While the level of Bcl-2 expression may be a critical difference between subpopulations with different memory potential, an important next step will be to determine what else distinguishes these subpopulations from one another. This could be achieved through protein or mRNA expression analysis, either on a large scale (i.e. microarray analysis) or by selecting potential targets. The goal of this comparison would be twofold: to identify other factors (if any) that contribute to the memory potential of the cells independently of Bcl-2 and to identify pathways that may lead to the differences in Bcl-2 expression. Several candidate targets and pathways could be selected based on previously described associations with T cell memory and/or Bcl-2 expression and activity. We observed that while all of the MPEC populations were identified as CD127hi, within the MPEC population, CD127 expression levels correlated with Bcl-2 expression, although in a less dynamic range. Thus IL-7 signaling likely contributes to the maintenance of Bcl-2 levels. The balance between STAT5 and PI3K/Akt activation downstream of cytokine signaling has been shown to be important in driving both Bcl-2 expression and a memory phenotype (Hand et al., 2010), and a close comparison of these signals between different Bcl-2-expressing populations would be worthwhile. The balance between the activity of the transcription factors T-bet and eomesedermin has also been shown to be critical for the formation of memory (Intlekofer et al., 2005; Joshi et al., 2007; Rao et al.), and there could be subtle differences in the activation of these factors between the populations described here. Given that Bim
has been connected with the apoptosis of activated T cells, in particular the CD127\textsuperscript{lo} subset (Hildeman et al., 2002; Wojciechowski et al., 2006), it would be worthwhile to examine the expression of other Bcl-2 family members within the different Bcl-2-expressing subsets to determine whether other proteins from this family might cooperate with or antagonize the effects of Bcl-2. Examining the differences in protein expression, signaling, and transcriptional pathways between the populations with different memory potential will not only allow us to further understand the mechanism by which memory cells survive the contraction period, it will also provide evidence for the precise signals that are needed to achieve “memory status.” In the future, the Bcl-2\textsuperscript{YFP} model could be used as a tool in experimentally manipulating the establishment or maintenance of T cell memory.
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Biography

Alexis Dunkle was born on March 15, 1980 in Palo Alto, California. She attended the University of California, Berkeley and graduated with a Bachelor of Arts in Molecular and Cell Biology with an emphasis in Immunology in 2002. She is the author of the following publications:


Alexis Dunkle, Ivan Dzhagalov, and You-Wen He (submitted). Cytokine-dependent and cytokine-independent roles for Mcl-1: Genetic evidence for multiple mechanisms by which Mcl-1 promotes survival in primary T lymphocytes.