THE UNIVERSITY OF CHICAGO

DISTINCT CELL DEATH SENSITIVITY AND ADAPTIVITY IN T CELL SUBSETS FOLLOWING MANIPULATION OF THE BCL-2 FAMILY OF PROTEINS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES
AND THE PRITZKER SCHOOL OF MEDICINE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

COMMITTEE ON CANCER BIOLOGY

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CHICAGO, ILLINOIS
DECEMBER 2019

To my mom and my friend Barb Ludwig.

Thank you for your constant support.

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ACKNOWLEDGMENTS

There are many people that I would like to acknowledge for contributing to the completion of my dissertation project as well as supporting my personal journey through graduate school. First and foremost, thank you to my PI and mentor, Dr. James LaBelle. Through the many ups and downs of graduate school, I was always able to rely on James for support, advice, and motivation. The passion that he has dedicated to the lab and the pursuit of scientific progress is truly inspiring, and the time I have spent working with James has made me a better scientist and a better person. I would also like to thank my thesis committee, Dr. Kay Macleod, Dr. Barbara Kee, and Dr. Bryan Dickinson, as well as former committee member Dr. Joel Collier. The feedback my committee has provided to me has improved how I think about scientific questions and helped shape my dissertation project into something I am truly proud of. I would also like to acknowledge the previous and current administrators in the Committee on Cancer Biology as well as the Department of Pediatrics that have helped ensure things run smoothly, including Emily Traw, Nadia Otikor, Laura Negrete, Sharon Dunn, and Kerri Pride-Fair. And many thanks to the staff of the Flow Cytometry Core Facility including David Leclerc for the use of their cell sorters and valuable expertise in flow data analysis.

I would also like to acknowledge all of the past and present members of the LaBelle Lab. It has been a true joy to work with everyone and to see so many interesting projects take shape and evolve. I am so grateful to have had the opportunity to work with such a smart and amazing group. Thank you to former postdocs, Rachel Eclov and Abbas Hadji for valuable feedback and advice as I established my project. And a special thank you to my fellow graduate students in the lab, Katrina Maxcy, Rosy Liao, and Mat Schnorenberg. It is truly fantastic to have such great labmates and more importantly, such great friends. And thank you to my fellow graduate students and friends that I have made along the way, Ashely Sample, Anna Chen, Beth Pollard, John Cao, James Goodman, Maya Springer, and Yuanyuan Li. I am so glad to have met all of you.

Finally I would like to say thank you to my family for supporting me through this process. To my brother and sister-in-law, Logan and Ashlin Ludwig, and to my nephew Brayden Ludwig. And thank you to my dog brother Max for always making me smile. Last but certainly not least, I would like to thank my mom, Barb Ludwig. I am eternally grateful for the support and confidence that she has given me. I have been able to pursue my dream career in scientific research, and I couldn't have done it without her.

ABBREVIATIONS

BCL-2: B cell lymphoma 2; BH3: BCL-2 homology 3; MCL-1: Myeloid cell leukemia 1; TNFR: Tumor necrosis factor receptor; MOMP: mitochondrial outer membrane depolarization; SMAC: second mitochondria-derived activator of caspases; DIABLO: direct IAP binding protein with low pI; APAF: apoptotic protease activating factor; PMA: phorbol myristate acetate; MEF: mouse embryonic fibroblast; SLE: systemic lupus erythematosus; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; TCR: T cell receptor; Treg: regulatory T cell; Tcon: conventional T cell; DN: double negative; DP: double positive; SP: single positive; CLL: chronic lymphocytic leukemia; CMML: chronic myelomonocytic leukemia; LPS: lipopolysaccharide; DLBCL: diffuse large B cell lymphoma; HDAC: histone deacetylase; NHL: Non-Hodgkin's lymphoma; HSV-1: herpes simplex virus 1; AML: acute myeloid leukemia; MM: multiple myeloma; ALL: acute lymphoblastic leukemia; CML: chronic myelogenous leukemia; KLH: keyhole limpet hemocyanin; MHC: major histocompatibility complex; GVHD: graft versus host disease; IL: interleukin; PBMCs: peripheral blood mononuclear cells; ALC: absolute lymphocyte counts; SEB: staphylococcal enterotoxin B; LCMV: lymphocytic choriomeningitis virus; IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked; GFP: green fluorescent protein; IRES: internal ribosome entry site; SCID: severe combined immunodeficiency; HSCT: hematopoietic stem cell transplant; NFAT: nuclear factor of activated T cells; CDK7: cyclin-dependent kinase 7; LCK: lymphocyte cell-specific protein tyrosine kinase; DMEM: Dulbecco's modified eagle media; FCCP: carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; WT: wild type; DMSO: dimethyl sulfoxide; CAR: chimeric antigen receptor

ABSTRACT

The BCL-2 family comprises an essential network of proteins that govern a cell's decision to live or die. An increasingly diverse number of BH3 mimetics have been developed to manipulate interactions between BCL-2 family proteins within diseased and normal cells. Based on lymphocyte dependency on these proteins for ontogeny and survival, we hypothesized that T cells would have unique differences in their BCL-2 repertoire at baseline and in response to BH3 mimetic treatment. Long-term targeting of BCL-2 proteins in T cell subsets and their ability to adapt to extensive BCL-2 family manipulation is largely unknown. We first used a genetic deletion model of *Bim*, a master BH3-only regulator of T cell survival, to evaluate long-term pressure. We demonstrate that BIM loss in T cells leads to upregulation of additional BH3-only proteins, including *Puma*, *Noxa*, and *Bmf*, with differential upregulation patterns in $CD8^+$ versus $CD4^+$ T cells. We then used the BCL-2 specific inhibitor ABT-199 to further assess differences in BCL-2 family regulation between $CD8^+$ T cells, Tcons, and Tregs. We find that $CD8^+$ T cells are more sensitive to BCL-2 inhibition than Tregs, likely due to a more BCL-2 dependent profile of $CD8^+$ T cells. Tregs appear be more effective at adapting to BCL-2 targeting and rely on other anti-apoptotic proteins for survival, including MCL-1 and BCL-X_L. This research provides new insight into the dynamic regulation and adaptivity of T cell subsets in response to genetic and small molecule targeting of the BCL-2 family proteins. This will contribute to an improvement in our ability to effectively use BH3 mimetics in the context of immune system modulation, which will lead to new therapeutic strategies for a myriad of diseases, including anti-cancer immune therapy, transplant tolerance, and autoimmunity.

CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Apoptosis is critical to maintain organismal homeostasis

Apoptosis, or programmed cell death, is the main process in which organisms eliminate unnecessary or malignant cells [1][1]. The apoptotic cascade can be divided into two main pathways, both of which culminate in the activation of effector caspases that cleave essential substrates and in turn mediate the ultimate destruction of the cell [2, 3]. The extrinsic pathway is initiated through external signals propagated via death receptors on the cell surface such as FAS (CD95) or other members of the tumor necrosis factor receptor (TNFR) family. Ligand-induced receptor trimerization initiates cellular demise through adaptor protein association and initiator caspase-8 activation [4, 5]. In contrast, the intrinsic pathway is activated in response to a variety of internal cellular stresses, and is mediated primarily by the BCL-2 family of proteins.

1.1.2 BCL-2 family proteins are regulators of the intrinsic apoptotic pathway

BCL-2 was first discovered as a part of a chromosomal translocation in B-cell lymphoma and was the first known oncogene to inhibit cell death as opposed to actively promoting proliferation [6, 7, 8]. The BCL-2 proteins share one to four highly conserved regions in both sequence and structure, termed BCL-2 homology (BH) domains. Based on these domains, and in conjunction with their activity profile, the BCL-2 family is divided into three functional subgroups: the multidomain anti-apoptotics (BCL-2, BCL-X_L, BCL-W, MCL-1,

1. Sections of this chapter were expanded and adapted from Lindsey M. Ludwig, Michele L. Nassin, Abbas Hadji, and James L. LaBelle. Killing two cells with one stone: Pharmacologic BCL-2 family targeting for cancer cell death and immune modulation. *Frontiers in Pediatrics*, 4:135, 2016.

BFL-1/A1), the multidomain pro-apoptotics (BAK, BAX, BOK), and the BH3-only proteins (BIM, BID, BAD, NOXA, PUMA, BMF, BIK, HRK) (Fig. 1.1). The BH3-only proteins, named so because they share only the third BH domain with the other BCL-2 family proteins, act as cellular sentinels that in times of stress bind discrete multidomain BCL-2 proteins and initiate the apoptotic cascade [9]. The contact interfaces between anti-apoptotic and BH3-only proteins have been elucidated through crystal structure analyses. This has led to increasing interest and ability to design drugs that recapitulate these interactions in an effort to overcome apoptotic resistance. While these efforts have mainly focused on inducing cell death in the context of cancer therapy, there is potential to use these compounds as immunomodulators based upon the differential BCL-2 family member dependencies of immune cells [10].

The process by which BH3-only proteins initiate apoptosis can occur through two known mechanisms. BH3-only proteins can bind anti-apoptotic BCL-2 members causing release of sequestered BAX and BAK [11]. These are indirectly activating BH3-only proteins (e.g. BAD and NOXA). In addition, other BH3-only proteins, such as BIM, BID, and PUMA, can not only bind anti-apoptotics but are also able to directly bind and activate BAK and BAX oligomerization [12]. Once oligomerized, BAK and BAX form pores in the outer mitochondrial membrane causing mitochondrial outer membrane permeabilization (MOMP) which leads to the release of cytochrome *c* and other pro-apoptotic factors such as SMAC/DIABLO from the inner mitochondrial membrane space [13, 14]. Cytochrome *c* associates with APAF and caspase-9 to form the apoptosome, which initiates the cleavage of effector caspases 3 and 7 leading to eventual cellular destruction[15].

1.1.3 Quantitative vs. Qualitative BCL-2 control over cell death

A major question in the field is if protein specificity or overall quantity is more important for the regulation of the intrinsic apoptotic pathway. In essence, are specific anti-apoptotic and BH3-only proteins key in regulating cell death decisions (qualitative model), or is it more

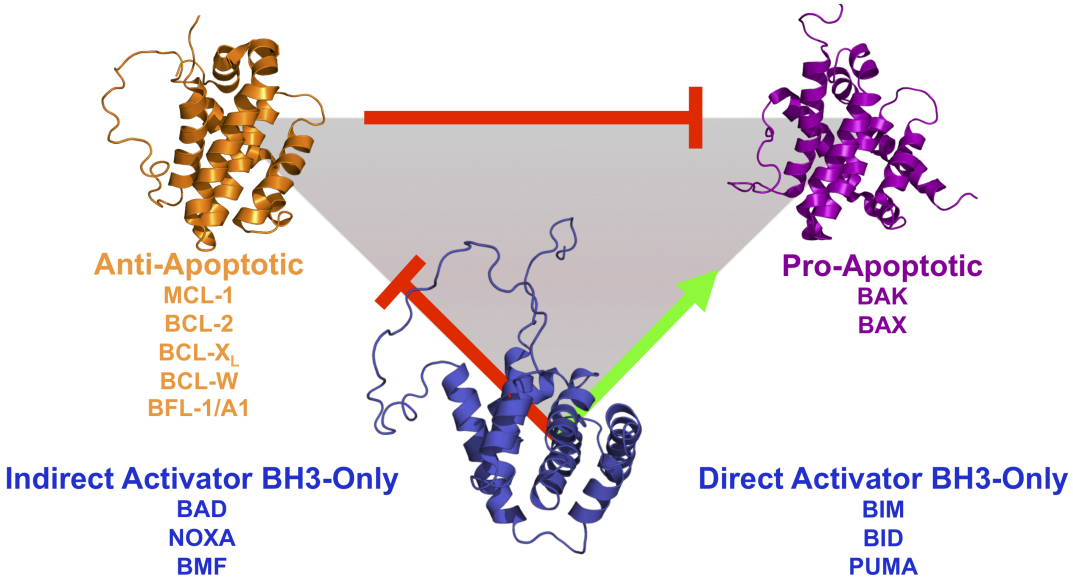

Figure 1.1: Overview of the BCL-2 family and interactions. The BCL-2 family is divided into three subgroups: the multidomain anti-apoptotics, the multidomain pro-apoptotics, and the BH3-only proteins. The anti-apoptotic proteins sequester the pro-apoptotic proteins BAX and BAK. In times of cellular stress, BH3-only proteins can either bind to the anti-apoptotic proteins and release the pro-apoptotics from their sequestration or directly bind and activate BAX and BAK. Once activated, BAX and BAK oligomerize and induce mitochondrial outer membrane permeabilization, leading to the release of other pro-apoptotic factors and eventual cellular destruction.

crucial that the overall balance of pro- and anti-apoptotic proteins reaches a certain threshold regardless of specificty (quantitative model) in order to initiate apoptosis? There is a large body of evidence that dictates roles for specific anti-apoptotic proteins to maintain survival in various cell types and contexts, including non-redundant roles in maintaining survival of various T cell subsets that will be discussed in subsequent sections. It is also known that certain cellular stresses preferentially induce the activation of distinct BH3-only proteins. For example ionomycin primarily induces apoptosis through BIM, while treatment with PMA induces apoptosis through PUMA [16, 17]. However, despite these distinctive patterns of BCL-2 regulation, there is a growing body of evidence suggesting that the quantitative regulation of these proteins is equally as important for dictating cell fate.

Most deletion models of a single anti-apoptotic or BH3-only protein have been in support of the qualitative model of BCL-2 family regulation. These models typically aim to determine whether or not there is a requirement for a specific protein in either prolonging survival or initiating apoptosis in distinct cell types. The qualitative role of specific anti-apoptotic proteins in T cells will be described further in the sections below. Recent data has started placing more emphasis on quantitative BCL-2 family regulation. For anti-apoptotic proteins, this has best been described through genetically deleting or therapeutically inhibiting multiple proteins at once and measuring how this effects apoptotic sensitivity [18]. Mice with deletion of one allele of MCL-1 were more sensitive to BCL-2 inhibition with ABT-199. Deletion of A1, which is thought to be relatively dispensable for the survival of most lymphocyte subsets, also lowered the apoptotic threshold in cells that were concurrently targeted with either BCL-2 inhibition or MCL-1 haploinsufficiency [18, 19]. Conversely, cells from MCL-1 haploinsufficient mice that were more apoptotically primed had reduced cell death when crossed with BCL-2 overexpressing mice [18]. This clearly demonstrates the additive effects anti-apoptotic proteins can have to maintain cell survival. Even loss of A1, a protein that has a less essential role in immune cell survival contributed to increased levels apoptosis in this quantitative model.

In the context of BH3-only proteins, models of double, triple, and even quadruple knockouts have demonstrated the additive effects that loss of multiple BH3-only proteins can lead to. Combined loss of BIM and PUMA causes a greater decrease in the proportion of double positive thymocytes and increased levels of mature cells in the spleen [20]. $Bim^{-/-}Bid^{-/-}$ double knockout mice have a larger accumulation of residual T cells after contraction of an immune response compared to the accumulation observed in $Bim^{-/-}$ single knockout mice [21]. Deletion of either *Puma* or *Noxa* also increases the accumulation of memory $CD8^+$ T cells after contraction [22]. The deletion of *Bmf* in conjunction with *Bim* did not contribute to abnormal thymocyte development, but double positive thymocytes isolated from these mice were more resistant to several apoptotic stimuli compared to single knockout mice, including cytokine deprivation and HDAC inhibition [23]. Triple knockout mice that lack BIM, BID, and PUMA have even more severe resistance to apoptosis compared to single ($Bim^{-/-}$) or double ($Bim^{-/-}Bid^{-/-}$) knockout animals [24]. A quadruple knockout with the additional loss of NOXA led to further increase in apoptotic resistance, but only in certain cell types, such as mouse embryonic fibroblasts (MEFs). T cells from quadruple knockout mice did not experience increased resistance to any apoptotic stimuli tested, but this may be because T cells express lower levels of NOXA at baseline [25].

Given the redundancy and dynamic regulation of the anti-apoptotic and BH3-only proteins, it is logical to propose that cells have the ability to adapt and shift their dependency to anti-apoptotic proteins that initially seemed irrelevant from a qualitative standpoint. However, there are still qualitative aspects to consider when assessing the BCL-2 repertoire in T cells subsets. For example, deletion of any BH3-only protein other than BIM causes minimal adverse effects on the T cell compartment [20, 23]. This emphasizes the critical, non-redundant role BIM plays in the regulation of T cell homeostasis, and quantitative deletion of multiple other BH3-only proteins is not sufficient to combat the role BIM plays in this context. Therefore, both qualitative and quantitative regulation of BCL-2 proteins will be important to consider when evaluating the BCL-2 repertoire in T cell subsets, especially

in the context of additional manipulation of this family using BH3 mimetics.

1.1.4 Role of BCL-2 family of proteins in immune ontogeny and homeostasis

Multidomain Pro-Apoptotics (BAX, BAK) The pro-apoptotic effector proteins BAK and BAX are considered to play redundant functional roles in the initiation of MOMP, as the deletion of either *Bak* or *Bax* alone leads to a minimal level of apoptotic defects [26]. However, deletion of both proteins leads to a high incidence of embryonic lethality with surviving mice having a host of developmental and neuronal defects. Not surprisingly, BAX/BAK deficient mice have a significant increase in both myeloid and lymphoid cells, leading to enlarged primary lymphoid organs and lymphocyte infiltration into peripheral organs [26]. Lymphocytes from these animals are resistant to known activators of the intrinsic apoptotic pathway, including cytokine deprivation, etoposide, and irradiation [26]. Mice with conditional T cell-specific *Bax/Bak* knockout have abnormal thymocyte development and increased accumulation of double negative cells in the thymus [27]. Thymocytes are resistant to apoptosis following treatment with γ-irradiation and animals develop T cell lymphoma with a median survival of only 10 months [27].

Because BAX and BAK activation is typically considered "the point of no return" in apoptosis induction, therapeutics that can directly activate their oligomerization would be potent initiators of apoptosis. However, there would be a lack of specificity in targeting these proteins directly and off-target effects may limit their clinical use. Direct BAX/BAK activators may find greater efficacy in combination with other, more specific BCL-2 family targeting agents. BH3 mimetics specific for discrete anti-apoptotic proteins could potentially lower the apoptotic threshold in a targeted subset of lymphocytes, allowing for lower doses of BAX/BAK activators to exclusively induce apoptosis in these cells. The greatest limitation to this, however, is the inability at the present time to target BH3 mimetics to specific cell populations.

Multidomain Anti-Apoptotics (BCL-2, BCL-X_L, BCL-W, MCL-1, BFL-1/A1)

BCL-2 was the first member of the anti-apoptotic subgroup to be extensively characterized. Over-expression of BCL-2 in Eμ-myc transgenic mice found that BCL-2 in conjunction with dysregulated c-myc promoted immature B cell proliferation and tumorigenesis. However, expression of BCL-2 alone allows for cell survival without any change in proliferation [8]. Constitutive BCL-2 expression ultimately leads to increased numbers of pre-B cells, plasma cells, and T cells, all of which demonstrate increased longevity in culture [28, 29]. BCL-2 overexpression in these animals leads to autoimmunity similar to that measured in patients with systemic lupus erythematosus (SLE) [28]. In contrast, global deletion of *Bcl-2* leads to a significant decrease in the number of double positive (DP) thymocytes and peripheral (splenic) B and T cells. TUNEL staining of the spleens and thymi of aged *Bcl-2* knockout mice reveals a significant increase in apoptotic cells. Thymocytes lacking BCL-2 are also more susceptible to a wide range of apoptotic stimuli [30].

MCL-1 also plays a significant role in immune ontogeny and maintenance. Global deletion of *Mcl-1* is embryonic lethal, therefore conditional knockout models have been utilized to determine the role of MCL-1 in various immune cell subsets [31]. Globally, MCL-1 and not BCL-2 expression is critical for maintaining hematopoietic stem cell survival. Inducible deletion of *Mcl-1* causes rapid bone marrow depletion and mice become moribund within several weeks. These animals rapidly develop severely reduced numbers of hematopoietic stem cells and other bone marrow progenitor populations [32]. T cell-specific *Mcl-1* deletion leads to a significant reduction in T lymphocytes, as well as an apparent blockade at the DN2/3 stage of thymocyte development [33]. Unlike BCL-2, MCL-1 expression remains constant or is slightly upregulated upon T cell receptor (TCR) stimulation [34, 35]. Another distinctive feature of MCL-1 is that it plays a key role in the maintenance of immunosuppressive regulatory T cells (Tregs). Because of this, mice with Treg specific *Mcl-1* deletion experience weight loss, inflammation, and death due to global autoimmunity within 4-8 weeks [36]. Additionally, B cell-specific MCL-1 loss leads to impaired B cell development beginning at

the pro-B cell stage [33]. MCL-1 is also essential for the formation of germinal centers and the survival of plasma cells [37, 38].

The remaining anti-apoptotic proteins have not been as well characterized in immune system homeostasis. Loss of BCL-X_L has a minimal effect on overall T cell survival. While mice lacking BCL-X_L have reduced numbers of double positive thymocytes, they have normal peripheral lymphocyte numbers indicating that BCL-X_L alone is not critical for lymphocyte homeostasis [39]. Deletion of *Bfl-1/A1* causes a decrease in DP thymocytes, and an increase in double negative (DN) and $CD8^+$ single positive thymocytes. However, BFL-1/A1 deficient lymphocytes have no significant increase in resistance to apoptotic stimuli [40]. Deletion of *Bfl-1/A1* in the myeloid lineage causes a decrease in the granulocyte population and causes increased levels of spontaneous apoptosis in both granulocytes and neutrophils in culture [40, 41]. BFL-1 has also been shown to be elevated in several human malignancies, including B cell chronic lymphocytic leukemia (CLL) and familial systemic lupus erythematosus [42, 43].

Based on the differential reliance on specific anti-apoptotic BCL-2 proteins in immune control, it may be feasible to target exclusive subsets of lymphocytes with agents having specificity to the BH3 binding domains of these proteins. Careful consideration will be needed in balancing BH3 mimetic dosing if given together with other chemotherapeutic agents that may lower the therapeutic threshold of immune cells or even alter their anti-apoptotic dependency during treatment. The long- and short-term effects of anti-apoptotic protein targeting of the immune system is unknown and how other proteins in this subclass may compensate is unclear. Rapid upregulation of non-targeted anti-apoptotic proteins has been found to occur in lymphocytes when treated with BH3 mimetics [44]. How this will impact immune effects clinically has yet to be determined.

BH3-Only Proteins (BIM, BID, PUMA, BAD, NOXA, BMF) Most data regarding the role of BH3-only proteins has involved studying the direct activator proteins BIM, BID, and PUMA though genetic deletion in murine models. BIM is considered the master

regulator of immune cell homeostasis. *Bim* deletion causes increased numbers of lymphoid and myeloid cells, defects in thymocyte development, and global lymphocyte resistance to multiple apoptotic stimuli [45, 16]. BIM is essential for negative selection of immature T cells in the thymus, and mice lacking BIM have shorter lifespans due to the development of fatal autoimmunity [45, 46]. BIM also plays an important role in regulating the survival of $CD4^+$ T cells in the periphery. Decreased BIM expression with age leads to longer-lived $CD4^+$ T cells that are prone to functional defects and become increasingly unable to properly respond to pathogens [47]. Deletion of additional BH3-only proteins exacerbates the immune dysfunction seen in *Bim* knockout animals. Combined deletion of *Bim* and *Puma* causes increased resistance to a wide range of apoptotic stimuli, and a subset of these mice develop spontaneous follicular B cell lymphoma [20]. Triple knockout of *Bim*, *Puma*, and *Bid* leads to increased lymphocytosis and profound, yet not complete, apoptotic resistance [24]. The prevalence of hematological malignancies has not yet been characterized in these triple knockout mice [24].

BID is a unique BH3-only protein because its structure is more similar to the multidomain anti-apoptotics and it provides functional crosstalk between the intrinsic and extrinsic apoptotic pathways. BID is cleaved to its functional form, tBID, by activated caspase-8, downstream of plasma membrane death receptor activation [48]. Young mice deficient in BID have no major hematological defects, whereas aged mice develop neutrophilia and many succumb to a hematopoietic malignancy resembling human chronic myelomonocytic leukemia (CMML) [49]. BID may also play a key role in survival of Langerhans cells, a unique subset of dendritic cells, as BID deficient Langerhans cells are more resistant to $CD4^+$ T cell-mediated apoptosis [50].

PUMA is regulated by the tumor suppressor p53 and has been implicated as a mediator of apoptosis in several subsets of immune cells [51, 52]. PUMA cooperates with BIM to regulate activated T cell contraction following an immune response. Mice lacking PUMA accumulate activated $CD8^+$ T cells in their spleen following herpes simplex virus (HSV-1)

infection and these cells are more resistant to cytokine deprivation in culture [53]. PUMA is also upregulated in activated B cells and *Puma* deletion leads to apoptotic resistance and B cell accumulation in addition to increased levels of memory B cells following antigen stimulation [54]. In the myeloid compartment, PUMA deficiency has been shown to impair the regulation of neutrophil contraction following an immune response, thus compromising the ability to properly respond to bacterial infections which can lead to lethal sepsis [55].

Like PUMA, the indirect activator NOXA is regulated by p53 [56]. *Noxa* knockout mice have no aberrations in thymocyte development, however NOXA may play a minor role in regulating the formation and maintenance of effector memory T cells during and following an immune response [57, 58]. NOXA has also been shown to play a key role in B cell activation and efficient generation of high affinity antibody clones. Loss of *Noxa* leads to an accumulation of low affinity B cells due to dysregulated apoptosis of these cells during immune response initiation [59].

Normal B and T cell development is maintained in cells lacking BAD and lymphocytes from BAD deficient animals retain normal sensitivity to apoptotic stimuli [60]. Currently, BAD appears most important in B cell ontogeny and maturation. Although incompletely understood, *Bad* knockout mice have reduced IgG production after lipopolysaccharide (LPS) stimulation and aged mice develop diffuse large B cell lymphoma (DLBCL) that is increasingly penetrant following ionizing radiation [60].

Lastly, although not as extensively studied as other BH3-only proteins, *Bmf* knockout mice maintain normal overall T cell counts and have no known abnormalities in thymocyte development but do experience B cell hyperplasia. BMF deficient T and pre-B cells are resistant to apoptosis in response to glucocorticoids or HDAC inhibition [61]. Mice lacking BMF also develop thymic lymphomas following exposure to γ-irradiation [61].

Extensive characterization of the BH3-only proteins in the immune system has revealed both overlapping and non-redundant roles for many of these proteins in specific immune cell subsets. Major focus has been placed on designing therapeutics that mimic the binding

of these proteins to multidomain apoptotic effectors. As development of these compounds increases for use as single agent or combination anti-cancer therapeutics, it will be essential to continue defining the exact roles of the BH3-only proteins in the ontogeny and maintenance of clinically relevant anti-viral, anti-bacterial, and anti-tumor immune responses. How these proteins are displaced following treatment and sequestered by expressed anti-apoptotics in immune cell subtypes is of particular interest. Harnessing their immune-based "off target" effects could allow for powerful modulation of the immune system alone or in concert with other emerging immune-based therapies.

1.1.5 BH3 mimetics

The BCL-2 family of proteins are heavily implicated in tumorigenesis and targeting their interactions shows great promise in overcoming apoptotic resistance in a vast array of malignancies [62, 63]. Given the importance of BH3-only proteins in regulating the crosstalk between the anti- and pro-apoptotic multidomain BCL-2 proteins, most drug development has centered on recapitulating their mechanism(s) of action in cells. These so called "BH3 mimetics" encompass an array of natural products, small molecules, and peptide therapeutics that mimic the BH3-domain-directed binding of BCL-2 proteins to either lower the apoptotic threshold or directly initiate the intrinsic apoptotic cascade. Most work has focused on the role of these compounds as anti-cancer therapeutics as described below. Less well understood is how these compounds can be used to modulate normal immune responses when used at the same dose or through dosing within a different therapeutic window. It is important to consider that cancer cells are "primed to die" due to their extreme dependence on thwarting apoptosis given their genetic and metabolic abnormalities. In contrast, although susceptible to these compounds, normal lymphocytes may have different sensitivities to these drugs as compared to malignant cells. It is critical that these potential differences are further elucidated in order to generate the most effective strategies for immune modulation.

Compounds in Clinical Trials

I. Antisense oligonucleotides

Oblimersen Sodium (genasense) Over 25 years ago it was demonstrated that an antisense oligonucleotide targeting BCL-2 could abrogate in vivo tumor growth [64]. These studies led to the development of the optimized antisense oligonucleotide oblimersen sodium (G3139; genasense; augmerosen), which demonstrated in vitro and in vivo efficacy against multiple hematological malignancies, including Non-Hodgkin's lymphoma (NHL), EBV-associated lymphoproliferative disorders, and Philadelphia chromosome-positive leukemia [65, 66, 67]. After showing promise in multiple pre-clinical models, oblimersen was tested in clinical trials for both hematological malignancies and solid tumors. Phase I and II clinical trials were encouraging against a wide range of cancer types, including hormone-refractory prostate cancer, CLL, and NHL [68, 69, 70]. Unfortunately, despite initial promise, several Phase III studies have found that oblimersen does not improve the response rate seen with the current standards of care [71, 72]. Oblimersen has not been FDA approved and subsequent attention has turned to the development of small molecules and peptide therapeutics that directly disrupt intracellular BCL-2 family protein:protein interactions.

II. Small Molecules

ABT-737 and ABT-263 (navitoclax) The first small molecule to effectively mimic the interaction between anti-apoptotics and BH3-only proteins was the BAD-derived BH3-only mimetic ABT-737 [73]. Discovered using a nuclear magnetic resonance (NMR)-based screening method, this small molecule has high affinity for the BH3 binding pocket of BCL-2, BCL-X_L, and BCL-W [73]. ABT-737 has been shown to disrupt BAX/BCL-2 complexes, leading to the release of cytochrome c and the initiation of the caspase cascade [74, 75]. BAX/BAK double knockout cells administered ABT-737 experience no significant decrease in viability, indicating that ABT-737 functions through on-target binding to anti-apoptotic proteins to induce the intrinsic mitochondrial apoptotic pathway [75]. ABT-737 has potent in vitro activity against a wide range of hematological malignancies, including acute myeloid leukemia (AML), multiple myeloma (MM), and acute lymphoblastic leukemia (ALL)

[75, 76, 77]. Subsequent studies in xenograft models of adult and pediatric hematological diseases have confirmed the on-target potency of ABT-737 against malignant cells [78, 79, 80]. The oral analogue of ABT-737, ABT-263 (navitoclax), has been tested in clinical trials for both hematological and solid tumors [81]. However, many patients experienced dose limiting thrombocytopenia due to the dependency of platelets on BCL-X_L and BAK [82, 83]. Another important caveat is that many cancers are refractory or become resistant to ABT-737 or ABT-263 due to upregulation of anti-apoptotic proteins (e.g. MCL-1, BFL-1) that lack specificity to either compound [75, 84, 85]. It will therefore be imperative to consider which anti-apoptotic proteins target cells express and understand their real-time compensatory capacity for apoptotic resistance before treatment with BH3 mimetics having limited anti-apoptotic protein specificity.

ABT-199 (venetoclax) To overcome the thrombocytopenia caused by ABT-263, a new BH3 mimetic was derived based on the X-ray crystal structure of BCL-2 and ABT-263 [86]. ABT-199 binds only to BCL-2 with sub-nanomolar affinity [86]. ABT-199 has demonstrated effective *in vitro* and *in vivo* cell killing in a range of cancers, including chronic myelogenous leukemia (CML), AML, and T-cell ALL [87, 88, 89]. Based on its efficacy in clinical studies, ABT-199 has recently gained FDA approval for the treatment of refractory 17-p-deleted CLL and AML, making it the first clinically approved small molecule targeting intracellular protein:protein interactions [90, 88]. Interestingly, there are reports describing ABT-199 inducing cell death in normal immune cell subsets in addition to its desired anti-cancer activity as was found in the case of normal mature B cells isolated from patients with CLL [91].

GX15-070 (obatoclax) The first pan inhibitor of the anti-apoptotic BCL-2 proteins was GX15-070 [92]. GX15-070 binds all anti-apoptotics with nanomolar to low micromolar affinity and, importantly, is able to overcome ABT-737 apoptotic resistance in cells with high MCL-1 expression [93, 94]. GX15-070 has efficacy against a range of solid tumors and hematological malignancies [94, 95, 96]. GX15-070 was well tolerated in Phase I clinical trials

for patients with CLL, refractory leukemia, and myelodysplasia [97, 98]. Unfortunately in phase II and phase I/II clinical trials GX15-070 did not improve outcomes in patients with myelofibrosis, mantle cell lymphoma, or AML [99, 100, 101]. On target specificity has been questioned regarding the mechanism of action of GX15-070, as BAX/BAK double knockout cells die when treated with this compound [95]. In fact, multiple modes of cell death have been measured in response to GX15-070 treatment. Canonical features of apoptosis, necrosis, and autophagy are seen in infant ALL (MLL-rearranged ALL) patient samples following treatment, indicating activation of related but not overlapping cell death mechanisms [102].

S63845 and AZD5991 MCL-1 has also been the target of selective inhibition through small molecule treatment, and two have demonstrated significant promise in clinical trials. S63845 is highly selective for MCL-1 and is efficacious in multiple models of lymphoma and CML, both *in vitro* and *in vivo* [103]. Similar results were also observed with a second selective MCL-1 inhibitor, AZD5991. This small molecule was tested in multiple hematological cancers and solid tumors, and had the most significant affect in hematological cancers such as multiple myeloma [104]. It was also demonstrated that in an *in vivo* model of AML, AZD5991 in combination with ABT-199 led to an even greater reduction in tumor burden compared to either compound alone [104].

III. Natural Products

Gossypol Family Gossypol is a natural phenolic pigment isolated from cottonseed. Its negative enantiomer R-(-)-gossypol, or AT-101, binds BCL-2, BCL- X_L, BCL-W, and MCL-1 and may have efficacy as an anti-cancer therapeutic [105, 106]. Like GX15-070, gossypol may not induce cell death exclusively via the intrinsic apoptotic pathway because BAX/BAK double knockout cells die following treatment [107]. Despite potential off-target effects, gossypol has shown efficacy against several hematological malignancies, including DLBCL, MM, and CLL [108, 109, 110]. Gossypol has entered several clinical trials, including a Phase II trial for small cell lung cancer, however, the results have not been promising [111]. Subsequent studies have focused on the development of small molecules derived from gossypol

in order to improve its potency and potential clinical efficacy [112, 113]. Interestingly, it has been demonstrated that gossypol induces apoptosis in polymorphonuclear leukocytes and monocytes isolated from healthy donors, suggesting a future potential for its use as an immune modulator [114].

Pre-Clinical Compounds

The promising clinical results of the aforementioned BCL-2 modulators have driven the discovery of a diverse range of small molecules and peptide therapeutics currently in clinical and pre-clinical development. New small molecules have been designed as specific inhibitors of single or multiple anti-apoptotic proteins. Single protein inhibitors will be useful for targeting malignant cells that are highly dependent on one anti-apoptotic protein with theoretically minimal off-target effects. However, malignant cells not initially killed have been shown to rapidly upregulate anti-apoptotic proteins lying outside of the primary compound's binding profile. Pan apoptotic inhibitors may have increased potency but run the risk of greater off-target cell killing. In the context of immune modulation, targeting single anti-apoptotics may be more desirable in manipulating the immune response over time and may allow for greater therapeutic dissection of specific myeloid, T, and B cell subpopulations.

In addition to small molecules, there is increasing interest in the use of peptide-based BCL-2 therapeutics that mimic the binding interface of specific BH3-only:anti-apoptotic complexes [115, 116, 117]. Isolated native BH3 helices are not attractive pharmaceutical compounds as they do not typically maintain their helical structure, are quickly degraded, and lack cellular penetrability [118]. Therefore, it is necessary to chemically modify these peptides in order to maintain their secondary structure, binding affinity, and protease resistance as has been done through chemical hydrocarbon stapling, chemical crosslinking, or peptide amphiphile/micelle incorporation [118, 119]. While BCL-2 peptides have yet to reach clinical testing, the ability to target larger surface areas of protein:protein interactions is a promising and highly specific targeting strategy.

1.1.6 BH3 mimetics as immune modulators

Most studies to date measuring the immune effects from BCL-2 modulation test ABT-737 in the context of autoimmunity or transplant tolerance and emphasize the compound's effects on lymphocytes (T and B cells). One of the first studies to examine the potential for BH3 mimetics to target the immune system found that treatment with ABT-737 induces apoptosis in lymphocytes and reduces the severity of disease in several murine models of autoimmunity [120]. Specifically, treatment with ABT-737 significantly reduces paw swelling in mice with collagen-induced arthritis and improves overall survival and renal function in mice with a systemic lupus erythematosus (SLE)-like syndrome [120, 121]. ABT-737 also suppresses immune responses to immunization against keyhole limpet hemocyanin (KLH), as T cells isolated and restimulated from ABT-737-treated mice have a significant reduction in proliferation upon re-exposure to KLH [120]. Subsequent studies on the effects of ABT-737 on normal hematopoietic compartments have shown that treatment causes a significant reduction in $CD4^+$ and $CD8^+$ T cells, B cells, and some subsets of dendritic cells, thus perhaps diminishing proper antigen presentation and T and B cell expansion [122]. In fact, treatment with ABT-737 leads to prolonged pancreatic islet allograft survival in a murine model of spontaneous diabetes and animals are able to maintain normal long-term control of blood glucose levels compared to vehicle treated controls [122]. While ABT-737 treatment alone is able to suppress allogeneic T cell responses in vitro, treatment with ABT-737 in vivo acts synergistically with cyclosporine A to reduce skin graft rejection in a MHC mismatched transplant model [123]. Interestingly, ABT-737 has also been shown to preferentially induce apoptosis in conventional T cells (Tcons) leading to a relative enrichment of immunosuppressive Tregs. This enrichment was found to slow progression in a murine model of graft-versus-host disease (GVHD) and improve overall survival following hematopoietic stem cell transplantation [124]. In addition to potential benefits in transplantation and autoimmunity, ABT-737 may be useful in the mediation of inflammatory diseases. ABT-737 induces apoptosis in T lymphocytes and lamina propria mononuclear cells in a BIM-dependent manner,

which is able to reduce levels of inflammation in spontaneous (*IL-10*$^{-/-}$) and acute models of colitis [125].

In addition to T and B cells, ABT-737 also has the potential to affect mature cells of other hematopoietic lineages. Mast cells are specialized myeloid cells that sense pathogens and initiate inflammatory responses. Their dysregulation can lead to aberrant inflammation and allergic reactions [126]. Different mast cell populations are sensitive to ABT-737 at varying dosages [127]. As expected, human and murine mast cell resistance to ABT-737 correlates with decreased BCL-2 and increased MCL-1 expression [127]. Interestingly, *in vivo* analysis indicates that while mast cells were highly sensitive to ABT-737, T cells isolated from the peritoneum of treated mice are unaffected by ABT-737. Other studies have demonstrated a marked reduction in T cells isolated from lymphoid organs and peripheral blood following ABT-737 administration, indicating that immune cell localization and tissue environment may be a critical factor in determining sensitivity to BH3 mimetics [122, 123]. These parameters should be carefully considered when testing the *in vivo* efficacy of these compounds prior to clinical translation for immune control.

Because of apparent Treg dependency on MCL-1, recent work has used GX15-070 to differentially target Tregs over Tcons. T cell sensitivity to GX15-070 appears to vary *in vitro* depending on a T cell's activation status. Mature human T cells that have undergone prolonged activation are more resistant to GX15-070 compared to lymphocytes in the early stages of activation [128]. This sensitivity profile extends to peripheral blood mononuclear cells (PBMCs) isolated from patients with ovarian cancer. *In vitro* treatment leads to significantly increased CD8$^+$:Treg and CD4$^+$:Treg ratios, indicating that GX15-070 preferentially induces apoptosis in the Treg subpopulation [128]. Depletion of Tregs while preserving Tcons is a promising therapeutic strategy for amplifying the anti-tumor immune response. These results support the finding that *in vivo* treatment with GX15-070 following vaccination leads to decreased lung metastases in a murine model of lung adenocarcinoma [129].

Although most published clinical reports using BCL-2 therapeutics have concentrated

on their antitumor effects, BH3 mimetic-induced manipulation of immune surveillance and activation could have profound ramifications for people suffering from a myriad of immunologically mediated conditions. Understanding the specific effects on leukocyte subsets of patients treated with these compounds will be paramount for their effective clinical translation. Beyond the results from testing these compounds *in vitro* and in preclinical animal models, most of what we currently know about the effects of these compounds on the human immune system must be extrapolated from oncology-based clinical trials.

Scrutinizing these studies indicate that targeted effects on the immune system include significant lymphopenias and neutropenias. Lymphopenia is a desirable effect in many cases, especially when treating hematologic malignancies like CLL. Patients with relapsed or refractory CLL treated with ABT-199 all had a >50% reduction in their absolute lymphocyte counts (ALC) and the majority had a 100% reduction. Neutropenia (grades 3 or 4) was reported in 35% of the patients with 6% of the patients experiencing serious infections and 2.5% developing autoimmune neutropenia [130]. While ABT-263 induces on-target thrombocytopenia, it also elicits neutropenia 28% of the time with many patients developing subsequent infectious sequelae [131, 132]. Similar findings have been observed in patients treated with other BH3 mimetics at antitumor dosing, including AT-101 and GX15-070 [97, 133].

Phase I clinical trials using the BCL-2 mRNA-targeting oligonucleotide oblimersen found that treatment with this compound led to global myelosuppression including neutropenia and lymphopenia [134, 135]. However, a caveat to these and other trials is that these studies included concurrent cytotoxic chemotherapy making it difficult to specifically analyze the effects of BCL-2 targeting alone on the immune system. Of note, oblimersen has been tested in pediatric patients with neuroblastoma making it the first compound targeting BCL-2 in children [69, 134, 135]. Dose escalation phase I studies evaluating the safety of the BCL-2 deoxyribonucleic acid inhibitor (DNAi) PNT2258 in patients with advanced solid tumors found rapid (within hours) decrease in lymphocytes with most patients having a >50% reduction. This phenomenon was dose dependent and used as a surrogate for efficacy [136].

Outside of the oncologic arena, BCL-2 family modulation using ABT-199 has been tested in patients with SLE. Most data remains preliminary, such as those presented at a recent American College of Rheumatology Annual Meeting (2015) demonstrating a dose-dependent reduction in total lymphocytes and B cells in particular. Neutropenia also occurred but was less consistent and correlated to different dosing thresholds (www.clinicaltrials.gov identifier NCT01686555) [137, 138]. These studies emphasize the promising immunomodulatory potential of BH3 mimetics. More extensive testing using a wider range of small molecules and peptide therapeutics with varying anti-apoptotic specificities will provide a fuller understanding of the mechanisms responsible for clinically effective immune control (Fig. 1.2).

1.1.7 Differential BCL-2 family dependency throughout T cell development and activation

After progressing from a hematopoietic stem cell to a common lymphoid progenitor, cells destined to become T cells migrate to the thymus for their continued differentiation [139]. The BCL-2 family of proteins fluctuate in regards to expression levels and overall importance throughout the multi-stage development T cells undergo. This section will focus on the anti-apoptotic proteins that dictate survival of conventional $CD4^+$ and $CD8^+$ T cells. BCL-2 proteins in Tregs will be discussed in a later section. While multiple BH3-only proteins can play a role in initiation of T cell apoptosis, BIM is considered to be the master regulator of apoptosis in T cells, and will be described in more detail in the next section.

In the thymus cells begin as $CD4^-CD8^-$ double negative cells and progress through four double negative stages: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$) [140]. Multiple anti-apoptotic proteins cooperate throughout the double negative stages of thymocyte development. MCL-1 helps maintain survival in earlier DN populations, as mice lacking MCL-1 experience a developmental blockade between the DN2 and DN3 stages [33]. Thymocytes in DN1-DN3 also have higher levels of BCL-2, and this decreases in DN4 thymocytes as cells begin to shift to a $CD4^+CD8^+$ double positive state

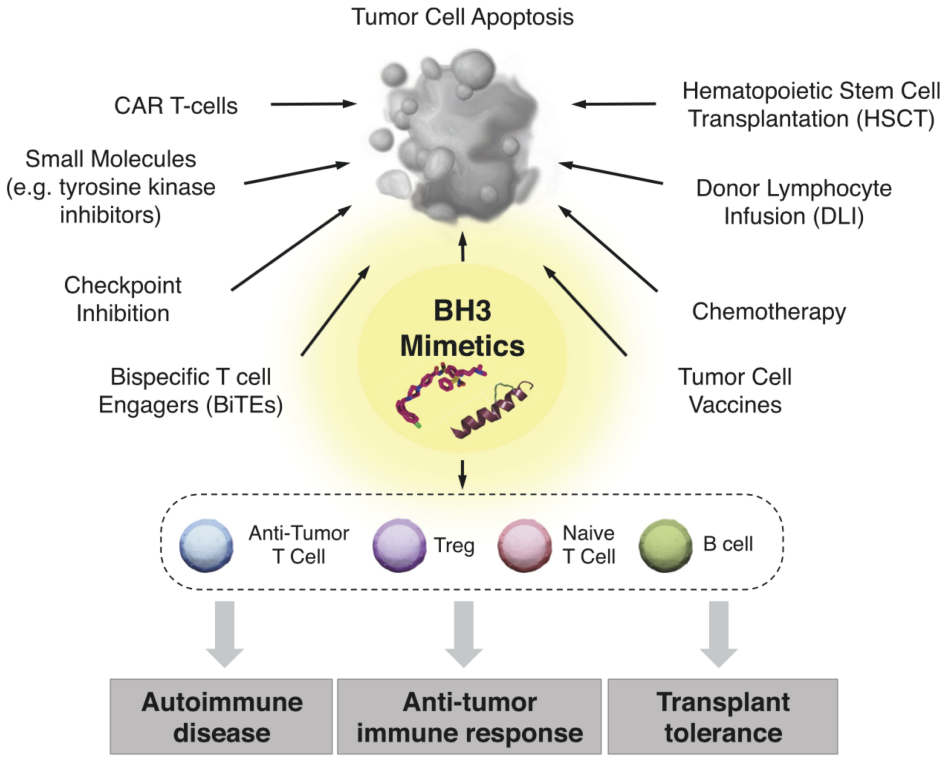

Figure 1.2: Clinical implementation of BH3 mimetics for immune modulation. In addition to their use as anticancer therapeutics, BH3 mimetics have promise for targeting specific immune cells subsets, which may provide therapeutic benefit in the context of antitumor immune responses, transplantation tolerance, and autoimmune diseases. Additionally, BH3 mimetics may be combined with both classical and cutting edge chemo- and immunotherapeutics to improve the standards of care in patients with a wide range of hematological malignancies.

[141]. Double positive thymocytes downregulate BCL-2 and upregulate BCL-X_L, and the BCL-X_L:BIM interaction is a primary regulator of negative selection, the process by which potentially autoreactive thymocytes are eliminated [39, 46]. As cells exit the double positive stage they become $CD4^+$ or $CD8^+$ single positive thymocytes and prepare to emigrate from the thymus into the periphery. Single positive thymocytes downregulate BCL-X_L and upregulate BCL-2 [141]. In addition to its role in the double negative populations, MCL-1 is also expressed at some level during all stages of thymocyte development and cooperates with the other anti-apoptotitic proteins to maintain survival [35].

Mature T cells in the periphery and lymphoid organs are naive until they encounter their specific antigen. Both naive $CD8^+$ T cells and $CD4^+$ Tcons are primarily dependent on BCL-2, as indicated by increased protein levels and sensitivity to BCL-2 inhibition [122, 142]. MCL-1 has also been implicated in naive T cell survival [33]. When T cells encounter their corresponding antigen and become activated, both $CD8^+$ T cells and $CD4^+$ Tcons upregulate multiple anti-apoptotic proteins, including MCL-1, BCL-X_L, and A1 [143, 144, 145]. BCL-2 is downregulated in activated T cells and stays at lower levels in shorter lived effector cells [146, 147]. At the end of the immune response, T cells experience a massive contraction, and approximately 90% of T cells undergo apoptosis [148]. The remaining cells are converted into memory T cells that can mount a secondary response if that specific antigen is encountered again. While short lived effector T cells downregulate BCL-2, cells destined for a memory phenotype, or memory precursor effector cells, maintain higher levels of BCL-2 [146, 149]. MCL-1, which is also reduced during T cell contraction, is re-expressed at higher levels in memory T cells as well [147]. These studies highlight the dynamic shifts in anti-apoptotic protein dependencies throughout multiple stages of T cell development, and emphasizes the importance of parsing these individual populations out when studying the effects of BCL-2 modulation in T cell subsets.

1.1.8 The role of BIM in T cells

BIM has been characterized as the most critical regulator of apoptosis in the lymphocyte compartment. There is no single deletion model of any other BH3-only protein that leads to the level of phenotypic defects observed in $Bim^{-/-}$ mice. Mice lacking BIM have an accumulation of lymphocytes that are more resistant to select apoptotic stimuli, including cytokine deprivation and ionomycin [45]. These mice have accelerated mortality, primarily due to the development of autoimmune kidney disease [45]. There are two areas in which BIM-mediated apoptosis of T cells is especially important, during thymocyte development and negative selection, and for initiation of T cell contraction and effective memory pool generation. BIM also plays a key role in the maintenance of Tregs, and this will be discussed further in the next section.

In mice lacking BIM, thymocyte development is compromised. In both global *Bim* deletion models and deletion specific to the hematopoietic compartment using a Vav-cre system, there is a reduction in the proportion of $CD4^+CD8^+$ double positive thymocytes, and an accumulation of $CD4^+$ and $CD8^+$ single positive cells, as well as $CD4^-CD8^-$ double negative cells [45, 16]. A critical checkpoint during T cell development is the process of negative selection, in which thymocytes that interact with self antigens are eliminated by the initiation of apoptosis [150]. The disruption of this process leads to escape of potentially autoreactive cells and an increased prevalence of autoimmunity. TCR ligation in double positive thymocytes causes the induction of apoptosis, however, thymocytes lacking BIM maintain normal cell numbers after ligation, and therefore do not undergo negative selection [46]. In normal cells, the initiation of negative selection increases the overall levels of BIM, as well as the interaction of BIM with BCL-X_L, the primary anti-apoptotic protein expressed in double positive thymocytes [46]. BIM loss also leads to an accumulation of double negative thymocytes that appear to be in the DN4 stage ($CD44^-CD25^-$). However, unlike normal DN4 cells, these cells express CD3 and TCRβ, indicating that they likely completed positive and negative selection, but lost both CD4 and CD8 expression and reverted back to a double

negative state [151].

BIM is also critical in culling the pool of effector T cells during the contraction of an immune response. Multiple infection models have been utilized to study the role of BIM in T cell contraction, the majority of which cause a rapid initial increase in effector cells followed by a decline back to baseline levels. In *Bim* knockout mice infected with staphylococcal enterotoxin B (SEB), T cell apoptosis after the immune response is almost entirely abrogated [152]. Similar results were also observed in models of viral infection. $CD8^+$ T cells from *Bim* knockout mice infected with herpes simplex virus (HSV-1) did not contract and were more resistant to cytokine deprivation *in vitro* [148]. Mice infected with lymphocytic choriomeningitis virus (LCMV) also did not have effective T cell contraction, and the prevalence of $CD127^{high}$ memory precursor cells was increased [153]. There is evidence that $CD127^{low}$ cells that were likely destined to undergo apoptosis can re-express CD127 and shift to a memory-like phenotype in mice lacking BIM [153]. In $CD4^+$ T cells isolated from aged mice, BIM levels decrease over time, and this leads to a longer lifespan in this T cell subset [47]. However, these cells tend to have reduced functionality, including lower levels of proliferation and reduced IL-2 production in response to antigen [47]. One caveat to this study is that these results were obtained from a global $CD4^+$ pool instead of Tcons alone. With the increased prevalence of Tregs, especially in aged *Bim* knockout mice as discussed below, it will be important to fully parse out these T cell subsets for a clear understanding of how *Bim* deletion affects functionality in $CD4^+$ Tcons. Other studies have also characterized the accumulation of CD4 responder cells with reduced function in terms of cytokine production and the ability to mount a secondary immune response [154]. Therefore, these studies point to a key role for BIM in limiting the memory pool to more potent T cells, leading to a more efficient response in the event of re-exposure to an antigen.

1.1.9 BCL-2 proteins in regulatory T cells

Regulatory T cells are key players in the suppression of T cells and preventing aberrant T cell activation and autoimmunity. The first evidence of T cells with suppressive capacity originated through characterization of mice that had undergone a thymectomy between days 2-4 after birth. These mice developed a severe autoimmune phenotype that could be rescued with the transfer of T cells from mature mice [155, 156]. It was later discovered that scurfy mice, which develop lethal autoimmunity within 3-4 weeks have a mutation in the scurfin protein, also known as FOXP3, and this transcription factor was found to be essential for the Treg lineage [157, 158, 159]. Mutations in FOXP3 were also linked to a similar disease phenotype in humans, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, or IPEX [160]. Since the discovery and characterization of the Treg population, a great deal of research has been done to elucidate the cellular mechanisms dictating their survival and function, including evaluation of the BCL-2 family proteins that Tregs are dependent on.

Like other T cell subsets, Tregs are primarily dependent on the BH3-only protein BIM as the key regulator for initiation of apoptosis. Tregs are highly dependent on IL-2, and IL-2 knockout mice lose their Treg population [161]. However, mice lacking BIM maintain normal levels of Tregs even in the absence of IL-2, demonstrating that BIM is the primary BH3-only protein that initiates the apoptotic cascade in Tregs [162]. While BIM deletion did rescue Treg survival, the Tregs had reduced functionality and were not able to prevent the development of autoimmunity [162]. Additional studies have further emphasized the importance of BIM in Treg regulation by observing Tregs in aged mice. Tregs accumulate in older mice, primarily by downregulating BIM [163]. These BIM^{low} Tregs likely accumulate due to prolonged IL-2 stimulation over time as IL-2 negatively regulates BIM expression, although additional work has also shown that other cytokines such as IL-15 and IL-6 play a role in Treg maintenance as IL-2 levels decline over time [163, 164, 165]. Of note, BCL-2 and MCL-1 levels were also found to decline in Tregs from older mice, suggesting that either

the extent of BIM reduction is more impactful on survival than the decreased levels of these two anti-apoptotic proteins, or that other anti-apoptotic proteins such as BCL-X_L or A1 are more critical for maintaining Treg survival in aged mice [163].

In regards to the anti-apoptotic proteins, initial studies demonstrated that BCL-2 was a sufficient promoter of Treg survival, and this was countered by BIM. Both BCL-2 over-expressing and *Bim* knockout mice have an accumulation of Tregs [166]. However, these Tregs downregulated other canonical Treg markers and had reduced function in an *ex vivo* suppression model and in an *in vivo* model of colitis. One caveat to note in regards to the loss of function observed in these Tregs was the use of FOXP3-GFP mice, in which GFP is directly conjugated to the FOXP3 protein. There is evidence that this direct conjugation leads to reduced Treg function, due to hindrance of FOXP3 activity, which may have partially led to the observed reduction in FOXP3 target genes [167]. A mouse model using a bicistronic system with a fluorescent reporter not conjugated directly to the protein has been generated and provides a more biologically relevant readout for the study of Treg function [168]. Additionally, while the BCL-2 overexpressing transgenic mouse indicated that BCL-2 was sufficient in prolonging Treg survival, it did not demonstrate that BCL-2 was necessary for maintenance of the Treg population. In fact, the generation of Treg-specific deletion models of BCL-2 and BCL-X_L demonstrated that both of these proteins were dispensable for Treg homeostasis [36]. However, loss of MCL-1 in Tregs led to reduced Treg numbers and the development of autoimmunity similar to that characterized in the scurfy mouse [36]. And while BCL-X_L was not shown to be necessary for Tregs at a level of general homeostasis, BCL-X_L is upregulated in Tregs that played a key role in inducing tolerance and preventing the development of disease in a murine model of systemic lupus erythematosus [169]. It is also important to consider the context that BCL-2 family levels in Tregs are being evaluated in, as different activation states and microenvironments are known to shift which BCL-2 proteins are most relevant in both murine and human Treg subsets. For example, BCL-X_L is transiently upregulated in human Tregs undergoing activation, which may be a

contributing factor to their increased fitness compared to naive Tregs [170]. And in a setting of chronic inflammation, it has been demonstrated that Tregs isolated from the sinovial fluid of patients with rheumatoid arthritis are more resistant to apoptosis than conventional T cells and have higher levels of BCL-2 [171]. To address the dynamic regulation of Tregs, Tcons and $CD8^+$ T cells described above, we aimed to evaluate BCL-2 family protein levels and dependency patterns in multiple states of activation. We utilize *ex vivo* expansion, *in vivo* treatment of a 'steady state' immune system, and congenic transplant models to more comprehensively determine how T cells adapt to manipulation of the BCL-2 family.

1.2 Significance

1.2.1 Modulating the immune system to improve transplant outcomes

Hematopoietic stem cell transplant is a critical therapeutic avenue for many hematologic malignancies, but a major obstacle that needs to be addressed in both hematopoietic and solid organ transplantation is the complication of aberrant immune responses. If the recipient immune system attacks a transplanted organ this can lead to organ rejection, conversely in hematopoietic stem cell transplants donor immune cells can recognize host organs as non-self and mount an immune response against them, leading to graft versus host disease. The current standard of care typically involves nonspecific immune suppression with drugs such as cyclosporin A, however this can lead to increased risk of opportunistic infection. Therefore, improving transplant tolerance while maintaining a robust immune response to foreign antigens is an imperative area of research. Through the use of BH3 mimetic treatment, we hypothesize that we can selectively target distinct immune cell subsets in order to improve transplant outcomes, due to the unique BCL-2 dependency patterns distinguishing Tregs from effector T cells.

One of the most promising therapeutic strategies to date is the infusion of immunosuppressive regulatory T cells. The infusion of Tregs in transplants has been shown to improve

tolerance and limit the development of GVHD. Early reports demonstrated that the addition of $CD4^+CD25^+$ T cells could prevent the development of GVHD in a C57BL/6 to BALB/c transplant model, but only in the treatment group where the Treg:Tcon ratio was 1:1 [172]. As Tregs are a relatively small proportion of Tcons and higher Treg:Tcon ratios were necessary for therapeutic benefit, *ex vivo* expansion of Tregs before transplant became a promising avenue to explore [173]. In contrast, depletion of $CD25^+$ CD4 cells exacerbated the development of GVHD in MHC mismatch models and transplanted SCID mice [173]. The addition of Tregs has also improved tolerance in the context of solid organ transplantation, delaying or even preventing the rejection of heart allografts in a heterotopic heart transplant model and skin in a humanized mouse model of skin grafting [174, 175, 176]. A key question for hematopoietic stem cell transplantation in the context of cancer therapy is whether or not the addition of a large number of immunosuppressive cells will hinder the graft versus tumor or graft versus leukemia response. Several studies have demonstrated that Treg enrichment does not prevent T cell mediated destruction of leukemia or lymphoma cells [177]. Even if Tregs were infused two days prior to bone marrow transplantation, the increased expansion time in this setting did not impair the graft versus leukemia response [178]. However, while these results are promising in regards to maintaining anti-tumor immunity with Treg infusion, there are some studies that call this into question. In a model of acute myeloid leukemia, an enrichment of Tregs led to decreased performance of cytotoxic T lymphocytes and poorer disease outcome [179]. Therefore the balance of Tregs to effector cells will have to be carefully considered in order to gain the benefit of preventing GVHD while maintaining graft versus leukemia responses.

The preclinical success of Tregs in reducing the severity of GVHD has led to the implementation of Tregs in clinical studies. Initial results have shown that Treg infusion in patients does not affect immune reconstitution and can prevent the development of acute GVHD [180]. Tregs isolated and expanded from umbilical cord blood were also effective at ameliorating symptoms of aGVHD, and addition of Tregs to the transplant regimen led to

accelerated reconstitution of total $CD4^+$ T cells [181]. A prospective analysis of immune reconstitution comparing T cell subsets in patients who developed chronic GVHD versus those that did not revealed that patients with disease had lower proportions of naive and stem cell memory Tregs compared to patients that did not develop cGVHD [182]. BCL-2 levels were unchanged in these populations suggesting that decreased survival is not the cause of lower Treg numbers, however, they did not evaluate levels of other anti-apoptotic proteins that are canonically more relevant to Treg survival [36, 170]. Due to promising initial results and prospective trials emphasizing the importance of Tregs in the context of transplantation, there has been increasing interest in improving the isolation and expansion process for Tregs to improve their survival and suppressive capacity [183, 184]. Low dose IL-2 leads to increased proliferation, higher levels of BCL-2, and increased resistance to apoptosis preferentially in Tregs compared to Tcons. There is also interest in bypassing the use of traditional calcineurin inhibitors such as cyclosporin because, while they are beneficial for general immune suppression, they have unfavorable effects on Tregs [185]. To circumvent this, the use of siroliumus, or rapamycin, in combination with OX40L blockade, a costimulatory molecule more prevalent to Tcons than Tregs, led to reduced Tcon expansion without effecting Tregs and reduced symptoms of aGVHD in a non-human primate model [186]. Sirolumus and tacrolimus have also been used in combination with freshly isolated Tregs in a small (n=7) clinical study, and none of the patients developed symptoms of acute or chronic GVHD [187].

Manipulation of the BCL-2 family of proteins in Tregs in order to prolong their survival in the context of HSCT [188] is an attractive therapeutic strategy. Treatment of mice post-transplant with ABT-737 and cyclosporin A led to a Treg-mediated delay in the development of aGVHD, as subsequent Treg depletion led to rapid onset of GVHD [124]. Transient upregulation of anti-apoptotic proteins such as BCL-2 or BCL-X_L with adenoviral vectors or Tat-fusion proteins leads to improved survival and expansion of hematopoietic stem cells, but this has not been evaluated in Tregs specifically [189, 190]. Another emerging area

of investigation is the presence of $CD8^+FOXP3^+$ Tregs, cells that appear to be a small population of induced Tregs [191]. These cells are less effective at reducing GVHD compared to $CD4^+$ Tregs, primarily due to their high levels of BIM, and BIM deletion led to improved prevention of GVHD by the $CD8^+$Treg population [192]. As we learn more about BCL-2 proteins in Tregs, targeting them therapeutically will be a promising strategy to improve Treg survival in the transplant setting. We were therefore interested in assessing how BCL-2 proteins in T cell subests are affected by BCL-2 targeting in a more in-depth manner, specifically in the context of T cell development in a congenic transplant model. By gaining a clear understanding of the BCL-2 regulation in this setting, we aim to translate our findings to improve therapeutic strategies for transplant tolerance and the amelioration of graft versus host disease.

1.2.2 Long-term BH3 mimetic treatment in cancer patients and following stem cell transplant

As BH3 mimetics become more widely used, there is a critical need to understand what the effects of long-term pressure to the BCL-2 family of proteins does in the context of, not only resistance in malignant cells, but also in terms of potentially reprogramming heathy cells susceptible to BCL-2 family targeting, such as immune cells. Many of the therapy regimens for BH3 mimetics will likely require their long-term use, and it is therefore imperative to understand how BH3 mimetic pressure over time modulates the BCL-2 family. This will improve our understanding of emerging resistance patterns and may allow us to intentionally modulate BCL-2 dependency patterns as part of new therapeutic strategies.

Most of what we understand about intrinsic and acquired resistance to BH3 mimetics is in the context of malignant cells. Many studies have been performed to elucidate baseline or cell-intrinsic sensitivities to BH3 mimetics, based on the initial expression patterns of BCL-2 proteins. The most common predictor of intrinsic ABT-737 or ABT-199 resistance is high expression of anti-apoptotic proteins that are not targeted by these compounds. In AML,

cells with high levels of MCL-1 or BCL-X_L are more resistant to ABT-737 or ABT-199 treatment respectively [75, 193]. BCL-2 phosphorylation can also lead to reduced potency of ABT-737 in AML cells [75]. Combined inhibition of MCL-1 or BCL-X_L using small molecules A-1210477 or A-1155463 respectively, could restore apoptotic sensitivity [193]. As the development of specific BH3 mimetics has continued increase, they have been utilized as a 'molecular toolkit' in order to assess initial sensitivity patterns. BH3 mimetics specific for BCL-2, BCL-X_L, and MCL-1 have been used to ascertain sensitivity to treatments in multiple cancer cell types, including multiple myeloma, AML, non-small cell lung cancer, and breast cancer [194, 195]. This strategy continues to improve as more potent inhibitors for the anti-apoptotic proteins are developed. For example, the development of a more potent MCL-1 inhibitor, S63845, has led to improved synergy with ABT-199 and this combination significantly delays tumor progression in patient derived xenografts of mantel cell lymphoma [196]. However, even with the combined inhibition of BCL-2 and MCL-1, tumors eventually began to increase in size, suggesting the emergence of a new resistance mechanism over time [196]. In healthy T cells little is known about the intrinsic resistance patterns of BCL-2 inhibition. Antigen-activated T cells become completely resistant to ABT-737, primarily through upregulation of A1 [44]. Because A1 upregulation in activated T cells occurs through the NFAT pathway, inhibition of NFAT by the calcinuerin inhibitor cyclosporin A was able to prevent resistance in this context [44]. T cells also become significantly more resistant to treatment with the small molecule ABT-737 if BIM is absent, again highlighting the critical role of BIM in mediating T cell apoptosis [142]. Based on deletion and transgenic overexpression models, other predictions can be made in terms of which T cell subsets are most likely to be sensitive to selective BCL-2 family inhibition, but extensive characterization of BH3 mimetic sensitivity patterns in discrete T cell subsets is an area of research that merits further investigation.

In addition to intrinsic sensitivity to BH3 mimetics, an emerging area of research is the evaluation of acquired resistance patterns, primarily in response to treatment of malignant

cells with ABT-737 or ABT-199. Most acquired resistance occurs through the upregulation of additional anti-apoptotic proteins. In lymphoma cell lines that were treated with ABT-737 long term to generate resistance, there was significant upregulation of MCL-1 or A1 and BIM was sequestered by these proteins to prevent apoptosis [85]. In this context they found increased transcription of MCL-1 and A1, but other studies have demonstrated that MCL-1 especially can also be modified at the protein level. In AML cells treated with ABT-199, MCL-1 had reduced transcription, but increased protein levels and a longer half-life [197]. Downregulation of MCL-1 from the use of DNA damaging agents sensitized cells to ABT-199, although the mechanism of how this occurs was not fully explored [197]. It is possible that the induction of DNA damage led to p53 activation and subsequent upregulation of NOXA, which can bind to MCL-1 and cause its ubiquitination and degredation [198]. The upregulation of MCL-1 to sequester BIM is a relatively common acquired resistance mechanism, and it has also been demonstrated in neuroblastoma cell lines and multiple myeloma [199, 200]. In neuroblastoma, cells with amplification of MYCN were found to be highly sensitive to ABT-199, due to MYCN-mediated upregulation of NOXA, which binds MCL-1 and inhibits the formation of MCL-1:BIM complexes in the context of ABT-199 treatment [201]. Targeting p53 can also be synergistic with ABT-199 due to p53 mediated degredation of MCL-1 through MCL-1 phosphorylation independent of NOXA upregulation [202]. With the development of more potent MCL-1 inhibitors there has been increased interest in using these compounds to mitigate the emergence of resistance to ABT-199 as well [203]. BCL-X_L upregulation has also been demonstrated to contribute to ABT-199 resistance that could be overcome with the use of BCL-X_L specific small molecules [200]. In double hit lymphoma, ABT-199 treatment eventually leads to resistance mediated by BFL-1 upregulation that can be overcome by the use of a BET bromodomain inhibitor that downregulates BFL-1 levels [204].

Besides upregulation of additional anti-apoptotic proteins, there are several other mechanisms of acquired resistance that may not be targetable by the combination of multiple BH3 mimetics. In resistant lymphoma lines, missense mutations were found in the BCL-2 BH3

binding pocket, leading to an inability of ABT-199 to effectively bind and inhibit BCL-2 protein interactions [205]. This study also characterized a missense mutation in BAX that prevented it from inserting into the mitochondrial membrane. Mutations in downstream pro-apoptotic proteins may present a particularly difficult challenge, due to impairment of the entire intrinsic apoptotic pathway, which many drugs are reliant on. In another model of lymphoma resistance, it was revealed that prolonged treatment of ABT-199 led to the selection of lymphoma cells that had lost the BCL-2 amplicon [206]. Combination treatment of ABT-199 with CDK7, which regulates RNA polymerase II, prevented ABT-199 resistance by suppressing transcriptional reprogramming and preserving the BCL-2 amplicon [206]. There is also evidence that extrinsic signals from the tumor microenvironment can lead to modified expression of anti-apoptotic proteins in malignant cells. CLL cells can acquire resistance to ABT-199 due to OX40 mediated stimulation via surrounding T cells in the tumor microenvironment, leading to upregulation of other anti-apoptotic proteins [207]. Treatment with receptor tyrosine kinase inhibitors resensitized cancer cells to ABT-199 in this context [207, 208]. In follicular lymphoma, resistance has been characterized, not by upregulation of anti-apoptotic proteins, but by almost complete loss of BIM, leading to an inability of these cells to initiate the apoptotic cascade [209].

Overall, both intrinsic and acquired resistance to BH3 mimetic treatment in the context of cancer is incredibly variable. For many malignancies there is no universal resistance mechanism, and apoptotic sensitivity can be quite variable even across individual cancer cells in a heterogeneous tumor [210, 211]. We predict that in healthy cells the mechanisms of evolving sensitivity to long-term BH3 mimetic treatment will be more definitive and uniform, however there is still a large amount of redundancy and dynamism one must take into consideration when manipulating the BCL-2 family of proteins. In order to drive the use of BH3 mimetics forward in the context of immune modulation, it will be critical to extensively evaluate how the BCL-2 proteins and overall immune cell repertoire adapt in response to BH3 mimetic treatment. The research we have performed in this study aims to

create a framework in which we will be able to dynamically evaluate changing sensitivity patterns in T cell subsets in response to long-term BH3 mimetic treatment, which will lead to an improvement in our ability to make clinical decisions regarding manipulation of the BCL-2 proteins in multiple disease settings.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

CD19^{CRE} Bim$^{fl/fl}$, LCK^{CRE} Bim$^{fl/fl}$, and LCK^{CREneg} (referred to hereafter as WT) mice were generated as described previously [27, 212, 213]. LCK^{CRE} Bim$^{fl/fl}$ mice were kind gifts from Loren Walensky, M.D., Ph.D., Dana-Farber Cancer Institute, Boston, MA and were generated through cross of LCK^{CRE} mice with Bim$^{fl/fl}$ animals [27, 213, 33]. FOXP3-IRES-GFP mice were gifts from Maciej Lesniak, M.D. CD45.1 C57/BL6 and WT (CD45.2) C57/BL6 mice were purchased from The Jackson Labs. Complete blood counts were obtained from peripheral blood using the ADVIA 120. All animal experiments were approved by and performed in accordance with the guidelines and regulations set forth by the Institutional Animal Care and Use Committee of the University of Chicago.

2.2 Fluorescence Activated Cell Sorting

Splenocytes and thymocytes were isolated from animals and single cell suspensions were generated by disruption of the spleen and thymus through 100μm and 70μm filters respectively as previously described[214]. Individual lymphocyte populations were sorted on a BD FACSAria (University of Chicago Flow Cytometry Core) using FOXP3-GFP, CD25-APC, 1:100 (BD), CD4-APC, 1:100 (BD) or CD4-PE, 1:100 (BD) and CD8-FITC, 1:100 (BD). Sorted populations were $\geq$98% pure.

2.3 Flow Cytometric Analysis

Single cell suspensions of splenocytes and thymocytes were prepared as described above. The following antibodies were used for staining at a dilution of 1:100 in PBS (All antibodies were from BD unless otherwise indicated): CD44-PE-Cy7, CD62L-PE, CD25-PerCPCy5.5, CD4-

APC, CD8-BUV395 and CD8-FITC. For intracellular FOXP3 and BCL-2 family staining, cells were fixed and permeabilized with the FOXP3 Fixation/Permeabilization kit (eBioscience) per the manufacturer's protocol. After fixation and permeabilization, cells were stained with FOXP3-FITC (eBioscience), BIM-PE (Cell Signaling Technology), BCL-X_L-PE (Cell Signaling Technology), MCL-1-PE (Cell Signaling Technology) at 1:50 in permeabilization buffer for 45 minutes in the dark on ice. BCL-2-PE (BD) was pre-diluted and used according to manufacturers instructions. Samples were analyzed using a LSRII cytometer (BD) or FACSAria (BD). Data analysis was performed using FlowJo (Tree Star).

2.4 Viability Assays

$CD4^+$ splenocytes, $CD8^+$ splenocytes, and $CD4^+CD8^+$ thymocytes were plated in 96 well round bottom plates at a density of 50,000 cells/well. Cells were cultured in cDMEM (DMEM with 10% fetal bovine serum, 1% Penicillin/Streptomycin, 1% L-glutamine, 1% non-essential amino acids, 1% HEPES and 10μM β-mercaptoethanol). Cells were treated with: cDMEM alone (cytokine deprivation), 1μg/mL ionomycin, 4ng/mL PMA, or 1μM etoposide. Cells were incubated for 24 and 48 hours, at 37°C, 5% CO_2. At the indicated timepoints, viability was assessed by staining cells with Annexin V-FITC (BD) and propidium iodide (Life Technologies) and analyzed via flow cytometry, with percent viable cells reported as Annexin V^{neg} PI^{neg} as previously reported [215].

2.5 qRT-PCR

Pure populations of $CD4^+$ splenocytes, $CD8^+$ splenocytes and $CD4^+CD8^+$ thymocytes were lysed with Trizol (Life Technologies) and mRNA extracted using the Direct-zol RNA MiniPrep kit (Zymo). mRNA concentration and purity was assessed using a DeNovix DS-11 spectrophotometer and reverse transcription was performed using the Superscript III first strand synthesis reverse transcription kit (Invitrogen) per the manufacturer's guidelines.

qRT-PCR was performed using TaqMan Master Mix and Gene Expression Probes (Applied Biosystems) for each of the BCL-2 family members as follows: Mcl-1: Mm00725832_s1; Bak1: Mm00432045_m1; Bcl2l11 (Bim): Mm00437796_m1; Bcl2a1a (Bfl-1/A1): Mm03646861_mH; Bbc3 (Puma): Mm00519268_m1; Pmaip1 (Noxa): Mm00451763_m1; Bad: Mm00432042_m1; Bid: Mm00477631_m1; Bcl-2: Mm00477631_m1; Bcl2l1 (Bcl-xL): Mm00437783_m1; Bcl2l2 (Bcl-w): Mm03053297_s1; Bax: Mm00432051_m1; Bmf: Mm00506773_m1; B2m: Mm00437762_m1. Samples were run on the 7500 Fast Real-Time PCR System (Applied Biosystems). Data was analyzed with the ExpressionSuite software using the $\Delta\Delta$CT method with B2m as the housekeeping gene and age-matched LCK^{CREneg} Bim$^{fl/fl}$ mice for the reference samples.

2.6 BH3 Profiling

BH3 profiling was performed as described previously [216, 217]. Briefly, cells were stained for CD4 and CD8 for 30 minutes, washed, and incubated with 75μM total peptide (NOXA + BAD comprised 37.5 μM each) with 20μg/mL oligomycin (Sigma) and 0.002% digitonin for 60 minutes. DMSO and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma) were used as negative and positive controls respectively. Cells were stained for an additional 30 minutes with 450nM rhodamine123 (Sigma) and analyzed via flow cytometry. % Depolarization caused by each BH3-peptide was calculated using the following formula: % Depolarization = (1-(sample-FCCP)/(DMSO-FCCP)) x 100.

2.7 T cell stimulation and expansion

Pure populations of CD8$^+$ T cells, CD4$^+$ Tcons, and CD4$^+$FOXP3$^+$ Tregs were cultured in cDMEM with the addition HEPES and gentamicin. For the stimulation and expansion of CD8$^+$ T cells and Tcons media was supplemented with CD3/CD28 Dynabeads (Life Technologies) at a 1:1 bead:cell ratio and 500U/mL of clinical grade IL-2. For Tregs, the media was supplemented with CD3/CD28 Dynabeads at a 3.5:1 bead:cell ratio and 2000U/mL IL-

2. Cells were expanded for 3-5 days before treating with DMSO or ABT-199 daily for five days.

2.8 ABT-199

ABT-199 was reconstituted to a concentration of 10mM in DMSO and further diluted with cDMEM to 500nM for *in vitro* treatment experiments. For *in vivo* treatment, ABT-199 was reconstituted using 10% ethanol, 30% PEG 400, and 60% Phosal and was administered via oral gavage at doses of 25mg/kg, 50mg/kg, or 100mg/kg.

2.9 Congenic transplant

CD45.1$^+$ mice were irradiated using a Cesium-137 gamma source at a fractionated dose of 550cGy (1100cGy total). 24 hours after irradiation, mice were transplanted with donor cells from CD45.2$^+$ donor mice. Bone marrow was isolated and T cell depleted (TCD) using a MACS column and CD3 microbeads (Miltenyi). Mice received 2x10^6 TCD bone marrow cells via retro-orbital injection.

2.10 Statistical Analysis

A one-way ANOVA test was used to evaluate statistically significant changes in young, middle, and old mice. For all other comparisons, an unpaired student t-test was performed and data are expressed as the means ± SEM. Plots were created using Prism (GraphPad Software). Expression data was depicted by RQ Min/Max with a 95% confidence level using the ExpressionSuite Software (Thermo Fisher/Life Technologies). Statistical significance was defined as *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

CHAPTER 3

LOSS OF BIM IN T CELLS RESULTS IN BH3-ONLY COMPENSATION BUT INCOMPLETE NORMALIZATION OF CELL DEATH SENSITIVITY

3.1 Introduction

Of the BH3-only proteins, BIM plays the most fundamental role in apoptotic regulation of the immune system, particularly with respect to T and B cells [218, 139, 10, 219, 220]. Young mice with global *Bim* deletion have increased white blood cell counts (WBC), abnormal thymopoiesis, and lymphocytes that are resistant to a wide range of apoptotic stimuli [45, 46]. By one year of age, over 50% of *Bim* null mice become moribund, typically due to B cell-mediated autoimmunity [45]. Targeted loss of BIM within the entire hematopoietic compartment recapitulates this phenotype, emphasizing the importance of BIM in immune stability and pointing to a lymphocyte-intrinsic mechanism by which BIM regulates immune cell ontogeny, homeostasis, and apoptotic resistance [16, 34].

Given the central importance of BIM in immune cell homeostasis, genetic ablation of *Bim* in specific lymphocyte subsets can provide a mechanistic framework to better understand how BCL-2 family compensation occurs in non-malignant lymphocytes, as what might occur in patients treated with BH3-mimetics [221, 222]. To evaluate if long-term deletion of *Bim* in T cells recapitulates the early phenotype measured in globally deleted animals we characterized the peripheral lymphoid compartment in aged mice harboring *Bim*-deficient T or B cells. Unlike $CD19^{CRE}$ $Bim^{fl/fl}$ mice, LCK^{CRE} $Bim^{fl/fl}$ mice experienced early lymphocytosis that normalized over time. We examined several potential mechanisms that may be responsible for this normalization, including global phenotypic changes in the T cell repertoire, apoptotic resistance patterns, and quantitative mRNA expression of the BCL-2 family of proteins. We found a consistent pattern of BH3-only mRNA upregulation, par-

ticularly of *Bmf*, in $CD4^+$ and $CD8^+$ T cells lacking Bim. Despite similarities in mRNA expression patterns, $CD4^+$ and $CD8^+$ T cells had differences in cell death sensitivities to apoptotic stimuli, suggesting divergent compensatory mechanisms. However there were no changes in anti-apoptotic BCL-2 dependence patterns between the two cell types. Our studies shed light on the importance of BH3-only proteins in T cell homeostasis and emphasizes the importance of BIM in T cell apoptotic control. This study also highlights the difference between qualitative versus quantitative BCL-2 family compensatory mechanisms in T cells, which may prove critical to the mechanistic understanding of normal immune cell sensitivities in patients treated with BH3 mimetics [18].

3.2 Results

3.2.1 Loss of BIM in T cells results in lymphocytosis that normalizes over time.

Previous work has confirmed BIM as a master BH3-only regulator of T and B cell homeostasis [45, 16]. To explore how loss of BIM affects the lymphocyte compartment during the natural lifespan, we analyzed the consequences of T and B cell-specific deletions of BIM in LCK^{CRE} and $CD19^{CRE}Bim^{fl/fl}$ animals, respectively. Complete blood counts (CBCs) from the peripheral blood of these animals were analyzed in young (6-12 wks), middle-aged (15-39 wks) and old-aged (>40 wks) mice. Loss of BIM in T cells (LCK^{CRE} $Bim^{fl/fl}$) resulted in a significant elevation of total white blood cells in young mice as previously described [45, 16]. However, this lymphocytosis normalized to levels equivalent to wild type (WT) mice by middle age and was maintained past 40 weeks of life (Fig. 3.1a). In contrast, $CD19^{CRE}$ $Bim^{fl/fl}$ mice developed a lower level of lymphocytosis when young, as previously reported, that remained relatively unchanged through old age (Fig. 3.1b) [212]. The changes in peripheral blood lymphocyte counts in LCK^{CRE} $Bim^{fl/fl}$ mice were also reflected in the absolute cell number from spleens and thymi indicating that the changes measured

were not isolated to peripherally circulating T cells (Fig. 3.1c-d). These data point to a BIM-independent compensatory mechanism responsible for lymphocyte normalization in LCK^{CRE} $Bim^{fl/fl}$ mice over time.

3.2.2 Thymocyte development remains abnormal in LCK^{CRE} $Bim^{fl/fl}$ mice as they age.

Normal thymocyte development is impaired in young mice with either global or hematopoietic cell-specific Bim deletion [45, 46, 16, 151]. Such animals harbor low numbers of $CD4^+CD8^+$ double positive (DP) cells and increased numbers of double negative (DN) and single positive (SP) $CD4^+$ and $CD8^+$ T cells [16, 151]. To determine if T cell contraction was due to changes in thymopoiesis in LCK^{CRE} $Bim^{fl/fl}$ animals, CD4 and CD8 expression was analyzed on thymocytes from young, middle-aged, and elderly mice. Thymocyte development remained abnormal in aged LCK^{CRE} $Bim^{fl/fl}$ mice, as reflected by reduced DP thymocytes and increased DN and SP cells, equivalent to what was observed in young mice (Fig. 3.2a-b). These proportions remained stable through all age groups (Fig. 3.2c-f). The overall decline in absolute thymocyte numbers in both WT and LCK^{CRE} $Bim^{fl/fl}$ mice was likely due to age-dependent thymic involution [223]. Our results are consistent with previous data showing that young native $Bim^{-/-}$ mice, aged 3-4 weeks, have larger thymi than wild-type animals but have similar cellularity to wild type animals by 8-12 weeks of age and a higher content of DN and SP cells $CD4^+$, and SP $CD8^+$ T cells [45, 151, 20]. Therefore, normalization of thymopoiesis does not appear to be responsible for peripheral WBC normalization in LCK^{CRE} $Bim^{fl/fl}$ mice as animals age.

3.2.3 Mature T cell numbers normalize in aged LCK^{CRE} $Bim^{fl/fl}$ mice.

Because there were no significant changes in thymopoiesis in aged $LCK^{CRE}Bim^{fl/fl}$ mice, we next turned our analysis to determine if splenic T cell numbers normalized over time

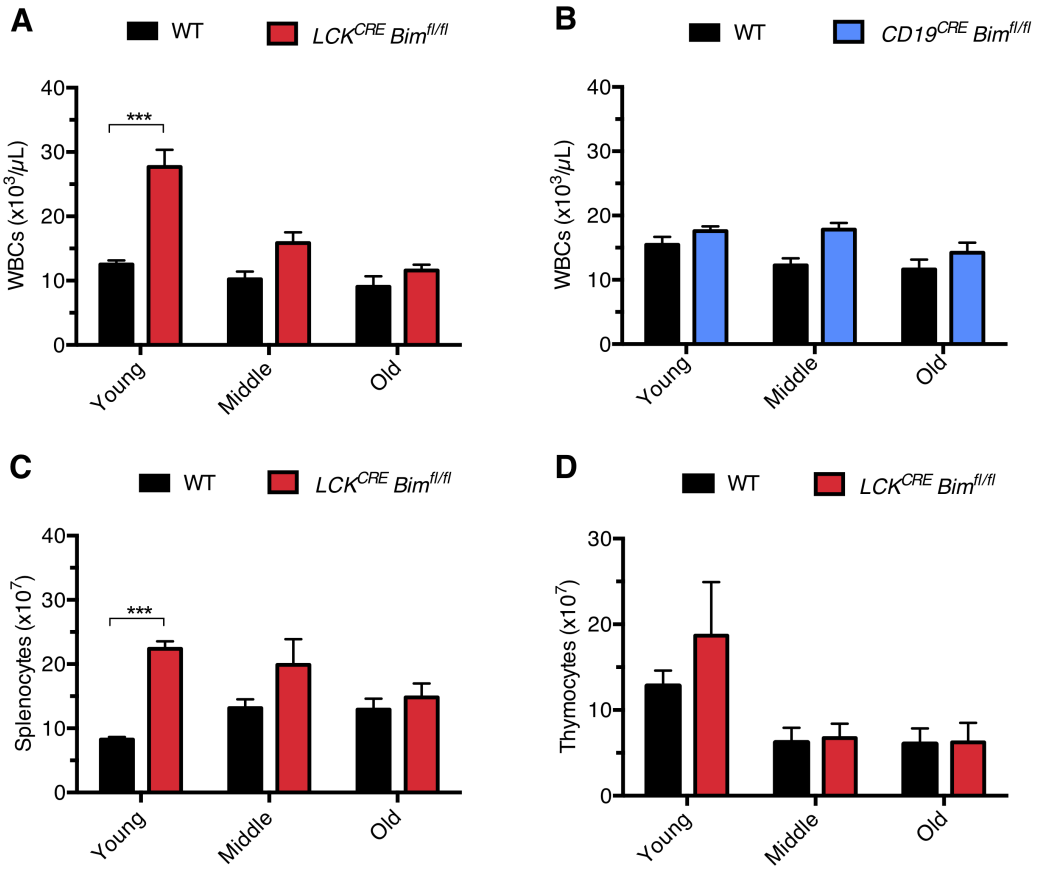

Figure 3.1: T cell-specific deletion of Bim leads to lymphocytosis early in life that normalizes as mice age. (a) CBC analysis was performed on peripheral blood from WT and $LCK^{CRE}\,Bim^{fl/fl}$ and (b) $CD19^{CREneg}$ and $CD19^{CREpos}$ (referred to hereafter as $CD19^{CRE}$) $Bim^{fl/fl}$ mice at the indicated ages. The peripheral blood lymphocytosis and normalization was also reflected in absolute cell numbers of (c) splenocytes and (d) thymocytes isolated from LCK^{CRE} $Bim^{fl/fl}$ animals. Young: 6-12 weeks of age, Middle: 15-39 weeks of age, Old: $\geq$40 weeks of age. LCK^{CRE} $Bim^{fl/fl}$ n$\geq$14, $CD19^{CRE}$ $Bim^{fl/fl}$ n$\geq$22, and WT n$\geq$5 for each age group. Data represented as means $\pm$SEM. *p$<$0.05, ** p$<$0.01, *** p$<$0.001.

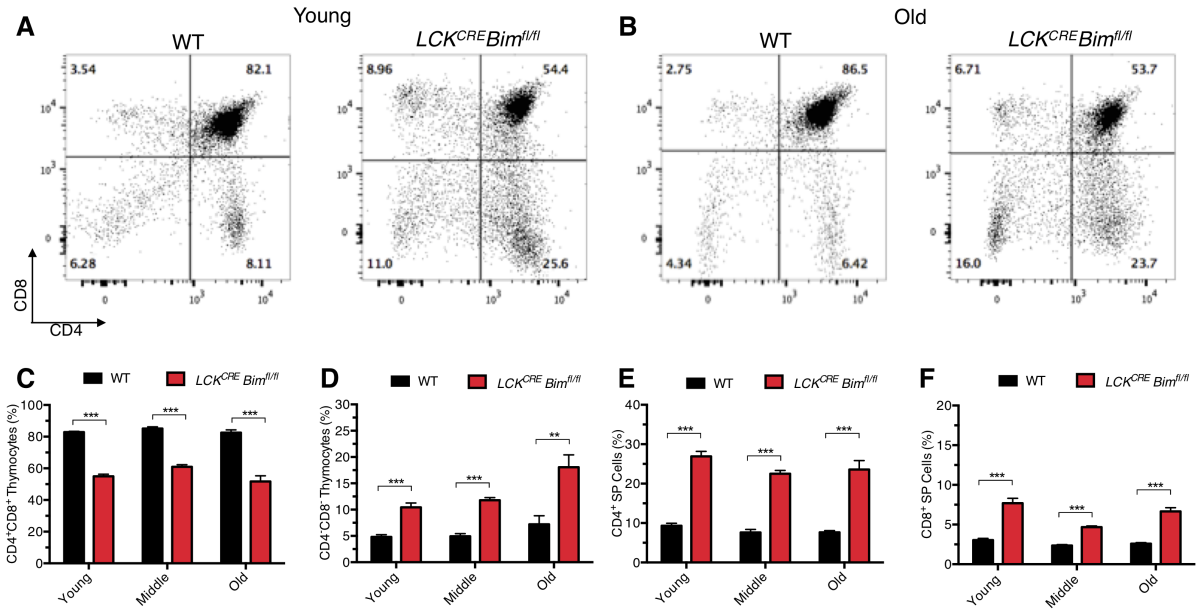

Figure 3.2: Thymocyte development remains abnormal as LCK^{CRE} $Bim^{fl/fl}$ mice age. Thymocytes were analyzed from WT and LCK^{CRE} $Bim^{fl/fl}$ mice at the indicated age groups. Representative flow plots of (a) young WT and LCK^{CRE} $Bim^{fl/fl}$ mice, as well as (b) old WT and LCK^{CRE} $Bim^{fl/fl}$ mice. (c) the percentage of DP $CD4^+CD8^+$, (d) DN $CD4^-CD8^-$, (e) SP $CD4^+$, (f) and SP $CD8^+$ cells. LCK^{CRE} $Bim^{fl/fl}$ and WT n$\geq$4 for each age group. Data represented as means $\pm$SEM. *p<0.05, ** p<0.01, *** p<0.001.

instead. The proportions of $CD4^+$ and $CD8^+$ T cells were lower in LCK^{CRE} $Bim^{fl/fl}$ mice compared to age-matched WT controls until they reached old age, at which point the proportions equalized (Fig. 3.3a-b). Despite lower proportions of T cells in young LCK^{CRE} $Bim^{fl/fl}$ animals, there were significantly higher absolute numbers of $CD4^+$ and $CD8^+$ T cells that normalized by middle age (Fig. 3.3c-d). These findings are consistent with the peripheral blood data from these animals (Fig. 3.1). Naïve T cells ($CD44^{low}CD62L^{high}$) decreased over time to a greater extent in LCK^{CRE} $Bim^{fl/fl}$ mice compared to WT mice

(Fig. 3.4a). Conversely, LCK^{CRE} $Bim^{fl/fl}$ mice accumulated more $CD4^+$ effector/memory T cells ($CD44^{high}CD62L^{low}$) as has been previously described in animals with global Bim deficiency (Fig. 3.4b) [20]. Like naïve $CD4^+$ T cells, naïve $CD8^+$ T cells decreased over time to a greater extent in LCK^{CRE} $Bim^{fl/fl}$ mice compared to WT mice (Fig. 3.4c). However unlike $CD4^+$ T cells, effector/memory $CD8^+$ cell percentages and absolute numbers were equivalent in all age groups (Fig. 3.4d). In contrast to conventional T cells, $CD4^+FOXP3^+$ T cells increased in proportion in both groups but more so in LCK^{CRE} $Bim^{fl/fl}$ mice. While Treg absolute numbers were elevated in LCK^{CRE} $Bim^{fl/fl}$ mice, their absolute numbers remained stable throughout aging (Fig. 3.4e). These data support previous studies demonstrating the importance of BIM in Treg ontogeny and the normal accumulation of Tregs as mice age [163, 224, 166]. Decreased levels of BIM in old versus young WT Tregs allows them to naturally accumulate and preferentially survive in older animals though chronic stimulation with IL-2 [163]. Taken together, $LCK^{CRE}Bim^{fl/fl}$ had higher total $CD4^+$ and $CD8^+$ cells than WT controls when young that normalized as they aged. As the overall numbers of these cells normalized, LCK^{CRE} $Bim^{fl/fl}$ mice accumulated memory $CD4^+$ T cells but not memory $CD8^+$ T cells. Both WT and LCK^{CRE} $Bim^{fl/fl}$ animals developed an increased percentage of $CD4^+$ Tregs throughout their lives but maintained overall constant Treg numbers.

3.2.4 Mature $CD4^+$ and $CD8^+$ LCK^{CRE} $Bim^{fl/fl}$ T cells have apoptotic sensitivity profiles reflecting partial BCL-2 family compensation.

One possibility for the normalization of overall T cell numbers in LCK^{CRE} $Bim^{fl/fl}$ mice is that they were able to compensate for the lack of BIM and normalize their apoptotic resistance patterns. $CD4^+$ and $CD8^+$ T cells from the spleens of young LCK^{CRE} $Bim^{fl/fl}$ mice were tested to determine if they were increasingly resistant to apoptotic stimuli given their longer, albeit variable, existence out of the thymus. This would allow for detection of BCL-2 family compensation in T cells that have survived longer than the ∼5 days it

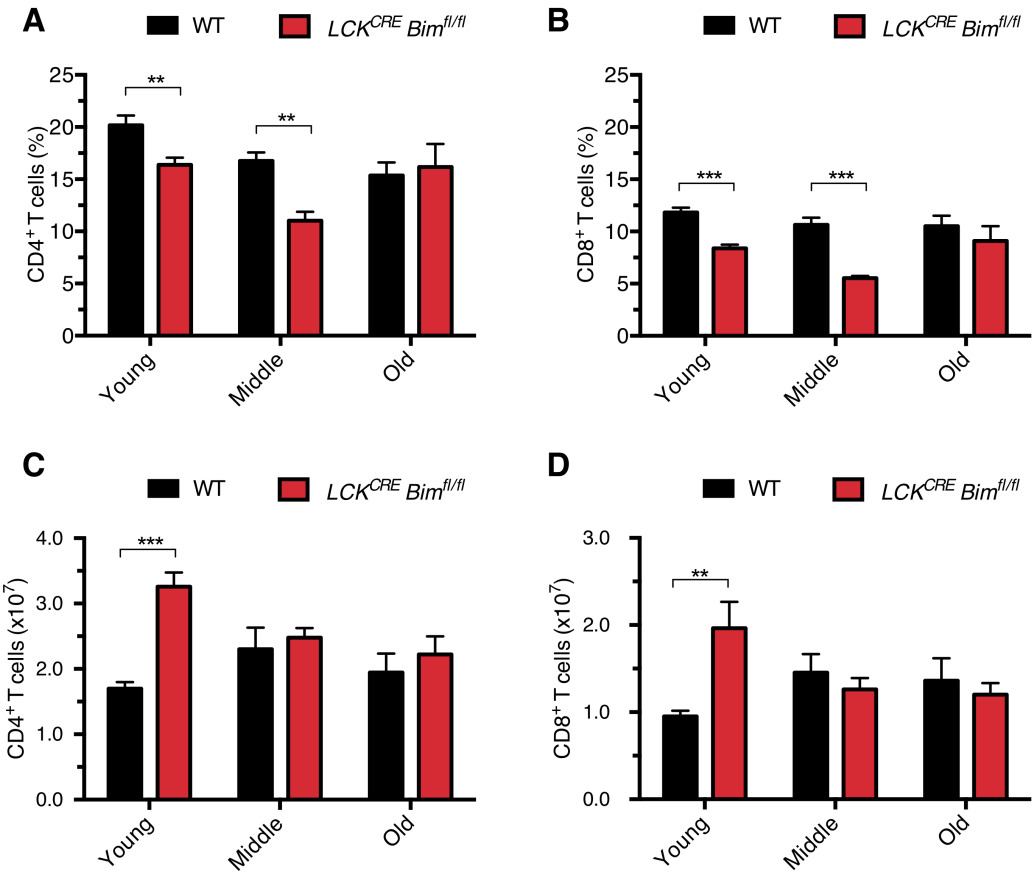

Figure 3.3: Mature CD4$^+$ and CD8$^+$ T cell numbers normalize over time in LCK^{CRE} $Bim^{fl/fl}$ mice. (a, b) Ratios of mature splenic CD4$^+$ and CD8$^+$ T cells from WT and LCK^{CRE} $Bim^{fl/fl}$ mice. (c, d) Absolute numbers of WT and LCK^{CRE} $Bim^{fl/fl}$ CD4$^+$ and CD8$^+$ T cells. LCK^{CRE} $Bim^{fl/fl}$ and WT n$\geq$3 for each age group. Data represented as means $\pm$SEM. *p$<$0.05, ** p$<$0.01, *** p$<$0.001.

takes for a SP T cell to mature and emigrate from the thymus [225, 226]. CD4$^+$ and CD8$^+$ T cells from young LCK^{CRE} $Bim^{fl/fl}$ mice were challenged to undergo cell death following treatment with cytokine deprivation (BIM, PUMA, BAD, HRK reliant death trigger), ionomycin (BIM reliant), phorbol 12-myristate 13-acetate (PMA; PUMA reliant), and etoposide (BID reliant) [16, 17]. BIM deficient CD4$^+$ and CD8$^+$ T cells were equally resistant to cytokine deprivation and etoposide treatment as age-matched controls (Fig. 3.5a-b). While LCK^{CRE} $Bim^{fl/fl}$ CD4$^+$ T cells were equally sensitive to ionomycin as WT con-

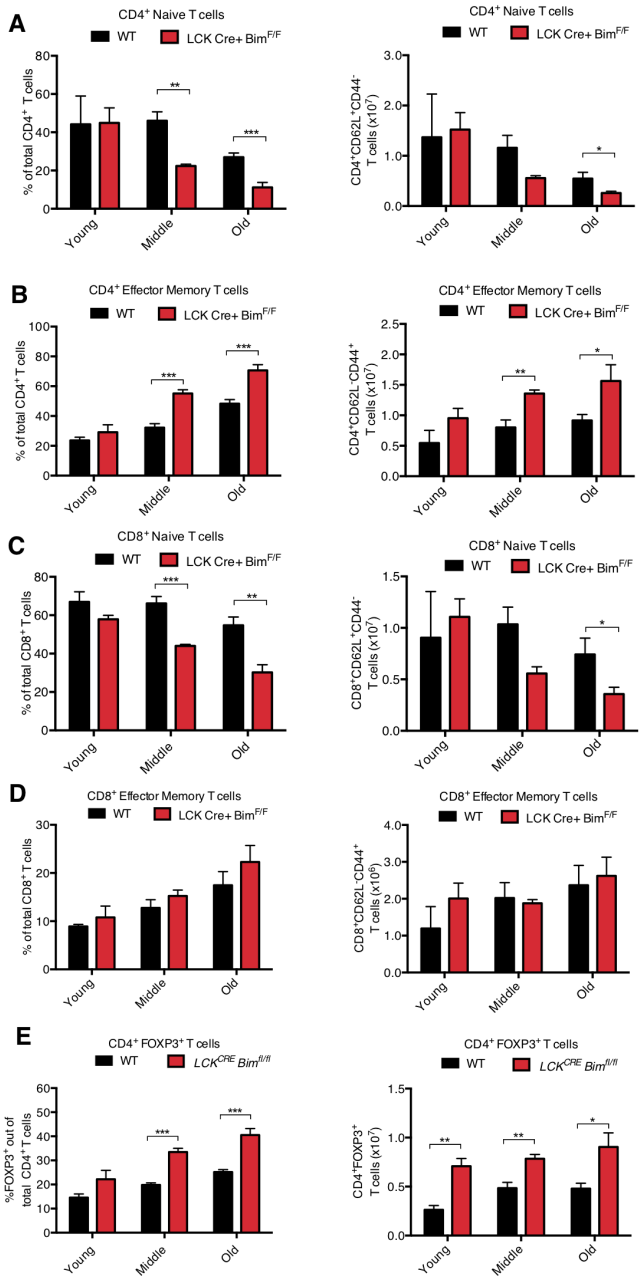

Figure 3.4: There is a decreased proportion of naïve CD4$^+$ and CD8$^+$ T cells and accumulation of CD4$^+$ effector/memory and regulatory T cells in aged LCK^{CRE} $Bim^{fl/fl}$ mice. (a, b) Proportions of naïve (CD44^{low}CD62L^{high}) CD4$^+$ and effector memory (CD44^{high}CD62L^{low}) CD4$^+$ T cells in LCK^{CRE} $Bim^{fl/fl}$ mice. (c, d) Proportions of naïve CD8$^+$ and effector memory CD8$^+$ T cells. (e) Percentage and absolute numbers of CD4$^+$FOXP3$^+$ regulatory Tregs. LCK^{CRE} $Bim^{fl/fl}$ and WT n$\geq$4 for each age group. Data represented as means $\pm$SEM. *p$<$0.05, ** p$<$0.01, *** p$<$0.001.

trols, LCK^{CRE} $Bim^{fl/fl}$ $CD8^+$ T cells were more resistant (Fig. 3.5a-b). The converse was measured against PMA treatment. These data indicate that mature $CD4^+$ and $CD8^+$ T cells lacking BIM were differentially capable of compensating against cell death triggers. We hypothesized that different BCL-2 family compensation mechanisms may underlie such distinctive responses in these cells. As such, we would expect thymic DP T cells to retain their apoptotic resistance patterns over time as thympoiesis remained abnormal in LCK^{CRE} $Bim^{fl/fl}$ mice as they aged. As expected, DP T cells from LCK^{CRE} $Bim^{fl/fl}$ mice were more resistant to all treatments compared to DP cells from WT control animals (Fig. 3.5c).

One possible explanation for our cell death results is that $CD4^+$ and $CD8^+$ T cells differentially altered their expression of BCL-2 family members in response to loss of BIM. Compared to age-matched WT controls, $CD4^+$ T cells from young LCK^{CRE} $Bim^{fl/fl}$ mice demonstrated a significant increase in the expression of *Bmf* (∼5x fold), *A1* (∼2x fold), *Puma* (2x fold) and *Noxa* (∼1.5x fold) (Fig. 3.6a). These transcripts remained elevated in $CD4^+$ T cells from older LCK^{CRE} $Bim^{fl/fl}$ mice, albeit with *Bmf* expression now ∼2x fold higher compared to WT controls. Surprisingly, $CD8^+$ T cells from LCK^{CRE} $Bim^{fl/fl}$ mice displayed a very similar pattern of BCL-2 family expression changes, but in contrast to $CD4^+$ cells, $CD8^+$ cells had a continued rise in expression of *Puma* (3x fold) and *Noxa* (2.5x fold) (Fig. 3.6b). Notably, the changes in BCL-2 family mRNA expression were largely confined to BH3-only proteins. $CD4^+$ and $CD8^+$ T cells had minimally increased expression (∼1.5x fold) of the anti-apoptotic BCL-2 protein BFL-1 (Fig. 3.6a-b).

The loss of BIM in T cells led to global changes in BH3-only expression but little alteration in anti-apoptotic BCL-2 protein transcript. This would predict that anti-apoptotic dependency patterns were similar in T cells from young LCK^{CRE} $Bim^{fl/fl}$ and WT animals despite clear changes in BH3-only protein profiles and partial normalization in cell death sensitivity (Fig. 3.5 and 3.6). In this regard, loss of BIM would have been compensated by other BH3-only proteins to regulate T cell homeostasis as has been measured with respect to functional upregulation of BCL-2 in response to loss of MCL-1 [18]. Such a quantitative

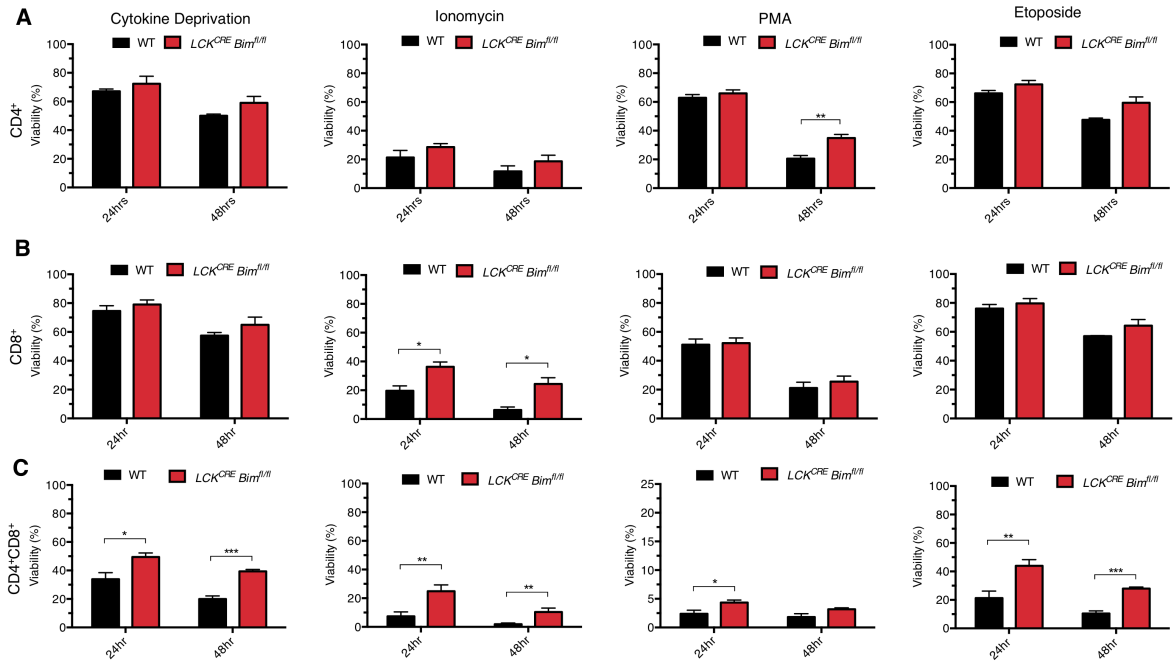

Figure 3.5: $CD4^+$ and $CD8^+$ T cells from $LCK^{CRE}\ Bim^{fl/fl}$ mice were differentially sensitive to apoptotic stimuli compared to age-matched WT controls. (a) $CD4^+$, (b) $CD8^+$, and (c) $CD4^+CD8^+$ DP thymocytes from young mice were cultured for 24 and 48 hours in the absence of cytokines, 1μg/mL ionomycin, 4ng/mL PMA, or 1μM etoposide. Viability was assessed by Annexin V/PI staining with Annexin $V^{neg}PI^{neg}$ cells considered viable. n$\geq$4 for each age group and genotype. Data represented as means $\pm$SEM. *p$<$0.05, ** p$<$0.01, *** p$<$0.001.

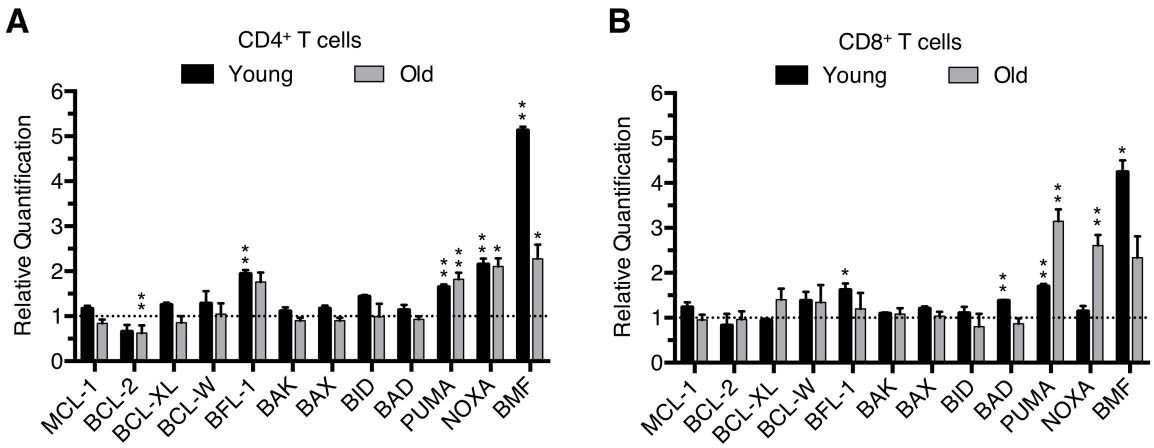

Figure 3.6: CD4$^+$ and CD8$^+$ T cells from LCK^{CRE} $Bim^{fl/fl}$ mice upregulate expression of other BH3-only proteins in response to loss of BIM. mRNA expression levels of BCL-2 proteins from (a) CD4$^+$ and (b) CD8$^+$ splenocytes from young and old LCK^{CRE} $Bim^{fl/fl}$ mice. Age-matched WT mice were used as a reference sample. Expression was normalized using B2M and analyzed using the $\Delta\Delta$CT method. n$\geq$3 for young and n$\geq$4 for old age group and genotype. Significance was compared to age-matched reference samples (normalized to 1, dashed line) Data represented as means $\pm$SEM. *p$<$0.05, ** p$<$0.01, *** p$<$0.001 as determined by calculating RQ Min/Max with a 95% confidence level using the ExpressionSuite Software.

model would predict that other BH3-only proteins are able to functionally compensate for the loss of BIM. To test this, we utilized BH3-profiling, a technique in which death domains of select BH3-only proteins with focused BCL-2 anti-apoptotic affinities are intracellularly introduced to determine the anti-apoptotic protein dependency patterns of individual cells (Fig. 3.7a) [216, 217, 227, 228]. Supporting our gene expression analyses, mitochondrial de-

polarization measurements from $CD4^+$, $CD8^+$ and DP thymocytes from LCK^{CRE} $Bim^{fl/fl}$ and WT animals showed no difference in BCL-2 family anti-apoptotic protein dependency (Fig. 3.7b). Despite this result, challenge with apoptotic stimuli found *Bim* deficient T cells to be variably resistant to cell death (Fig. 3.5). To determine if this cell death resistance was also reflected at the level of the mitochondrion, BIM BH3 titration was used to compare apoptotic priming to the intrinsic apoptotic pathway in $CD4^+$, $CD8^+$ and DP thymocytes from LCK^{CRE} $Bim^{fl/fl}$ and WT animals. Mitochondria of T cells from LCK^{CRE} $Bim^{fl/fl}$ mice were progressively resistant to depolarization following treatment with decreasing doses of BIM BH3 (Fig. 3.7c). Akin to our cell death data, these results suggest that T cells lacking BIM upregulate other BH3-only members and that these proteins partially compensate for apoptotic sensitivity in mature T cells lacking BIM.

3.3 Conclusions

Understanding the roles that BCL-2 family proteins play in the regulation of immune cell viability and function has become increasingly important as the clinical use of highly-specific small molecule and peptide BH3-mimetics increases. The role of BCL-2 anti-apoptotic proteins in regulating death and survival of various lymphocyte populations has been widely studied [18, 229]. Less well characterized however is the role that BH3-only proteins play in long-term immune homeostasis of these same populations. In this report, we have characterized how the long-term loss of *Bim* in T cells affects total lymphocyte numbers and T cell apoptotic sensitivity over time. We and others confirm that BIM is the master BH3-only regulator of T cell homeostasis, as its deletion leads to abnormal thymocyte development, elevated white blood cell counts, and resistance to apoptotic stimuli [16]. These phenotypes are recapitulated in mice with deletion of *Bim* restricted to T cells. Importantly, the elevation in peripheral lymphocytes in LCK^{CRE} $Bim^{fl/fl}$ mice normalized as they aged suggesting the existence of T cell-specific BCL-2 family compensatory mechanism as a result of BIM loss. Our study thereby supports the fundamental importance of BIM in T cell apoptotic

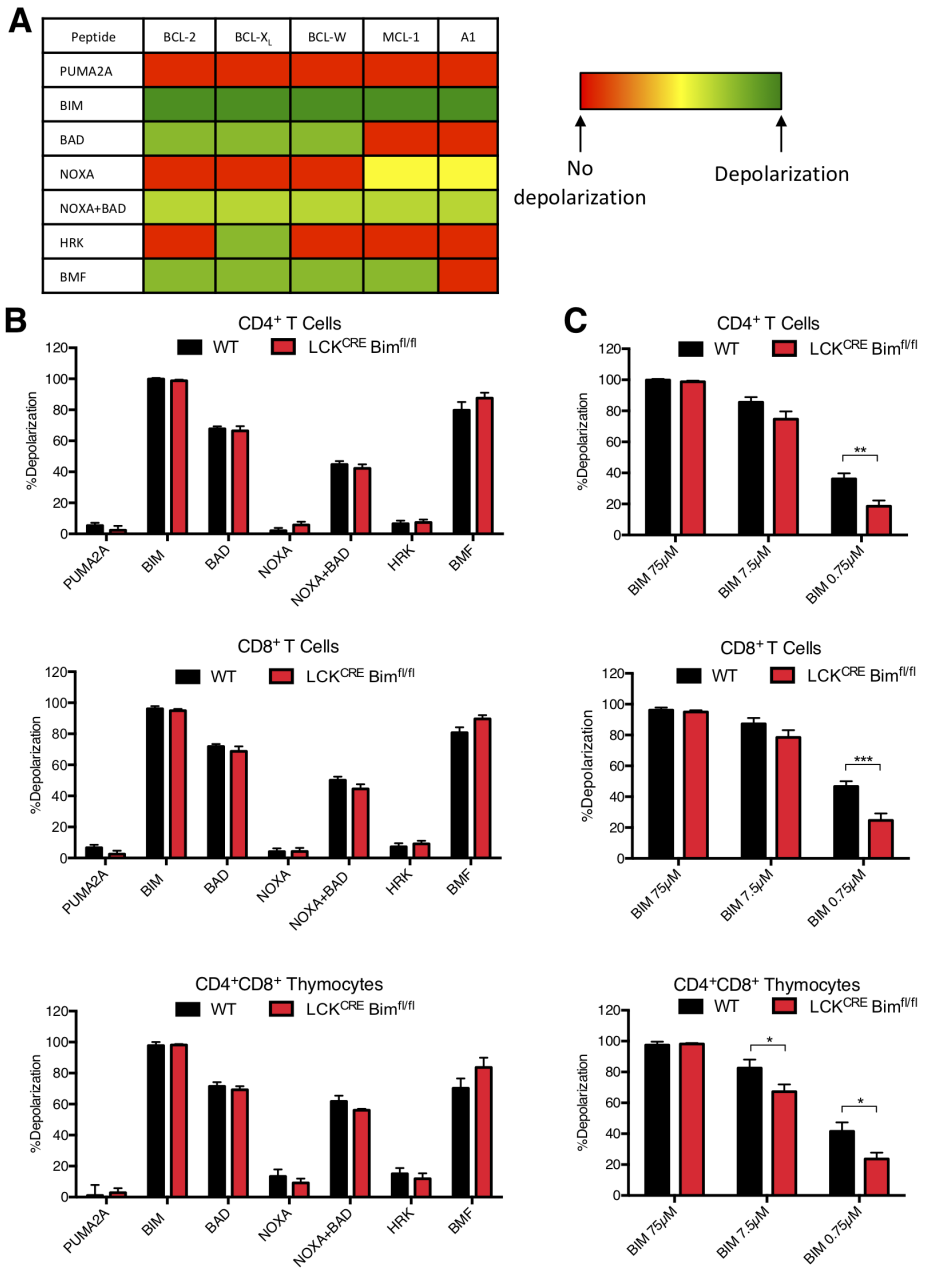

Figure 3.7: T cells lacking BIM do not shift their anti-apoptotic dependency and remain less primed to die compared to WT T cells. (a) Table of BH3 peptides used for BH3 profiling and expected mitochondrial depolarization patterns. (b) BH3 profiling of mature $CD4^+$, $CD8^+$, or $CD4^+CD8^+$ DP T cells. (c) To measure apoptotic priming, cells were treated with decreasing concentrations of the BIM BH3 peptide. n$\geq$4 for each genotype. Data represented as means $\pm$SEM. *p$<$0.05, ** p$<$0.01, *** p$<$0.001.

homeostasis and shows that, unlike BCL-2 anti-apoptotic proteins, BH3-only proteins are unable to fully functionally replace BIM in T cells [230]. Therefore, our work corroborates that of other's pointing to the notion that BH3-only proteins, like anti-apoptotic BCL-2 proteins, can contribute at least somewhat quantitatively to the survival of T cells [18, 229].

Global, conditional hematopoietic (*VAV-CreER*), and T cell-specific (LCK^{CRE} $Bim^{fl/fl}$, as shown here) deletion of BIM results in abnormal maturation of thymocytes, including an increase of DN thymocytes and increased time in the DN phases [45, 46, 16, 34]. If compensatory mechanisms leading to white count normalization occurred in the thymus, aged LCK^{CRE} $Bim^{fl/fl}$ mice would have been expected to develop an increasingly normal distribution of DN and DP thymocytes. However, thymocyte development remained abnormal in aged animals, pointing to a normalization mechanism that occurred over a longer period of time than that needed for a mature T cell to exit the thymus.

While $CD4^+$ and $CD8^+$ splenocyte numbers approached those of WT animals over time, there was an increase in the percentage of $CD4^+$ T cells that were $FOXP3^+$ Tregs in both WT and LCK^{CRE} $Bim^{fl/fl}$ animals as they age (Fig. 3.4e). BIM is a critical regulator of Treg levels and partially mediates their accumulation in aged mice through decreased BIM expression [163, 166]. Treg accumulation may rely predominantly on BIM rather than anti-apoptotics as Tregs from older animals have decreased expression of both BCL-2 and MCL-1, the major partners of BIM in T cells [18, 142]. Tregs from aged mice have increased suppressive capacity compared to Tregs from young mice, suggesting that the increased proportion of Tregs may be a contributing factor to the normalization of lymphocyte counts in aged mice [231]. We found that Treg accumulation was more robust in LCK^{CRE} $Bim^{fl/fl}$ mice but did not increase significantly with age compared to WT animals. Perhaps the accumulation of Tregs in LCK^{CRE} $Bim^{fl/fl}$ animals is partially responsible for the normalization of CD4+ and CD8+ T cells deficient in Bim. However, a presumed natural rise in Tregs was unable to normalize WBC in 20-25 week aged $CD19^{CRE}$ $Bim^{fl/fl}$ or $E\mu^{CycD1}CD19^{CRE}$ $Bim^{fl/fl}$ animals indicating a T cell-specific phenomenon [212]. Additionally, there were equivalent

$CD4^+$ T cells in older mice from both groups (Fig. 3.4a-b and Fig. 3.4e), suggesting that the mechanism for WBC normalization does not depend, at least fully, on Tregs. The direct role that BIM plays in Treg-specific maintenance and function continues to be an active area of investigation.

We observed a consistent upregulation in other BH3-only proteins in response to loss of BIM. This likely played a predominant role in lymphocyte number compensation and partial normalization to cell death stimuli as there were no significant changes in anti-apoptotic expression or difference in BH3 profiling between T cells from LCK^{CRE} $Bim^{fl/fl}$ and WT animals (Fig. 3.6 and 3.7). Mature peripheral T cells had similar sensitivity profiles to WT animals, further supporting that a qualitative compensation mechanism takes either time or cell death pressure to develop. The exception was $CD8^+$ T cells, which remained significantly resistant to ionomycin suggesting that there are fundamental differences in how $CD4^+$ and $CD8^+$ cells respond to the loss of BIM. This distinction may reflect previously unresolved discrepancies in cell death sensitivity profiles and BCL-2 family compensation in non-malignant T cells from patients treated with BH3-mimetics where the primary mechanism is disruption of BIM binding to various anti-apoptotic BCL-2 proteins [228, 232, 233].

This study, and that of others, continues to support the notion that the full functionality of BIM in lymphocytes is irreplaceable by other BH3-only proteins. There may also exist different levels of BH3 compensation in the setting of loss of BIM. This may perhaps be best reflected in work showing that mice expressing BIM protein with altered BH3-defined specificities (Bim^{Bad}, Bim^{Puma}, BIM^{Noxa}) show partial, yet incomplete, WBC normalization compared to mice lacking Bim [230]. DP thymocytes from these animals showed partial *in vitro* normalization to cell death stimuli. Mature $CD4^+$ and $CD8^+$ T cells were not examined nor was Bim^{Bmf}. The supremacy of BIM for initiation of apoptosis extends to its capacity to directly activate BAX and BAK to a higher degree than other potential direct activators, such as BID and PUMA [24, 234]. According to this model, the BH3 region of these proteins lead to "very substantial" apoptosis but "maximal" apoptosis was only manifested by BIM

[230]. This idea is supported by our data and mechanistically by the ability of BIM BH3 peptide to fully depolarize mitochondria when used in BH3 profiling, and thus serve as a positive control, independent of a cell's anti-apoptotic dependency patterns [216, 228, 235].

While there were slightly different patterns of BH3-only protein upregulation in $CD4^+$ and $CD8^+$ T cells lacking *Bim*, the proteins involved were identical, indicating a conserved T cell mechanism of BH3 protein cell death control (Fig 3.6). In particular, there was increased transcription of *Puma* and *Noxa*, but *Bmf* was the most consistently upregulated BH3-only transcript. Several studies have suggested BIM and BMF may have evolved from a common ancestry with shared transcriptional control and overlapping functions and regulatory programs [25, 236]. Both proteins are normally sequestered by the cytoskeleton and global deletion of either leads to defects in the immune system, although *Bmf* deletion leads to a more dramatic effect in B cells [61, 23]. Like loss of BIM, BMF deficiency also leads to lymphocyte accumulation through a cell autonomous mechanism and results in increased B cell accumulation compared to T cells [61]. $Bim^{-/-}Bmf^{-/-}$ double knockout mice develop thymocytes more resistant than those from single knockout mice to a number of agents, including steroids and HDAC inhibition [61, 23]. However, apoptotic resistance in mature SP T cells was completely BIM dependent [23]. Additionally, combined loss of BIM and BMF led to a compounded increase in B cells but T cell numbers were not increased above single $Bim^{-/-}$ animals, suggesting a redundant mechanism of apoptotic control in B cells and not T cells. These studies support our observation that normal T cell death regulation and numbers are predominantly regulated by BIM. Given their functional overlap, it is conceivable that BMF compensates for loss of BIM in aged LCK^{CRE} $Bim^{fl/fl}$ animals. T cell-specific genetic regulation and possible function for BMF has not been previously reported. We also cannot discount a combined cooperative compensation by multiple BH3 proteins in the absence of BIM that are able to control some, but not all, aspects of cell death control.

Identification of how BCL-2 proteins compensate for functional or deletional loss of one another could have important clinical implications. This study provides new insight into

the redundancy and fluidity of the BH3 subset of the BCL-2 family of proteins. It will be essential to learn more about how these proteins are able to compensate for one another in response to long-term pressure such as encountered during prolonged treatment with BH3 mimetics.

CHAPTER 4

MANIPULATION OF BCL-2 PROTEINS AND DEPENDENCY PATTERNS IN T CELLS USING ABT-199

4.1 Introduction

In the last section we highlighted the essential role the BH3-only protein BIM plays in regulation of apoptosis in T cells. The BH3-only proteins are the main initiators of the apoptotic cascade. In times of cellular stress, the BH3-only proteins can either inhibit anti-apoptotic proteins or directly bind to and activate the pro-apoptotic proteins BAX and BAK [11, 12]. Due to subtle differences in the binding pockets of anti-apoptotic proteins, there is a degree of selectivity that individual BH3-only proteins have for each anti-apoptotic [11]. As our understanding of these dynamic protein:protein interactions becomes clearer, many small molecules and therapeutic peptides are being developed to mimic the action of BH3-only proteins [63]. Like BH3-only proteins these compounds, termed BH3 mimetics, have varying potencies and specificities to the anti-apoptotics. Many BH3 mimetics have had success in clinical trials and ABT-199 (venetoclax/venclexta) has received FDA approval for treatment of chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) [87, 88]. Because many cancers upregulate one or more of the anti-apoptotic proteins as a key survival mechanism, targeting the apoptotic pathway is an attractive therapeutic strategy for many solid tumors and hematological malignancies [63, 237].

As the use of BH3 mimetics in the context of cancer continues to expand, one area of research that requires further exploration is how the administration of these compounds can affect healthy cells that are also sensitive to BCL-2 modulation, including immune cells. There is a growing body of evidence demonstrating that multiple immune cell subsets are effected by BH3 mimetic treatment, at doses at or lower than what would typically be used in the context of cancer [91, 18]. It is therefore imperative to gain a clearer understanding of how BH3 mimetics administered long-term could potentially affect the global immune

repertoire. Additionally, because the BCL-2 family has a high level of redundancy and dynamism, it will be vital to evaluate how the BCL-2 family changes in individual cell types if exposed to BH3 mimetic pressure.

We demonstrate here that $CD8^+$ T cells, $CD4^+$ Tcons, and $CD4^+FOXP3^+$ Tregs have differential sensitivities to ABT-199 treatment if expanded in the presence of ABT-199, and have diverging changes in BCL-2 family protein levels and dependency patterns. *In vivo*, $CD8^+$ T cells were most sensitive to ABT-199 treatment, and Tregs were resistant at all doses tested. $CD8^+$ T cells and Tcons upregulated BCL-2 while Tregs primarily upregulated BCL-X_L in response to to ABT-199 treatment both at steady state and after long-term treatment in a congenic transplant model. Overall, this study contributes to our understanding of T cell adaptivity in response to BCL-2 inhibition, which has important implications for long-term BH3 mimetic use, especially in the context of combinatorial immunotherapies and in the transplant setting.

4.2 Results

4.2.1 T cell expansion results in differential sensitivity patterns across T cell subsets in response to ABT-199

T cell expansion is a critical step in adoptive T cell transfer, a therapeutic strategy that has gained prevalence for the treatment of multiple hematological malignancies. In addition to more traditional regimens of hematopoietic stem cell transplantation, there is growing interest in manipulation of T cells *ex vivo* to further increase their potency and therapeutic efficacy, including potentially prolonging their survival by modifying their apoptotic pathway [189, 190]. It has been well characterized that T cells change their patterns of BCL-2 family expression upon stimulation and expansion [143, 144, 145, 146]. We therefore hypothesized that the process of T cell expansion would alter the baseline sensitivity of T cells to ABT-199 treatment. $CD8^+$ T cells, $CD4^+$ Tcons, and $CD4^+FOXP3^+$ Tregs were

isolated from FOXP3-IRES-GFP mice. T cells were then stimulated and expanded for five days with CD3/CD28 and high dose IL-2 and sensitivity to ABT-199 was assessed. Compared to unstimulated controls, $CD8^+$ T cells had a 50-fold increase in ABT-199 resistance (EC_{50} 40nM±0.038 to 2020nM±0.054), while Tcons had close to a 10-fold increase (EC_{50} 68nM±0.020 to 611nM±0.043). Tregs did not have any increase in resistance to ABT-199 after stimulation and expansion (EC_{50} 169nM±0.070 to 142nM±0.137) (Fig. 4.1a). To determine if the observed resistance to apoptosis was dependent on BCL-2 targeting, BH3 priming was performed to measure global changes in apoptotic sensitivity. As expected, $CD8^+$ T cells and Tcons had reduced levels of depolarization after expansion, and Tregs did not, indicating that $CD8^+$ T cells and Tcons but not Tregs were less primed to die (Fig. 4.1b). This data suggests that $CD8^+$ T cells and, to a lesser extent, Tcons shift away from BCL-2 dependency during expansion, and become globally more resistant to apoptosis. Tregs do not experience this shift from BCL-2 and remain equally sensitive to BCL-2 targeting after stimulation and expansion.

Stimulation and expansion caused a large decrease in sensitivity to ABT-199 in $CD8^+$ T cells and Tcons but not Tregs. We therefore hypothesized that these expanded T cell subsets would have distinct changes in their BCL-2 repertoire. To determine if there were any shifts in anti-apoptotic dependency, BH3 profiling was performed. Compared to unstimulated controls, all three T cell subsets had decreased mitochondrial depolarization in response to either the BAD peptide alone ($CD8^+$ T cells and Tregs) or the BAD+NOXA combination treatment ($CD8^+$ T cells and Tcons), suggesting a shift away from BCL-2/BCL-X_L/BCL-W dependency (Fig. 4.2a). However, profiling did not reveal any distinct differences between $CD8^+$ T cells, Tcons, and Tregs that would explain their divergent sensitivities to ABT-199 post stimulation and expansion. We next evaluated changes in expression of BCL-2 family members across T cell subsets. Relative to unstimulated controls, $CD8^+$ T cells and Tcons had a 4-fold and 10-fold increase in *Bcl-2* expression respectively, and both had a 10-fold increase in *Bcl-X_L* expression. In contrast, Tregs only upregulated *Bcl-2* 2-fold.

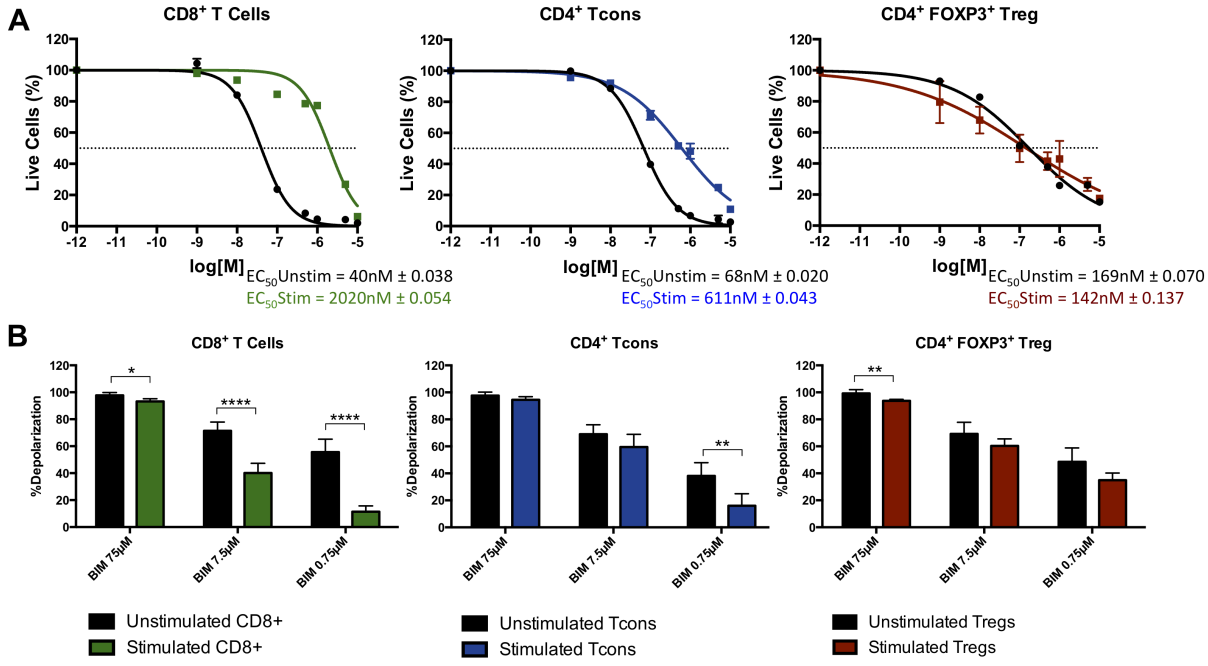

Figure 4.1: Expanded CD8$^+$ T cells become resistant to ABT-199 and expanded Tregs do not. (a) CD8$^+$ T cells, CD4$^+$ Tcons, and CD4$^+$FOXP3$^+$ Tregs were incubated with increasing doses of ABT-199 for 24 hours directly after isolation or after 5 days of expansion. Viability was assessed by Annexin V/PI staining with Annexin V^{neg}PI^{neg} cells considered viable. (b) Freshly isolated, unstimulated T cells and expanded T cells were permeabilized and incubated with increasing concentrations of BIM peptide to determine apoptotic priming. Data is representative of 3 independent replicates. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

$CD8^+$ T cells and Tcons also upregulate expression of multiple BH3-only proteins while Tregs exclusively upregulate *Noxa* (Fig. 4.2b). To determine if protein levels correlated with mRNA expression levels, intracellular flow cytometry was performed on select BCL-2 proteins. All three T cell subsets had increased levels of MCL-1, BCL-X_L, and BCL-2, as well as the BH3-only protein BIM. Compared to unstimulated controls, Tregs had a higher ratio of BCL-2 than $CD8^+$ T cells and Tcons (Fig. 4.2c). This increase in BCL-2 may be why expanded Tregs do not experience increased resistance to ABT-199. These findings demonstrate that expanded T cell subsets have distinct changes in their BCL-2 repertoires, and this is reflected by differing levels of sensitivity to treatment with ABT-199.

4.2.2 T cell expansion in the presence of ABT-199 leads to increased resistance in Tregs but not $CD8^+$ T cells

Expansion of T cells in the presence of BH3 mimetic pressure in order to modulate the BCL-2 repertoire before infusion is an interesting therapeutic strategy. Manipulation of the apoptotic pathway in T cells may allow for selective killing or enrichment of favorable populations based on their unique BCL-2 family repertoires. Therefore we wanted to determine how T cell subsets were able to adapt if expanded in the presence of ABT-199. $CD8^+$ T cells, Tcons, and Tregs were stimulated and expanded for five days in the presence of DMSO or 500nM of ABT-199. We predicted that this dose would allow for a sublethal amount of BH3 mimetic pressure, enough to elicit changes in the BCL-2 proteins in each T cell subset without causing an extensive amount of apoptosis. In contrast to expansion alone, $CD8^+$ T cells had only a $\sim$1.5-fold increase in resistance (2020nM$\pm$0.054 to 2870nM$\pm$0.034), Tcons had a 4-fold increase in resistance(611nM$\pm$0.043 to 2440nM$\pm$0.062), and Tregs had a 50-fold increase in resistance to ABT-199 treatment (142nM$\pm$0.137 to 7020nM$\pm$0.074) (Fig. 4.3a). Despite the increased resistance to ABT-199 in Tcons and Tregs, all three T cell subsets had an increased level of apoptotic priming compared to cells expanded with DMSO control (Fig. 4.3b).

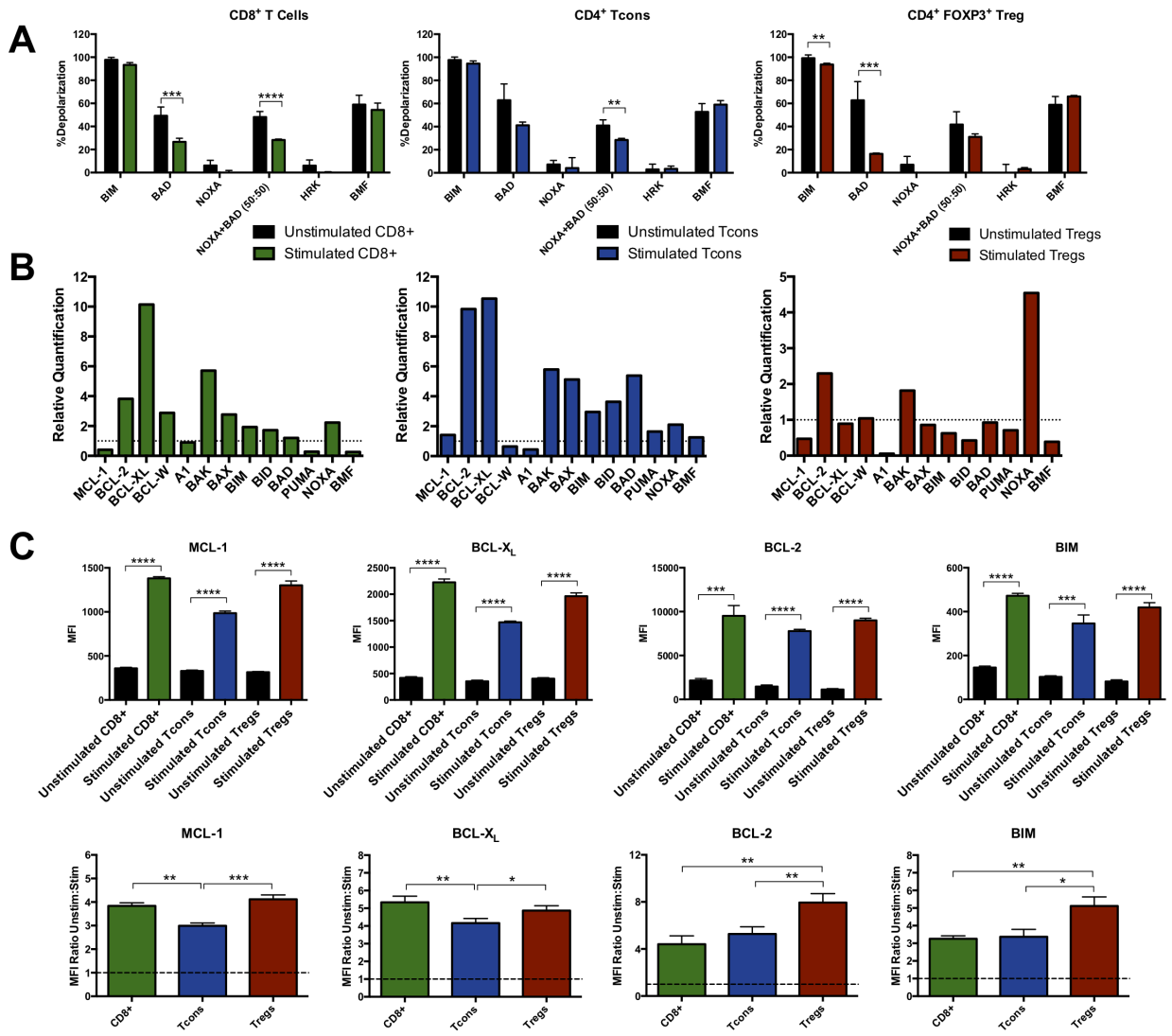

Figure 4.2: T cell subsets have differential shifts in their BCL-2 family repertoires in response to stimulation and expansion. (a) Freshly isolated, unstimulated T cells and expanded T cells were permeabilized and incubated with BH3-only peptides to determine their anti-apoptotic dependencies. (b) mRNA expression levels of expanded $CD8^+$ T cells, $CD4^+$ Tcons, and $CD4^+FOXP3^+$ Tregs. Unstimulated T cells were used as a reference sample. Expression was normalized using UBC and analyzed using the $\Delta\Delta$CT method. (c) Protein levels of unstimulated and stimulated $CD8^+$ T cells, $CD4^+$ Tcons, and $CD4^+FOXP3^+$ Tregs. Data is shown as both MFI (top) and as ratios of stimulated protein levels compared to unstimulated. Data is representative of 3 independent replicates.Data represented as means $\pm$SEM. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

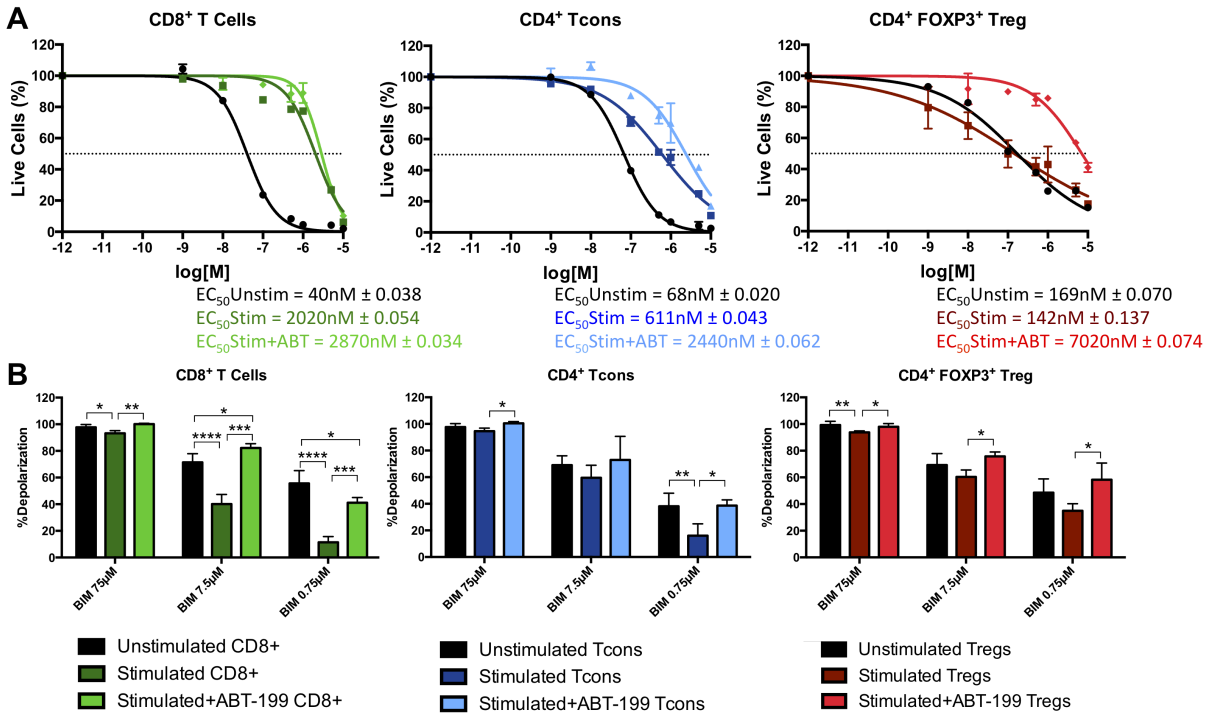

Figure 4.3: Expansion with ABT-199 pressure leads to increased ABT-199 resistance in Tregs but not CD8$^+$ T cells. (a) CD8$^+$ T cells, CD4$^+$ Tcons, and CD4$^+$FOXP3$^+$ Tregs were incubated with increasing doses of ABT-199 for 24 hours directly after isolation or after 5 days of expansion as shown in Figure 4.1. An additional group of cells for each subset was expanded and treated with 500nM of ABT-199 daily for 5 days and the viability assay was repeated. Viability was assessed by Annexin V/PI staining with Annexin V^{neg}PI^{neg} cells considered viable. (b) Freshly isolated, unstimulated T cells, expanded T cells, and T cells expanded with ABT-199 pressure were permeabilized and incubated with increasing concentrations of BIM peptide to determine apoptotic priming. Data is representative of 3 independent replicates. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

In order to determine how the addition of ABT-199 during expansion affected the BCL-2 family in T cells, we reevaluated changes in anti-apoptotic dependency, mRNA expression, and overall protein levels. Compared to cells expanded with DMSO, $CD8^+$ T cells expanded in the presence of ABT-199 had decreased depolarization with the NOXA+BAD peptide treatment, likely indicating a shift away from BCL-2/BCL-X_L/BCL-W dependency (Fig. 4.4a). However, there were no peptides other than BMF that caused increased depolarization in $CD8^+$ T cells, meaning there are no clear shifts towards another anti-apoptotic protein in response to ABT-199 treatment. Conversely, Tcons and Tregs expanded with ABT-199 pressure have increased depolarization in response to the NOXA/NOXA+BAD, and HRK peptide treatments, indicating shifts towards MCL-1/A1 and BCL-X_L respectively (Fig. 4.4a). Tregs also have increased depolarization in response to the BAD peptide, which could be increased dependency on BCL-2, BCL-X_L, or BCL-W. In this case it is most likely BCL-X_L due to the same increase in depolarization observed with the HRK peptide. $CD8^+$ T cells and Tcons treated with ABT-199 had only minor changes in BCL-2 family expression relative to cells expanded with DMSO, while Tregs upregulate the anti-apoptotics *Bcl-w* and *A1*, as well as downregulate the BH3-only proteins *Bim*, *Bid*, and *Bmf* (Fig. 4.4b). At the protein level, all three T cell subsets upregulate MCL-1, BCL-X_L, and BCL-2, but Tregs upregulate BCL-X_L more than Tcons and $CD8^+$ T cells (Fig. 4.4c-d). Overall, these results suggest that expanded Tregs, and to a lesser extent Tcons, are either intrinsically less dependent on BCL-2, or are able to more effectively shift away from BCL-2 dependency to other anti-apoptotic proteins in order to survive in the presence of ABT-199.

4.2.3 $CD8^+$ T cells are highly sensitive to ABT-199 in vivo, while Tregs are relatively resistant

Compared to $CD8^+$ T cells, Tregs and Tcons expanded in the presence of ABT-199 became more resistant to additional ABT-199 treatment *in vitro*. We therefore hypothesized that Tcons and Tregs would also be more resistant to ABT-199 *in vivo*. Mice were treated for

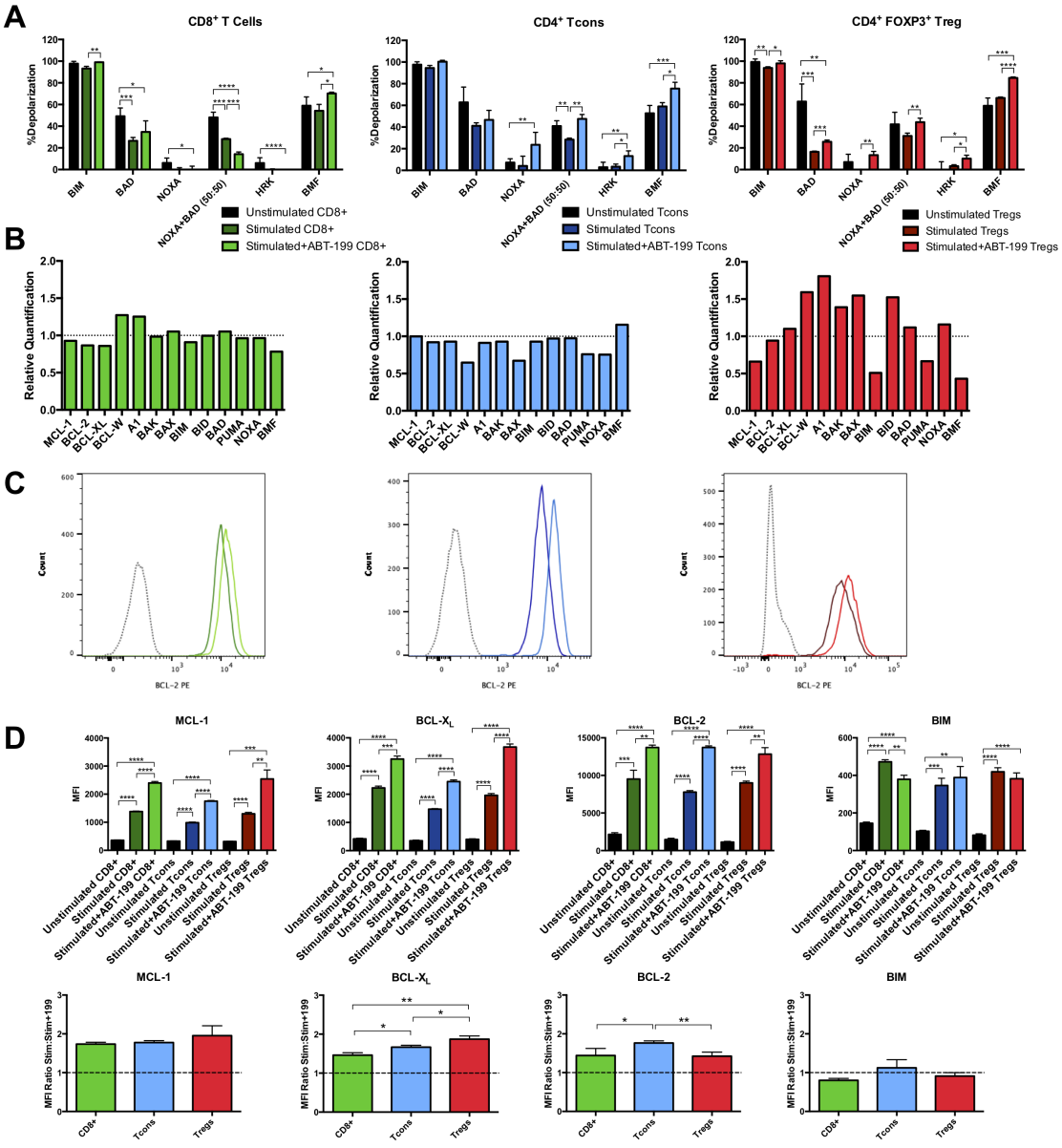

Figure 4.4: Tregs have a greater shift away from BCL-2 dependency compared to CD8$^+$ T cells. (a) T cell subsets were unstimulated, stimulated, or stimulated with 500nM ABT-199 pressure for 5 days, and after treatment, incubated with BH3-only peptides to determine anti-apoptotic dependencies. (b) mRNA expression levels of CD8$^+$ T cells, CD4$^+$ Tcons, and CD4$^+$FOXP3$^+$ Tregs treated with 5 days of ABT-199 pressure. Stimulated, DMSO treated cells were used as a reference sample. Expression was normalized using UBC. (c) Representative flow of BCL-2 for CD8$^+$ T cells (green), Tcons (blue) and Tregs (red) stimulated alone and with 500nM ABT-199. IgG isotype control is in black. (d) Protein levels of unstimulated, stimulated, and stimulated with ABT-199 pressure T cell subsets. Data is shown as both MFI (top) and as ratios of stimulated protein levels compared to unstimulated. Data is representative of 3 independent replicates. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

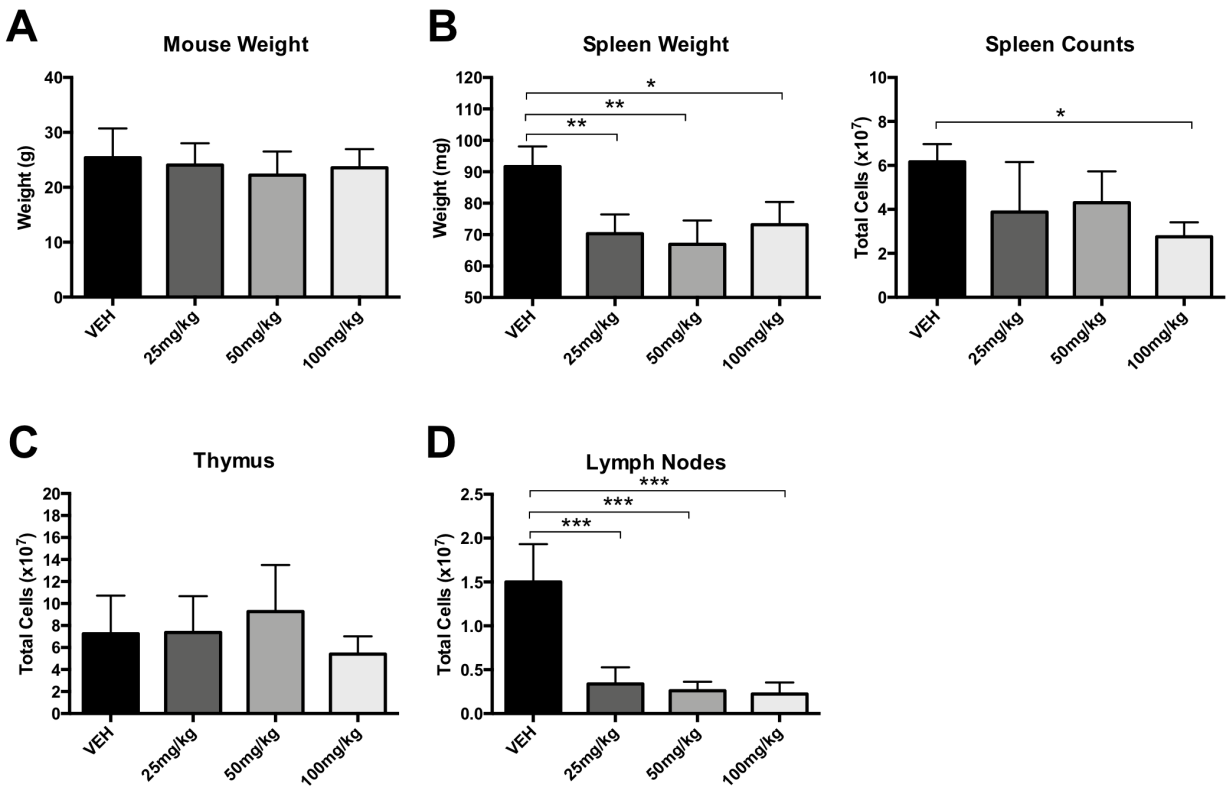

Figure 4.5: Mice treated with ABT-199 have decreased lymphocyte counts. FOXP3-IRES-GFP mice were treated with increasing doses of ABT-199 administered orally daily for seven days. (a) Mouse body weight. (b) Spleen weight and total splenocyte count (c) Total cell counts from the thymus and (d) lymph nodes. n$\geq$3 for each group. Data represented as means $\pm$SEM. *p$<$0.05, ** p$<$0.01, *** p$<$0.001, **** p$<$0.0001.

seven days with 25, 50, or 100mg/kg of ABT-199. ABT-199 was overall well tolerated as treatment did not cause significant body weight loss at any dose (Fig. 4.5a). Mice did experience a reduction in spleen weight at all doses tested, as well as a reduction in total splenocyte count at the highest dose (Fig. 4.5b). Thymocyte counts were not affected at any dose, suggesting that thymocytes are more resistant than mature lymphocytes (Fig. 4.5c) [238, 18]. The total cell number in the lymph nodes were also significantly reduced even at the lowest dose of ABT-199 treatment (Fig. 4.5d).

As predicted, mice treated with ABT-199 had a significant decrease in the proportion and absolute number of $CD8^+$ T cells (Fig. 4.6a-b). The percentage and cell number of total $CD4^+$ T cells stayed relatively stable, but there was a decrease in the percentage of

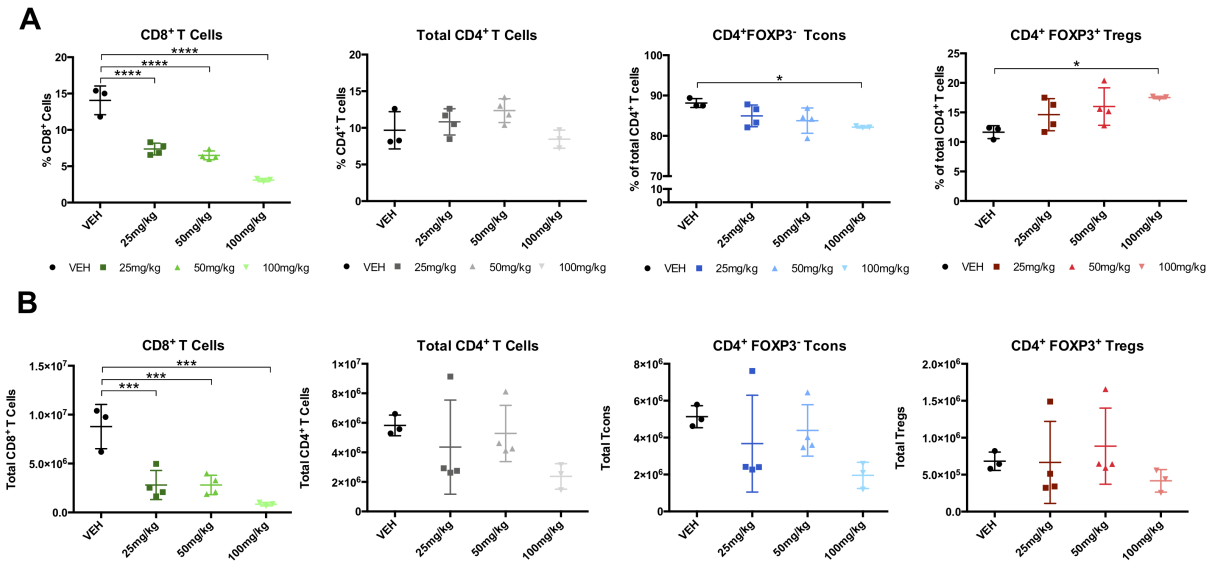

Figure 4.6: $CD8^+$ T cells are highly sensitive to ABT-199, while Tregs are relatively resistant. (a) Percentage and (b) Absolute number of $CD8^+$ T cells, Tcons, and Tregs, shown as subsets of total $CD4^+$ T cells isolated from the spleen. n≥3 for each group. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Tcons and increase in the percentage of Tregs at the highest treatment dose (Fig. 4.6a). At this dose the absolute numbers of both Tcons and to a lesser extent Tregs decreased slightly, but this was not statistically significant (Fig. 4.6b). This data is in agreement with the resistance patterns observed *in vitro*, with $CD8^+$ T cells>Tcons >Tregs in terms of ABT-199 sensitivity.

For *in vitro* experiments on primary T cells, it is necessary to stimulate and expand cells for them to survive for the duration of the experiment. This expansion leads to a homogeneous population of T cells with an effector memory phenotype. Therefore we wanted

to assess if naive, effector memory, and central memory T cells were differentially sensitive to ABT-199 *in vivo*. $CD8^+$ T cells, Tcons, and Tregs all had an increase in the proportion of effector memory ($CD44^{high}CD62L^{low}$) cells (Fig. 4.7a-c). In $CD8^+$ T cells, the percentage of naive ($CD44^{low}CD62L^{high}$) cells stayed relatively stable, but there was a significant decrease in absolute numbers of naive $CD8^+$ T cells. There was also a decrease in percentage and absolute number of central memory ($CD44^{high}CD62L^{high}$) $CD8^+$ T cells (Fig. 4.7a). In contrast, naive Tcons and Tregs decreased in both percentage and absolute number, while the percentage and absolute numbers of central memory cells stayed relatively stable or even slightly increased (Fig. 4.7b-c). This would suggest that, regardless of T cell type, effector memory T cells are less dependent on BCL-2 for survival and therefore resistant to ABT-199.

BCL-2 proteins are important regulators of thymocyte development, therefore we investigated the possibility that ABT-199 could impair T cell maturation in the thymus. After seven days of treatment, there were no overt differences in the proportions of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP, and $CD4^-CD8^-$ DN thymocytes (Fig. 4.8a-b). There was a decrease in the percentage of DN1 cells, but no significant changes in the proportions of DN2-DN4 cells (Fig. 4.8c). Therefore while DN1 cells are slightly sensitive to the drug, it is not to an extent that leads to diminishment of the global DN population or a reduction in total thymocyte number.

The sensitivity patterns of $CD8^+$ T cells, Tcons, and Tregs treated with ABT-199 *in vivo* were in agreement with what we predicted based on our *in vitro* data. We next wanted to ascertain if the changes in the BCL-2 family repertoire were recapitulated in this setting. To evaluate the BCL-2 repertoire in cells remaining following treatment, mice were treated for seven days with 50mg/kg ABT-199. BH3 priming revealed that all three T cell subtypes were slightly less primed to die after treatment, with Tregs experiencing a slightly greater reduction in priming compared to $CD8^+$ T cells (21.0% vs. 15.9% reduction in depolarization for $CD8^+$ T cells vs. Tregs) (Fig. 4.9a). Unlike cells stimulated and expanded *in vitro*, BH3 profiling did not indicate a clear shift to other anti-apoptotic proteins in any of the T cell

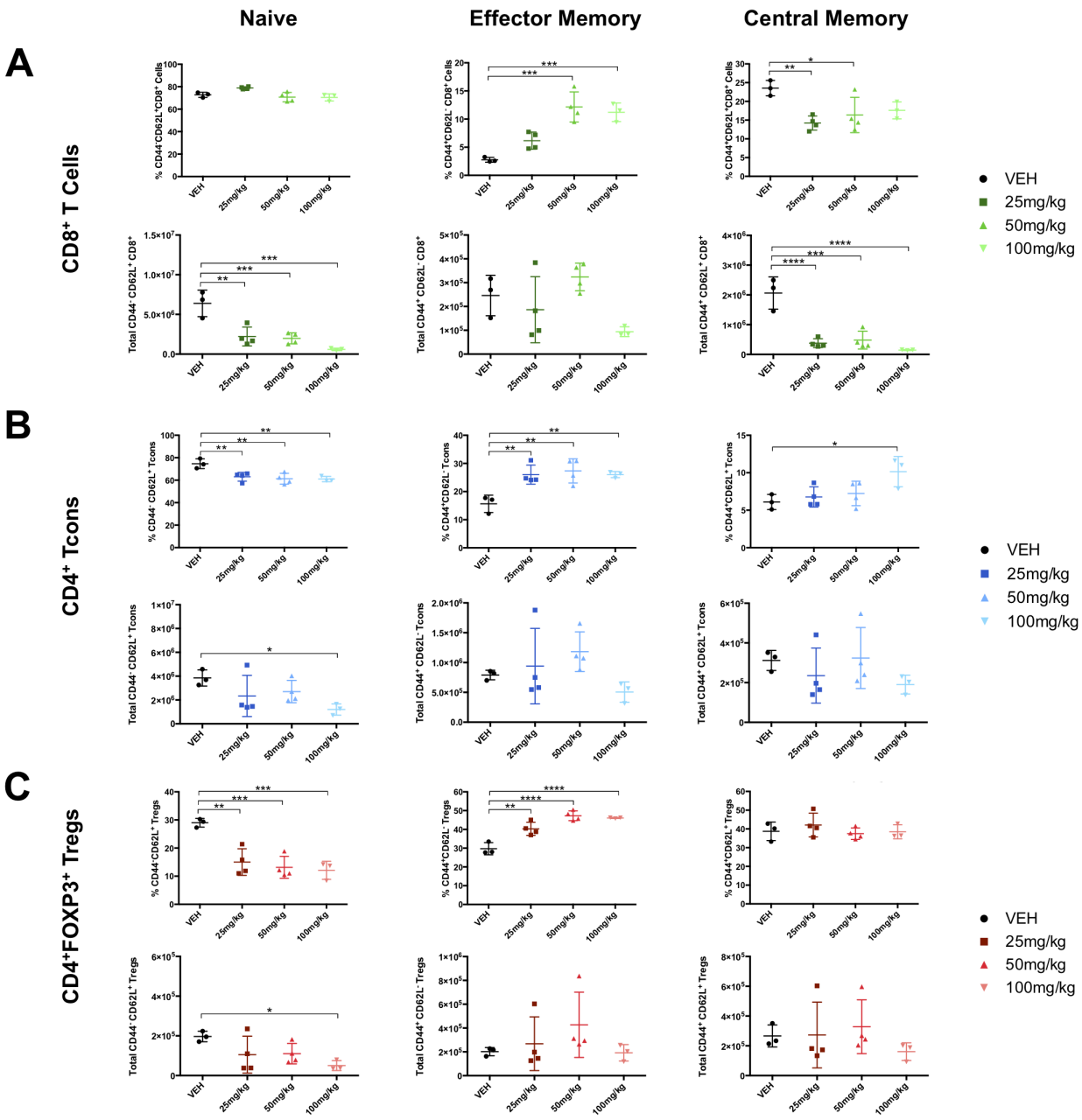

Figure 4.7: There is a decrease in naive T cells while effector memory T cell subsets are enriched in mice treated with ABT-199. Proportions of naive ($CD44^{low}CD62L^{high}$), effector memory ($CD44^{high}CD62L^{low}$), and central memory ($CD44^{high}CD62L^{high}$) T cells, shown as percentages (top) and absolute numbers (bottom). (a) $CD8^+$ T cells (b) Tcons (c) Tregs. n≥3 for each group. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

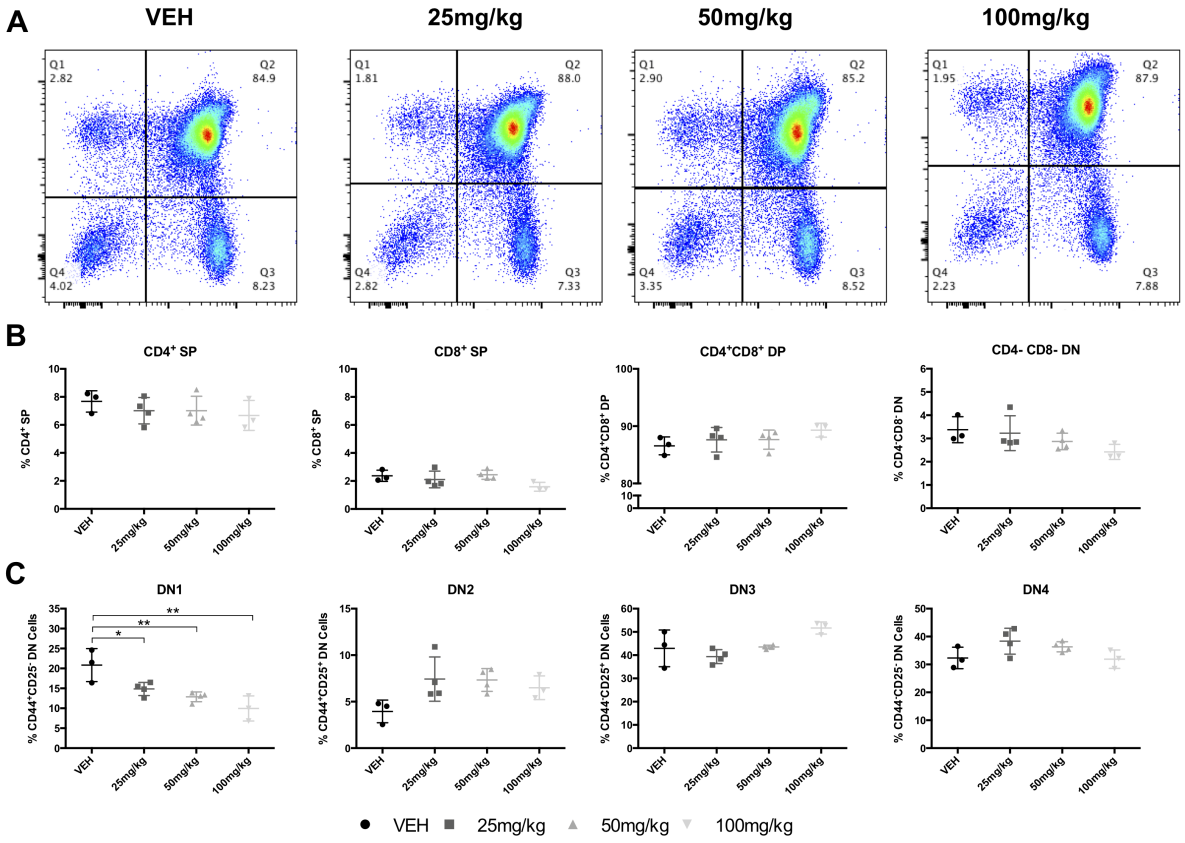

Figure 4.8: T cell development in the thymus is only minimally affected by ABT-199 treatment. (a) Representative flow plots of thymi from each treatment group. (b) Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP, and $CD4^-CD8^-$ DN thymocytes. (c) Percentages of DN subsets: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). $n \geq 3$ for each group. Data represented as means $\pm$SEM. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

subsets. However, all T cells did have decreased depolarization in response to the BAD and NOXA+BAD peptide treatments, which is likely indicative of a shift away from BCL-2. Tcons and Tregs had a greater reduction in depolarization from the BAD peptide compared to $CD8^+$ T cells (Fig. 4.9b). Intracellular flow of the surviving cells showed that all three T cell subsets had increased levels of MCL-1 and BCL-X_L, as well as decreased levels of BIM. However, while $CD8^+$ T cells and Tcons also had significantly higher levels of BCL-2, Tregs did not upregulate BCL-2 after ABT-199 treatment (Fig. 4.9c-d). This data indicates that $CD8^+$ T cells, Tcons, and Tregs respond differently to ABT-199 at the level of BCL-2 family proteins.

4.2.4 T cells that have developed in the presence of ABT-199 maintain normal T cell proportions but have long-term reprogramming of their BCL-2 repertoire

It has been well characterized that the BCL-2 proteins have differing levels of expression and influence throughout T cell development [142, 143, 144, 146, 147]. We therefore wanted to determine if the addition of a BCL-2 inhibitor from hematopoietic stem cell to mature T cell would lead to global changes in the T cell repertoire or long-term reprogramming of the BCL-2 family. To evaluate this we utilized a congenic transplant model. Host $CD45.1^+$ mice were given a lethal dose of γ-irradiation and transplanted with T cell-depleted bone marrow from donor $CD45.2^+$ mice. Mice were then treated with 25mg/kg or 50mg/kg of ABT-199 daily for 28 days post-transplant. ABT-199 was well tolerated for the duration of the treatment period, as mice did not experience weight loss during treatment, and did not weigh less than vehicle controls two and three months after the transplant. Spleen weights were also not affected by ABT-199 up to three months post-transplant (Fig. 4.10a). Mice did have reduced thymocyte counts one month post-transplant, and lower splenocyte counts two and three months post-transplant. Lymph node cell counts were not significantly affected,

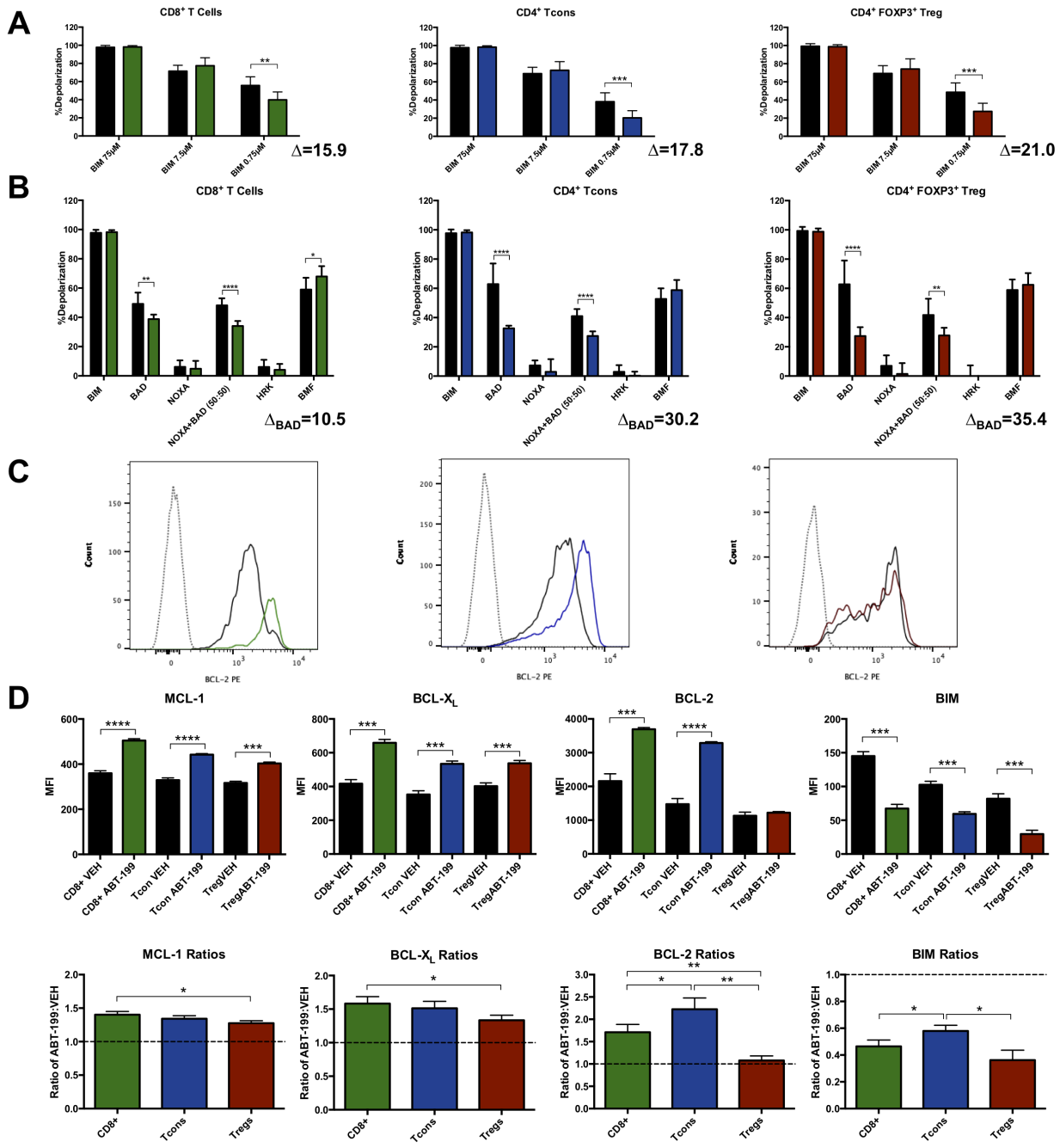

Figure 4.9: After 7 days of ABT-199 treatment, $CD8^+$ T cells and Tcons have increased BCL-2 protein levels, but Tregs do not. FOXP3-IRES-GFP mice were treated daily for seven days with 50mg/kg ABT-199 or vehicle control. (a) $CD8^+$ T cells, Tcons, and Tregs were incubated with increasing concentrations of BIM peptide to determine apoptotic priming and (b) specific BH3-only proteins to determine anti-apoptotic dependency. (c) Representative flow plots of BCL-2 MFI for $CD8^+$ T cells (green), Tcons (blue) and Tregs (red). IgG isotype control is in black. (d) Protein levels of $CD8^+$ T cells, Tcons, and Tregs. Data is shown as both MFI (top) and as ratios of stimulated protein levels compared to unstimulated. n=3 mice for each group. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

although there was a trend towards slightly reduced cell numbers in the lymph nodes as well (Fig. 4.10b).

T cell chimerism was determined via flow cytometric analysis post-transplant through cell surface expression of CD45.2 (donor) and CD45.1 (host). One month after transplant, there was a relatively high proportion of residual host cells compared to donor cells in the spleen and lymph nodes, but the thymus was entirely donor cells, suggesting that the remaining host cells were not new but residual mature cells that survived the irradiation (Fig. 4.11a). By months 2 and 3, as the donor cells continued to engraft, the percentage of host lymphocytes was vastly reduced (Fig. 4.11a). Flow cytometric analysis of the spleen revealed that the residual host cells were primarily T cells (Fig. 4.11b). The number donor T cells was low one month post-transplant, but they returned to more normal levels by two months post-transplant (Fig. 4.11b) [239].

We next wanted to determine if the presence of ABT-199 pressure throughout the entirety of T cell development would recapitulate the changes in T cell repertoire observed in the 7 day treatment (Figs. 4.6-4.8). Unlike the 7 day treatment, mice treated for 28 days following transplant did not not have any significant differences in proportions of $CD8^+$ T cells, Tcons, and Tregs one month post-transplant (Fig. 4.12a). There was a slight decrease in the proportion of $CD8^+$ T cells two months post-transplant, but this did not persist as treated mice analyzed three months post-transplant had equal percentages of $CD8^+$ T cells compared to vehicle control mice. There was also a decreased proportion of total $CD4^+$ T cells at the three month timepoint, but no change in the percentage of Tcons versus Tregs. The most notable difference was a reduction in absolute cell numbers two months post-transplant for all three T cell subsets (Fig. 4.12b). In sum, ABT-199 treatment for one month following transplant did not cause any overt changes in T cell subsets, but it did cause a non-specific reduction in cellularity that was observable two months post-transplant.

We also assessed changes in naive, effector memory, and central memory within each donor T cell subset. Percentages of these populations remained unchanged for all three T cell

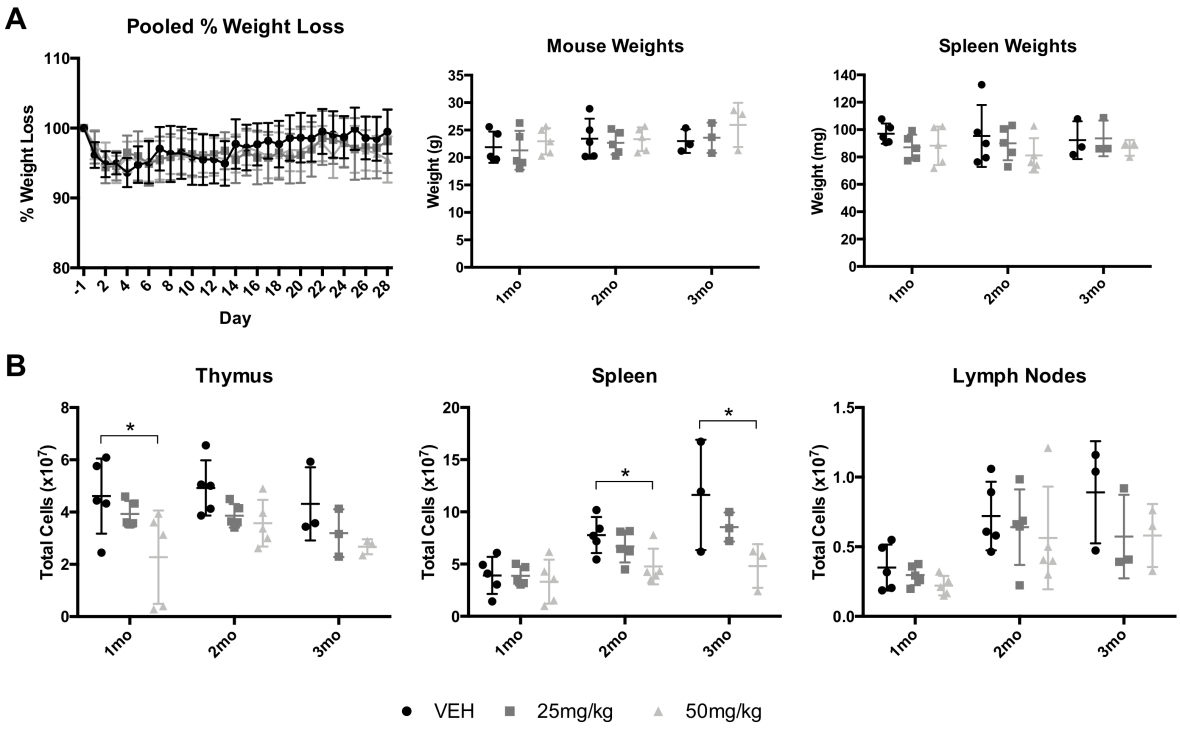

Figure 4.10: Mice treated with ABT-199 have reduced thymocyte counts one month post-transplant, and reduced splenocyte counts 2 and 3 months post-transplant. CD45.1$^+$ recipient mice were irradiated and transplanted with CD45.2$^+$ bone marrow. Mice were treated with 25mg/kg or 50mg/kg ABT-199 daily for 28 days. (a) Mouse body weights during daily ABT-199 treatment as well as body and spleen weights 1, 2, and 3 months post-transplant. (b) Total cell counts from the thymus, spleen, and lymph nodes 1,2, and 3 months post-transplant. n=5 mice/group for the 1 and 2 month timepoints and n=3 for the 3 month timepoint. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

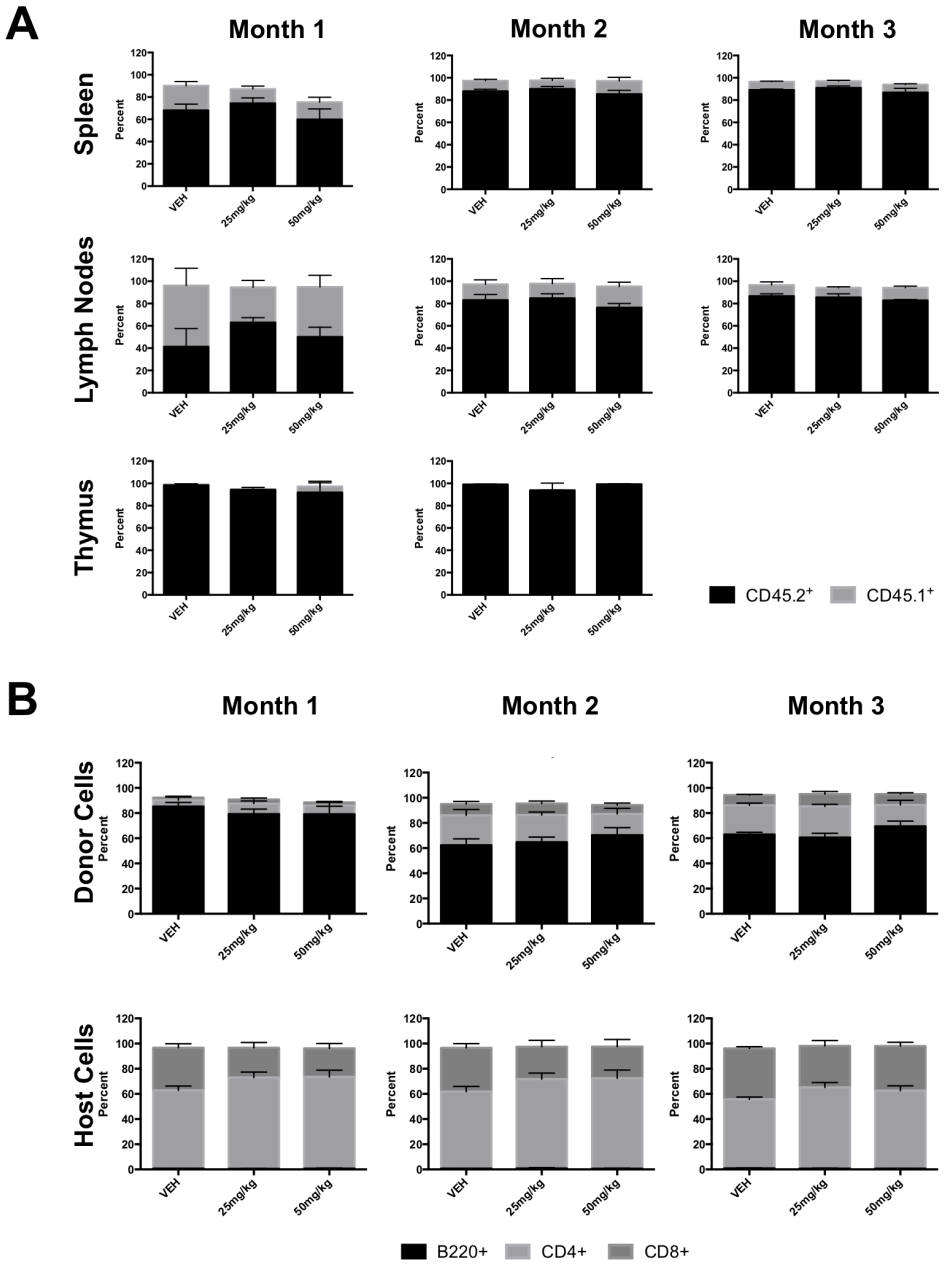

Figure 4.11: By 2 months post-transplant, most lymphocytes are donor-derived with a small subset of remaining host T cells. (a) Proportions of donor ($CD45.2^+$) and host ($CD45.1^+$) lymphocytes from the spleen, lymph nodes, and thymus of transplanted mice. (b) Proportions of $B220^+$ B cells, $CD4^+$ T cells, and $CD8^+$ T cells in the donor and host lymphocyte populations. n=5 mice/group for the 1 and 2 month timepoints and n=3 for the 3 month timepoint. Data represented as means ±SEM. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

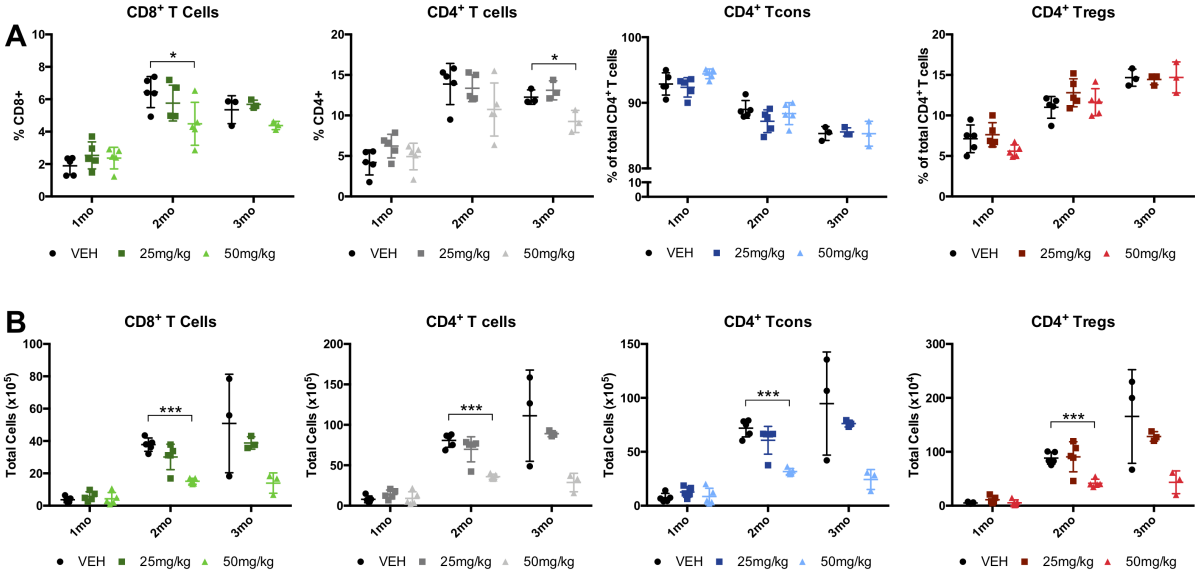

Figure 4.12: The proportions of CD8$^+$ T cells, Tcons, and Tregs stay consistent with ABT-199 treatment but absolute numbers decrease for all subsets. (a) Percentage and (b) Absolute number of CD8$^+$ T cells, Tcons, and Tregs, shown as subsets of total CD4$^+$ T cells isolated from the spleen at 1, 2, and 3 months post transplant. n=5 mice/group for the 1 and 2 month timepoints and n=3 for the 3 month timepoint. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

subsets (Fig. 4.13a-c). As observed in the global $CD8^+$ T cell, Tcon, and Treg compartments, total numbers of naive, effector memory, and central memory cells from treated mice were reduced compared to controls two months post-transplant (Fig. 4.13a-c). This data further indicates that long-term ABT-199 treatment during T cell development and maturation does not skew the proportions of T cell subsets over time. Lastly, thymocyte development was evaluated one, two, and three months after bone marrow transplant. There were no overt changes in the proportions of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP or $CD4^-CD8^-$ DN thymocytes (Fig. 4.14a-b). There were slight differences in the DN3 and DN4 population, but they were not dose dependent (Fig. 4.14c.) Overall, the percentages of both developing and mature T cells were relatively unchanged even after 28 days of ABT-199 pressure in a transplant setting, and the only significant difference observed was a nonspecific decrease in lymphocyte numbers two months post-transplant.

Although there were no overt differences in the percentages of $CD8^+$ T cells, Tcons, and Tregs, we wanted to ascertain if there were any changes in these T cell subsets at the level of the BCL-2 family. As observed in the seven day treatment model, donor $CD8^+$ T cells and Tcons significantly upregulated BCL-2. Donor Tregs also upregulated BCL-2 but to a lesser extent, and primarily upregulated BCL-X_L(Fig. 4.15a). $CD8^+$ T cells and Tcons, but not Tregs also upregulated BIM at the highest treatment dose (50mg/kg). Because ABT-199 was administered for the entirety of T cell development, we also wanted to evaluate if the BCL-2 proteins were affected in developing thymocytes. Similar to mature cells in the spleen, treated CD8 and CD4 SP thymocytes had significantly higher levels of BCL-2 compared to vehicle control. DN cells also upregulated BCL-2 levels, while DP cells, which downregulate BCL-2 and upregulate BCL-X_L, do not have an increase in BCL-2 in response to treatment (Fig. 4.15b) [141]. DN and SP cells also have increased levels of MCL-1, and DP thymocytes slightly upregulate BCL-X_L. Therefore, despite the proportions of T cell subsets remaining largely unchanged, we do observe differences in the BCL-2 family repertoire of both mature and developing T cells in response to ABT-199 pressure post-transplant.

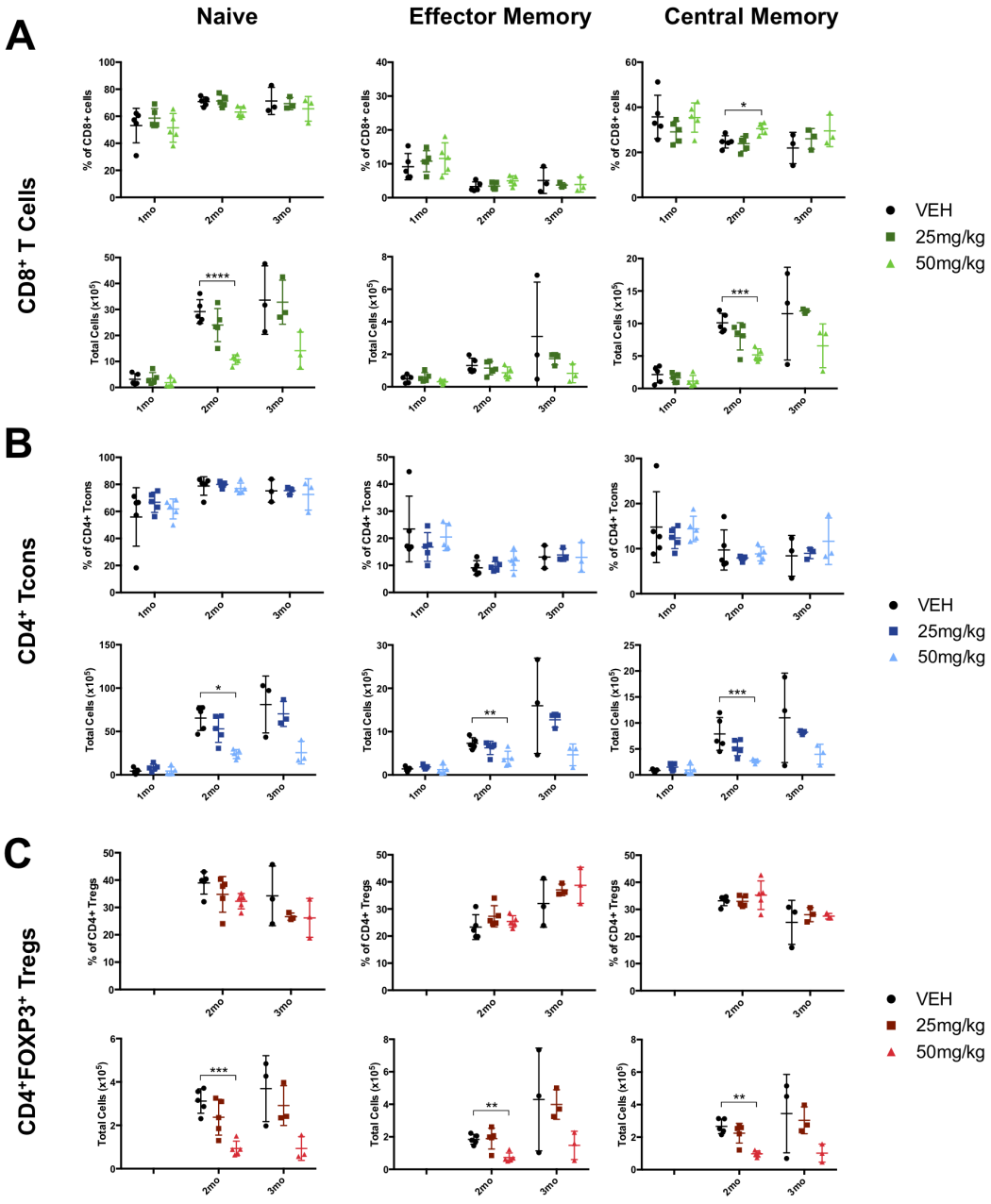

Figure 4.13: ABT-199 does not affect naive, effector memory, or central memory T cell subsets but does reduce overall cell numbers of all subsets. Proportions of naive ($CD44^{low}CD62L^{high}$), effector memory ($CD44^{high}CD62L^{low}$), and central memory ($CD44^{high}CD62L^{high}$) T cells, shown as percentages (top) and absolute numbers (bottom). (a) $CD8^+$ T cells (b) Tcons (c) Tregs. n=5 mice/group for the 1 and 2 month timepoints and n=3 for the 3 month timepoint. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

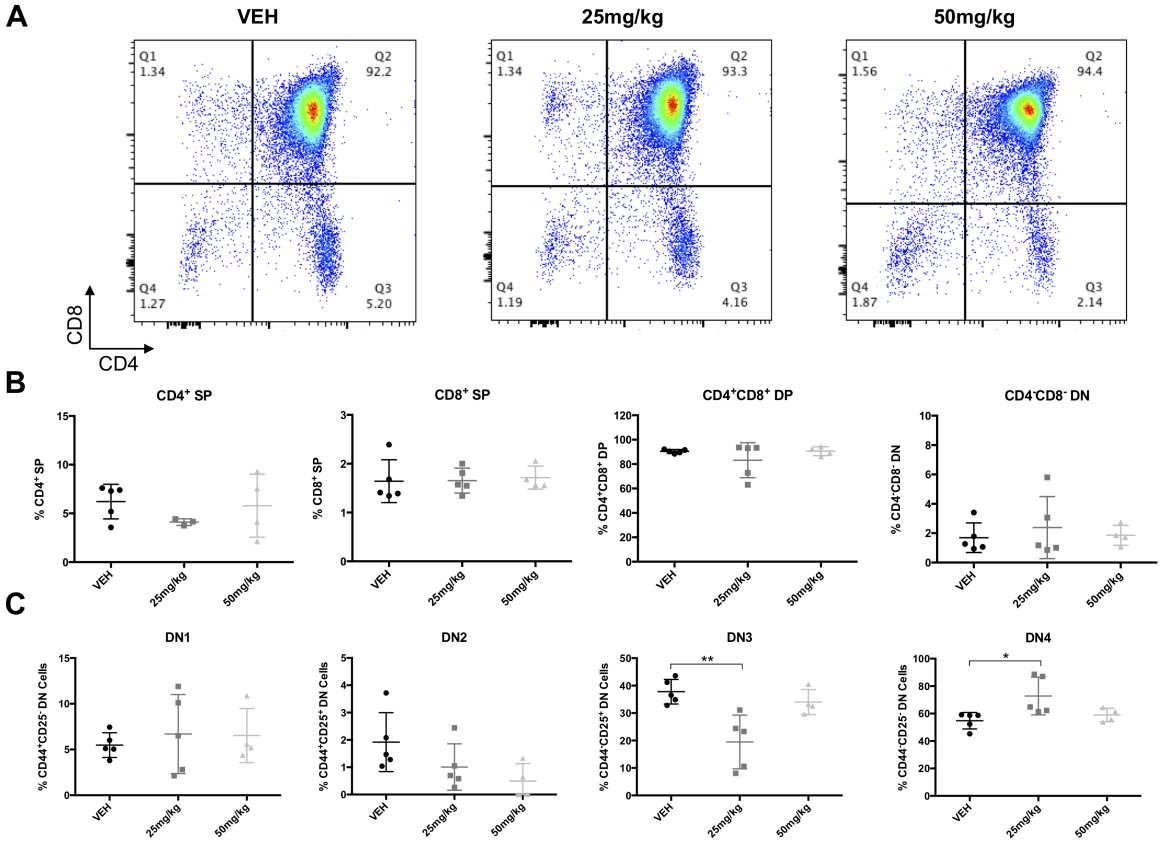

Figure 4.14: ABT-199 treatment during immune reconstitution does not affect thymocyte development. (a) Representative flow plots of thymi from each treatment group. (b) Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP, and $CD4^-CD8^-$ DN thymocytes. (c) Percentages of DN subsets: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). n=5 mice/group for the 1 and 2 month timepoints and n=3 for the 3 month timepoint. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

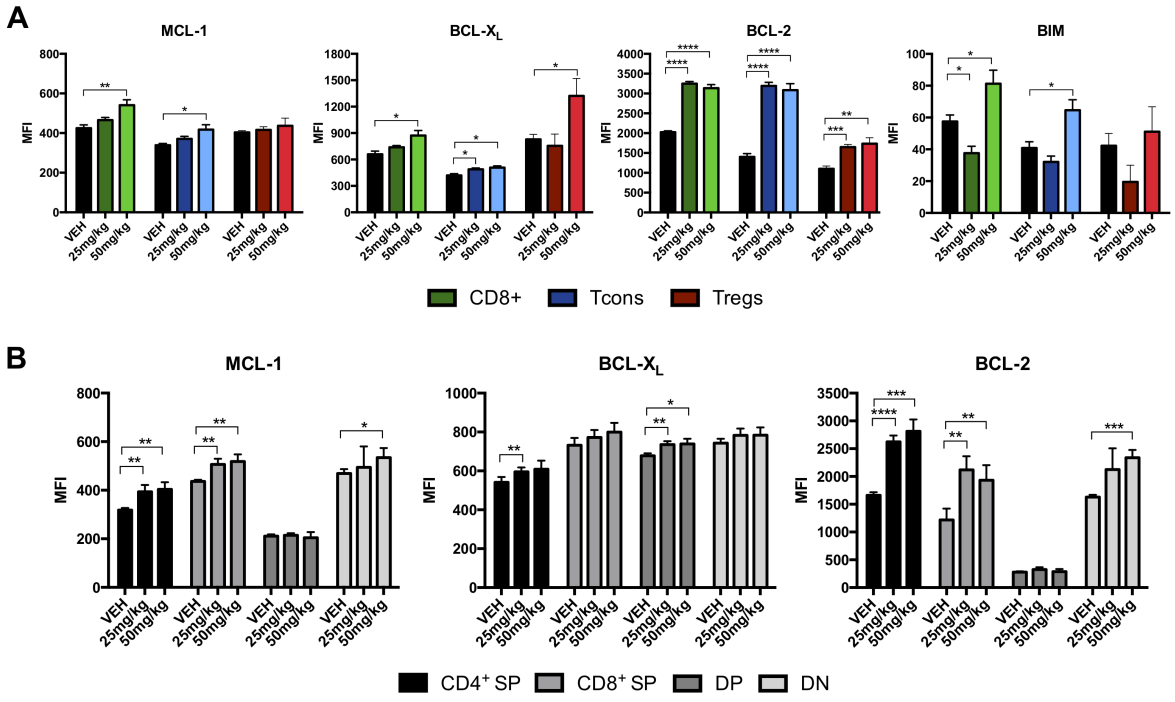

Figure 4.15: CD8$^+$ T cells and Tcons have increased protein levels of BCL-2, while Tregs primarily increase BCL-X$_L$ (a) Protein levels of CD8$^+$ T cells, Tcons, and Tregs isolated from the spleen 1 month post-transplant. (b) Protein levels of CD4$^+$ SP, CD8$^+$ SP, CD4$^+$CD8$^+$ DP, and CD4$^-$CD8$^-$ DN thymocytes 1 month post-transplant. Data is shown as MFI of each antibody subtracted from the IgG isotype control. n=5 mice/group. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

We next wanted to determine if any of the changes to the BCL-2 repertoire persisted long-term after the cessation of ABT-199. After treating for 28 days post-transplant, mice were monitored for an additional two months, then T cells were isolated and evaluated. Mature donor $CD8^+$ T cells and Tregs isolated from the spleen still had increased levels of all three anti-apoptotic proteins, while Tcons had slightly increased levels of MCL-1 and BCL-X_L, but it was not statistically significant (Fig. 4.16a). BIM protein levels had returned to baseline. In the thymus, the levels of BCL-2 had normalized, but interestingly the levels of BCL-X_L were higher in all developing populations, and there was an increase in MCL-1 in DN cells (Fig. 4.16b). All three mature T cell subsets were more primed to die compared to vehicle controls, but $CD8^+$ T cells increased their priming to a slightly higher extent than Tregs (23.3% vs. 16.0% increase in depolarization for $CD8^+$ T cells vs. Tregs) (Fig. 4.16c). We also assessed if there were any long-term changes in anti-apoptotic dependency with BH3 profiling. $CD8^+$ T cells had no significant changes in depolarization with any of the peptide treatments, however Tcons and Tregs did have a slight increase in depolarization with the BAD+NOXA treatment, suggesting a slight shift towards MCL-1/A1 dependency (Fig. 4.16c). Therefore, even two months after ceasing ABT-199 treatment, we observed long-term changes to the BCL-2 repertoire in developing and mature T cells after exposing them to long-term pressure post-transplant.

4.3 Conclusions

The BCL-2 family of proteins are important regulators of immune system homeostasis, and they play key roles throughout T cell development, activation, and contraction [18, 240]. As BH3 mimetics such as ABT-199 continue to gain clinical relevance, it is critical to evaluate how long-term treatment of patients with BCL-2 family modulators affects the immune system over time. We hypothesized that expanding T cell subsets would have diverging sensitivities to ABT-199, due to the differential dependency patterns of $CD8^+$ T cells, Tcons, and Tregs. Most of what is known about BCL-2 family expression during T cell activation

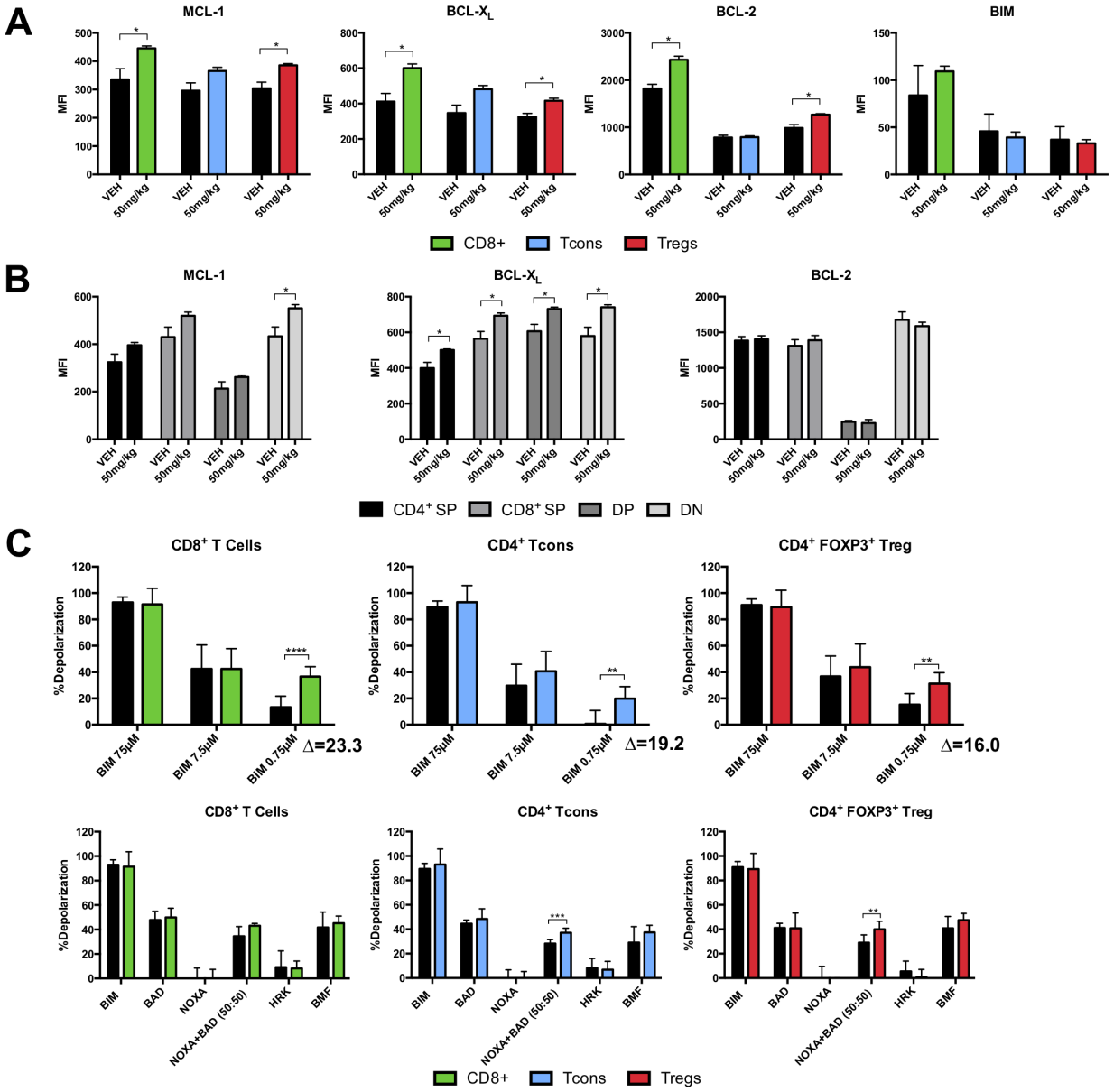

Figure 4.16: There are long-term shifts in the BCL-2 family repertoire in T cell subsets 3 months post-transplant. (a) Protein levels of CD8$^+$ T cells, Tcons, and Tregs isolated from the spleen 3 months post-transplant. (b) Protein levels of CD4$^+$ SP, CD8$^+$ SP, CD4$^+$CD8$^+$ DP, and CD4$^-$CD8$^-$ DN thymocytes 3 months post-transplant. Data is shown as MFI of each antibody subtracted from the IgG isotype control (c) CD8$^+$ T cells, Tcons, and Tregs were permeabilized and incubated with increasing concentrations of BIM peptide to determine apoptotic priming (top) as well as specific BH3-only proteins to determine their anti-apoptotic dependency (bottom) 3 months post-transplant. n=3 mice/group. Data represented as means ±SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

and expansion is from the study of $CD8^+$ T cells. Naive $CD8^+$ T cells are thought to depend primarily on BCL-2 and MCL-1 [122, 142, 33]. During activation and expansion, $CD8^+$ T cells upregulate MCL-1 and BCL-X_L [57, 143, 144]. But while BCL-X_L is upregulated, it may be redundant due to the fact that *Bcl-x* knockout mice can mount a normal immune response and generate effector and memory $CD8^+$ T cells [241]. BCL-2 has a more diverse role in the expansion and contraction of $CD8^+$ T cells. While short lived effector cells primarily downregulate BCL-2, cells destined to become memory cells, or memory precursor effector cells, continue to express BCL-2 at high levels, likely to combat the increased level of BIM that occurs during the contraction phase of the immune response [146, 147, 149]. Once cells differentiate to the memory phenotype, they appear to be more reliant on MCL-1 [147]. Like naive $CD8^+$ T cells, naive Tcons are also reliant on BCL-2 and MCL-1 [122, 142, 33]. Tcons also upregulate MCL-1 and BCL-X_L when activated, but the BCL-2 proteins that dictate memory cell survival in Tcons during contraction of an immune response are not as clear [143, 145]. Even less well characterized are the BCL-2 proteins that govern Treg survival during activation and memory cell generation. Genetic deletion models have demonstrated that MCL-1 is critical for maintaining the Treg population, while BCL-2 and BCL-X_L are largely dispensable [36]. There is evidence that, like $CD8^+$ T cells and Tcons, human Tregs also transiently upregulate BCL-X_L in response to stimulation [170]. However, the role specific BCL-2 proteins play in Treg development and activation is still an ongoing area of research.

As expected, *ex vivo* stimulated and expanded $CD8^+$ T cells, Tcons, and Tregs differ in their resistance to ABT-199 (Fig. 4.1). Unstimulated cells for all three subsets had relatively similar EC_{50} values for ABT-199, but stimulation and expansion with CD3/CD28 and IL-2 led to a large decrease in ABT-199 sensitivity for $CD8^+$ T cells and a smaller shift in Tcons but no change in Tregs. This is consistent with the fact that T cell activation leads to upregulation of other anti-apoptotic proteins such as MCL-1 and BCL-X_L, therefore targeting BCL-2 would be less effective in cells shifting from primarily naive to effector and

memory T cells (Fig. 4.2) [57, 143, 144]. Naive Tregs are already less dependent on BCL-2, but interestingly we observed a greater increase in the ratio of BCL-2 protein levels in Tregs compared to their unstimulated counterparts (Fig. 4.2c). This may partially explain why Tregs did not experience increased resistance when BCL-2 was targeted, as cells that have increased BCL-2 are more sensitive to BCL-2 inhibition [238].

The upregulation in protein levels of BCL-2 correlates with increased mRNA expression for all three T cell subsets (Fig. 4.2b). MCL-1 protein was also high, however, mRNA expression of MCL-1 was minimally affected or even downregulated after stimulation. It is possible that MCL-1 protein was not due to increased expression, but stabilization by interaction with other BH3-only proteins. It has previously been demonstrated that while NOXA binding to MCL-1 leads to its ubiquitination and subsequent degradation, BIM binding MCL-1 leads to increased protein stability [242]. In fact, BIM:MCL-1 interaction after treatment with ABT-199 has been characterized as a resistance mechanism to effective therapy in AML [197]. It is therefore possible that increased levels of BIM in response to expansion, as well as the blockade of the BIM:BCL-2 interaction by ABT-199, is enough to stabilize MCL-1, leading to increased protein without any upregulation in mRNA expression (Fig. 4.2b-c). BCL-X_L was upregulated in mRNA for $CD8^+$ T cells and Tcons but not Tregs. However, all three T cell subsets had increased protein levels. One possibility is that Tregs upregulate BCL-X_L more transiently, but the presence of BCL-X_L is more stable long-term due to its longer half-life [170]. A greater understanding of the dynamic changes in BCL-2 family dependency across T cell subsets as they differentiate from naive to memory cells will allow us to further understand and predict how BH3 mimetic treatments may affect the global T cell repertoire.

We next explored how the addition of ABT-199 throughout the expansion process affected T cell sensitivity to the drug. Interestingly, we show that expansion in the presence of ABT-199 leads to increased resistance to ABT-199 in Tregs and to a lesser extent Tcons, but not in $CD8^+$ T cells (Fig. 4.3). This was in direct contrast to the resistance patterns observed

in T cells expanded without ABT-199. We hypothesized that Tregs were more effective at adapting in response to the presence of ABT-199 compared to $CD8^+$ T cells. BH3 profiling revealed shifts towards BCL-X_L and MCL-1 dependency in Tregs and Tcons, but not $CD8^+$ T cells (Fig. 4.4a). Evaluation of protein levels also confirmed that, compared to $CD8^+$ T cells, Tregs and Tcons had a greater increase of BCL-X_L relative to cells expanded without ABT-199. Tcons also maintained higher levels of BCL-2, which may explain the smaller increase in resistance observed in Tcons. MCL-1 was upregulated as well, however there were no major differences in the extent of MCL-1 upregulation across T cell subsets (Fig. 4.4c). At the mRNA level, $CD8^+$ T cells and Tcons experience relatively few changes in expression relative to cells expanded without ABT-199. However, Tregs slightly upregulate *A1* and *Bcl-w*. Little has been described about the role of BCL-W and A1 in T cell survival. A1 is transiently upregulated during TCR engagement and T cell activation, but is likely redundant along with the upregulation of other anti-apoptotic proteins such as MCL-1 and BCL-X_L, and its role as a potential resistance factor to BH3 mimetic treatment has only been minimally explored [243, 85, 204]. However, in a quantitative model of BCL-2 family regulation, the additional increase of A1 may be sufficient for Tregs to remain below the apoptotic threshold in the presence of ABT-199 [18]. A lack of reliable antibodies has made the study of A1 and BCL-W more difficult than the more well characterized anti-apoptotic proteins, but these results emphasize the importance of simultaneous evaluation of all BCL-2 proteins, especially in the context of manipulating these proteins with the use of BH3 mimetics. As we learn more about the regulation of this dynamic family of proteins, it may become an attractive therapeutic strategy to modulate the survival capacity of T cells expanded for infusion. This could be further extrapolated to emerging therapies such as CAR T cells [244].

As we predicted based on our *in vitro* data, $CD8^+$ T cells were highly sensitive to ABT-199 *in vivo* at even the lowest treatment dose (Fig. 4.6). This corresponds to the fact that naive $CD8^+$ T cells are primarily dependent on BCL-2 for their survival [122, 142]. Tcons

and Tregs treated *in vivo* were less affected by ABT-199 treatment, and the percentage of Tregs was actually enriched at the highest treatment dose. This again correlates with Tregs being less dependent on BCL-2 compared to other T cell subsets [36]. At the protein level, both $CD8^+$ T cells and Tcons had significantly higher BCL-2 levels in response to seven days of ABT-199 treatment, while Tregs did not. This coincided with Tregs having a sightly greater reduction in apoptotic priming and a greater decrease in depolarization in response to the BAD peptide (Fig. 4.9). The lack of BCL-2 upregulation in Tregs is perhaps one reason why Tregs are not as affected by BCL-2 targeting *in vivo*. Whether this is due to Tregs lower dependency on BCL-2 at baseline or the possibility that Tregs are able to more effectively adapt in response to ABT-199 treatment remains to be explored [122, 142, 36]. Tregs from MCL-1 haploinsufficient mice had increased sensitivity to ABT-199 *in vivo*, however, this was measured after only a single high dose of ABT-199 [18]. Long-term single and combination targeting of BCL-2 family proteins in different T cell subsets is an ongoing area of research.

Unlike our *in vitro* studies, in which all T cells were pushed to an effector memory phenotype, our *in vivo* studies allowed us to investigate a more biologically relevant distribution of naive, effector memory, and central memory T cells. We found that effector memory cells were resistant to ABT-199 for all three T cell subsets, which is consistent with previous reports examining effector cells treated with ABT-737 [44, 245]. This also coincides with other anti-apoptotic proteins becoming more relevant for T cell survival during the transition from a naive to an effector memory phenotype, such as MCL-1, BCL-X_L, and A1 [57, 143, 243]. We also observed that central memory $CD8^+$ T cells were sensitive to ABT-199, while central memory Tcons and Tregs were not. BCL-2 family regulation of central memory T cell subsets has been less well characterized, but this may merit further investigation due to the diverging sensitivity patterns observed here. In addition to observing the effects of ABT-199 on mature T cell subsets, we also evaluated potential effects of BCL-2 inhibition on developing T cells in the thymus. We determined that thymocyte development was not overtly affected by seven days of ABT-199 treatment (Fig. 4.8). This is compatible with data from

murine models of BCL-2 deletion that have normal thymic development [30]. Although BCL-2 is expressed at higher levels in DN and CD4 and CD8 SP cells, these cells are likely dependent on additional anti-apoptotic proteins such as MCL-1 for their survival [35, 33]. DP thymocytes downregulate BCL-2 and primarily depend on BCL-X_L for their survival, so it was foreseeable that this population was resistant to ABT-199 [39]. The only appreciable change observed in developing thymocytes was a decrease in DN1 cells (Fig. 4.8c). Because BCL-2 levels are elevated in the DN1-3 populations before beginning to decline as cells transition to the DP phase, it is understandable that these cells were slightly sensitive to BCL-2 targeting [141]. Despite a reduction in DN1 thymocytes, the overall proportion of DN cells was not affected, nor was the global number of thymocytes.

We next wanted to observe how T cell populations would change if cells developed and matured in the presence of ABT-199 from early progenitor to mature T cell. To explore this, we employed a congenic transplant model, which allowed us to monitor engrafted donor cells that developed with ABT-199 treatment. Interestingly, we did not observe a reduction in any specific proportion of T cells after 28 days of ABT-199 treatment. Despite the fact that peripheral $CD8^+$ T cells were acutely sensitive to ABT-199 in our seven day treatment model, $CD8^+$ T cells that developed in the presence of ABT-199 had no apparent survival disadvantage compared to other T cell subsets (Fig 4.12, 4.13). The only difference observed in the treatment group was a global reduction in splenocyte count (Fig. 4.10). At the level of the BCL-2 family, $CD8^+$ T cells and Tcons had heightened levels of BCL-2, which was consistent with the seven day treatment model. Tregs only slightly upregulated BCL-2, and primarily upregulated BCL-X_L (Fig. 4.15). Our data also correlates with a recent study that looked at the apoptotic sensitivity of $CD8^+$ T cells, Tcons, and Tregs from patients post-stem cell transplant. Tregs isolated from these patients had lower levels of BCL-2 and higher levels of BIM, which made them more primed to die [216]. However, we hypothesize that treating patients with a BCL-2 specific BH3 mimetic such as ABT-199 may skew the T cell repertoire more towards Tregs, due to their low sensitivity to BCL-2 targeting, and

this may reduce the chance of developing GVHD.

In order to exclusively observe changes in T cells that had developed fully in the presence of ABT-199, we gated out any host cells that survived irradiation. However, we do observe a residual population of host lymphocytes, comprised almost entirely of T cells (Fig. 4.11). Therefore an interesting component of this model that may merit further investigation is the presence of host T cells post-transplant. There is evidence that the levels of remaining host T cells versus engrafting donor cells can be a mediator of both graft versus tumor response and GVHD [246]. It has also been demonstrated that Tregs are more resistant to irradiation induced cell death compared to other T cell subtypes and are therefore likely an enriched population of the residual host T cell population [247, 248, 249]. It may be interesting to further characterize this population and determine how long-term ABT-199 treatment affects these mature T cells as well.

The idea of modulating the BCL-2 family of proteins to improve transplant outcomes has been gaining popularity [188]. It has been characterized in multiple transgenic models that overexpression of BCL-2 can lead to increased levels of HSCs and a survival advantage in a transplant setting [250, 251]. Several studies have also demonstrated the efficacy of a transient increase in the anti-apoptotic proteins BCL-2 and BCL-X_L [189, 190]. The use of a Tat-BCL-2 fusion protein led to an increased expansion of the hematopoietic compartment from HSCs [189]. A BCL-X_L fusion protein, as well as the transient upregulation of BCL-X_L through the use of an adenoviral vector, was effective in conferring a survival advantage in a competitive transplant model [190]. However, these studies did not fully dissect the effects of these strategies on Tregs specifically. Recent work has demonstrated that treatment with ABT-737, which targets BCL-2, BCL-X_L, and BCL-W in combination with cyclosporin A post-transplant delayed the progression of GVHD and could promote skin graft survival in two distinct transplant models [124]. They also confirm that Tregs are more resistant to ABT-737 treatment and have higher levels of MCL-1. However, targeting BCL-2 and BCL-X_L in Tregs may be a less effective strategy than targeting BCL-2 alone. Recent data

has demonstrated that in human Tregs, BCL-X_L is also upregulated during activation and expansion, meaning that BCL-X_L may also play a role in maintaining Treg fitness in the transplant setting [170]. A more complete understanding of the dynamics of BCL-2 proteins in transplanted progenitor cells and developing T cells will lead to more robust and effective strategies to manipulate this family for therapeutic benefit.

Because we were administering ABT-199 through the entirety of T cell development, one interesting potential consequence was long-term reprogramming of the BCL-2 repertoire. In fact, analysis of T cells two months after cessation of ABT-199 treatment revealed that mature T cells maintained higher levels of MCL-1, BCL-X_L, and BCL-2. BH3 profiling also demonstrated that Tcons and Tregs may have shifted slightly towards MCL-1/A1 dependency compared to cells treated with vehicle control (Fig. 4.16). Interestingly, thymocytes also had long-term changes in their BCL-2 repertoire, as DN, DP, CD4 SP, and CD8 SP thymocytes all had increased levels of BCL-X_L, and DN cells had additional upregulation of MCL-1. By two months post-treatment, all of the cells developing in the thymus in the presence of ABT-199 would have matured and emigrated [226]. Therefore, there is a possibility that ABT-199 also affects earlier progenitor cells such as common lymphoid progenitors or even hematopoietic stem cells. HSCs have been described to be primarily dependent on MCL-1 [32]. We therefore would not predict that targeting BCL-2 would have a completely ablative effect on the HSC compartment. However, it is possible that inhibition of BCL-2 at this stage did not induce apoptosis but did lead to transcriptional and/or translational changes in the BCL-2 repertoire as a compensatory mechanism in response to the presence of ABT-199 in these cells. The implications of reprogramming hematopoietic stem cells with long lasting effects in a transplant setting is an area of research that would be interesting to further explore.

In conclusion, we have demonstrated here that $CD8^+$ T cells, Tcons, and Tregs have differential sensitivities to the BH3 mimetic ABT-199. We show that $CD8^+$ T cells tend to be more sensitive to ABT-199, and are less capable of becoming resistant to ABT-199

treatment during stimulation and expansion. In contrast, Tregs are at baseline more resistant to ABT-199, and also become more resistant to treatment during expansion. This work highlights the dynamism and adaptive capabilities of T cells in response to inhibition of BCL-2, and promotes further exploration of utilizing the expanding toolkit of BH3 mimetics to evaluate the roles of additional anti-apoptotic and BH3-only proteins in specific T cell subsets. This research contributes to a growing body of evidence that the immune system can be manipulated using BH3 mimetics, and this has a wide range of therapeutic implications in anti-cancer immune therapy, transplant tolerance, and autoimmunity. These studies provide critical groundwork for additional exploration of the immune system in response to BH3 mimetic pressure, both at a global immune repertoire level and at a cellular level in regards to the dynamic regulation of the BCL-2 proteins.

CHAPTER 5

FUTURE DIRECTIONS AND CONCLUSIONS

This research provides an important framework for the comprehensive assessment of the BCL-2 proteins in multiple T cell subsets. We have explored how T cell subsets are able to adapt over time to both genetic manipulation and small molecule inhibition targeting the apoptotic pathway (Figure 5.1). The results generated here have led to several interesting questions and experimental plans in order to further understand how BCL-2 modulation influences T cell homeostasis and function. This includes evaluating regulation of BCL-2 family proteins beyond the BCL-2 level, and assessing how T cell functionality is affected by long-term BH3 treatment. We also predict that ABT-199 treatment will be efficacious in reducing the severity of graft versus host disease, and that evaluation of human T cell samples to corroborate our murine data will contribute greatly to the significance of this research.

5.1 Elucidating upstream regulators of BCL-2 proteins in response to long-term pressure

At this time, we have comprehensively evaluated how ABT-199 treatment affects multiple T cell subsets, and how BCL-2 protein levels and dependency patterns change in $CD8^+$ T cells, Tcons, and Tregs. One interesting question is how upstream regulation of these proteins may change in response to BH3 mimetic treatment, and if different T cell subsets have discrete patterns of transcriptional reprogramming in this context. By isolating pure populations of $CD8^+$ T cells, Tcons, and Tregs from mice that have been treated with ABT-199 either at steady state or post-transplant, we could perform RNA sequencing or ATAC sequencing to look for evidence of transcriptional reprogramming of these T cell subsets. Furthermore, advances to these techniques have also allowed for the use of lower cell numbers, meaning we can further parse out discrete populations of naive, effector memory, and central memory

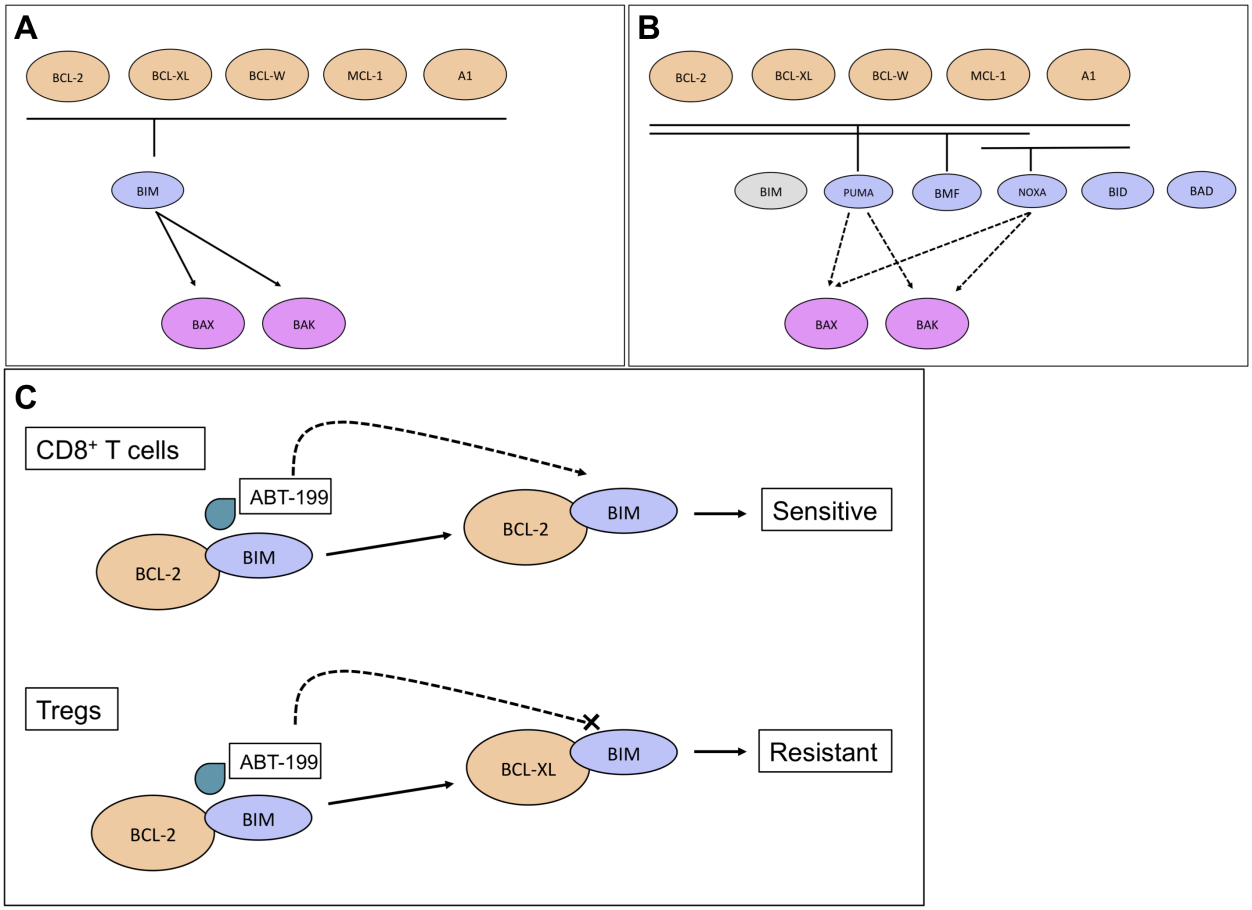

Figure 5.1: Adaptivity of T cells in response to BCL-2 family modulation. (a) and (b) T cells with an impaired ability to initiate apoptosis through loss of the BH3-only master regulator BIM upregulate additional BH3-only proteins including PUMA, BMF, and NOXA. These proteins are able to effectively bind all anti-apoptotic proteins as well as directly activate BAX and BAK. (c) T cell subsets have differing sensitivity to BCL-2 inhibition through ABT-199 treatment. In response to ABT-199, $CD8^+$ T cells primarily upregulate BCL-2 and remain sensitive to BCL-2 inhibition, while Tregs primarily upregulate and depend on anti-apoptotics that ABT-199 does not bind to, such as BCL-X_L, and are therefore more resistant to treatment.

cells. This will give us an even clearer understanding of how transcriptional regulation and signaling pathways upstream of BCL-2 regulation may differentially change across T cell subsets in response to BH3 mimetic pressure, or if these pathways change at all. Another interesting facet to explore is if the global cytokine profile changes in response to BCL-2 modulation. Because ABT-199 treatment leads to reduction of selective populations of T cells, and may also preferentially target subsets of other cytokine secreting cells that have not been fully elucidated in this project to date, there is a possibility that the cytokine profiles of these mice also change over time. This could lead to numerous differences in the regulation of T cell populations, including feedback loops from cytokines that are known regulators of T cell survival through modulation of BCL-2 family regulation such as IL-2, IL-10, and TGF-β. Cytokine analysis from serum isolated from mice treated with ABT-199 through ELISAs or multiplex cytokine arrays will be another interesting upstream regulator of BCL-2 family modulation to explore. Overall, transcriptional evaluation and cytokine profiling assays will lead to a more detailed mechanistic understanding of how upstream signaling cascades may be adapting in response to BCL-2 family modulation over time.

5.2 Determining T cell functionality after long-term BH3 mimetic treatment

We have demonstrated diverging sensitivity patterns of various T cells subsets in response to ABT-199 treatment. However, despite the fact that some cell subsets, such as Tregs and effector memory cells, are more resistant to ABT-199, a key question that needs to be addressed is whether these cells, and any remaining cells in sensitive populations, retain their functionality after treatment. There are multiple functional assays that will be performed in order to determine if BCL-2 inhibition alters T cell function, and having a clear understanding of these results will be critical before moving forward with BCL-2 modulation in a clinical setting.

One of the most important functional tests that will need to be performed is the evaluation of Treg suppression. Based on the limited cell death we observe in the Treg population after ABT-199 treatment, we hypothesize that Treg suppressive function will be retained. This is also the ideal outcome in the context of enriching Tregs to induce tolerance and prevent GVHD, as it will be key that Tregs are capable of suppressing the proliferation of activated autoreactive cells. We will therefore perform *ex vivo* suppression assays on Tregs that have been treated *in vivo* with ABT-199, either in a steady state model or after bone marrow transplant. If suppressive function of Tregs is in fact impaired due to ABT-199 treatment, we may need to further evaluate the timing and dosing of ABT-199 or look into possible combination therapies to find an optimal treatment strategy that maintains, not only Treg numbers, but their function as well.

Because of the critical role BCL-2 proteins play in thymocyte development, another interesting question is whether or not we are affecting negative selection in the thymus. We observed no overt changes in double positive and single positive thymocyte populations, but there were subtle changes in the DN1 population in the seven day treatment model, and in the transplant setting BCL-2 protein levels in thymocytes were modified in response to ABT-199 treatment. We therefore want to evaluate if the diversity of the T cell repertoire is affected in response to ABT-199 treatment. We will isolate mature T cells after they emerge post-transplant and perform TCR-Vβ screening. We hypothesize that we may see an increase in diversity if BCL-2 treatment has impaired the negative selection process, and the potential consequences of that will be explored in future experiments.

Because we observe differences in apoptotic sensitivity via BH3 priming in response to ABT-199, an additional area of interest is how T cells may change in response to more diverse apoptotic stimuli. This could include a multitude of different therapeutically relevant treatments, including but not limited to: cytokine deprivation, ionomycin, irradiation, cyclosporin, and other BH3 mimetics such as ABT-263, MCL-1 or BCL-X_L-specific small molecules, and various stapled peptide therapeutics. It will be especially interesting if $CD8^+$

T cells, Tcons, and Tregs experience diverging sensitivity patterns to any of the stimuli tested, further indicating differential mechanisms of adaptivity in response to ABT-199 treatment. These studies could even be further extrapolated by treating T cell subsets with BH3 mimetics other than ABT-199. For example, would treatment with a small molecule that targets multiple anti-apoptotic proteins, such as ABT-263, have similar patterns of sensitivity and resistance? Addressing these questions in more detail will provide valuable insights into how the inhibition of one or more anti-apoptotic proteins affects global apoptotic pathway regulation in T cell subsets.

5.3 Improving efficacy of BH3 mimetics in an acute graft versus host disease model

Treatment with ABT-199 *in vivo* induces apoptosis in $CD8^+$ T cells but not Tregs. The enrichment of immunosuppressive cells such as Tregs in hematopoietic stem cell transplantation is gaining prevalence as a therapeutic strategy to reduce the incidence and severity of graft versus host disease. As described above, there is a growing body of evidence to support the enrichment of the Treg population during transplant in order to reduce or prevent GVHD. Most commonly used treatment regimens to date cause general immune suppression (such as calcineurin inhibition via cyclosporin), but it would be more efficacious to target naive cytotoxic cells directly while sparing immunosuppressive populations.This makes the use of BH3 mimetics in this context particularly interesting. Specifically ABT-199, as we and others have demonstrated ABT-199 can preferentially induce apoptosis in $CD8^+$ T cells but not Tregs. We also demonstrate that with ABT-199 treatment effector memory cells are enriched while naive cells are targeted, which is also preferred in the context of GVHD [252]. We therefore will investigate if the addition to ABT-199 treatment in a complete mismatch model of acute GVHD provides prolonged survival or reduced disease severity. We will re-evaluate the global immune cell repertoire and determine changes in naive, effector,

and memory T cells, as well as determine if other myeloid or lymphocyte subsets change. Because the development of GVHD can depend on multiple subsets of the immune system, including T cells, B cells, and other antigen presenting cells, it will be important to comprehensively elucidate the effects of ABT-199 on global immune homeostasis in this context. We will also determine changes in BCL-2 protein levels and dependency patterns in this system, as the increased activation of T cells may affect how these proteins are regulated in response to BH3 mimetic pressure.

There are several key questions that will need to be further explored during the development of this model. First, timing and dosing of ABT-199 administration will be an important factor to consider. The donor immune cells begin to expand rapidly after transplant, and most models of GVHD require the transplantation of mature T cells in addition to bone marrow. Activated T cells are much more resistant to ABT-199 due to the upregulation of additional anti-apoptotic proteins, therefore targeting naive $CD8^+$ cells for apoptosis before there is significant activation will likely be critical [143, 144]. Another important question will be if ABT-199 has the ability to prevent apoptosis as a single agent, or if combination with other therapies will be more efficacious. We do see significant cell death *in vivo* in naive $CD8^+$ T cells with ABT-199 alone, however, we will need to assess if these results also translate to a more activated immune system. It may be synergistic to use ABT-199 with more traditional GVHD therapies such as cyclosporin, and it may be possible to use lower doses of both compounds to limit potential toxicity or off target effects if they have complimentary activity in this setting. Additionally, there are multiple described models of GVHD, ranging in severity and levels of MHC mismatch. It may be prevalent to test the efficacy of ABT-199 in multiple disease models as well.

5.4 Translation to human T cell subsets and patient samples

It will be crucial to evaluate how this data translates to human T cell subsets. There is a high level of conservation between the BCL-2 family in mice and humans, but there are structural

differences between some proteins that may lead to conflicting results between murine and human samples. For example, MCL-1 and A1/BFL-1 are less well conserved between mice and humans, and it has made the evaluation of MCL-1 and BFL-1 specific inhibitors more difficult in the context of immune modulation. Steps have been taken to circumvent the problem of targeting MCL-1 in mice by generating mice with humanized MCL-1, however it would be more impactful to directly evaluate the effects of BH3 mimetics on T cells isolated from patient samples or healthy volunteers [253]. We will begin by isolating T cells from healthy volunteers and expanding them *ex vivo* in the presence or absence of BH3 mimetic pressure and evaluating if our murine results agree with the changes observed in human T cell subsets. An area of future interest will be to isolate T cells from patient samples, especially patients that have been treated with ABT-199 or ABT-263, and evaluating the BCL-2 repertoire and dependency patterns in those cells as well. While sample collection and heterogeneity might provide a challenge in this setting, as BH3 mimetic treatment continues to gain prevalence in the clinical setting this will be of significant interest to evaluate in more detail.

5.5 Conclusions

The BCL-2 family of proteins are critical regulators of apoptosis and targeting these protein interactions to induce cell death has continued to gain relevance. BH3 mimetics developed for use as anti-cancer therapeutics have demonstrated significant preclinical and clinical success, and there is growing interest in repurposing these compounds for use in modulating BCL-2 proteins in other susceptible cells. However, relatively little information is available regarding the effects long-term BCL-2 manipulation has over time, in the context of the global immune landscape as well as at the BCL-2 protein level in individual cells.

We have now characterized two different models of BCL-2 family manipulation in T cells: genetic deletion of *Bim*, and BCL-2 inhibition using ABT-199. We have demonstrated that loss of BIM in T cells leads to compensation through upregulation of multiple other BH3-only

proteins, and interestingly this compensation was not identical between $CD8^+$ T cells and $CD4^+$ T cells. These findings led us to postulate that differential compensatory mechanisms likely existed between T cell subsets, and this would create a therapeutic window that would allow for the targeting of specific T cells while sparing others. Indeed, BCL-2 inhibition with ABT-199 preferentially induced apoptosis in $CD8^+$ T cells while Tregs remained relatively resistant. Analysis of anti-apoptotic protein levels and dependency patterns revealed divergent changes in the BCL-2 family of these T cell subsets, with Tregs shifting away from BCL-2 dependency to a greater extent than $CD8^+$ Tcons.

This research provides a crucial framework for the evaluation of BCL-2 family interactions in T cell subsets in response to BH3 mimetic treatment. It also contributes to a growing body of work demonstrating the potential clinical benefit that can be obtained from utilizing BH3 mimetics in the context of immune system modulation. This has implications for a multitude of therapies, including anti-tumor immunotherapy, autoimmunity, transplant tolerance, and graft versus host disease.

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