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Bcl-2 Family Protein Regulation in Differentiating and Malignant Plasma Cells

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Bcl-2 Family Protein Regulation in Differentiating and Malignant Plasma Cells

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Abstract

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The maturation, selection and extended survival of plasma cells requires the dynamic interactions of the Bcl-2 family of proteins that control the induction of apoptosis. Cellular processes such as proliferation, differentiation and stress response increase the pro-apoptotic signaling in a cell. Cells must counter pro-apoptotic signals with anti-apoptotic signaling in order to survive. Thus the cell establishes apoptotic checkpoints to guard against diseased and malignant cells.

One such checkpoint occurs during plasma cell differentiation when the cell must activate the unfolded protein response (UPR) in order to increase the capacity of the endoplasmic reticulum (ER). In both a murine lymphoma cell line and a primary murine B cell model of plasma cell differentiation we observed activation of the UPR including ATF4 and CHOP activation. This signaling included induction of Bim and inhibition of Bcl-2 and Mcl-1. Differentiation also induced Bcl-x_L, which was necessary to protect from CHOP-induced apoptosis. These studies demonstrate a previously undescribed Bcl-x_L-dependent state in plasma cell differentiation.

Waldenström macroglobulinemia (WM) is a malignancy of lymphoplasmacytoid cells that secrete monoclonal pentameric IgM. Because the disease has a high prevalence of

activating mutations that signal through NF-κB and consists of cells at the intersection between B cell and plasma cell we hypothesized that WM cells would display a Bcl-x_L-dependency similar to differentiating plasma cells. In our examination of WM patient expression data we observed that plasma cell phenotype WM cells express both pro- and anti-apoptotic Bcl-2 family members at levels similar to normal plasma cells and lower than with multiple myeloma malignant plasma cells. WM cell lines displayed similarly low expression of pro-apoptotic Bcl-2 proteins. In two cell lines, low expression of Bim was due to overexpression of miR-155, which is common in WM and inhibits FoxO3a, an important transcription factor in the induction of Bim.

These studies demonstrate how the apoptotic threshold of a malignant cell is set by both its normal physiology and its cancer physiology and that agents that induce mitochondrial priming by targeting both physiological and cancer-specific pathways, are necessary to efficiently lower the apoptotic threshold and kill the cancer cell.

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I. INTRODUCTION

Lasting humoral immunity is a necessary function of the adaptive immune system in all jawed vertebrates. This response is dependent on the production and survival of long lived plasma cells which have been shown to have extensive half-lives and persist long after vaccination [1]. The consequence of creating cells with such prolonged life spans is that they are inherently resistant to apoptosis. This is normally not problematic since plasma cells normally do not retain the ability to proliferate. It is when aberrant generation or proliferation of these cells occurs that their long survival can contribute to disease. Because long-term survival is rooted in their cellular program, plasma cells need not acquire survival signaling through oncogenic transformation unlike many cancers. Conversely, the malignant plasma cell must acquire a proliferative signal through oncogene activation. Therefore it is imperative that the survival of both normal and malignant plasma cells be studied.

Unlike many malignancies, plasma cell cancers are not likely to de-differentiate and stop the normal processes of a plasma cell [2]. These processes include the activation of stress pathways associated with the production of large amounts of protein. These pathways imprint a survival signature upon the cell that hinges upon the interactions of the Bcl-2 family of proteins which control the process of apoptosis on the cellular level. In many ways B cell and plasma cell malignancies have Bcl-2 family expression that is dependent on the biology of their normal cell counterparts and this is integral to understanding their response to therapeutic agents.

A. Plasma cell differentiation

Mature B cells occupy distinct functional niches and possess differing abilities to respond to stimuli and further differentiate to antibody secreting plasma cells. In recent years it has become apparent that both the qualities of these niches and the maturation pathways traversed by these subtypes contribute to their differentiation [3]. Follicular B cells are found recirculating in the B cell areas of the spleen and bone marrow as well as Peyer's patches and lymph nodes. They require B cell receptor (BCR) ligation and CD40 signaling for differentiation to plasma cells and thus respond to T-dependent antigens [4]. Follicular B cells along with T cells and antigen presenting cells form germinal center reactions that yield class switched high affinity plasma cells[5]. Marginal zone B cells reside near the marginal sinuses of the spleen. Due to their localization and high expression of major histocompatibility complex (MHC) class II they are efficient presenters of antigen to activated T cells in the lymphoid follicle [6]. Importantly, these cells can also be induced to differentiate to IgM-secreting plasma cells via toll-like receptor (TLR) ligation by T-independent antigen such as lipopolysaccharide (LPS) [7]. In rodents there are also B-1 B cells in the pleural and peritoneal spaces that are able to respond to T-independent antigens such as phosphocholine to differentiate to IgM-secreting plasma cells [3]. There is also evidence that antigen specific B-1 B cells can be induced to class switch to IgA-secreting plasma cells at mucosal sites in the absence of T cell help [8].

While BCR ligation and CD40 signaling is required for terminal differentiation of follicular B cells, TLR signaling can contribute to the tuning of the response. In both marginal zone and B-1 B cells, TLR signaling alone is sufficient to cause differentiation.

Regardless of the stimuli or the type of plasma cell derived, there are commonalities in the differentiation programs activated in these cells (Figure 1). The transcriptional programs activated in plasma cell differentiation are governed by the master regulator B-lymphocyte induced maturation protein 1 (BLIMP1) which is necessary in B cells for differentiation to antibody secreting cells (Figure 2) [9]. Signaling through the BCR with CD40 co-stimulation or cytokine signaling with IL-4, IL-5, or IL-6 or signaling through toll-like receptors leads to degradation of B cell CLL/lymphoma 6 (BCL6) and induction of BLIMP1 which are mutually inhibitory [10]. Many of these stimuli signal through nuclear factor κB (NF-κB) and it has been shown that NF-κB binding to the *prdm1* promoter is essential for induction of BLIMP-1 and differentiation of plasma cells [11]. BLIMP-1 inhibits the transcriptional program of paired box 5 (PAX5) and its downstream effect such as the BCR complex, B cell linker (BLNK), and activation-induced cytidine deaminase (AID), which are essential components of the B cell as well as relieving PAX5-mediated repression of IgH, IgL, J-chain and x-box binding protein 1 (XBP1) [12, 13]. BLIMP-1 also induces cell cycle arrest via inhibition of the *c-myc* transcriptional program [14]. XBP1 is necessary for the formation of plasma cells but must be activated post transcriptionally via activation of inositol requiring enzyme 1 (IRE1) through the unfolded protein response (UPR) [15, 16].

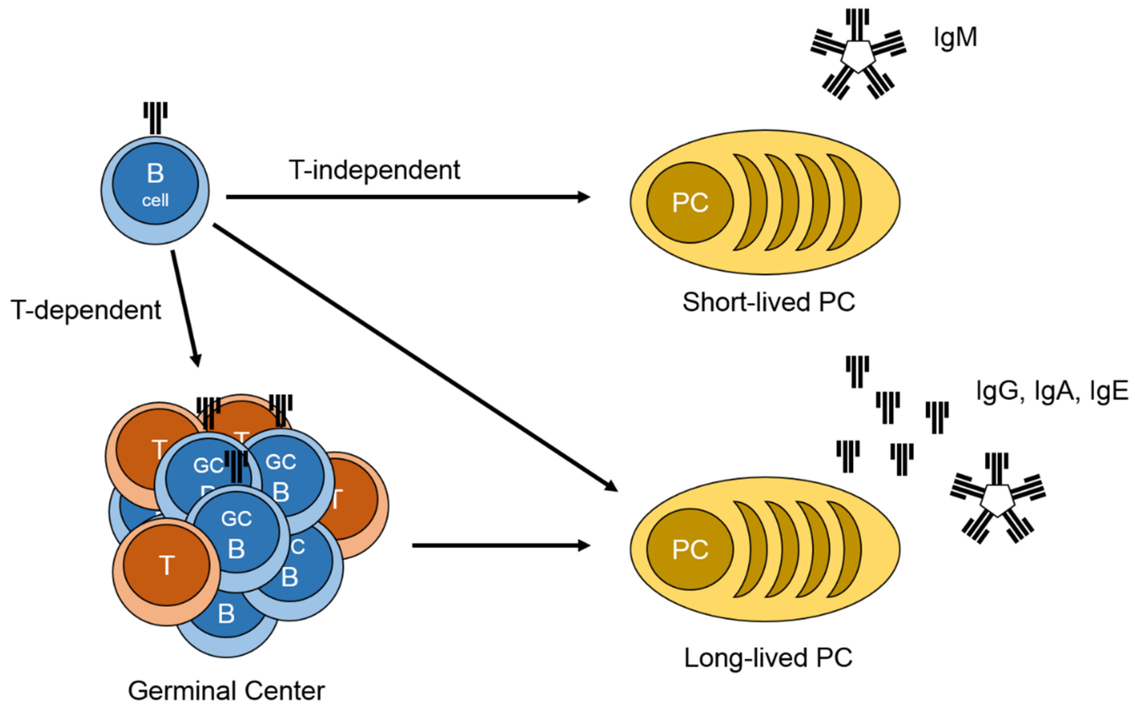


Figure 1. Plasma cell differentiation proceeds through many pathways.

Mature B cells become plasma cells through a variety of pathways that are both T cell-dependent and T cell-independent. Regardless of the antigen type and signaling pathways activated the cell must undergo vast morphological changes that include the building of an extensive network of rough endoplasmic reticulum which is dependent on activation of the unfolded protein response.

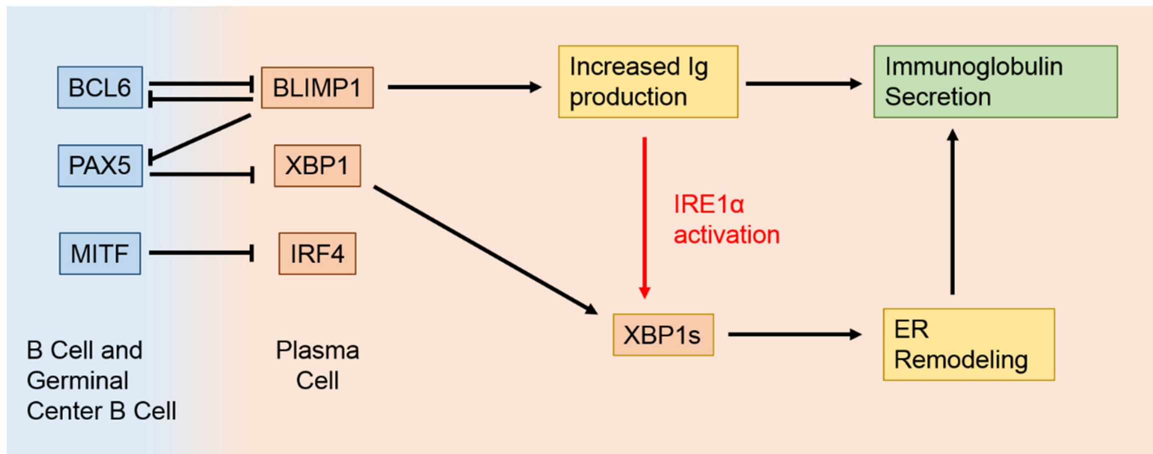


Figure 2. Master transcriptional regulators of plasma cell differentiation

The BCL6-mediated transcriptional protein of the mature B cell gives way to the BLIMP1-mediated transcriptional program of the plasma cell through mutually-inhibitory interactions. Importantly it is crucial that the mRNA for the transcription factor XBP1 must be spliced by activated IRE1 in order for successful differentiation into the antibody secreting cell.

B. The Unfolded Protein Response

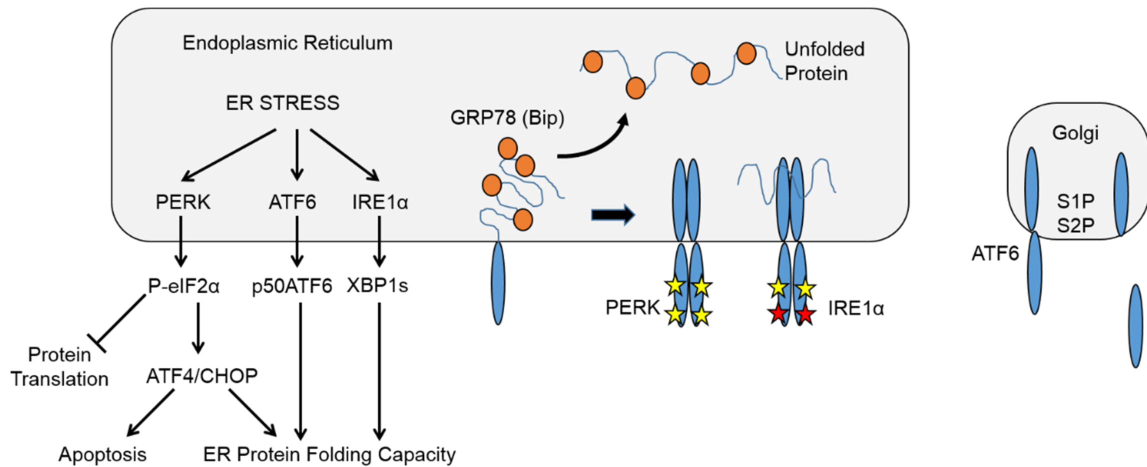
1. Mechanics of the UPR – The unfolded protein response is a set of signaling cascades activated when secreted protein load in the endoplasmic reticulum (ER) exceeds the protein folding capacity of the ER (Figure 3A). These pathways are found in all eukaryotic cells in some form. In mammalian cells, the UPR consists of signaling downstream of three main sensor ER transluminal proteins, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and protein kinase R-like ER kinase (PERK) [17, 18]. The activation of these three sensors follows a similar mechanism in that the luminal tails of the sensors are bound by the ER chaperone Bip which keeps them inactive. When the level of unfolded protein in the ER increases, Bip is titrated off ATF6, IRE1 and PERK [19]. The luminal tail of ATF6 is then allowed to properly fold and translocate to the golgi apparatus where S1P and S2P collaborate to cleave off and release the soluble p50ATF6 which is itself a transcription factor that binds to ER stress-responsive elements (ERSE) [20-22]. Activation of IRE1 requires the release of Bip as well but there is evidence that the luminal tail of IRE1 may be further activated by binding unfolded protein itself and creating active oligomers [23]. This then allows for activation of both a cytoplasmic kinase and ribonuclease domain which then performs a unique splicing of the mRNA for XBP1 which yields a transcript for a potent activator of ERSE and unfolded protein response elements (UPRE) [24-26]. Release of Bip from the luminal tail of PERK allows for its oligomerization and trans-phosphorylation which activates its cytoplasmic eukaryotic translation initiation factor 2A (eIF2A) kinase domain [27]. Phosphorylation of eIF2A inhibits global protein translation but favors the translation of mRNA for activating transcription factor 4 (ATF4) which binds to amino-acid-regulatory

elements (AARE) [28]. Activation of C/EBP homologous protein (CHOP) is a downstream effect of ATF4 as its promoter contains two AARE motifs. The CHOP promoter also contains two ERSE elements and though binding of p50ATF6 and XBP1s enhance its activation, CHOP induction is not seen in the absence of ATF4 binding to AARE elements [29]. CHOP is implicated as a link to apoptosis from prolonged ER stress activation as it has been shown to induce the pro-apoptotic BH3-only protein Bim and inhibit the expression of the anti-apoptotic protein Bcl-2, and overexpression of CHOP induces apoptosis [29-31]. While activation of genes containing UPRE and ERSE elements is broadly protective, yielding increases in ER chaperone production, lipid biogenesis, and redox control to allow for more ER folding capacity in addition to increasing ER-associated degradation (ERAD) of unfolded protein, activation of CHOP has both protective and cytotoxic effects.

2. The physiological UPR of plasma cell differentiation – The process by which a B cell becomes a plasma cell makes use of the UPR as a mechanism to increase the protein folding capacity to the level necessary for a professional antibody secreting cell. Though activation of ATF6 and IRE1 is seen in plasma cell differentiation, PERK activation is not observed at significant levels (Figure 3B) [17, 18]. There are also distinct mechanisms of feedback inhibition of the PERK-ATF4-CHOP arm of the UPR. CHOP activation itself is shown to induce protein phosphatase 1 regulatory subunit 15A (PPP1R15A), also known as GADD34, which combines with protein phosphatase 1 (PPP1) to dephosphorylate eIF2A [32]. Activation P58IPK, the promoter of which contains an ERSE element, is able to deactivate PERK by binding to its ER-luminal tails and blocking

oligomerization [33, 34]. Furthermore, *Perk*$^{-/-}$ animals have functional plasma cells [35]. This would seem to solve the problem of overcoming tanslation inhibition and induction of CHOP-dependent apoptosis in plasma cells but it has been observed that though *Chop*$^{-/-}$ animals can make plasma cells, the resulting cells are not fully capable of secreting immunoglobulin to the levels of wild type cells, suggesting that some level of CHOP activation is necessary in plasma cell differentiation [36].

A.



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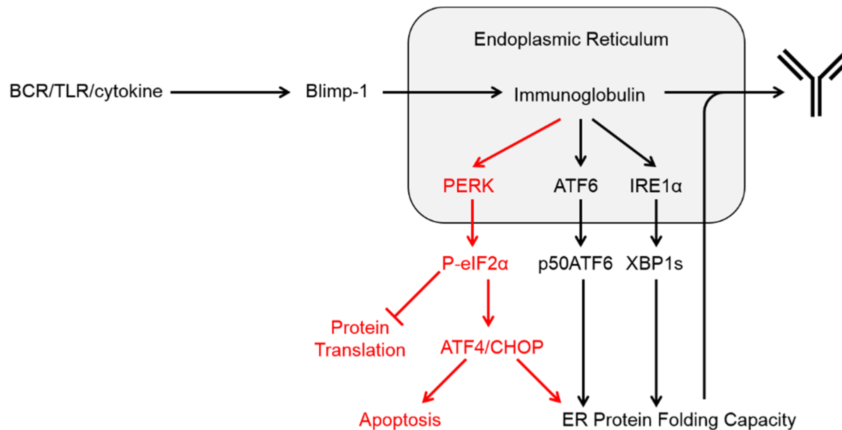

Figure 3. The Unfolded Protein Response in mammalian cells and plasma cells.

The UPR is a set of signaling pathways that is activated upon induction of ER stress. (A) In all mammalian cells the UPR consists of the PERK, ATF6 and IRE1 signaling pathways. These are activated when unfolded protein accumulates in the ER and the chaperone Bip is titrated off the sensor molecules' luminal tails. (B) In the physiological UPR of plasma cell differentiation, it has been shown that activation of the PERK arm of the UPR is not seen and is not necessary for the developing plasma cell.

C. Apoptosis and the Bcl-2 Family

Apoptosis is a highly regulated form of cellular suicide by which an organism can delete cells that are diseased or unneeded by an immunologically silent mechanism [37-39]. The cell achieves this by activation of cysteine-dependent aspartate-directed proteases called caspases. Caspases are present in the cell in the form of inactive zymogens and once activated cleave many different substrates which culminates in the breakdown and packaging of the cell into apoptotic bodies to be dispensed with by phagocytic cells [40]. The process by which caspases are activated can be either through cell-intrinsic stimuli such as nutrient deprivation and DNA damage or through cell extrinsic stimuli such as death receptor ligation [41].

1. Intrinsic pathway of apoptosis – The intrinsic pathway culminates in mitochondrial outer membrane permeabilization (MOMP) which releases cytochrome c and other pro-apoptotic factors such as second mitochondria-derived activator of caspase (SMAC) and OMI/HtrA2 from the mitochondrial intermembrane space into the cytosol. This activates a caspase cascade beginning with the Caspase-9, APAF1 and cytochrome c containing apoptosome and continuing through activation of Caspase-3 and -7 and leading to apoptosis and cell death [37, 42]. This is thought of as a point of no return for the cell as blocking downstream caspases still eventually leads to the death of the cell due to mitochondrial dysfunction [43]. Regulation of MOMP involves the complex interaction of both pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins. All Bcl-2 family proteins contain at least one Bcl-2 homology domain (BH1-4) with only the BH3

domain being common to the entire family (Figure 4). Bcl-2 family regulation of apoptosis culminates in the pro-apoptotic BH3-only proteins Bim, Bid and Puma transiently associating with and causing a conformational change in the multi-domain pro-apoptotic proteins Bax and Bak [44]. Upon their activation, Bax and Bak homo-oligomerize and initiate MOMP [45]. The ability for Bid to activate Bak and to a lesser degree Bax is greatly increased by cleavage by Caspase-8 into tBid which then readily associates with the mitochondrial membrane placing it in proximity to the membrane bound Bak [46]. Contrastingly, Bim needs no further processing to activate Bax and Bak. When Bim or tBid are present a cell must also express anti-apoptotic Bcl-2 family proteins to block apoptosis. The set of known anti-apoptotic Bcl-2 family proteins now includes Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, Bcl-b and A1/Bfl-1, which are capable of binding both Bim, tBid and additionally activated monomers of Bax and Bak [47]. Ectopic expression of these proteins is capable of indefinitely blocking apoptosis provided they exceed the load of downstream pro-apoptotic proteins. This balance can be shifted, however, by the expression of pro-apoptotic BH3-only proteins termed sensitizers including Hrk, Bad, Bik, Bmf, Noxa and Puma. Sensitizers bind the anti-apoptotic Bcl-2 proteins with varying affinities and are capable of displacing Bim, tBid, Puma or activated Bax and Bak leading to MOMP and apoptosis [48]. The interactions of these proteins is shown graphically in Figure 5.

2. Extrinsic pathway of apoptosis – The extrinsic pathway of apoptosis utilizes some of the same machinery as the intrinsic pathway but is initiated by signaling through death receptors on the cell surface. Ligation of death receptors brings together a complex

containing FAS-associated death domain protein (FADD) and Caspase-8 known as the death inducing signaling complex (DISC). Once brought together Caspase-8 dimers become activated and can cleave downstream substrates including Caspase-3 [39]. In some cells this is sufficient to initiate apoptosis but most cells require Caspase-8 cleavage of Bid which induces Bax/Bak-dependent MOMP and releases factors such as SMAC and OMI which are necessary to relieve X-linked inhibitor of apoptosis protein (XIAP)-mediated inhibition of the executioner Caspases-3 and -7 [49-51]. Importantly, there are mechanisms of Caspase-8 activation via an intrinsic DISC or iDISC leading to Bid cleavage and the initiation of apoptosis [52-54].

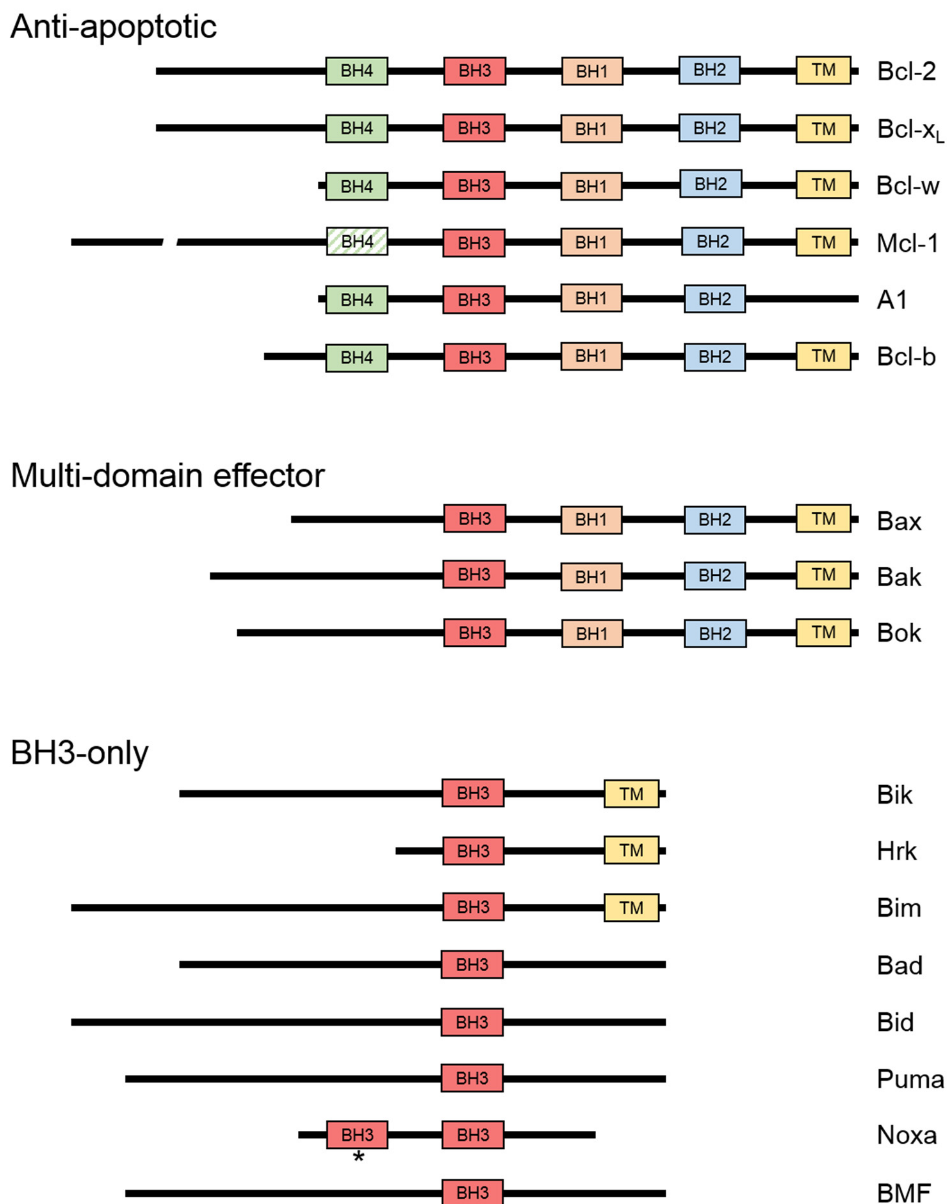

Figure 4. The domain structure of the Bcl-2 family

Bcl-2 family proteins contain at least one of four Bcl-2 homology domains (BH1-4). The anti-apoptotic family members contain BH1 – BH4 domains with the BH4 domain of Mcl-1 being less well conserved. Multi-domain effectors or Bax/Bak-like Bcl-2 family members contain BH1-BH3 domains and are pro-apoptotic proteins capable of causing mitochondrial pores upon activation leading to MOMP. The BH3-only proteins are either classified as activators (Bim, Bid and Puma) which directly activate multi-domain effectors or sensitizers which antagonize anti-apoptotic proteins. * The second BH3 domain of Noxa is present in the mouse but not human form of the protein.

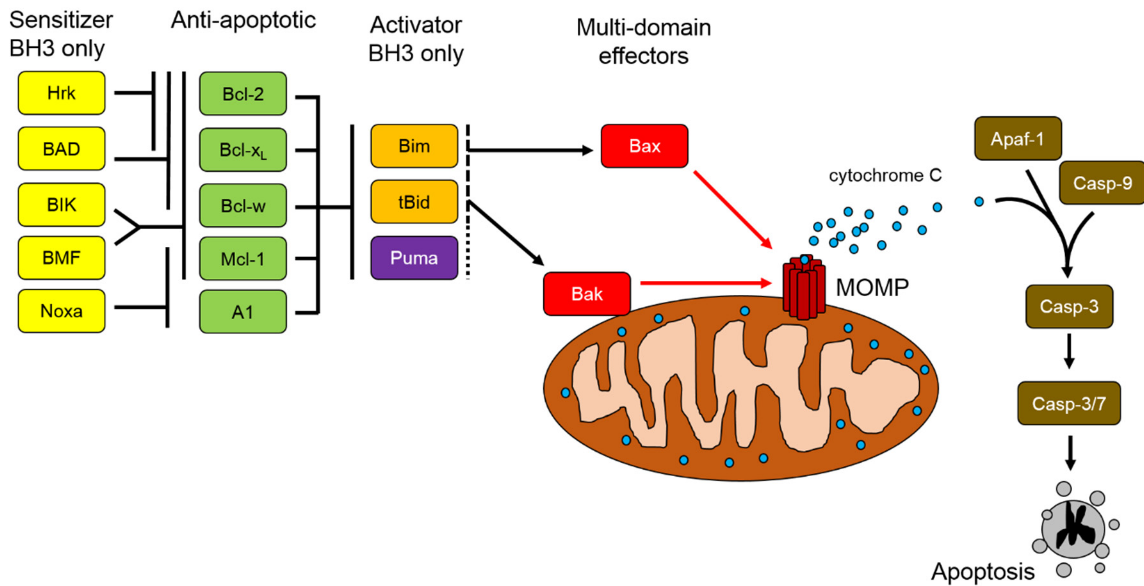

Figure 5. Bcl-2 Family Regulation of Intrinsic Apoptosis

The interactions of the members of the Bcl-2 family of proteins control the activation of mitochondrial outer-membrane permeableization (MOMP). Sensitizer BH3-only proteins bind with varying affinities to and antagonize anti-apoptotic Bcl-2 proteins. The anti-apoptotic family members are able to bind to and inhibit both direct activator BH-3 only proteins and activated monomers of Bax and Bak (not drawn). Free activator BH-3-only proteins are able to transiently associate with Bax and Bak causing a conformational change that allows for the homo-oligomerization of Bax or Bak leading to MOMP. This allows for pro-apoptotic factors including cytochrome c to enter the cytosol leading to the formation of the apoptosome, a complex consisting of cytochrome c Apaf-1 and Caspase-9 which activates executioner caspases leading to apoptosis.

D. B Cell Development and the Bcl-2 Family

The generation of the adaptive immune system requires the production of a vast repertoire of cells capable of recognizing and responding to any foreign antigen while sparing the self. This process involves the repeated expansion, selection and deletion of cells. At all points in B lymphocyte development the Bcl-2 family of proteins is integral to this process (Figure 6) [55-57]. From as early on as the hematapoetic stem cell (HSC) and common lymphoid progenitor (CLP) stage these cells show dependence on Mcl-1 for survival [58]. At the Pro-B cell stage, IL-7 signaling is required to protect from Bim-induced apoptosis and this protective effect is once again Mcl-1 expression [59, 60]. Positive selection at the Pre-B cell stage involves a checkpoint at which the cell must express a valid PreBCR or be sacrificed. This is accomplished through expression of Bcl-2 and Mcl-1 downstream of tonic Pre-BCR stimulation which inhibits pro-death expression of Bim and Puma [59, 61, 62]. Having survived positive selection, the immature B cell must undergo negative selection to ensure that the newly minted BCR is not auto-reactive. This is accomplished when strong signaling through the BCR leads to Bim-dependent apoptosis whereas $Bim^{-/-}$ animals have large amounts auto-reactive B cells [63]. Once the cells have reached the mature B cell stage they still must contend with the expression of pro-apoptotic factors and are dependent on expression of Bcl-2, Mcl-1 and Bcl-x_L downstream of BAFF signaling and NF-κB activation [64, 65]. Finally, it has been shown that Mcl-1 is necessary for the survival and maintenance of the terminally differentiated plasma cell [66]. Importantly, in a murine immunization model it was observed that new plasma cells could not be formed in the presence of the Bcl-2/Bcl-x_L inhibitor ABT-737, suggesting an intermediate that is not Mcl-1 dependent [67].

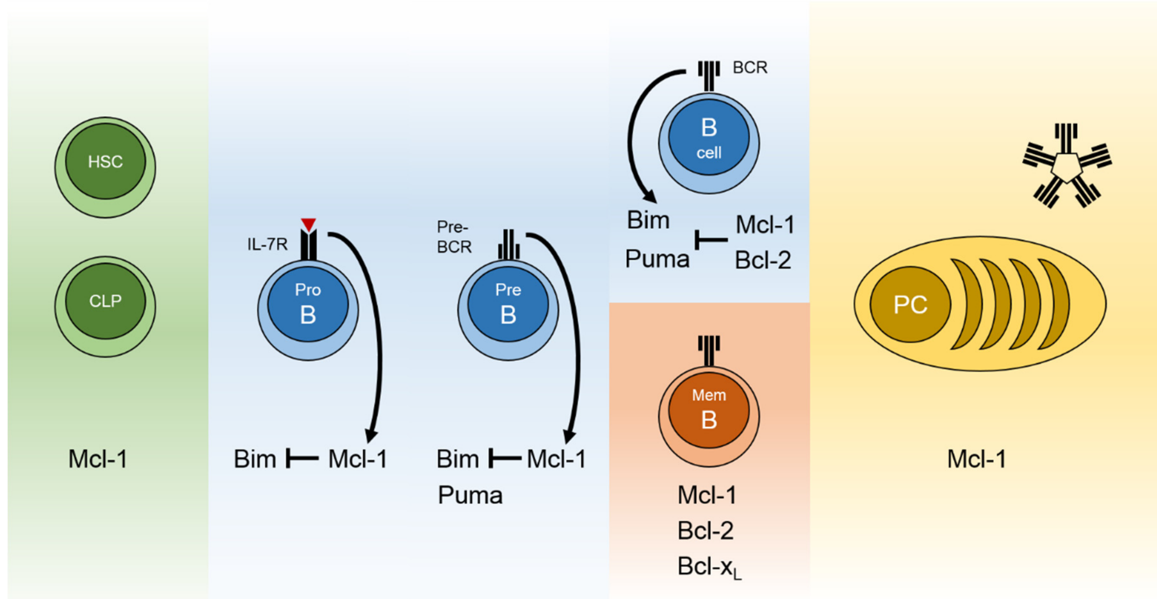

Figure 6. Bcl-2 family interactions in B cell development.

The positive and negative selection of B cell precursors depends on the interactions of the Bcl-2 family of proteins. Mature B cells, memory B cells and the terminally differentiated effectors, plasma cells, are dependent on members of the Bcl-2 family for survival. Notably it has been shown that Mcl-1 is important in the survival of B lineage cells at every stage of differentiation.

E. Mitochondrial Priming and Apoptotic Threshold.

As cells enter states of proliferation and differentiation the expression and interaction of the Bcl-2 proteins changes such that cells increase their levels of pro-apoptotic or death signaling. This sets up the opportunity for selection or checkpoints in which a cell must then increase its expression of anti-apoptotic proteins to counter the death signaling. For example, enforced expression of the proliferative oncogene *c-myc* leads to proliferation but also leads to Bim-dependent apoptosis. However, co-expression Bcl-2 or any other anti-apoptotic family member with *c-myc* rescues this cell death resulting in-vivo in tumor formation [68-70]. Because of this we can think of activated, proliferating, and differentiating cells as closer to apoptosis, or primed. By this method it takes less death signaling to initiate apoptosis in a primed cell as compared with a resting cell. This is the foundation of the organism's guard against aberrant proliferation and cancer. For tumors to develop they must first break these checkpoints in many cases by increasing the expression of an anti-apoptotic Bcl-2 protein, which in this case becomes and oncogene. Cancer cells of many types are characterized by higher expression of both pro- and anti-apoptotic Bcl-2 proteins. The work of Letai et. al. has demonstrated that the amount of pro-apoptotic BH3-only protein in a cell or priming can be used to determine how much death signaling will be needed to induce MOMP in a cell, and that this mitochondrial priming directly correlates with sensitivity to a variety of inducers of apoptosis [48, 71]. We can think of this as the distance to a threshold where the death signaling induces MOMP and the cell passes a point of no return and commits to apoptosis. The more primed a cell is the lower its apoptotic threshold. The differential in apoptotic threshold between a

cancer cell with a high level of priming and a healthy cell with a low level of priming provides for the therapeutic index in cancer treatment.

After the characterization of the various pro- and anti-apoptotic Bcl-2 proteins came the discovery that not all BH3 helices and binding pockets were equal and that the binding affinities of BH3-only proteins to the various anti-apoptotic proteins varied greatly [48]. This then led to the development of a new class of drugs designed to perturb the binding interactions of specific members of the Bcl-2 family. Small molecule inhibitors termed BH3-mimetics are designed to bind with higher affinity to the anti-apoptotic proteins than their pro-apoptotic counterparts. An early success in this line of drugs was ABT-737 which is a mimetic of the BH3-only protein Bad and therefore binds with high affinity to Bcl-x_L, Bcl-2 and Bcl-w [72]. In addition to promising pre-clinical outcomes these new drugs also proved to be useful tools in examining the specific anti-apoptotic protein dependencies of various cell types and even separate individual cancer lines within a given cancer type by anti-apoptotic protein dependency [67, 73].

F. Plasma Cell Malignancy.

Two important hallmarks of any cancer cell are the ability to proliferate beyond the scope of its tissue type and the ability to evade the apoptotic program normally initiated due to aberrant proliferation [74, 75]. In other words it must break an apoptotic checkpoint. Most often this involves the induction of both a proliferation gene and a survival gene. This need is equally true for plasma cell malignancies, two of which are multiple myeloma (MM) and Waldenström macroglobulinemia (WM). These are two separate diseases characterized by aberrant proliferation of antibody secreting plasma cells and as such have some basic similarities but, importantly, are quite different in their generation, disease manifestation, and basic biochemical makeup including Bcl-2 family expression patterns.

1. Multiple myeloma – In many ways multiple myeloma (MM) cells inherit much of their phenotype and biochemical makeup from their healthy cell counterpart, the long-lived plasma cell, and as such, therapies that take into account their normal biology as well as their cancer biology work best [2]. The foremost dissimilarity between a normal plasma cell and a MM cell is that the MM cell is able to self-renew and proliferate whereas a long lived plasma cell cannot. Much of the other functions of a plasma cell are still carried out by the MM cell such as production of immunoglobulin, interaction with the stromal niche, and even longevity. MM cells are at least partially dependent on Mcl-1 expression for survival in much the same way that plasma cells are [66, 76, 77]. The disease is characterized by the proliferation, initially in the bone marrow, of plasma cells

producing a monoclonal antibody or light chain immunoglobulin. Disease manifestation occurs due to proliferation of cancer cells and to high amounts of inappropriate secreted proteins in the form of hypercalcemia, renal failure, anemia and lytic bone lesions lending itself to the CRAB acronym [78]. The disease progresses through stages of monoclonal gammopathy of undetermined significance (MGUS), smoldering or asymptomatic MM, symptomatic MM and finally extra-medulary MM at which point the cancer cells grow beyond dependence on their stromal niche. Regardless of the staging and presentation, nearly all MM cells share the quality that they derive from cells that have undergone the processes of VDJ recombination, somatic hyper mutation and class switch recombination on their way to becoming plasma cells. Because of this, MM cells often present with common translocations in which genes are recombined so that an oncogene is driven by an enhancer of the immunoglobulin locus. While study of these oncogenic events has led to information on which MM cells might be more sensitive to a given anti-cancer treatment, the most successful therapies to date are those that target the protein catabolism of this antibody secreting cell [79].

2. Waldenström macroglobulinemia – Though also characterized by aberrant proliferation of monoclonal antibody-secreting cells, WM differs from MM in many important ways. First described by Jan Gosta Waldenström in 1944, the disease presents with bleeding disorders and organ problems due to high serum viscosity as well as anemia due to infiltration of the bone marrow by lymphoplasmacytic cells [80]. WM cells are phenotypically lymphoplasmacytic in that their morphology can be that of a lymphocyte, a plasma cell or intermediate. The secretion of monoclonal, pentameric IgM is important

in the pathology of the disease. While there are common cytogenetic abnormalities such as deletion of chromosome 6q [81], the most striking genetic anomaly in WM is the expression of an activating somatic mutation in MyD88 (L265P) in 91% of patients [82]. Importantly MyD88 signaling results in activation of NF-κB which itself drives plasma cell differentiation and survival signaling. Another important discovery in WM has been in its epigenetic regulation. A signature of dysregulated microRNAs exists in WM including upregulation of 6 miRs (miR-363*, -206, -494, -155, -185, and -542-3p) and downregulation of miR-9* [83]. miR-155 in particular has been shown to regulate proliferation of the cells and growth in-vivo as xenografts which regressed with inhibition of mir-155 [84].

Phenotypically, WM cells differ from MM in that they retain the expression of B-cell markers such as CD19, B220 and CD20 [85]. This is important in the treatment of WM because the use of rituximab or anti-CD20 monoclonal antibody is possible. In fact the standard of care in WM involves rituximab in combination with low dose dexamethasone or cytotoxic agent. Interestingly, rituximab as a single agent offers no therapeutic advantage over any other single agent and often relapse occurs eventually necessitating the development of other therapies, one of which that shows promise is proteasome inhibition [86]. The similarity between MM and WM that they both secrete large amounts of protein and therefore are sensitive to proteotoxic stress may inform us on the mechanism behind the success of proteasome inhibition in WM. A distinction from MM is that there is not much known about the Bcl-2 family protein expression or interaction in WM in order to inform the use of therapies that specifically target the apoptotic regulation of the cell.

G. Statement of Problem

The survival of plasma cells is important in both the generation of long lasting humoral immunity and in aberrant persistence and proliferation of plasma cells in malignancy and autoimmunity. The dynamics of B cell maturation and plasma cell differentiation contain inherent checkpoints that guard against the generation of auto-reactive cells and against hyper-proliferation of cells not needed for the immune response. At times these checkpoints are broken and cells proliferate and survive causing disease. In the case of malignancy, a cell must both proliferate and resist apoptosis to survive outside the rules set by these checkpoints. The study of diseases of malignant plasma cells such as multiple myeloma and Waldenström macroglobulinema has given us the knowledge that though cancerous, MM and WM cells both continue to function much the same as their non-malignant counterpart, the plasma cell. They continue to produce and secrete large amounts of monoclonal antibody, the hallmark of a plasma cell, they remain in large part dependent on the survival niche inherent to the plasma cell, yet they are able to proliferate and cause disease. For this reason it is imperative that we study both the normal physiology and survival signaling of plasma cells alongside their malignant cousins.

Study of survival signaling in plasma cells must address both the activation of stress pathways inherent in the biology of the antibody secreting cell and the effect on the dynamics of the ultimate downstream regulators of apoptosis, the Bcl-2 family of proteins. It is known that differentiating plasma cells are dependent on the activation of a physiological UPR in order to prepare the ER for secretion of large amounts of immunoglobulin [18, 87]. This is a UPR that includes necessary activation of the ATF6 and

IRE1 signaling branches but is reported to not include activation of the PERK-ATF4-CHOP arm of the UPR [32, 36]. Previous work published by our group has shown that multiple myeloma cells treated with a proteasome inhibitor activate a full or terminal UPR, characterized by PERK-ATF4-CHOP activation which signals to Bcl-2 family-dependent apoptosis [79]. Given these observations we endeavored to examine the control of UPR signaling in the differentiation of normal plasma cells and how it informs the survival signaling in the generation of long lived antibody secreting cells.

In the following chapters we detail work examining the activation of the UPR in an in-vitro murine plasma cell differentiation model. We show that cytokine and LPS-driven differentiation induces a UPR that includes activation of the physiological UPR in addition to previously undescribed ATF4 and CHOP activation in the absence of PERK-eIF2A signaling. We further show that this signaling induces a shift in the Bcl-2 family dynamics such that Bim is induced and Bcl-2/Mcl-1 expression is decreased which nets a pro-apoptotic effect. This is then countered by an induction of Bcl-x_L which sequesters Bim and is necessary to protect from CHOP-dependent apoptosis. This work provides mechanism to a previously reported defect in the ability to produce new plasma cells in the presence of Bcl-2/Bcl-x_L inhibition [67].

Having demonstrated a previously undescribed transient Bcl-x_L-dependent state in plasma cell differentiation, we sought to determine what effect this has on the survival of plasma cell phenotype cells in Waldenström macroglobulinemia. WM is a disease characterized by a high prevalence of activating mutations in pathways that signal through NF-κB similar to those we exploited in our model of differentiation. These cells also bear a phenotype similar to the product of our differentiation experiments, namely

IgM-secreting CD19^{+}, CD20^{+}, CD138$^{+,-}$, plasma cell phenotype cells. Our investigation of WM patient derived expression data provide striking evidence that Bcl-2 family expression in WM is more similar to normal plasma cells than malignant plasma cells. Whereas multiple myeloma cells express pro-apoptotic Bcl-2 family members at high levels which is countered by high expression of anti-apoptotic family members, indicative of a cell breaking an apoptotic checkpoint, WM cells express both pro- and anti-apoptotic Bcl-2 family members at low levels. Using three available WM cell lines we demonstrate that the low expression of Bim, a protein that is expressed highly in MM cells, is mechanistically due to epigenetic regulation by miR-155, which is commonly over-expressed in WM. This expression pattern of Bcl-2 family members causes the WM cell to display less mitochondrial priming which is important in the response to treatment with any therapies that induce apoptosis in cancer cells. This work provides information important in the selection of therapeutic agents that induce priming in WM cells, thereby contributing to the efficacy of any agents used in combination therapies.

II. BCL-2 FAMILY DYNAMICS DURING NORMAL MURINE PLASMA CELL DIFFERENTIATION

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Bcl-x_L Protects From CHOP-Dependent Apoptosis During Plasma Cell Differentiation.

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*Running title: *Bcl-x_L protects from upr-associated apoptosis*

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Key Words: plasma cell differentiation; UPR; apoptosis

Background: UPR-associated death signals are activated during plasma cell differentiation.
Results: Bcl-x_L sequesters Bim during plasma cell differentiation preventing apoptosis.
Conclusion: Bcl-x_L dependence during plasma cell differentiation protects the cell from the UPR activation necessary for ER remodeling.
Significance: Bcl-2 family dynamics are crucial for both the development of plasma cells and the treatment of diseases of abnormal plasma cells.

A. Abstract

While it is known that the unfolded protein response plays a significant role in the process of plasma cell differentiation, the contribution of the individual sensors of the UPR to this process remains unclear. In this study we examine the death signals and compensatory survival signals activated during B cell activation and the first stages of plasma cell differentiation. During in-vitro differentiation of both primary murine B cells and the Bcl1 cell line, we demonstrate that in addition to activation of the physiological UPR, changes in the expression of several Bcl-2 proteins occur, that are consistent with a lowering of the apoptotic threshold of the cell. Specifically, we observed decreased expression of Bcl-2 and Mcl-1 and increased expression of the pro-apoptotic protein Bim. However, these changes were countered by Bcl-x_L induction, which is necessary to protect differentiating cells from both ER stress-induced death by tunicamycin and from the death signals inherent in differentiation. Consistent with differentiating cells becoming dependent on Bcl-x_L for survival, the addition of ABT-737 resulted in apoptosis in differentiating cells through the inhibition of sequestration of Bim. Confirming this result, differentiation in the context of RNAi-mediated Bcl-x_L knockdown also induced apoptosis. This cell death is CHOP-dependent connecting these events to the UPR. Thus plasma cell differentiation proceeds through a Bcl-x_L-dependent intermediate.

B. Introduction

Plasma cell differentiation involves striking changes in the morphology and physiology of the cell. In order to become a professional antibody secreting cell, the B cell must increase the size and capacity of the endoplasmic reticulum (ER) and secretory apparatus.[17] In order to accomplish this end, the cell activates a set of signaling pathways known as the unfolded protein response (UPR).[87] During periods of ER stress when the protein load in the ER outweighs its folding capacity, three sensors of ER stress, activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1) and PKR-like ER kinase (PERK), are activated.[89] These sensors reside across the ER membrane with their luminal tails bound to the ER chaperone Bip (GRP78) keeping them in an inactive state. When unfolded protein builds in the ER, Bip is titrated off the luminal tails of these signaling molecules. ATF6 translocates to the golgi where it is cleaved to yield p50ATF6, a transcription factor that upregulates ER quality control and capacity.[20-22, 90, 91] IRE1 oligomerizes and activates its ribonuclease which splices x-box binding protein 1 (XBP1) mRNA.[19, 24, 92-94] This spliced XBP1 mRNA yields a highly active transcription factor that upregulates chaperones and expands ER function and capacity.[26, 95] PERK oligermizes and phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α).[27] Phosphorylated eIF2α inhibits global protein translation while favoring translation of activating transcription factor 4 (ATF4) mRNA.[28] ATF4 also increases ER capacity as well as strongly inducing C/EBP homologous protein (CHOP).[28, 96, 97] Activation of these three signaling molecules results in the upregulation of ER protein folding capacity, quality control and ER associated degradation (ERAD) while delaying

further translation of mRNA. If ER stress is not resolved through the UPR, CHOP induces apoptosis by inhibition of Bcl-2 and induction of Bim.[98, 99]

The expansion of the secretory capacity necessary for a B cell to become a professional antibody secreting cell makes use of the UPR machinery already present in all cells. It must however overcome effects of PERK activation including translation inhibition and apoptosis. ATF6 and IRE1 are activated and XBP1 is spliced during plasma cell differentiation and they are necessary to form plasma cells in vivo as animals deficient in any of these genes lack plasma cells.[15, 25, 87, 100] Conversely, *Perk*$^{-/-}$ animals have plasma cells and PERK is not significantly activated during plasma cell differentiation.[32, 35] While it is true that ATF4, p50ATF6 and XBP1s all bind to the promoter of CHOP, only ATF4 has been shown to be necessary for CHOP activation.[96] Therefore it stands to reason that inactivation of PERK would inhibit ATF4 and CHOP relieving the cell of translation inhibition and the pro-apoptotic effects of CHOP. However, it has also been shown that some activation of CHOP is necessary for production of a maximally efficient plasma cell as *Chop*$^{-/-}$ animals have plasma cells that secrete immunoglobulin at a lower rate than wild type animals.[36] Therefore the pro-apoptotic aspects of CHOP signaling must be overcome for optimal plasma cell differentiation.

During all stages of B cell development there are pro-survival and pro-apoptotic proteins responsible for determining which B cells are propagated and which are deleted from the repertoire. The Bcl-2 family of proteins responsible for regulating the intrinsic pathway of apoptosis play a major role in this survival signaling.[38, 65, 101] This is true in mature B cells and in plasma cells but less is known about this pathway during the transition between these cell types. The multi-domain anti-apoptotic proteins Bcl-2, Mcl-1 and

Bcl-x_L are important in B cell survival. They function by binding and sequestering pro-apoptotic BH3 only proteins, such as Bim.[47, 102] This prevents Bim from activating Bax and Bak, which when active induce mitochondrial outer membrane permeabilization (MOMP), resulting in cytochrome *c* release that leads to downstream caspase activation and apoptosis. This function of anti-apoptotic proteins can now be targeted by a class of drugs called BH3 mimetics. These are small molecules that bind to and antagonize the antiapoptotic proteins. ABT-737, a mimetic of the BH3-only protein Bad, binds to Bcl-x_L, Bcl-2 and Bcl-w, displacing Bim and leading to apoptosis in cells that are dependent one of these proteins for survival.[72] ABT-737 does not bind Mcl-1 and therefore will not cause apoptosis in a cell that is dependent on Mcl-1 for survival. In murine immunization models it has been shown that germinal center B cells and existing plasma cells are insensitive to ABT-737.[67] Accordingly, it has also been shown that these cell types are dependent on Mcl-1 for survival.[65, 66] These studies did show that there was a deficit in newly formed plasma cells in the presence of ABT-737, however the molecular basis for this deficit was not fully defined. In this study we define the molecular basis of differential Bcl-2 family dependence during plasma cell differentiation.

C. Experimental Procedures

Cell Culture - The Bcl1 cell line, clone CW13.20.3B3, was acquired from ATCC (CRL-1699). Primary murine B cells were prepared from splenocytes isolated from C57BL/6 spleens and depleted of non B cells and activated B cells by magnetic bead column separation (Miltenyi B cell isolation kit 130-090-862, LS columns). All cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 μM L-glutamine, 100 I.U. penicillin/streptomycin, 10 mM HEPES buffer, 1 mM sodium pyruvate, non-essential amino acids and 50 μM 2-mercaptoethanol.

In-vitro differentiation: Bcl1 cells were cultured at a concentration of 0.3 $X10^6$ cells / mL in complete growth media supplemented with 10 ng/mL IL-5 (R&D Systems) and 10 μg/mL lipopolysaccharide (Sigma L-4391) for up to 96 hours. UPR was activated with 0.5 μg/mL tunicamycin (Sigma T7765) followed by replacement with complete growth medium. Primary C57BL/6 B cells were cultured at a concentration of $1X10^6$ cells / mL in complete growth medium supplemented with 20 ng/mL IL-4 (Peprotech) and 20 μg/mL lipopolysaccharide (Sigma L-6216) for up to 96 hours. ABT-737 and ABT-199 were a generous gift of Abbvie (North Chicago, Il).

Lentiviral knockdown: Virus was prepared in 293T cells using the MISSION shRNA TORC1 system (Sigma SHCLNG-NM_004083) or with pLKO.1 vector control. Bcl1 cells were then infected and selected with puromycin. Transductants were kept in selection during passage and verified by western blot and qRT-PCR. Experiments were carried out in the absence of puromycin.

Flow cytometry - Cells were collected at the various time points and treatments. 0.25-0.5 million cells were washed with PBS and resuspended in 100 µl FACS buffer (1% BSA in PBS containing 0.01% sodium azide) and the appropriate amount of antibody for 30 m at 4˚C. Cells were then washed in FACS buffer and resuspended in 0.5 mL FACS buffer with 5 µl 7AAD and incubated at room temperature for 5 m. Samples were then assayed on a BD FACSCanto II.

Antibodies: CD19-V450 (560375), CD138-PE (553714), CD44-FITC (553133) and 7AAD were purchased from BD Biosciences. IgM-FITC (69819) was purchased from Santa Cruz Biotechnology.

Apoptosis was assayed with Annexin-V-FITC (Biovision 1001-1000) and propidium iodide (2 µg/mL Sigma) staining and measured with a BD FACScanto II as previously described.[103]

Immunoblotting and Immunoprecipitation

Western blotting was performed on lysates collected at the various time points and treatments as previously described.[103] Samples were run on 4-15% Bio-Rad TGX minigels. Gels were transferred to nitrocellulose membrane and blocked as previously described.[103]

Antibodies: ATF4 rabbit polyclonal was purchased from Abcam. Bcl-2 hamster (3F11), and Syrian and Armenian hamster-HRP mouse were purchased from BD Biosciences. Bcl-2 rabbit (50E3), Bip rabbit (C50B12), CHOP rabbit (D46F1), p-eIF2α (Ser 51) rabbit (D9G8), p-eIF2α (Ser 51) rabbit (119A11), eIF2α mouse (L57A5), GRP94 rabbit polyclonal, p58IPK rabbit (C56E7) and Mcl-1 rabbit (D35A5) were purchased from Cell

Signaling Technology. Rabbit IgG-HRP donkey and Mouse-IgG-HRP sheep were purchased from GE Healthcare. Bim rabbit polyclonal was purchased from Milipore. β-Actin mouse (AC-15) and β-Actin rabbit polyclonal were purchased from Sigma Aldrich. Bcl-x_L mouse (2A1) and Bcl-x_L rabbit polyclonal (13.6) have been previously described.[104]

Co-immunoprecipitation was performed with the Immunocruz Optima C kit (Santa Cruz) as previously described.[103] Lysates were prepared in 2% Chaps buffer 100 μg of protein was used for each antibody for each sample. Samples were pre-cleared with protein G and preclearing matrix for two hours. IP matrices were prepared using 3 μg of the appropriate antibody. Pre-cleared lysates were rocked in IP matrices overnight. Samples were eluted in 2:1 RIPA: 6x loading dye under non-reducing conditions after 5 m incubation at 95˚C.

Antibodies: Mcl-1 mouse (B6) was purchased from Santa Cruz Biotechnology. Bcl-2 mouse (Bcl/10C4) was purchased from Novus Biologicals. Bcl-x_L mouse (7B2.5) was previously described.[104]

Gene Expression - cDNA was prepared from RNA harvested at specified time points using ABI high capacity cDNA kit (Applied Biosystems). Realtime PCR was performed using Taqman gene expression master mix (ABI 4368814) with an ABI 9600 Fast thermocycler as previously described.[73]

Probes: Mcl-1 (mcl1) Mm00725832_s1, Bcl-2 (bcl2) Mm00477631_m1, Bcl-x_L (bcl2l1) Mm00437783_m1, Bim (bcl2l11) Mm00437796_m1, Blimp-1 (prdm1)

Mm004761289_m1, Bcl-6 (bcl6) Mm00477633_m1 and GAPDH 4352932-0912031 were purchased from Applied Biosystems.

ELISA - ELISA was performed according to protocol using mouse IgM ELISA kit (Bethyl Labs). Supernatants were diluted 1:4 and loaded at 25 µl per well then calibrated to live cell number counted using trypan blue exclusion. Lysates were loaded at 2.5 µg total protein per well. TMB substrate (Invitrogen) and color change was used to measure concentration. Color change was measured as A450-A550. IgM concentrations were reported as IgM per cells in culture or IgM per cells contributing to the lysate loaded and were calculated against the four parameter logistic curve fit of dilutions of standard mouse serum with known IgM concentration.

Xbp1 splicing assay - cDNA was prepared from RNA collected from samples at specified time points with the various treatments. The region of the splice site was amplified using primers flanking the splice site. Products were amplified using the DreamTaq kit (Fermentas) and a custom PCR routine: 94˚C/4 m, 40 cycles of 94˚C/30 s, 65˚C/30s, 72˚C/30s, then 72˚C/10m and 4˚C/hold. PCR products were run on 3% high resolution agarose gels (Invitrogen). 10 cm gels were run at 50V for 6 hours.

Primers:

fwd: 5’-GAACACGCTTGGGAATGGACAC-3’

rev: 5’-AGAAAGGGAGGCTGGTAAGGAAC-3’

D. Results

IL-5 and LPS treatment induces differentiation in Bcl1 cells - The murine B cell leukemia cell line Bcl1 can be stimulated to differentiate to an antibody secreting cell using lipopolysaccharide (LPS) and cytokines.[105, 106] We tested combinations of previously described differentiation stimuli including IL-2, IL-5 and LPS and found that the combination of IL-5 and LPS was as efficient a differentiation stimulus as the combination of all three (not shown). Consistent with published data,[106] this cell line has high basal expression of the IL-5 receptor (not shown). Differentiating Bcl1 cells display phenotypic changes associated with the early stages of plasma cell differentiation including upregulation of CD138, CD19 and CD44 along with down regulation of surface IgM (Figure 1A). To examine the effects of UPR activation on plasma cell differentiation we induced ER stress with an inhibitor of N-linked glycosylation, tunicamycin. A five hour pulse of tunicamycin activated the UPR as demonstrated by increased expression of CHOP and XBP1 (not shown). These cells rapidly underwent apoptosis unless LPS was included in the differentiation stimulus following the tunicamycin pulse (Figure 1B, and not shown). While IL-5 was not sufficient on its own to protect cells, only those cells that received both LPS and IL-5 produced and secreted antibody at the rate similar or greater than cells that did not receive tunicamycin pretreatment (Figure 1 C,D, and not shown). These data demonstrate that Bcl1 cells are sensitive to ER stress-induced cell death yet are protected by LPS signaling during differentiation.

Differentiating Bcl1 cells activate CHOP independent of PERK - Since plasma cell differentiation induces parts of the unfolded protein response we chose to further examine

the activation of this pathway during ER stress and differentiation. All cells treated with the combination of LPS and IL-5 displayed a large increase in the ER chaperones GRP94 and GRP78 (Bip) regardless of tunicamycin treatment (Figure 2A). Consistent with this observation, activation of IRE1, as measured by XBP1 splicing, was highest in cells treated with both LPS and IL-5 throughout the time course (Figure 2B). Together these data suggest that differentiation of Bcl1 cells results in the activation of a physiologic UPR and that tunicamycin does not influence this response. In cells pulsed with tunicamycin for five hours we observed phosphorylation of eIF2α at higher levels than untreated cells at 24 and 48 hour time points by western blot (Figure 2A). Phosphorylated eIF2α was not observed when cells were differentiated in the absence of tunicamycin, a finding consistent with PERK activation not being part of plasma cell differentiation. Protein levels of ATF4 and CHOP, both downstream effectors of the PERK arm of the UPR, were also increased by tunicamycin addition. Cells that received tunicamycin without differentiation stimulus do not show as robust activation of this arm downstream of eIF2α phosphorylation, but it is important to note that the viability of these cells was very low at these timepoints (Figure 1B). Surprisingly, all cells treated with LPS and IL-5 in the absence of tunicamycin also demonstrated robust and prolonged expression of both ATF4 and CHOP in the absence of eIF2α phosphorylation. Moreover, cells receiving both IL-5 and LPS also showed induction of the negative regulator of PERK, $p58^{ipk}$ (Figure 2A). These data demonstrate that during differentiation, Bcl1 cells activate a robust UPR including downstream elements of XBP1 activation and the ATF4/CHOP arm of the UPR, without measurable activation of PERK.

Bcl-x_L is induced during cytokine and LPS-driven differentiation - Having observed CHOP activation in differentiating Bcl1 cells and given that CHOP induction can lead to apoptosis, we investigated the survival signaling inherent to the differentiation stimuli. During a four day time course of IL-5 and LPS-driven differentiation in Bcl1 cells, Bcl-x_L mRNA increased more than 10 fold while mRNA for both Bcl-2 and Mcl-1 decreased (Figure 2C). At the protein level, Mcl-1 decreased dramatically when cells were treated with both IL-5 and LPS, while Bcl-x_L reciprocally increased (Figure 2D). Bcl-2 protein levels did not change significantly. While IL-5 and LPS treatment alone did not induce Bim, cells that were pulsed with tunicamycin prior to IL-5 and LPS treatment displayed large increases in Bim protein by 72 hours. Interestingly, this was not sufficient to cause significant apoptosis in cells also treated with LPS and IL-5 suggesting that induction of Bcl-x_L was sufficient to protect these cells.

Bcl-x_L sequestration of Bim protects differentiating Bcl-1 cells - Since the expression patterns of the Bcl-2 family of proteins is consistent with B cells traversing a Bcl-x_L-dependent state during plasma cell differentiation, we determined the effects of the Bcl-x_L inhibitor ABT-737 on Bcl1 differentiation. ABT-737 binds to the BH3 binding pocket of Bcl-2 and Bcl-x_L but not Mcl-1 blocking the interaction with proapoptotic factors such as Bim. Bcl1 cells were pulsed with tunicamycin for 5 hours and then treated with IL-5, LPS or the combination in the presence or absence of 600nM ABT-737. At 24 hours post treatment, LPS was able to protect cells from tunicamycin-induced apoptosis (Figure 3A). This LPS-mediated protection was completely abrogated in the presence of ABT-737 demonstrating that the protective factor was Bcl-x_L or Bcl-2. We then examined the survival signaling during differentiation in the absence of tunicamycin. Bcl1 cells were

given IL-5 and LPS over a 96 hour time course. Cells were treated with varying doses of ABT-737 only during the last 24 hours prior to collection and apoptosis was determined. ABT-737 sensitivity increased as the cells differentiated through the 72 hour and 96 hour time points suggesting the cells become more Bcl-2 or Bcl-x_L-dependent as they differentiate (Figure 3B).

To interrogate the mechanism behind this protection, co-immunoprecipitation was performed on cells treated with IL-5 and LPS for 72 hours in the presence or absence of ABT-737. We immunoprecipitated the antiapoptotic proteins Bcl-x_L, Mcl-1 and Bcl-2 and blotted for Bim to determine the Bim binding pattern (Figure 3C). In untreated Bcl1 cells, Bim was primarily bound to Mcl-1 and Bcl-2. However, in cells treated with IL-5 and LPS Bim was almost completely bound to Bcl-x_L. Interestingly this was not due to changes in Bcl-2 expression, which are unchanged during differentiation (Figure 2D). Association of Bim with Bcl-x_L suggests that these cells are Bcl-x_L-dependent, a finding that is consistent with the change in ABT-737 sensitivity during differentiation. To further investigate Bcl-x_L dependence during B cell differentiation we determined the effects of ABT-737 on Bim binding to Bcl-2 proteins during differentiation. As seen in Figure 3C, addition of ABT-737 in undifferentiated cells resulted in a shift of Bim from being bound by both Bcl-2 and Mcl-1 to exclusive binding to Mcl-1. This explains why these cells are not sensitive to ABT-737 (Figure 3A). In contrast when cells are differentiated, Bim is now exclusively bound to Bcl-x_L and addition of ABT-737 results in release of Bim. However, in these cells there is no Mcl-1 available to sequester Bim, allowing for induction of apoptosis.

Bcl-x_L induction in differentiating primary murine B cells - To validate these findings in a non-transformed cell we performed in-vitro differentiation of primary murine B cells. Of the various treatments examined for differentiation IL-4 and LPS in combination yielded the most robust response as shown by increased Blimp-1 and Bcl-x_L expression (not shown). Purified primary resting B cells were cultured with IL-4 and LPS for up to 96 hours to induce differentiation of antibody secreting cells. We first examined the mRNA levels of the Bcl-2 family proteins. As seen with Bcl1 cells, mRNA for both Mcl-1 and Bcl-2 decreased and Bcl-x_L mRNA increased with treatment (Figure 4A). Increased Blimp-1 and decreased Bcl-6 expression were also observed (Figure 4B). Western blotting demonstrated a loss in Bcl-2 protein accompanied by a reciprocal increase in Bcl-x_L protein (Figure 4C). Mcl-1 protein expression did not decrease along with the loss of mRNA or as it had in the Bcl1 cell line. Also somewhat unlike the Bcl1 cell line, differentiation stimulus alone was sufficient to induce Bim in primary cells. ATF4 and CHOP protein expression were observed by western blot after 72 and 96 hours of differentiation, respectively, indicating UPR activation (Figure 4D). Intracellular light chain expression also increased throughout the treatment course indicating an increase in immunoglobulin production (Figure 4D). We performed a co-immunoprecipitation to examine Bim binding in these differentiating primary cells (Figure 4E). In primary murine B cells, at time of harvest, Bim was bound to Bcl-2. After 48 hours of treatment with IL-4 and LPS, Bim was bound to Bcl-2 and to a lesser extent Mcl-1. After 72 hours of treatment, Bim was found almost exclusively on Bcl-x_L. Importantly, when ABT-737 was added during the last 24 hours of treatment, Bim displaced from Bcl-2 by ABT-737

was able to bind Mcl-1 at 48 hours. However, at 72 hours Bim displaced from Bcl-x_L by ABT-737 did not in great measure bind to Mcl-1.

We then examined the ABT-737 sensitivity of these differentiating primary B cells. Primary B cells were treated with IL-4 and LPS for a period of 72 hours. ABT-737 was added to the cells during the last 24 hours of differentiation. ABT-737 treatment caused a loss of large, differentiating, CD138+ cells and a gain in small B cells in the live cell subset (Figure 5A, B). Furthermore, the viability of total CD138+ cells was significantly decreased while the change in viability of CD138- and total cells did not meet significance (Figure 5C-E). This indicates an increase in ABT-737 sensitivity during plasma cell differentiation.

ABT-737-induced apoptosis is CHOP-dependent - In order to investigate the link between UPR activation and Bcl-x_L dependence during differentiation, we silenced ATF4 and CHOP in Bcl1 cells. Sets of five short hairpin lentiviral constructs were stably infected and tested for both ATF4 and CHOP. The two constructs that displayed the best knockdown of ATF4 or CHOP following treatment with IL-5 and LPS were used for further experiments (not shown). The cells were treated with IL-5 and LPS for 72 hours. ABT-737 was added to the cells 24 hours prior to collection. Knockdown of ATF4 was able to block a significant, though modest amount of ABT-737-induced apoptosis in differentiating cells (Figure 6A) while knockdown of CHOP reduced apoptosis in ABT-737-treated differentiating cells from 50% in pLKO.1 vector control cells to 30% in shCHOP cells (Figure 6B). This protection from ABT-737-induced apoptosis corresponded to the level of knockdown of ATF4 and CHOP as measured by western blot

(Figure 6C, D). This indicates that activation of ATF4/CHOP is responsible for cell death by Bcl-x_L/Bcl-2 inhibition during LPS and cytokine driven differentiation.

Bcl-x_L silencing sensitizes cells to differentiation-induced apoptosis – To verify the protective factor induced by differentiation was Bcl-x_L, we used shRNA to knock down Bcl-x_L expression in Bcl1 cells. We tested five shRNA hairpins for Bcl-x_L knockdown during differentiation against a hairpin targeting GFP as a control and chose the two with the best knockdown for further experiments (Figure 6E and not shown). Differentiation with IL-5 and LPS treatment alone induced apoptosis in Bcl1 cells expressing shRNA to Bcl-x_L but not in control cells expressing shRNA to GFP (Figure 6F). In shBcl-x_L cells but not shGFP cells, addition of ABT-199, which binds to and inhibits only Bcl-2[107], increased apoptosis to levels comparable to cells treated with ABT-737 during differentiation regardless of hairpin. This suggests that though differentiation favors Bim sequestration by Bcl-x_L, in conditions where Bcl-x_L expression is abrogated, Bim can bind to available Bcl-2.

E. Discussion

While it is established that the UPR is integral to the process of plasma cell differentiation, the precise role and activation of components of this pathway have not been completely elucidated. It stands to reason that activation of the downstream effectors of the ATF6 and IRE1/XBP1 arms of the UPR are beneficial to the process of becoming a professional protein secreting cell such as a plasma cell. However, activation of the PERK arm includes aspects of the UPR that are in direct contrast to the needs of the plasma cell, namely translation inhibition and apoptosis. Consistent with this model, PERK is neither necessary nor is it activated during plasma cell differentiation despite having an activation step nearly identical to IRE1.[32, 35] However, it has been shown that one of the main downstream effectors of PERK, CHOP, is necessary for a maximally efficient plasma cell.[36] Activation of CHOP has also been shown to induce Bim and inhibit Bcl-2 leading to apoptosis.[98, 99] In this study we have verified that the UPR of the differentiating Bcl1 cell includes activation of ATF4 and CHOP but lacks phosphorylation of eIF2α (Figure 2A). Examining the effects of PERK activation on this differentiation, we demonstrated that Bcl1 cells were very sensitive to tunicamycin-induced apoptosis, yet were protected when they were subsequently given LPS as a differentiation stimulus (Figure 3A). This led us to hypothesize that there was survival signaling inherent in the differentiation contributing to this protection. We examined the Bcl-2 family of antiapoptotic proteins and showed that Bcl-x_L was induced by differentiation in both the Bcl1 cell line and in primary C57BL/6 B cells (Figure 2B,D, 4A,C). Additionally, in primary B cells we saw the decrease of Bcl-2 and increase of Bim levels along with CHOP activation (Figure 4D). These data suggest that the

differentiating B cells become dependent on Bcl-x_L and consistent with this possibility, differentiating cells are more sensitive to ABT-737. While LPS treatment provided immediate protection from ER stress-induced apoptosis (Figure 3A), only after 72 hours of stimulation was there a shift to Bcl-x_L dependence (Figure 3B), suggesting that differentiation is necessary for this shift. This is consistent with previous studies demonstrating that newly formed plasma cells are lost in animals treated with ABT-737 while no loss occurs in mature B cells in the germinal centers and spleen or in long lived bone marrow plasma cells.[65-67] This would suggest that Bcl-x_L-dependence only occurs at an intermediate stage of plasma cell differentiation. The sensitive population of differentiating primary B cells were $CD138^{+}$, $CD19^{High}$, indicative of an early plasma blast phenotype (Figure 5 and data not shown). The molecular basis for the change in ABT-737 sensitivity was due to a change in sequestration of Bim by Bcl-2 and Mcl-1 to Bcl-x_L during this stage of differentiation.

From these data we propose a model in which differentiation induces a loss of Mcl-1 and Bcl-2 and induction of Bim associated with UPR activation. While we have not directly demonstrated the mechanism of differentiation-induced apoptosis, we have shown that it is at least partially dependent on ATF4 and CHOP, which are part of the UPR. However, differentiation also induces Bcl-x_L, which is sufficient to sequester Bim preventing apoptosis (Figure 7). Presumably this stage is short lived as it has been shown that long-lived plasma cells are reliant on enforced expression of Mcl-1 along with survival signals provided by their stromal niche.[66] While some of this change in dependence is certainly due to changes in expression, there is evidence that expression alone does not determine Bim binding.[73] This may explain why in normal B cell differentiation, Bim released from

Bcl-x_L is unable to bind to Mcl-1, yet prior to this point in differentiation, Bim released from Bcl-2 can bind to Mcl-1. It may also explain why we see changes in the expression of anti-apoptotic Bcl-2 proteins throughout lymphocyte development and differentiation, even though these proteins all have the same basic function in regulating apoptosis.

F. Acknowledgements

The authors thank Ned Waller and Kasia Darlak for assistance with mouse spleens and the Boise lab for critical feedback on the data. The work was supported by R01 CA127910 and R01 CA129968 as well as funding from the TJ Martell Foundation (LHB). LHB is a GRA Distinguished Cancer Scientist.

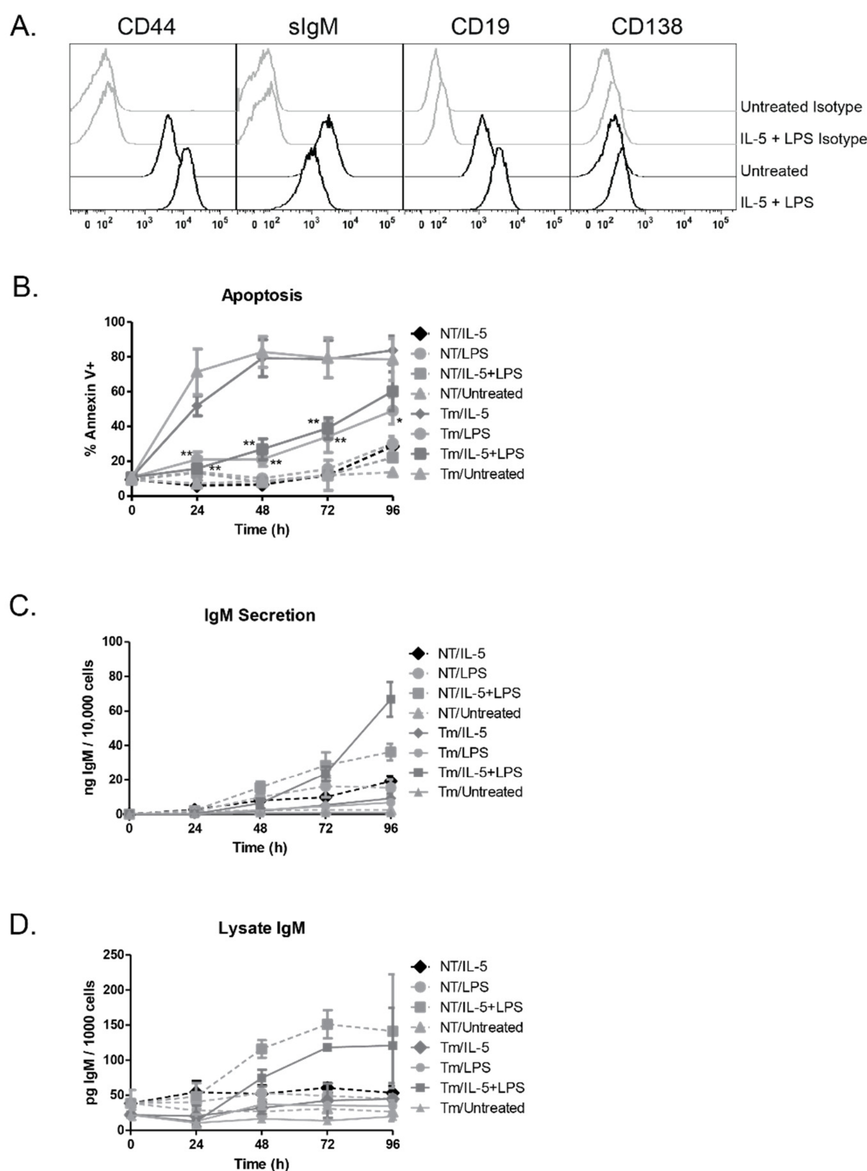

FIGURE 1. Bcl1 cells differentiate with IL-5 and LPS treatment. (A) Bcl1 cells were treated with IL-5 and LPS for 72 hours prior to collection and staining. B cell and plasma cell markers were measured by flow cytometry. (B-D) Bcl1 cells were untreated (NT) or pretreated with tunicamycin (Tm) for 5 hours prior to treatment with IL-5 and/or LPS for 96 hours. (B) Apoptosis was measured by Annexin V and PI staining. (C) IgM was measured by ELISA of the supernatant of the various treatment groups and is reported as a function of the live cell number. (D) IgM was measured by ELISA of the lysates of the various treatment groups and is reported as a function of the live cell number contributing to the lysate. Data are represented as the mean ± SE of 3 independent experiments (*p < .05, **p < .01)

Figure 2

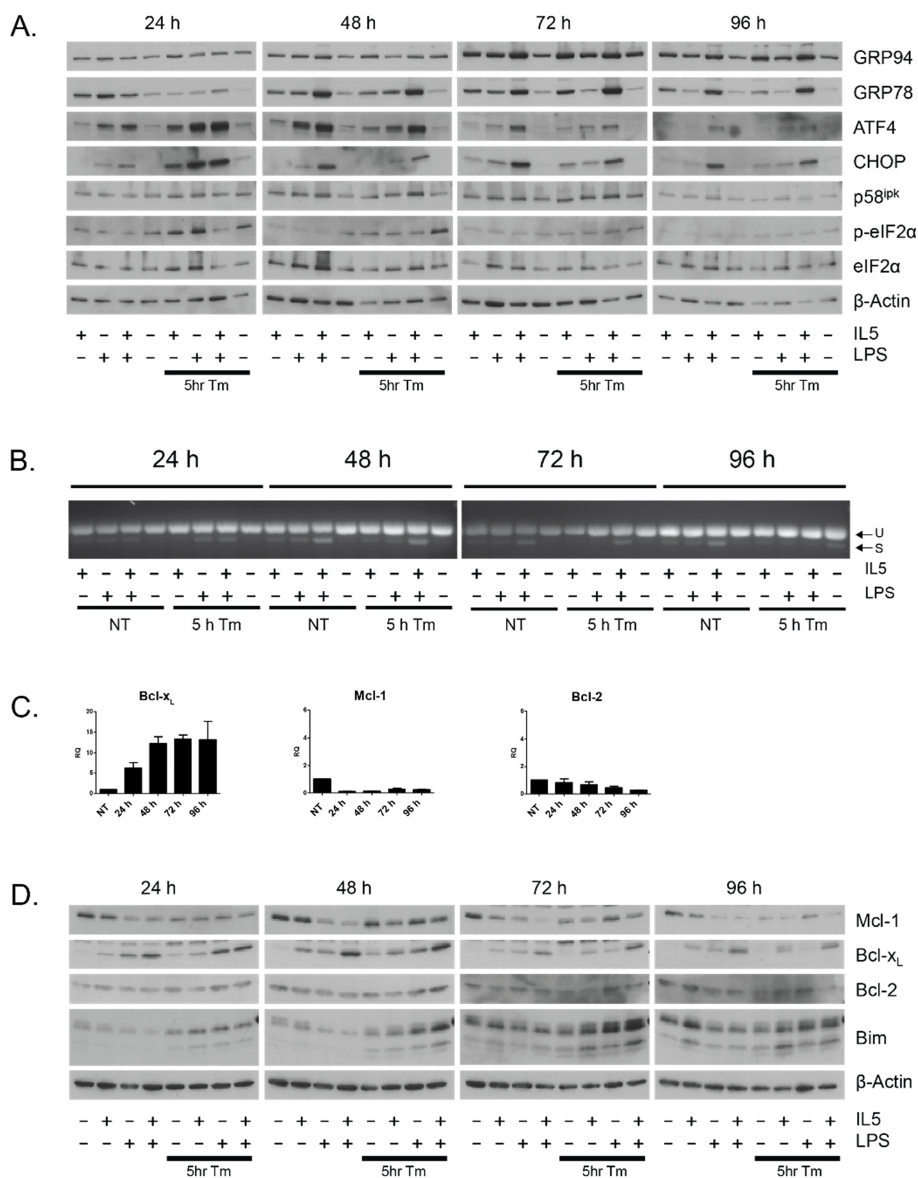

FIGURE 2. Differentiating Bcl1 cells activate the unfolded protein response and upregulate Bcl-x$_L$. Bcl1 cells were pretreated with tunicamycin for 5 hours prior to treatment with IL-5, LPS or the combination for 96 hours. (A) Protein levels of UPR-related proteins were determined by Western blot. (B) XBP1 splicing was determined by amplification of the spliced region and running the PCR products on agarose gels. "U" and "S" designate unspliced and spliced PCR products respectively. (C) Bcl1 cells were treated IL-5 and LPS for 96 hours. qRT-PCR was performed on RNA collected at 24 hour periods. Cycle numbers were normalized to GAPDH and presented as RQ as a function of untreated samples. (D) Bcl1 cells were pretreated with tunicamycin for 5 hours prior to treatment with IL-5, LPS or the combination for 96 hours. Protein levels of Bcl-2 family proteins were determined by Western blot.

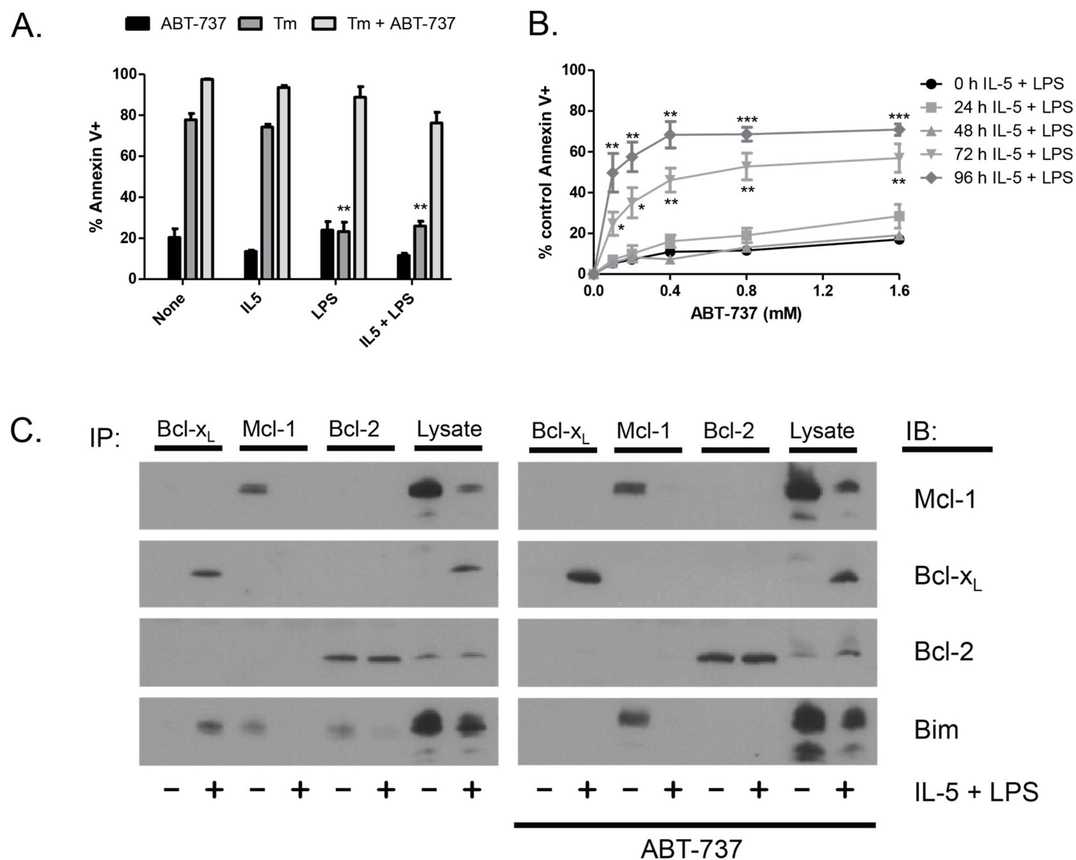

FIGURE 3. Bcl-x$_L$ mediates protection from ER stress- and differentiation-induced apoptosis in Bcl1 cells. (A) Bcl1 cells were pretreated with tunicamycin for 5 hours prior to treatment with IL-5, LPS or the combination for 24 hours with or without 600 nM ABT-737. Apoptosis was measured using annexin V and PI staining. (B) Bcl1 cells were treated with IL-5 and LPS for 96 hours. Samples were treated with varying doses of ABT-737 for the final 24 hours prior to collection and apoptosis was measured by annexin V and PI staining. (C) Bcl-1 cells were treated with IL-5 and LPS with or without 600 nM ABT-737 for 72 hours. Immunoprecipitation was performed on lysates prepared from individual treatments. Data are presented as the mean ± SE of 3 independent experiments (*p<.05, **p<.01, ***p<.001)

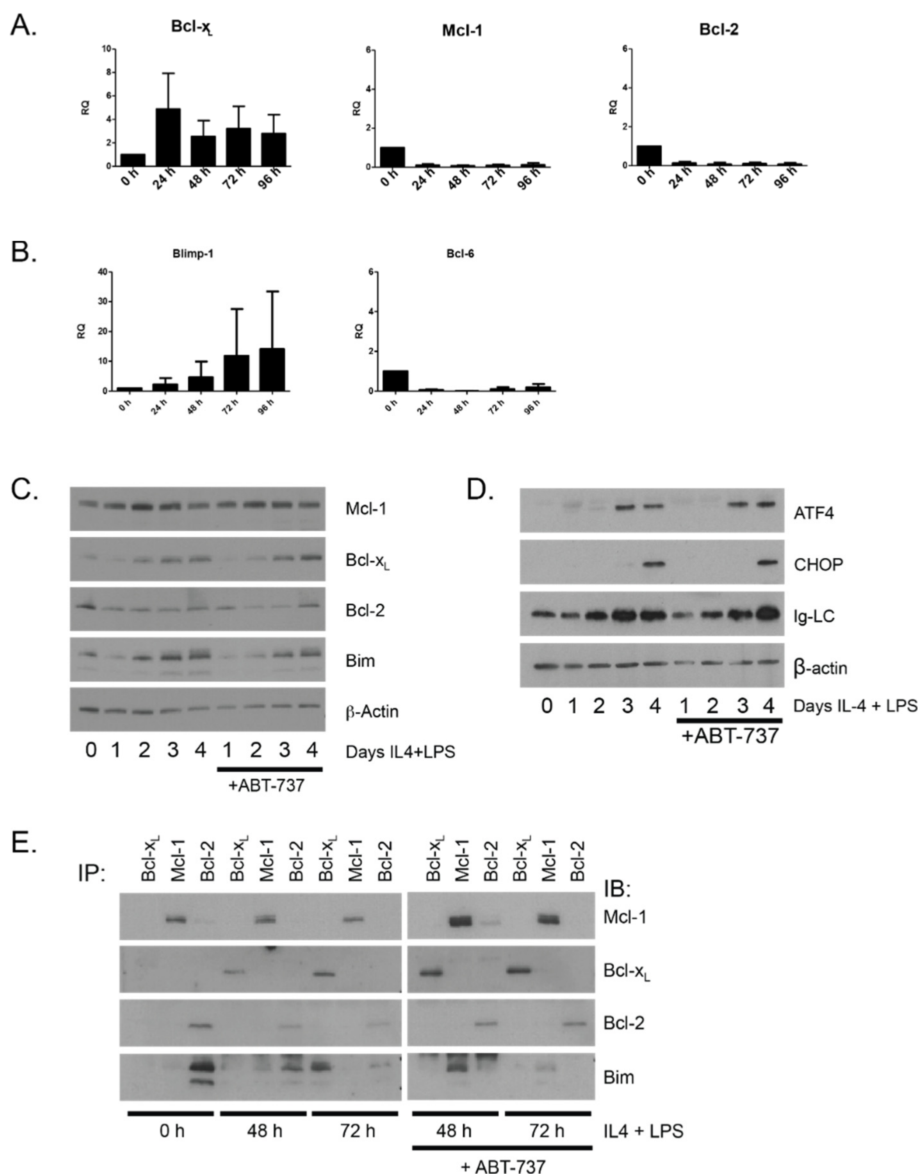

FIGURE 4. Bcl-x$_L$ is induced during plasma cell differentiation in primary B cells. (A, B) Primary murine B cells were treated with IL-4 and LPS for 96 hours. qRT-PCR was performed on RNA prepared from samples collected at 24 hour intervals. Cycle numbers were normalized to GAPDH and presented as RQ as a function of untreated time zero samples. (C,D) Primary murine B cells were treated with IL-4 and LPS for 4 days. 600 nM ABT-737 was added to cultures 24 hours prior to collection. Bcl-2 family members were assayed by Western blot at the indicated time. (E) Primary murine B cells were treated with IL-4 and LPS for 72 hours. ABT-737 was added to the cultures in the final 24 hours of treatment. Co-immunoprecipitation was used to assess Bim binding at 0, 48 and 72 hour time points.

Figure 5

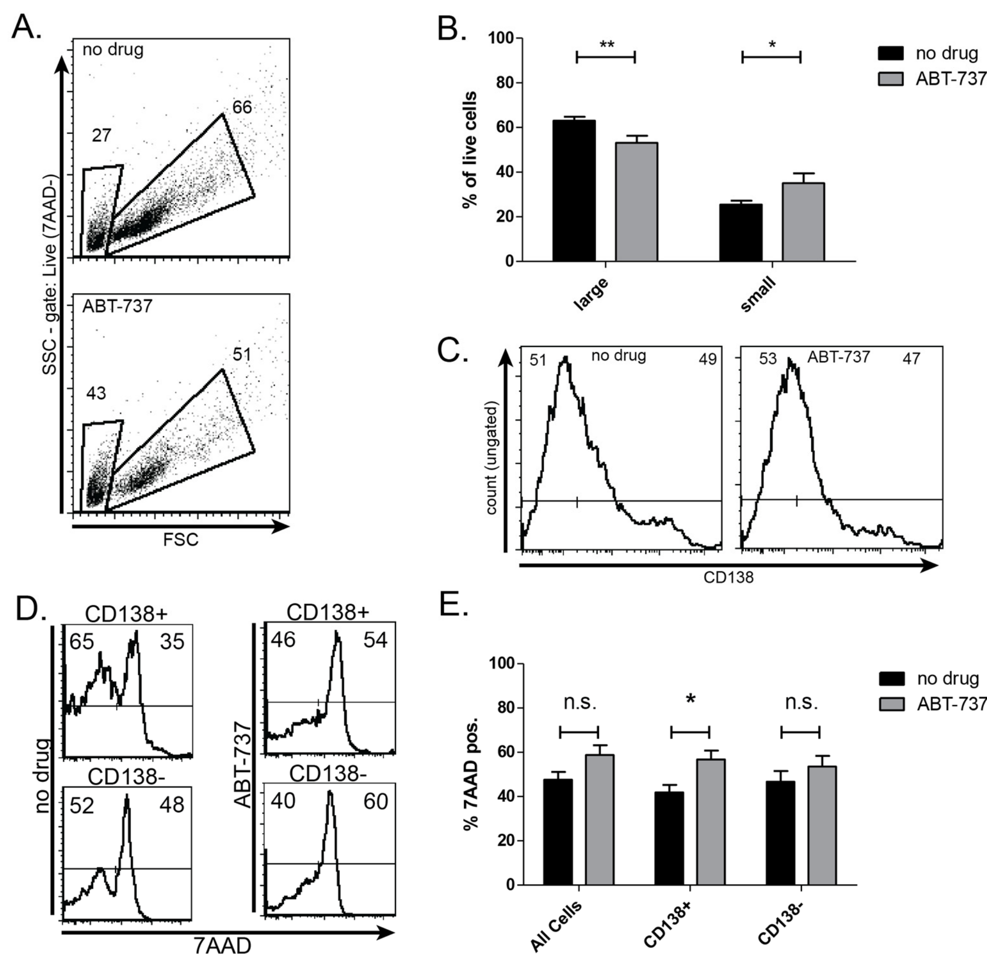

FIGURE 5. ABT-737 sensitizes primary B cells to differentiation-induced apoptosis. Primary murine B cells were treated with IL-4 and LPS for 96 hours. ABT-737 was added during the final 24 hours prior to collection. Live cells determined by negative 7AAD staining were gated into small B cells or large differentiating cells (representative experiment, A) and are quantified in B. Total cells were gated into CD138 positive and negative fractions (C is representative of gating) and 72 hour cell death was measured as the proportion of 7AAD positive cells (representative experiment, D) and is quantified in E. Data in B and E are presented as the mean ± SE of 6 independent experiments. (*p<.05, **p<.01)

Figure 6

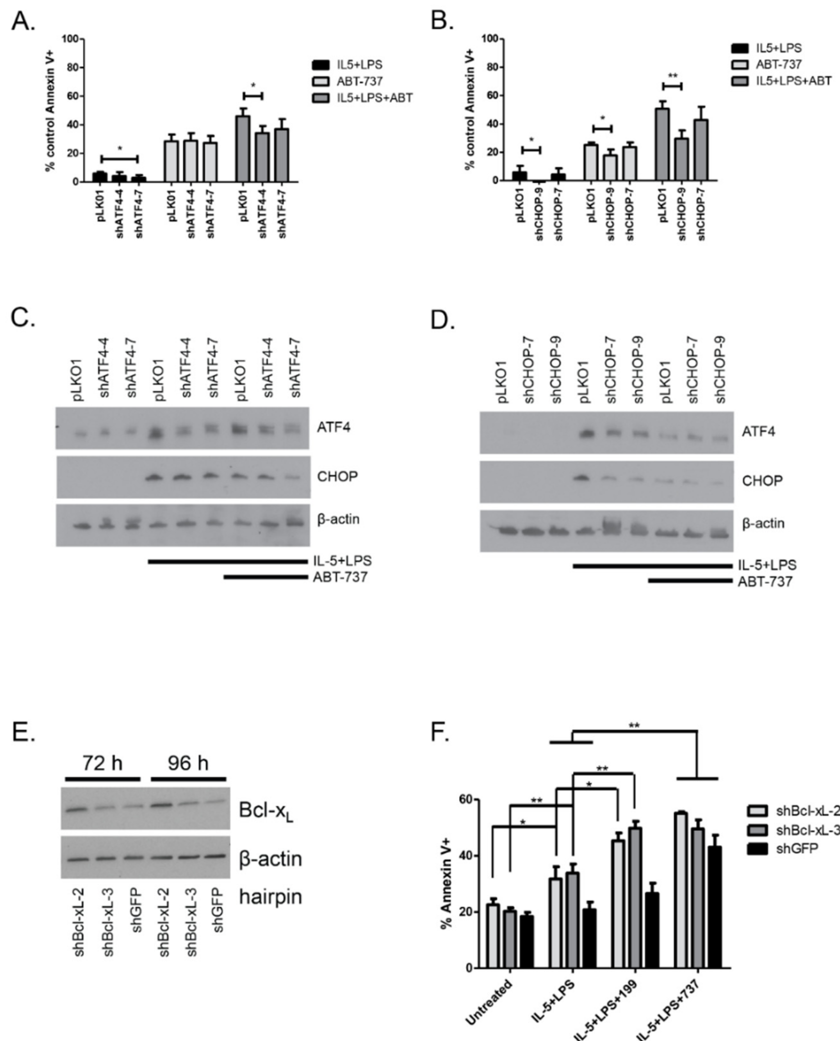

FIGURE 6. Knockdown of CHOP abrogates differentiation induced ABT-737 sensitivity. Bcl1 cells infected with shATF4, shCHOP or pLKO1 vector control lentivirus were treated with IL-5 and LPS for 72 hours. 600 nM ABT-737 was added 24 hours prior to collection. (A, B) Apoptosis was measured at 72 hours. Western blots were prepared at 72 hours (representative experiments C, D). Data are presented as the 4 independent experiments. (*$p<.05$, **$p<.01$)

Knockdown of Bcl-xL sensitizes Bcl1 cells to differentiation-induced apoptosis
Bcl1 cells stably infected with shBcl-x_L or shGFP lentivirus were untreated or treated with IL-5 and LPS for 72 hours. ABT-737 (600nM) or ABT199 (600nM) were added 24 hours prior to collection. Western blots were prepared at 72 h and 96 h time points to verify knockdown (E). Apoptosis was measured at 72 hours (F). Data in F are presented as the mean ± SE of 3 independent experiments. (*$p<.05$, **$p<.01$)

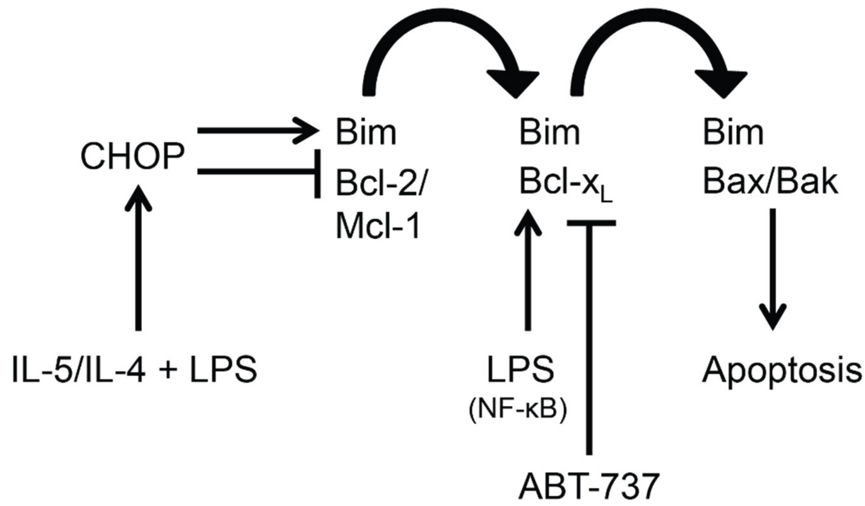

FIGURE 7. Schematic of proposed Bcl-2 family BH3 protein interactions in differentiation. LPS and cytokine-driven differentiation induces Bim while decreasing expression of Mcl-1 and Bcl-2. Bcl-x$_L$ is induced and binds Bim that is displaced. ABT-737 blocks Bcl-x$_L$ releasing Bim which is then free to activate Bax and Bak, resulting in the induction of apoptosis.

III. BCL-2 FAMILY REGULATION OF APOPTOSIS IN WALDENSTRÖM MACROGLOBULINEMIA

Low Expression of Pro-Apoptotic Bcl-2 Family Proteins Sets the Apoptotic Threshold in Waldenström Macroglobulinemia.

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A. Abstract

Waldenström macroglobulinemia (WM) is a proliferative disorder of IgM secreting, lymphoplasmacytoid cells that inhabit the lymph nodes and bone marrow. The disease carries a high prevalence of activating mutations in MyD88 (91%) and CXCR4 (28%). Because signaling through these pathways leads to Bcl-x_L induction, we examined Bcl-2 family expression in WM patients and cell lines. Unlike other B-lymphocyte-derived malignancies, which become dependent on expression of anti-apoptotic proteins to counter expression of pro-apoptotic proteins, WM samples expressed both pro- and anti-apoptotic Bcl-2 proteins at low levels similar to their normal B-cell and plasma cell counterparts. Three WM cell lines expressed pro-apoptotic Bcl-2 family members Bim or Bax and Bak at low levels which determined their sensitivity to inducers of intrinsic apoptosis. In two cell lines, miR-155 upregulation, which is common in WM, was responsible for inhibition of FOXO3a and Bim expression. Both antagonizing miR-155 to induce Bim and proteasome inhibition to cleave Bid increased the sensitivity to ABT-737 in these lines indicating a lowering of the apoptotic threshold. In this manner, treatments that increase pro-apoptotic protein expression increase the efficacy of agents treated in combination in addition to direct killing.

B. Introduction

Since the discovery of B-cell lymphoma-2 (Bcl-2), nearly three decades ago, the Bcl-2 family of proteins has been known to regulate the process of apoptosis in all mammalian cells. Bcl-2 family regulation of apoptosis culminates in the pro-apoptotic BH3-only activator proteins Bim, tBid and Puma transiently associating with and causing a conformational change in the multi-domain pro-apoptotic proteins Bax and Bak[44]. Upon their activation, Bax and Bak homo-oligomerize and initiate mitochondrial outer membrane permeabilization (MOMP)[45]. When Bim or tBid are present a cell must also express anti-apoptotic Bcl-2 family proteins to block apoptosis. The set of known anti-apoptotic Bcl-2 family proteins now includes Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, Bcl-b and A1/Bfl-1, which are capable of binding BH3-only activators as well as activated monomers of Bax and Bak[47]. Expression of these proteins is capable of blocking apoptosis provided they are expressed at levels in excess of pro-apoptotic proteins. This balance can be shifted, however, by the expression of pro-apoptotic BH3-only proteins termed sensitizers including Hrk, Bad, Bik, Bmf and Noxa. Sensitizers bind the anti-apoptotic Bcl-2 proteins with varying affinities and are capable of displacing Bim, tBid, Puma or activated Bax and Bak leading to MOMP and apoptosis[48].

Processes of proliferation and differentiation induce death signals in the form of pro-apoptotic protein expression that are countered by survival signals that induce expression of anti-apoptotic proteins. For example, enforced expression of the proliferative oncogene *c-myc* leads to proliferation but also leads to apoptosis, however co-expression of Bcl-2 or any other anti-apoptotic family member with *c-myc* rescues this cell death resulting in tumor formation[68, 69]. In this manner a cancer cell that breaks a

differentiation or proliferation checkpoint must then compensate for the inherent activation of pro-apoptotic Bcl-2 family members with increased expression of anti-apoptotic family members. This has come to be known as mitochondrial priming in that cancer cells become primed for death by increased abundance of pro-apoptotic protein being sequestered by anti-apoptotic proteins[48]. In this way the apoptotic threshold of a cancer cell is lowered because it requires less death signaling to engage mitochondrial-dependent apoptosis. Furthermore, it has been shown that the level of priming of a variety of cancers and healthy tissues determines their response to various anti-cancer agents illustrating a basis for the therapeutic index seen in-vivo[71].

Waldenström macroglobulinemia (WM) is a low grade lymphoproliferative disorder characterized by clonal, lymphoplasmacytoid, IgM-secreting cells[85, 108]. The clonal cancer cells exist at the point of differentiation between a B-cell and plasma cell. Two activating mutations have been shown to be common in WM. The MyD88 (L265P) mutation is found in 91% of WM cases[82, 109] and the CXCR4 (S338X) mutation is found in nearly a third of WM cases. Since both MyD88 and CXCR4 signaling lead to downstream activation of NF-κB which induces Bcl-x_L, and since we have shown that differentiating plasma cells proceed through a Bcl-x_L-dependent intermediate[88], we hypothesized that WM cells are dependent on Bcl-x_L for survival. In this study we examined the Bcl-2 protein expression in WM patient samples and observed that WM cells are characterized by low expression of both pro- and anti-apoptotic Bcl-2 family proteins. This is in sharp contrast with the plasma cell tumor, multiple myeloma (MM), which is characterized by increased expression of anti-apoptotic Bcl-2 family members to compensate for increased expression of Bim. These data provide evidence that the

apoptotic threshold in WM cells is high due to low expression of pro-apoptotic Bcl-2 family members not due to high expression of anti-apoptotic proteins.

C. Methods

Gene Expression Array Analysis – Normalized (Affymetrix HG-U133A Plus 2.0 Microarray) expression data was obtained from GEO database (GSE6691; PMID: 17252022) for the study[110]. The expression data was RMA normalized and log2 transformed, to examine the gene expression differences between B-cell like and plasma-cell like patient groups. Unsupervised hierarchical clustering of 9 BCL-2 genes microarray derived expression was performed to demonstrate the up- and down-regulated expression differences at the gene and probe level between B-cell like and plasma-cell like patient samples. Euclidean distance measure with agglomerative average-linkage was used for clustering row normalized expression data.

Cell Culture – BCWM.1, MWCL-1 and RPCI-WM1 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 I.U. penicillin/streptomycin, 1 mM sodium pyruvate and non-essential amino acids. Bortezomib was purchased from LC Laboratories. ATO was purchased from Sigma Aldrich. ABT-737 was generous gift of Abbvie (North Chicago, IL).

Lentiviral anti-mIR expression - Virus encoding anti-mIR-155 or control *Arabidopsis thaliana* anti-mIR was purchased from Sigma Aldrich. WM cell lines were then infected and selected with puromycin. Transductants were kept in selection during passage and verified by western blot and qRT-PCR of downstream targets. Experiments were carried out in the absence of puromycin.

Apoptosis was assayed with Annexin-V-FITC (Biovision 1001-1000) and propidium iodide (2 μg/mL Sigma) staining and measured with a BD FACScanto II as previously described[103].

Gene Expression - cDNA was prepared from RNA harvested at specified time points using ABI high capacity cDNA kit (Applied Biosystems). Realtime PCR was performed using Taqman gene expression master mix (ABI 4368814) with an ABI 9600 Fast thermocycler as previously described[73]. Probes: Bim (bcl2l11) Hs00708019_s1, Bak (bak1) Hs00832876_g1, Bax (bax) Hs00180269_m1, FOXO3a (foxo3) Hs00818121_m1, GAPDH 4333764.

MicroRNA Expression - RNA was extracted and enriched for small RNA using the Ambion Mirvanna kit (Life Technologies AM1561) according to protocol. cDNA was prepared using a specific primer pool of 8 target microRNAs plus 4 small control RNAs (Applied Biosystems). Realtime PCR was performed using TaqMan Universal Master Mix II (4440040) according to protocol. Endogenous control CT_{ec} was determined by average of the four control small RNA CT values. RQ was determined by the CT value of the experimental miR normalized to the endogenous control ($\Delta CT = CT - CT_{ec}$) then normalized to the experimental control ($\Delta\Delta CT = \Delta CT - \Delta CT_{c}$) and expressed as the relative quantity ($RQ = 2^{-\Delta\Delta CT}$). Probe and primer sets: (PN4427975) hsa-mir-9*, hsa-mir-155, hsa-mir-184, hsa-mir-206, hsa-mir-363, hsa-mir-363*, hsa-mir-494, hsa-mir-542-3p, RNU6B, RNU44, RNU48, U47.

Immunoblotting - Western blotting was performed on lysates collected at the various time points and treatments as previously described[88, 103]. Antibodies: Rabbit IgG-HRP

donkey and Mouse-IgG-HRP sheep were purchased from GE Healthcare. Mcl-1 rabbit polyclonal was purchased from Enzo Life Sciences. Bcl-2 hamster (6C8) was purchased from BD Pharmingen. Bax, Caspase-3, Caspase-7, Bid and FOXO3a rabbit polyclonal and Caspase-8 mouse (1C12) were purchased from Cell Signaling Technology. Bim and Bak rabbit polyclonal were purchased from Milipore. β-Actin mouse (AC-15) and Parp rabbit polyclonal were purchased from Sigma Aldrich. Bcl-x_L mouse (2A1) and Bcl-x_L rabbit polyclonal (13.6) have been previously described[111].

D. Results

We examined Bcl-2 protein expression in a published expression database containing 10 WM patients along with 11 chronic lymphocytic leukemia (CLL) patients, 12 multiple myeloma (MM) patients, 8 normal B-cell (NBL) donors and 5 normal plasma cell (NPC) donors[110]. The WM cells were separated pairwise by patient based on their B-cell-like (WBL) or plasma cell-like (WPC) phenotype. We performed an unsupervised hierarchical clustering of nine Bcl-2 family genes in all samples (Figure 1A). Interestingly, these nine Bcl-2 family genes alone were sufficient to cluster the various cell types[110]. The greatest separation based on gene expression of the cell types was between the B-cell-like (NBL, CLL, WBL), and plasma cell-like (WPC, NPC, MM) groups indicating that Bcl-2 family expression is primarily driven by the state of differentiation, not transformation. We therefore split these groups and performed an unsupervised hierarchical clustering of these same nine genes on the set of B-cell like or plasma cell like groups separately. In the B-cell-like group, we observed a pattern where NBL samples expressed lower levels of Bcl-2 proteins than CLL samples and WBL samples were split between being similar to NBL and CLL samples (Figure S1A). The only gene differentially expressed in WBL samples was Noxa which was expressed in WBL samples at significantly higher levels than NBL samples and nearly significantly higher levels than CLL samples (Figure S1B).

We observed that WPC samples clustered more closely with NPC samples not MM samples and that in general WPC and NPC samples expressed lower levels of Bcl-2 family proteins than MM samples (Figure 1B). Analysis of specific genes revealed that WPC samples expressed less Bad than both MM samples and possibly less Bcl-2

although only one of four probes displayed this trend (Figure 1C and not shown). Both WPC and NPC groups expressed Bid at significantly higher levels than MM samples (Figure 1D). MM samples displayed higher expression of Bim than both NPC and WPC samples (Figure 1E). These data demonstrate that plasma cell phenotype WM cells express Bcl-2 family proteins at levels more similar to normal plasma cells than MM which is indicative of WM cells having a higher apoptotic threshold.

In order to determine the effects of Bcl-2 family protein expression on WM cell survival, we obtained three previously described WM cell lines, BCWM.1, MWCL-1 and RPCI-WM1[112-114]. We then assessed Bcl-2 family protein expression in three WM cell lines compared to two MM cell lines. We observed low to moderate levels of the anti-apoptotic proteins Bcl-x_L, Mcl-1 and Bcl-2, the last of which was nearly undetectable in RPCI-WM1 cells (Figure 2A). All three WM cell lines expressed Bid at higher levels than the MM cell lines and both BCWM.1 and MWCL-1 cells expressed moderate levels of Puma (Figure 2B). Strikingly, we observed marked defects in the expression of Bak, Bax and Bim in these cell lines (Figure 2B). BCWM.1 cells expressed Bim at a very low level and MWCL-1 cells expressed no detectable Bim. RPCI-WM1 cells expressed Bim at moderate levels compared to MM cell lines however expressed very little Bak and no detectable Bax. We then examined the mRNA expression of these genes in the cell lines and observed very low levels of Bim mRNA in both BCWM.1 and MWCL-1 cells (Figure 2C). While RPCI-WM1 cells displayed levels of Bak mRNA expression comparable to the other cell lines, only very low levels of Bax mRNA was detected indicating distinct mechanisms of down regulation of these two proteins (Figure 2D, E). We then examined the expression of Bim, Bak and Bax with bortezomib and arsenic

trioxide (ATO) treatment. We observed that Bim was induced by ATO treatment in BCWM.1 but not MWCL-1 cells (Figure 2F). Bortezomib treatment did not increase protein expression of Bim, Bax or Bak in these cell lines indicating that downregulation was not due to increased proteasomal degredation as compared with the proteasome substrate Mcl-1 (Figure 2F). The expression of Bcl-2 family members in these WM cell lines indicate distinct mechanisms leading to a common defect in the intrinsic apoptotic pathway at the point of Bim activation of Bax and Bak.

Given the deficiencies in proteins required to activate mitochondria-dependent apoptosis, we then determined the sensitivity of these cells to agents known to activate this pathway. We first examined sensitivity to the Bcl-2 and Bcl-x_L inhibitor, ABT-737[72]. Importantly, treatment with ABT-737 alone, which relies on the priming of a cell to activate apoptosis, induced only very low levels of apoptosis in BCWM.1 and MWCL-1 cells as compared to the ABT-737-sensitive MM cell line MM.1s (Figure 3A). No detectable apoptosis was induced by ABT-737 treatment in RPCI-WM1 cells (Figure 3A). Treatment with the proteasome inhibitor, bortezomib induced moderate apoptosis in both BWCM.1 and MWCL-1 cells compared with MM.1s but once again induced no apoptosis in RPCI-WM1 cells (Figure 3B). Treatment with ATO, which induces mitochondria-dependent apoptosis at low concentrations, induced only low levels of apoptosis in BCWM.1 and MWCL-1 cells and no apoptosis in RPCI-WM1 unless the concentration was increased to levels of toxicity (Figure 3C). Treatment with Dexamethasone induced no cell death in all three cell lines (not shown). Interestingly, treatment with bortezomib or ATO alone at a concentration that induced moderate apoptosis, induced more than additive amounts of

apoptosis with co-treatment with ABT-737 in both BCWM.1 and MWCL-1 cells yet induced no additional apoptosis in RPCI-WM1 cells (Figure 3D-F).

Since RPCI-WM1 cells were insensitive to agents that induced death in BCWM.1 and MWCL-1 cells, we treated RPCI-WM1 cells with up to 100 nM bortezomib for 48 hours and observed cell death (Figure 4A). We then treated the cells with bortezomib in the presence of the pan-caspase inhibitor BocD-FMK (BocD). In BCWM.1 and MWCL-1 cells the presence of BocD abrogated approximately 50% of the cell death whereas it was unable to block any cell death induced by high dose bortezomib in RPCI-WM1 cells (Figure 4B). Furthermore, the annexin-V and PI staining pattern of both bortezomib treated BCWM.1 and MWCL-1 cells is indicative of apoptosis with cells becoming annexin-V positive and then annexin-V and PI double positive, while the staining pattern for RPCI-WM1 cells treated with bortezomib is not indicative of apoptosis with cells becoming PI positive and then PI and annexin-V double positive (Figure 4C-E). Examination of apoptosis-related proteins by western blot indicated the loss of full length Caspases 3 and 7 and Bid as well as PARP cleavage in BCWM.1 and MWCL-1 cells but not in RPCI-WM1 cells (Figure 4F). Together these data show BCWM.1 and MWCL-1 cells, though insensitive to agents acting with a Bim-dependent mechanism such as ABT-737 and Dexamethasone, are capable of undergoing apoptosis with bortezomib treatment while RPCI-WM1 cells are insensitive to all these agents and are completely apoptosis deficient.

In order to further investigate the mechanism of dysregulation of Bim, Bax and Bak in the WM cell lines, we first examined the genomic copy number data available in WM patient samples and the cell lines. Array-CGH analysis of the RPCI-WM1 cell line, the

original donor patient sample and an age and sex-matched normal donor showed no genomic copy number variation of either Bak, Bax or Bim (Table S1 and not shown). We then examined a SNP copy number array of 46 WM patients at the loci for Bak, Bax and Bim. This data set contained only one copy number gain for each Bak and Bax and no losses in any of the three genes nor did it show evidence of uniparental disomy in these genes (Table S2). These data provide no evidence that low expression of Bax, Bak or Bim protein in either the cell lines or WM patients is due to genomic copy number variation.

With no evidence or changes in genomic copy number variation and no evidence of modulation in protein stability as proteasome inhibition did not cause a recovery of Bim, Bax or Bak protein in the cell lines (Figure 2F), we then examined mechanisms of epigenetic regulation of expression. We first examined the methylation state of promoter CpG islands in Bax, Bak and Bim in BCWM.1, MWCL-1 and RPCI-WM1 cells. PCR amplification of bisulfite treated samples from all three cell lines yielded only products of primers targeted to unmethylated DNA and therefore converted promoters (Figure S2A). Similarly, bisulfite sequencing of Bax and Bim promoters displayed no evidence of hypermethylation of CpG islands in the promoters of these genes in these WM cell lines (Figure S2B, C).

We next examined the microRNA expression in these cell lines. We first analyzed the set of seven commonly dysregulated microRNAs in WM[83] for miRs targeting Bim, Bax and Bak and found that only miR-494 had a low probability 7mer site in the 3' UTR of Bim[115] (Figure S3A). While it was reported that miR-363* is upregulated in WM, its sister mature microRNA, miR-363 had two moderately strong target sites in the 3' UTR

of Bim so we added it to our analysis (Figure S3B). miR-494 was not expressed at higher levels in MWCL-1 cells than RPCI-WM1 cells and only slightly higher levels in BCWM.1 cells were detected (Figure 5A). Though miR-363* was undetectable in all three WM cell lines, miR-363 was expressed at high levels in BCWM.1 and to a lesser degree in both MWCL.1 and RPCI-WM1 (Figure 5B, C). Since the pattern of these two possible Bim targeting microRNAs did not fit the pattern of Bim expression in the three WM cell lines, we examined the rest of the commonly upregulated microRNAs (Figure 5D-G) and one downregulated microRNA (Figure 5H). Only the miR-155 expression pattern was consistent with suppression of Bim (Figure 5D). None of the set of commonly upregulated microRNAs had predicted targets in the 3'UTRs of Bax or Bak (not shown) and none were expressed at higher levels in RPCI-WM1 than in Bax/Bak expressing cells (Figure 5).

miR-155 has been shown to both directly and indirectly target the transcription factor FOXO3a of which Bim is a downstream target (Figure S3C)[84, 115-117]. We therefore examined the expression of FOXO3a protein in the WM cell lines, with and without treatment with bortezomib and ATO, and observed that FOXO3a expression mirrored the Bim expression pattern (Figure 6A, B). Having observed the expression patterns of miR-155, FOXO3a and Bim, we used a lentiviral vector to stably deliver either an anti-miR targeting miR-155 or a control anti-miR to the three WM cell lines in order to determine its role in Bim expression control. In both BCWM.1 and MWCL-1 cells, along with increased doubling time (data not shown), we saw an increase in mRNA for FOXO3 and Bim and an increase in Bim protein with anti-miR-155 virus (Figure 6C-E). While there was no increased apoptosis in untreated anti-miR-155 expressing BCWM.1 or RPCI-

WM1 cells there was a small but significant increase in apoptosis in MWCL-1 cells expressing anti-miR-155 (Figure 6F). We then treated these cells with ABT-737 and observed an increase in apoptosis in both BCWM.1 and MWCL-1 cells in the absence of any other death signal (Figure 6F). These data demonstrate that increased expression of miR-155 in BCWM.1 and MWCL-1 cells decreases expression of Bim and its transcription factor FOXO3a abrogating activation of intrinsic apoptosis.

E. Discussion

All cancer treatment requires a therapeutic index that dictates that the tumor cells will die before, and in greater numbers than the patient's healthy tissues. It is sometimes acceptable to use therapy that targets the normal biology of the tumor as in the case of rituximab targeting CD20-expressing cells. Since the normal cells that express CD20 are not essential for life, targeting this biology has limited toxicity. However, most chemotherapeutic agents target all cells, yet kill cancer cells at lower concentrations than healthy cells. In the case of these treatments, the therapeutic index relies on the concept of mitochondrial priming[71]. This priming is the load of pro-apoptotic protein being sequestered in a given cell. The more primed a cell is the less death signaling it needs to cross the apoptotic threshold. On the whole, cancer cells exhibit higher priming due to breaking proliferative and differentiation checkpoints. In this study we examined the expression of Bcl-2 family proteins in Waldenström Macroglobulinemia and its effect on apoptotic threshold.

Based on our studies of plasma cell differentiation, we had hypothesized that a disease harboring the MyD88 (L265P) mutation would have increased expression of Bcl-x_L acting as a protective oncogene[88]. At the very outset we observed that this was not the case in WM as patient gene expression data showed that Bcl-x_L expression was at levels similar to normal tissues (Figure 1). Furthermore, treatment with ABT-737 did not induce significant apoptosis in WM cell lines (Figure 2A) which would also suggest that there is not pro-apoptotic protein being sequestered by either Bcl-x_L or Bcl-2. Both in patient expression data and in BCWM.1 and MWCL-1 cells we observed low expression of Bim (Figure 3, 4). In contrast, the RPCI-WM1 cell line expressed Bim at higher levels

yet was deficient in both Bax and Bak which rendered it apoptosis-deficient in all conditions tested (Figure 3 - 5). Thus all three cell lines resist apoptosis through loss of pro-apoptotic proteins not upregulation of anti-apoptotic Bcl-2 family members. However, both BCWM.1 and MWCL-1 cells did undergo apoptosis provided that it was Bim-independent. Bortezomib treatment has been shown to induce Caspase-8-mediated cleavage of Bid which then synergizes with ABT-737 treatment[118], which we observed in MWCL-1 and BCWM.1 cells (Figure 4). In line with this observation, WM patient samples displayed Bid expression at levels similar to normal plasma cells and higher than MM patient samples (Figure 1B, D). However, Bid expressed in WM and normal plasma cell requires cleavage by Caspase-8 for full activity and thus does not contribute to mitochondrial priming without treatment.

Low levels of mitochondrial priming in WM implies that treatments that require differential priming for efficacy may not be active against the disease unless priming can be induced. It has been previsously published that miR-155 antagonism also slows growth of WM cells and leads to xenograft regression[84]. We observed that while antagonizing miR-155 slowed growth it also induced Bim priming in BCWM.1 and MWCL-1 which increased their sensitivity to ABT-737 in absence of any other death signal (not shown and Figure 6). We also observed that inducing cleavage of Bid with proteasome inhibition increased sensitivity to ABT-737 suggesting an increase in priming through a tBid-dependent mechanism (Figure 2). Therefore, though the mitochondria of WM cells may not be primed with pro-apoptotic protein, there are treatments that can induce priming which in turn increases their sensitivity to a variety of anti-cancer agents.

In Figure 8 we present a model for apoptotic threshold in WM. MM cells express both pro- and anti-apoptotic Bcl-2 proteins at higher levels than healthy cells (Figure 7A). This sets a lower apoptotic threshold as it takes less death signaling to induce pro-apoptotic proteins sufficient to shift the balance and activate Bax and Bak and induce apoptosis (Figure 7B). In the case of WM, both pro- and anti-apoptotic proteins are expressed at low levels similar to normal plasma cells which sets a higher apoptotic threshold (Figure 7C). Mechanistically this occurs through the expression of miR-155 inhibits expression of Bim resulting in decreased. The pro-apoptotic Bcl-2 protein Bid is expressed in WM (Figure 1B, D), but it requires processing before becoming fully able to activate Bak. This means that more death signaling is required to cause MOMP and therefore apoptosis in WM. However, activation of Caspase-8 by bortezomib treatment, or antagonizing miR-155 to relieve inhibition of Bim lowers the apoptotic threshold which allows activation of apoptosis with less death signaling (Figure 7C). Finally, the RPCI-WM1 line, which does not conform to the pattern of WM expression seen in the patient data or the other two cell lines, expresses Bim at moderate levels but is unable to activate mitochondria-dependent apoptosis as it lacks Bax and Bak (Figure 7D). Therefore its apoptotic threshold is essentially infinite. Though the mechanism of Bax and Bak inhibition in these cells remains to be determined, restoring Bak or Bax expression in these cells should be sufficient to allow for the induction of apoptosis (Figure 7D).

The current standard of care in WM includes rituximab which targets the B-cell biology of the WM cell and depends more on complement and immune killer cell-mediated mechanisms than differential mitochondrial priming for action[86]. However, rituximab as

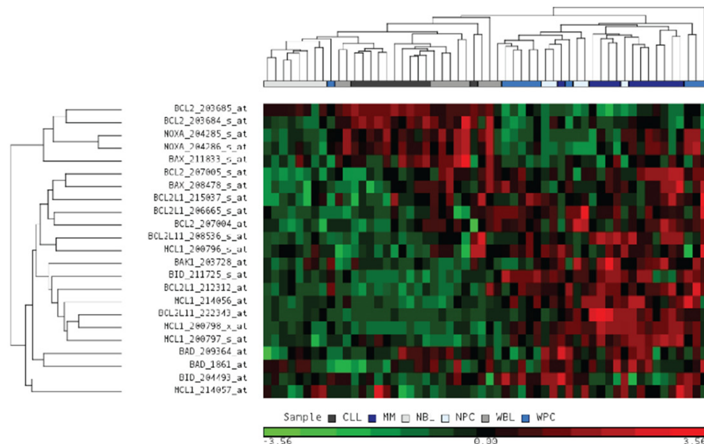
a single agent offers no advantage over other single agent treatments in WM patients[86]. Virtually all WM patients are now treated with some form of combination therapy. Knowledge of the state of mitochondrial priming in WM can provide direction in choosing the combination therapies likely to be effective in treatment. Treatment modalities showing promise in WM are those combinations including a proteasome inhibitor[119]. Targeting the proteasome in WM allows for the induction of mitochondrial priming because of both the Bcl-2 family expression and the biology of the cell. Proteasome inhibitors attack the WM cell's dependency on both the constitutive and immunoproteasome causing both proteotoxic stress and inhibiting immune functions of the cell in addition to more broadly targeted effects like NF-κB inhibition and cell cycle modulation[120, 121]. The data presented in this study show that WM cells express pro-apoptotic Bcl-2 family members at low levels which makes them less sensitive to apoptosis-inducing agents. However we provide evidence that mitochondrial priming can be induced in WM by antagonism of miR-155 or cleavage of Bid, which is expressed in WM. In this way, including agents that are capable of inducing priming and therefore lowering the apoptotic threshold as part of a combination therapy will increase the efficacy of a variety of other co-treatments.

F. Acknowledgements

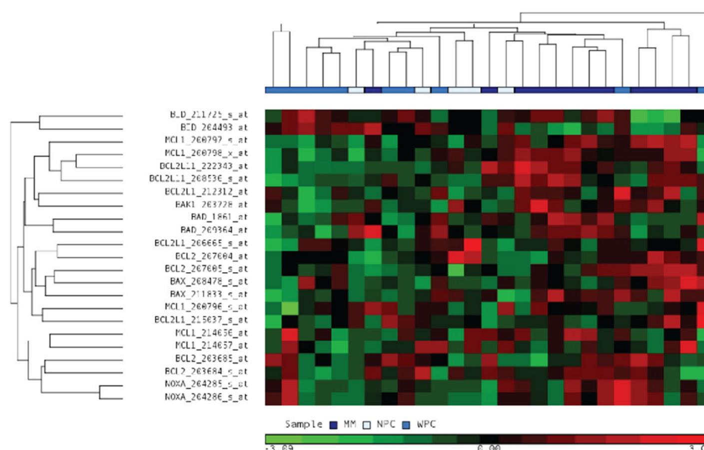
We thank Stephen Ansell and Kelvin Lee for cell lines and reagents. This work was supported by R01 CA127910 and R01 CA129968 as well as funding from the TJ Martell Foundation (LHB), the Leukemia & Lymphoma Society (AAC), the International Waldenstrom Macroglobulinemia (AAC) and the Comité du Septentrion de la Ligue contre le Cancer (XL). LHB is a GRA Distinguished Cancer Scientist.

Figure 1

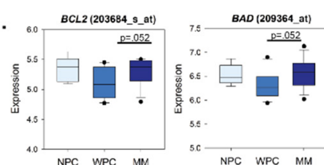
A.



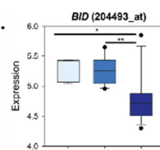
B.



C.



D.



E.

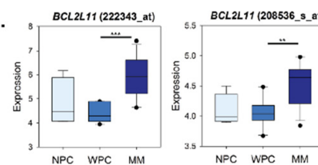


Figure 1: Bcl-2 family clustering of B cell malignancies including WM. (A) Unsupervised hierarchical clustering of B-cell (CLL, WBL, and NBL) and Plasma-cell (MM, WPC, and NPC) based on expression data for 9 Bcl-2 family genes [110]. (B) Unsupervised hierarchical clustering of plasma cell phenotype samples. (C) Statistically significant probes, *BCL2* (203684_at) and *BAD* (1861_at, 209364_at) that are under-expressed in WPC *vs*. NPC and MM (D) Statistically significant probes in *BID* (204493_at) that is under-expressed in MM compared to WPC and NPC. (E) Statistically significant probes, *BCL2L11* (222343_at, 208536_s_at), that are over-expressed in MM when compared to WPC and NPC. *p-value is calculated by Wilcoxon rank-sum test. (*p < .05, **p < .01, *p < .001)

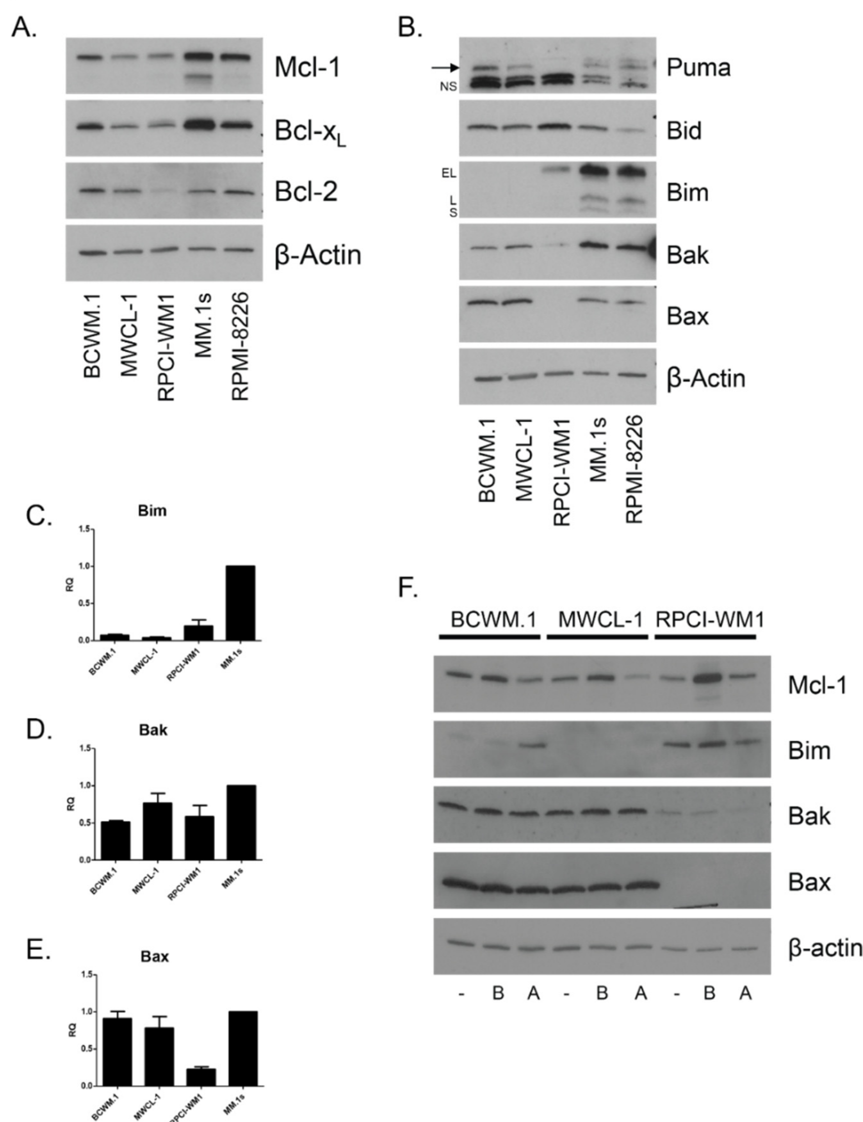


Figure 2: WM cell lines aberrantly express pro-apoptotic BH3 proteins. (A, B) Western blots were prepared from lysates of WM and MM cell lines. In (B), "→" denotes Puma band, "NS" denotes two non-specific bands. "EL", "L" and "S" denote isoforms of Bim. (C – E) qRT-PCR was performed on RNA isolated from untreated WM cells. Data is presented as relative quantity (RQ) compared to MM.1s cells (1) normalized to GAPDH endogenous control. (F) WM cell lines were left untreated [-], treated with bortezomib [B] at 5 nM for BCWM.1 and MWCL-1 cells and 20 nM for RPCI-WM1 cells or arsenic trioxide [A] at 5 μM for BCWM.1 and MWCL-1 cells and 10 μM for RPCI-WM1 cells. Western Blots were prepared from lysates of the treated cells at 24 h.

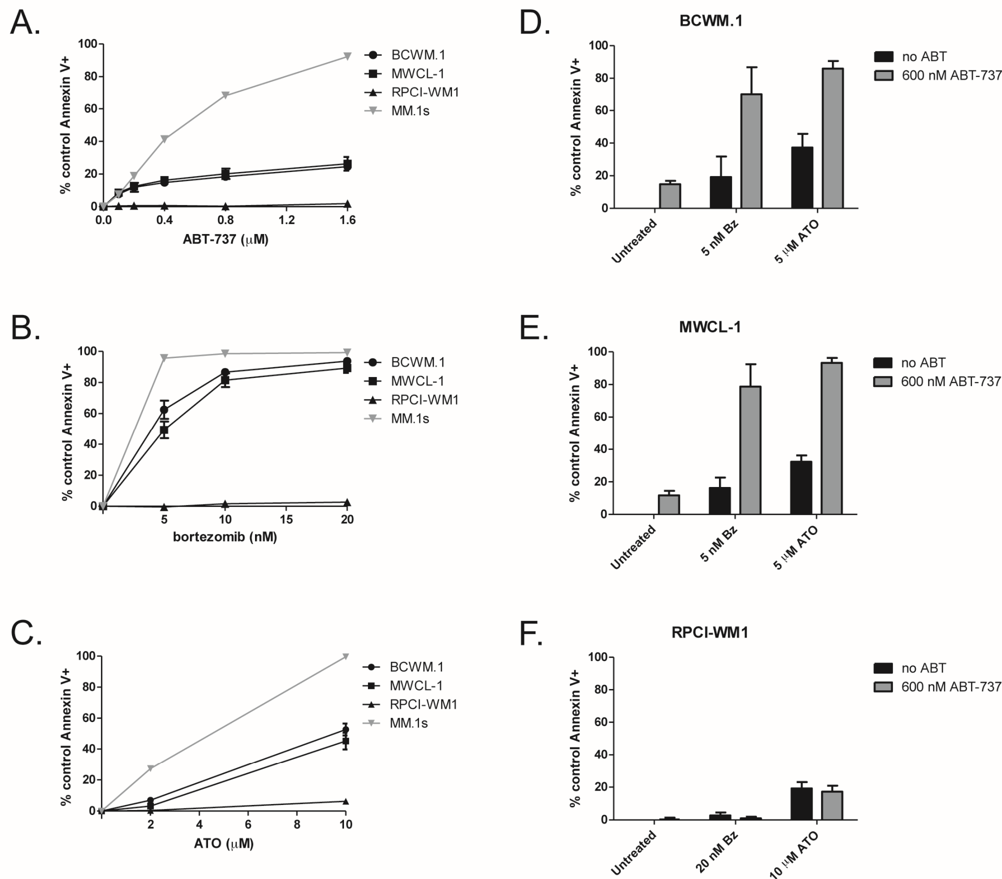


Figure 3: WM cell lines are insensitive to inducers of intrinsic apoptosis. WM cell lines were exposed to varying concentrations of ABT-737 (A), bortezomib (B) or arsenic trioxide (ATO) (C) for 24 hours. Apoptosis was measured by annexin V and PI staining and flow cytometry. Bortezomib and ATO sensitize BCWM.1 and MWCL-1 but not RPCI-WM1 cells to ABT-737-induced apoptosis. BCWM.1 (D), MWCL-1 (E) and RPCI-WM1 (F) cell lines were treated with bortezomib or ATO in the presence or absence of ABT-737 for 24 hours. Apoptosis was measured by annexin V and PI staining and flow cytometry. Data are presented as the mean ± SE of three independent experiments.

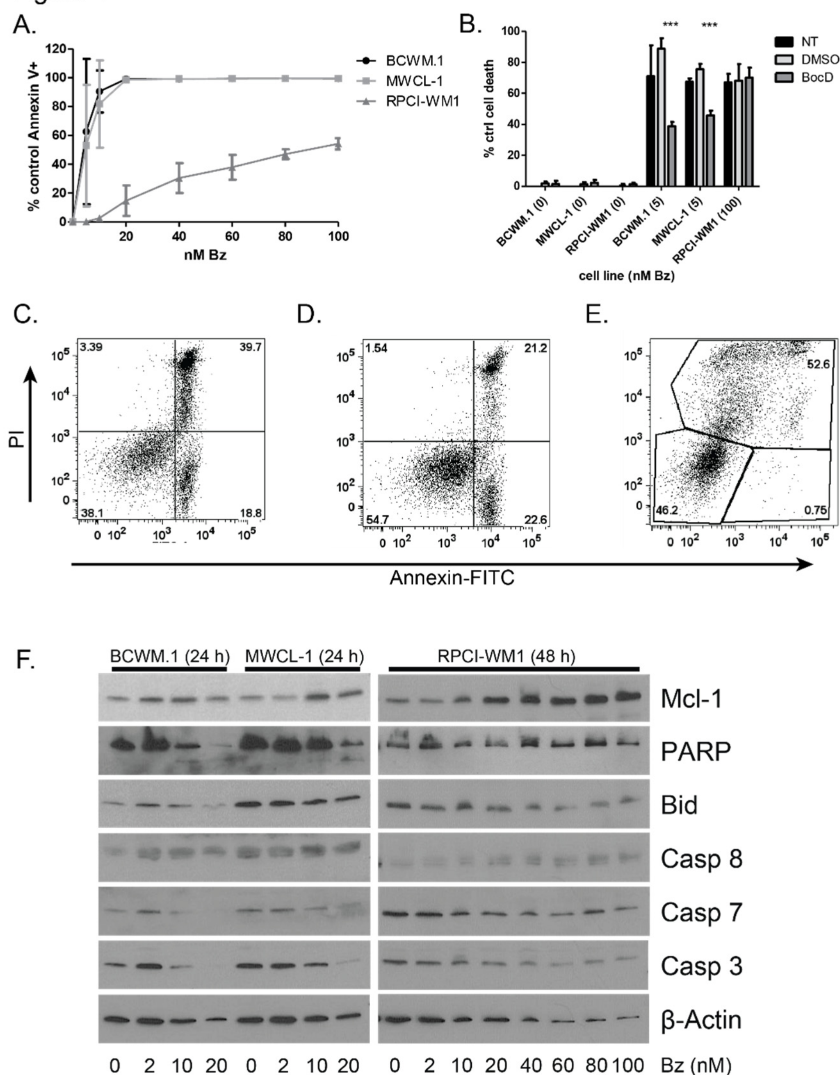


Figure 4: BCWM.1 and MWCL-1 cells but not RPCI-WM1 cells activate apoptosis during cell death. (A) WM cell lines were treated with various concentrations of bortezomib for 48 h. (B) WM cell lines were treated with high dose bortezomib in the presence of BocD-fmk or vehicle for 48 hours. Cell death was measured by annexin V and PI staining and flow cytometry. Data in A and B are presented as the mean ± SE of three independent experiments. (***p < .001) (C-E) Representative FACS plots from WM cells treated with bortezomib. Apoptosis was determined for BCWM.1 (C) and MWCL-1 (D) cells as percent of control annexin-V+ events after treatment with 10 nm bortezomib for 24 hours. Cell death was determined in RPCI-WM1 (E) cells as percent of control PI+ events after treatment with 100 nM bortezomib for 48 h. (F) Western Blots were prepared from lysates of WM cell lines treated with indicated concentrations of bortezomib sufficient to induce cell death for 24 h (BCWM.1 and MWCL-1) or 48 h (RPCI-WM1).

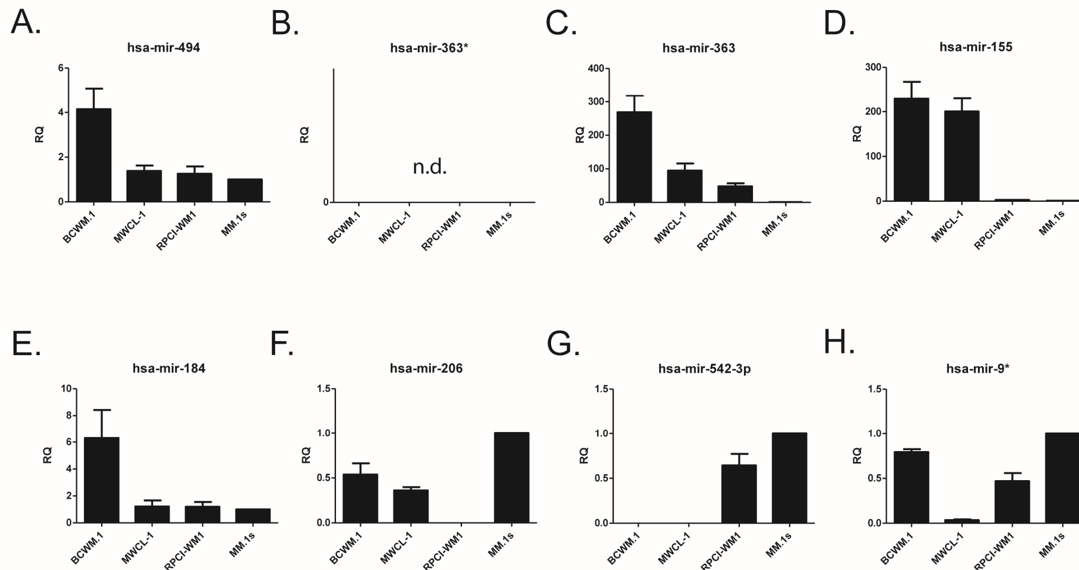


Figure 5: miR-155 is up-regulated in BCWM.1 and MWCL-1 cells but not RPCI-WM1 cells. microRNA enriched RNA was prepared from untreated WM and MM cells and cDNA was prepared using a primer pool containing 8 microRNA and 4 small nuclear or non-coding RNA primers (endogenous controls). qPCR was then performed using probes for these 12 targets. Cycles to threshold were first normalized to the mean of the 4 endogenous controls and then to the MM.1s cell line. Data are presented as the mean ± SE of three independent experiments.

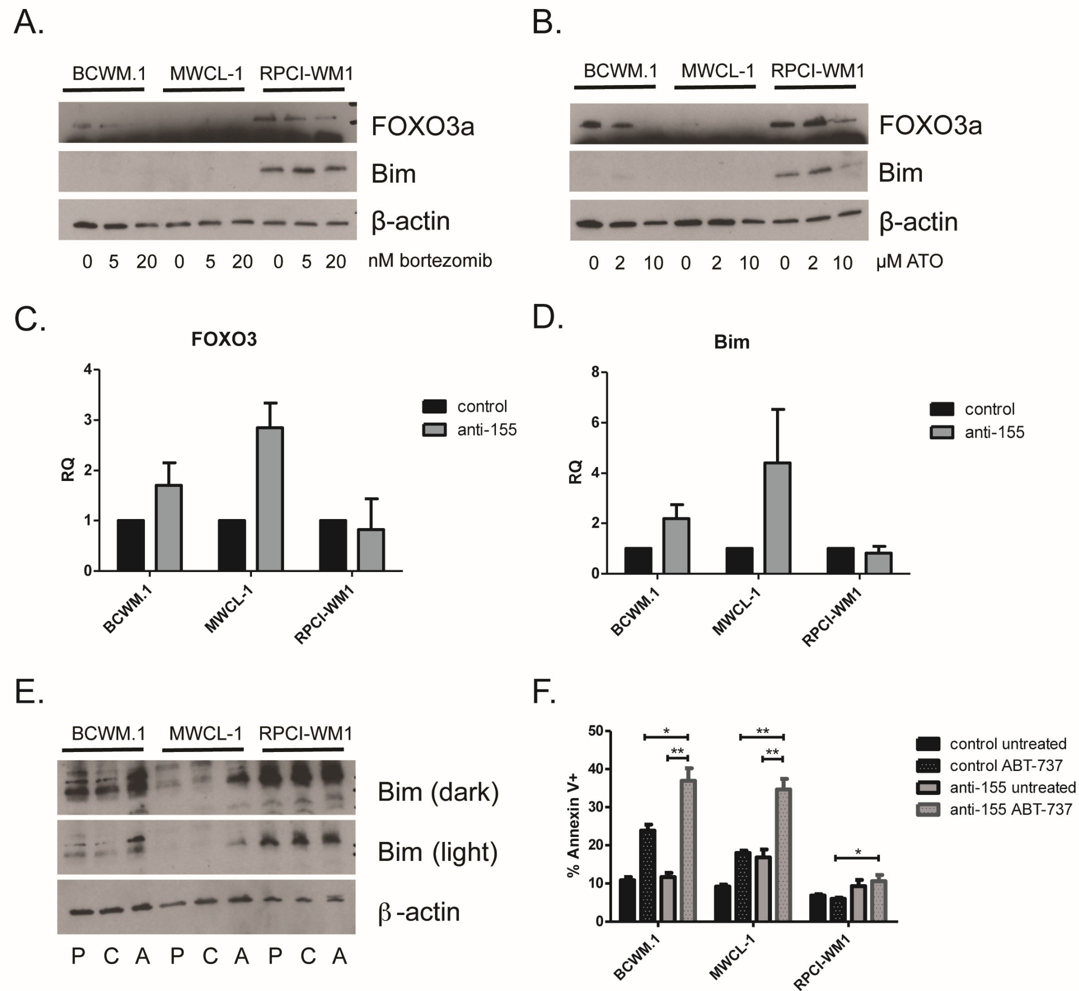
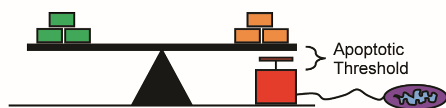


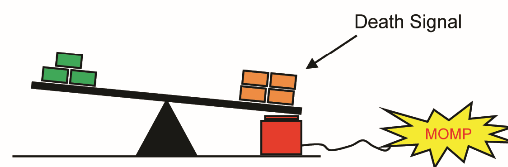
Figure 6: WM cell lines were treated with indicated doses of bortezomib (A) and ATO (B) for 24 hours. Western blots were prepared from samples collected at 24 h post treatment. miR-155 inhibits Bim expression. (C, D) qRT-PCR was performed on RNA prepared from WM cell lines stably expressing either anti-miR-155 or control anti-miR. Data is presented as relative quantity (RQ) compared to control anti-miR (1) normalized to GAPDH endogenous control. (E) Western blots performed on protein prepared from untreated parental [P], control anti-miR [C] or anti-miR-155 [A] cells. (F) WM cells stably expressing either control anti-miR or anti-miR-155 were treated with 0.4 μM ABT-737 for 24 hours. Apoptosis was measured by annexin-V and PI staining and flow cytometry. Data in C, D and F are presented as the mean ± SE of three independent experiments. (*p < .05, **p < .01, *p < .001)

Figure 7

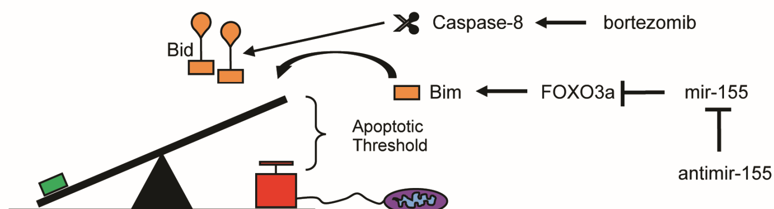
A. Multiple Myeloma



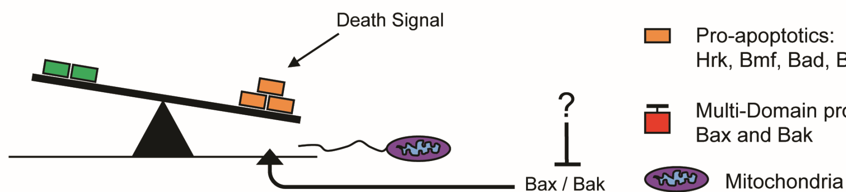
B. Apoptosis



C. WM, BCWM.1, MWCL-1



D. RPCI-WM1



Legend:

Anti-apoptotics: Bcl-2, Bcl-x_L, Mcl-1, Bfl1/A1, Bcl-w, Bcl-b

Pro-apoptotics: Bim, Bid, Puma, Hrk, Bmf, Bad, Bik, Bmf, Noxa

Multi-Domain pro-apoptotics: Bax and Bak

Mitochondria

Figure 7: A model of apoptotic threshold in Waldenström Macoglobulinemia. See Discussion text.

G. Supplemental Methods

Methylation–specific PCR and genomic bisulfite sequencing

Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen) and bisulfite modified (2 µg) as previously described (Herman et al., 1996). Methylation-specific PCR (MSP) was performed using 50 ng bisulfite- modified DNA under the following reaction conditions: 67mM Tris-HCL (ph8.8), 16.6 mM $(NH_4)_2SO_4$, 10mM 2-mercaptoethanol, 6.7 mM $MgCl_2$, 1.5mM dNTP's, 0.5U Taq, and 1µM of each primer. A hot start was performed (5min, 95°C) followed by 32 cycles of PCR (95°C, 30 s; 61°C, 30 s; 72°C, 30 s) with a final extension of 4 min. at 72°C. Primers were designed to overlay 3 CpG sites each and parallel PCR reactions performed with primers that selectively anneal to the interpolated methylated and unmethylated sequences after bisulfite modification. DNA methylated or unmethylated at all CpG sites (Universal Unmethylated/Methylated DNA, Chemicon) were used as controls. For genomic bisulfite sequencing, 50 ng bisulfite-modified DNA was PCR amplified for 30 cycles using the reaction conditions described above, except that a 58°C annealing temperature and a 10 min extension were performed. Primers were designed to avoid CpG sites so that unmethylated and methylated sequences are amplified as a single pool. The resulting pools were cloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen) and 8-10 individual subclones per amplicon pool isolated and sequenced. Locus-specific primers used for MSP and bisulfite sequencing are listed in **Table S3**.

Supplemental Tables

Table S1: Array-CGH analysis of RPCI-WM1 cell line and donor patient sample for Bak and Bax loci.

Bax	Chromosome	Start	Stop	Start Band	Stop Band	Call
Patient	chr19	39254322	55230497	q13.11	q13.33	0
RPCI-WM1	chr19	50108169	63305540	q13.32	q13.43	0

Bak	Chromosome	Start	Stop	Start Band	Stop Band	Call
Patient	chr6	163023	92257317	p25.3	q16.1	0
RPCI-WM1	chr6	163023	92257317	p25.3	q16.1	0

Table S2: SNP-copy number analysis for 46 WM patients at loci for Bax, Bak and Bim

Gene	Chromosome	Copy number variation	Uni-parental disomy
Bax	19q13	1 / 46 (gain)	0 / 46
Bak (Bak1)	6q21	1 / 46 (gain)	0 / 46
Bim (Bcl2l11)	2q13	0 / 46	0 / 46

Table S3: PCR Primers used for Methylation Analyses

Locus	Method*	Primer	Sequence (5' – 3')	Chrm Position (Hg19)
BAK1	MSP	Methylated (sense)	TTTTATAGGTTGTCGGTTTGTGC	chr6:33548071-33548093
(Bak)		Methylated (antisense)	AAAATAAACGTCAATACATTCCCG	chr6:33547908-33547931
		Unmethylated (sense)	TATTTTATAGGTTGTTGGTTTGTGT	chr6:33548071-33548095
		Unmethylated (antisense)	AAAAATAAACATCAATACATTCCCA	chr6:33547907-33547931
BAX	MSP	Methylated (sense)	GTTACGTGACGGGATTAAATTTTTC	chr19:49457992-49458016
		Methylated (antisense)	GTAAAAACCCCGCTAAACGTACG	chr19:49458098-49458120
		Unmethylated (sense)	AGTTATGTGATGGGATTAAATTTTTT	chr19:49457991-49458016
		Unmethylated (antisense)	ACATAAAAACCCCACTAAACATACA	chr19:49458098-49458122
	BSS	Sense	TTGTTAGATTTAGGTTTTTGTAAAAA	chr19:49457736-49457761
		Antisense	TAACTATCCAATAAACATCTCCC	chr19:49458308-49458332
BCL2L11				
(Bim)	MSP	Methylated (sense)	ATTTCGGTAAATACGTTAGGGAC	chr2:111878262-111878284
		Methylated (antisense)	GCGCGAACTAACAAACTCCG	chr2:111878376-111878395
		Unmethylated (sense)	GTTATTTTGGTAAATATGTTAGGGAT	chr2:111878259-111878284
		Unmethylated (antisense)	ACACACAAACTAACAAACTCCA	chr2:111878376-111878397
	BSS	Sense	GGAAGAAAAGTTGGAGAGTTTTTG	chr2:111877858-111877881
		Antisense	TCACTATACCCTAAATTTCTAAACC	chr2:111878056-111878080

*MSP-Methylation-specific PCR; BSS – Bisulfite genomic sequencing

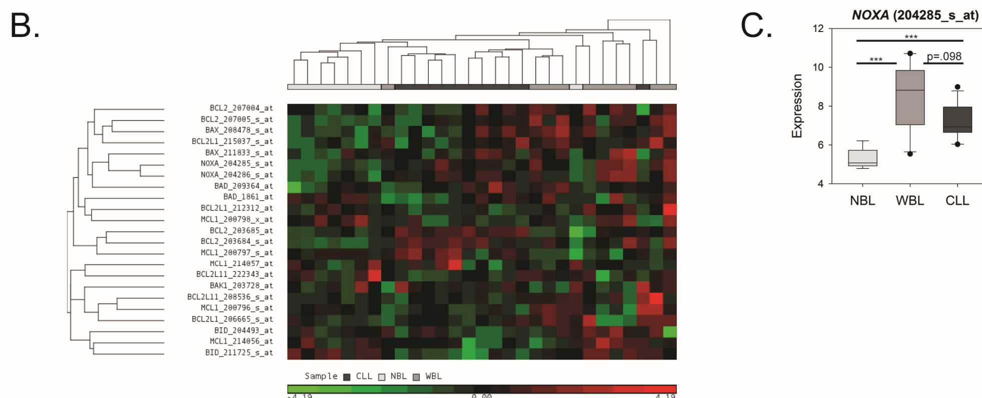


Figure S1 – (A) Unsupervised hierarchical clustering of B-cell phenotype cells. (B) Statistically significant increased expression of *NOXA* (204285_s_at) in WBL vs. NBL and CLL. For (A) the down-regulation and up-regulation of genes expressed across the samples are shown in gradient from green to red, respectively. The samples, CLL, WBL, NBL, MM, WPC, and NPC are shown in dark gray, gray, light gray, dark blue, blue, and light blue, respectively. *p-value is calculated by Wilcoxon rank-sum test. (*p < .05, **p < .01, *p < .001)

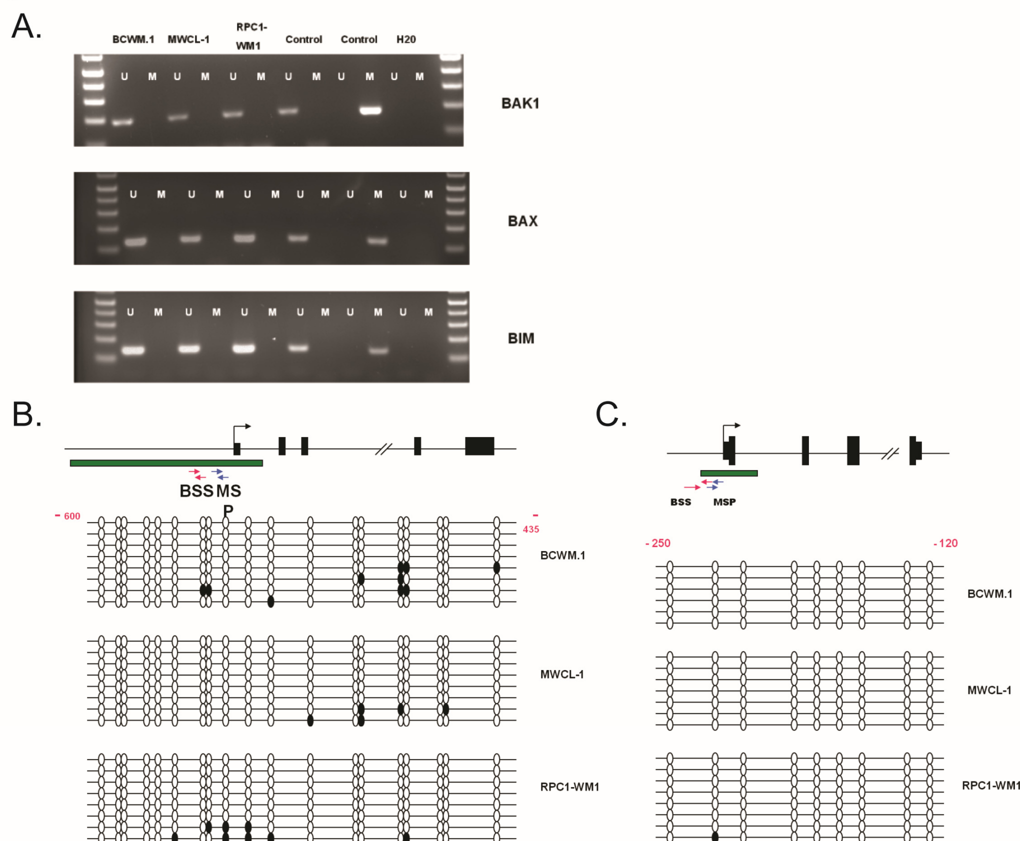


Figure S2 - Methylation status of the Bim, Bax, and Bak promoters in WM cell lines. (A) Methylation-specific PCR analysis of the BCL2L11 (Bim), BAX, and BAK1 (Bak) promoters [122]. DNA was isolated from the indicated cell line, bisulfite modified and amplified in parallel with primers recognizing the unmethylated (U) or methylated (M) DNA. Genomic DNA universally methylated or unmethylated at all CpG sites was included as a control.s (B, C) Bisulfite sequence analysis of the BCL2L11 (B) or BAX (C) locus. Bisulfite-modified DNA was amplified with the indicated primer set, subcloned and sequenced. Each row represents an individual allele. Filled circles, methylated CpGs; open circles, unmethylated CpGs. The relative positions of the CpG island (green bar), the transcription start site (TSS) and primers used in the bisulfite sequencing (BSS) or methylation-specific PCR (MSP) analyses are shown for reference. Nucleotide positions are relative to the TSS.

Figure S3

A.

<table>
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||||||
3' CUCCAAAGGGCACAUACAAAGU</td><td>7mer-1A</td><td>-0.074</td><td>-0.002</td><td>-0.024</td><td>0.101</td><td>0.025</td><td>0.038</td><td>> -0.01</td><td>19</td><td>2.299</td><td>N/A</td></tr>
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B.

<table>
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<tr><td>Position 2695-2701 of BCL2L11 3' UTR
hsa-miR-363</td><td>5' ...GAGCCAAAUGUCUGUGUGCAAUU...
|||||||
3' AUGUCUACCUAUGGCACGUUAA</td><td>7mer-m8</td><td>-0.120</td><td>-0.007</td><td>-0.042</td><td>0.151</td><td>0.000</td><td>0.015</td><td>> -0.02</td><td>21</td><td>3.261</td><td>0.78</td></tr>
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|||||||
3' AUGUCUACCUAUGGCACGUUAA</td><td>8mer</td><td>-0.247</td><td>0.013</td><td>-0.030</td><td>-0.096</td><td>0.001</td><td>0.019</td><td>-0.34</td><td>96</td><td>3.923</td><td>0.97</td></tr>
</table>

C.

<table>
<tr><th></th><th>predicted consequential pairing of target region (top) and miRNA (bottom)</th><th>seed match</th><th>site-type contri-bution</th><th>3' pairing contri-bution</th><th>local AU contri-bution</th><th>position contri-bution</th><th>TA contribution</th><th>SPS contribution</th><th>context+ score</th><th>context+ score percentile</th><th>conserved branch length</th><th>P_{CT}</th></tr>
<tr><td>Position 1497-1503 of FOXO3 3' UTR
hsa-miR-155</td><td>5' ...UUUCUUUGCAUAAAAAGCAUUAG...
|||||||
3' UGGGGAUAGUGCUAAUCGUAAUU</td><td>7mer-m8</td><td>-0.120</td><td>0.003</td><td>-0.075</td><td>0.150</td><td>-0.008</td><td>0.046</td><td>> -0.02</td><td>26</td><td>1.488</td><td>0.27</td></tr>
</table>

D.

<table>
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<tr><td>Position 424-430 of FOXO3 3' UTR
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|||| ||||||
3' UGGGGAUAGUGCUAAUCGUAAUU</td><td>7mer-1A</td><td>-0.074</td><td>-0.009</td><td>-0.004</td><td>-0.004</td><td>-0.007</td><td>0.030</td><td>-0.07</td><td>47</td><td>1.043</td><td>< 0.1</td></tr>
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|||||||
3' UGGGGAUAGUGCUAAUCGUAAUU</td><td>8mer</td><td>-0.247</td><td>0.003</td><td>0.062</td><td>0.143</td><td>-0.011</td><td>0.069</td><td>> -0.03</td><td>18</td><td>0.404</td><td>< 0.1</td></tr>
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</table>

Figure S3: Targetscan [115] output displaying putative microRNA sites in the 3'UPR of Bim and FOXO3. (A) Alignment of hsa-mir-494 site in 3'UTR of Bim. (B) Alignment of 2 sites in the 3'UTR of Bim for hsa-mir-363. (C, D) Highly conserved (C) and less conserved sites in the 3'UTR of FOXO3 for hsa-mir-155.

IV. DISCUSSION

A. Implications of Plasma Cell Differentiation Studies

In our study of plasma cell differentiation we observed a state in which the differentiating cell is protected from CHOP-dependent apoptosis by Bcl-x_L-mediated sequestration of Bim (Chapter 2). Previous work in this area has described un-equal activation of the three arms of the UPR in plasma cell differentiation, with activation of IRE1 and ATF6 occurring in the absence of PERK activation [32, 87]. While we did confirm previously described suppression of PERK activation and eIF2A phosphorylation in the form of P58IPK induction, we observed a previously undescribed robust activation of ATF4 and CHOP in the absence of eIF2A phosphorylation (Chapter 2, Figure 2A). This suggests that though PERK activation and subsequent eIF2A phosphorylation are avoided, ATF4 and CHOP serve a purpose in the differentiating cell, which is in line with the published observation that *Chop*$^{-/-}$ animals produce suboptimal plasma cells [36]. CHOP activation necessitates that the cell must cope with downstream apoptotic effects [123], which we observed in the form of lowered expression of Mcl-1/Bcl-2 and induction of Bim (Chapter 2, Figure 2C, D, 4A, C). Furthermore, we observed that the differentiation induces Bcl-x_L, which is necessary to sequester Bim and prevent apoptosis (Chapter2, Figure 2C, D, 3C, 4C, E, 5F). While the induction of Bcl-x_L was sufficient at 24 hours of LPS treatment to block apoptosis due to acute ER stress induced by tunicamycin treatment, it was not until 72 hours of differentiation that cells became sensitive to Bcl-x_L inhibition by ABT-737 treatment (Chapter 2, Figure 3A, B). This is an important distinction as Bcl-x_L has a strong anti-apoptotic effect throughout the treatment but it is the differentiation of the cell that induces Bcl-x_L dependence. In this manner the cell is

able to activate a full UPR, without the effect translation inhibition, and compensate for pro-apoptotic signals (Figure 1).

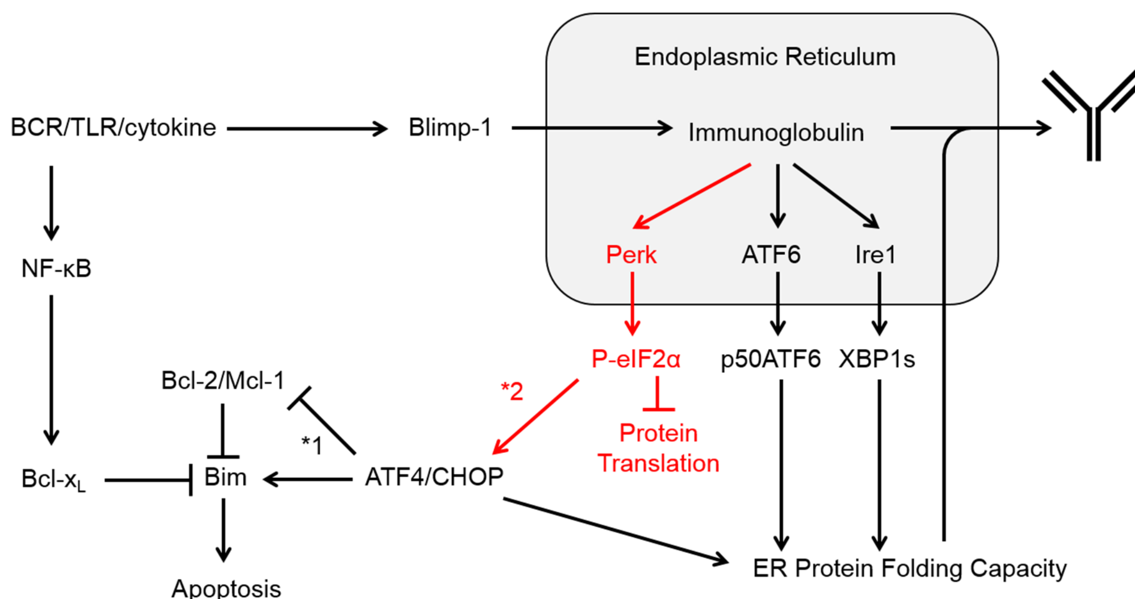

Figure 1: Activation of the UPR in plasma cell differentiation

The eIF2A-independent activation of ATF4 and CHOP allows for the efficient remodeling of the ER without prolonged translation inhibition. Contrary to previous publication, this includes pro-apoptotic signaling including induction of Bim and inhibition of Bcl-2/Mcl-1. The differentiation signal provides for the induction of Bcl-x_L which is necessary to counter the pro-apoptotic signals. *1: The mechanism for CHOP-mediated induction of Bim and inhibition of Mcl-1 and Bcl-2 though previously described, requires verification in this model. *2: The mechanism behind eIF2A-independent translation of ATF4 remains unknown and must be further researched.

1. Caveats and opportunities for further study

While this work was completed both in a transformed cell line and in primary murine spenocytes, it is important to consider that all of the differentiation reactions were completed in-vitro. However, taking together the previously described block in plasma cell formation in the context of ABT-737 treatment [67] with the phenotype of plasma cell differentiation in *Perk*$^{-/-}$ and *Chop*$^{-/-}$ animals [35, 36], it is likely that the effects we observed in-vitro are relevant to and perhaps lend mechanism to observations made in-vivo. Another concern with these in-vitro models is that they are limited to murine plasma cell differentiation. Though in many cases murine immunology is a valid surrogate for human immunology, the case can surely be made that these differentiation studies could be done with a human B cell line or primary human B cells. While we did attempt differentiation of a human lymphoma cell line that was previously reported to differentiate with cytokine treatment, we were not able to observe differentiation in this model (data not shown). Primary human cells present a further complexity that adds both difficulty and opportunity in that humans present more genetic diversity as compared with inbred strains of mice. While it is important that studies in human cells be done, the study of plasma cell differentiation in the less variable murine system is a valuable first step as it allows for the testing of individual variables without the complexity of the outbred system. Furthermore, the use of a murine system provides a way toward a next step in this investigation. Much is known about different subsets of mature B cells in the mouse and their varying response to differentiation stimuli. It will be important to continue the study of the activation of the UPR and associated changes in survival signaling with the various subsets of B cells. As these subsets respond differently to

different combinations of stimuli such as BCR ligation, TLR signaling, CD40 signaling and cytokine signaling, it will also be important to examine these various signals in each subset.

There are also technical limitations to the studies presented here that should be discussed. In this study we used shRNA to knockdown the expression of ATF4 and CHOP to show that the pro-apoptotic signals were UPR-dependent. In both cases the knockdown was incomplete and the protection was only limited (Chapter 2, Figure 6A-D). Because both of these proteins are induced by the differentiation stimuli, achieving a more thorough knockdown with this method was not possible. While the knockdown of Bcl-x_L was better, it was also incomplete and therefore the effect was also limited (Chapter 2, Figure 6E, F). While it would be possible to perform differentiation experiments with primary B cells from *Chop*$^{-/-}$ or possibly *Atf4*$^{-/-}$ animals, a more precise way to achieve complete knockdown of these proteins without affecting the generation of the mature B cell would be the more recently developed CRISPR Cas9 system which is capable of targeted complete inactivation of a gene in a cell line [124].

2. Implications for future research

While we show that the induction of Bcl-x_L is indeed protective against UPR-induced apoptosis in these studies, we did not uncover the signaling behind the Bcl-x_L induction. It is known that the downstream effects TLR4 signaling and cytokine signaling namely NF-κB and STATs can induce Bcl-x_L [125], however, we have not explicitly shown this link. Further investigation of these signals may prove useful in examining how different

types of antigens signaling through varying combinations of pathways may elicit differing quantity and quality of plasma cells and whether this is linked to the survival signaling at the point of activation and differentiation. Similarly, it has been shown that CHOP activation induces Bim and inhibits Bcl-2 and Mcl-1 [30, 31, 126], and though we observed these changes, we did not determine the signaling behind them. Examining the effect of knockdown of ATF4 and CHOP on the protein levels of Bim, Bcl-2 and Mcl-1 should be a first step at examining the relationship between these pathways. In moving from the B/alb c-derived Bcl1 cell line to the C57Bl/6-derived primary B cells we observed a difference in the effect of differentiation on Bcl-2 and Mcl-1 expression. In Bcl1 cells treatment with LPS and IL-5 or IL-4 induced a reduction of Mcl-1 expression (Chapter2, Figure 2C, D and not shown), while in primary C57Bl/6 B cells treatment with LPS and IL-4 induced a reduction in Bcl-2 (Chapter 2, Figure 2 A, C). It remains unclear if the reason for this difference is due to the transformation state of the Bcl1 cell or the difference in the genetic background of the donor mice themselves. To address this problem, the differentiation should be performed in primary B cells isolated from B/alb-c mice or other background.

Perhaps the most interesting question brought up by this work is the induction of ATF4 in the absence of eIF2A phosphorylation. The efficiency of translation of ATF4 message is greatly increased by phosphorylation of eIF2A, which reduces the likelihood that the ribosome recognizes the inhibitory codon in an upstream open reading frame of the ATF4 mRNA [127]. Without this, translation efficiency of ATF4 is low. However, in our system we see robust induction of ATF4 at the protein level without measurable increase in phosphorylation of eIF2A (Chapter 2, Figure 2A, 4D). The mechanism for this

expression of ATF4 may be an as yet undescribed mechanism of avoiding PERK activation by expressing a downstream protein that causes feedback inhibition of PERK-mediated phosphorylation of eIF2A via both GADD34 and P58IPK expression. One hypothesis for this observation would be that ATF4 is increased at the mRNA level which overcomes the low efficiency of translation of the message. To this end, we examined the mRNA level of ATF4 during treatment with IL-5 and LPS in Bcl1 cells and observed only a modest increase in mRNA (data not shown). Another hypothesis for this would be a mechanism similar to observations in pancreatic β cells where cAMP and protein kinase A signaling increase the efficiency of ATF4 translation in conjunction with eIF2A phosphorylation. This greatly increases ATF4 protein expression with relatively low levels of eIF2A phosphorylation, which further feeds back, inhibiting PERK-mediated eIF2A phosphorylation [127, 128]. Finally, a third hypothesis for altering the expression pattern of ATF4 to be examined is post-translational modification of ATF4 which affects its stability. It has been shown that phosphorylation-dependent interaction with the F-box protein β-TrCP directs ATF4 to be ubiquitenated and degraded by the proteasome [129]. Examining the levels of β-TrCP or similar proteins during differentiation will be necessary to answer this question.

B. Implications from Waldenström Macroglobulinemia Studies

In our study of Bcl-2 family expression in Waldenström Macroglobulinemia we observed that when compared with another plasma cell phenotype cancer, multiple myeloma, WM cells display less mitochondrial priming. This is important because the increased priming of cancer cells is responsible for the therapeutic index in cancer treatment [71]. When we compared Bcl-2 family gene expression data from plasma cell phenotype WM samples with MM samples and normal plasma cell samples, we observed that the WM samples expressed pro-apoptotic Bcl-2 proteins at levels similar to normal plasma cells and lower than MM samples (Chapter 3, Figure 1B). Also similar to normal plasma cells, WM samples displayed higher levels of Bid expression than MM samples (Chapter 3, Figure 1B, D). However, when taken as a whole, the samples from all six donor groups, CLL, WBL, NBL, MM, WPC, and NPC all clustered based on their Bcl-2 protein expression into either B lymphocyte or plasma cell groups suggesting that their Bcl-2 expression is derived from their normal cellular program as opposed to their cancer cell program (Chapter 3, Figure 1A).

When we obtained the only three characterized WM cell lines so that we could examine Bcl-2 family interactions more in-depth we found that the cell lines harbored very interesting apoptotic programs. We observed deficiencies in intrinsic apoptosis in all three lines at the same point of regulation. Both BCWM.1 and MWCL-1 cells lack the expression of Bim and, though RPCI-WM1 cells express Bim, they lack expression of Bak and Bax (Chapter 3, Figure 2). Together these observations indicate that WM cells avoid apoptosis by under-expressing pro-apoptotic proteins not over-expressing anti-apoptotic proteins as is common in most cancers. We investigated the mechanism behind

the low expression of Bim in BCWM.1 and MWCL-1 cells and found that the over-expression of miR-155, which is common in WM patients, inhibits the transcription factor FoxO3 which is important in the induction of Bim (Chapter 3, Figure 5C, 6A-D). This is important because the lack of pro-apoptotic Bcl-2 family protein expression in WM gives it a high apoptotic threshold. Therefore antagonism of miR-155 would lower this threshold by relieving inhibition on Bim expression thereby lowering the apoptotic threshold. This in turn would improve the efficacy of a variety of therapeutics.

1. Caveats and opportunities for further study

One of the largest hurdles in the study of WM is the inherent rarity of the disease. The low availability of patient samples means that there is not much expression data available from primary cancer cells. Because of this we were limited to use of one available data set that contained comparative data. While we were able to investigate the mechanism behind some aspects of Bcl-2 family expression in the available cell lines, it is important to make the distinction between cell lines and primary patient samples. However, since two of the three cell lines shared a common defect that has been shown in primary samples too, namely miR-155 overexpression, it can be inferred that this phenotype is relevant to the disease. While this work examines previously unreported Bcl-2 family expression and dynamics, the samples size of the available database is small and the in-vitro data is limited to cell lines. Future research in this area should examine the expression of the Bcl-2 family in additional primary patient samples.

Though the use of anti-miR-155 lentiviral constructs to antagonize miR-155 allowed us to recover Bim expression in the BCWM.1 and MWCL-1 cell lines, it was not possible in our system assay the level of miR-155 antagonism empirically. The anti-miR itself bound the qRT-PCR probe increasing the apparent level of miR-155 expression artificially (data not shown) thereby making this manner of detection impossible. However, we were able to see the effect of antagonism of miR-155 in the increase of message for the target, FoxO3 (Chapter 3, Figure 6C). While we did observe similar increases in Bim protein in both BCWM.1 and MWCL-1 cells which corresponded to increases in apoptosis induced by ABT-737, there did seem to be a qualitative difference in the effect in the two cell lines (Chapter 3, Figure 6E, F). In BCWM.1 cells there is low expression of Bim at baseline which was increased with miR-155 antagonism, however, in MWCL-1 Bim is undetectable at baseline and increased with miR-155 antagonism (Chapter 3, Figure 6E). In order to determine whether the miR-155-dependent regulation of Bim is by direct inhibition by the miR on the mRNA for FoxO3 or indirect via increased AKT signaling, the relative amounts of both FoxO3a and phosphorylated FoxO3a protein in these cells should be examined.

Finally, we show evidence that proteasome inhibition in MWCL-1 and BCWM.1 cells increases the sensitivity of these cells to ABT-737 treatment indicating that they have increased priming (Chapter 3, Figure 3D, E). We hypothesize that the better than additive levels of apoptosis in these co-treated cells is due to the cleavage of Bid by Caspase-8. The loss of full length Bid in proteasome inhibited cells is evidence of this (Chapter 3, Figure 4F), however, better experimental proof of this concept would be the

use of shRNA targeting Bid during proteasome inhibition to determine if the death is Bid-dependent.

2. Implications for future research

Previous investigation into Bcl-2 family dynamics in WM has been limited to identification of a pattern of Bcl-2 overexpression in WM and investigation into methods of inhibition Bcl-2 with either anti-sense oligonucleotides or AT-101 [130-132]. The work we present here shows evidence that inhibiting the anti-apoptotic Bcl-2 family members in WM can only be effective if appropriate agents are used in conjunction that increase pro-apoptotic protein expression. Future research in WM should include examining the mechanisms of Bcl-2 family regulation on a broader scale to determine if the over-expression of miR-155 mediates Bim inhibition in patients as this work suggests it might. Therefore development of a treatment that inhibits miR-155 will not only slow the growth of affected WM cancers as shown in xenograft experiments [84], it will induce Bim priming thereby increasing the efficacy of agents used in conjunction. Similarly, further research should be done regarding the combination of other agents that have the ability to induce priming in WM cells with agents that specifically target primed cells like BH3 mimetics. Proteasome inhibitors, which can induce Bid cleavage to prime cells with tBid, might be particularly effective in cells that express Bid at high levels as we observed in WM samples (Chapter 3, Figure 1B, E).

WM is a disease that is characterized by high prevalence of activating mutations that signal through NF-κB, such as MyD88 (L265P) [82, 109] and CXCR4 (S338X) [133, 134], yet in

our investigations we have not seen a dependence on Bcl-x_L downstream of this signaling. While there is also published work investigating the combination of proteasome inhibitors with NF-κB inhibitors [135], mechanistic investigation of the effects these activating mutations has been lacking. An interesting hypothesis to test regarding NF-κB signaling in WM comes from what we know about plasma cell differentiation stimuli. If clonal WM cells are both lymphocytic and plasmacytic in morphology, do the driving mutations in the NF-κB pathways cause this differentiation? Future studies should examine if the silencing of signaling downstream of these mutations also lowers the incidence of plasma cell phenotype cells. This could be therapeutically important since much of the disease manifestation is due to secretion of the monoclonal, pentameric IgM.

Another interesting avenue of research not yet described in WM is the possible link between the common mutations that drive NF-κB such as MyD88 (L265P) or CXCR4 (S338X) and the overexpression of miR-155. Importantly miR-155 is important not only in malignancy but also in the normal differentiation of B lymphocytes in regulation of Pax5 expression [136]. It has been shown in diffuse large B cell lymphoma (DLBCL) that increased NF-κB signaling correlates with increased miR-155 expression [137-139], and that LPS signaling induces higher expression of the miR in DLBCL cell lines [140]. Also there is some mechanistic evidence NF-κB driving miR-155 in the context of Epstein Barr virus latency membrane protein 1 (EBV LMP-1) expression where miR-155 upregulation is blocked with NF-κB or p38/MAPK inhibitors [141]. This evidence points to a possible link between MyD88 or CXCR4 activation and miR-155 overexpression in WM. This

can be investigated using inhibitors of NF-κB signaling in WM cell lines and examining the expression level and downstream effectors of miR-155, including FoxO3a and Bim.

C. Apoptotic Checkpoints in Normal and Malignant Plasma Cells

The Bcl-2 family is responsible for orchestrating the dynamic process of regulation of apoptosis in all cells, healthy and otherwise. In order to remove cells that are diseased or malfunctioning, checkpoints are set up such that pro-apoptotic signaling occurs during times of proliferation and stress and must be met with anti-apoptotic signals if the cell is to survive. A cell proliferating as part of its normal biology induces the same pro-apoptotic signals induced when a cancer cell divides inappropriately. The difference is that the healthy cell program includes the compensatory anti-apoptotic protein induction whereas the cancer cell must attain a second transformative event to induce these survival signals in the form of oncogenic survival signaling. This is also true in the activation of stress pathways. A cell undergoing ER stress unusual to its normal state will activate pro-apoptotic signals and if the stress is unresolved it will undergo apoptosis. A cell undergoing stress as a function of a differentiation program will activate the same pro-apoptotic signals but must also activate compensatory survival signals. The pro-apoptotic signaling in a cell due to these processes leads to the accumulation of pro-apoptotic Bcl-2 proteins. The sum of these proteins leads to mitochondrial priming and gives us the therapeutic index required in cancer treatment which dictates that cancer cells treated with the same dose of an agent will undergo apoptosis before healthy cells.

1. The ER stress-induced apoptotic checkpoint in plasma cell differentiation

In our investigation of plasma cell differentiation we observed an apoptotic checkpoint induced at the point of differentiation that hinges on the activation of the ER stress

response pathways of the UPR. We observed the induction Bim and inhibition of Bcl-2/Mcl-1 which was managed by the induction of Bcl-x_L which protected the cell from apoptosis. This knowledge may not directly aid in the treatment of disorders of mature plasma cells like multiple myeloma since we know that this state of the cell is transient as mature plasma cells are known to be Mcl-1-dependent [66]. In-vivo, Mcl-1 is induced downstream of B-cell survival factors that signal through B cell maturation antigen (BCMA) and through stromal-dependent cytokine signals like IL-6. However, these signals do not protect the newly differentiating plasma cell. Since the signaling downstream of the various TLRs that are activated downstream of adjuvant signaling in vaccine strategies is similar yet varied, it may be possible that some adjuvants are better at activating Bcl-x_L than others and may be necessary with antigens that strongly activate the production of immunoglobulin thus activating a robust UPR.

Conversely, the appreciation of a Bcl-x_L-dependent intermediate in plasma cell differentiation may prove useful in the treatment of disorders of aberrant plasma cell differentiation. Carrington et. al. proposed that the ABT-737-sensitive intermediate in plasma cell differentiation could provide for the use of this BH3 mimetic to modulate immune responses and stem plasma cell-dependent autoimmunity [67]. Our findings provide a mechanism to this finding showing Bcl-x_L protection from UPR-dependent apoptosis to be a possible target for treating autoimmune conditions. While Bcl-x_L inhibition in patients has led to throbocytopenia and is not likely to be beneficial to those with blood cancers [142], for a patient with acute autoimmunity, the benefits might outweigh the detriments.

2. Low expression of pro-apoptotic protein in WM redefines the apoptotic checkpoint.

By inhibiting the expression of pro-apoptotic Bcl-2 protein, WM cells raise their apoptotic threshold by effectively removing an apoptotic checkpoint. Contrary to the way in which MM cells break an apoptotic checkpoint by increasing their expression of anti-apoptotic proteins to counter increased pro-apoptotic protein due to proliferative signals, WM cells express Bcl-2 proteins similar to normal cells (Chapter 3, Figure 1). For example, WM cells subvert the checkpoint by way of miR-155 overexpression-mediated inhibition of Bim. Importantly this not only allows them to proliferate unchecked it leaves them less primed and thus having a higher apoptotic threshold (Chapter 3, Figure 7). Thus the WM cell requires similar amounts of death signals to normal cells in order to initiate apoptosis. Therefore, it is the normal cellular biology that must be examined for successful treatment.

3. What is normal and what is cancer

A large amount of research has gone into fighting cancer based on its cancer-specific biology. This is true as well in the ways the biology of a cancer cell dictate the relationships of the Bcl-2 family of proteins. BH3 mimetics function solely based on the differences in Bcl-2 family dynamics in a cancer cell versus their normal counterparts. In fact a whole variety of cancer therapeutics rely on the principle that because of their traversing apoptotic checkpoints, cancer cells are more primed for death than normal cells [48, 71]. The data presented here illustrate that there is a need and a benefit to taking into account the normal biology of the cell as well as its cancer biology. Just as

proliferative signals induce pro-apoptotic signaling that can be taken advantage of in many cancers, the reliance on stress pathway activation to protect from proteotoxic stress can be taken advantage of in highly secretory cancer cells. Plasma cells traverse an apoptotic threshold associated with the activation of the UPR that results in the increased expression of Bim [88]. Because their normal cell biology is that of a plasma cell, in MM cells proteasome inhibition induces UPR-associated pro-apoptotic signals that lower the apoptotic threshold [79]. WM cells are both antibody secreting and have activated NF-κB which makes them dependent on two pathways that are affected by the proteasome. In this way a therapeutic agent such as a proteasome inhibitor that acts both on the cancer biology and normal cell biology is an effective strategy for treatment. In a highly secretory cell that is also dependent on NF-κB signaling such as WM, proteasome inhibition can induce ER stress and autophagy as well as inhibit pro-survival signals downstream of NF-κB (Figure 2). In this way the apoptotic threshold can be lowered by three distinct pathways making the cell more sensitive to all agents that induce apoptosis (Figure 3). Targeting the normal biology of a cell is not novel in cancer treatment and can be effective so long as the normal biology that is targeted is not necessary for the life of the organism. Two successful examples of this in non-B cell diseases are anti-androgen therapy in prostate cancer and anti-estrogen therapy in breast cancer [143, 144].

The Bcl-2 family dynamics in WM are different to that in MM in that they do not exhibit the increased priming seen in many cancers. This means that their response to agents that kill cells based solely on their cancer biology will not work as well as agents that target the normal cell biology of WM. Importantly, agents that target the normal cell biology need not induce apoptosis on their own to be effective treatments. Any treatment that

induces pro-apoptotic signals will lower the apoptotic threshold of the cell thus increasing the efficacy of co-treatments. The efficacy of rituximab in WM is illustrative of this point. It is successfully used as a first line therapy only in conjunction with other treatments and not as single agent therapy [86]. It is also important that eventual relapse is often seen with rituximab treatment [86]. Therefore it is important to examine other methods of inducing priming in WM cells. We have shown here that antagonizing the overexpression of miR-155 and proteasome inhibition can induce priming in WM cells as they both increase the activity of ABT-737, an agent that requires mitochondrial priming for activity. Both the overexpression of miR-155 and reliance on protein catabolism are integral to the specific biology of the WM cell and we hypothesis that this is precisely why these strategies will prove effective in the treatment of this disease when used in conjunction with a variety of other agents. If the overexpression of miR-155 is indeed a consequence of the increased NF-κB activation in WM, targeting this pathway as a driver mutation in WM may also lower the apoptotic threshold by reducing miR-155-dependent inhibition of FoxO3a and Bim.

In the treatment of cancer and especially hematologic malignancies, where often a cancer cell retains the phenotype of its analogous healthy cell, it is important that we consider both the cancer biology and the normal biology of the cell when designing treatments. While proliferative driving mutations provide attractive targets in cancer, we must also consider that just as these lower the apoptotic threshold of the cell so do the many normal physiological processes. It is the combination of these strategies that will lead to successful treatment modalities in the future.

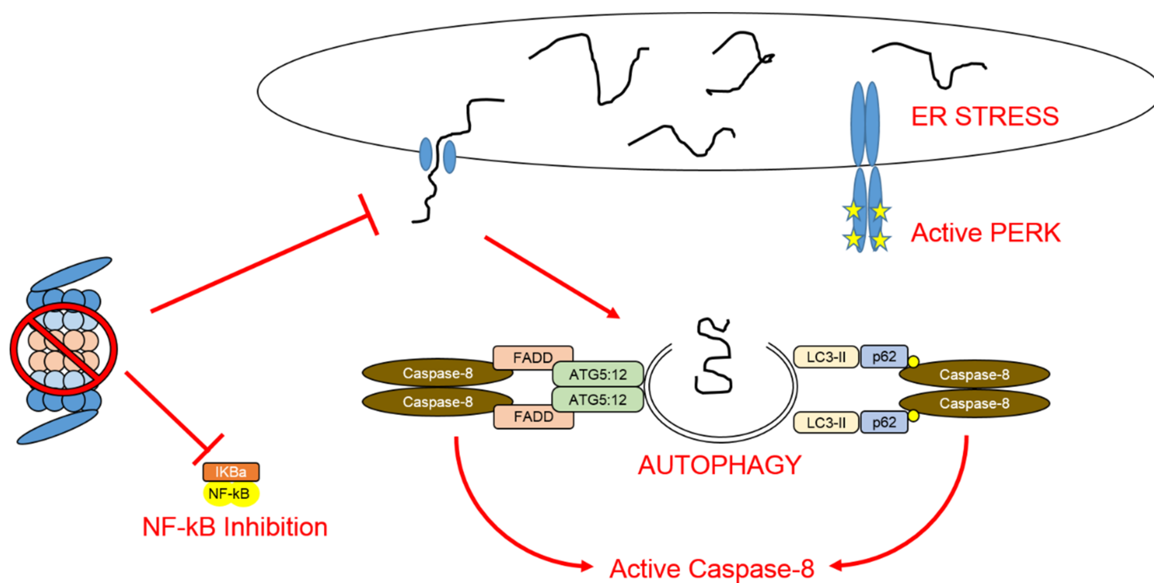

Figure 2: Proteasome inhibition affects multiple cellular processes in WM. Proteasome inhibition inhibits the NF-κB pathway by blocking the degradation of IκBα. Additionally, in highly secretory cells which are dependent on ERAD to maintain ER homeostasis, proteasome inhibition can cause both ER stress including PERK activation and the induction of autophagy which can trigger Caspase-8 activation.

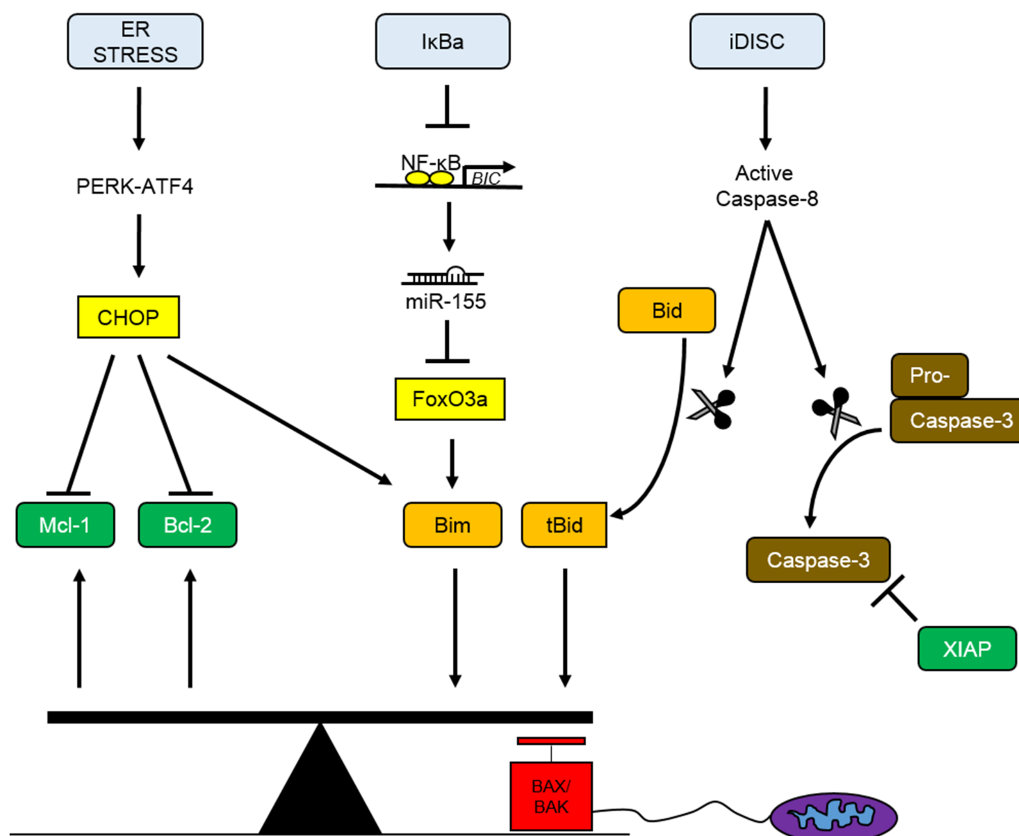

Figure 2: Proteasome inhibition acts through three distinct pathways to lower the apoptotic threshold in WM. Antibody secreting cells are dependent on ER-associated degredation (ERAD) to maintain ER homeostasis. Proteasome inhibition blocks the destruction of retrotranslocated proteins which feeds back on the ER causing ER stress. This activates the terminal UPR including the PERK-ATF4-CHOP arm[79]. CHOP inhibits the expression of Mcl-1 and Bcl-2 and induces Bim [88]. Another proteasome substrate, IκBα cannot be degraded under proteasome inhibition and therefore inhibits downstream NF-κB signaling[145, 146]. In WM this may block signaling downstream of common driver mutations including MyD88 (L265P). The B cell integration cluster (BIC) that encodes miR-155 is a target of NF-κB[140, 141], and therefore may be inhibited during proteasome inhibition therefore relieving inhibition of FoxO3a and Bim. Finally, protein products of ERAD that cannot be degraded by the proteasome can form aggresomes which are brought to the autophagolysosome which may initiate the intrinsic death-inducing signaling complex (iDISC) which yields activated Caspase-8 [53]. Activated Caspase-8 then is able to cleave Bid into tBid. In this way the cell increases its load of pro-apoptotic BH3-only proteins (Bim and tBid) at the same time it decreases expression of anti-apoptotic protein which lowers its apoptotic threshold. Importantly, activated Caspase-8 also may cleave Caspase-3. In most cells this cleaved Caspase-3 remains inhibited by XIAP but will be quickly activated upon the induction of MOMP and release of the XIAP inhibitors, SMAC and OMI[51].

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