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The life and death of immune cell types: the role of BCL-2 anti-apoptotic molecules

Emma M Carrington[1,2], David M Tarlinton[3], Daniel H Gray[1,2], Nicholas D Huntington[1,2], Yifan Zhan[1,2]*, Andrew M Lew[1,2,4]*

[1]The Walter and Eliza Hall Institute of Medical Research, Parkville, 3052 Australia [2]Department of Medical Biology, University of Melbourne, Parkville, 3010, Australia [3]Department of Immunology & Pathology, Monash University, Melbourne, 3004, Australia
and [4]Department of Microbiology & Immunology, University of Melbourne, Parkville, 3010, Australia.

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*Co-senior authors

Correspondence: Emma Carrington, Immunology Division, The Walter & Eliza Hall Institute of Medical Research, 1G Royal Parade, Melbourne, VIC 3052, Australia; Tel (+61 3) 9345 2563; Fax: (+61 3) 9347 0852. E-mail: carrington@wehi.EDU.AU

ABSTRACT

Targeting survival mechanisms of immune cells may provide an avenue for immune intervention to dampen unwanted responses (e.g. autoimmunity, immunopathology and transplant rejection) or enhance beneficial ones (e.g. immune deficiency, microbial defence and cancer immunotherapy). The selective survival mechanisms of the various immune cell types also avails the possibility of specific tailoring of such interventions. Here, we review the role of the BCL-2 anti-apoptotic family members (BCL-2, BCL-XL, BCL-W, MCL-1 and A1) on cell death/survival of the major immune cell types e.g. T, NK, B, dendritic cell (DC) lineages. There is both selectivity and redundancy among this family. Selectivity comes partly from the expression levels in each of the cell types. For example, plasmacytoid DC express abundant BCL-2 and are susceptible to BCL-2 antagonism or deficiency, whereas conventional DC express abundant A1 and are susceptible to A1 deficiency. There is, however, also functional redundancy; for example, over-expression of MCL-1 can override BCL-2 antagonism in plasmacytoid DC. Moreover, susceptibility to another anti-apoptotic family member can be unmasked, when one or other member is removed. These dual principles of selectivity and redundancy should guide the use of antagonists for manipulating immune cells.

INTRODUCTION

Apoptosis is such a general pathway and the BCL-2 family of molecules expressed so widely that it was deemed that therapy exploiting such a fundamental pathway would cause major toxicity. However, we now know that haematopoietic cells are especially sensitive to manipulation of this pathway. For example, doses of the BCL-2 antagonists ABT-199/Venetoclax or ABT-737 that virtually eliminate all naïve T cells in mice have little effect on the rest of the animal [1, 2] demonstrating that cell selectivity can be achieved by manipulating drug dosage. Moreover, whilst some immune cell types have a selective dependence on all of the five BCL-2 anti-apoptotic molecules, others express only a restricted few, further offering the opportunity for selective targeting within different immune cells via this pathway. This review will appraise recent literature on the influence of anti-apoptotic BCL-2 family proteins on the life and death of immune cell types including various cells of the T, B, NK and dendritic cell (DC) lineages. Such studies rely on genetic deletion models or the use of antagonists of BCL-2 family members or both. The BCL-2 antagonists avail a way of not only interrogating the survival requirements of immune cells but also provide an exciting avenue for intervening therapeutically in immune-mediated pathology.

THE BCL-2 FAMILY AND APOPTOSIS

The BCL-2 family has three sub-families, comprising two sets of pro-apoptotic ("initiators", "effectors") and one set of anti-apoptotic ("guardians") molecules [3, 4](Fig. 1). The BCL-2 family proteins share up to four distinct regions of homology termed BCL-2 homology (BH) domains (BH1-4) (Fig. 1a). The five known anti-apoptotic members (BCL-2, BCL-XL, BCL-W, MCL-1 and A1/BFL1) show similarity in all four BH domains. BCL-2, BCL-XL and BCL-W are relatively long-lived proteins (half-life ~20h), whilst the half-life of MCL-1 and A1 are much shorter l (0.5-3h) [5-7]. The pro-apoptotic "initiators" consist of at least eight members (BID, BIM, PUMA/BBC3, BAD, NOXA/PMAIP, BIK/BLK/NBK, BMF, HRK/DP5) and share homology only in the BH3 region (aka BH3-only proteins). The pro-apoptotic "effectors" have two members (BAX and BAK)

and share similarities in all four BH domains. BAX/BAK permeabilize the outer membrane of mitochondria, releasing cytochrome c (and other apoptogenic factors, e.g. SMAC/DIABLO) into the cytosol to assemble with APAF-1 and pro-caspase 9 to form the apoptosome "wheel of death". This complex induces a 'caspase activation cascade', resulting in demolition of the doomed cell. In the steady state, the anti-apoptotic BCL-2 family members bind and restrain BAX/BAK. Upon stress, the "initiators" are induced/activated and compete for binding the anti-apoptotic members, thereby releasing BAX/BAK. It has also been reported that some "initiators" (eg. BIM) may also activate BAX/BAK by direct binding [3]. Our most recent studies suggest that immune cell survival is controlled by the quantitative participation of multiple anti-apoptotic proteins, rather than any one protein, which collectively function to maintain cell survival [2]. Thus, it is the sequential loss of function of each protein that ultimately dictates the timing of apoptosis initiation, when a predetermined threshold for death is reached.

Appropriately controlled cell death is integral to a healthy functioning immune system with the BCL-2 family of proteins central to this process. Controlling when immune cells die not only limits numbers at a gross level, but also provides a means to generate the most suitable immune repertoire. By continually purging unwanted, useless or harmful cells, the immune response can be continually shaped, evolving to the needs of a niche by removing auto-reactive, defective or superfluous cells, whilst retaining and refining an appropriate adaptive immune cell arsenal at the ready. Of the three BCL-2 sub-families, it is the anti-apoptotic proteins that convey differential regulation of immune cell survival, whilst the pro-apoptotic "initiators" or "effectors" appear to have more global effects. For example, the targeted disruption of pro-apoptotic proteins increase the numbers of all DC populations [8] and does not seem to confer the differential survival effects instilled by, for example, deletion of anti-apoptotic proteins BCL-2 or A1, which selectively impact the plasmacytoid (pDC) and conventional DC (cDC) populations, respectively [9]. A summary of reported immune cell dependency on individual anti-apoptotic BCL-2 proteins is provided (Table 1). Herein we review

the role of the five known anti-apoptotic proteins in different immune cell populations, highlighting their importance as targets for selective immune cell intervention.

T CELL LINEAGE

Thymic T cells

Developing T cells in the thymus progress through a series of apoptotic 'check-points' to ensure the export of safe and effective mature T cells into the periphery. Consistent with this concept, both the type and amount of anti-apoptotic BCL-2 family proteins fluctuate to facilitate this process. Following seeding from bone marrow (BM), double negative (DN) progenitors progress through a series of stages (DN1-4) until they become double positive cells, expressing both CD8 and CD4 (DP) and a newly rearranged T-cell receptor (TCR). These cells audition for positive selection within the thymus based on the ability of their TCR to recognize peptide-self MHC. Those cells possessing a TCR complex that can engage peptide/MHC complexes and receive appropriate TCR signalling continue through differentiation, whilst those that do not, die through apoptosis in a process termed 'death by neglect'. Likewise, maturing T cells that receive a TCR signal that is too strong are also purged from the repertoire by apoptosis (deletion, one mechanism of negative selection), ensuring the fully mature single positive (SP) $CD4^+$ or $CD8^+$ T cells that finally emerge into the periphery are suitably tolerant of self.

Genetic deletion in animal models has revealed distinct requirements for different anti-apoptotic proteins at different stages of T cell development [10]. Surprisingly, even though BCL-2 is highly expressed in both very immature DN and fully mature SP $CD4^+$ and $CD8^+$ T cells (but not in DP until they have been positively selected), its global deletion had little effect on the developing T cells within the thymus [11-13]. BCL-2 deficient mature T cells were lost only once exported to the periphery (see below). This finding is also consistent with data from treatment with the pharmacological BCL-2 antagonists ABT-199/Venetoclax and ABT-737 [1, 2, 14]. Similarly, deletion of BCL-XL, which is upregulated upon transition to DP stage (but is low in all other stages), also

surprisingly has little affect on T cell development [15-17]. However, consistent with our proposed quantitative model of anti-apoptotic Bcl-2 protein function, DP thymocytes from these animals were abnormally sensitive to death during *in vitro* culture, arguably coincident with the loss of shorter half-life proteins such as MCL-1 which may not be maintained in culture [18]. Likewise, although BCL-W is detected in all 4 major thymocyte populations [19], it was also found to be redundant upon global deletion *in vivo*, in all immune cells and tissues examined [20].

In contrast, MCL-1 is expressed at all stages of T cell development, and its conditional deletion under direction of the Lck promotor arrests T cell development at the DN stage [21]. Interestingly, as noted by Opferman and colleagues, this defect was most profound in the DN2/3 stages, a period when TCR rearrangement begins and developing cells become highly dependent on cytokine signalling (eg. IL-7) for survival [22]. Notably, this is also the point at which developing T cells down-regulate BCL-2 [23-25]. Arguably, by switching survival away from the long-lived BCL-2 protein, to a survival programme weighted heavily toward shorter-lived MCL-1, this shifts survival dependence to external signals such as those provided through the pre-TCR, which may be required to sustain MCL-1 expression. This MCL-1 weighted survival programme is then maintained throughout the selection process until cells are fully mature SP $CD4^+$ or $CD8^+$ lymphocytes ready for export to the periphery. Signalling though IL-2 receptor (CD25) during this late period of thymic T cell differentiation may also give developing regulatory T (Treg) cells a survival advantage, facilitating retention of these higher affinity, auto-reactive cells prior to induction of the FOXP3 regulatory program[26]. Collectively, one interpretation of these data is that MCL-1 shoulders most the anti-apoptotic burden during thymic T cell differentiation, functioning collectively with other anti-apoptotic proteins at various stages. Because of this, the function of the other proteins is masked and appears insignificant. However, MCL-1 having a short half-life requires continuous production to maintain its concentration. Therefore, if cells are placed in a

setting where MCL-1 is not maintained (e. g. cell culture), then the contribution of the other proteins becomes more apparent.

Conventional T cells

Different T cell subsets rely on different combinations of anti-apoptotic BCL-2 family molecules presumably to accommodate their changing survival needs. For example, it has long been appreciated that the survival requirements of naïve T cells differ from their memory counterparts [27]. Although T cells do not require BCL-2 during development, once they mature and export to the periphery this dependency becomes strikingly apparent; both $CD4^+$ and $CD8^+$ T cells are rapidly lost from the periphery of globally BCL-2 deficient animals over time [12, 13]. Accordingly, a similar effect is seen in naïve T cell populations following treatment with the selective pharmacological BCL-2 antagonist ABT-199 [2], or the combined BCL-2/BCL-XL/BCL-W inhibitor ABT-737 [1, 28, 29]. Notably, most recent data suggest the action of ABT-737, at least in lymphoid cells, is primarily through inhibition of BCL-2, with suboptimal BCL-XL and BCL-W antagonism in cells expressing multiple proteins [30, 31]. Likewise, inducible deletion of MCL-1 led to rapid loss of established peripheral T cell populations [21], with T cell survival also impaired in mice with global MCL-1 haplo-insufficiency [2]. Surprisingly, depletion of endogenous T cell populations was not reported in mice treated with a recently described, highly specific MCL-1 inhibitor *in vivo* at a dose that abrogated the growth of transplanted syngeneic haematopoietic tumours [32]. However, cellularity in this study was assessed nine days post treatment, possibly allowing rebound of affected populations as has been previously described [1, 33]. Autonomous loss of A1 [34, 35], BCL-XL[15, 16] or BCL-W[20] had little effect on T cell survival unless in combination with additional loss of BCL-2 or MCL-1, further highlighting the quantitative nature of anti-apoptotic protein function [2].

In contrast to naïve T cells, most memory/activated T cell subsets are largely resistant to pharmacological BCL-2 inhibition [1, 2, 28, 29]. Indeed, this resistance was found to correspond with

down-regulation of BCL-2, and coincidental upregulation of A1 and BCL-XL dependent on TCR signal strength [29]. Despite this, deficiency in either A1 or BCL-XL protein alone does not appreciably impact the ability to form T cell memory *in vivo* [17, 34, 35]. On the other hand, amounts of MCL-1 appear to be critical for T cell survival at all stages of development. Further to this, recent studies have shown that deficiency in the pro-apoptotic protein NOXA *in vivo*, a specific inhibitor of MCL-1 and A1 function, led to the generation of larger memory T cell pools [36] with increased clonal diversity, yet were ultimately impaired due to a strikingly reduced TCR affinity [36]. Thus, reminiscent of positive selection of T cells in the thymus during development, it appears that following T cell stimulation, T cells switch away from a long half-life BCL-2 dominated survival program in favour of short-half-life proteins, which may shift dependence of T cell survival to external signals and generate a competitive environment to achieve selection of the better suited, higher affinity clones. Additionally, it was shown that TCR signal strength was directly proportional to the surface expression of IL-2R on competing clones, enabling these clones to better compete for limited IL-2 during this period in order to maintain MCL-1 amounts and stay alive [36]. By impairing apoptosis (by removing NOXA), selective pressure among the clones was relieved, allowing the survival and thus retention of ‘less fit’ low affinity clones within the memory T cell pool [36].

Regulatory T cells (Treg)

Treg cells are a special class of $CD4^+$ T cells that governs the reactivity and homeostasis of their conventional counterparts. They are characterized by a transcriptional and epigenetic landscape that orchestrates their suppressive function and unique homeostatic properties, in part induced by the transcription factor FOXP3. There are at least three subsets of Treg cells: 1) central Treg cells, which recirculate through lymphoid tissues, 2) effector Treg cells that have undergone activation via high avidity TCR and co-stimulatory signals, and which attain the capacity to home to tissues, proliferate extensively and exert increased suppressive activity [37], and, 3) tissue-resident Treg cells

that lodge in tissues early in life and control local immune and metabolic function [38]. The question of how the number of these Treg cell subsets is controlled is important: too few Treg cells unleashes damaging immune pathology and autoimmunity, while too many induce immunosuppression.

A feature of the Treg cell population overall is that a high rate of turnover and flux maintains their homeostasis at steady-state. BrdU labeling studies estimate that half of the population is turned over within 10 days [33]. This property imparts the capacity for the population to swiftly adapt to the changes that occur during immune responses [39]. Surprisingly, the pro-survival activities of BCL-2, A1 or BCL-XL are dispensable for the survival of the bulk Treg cell population [33, 34]. Rather, it is the maintenance of MCL-1 expression by IL-2 that is essential for Treg cell survival and immune homeostasis [33, 39]. Consistent with these observations, Treg cells are largely resistant to pharmacological BCL-2 inhibitors ABT-199/ABT-737, unless they also have reduced amounts of MCL-1 [2]. Thus, the lifespan of most Treg cells is likely to be limited by the levels of this short-lived, pro-survival protein. This feature would explain the constant imperative for central Treg cells to receive IL-2R signals, transduced by the Signal Transducer and Activator of Transcription (STAT) 5, in proximity to partially activated conventional T cells [40]. On one hand, these signals reduce the pro-apoptotic activities of BIM and FOXP3 itself [41] and, on the other, restore the levels of MCL-1 [33] to rescue the cell from cell death.

The situation is different for the effector Treg that populate non-lymphoid tissues. These cells do not access IL-2-rich areas but, rather, require signals via ICOS and GITR to support their survival [42, 43]. Accordingly they express lower CD25 and are insensitive to IL-2 blockade in comparison to their IL-2 dependent central Treg counterparts [43]; Lower BCL-2 (protein) and MCL-1 (mRNA) and increased propensity for spontaneous death in short term culture was also reported [43]. Likewise, tissue-resident Treg cells appear to rely on distinct growth factors provided by their environment, such as lipid and IL-33/ST-2 interactions in adipose tissue or short-chain fatty acids in the gut [38].

Precisely how these different signals support effector and tissue-specific Treg cell survival remains unclear. Nevertheless, the interplay between host tissue factors and pathological conditions, such as chronic viral infection or malignancy, exert local control of Treg cell homeostasis. Understanding this interplay will be critical if targeting this crucial immune-modulatory population is to be clinically applicable.

Natural Killer cells

Natural killer (NK) cells were identified in the early 1970's for their spontaneous cytolysis of both syngeneic and allogeneic leukemia cells [44]. NK cells and their related innate lymphoid cell (ILC) family members were dependent on cytokines that signalled via the common gamma chain and the transcriptional regulator ID2 (inhibitor of DNA-binding 2). Amongst developing lymphoid progenitors, ID2 marks commitment to the ILC lineage. Upon commitment to the NK cell lineage, progenitors become dependent on IL-15 and continual high ID2 expression was found to prevent the aberrant expression of E-protein target genes including suppressor of cytokine signalling 3 (*Socs3*). Thus in the absence of ID2, NK cells became refractory to IL-15 signalling and underwent apoptosis, disappearing entirely from peripheral tissues. Consistent with this observation, NK cells failed to survive when adoptively transferred into *Il15*$^{-/-}$ mice indicating that IL-15 was essential for NK cell survival *in vivo* [45, 46]. IL-15 binding to IL-15Rγ/β on NK cells results in the phosphorylation of Janus kinases (JAK1 and JAK3) and the recruitment and phosphorylation of STAT 5. Phospho-STAT5 dimers then translocate to the nucleus and bind STAT5-responsive genes. Whilst the survival effect of IL-15 on NK has long been linked to expression of BCL-2 family proteins, it was not until genetic deleter mouse models became available that the relative importance of each protein could be assessed.

Although germ-line ablation of *BCL-2* resulted in lymphopenia in mice, the intrinsic effect of BCL-2 on NK cell survival was only revealed using a hypomorphic BCL-2 mutant (*what else*; *WE (Bcl2*$^{WE/WE}$) or conditional deletion of *Bcl-2* in NK cells (*Bcl-2*$^{fl/fl}$*Ncr1*$^{iCre)}$ [47]. The latter resulted in the

loss of 90% peripheral NK cells. Conditional deletion of MCL-1 (*Mcl-1*$^{fl/fl}$*Ncr1*^{iCre}) was even more dramatic, leading to a total lack of NKp46^{+} NK cells, ILC3 and ILC1 [48, 49]. STAT-5 was found to directly drive MCL-1 expression by binding its 3' UTR, linking MCL-1 levels to cytokine responsiveness (eg. IL-15) and subsequent NK cell survival [49]. This anti-apoptotic protein dependence could also be recapitulated following treatment with pharmacological BCL-2 inhibitors ABT-737 or ABT-199, with NK cells rapidly lost following treatment both *in vitro* and *in vivo* [2, 50]. Drug-mediated depletion could be further exacerbated by genetically reducing MCL-1 due to haplo-insufficiency (*Mcl-1*$^{+/-}$) [2]. Intriguingly, NK cells that were resistant to apoptosis in the absence of BCL-2 (*Bcl-2*$^{fl/fl}$*Ncr1*^{iCre} NK cells) were mostly proliferating (~70% Ki67 compare to ~10% in control mice) [49]. Intracellular analysis revealed that cycling NK cells expressed significantly higher amounts of MCL-1 than their non-cycling counterparts. Thus, cycling NK cells appear resistant to apoptosis in the absence of BCL-2 due to higher MCL-1 expression. Similarly, continuous exposure to the BCL-2 inhibitor ABT-737 appeared to render NK cells more resistant to treatment *in vivo*, potentially via a similar mechanism [1]. BCL-XL is dispensable for NK cell development *in vivo*, despite clear protein expression, as mice with specific deletion had normal proportion and number of NK cells in both lymphoid and non-lymphoid tissues [49]. Thus, taken together, BCL-2 and MCL-1 are both key inhibitors of NK cell apoptosis *in vivo*. The dominant role for MCL-1 suggests that it may be a more dynamic regulator of NK cell survival during homeostasis and immune responses, since amounts of MCL1 can be rapidly altered by IL-15 in the tissue microenvironment.

B CELL LINEAGE

Naïve B cells

B-cell development proceeds from the common lymphoid progenitor through to naïve, mature B cells in stages, starting in the BM and finishing in the periphery. It includes stages of proliferation interspersed with quiescence that achieve the expansion, distribution and persistence of B cell receptor (BCR) positive cells throughout the body, in anticipation of responding to their cognate,

foreign antigen (Ag). The B-cell response to foreign Ag recapitulates aspects of development in that there are periods of intense proliferation followed by periods of quiescence, reflecting the germinal centre (GC) reaction followed by that of memory B cells and long-lived plasma cells (PC), the latter providing lifelong high affinity immunity [51]. How homeostasis of the different B-cell compartments, both pre- and post-Ag, is maintained has been the subject of investigation over many years. An avenue of research we have pursued has been to assess the stage-specific expression of the individual pro-survival members of the BCL-2 family, and if that expression was correlated with a requirement for the survival of the cell [52]. This research, using both genetic and pharmacological means, has identified mechanisms of B-cell survival and thus revealed how particular populations might be targeted for therapeutic outcomes.

Peripheral B cells exist in one of several subsets defined by phenotype, location, lifespan and reactivity to Ag stimulation. The earliest developmental stage in the periphery of mice and humans is the so-called transitional stage, which in mice is further divided into subsets transitional 1 and 2 (T1, T2). T1 are the most recent emigrants from the bone marrow and give rise to T2 B cells. T2 cells in mice are the precursors for the marginal zone (MZ) B cells and follicular (Fo) B-cells, which differ in phenotype, distribution, recirculation and responsiveness to Ag. In mice a population of mature Fo B-cells are present in the BM. Finally, mice contain also a distinct B-cell population in their peritoneal and pleural cavities, designated B1 cells [53]. B1 cells are distinct in phenotype and developmental origin from conventional B cells (also called B2 or Fo B cells), arising predominantly from foetal precursors and persisting as mature B cells in the adult rather than being continuously generated from BM precursors as happens for B2 cells [54].

After exposure to Ag, additional subsets of B cells become identifiable [51]. This includes antibody secreting cells (ASC), which may be either short- or long-lived and located in either peripheral lymphoid organs or BM; germinal centre (GC) B cells, which are undergoing somatic

hypermutation and selection as part of the process of affinity maturation of the B cell response to Ag; and finally memory B cells, which are the quiescent, recirculating products of the GC, usually isotype switched and of improved affinity for the immunising Ag.

A dissection of the survival mechanisms of naïve B cells has recently been published [14], describing BCL-XL and BCL-2 promoting survival of immature and mature B cells respectively. MCL-1 was found important at all stages of B cell development [14, 21]. Instead, we will focus on the survival mechanisms of the post-Ag compartments (GC B cells and plasma cells) especially given their association with B cell cancers, immunity to vaccines and autoimmune diseases.

GC B cells

Transgene-mediated over-expression of BCL-2 or BCL-XL in B cells had profound effects on the B cell response. Over-expression of BCL-2 was reported at first to promote substantial increases in the size of the memory B cell compartment together with what appeared to be inappropriate retention of low affinity B cells [55]. Additionally, the extra-follicular foci of ASC, which typically involute due to apoptosis within days of formation, were preserved beyond their normal lifespan. The consequences of BCL-XL transgenesis were more subtle [56] with more diverse clones entering the GC at the start of the response and persisting into the long-lived BM ASC population, despite secreting low affinity antibody. A subsequent and more detailed study of the immune response in the BCL-2 transgenic mice revealed that in contrast to the expansion of the memory compartment with low affinity clones, the BM ASC compartment remained stringently selected for high affinity [57]. The difference between the BM ASC in these two models might indicate a difference in the role of BCL-2 and BCL-XL in the appearance of these cells. These results demonstrated that the GC relied on unimpeded implementation of apoptosis to function normally and that this could be blocked by increased expression of the pro-survival genes BCL-2 and BCL-XL. They did not,

however, indicate which of the multitude of possible pro-survival factors was normally modulated in GC B cells to achieve these outcomes.

The identification of the pro-survival gene required for B cell survival in the GC used a system in which candidate genes were deleted at the onset of the GC reaction and the consequences on cell survival determined [58]. This study deleted BCL-XL or MCL-1 (both of which were abundantly expressed in GC B cells) from B-cells as they formed GC following Ag exposure. MCL-1 was revealed to be absolutely required while BCL-XL was apparently redundant. Interestingly, complete loss of BCL-XL or partial loss of MCL-1 had no impact on affinity maturation, suggesting this process was driven more by proliferation of high affinity cells than the active death of low affinity cells. These genetic studies were supported by parallel studies using the drug ABT-737 in immunised mice [1], which showed that blocking BCL-2 and BCL-XL (BCL-W is not expressed in GC B cells), had no impact on existing GC B cells, indicating their independence of either pro-survival protein.

Plasma Cells

The loss of GC caused by deletion of MCL-1 resulted in a failure to produce GC-derived ASC [59], which ultimately populate the BM and persist at length in both mice and humans, leaving the question of the survival mechanism of these ASC unresolved. Treatment of mice post immunisation with ABT-737 did not reduce the frequency of BM ASC, again indicating neither BCL-2 nor BCL-XL was critical [1]. The most expressed pro-survival proteins in long-lived ASC in both spleen and BM were BCL-2 and MCL-1[59]. Furthermore, amounts of MCL-1 in BM ASC were found to be sensitive to signalling by the ASC survival factor APRIL (a proliferation inducing ligand). When MCL-1 was inducibly deleted from existing ASC, effectively all such cells were depleted from the animals irrespective of location or state of maturation [59]. Thus, MCL-1 was found to be essential for the survival of ASC.

Interestingly, a nuance in the ABT-737 treatment experiments indicated that there may be variation in the use of pro-survival proteins during the maturation and distribution of ASC following immunisation [1]. Specifically, when mice were subjected to treatment while undergoing an immune response, there was a significant reduction in the recruitment of Ag specific ASC into the BM despite normal numbers in the spleen. This suggested that at some point between production in the GC and acquiring residency in the BM, ASC were sensitive to the effects of ABT-737. This might mean that while in transit through the blood, ASC rely on either BCL-XL or BCL-2 for their survival. The extended half-life of these proteins (cf. the short-lived MCL-1) may allow these nascent, migrating ASC sufficient time to be recruited into long term survival niches in the BM [60].

The requirement for continued expression of MCL-1 in ASC independent of their location has a number of important implications. First, it suggests that irrespective of the various survival signals ASC receive from the plasma cell survival niche over time and location, they all converge on sustaining MCL-1 expression. Second, targeting MCL-1 is highly likely to be effective in treating plasma cell dyscrasias (eg. Myeloma) [32].

Memory B cells

The prolonged survival of memory B cells, sometimes lifelong, has been an enigma in immunology [61]. Despite intensive investigation, no external factors have been identified that are absolutely required to sustain memory B cells. While MCL-1 was found to be essential for memory B cells, their rate of loss following its deletion was no different to that of the shorter-lived Fo B cells, indicating that the requirement for MCL-1 was no greater in memory B cells than in Fo B cells [58]. Treatment of mice with ABT-737 post-immunisation, however, did differentially reduce the frequency of memory compared to naive B cells suggesting that these cells were more sensitive to the loss of BCL-2/XL than were Fo B cells and certainly ASC [1]. Although little progress on

memory B cell survival has been made since these observations, it remains an area of active research.

MYELOID LINEAGE

Conventional DC (cDC)

DC are critical stimulators of the adaptive immune system, charged with implementing robust defence against invading pathogens and cancer, whilst ensuring tolerance to healthy self-tissue. Thus, DC homeostasis is paramount to a safe and effective immune system; too few DC abrogates adaptive immune responses to exogenous Ag, whilst too many can initiate autoimmunity. Due to the cellular complexity of the DC network and the myriad of functional roles DC are required to perform, it is now clear, as with many of the other immune subsets, that there is no “one rule for all” regarding regulation of survival of the various DC subsets. Different DC subsets clearly have different cellular requirements, and this appears to be reflected in the proteins they need for survival.

cDC are the classical professional antigen-presenting cells. They not only express readily detectable amounts of BCL-2, MCL-1 and BCL-XL, but also constitutively express A1 protein [2]. Despite this cache of survival proteins, only genetic deficiency in MCL-1 [2,9], or to a lesser extent A1 [2,34], reduces the survival of cDC *in vivo.* Moreover, treatment with pharmacological inhibitors ABT-737 (specificity for BCL-2, BCL-XL, BCL-W), ABT-199 (BCL-2 selective) or WEHI-539 (BCL-XL selective) had minimal impact on cDC numbers *in vitro* or *in vivo* [1,2,9,62]. Surprisingly, treatment with a highly selective MCL-1 specific compound also did not induce cDC death *in vitro* [2]. Despite the underwhelming effect of deficiency or antagonism of the anti-apoptotic BCL-2 family members individually, combined impairment significantly reduced cDC numbers, highlighting the quantitative and additive nature of the family members. For example, $A1^{-/-}$ cDC were significantly more sensitive to BCL-2 or MCL-1 antagonism, unmasking a role for these proteins in maintaining cDC survival [2]. Furthermore, impairment of all three proteins ($A1^{-/-}.Mcl\text{-}1^{+/-}$ with ABT-199

treatment) reduced cDC survival conspicuously [2]. Thus, it appears in cDC (and many other immune cells) that induction of intrinsic apoptosis is regulated by the quantitative participation of all anti-apoptotic BCL-2 proteins present in the cell, which collectively function to neutralize pro-apoptotic proteins and maintain cell viability.

Plasmacytoid DC (pDC)

pDC are major producers of type I interferon (reviewed in[63]). Unlike cDC which are continuously processing and presenting Ag, pDC only acquire the ability to do this following activation such as via CD40 ligation or exposure to viral or bacterial stimuli [64]. Whilst activation was found to prolong pDC survival in culture [65, 66], *in vivo* evidence for this is somewhat contradictory [67] and may be hampered by sequestration of activated pDC in inflamed tissue [68]. pDC express abundant BCL-2 and MCL-1, with a small amount of BCL-XL protein [9, 66]. They did not express A1 (like their cDC counterparts) or BCL-W protein, even following TLR agonism [9]. Accordingly, mice deficient in A1 did not show reduced pDC *in vivo* [9, 34], nor impaired response to viral challenge [35]. Likewise, no defect in pDC (or any other immune cell) has been reported in BCL-W deficient animals [19, 20].

On the other hand, pDC are exquisitely sensitive to inhibition of BCL-2 or MCL-1. Mice globally haplo-insufficient for *Mcl-1* (*Mcl-1*$^{+/-}$) had significantly reduced pDC *in vivo*, as did mice with DC-specific MCL-1 deletion (*CD11c-cre.Mcl-1*$^{fl/fl}$)[2, 9]. Furthermore, pDC were also highly sensitive to apoptosis induced with a pharmacological MCL-1 antagonist *in vitro* [2]. Similarly, treatment with BCL-2 inhibitors ABT-199 or ABT-737 also led to rapid loss of pDC both *in vitro* and *in vivo.* pDC activation only offered marginal protection against depletion using these agents, a finding consistent with the ability of these drugs to deplete pDC from humans and animals with active pDC-mediated disease such as lupus [66]. Surprisingly, ABT-199/Venetoclax has also been used to treat two patients with refractory Blastic Plasmacytoid Dendritic cell Neoplasm with significant disease responses [69].

Consistent with the quantitative participation of BCl-2 and MCL-1 in maintaining pDC viability, ABT-199 treatment ablated almost all MCL-1 haplo-insufficient pDC *in vitro* and *in vivo* [2].

Neutrophils

Neutrophils are remarkably short-lived under steady-state conditions, with a lifespan of hours following their release from BM into the circulation as terminally differentiated effectors [70]. Following pathogen encounter they are rapidly deployed to tissue sites of infection, where they initiate broad anti-microbial activity including production of proteolytic enzymes, reactive oxygen species and an array of cytokines and chemokines before their untimely death. However, the lack of specificity of effector function avails collateral damage and presumably short life span reduces this potential for major injury.

In line with a shorter lifespan, mature neutrophils express very little BCL-2, BCL-W or BCL-XL but readily detectable MCL-1 and A1 [18, 71, 72]. Accordingly, genetic ablation of BCL-2 or BCL-W did not significantly impair neutrophil survival *in vitro* or *in vivo* [73], nor did treatment with BCL-2/XL/W antagonist ABT-737 [1]. Mice lacking *A1a*, one of the three active isoforms of A1 in mice, had normal neutrophil numbers but they died more rapidly *in vitro* over 24 hours [74]. However, we found no such defect in neutrophils when all functional A1 isoforms were ablated [34]. MCL-1 appears to be a chief anti-apoptotic regulator of neutrophil survival. It is consistently detected at both mRNA and protein level, with the amount of protein correlating closely with neutrophil survival [18, 71, 72, 75]. Accordingly, mice with conditional deletion of MCL-1 display severely impaired mature neutrophil survival [76, 77]. Neutrophil survival can be greatly extended following exposure to inflammatory cytokines GM-CSF or TLR agonists such as LPS [72, 78]; a finding attributed, at least in part, to increased MCL-1 protein stability [78].

CONCLUDING REMARK

Identifying the combinations of proteins responsible for maintaining immune cell viability *in vivo* has tremendous clinical implications. With the advent of highly specific and well-tolerated small mimetic inhibitors, different immune cells may be specifically targeted based on these requirements, effectively tailoring the treatment to the disease. This "personalized" therapy could facilitate specific targeting of detrimental immune responses, such as those associated with immunopathology or hematological malignancy, whilst preserving the function of others, thus minimizing unwanted side effects associated with global immunosuppression.

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FIGURE LEGENDS

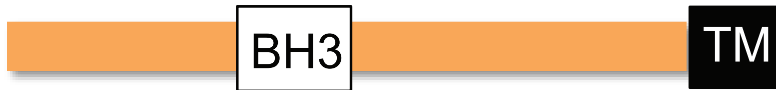
Figure 1. The Bcl-2 family proteins and their pharmacological inhibitors. A) The structure of Bcl-2 family proteins. Bcl-2 family proteins contain between one and four BCL-2 Homology (BH) domains (BH1-4) that facilitate interactions between proteins. The anti-apoptotic “guardians” (BCL-2, BCL-XL, BCL-W, MCL-1 and A1/BFL1) and pro-apoptotic “effectors” BAX/BAK show similarity in all four BH domains. The pro-apoptotic “initiators” consist of at least eight members (BID, BIM, PUMA/BBC3, BAD, NOXA/PMAIP, BIK/BLK/NBK, BMF, HRK/DP5) and share homology only in the BH3 region (aka BH3-only proteins). Most BCL-2 family members contain a

transmembrane (TM) domain. B) Schematic of the BCL-2 apoptotic pathway. Pro-apoptotic and anti-apoptotic members selectively interact to govern activation of BAX/BAK and induction of apoptosis. Direct activation of BAX/BAK by BIM, PUMA and tBID may not be obligatory for death induction and hence is displayed as a dashed arrow. **C)** Schematic of the specificity of the BH3 mimetic compounds for antagonising various members of the anti-apoptotic BCL-2 family.

Table 1. Reported dependence of immune cell types on individual anti-apoptotic BCL-2 family molecules for survival.

Critical molecule	Immune cell type	Ref
BCL-2	Naïve T, naïve B, pDC, NK	[1, 9, 14, 47]
BCL-xL	Immature B	[14]
MCL-1	Treg, NK, immature and mature naïve B, immature and mature T cells, GC B cell, plasma cell, pDC, cDC, neutrophil	[1, 9, 21, 33, 39, 48, 49, 58, 59, 76, 77]
A1	cDC	[2, 34]

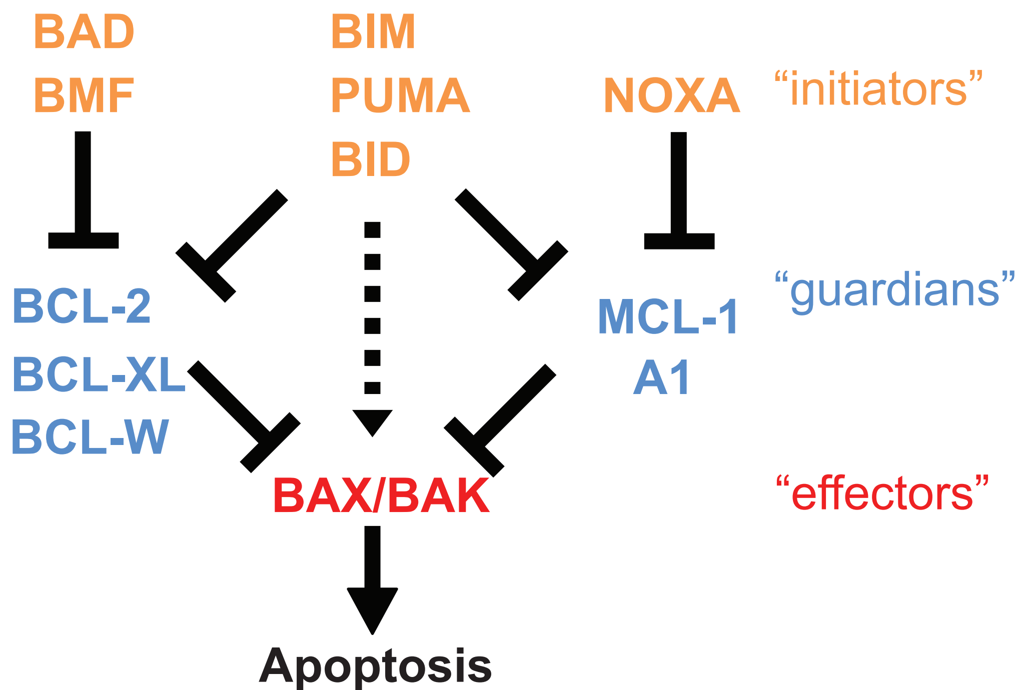
A) Pro-apoptotic BH3-only “initiators”:



Anti-apoptotic “guardians”

Pro-apoptotic “effectors”

B)



C)