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Anti-apoptotic proteins BCL-2, MCL-1 and A1 summate collectively to maintain survival of immune cell populations both *in vitro* and *in vivo*

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Running title: BCL-2 proteins quantitatively control survival

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Abstract

Survival of various immune cell populations has been proposed to preferentially rely on a particular anti-apoptotic BCL-2 family member e.g. naïve T cells require BCL-2, while regulatory T cells require MCL-1. Here we examined the survival requirements of multiple immune cell subsets *in vitro* and *in vivo*, using both genetic and pharmacological approaches. Our findings support a model in which survival is determined by quantitative participation of multiple anti-apoptotic proteins rather than by a single anti-apoptotic protein. This model provides both an insight into how the sum of relative levels of anti-apoptotic proteins BCL-2, MCL-1 and A1 influence survival of T cells, B cells and dendritic cells, and a framework for ascertaining how these different immune cells can be optimally targeted in treatment of immunopathology, transplantation rejection or hematological cancers.

Introduction

The BCL-2 family can be envisaged as a tripartite apoptosis control system comprising one set of anti-apoptotic and two sets of pro-apoptotic proteins, which interact to determine whether cells live or die in many pathophysiological states [1, 2, 3, 4]; **Figure 1A**). The five known anti-apoptotic members (BCL-2, BCL-XL, BCL-W, MCL-1 and A1/BFL1) share similarity in four BCL-2 homology (BH) domains. The pro-apoptotic members can be further sub-divided into "initiators" that share homology only in the BH3 region (e.g. BIM, PUMA; see below) and "effectors" that share similarities in all four BH domains, viz. BAX and BAK. BAX/BAK elicit mitochondrial outer membrane permeabillization (MOMP), releasing cytochrome c (and other apoptogenic factors; e.g. SMAC/DIABLO) into the cytosol to promote apoptosome formation and activation of the caspase cascade causing cellular demolition [5]. The main function of anti-apoptotic BCL-2 proteins is to restrain pro-apoptotic BAX/BAK, thus preserving mitochondrial outer membrane integrity. This is achieved by direct binding and sequestration of pro-apoptotic BH3-only proteins that possess the ability to directly or indirectly activate BAX/BAK. The BH3-only proteins include at least 8 members (BID, BIM, PUMA/BBC3, BAD, NOXA/PMAIP, BIK/BLK/NBK, BMF, HRK/DP5). Upon stress these proteins, transcriptionally induced or post-transcriptionally activated, bind anti-apoptotic members thereby unleashing BAX/BAK from their restraint [6]. Certain BH3-only proteins (e.g. BIM, PUMA) have been reported to also directly activate BAX/BAK, although this may not be obligatory [6, 7, 8, 9].

Binding studies have revealed that the capacity for different BH3-only proteins to bind anti-apoptotic proteins is not equal [8, 10]. Some BH3-only proteins (BIM, PUMA,

truncated BID (tBID)) bind avidly to all five anti-apoptotic proteins, whereas others are more selective. For example, BAD only binds to BCL-2, BCL-XL and BCL-W but not MCL-1 or A1/BFL-1, whereas NOXA does the converse. Moreover, it has been reported that distinct anti-apoptotic proteins also differ in their ability to restrain BAX or BAK; for example BCL-2 only being able to bind and restrain BAX but not BAK [11]. Simplistically, this would imply there should be qualitative differences in the capacity of at least some anti-apoptotic proteins to block death, a finding indirectly supported by the reciprocal pattern of expression often observed between different anti-apoptotic proteins throughout immune cell development (e.g. low MCL-1 and high BCL-2 in naïve B cells, but high MCL-1 and low BCL-2 in germinal centre B cells) [12, 13, 14].

BH3-only protein binding selectivity has formed the basis of rational drug design strategies to antagonize distinct anti-apoptotic BCL-2 family members. First generation compounds ABT-737 and ABT-263/navitoclax antagonize BCL-2, BCL-XL and BCL-W [15, 16, 17]. The second generation compound ABT-199/venetoclax/venclexta, selective for BCL-2 alone [18], has proven highly effective in the treatment of certain leukemias and lymphomas [19, 20, 21] and received FDA approval in April 2016 for treating refractory chronic lymphocytic leukaemia (CLL) with 17p chromosomal deletion. However, factors predicting cell sensitivity, particularly *in-vivo*, and what role each anti-apoptotic member plays are not definitively understood.

Besides selectivity of binding between some BH3-only proteins to anti-apoptotic BCL-2 family members, arguably the most cogent case for qualitative differences in the

anti-apoptotic molecules is the apparent differential dependence by many immune cell subsets on individual proteins for survival. For example, platelets were found to be reliant on BCL-XL [22, 23]. Naïve T cells, naïve B cells and plasmacytoid dendritic cells (pDC but not conventional DC, cDC) rely on BCL-2 [24, 25, 26, 27]. Conversely, germinal center B cells [14], plasma cells [28], NK cells [29] and regulatory T cells [30] depend on MCL-1 (summarized in **Figure 1B**). One conspicuous extrapolation of these studies is that the anti-apoptotic proteins play distinct roles in these subsets (qualitative model). The alternative model is that cell survival is safeguarded by quantitative expression of the total set of BCL-2 family members (quantitative model), but because of differential expression among the subsets, one family member may appear more important than another. This latter model would predict that reduced levels or antagonism of one anti-apoptotic member may be compensated for by increased levels of another; i.e. implying there is functional redundancy (at least partially) among the anti-apoptotic BCL-2 proteins.

Herein we looked at the impact of manipulating the amount of different anti-apoptotic BCL-2 family members in immune cells. We found that by reducing endogenous MCL-1 and/or A1, we could sensitize cells to death by BCL-2 antagonism. Conversely, increasing BCL-2 or MCL-1 could compensate for reduced levels of either protein. These findings reveal that rather than autonomously controlling survival of a particular cell type, individual anti-apoptotic proteins contribute quantitatively to survival outcome.

Results

Deficiency of MCL-1 can be compensated by increased levels of BCL-2

We have previously shown that haplo-insufficiency of *Mcl-1* (*Mcl1*$^{+/-}$) significantly reduced DC populations *in vivo* [27]. To determine whether this loss was MCL-1-specific, we tested whether increasing the level of BCL-2 could compensate *Mcl-1* haplo-insufficiency. We crossed *Mcl-1*$^{+/-}$ mice to mice overexpressing BCL-2 in all hematopoietic cells (*BCL-2* tg) and then generated BM chimeras from resultant offspring. *Mcl-1* haplo-insufficiency within the immune cell compartment resulted in reduced numbers of both pDC and cDC (**Figure 2A**), as previously reported [27]. B cell but not T cell numbers were also significantly decreased. Upon BCL-2 over-expression, the numbers of all cell populations that were diminished in the *Mcl-1*$^{+/-}$ mice were equivalent to or even above those seen in wt (*Mcl-1*$^{+/+}$) controls. Enhanced production of pDC and B cells in BM did not appear to be responsible for restoration of cells in the periphery as concurrent organ analysis revealed BM was only mildly affected by BCL-2 overexpression compared to spleen and other peripheral sites such as blood (**Supp Figure 1**). Importantly, Western blot analysis of purified B cells, pDC and cDC confirmed reduced MCL-1 and increased BCL-2 expression in cells from *BCL-2 tg/Mcl-1*$^{+/-}$ mice. There was no compensatory increase in A1, discounting this as a factor contributing to rescue (**Figure 2B**). This is an important finding as A1 has more similar binding specificity to MCL-1 than other anti-apoptotic proteins (e.g. they both bind NOXA but not BAD). Interestingly, BCL-2 overexpression in B cells and pDC appeared to coincide with reduced MCL-1 protein (**Figure 2B**). Overexpression of BCL-2 could also fully protect B cells and pDC in culture from apoptosis induced by pharmacological inhibition of MCL-1 by a recently described highly specific BH3 mimetic [36] (**Figure 2C**). Importantly, sensitivity to this inhibitor was preserved under conditions of reduced MCL-1 (*Mcl-1*$^{+/-}$) suggesting that

overexpression of BCL-2, and not deviation from MCL-1 dependency, was responsible for protection in the *BCL-2* tg/ *Mcl-1*$^{+/-}$ cells (**Figure 2C**). All cDC tested were refractory to MCL-1 inhibitor (**Figure 2C**), suggesting that although initially dependent on MCL-1 [27], once fully differentiated, cDC are no longer fully reliant on MCL-1 for their survival. Taken together, these data suggest that despite being the targets of different BH3-only proteins (e.g NOXA but not BAD binds MCL-1; the converse for BCL-2) increasing levels of BCL-2 could compensate for reduced amounts of MCL-1.

Partial deficiency in *Mcl-1* unmasks the sensitivity of cells to BCL-2 antagonism

Since increased BCL-2 could mitigate the effect of haplo-insufficiency in *Mcl-1*, we hypothesized that antagonism of endogenous BCL-2 would exacerbate the effect of partial *Mcl-1* deficiency. Indeed, when *Mcl-1*$^{+/-}$ splenocytes were cultured in the presence of the BCL-2 inhibitor ABT-199 [18], B cells, T cells and pDC died significantly more over a range of doses than the corresponding wt control cells treated with ABT-199 (**Figure 3A**). This was also true *in vivo* when mice were given a single 100 mg/kg body weight dose of ABT-199 and spleens analyzed 24 h following treatment, particularly in the pDC compartment where inhibition of BCL-2 ablated almost all pDC in *Mcl-1*$^{+/-}$ mice (**Figure 3B**). Whereas global *Mcl-1* haplo-insufficiency *per se* approximately halved the splenic T cell numbers *in vivo* (**Figure 3B**), additional inhibition of BCL-2 by ABT-199 treatment substantially increased death of these cells, with a further 4-fold reduction in CD4^{+} and CD8^{+} T cells (**Figure 3B**). This effect was also seen in B cells, but was less remarkable in cDC; i.e. there was only a modest albeit significant reduction in the cDC numbers seen in the

spleens of ABT-199 treated *Mcl-1*$^{+/-}$ mice compared to vehicle treated *Mcl-1*$^{+/-}$ mice (**Figure 3B**).

We had previously found that MCL-1 is critical for NK cell [29] and Treg cell [30] survival by demonstrating that these leukocyte subsets are selectively ablated by *Mcl-1* deletion using cell type restricted Cre transgenes (*Nkx-Cre* or *FoxP3-Cre*). Therefore, we decided to test the impact of ABT-199 (BCL-2 inhibitor) treatment on these "MCL-1 dependent" cell subsets. We found that for NK cells, either BCL-2 inhibition or *Mcl-1* haplo-insufficiency significantly reduced numbers *in vivo* compared to vehicle treated controls; however, the combination of BCL-2 inhibition and *Mcl-1* haplo-insufficiency reduced NK numbers even further compared with either survival impairment alone (**Figure 3C**). Similarly, Treg numbers were more substantially reduced by combined ABT-199 treatment and *Mcl-1* haplo-insufficiency than by either impairment alone (**Figure 3D**).

Collectively, these results reveal that in multiple cell types endogenous BCL-2 and MCL-1 have overlapping, rate-limiting functions in cell survival.

BCL-2 inhibition can be compensated by increased MCL-1 levels

We had shown above that BCL-2 overexpression could compensate for reduced MCL-1 levels. Next, we wanted to determine whether increased MCL-1 levels could protect cells from apoptosis induced by antagonism of endogenous Bcl-2. As we had previously shown that B cells, T cells and pDC, but not cDC, were sensitive to BCL-2 inhibition [26, 27], we examined the *in vitro* response of these cell types to ABT-199, when MCL-1 was overexpressed (*Mcl-1* tg). All three ABT-199 sensitive cell types (B cells, T cells, pDC) were protected from death over a range of doses with significantly more *Mcl-1* tg cells recovered after treatment compared to wt control cells (**Figure**

4A). This protection was also evident *in vivo* when animals were gavaged with a high dose of ABT-199 (100 mg/kg body weight; 5 daily doses), such that all cell types were completely protected from depletion in *Mcl-1* tg animals whereas substantial portions of these cell types were lost in the ABT-199 treated wt controls (**Figure 4B**). This was particularly remarkable for pDC, which are normally exquisitely sensitive to ABT-199 but were completely protected by MCL-1 overexpression both *in vitro* (**Figure 4A**) and *in vivo* (**Figure 4B**). As expected [27], wt cDC were minimally affected by ABT-199 treatment at all doses tested. Taken together these data demonstrate that increased levels of MCL-1 can compensate for loss of BCL-2 function, both *in vitro* and *in vivo*.

Loss of A1 unmasks the sensitivity of cDC to BCL-2 antagonism

The one immune cell whose survival is known to be reliant on A1 is the cDC, a finding replicated in both the A1 knockdown mouse [27] and genetically deficient mouse models [37] and consistent with the inability of BCL-2 inhibitors to kill this subset (**Figure 3, 4**; [27]). Therefore, we assessed whether A1 deficiency (loss of all A1 isoforms;[37]) could sensitize cDC to BCL-2 antagonism. We first measured the amount of A1, MCL-1 and BCL-2 in splenic cDC from wt and *A1*$^{-/-}$ animals confirming complete loss of A1 protein and no major expression differences of BCL-2 and MCL-1 in the A1$^{-/-}$ cDC compared to wt (**Figure 5A**). When splenocytes were cultured in the presence of ABT-199, *A1*$^{-/-}$ cDC had significantly impaired survival (LD50 ~30 nM) compared to wt cDCs, which remained unaffected by ABT-199 (**Figure 5B**). As expected [27], cDC numbers *in vivo* were unaffected after ABT-199 gavage in wt mice. Untreated or vehicle treated *A1*$^{-/-}$ mice had modestly lower cDC numbers than wt animals as previously described [37]. However, BCL-2 inhibition by ABT-199 gavage in

A1$^{-/-}$ mice caused a dramatic loss of cDC numbers, reducing them to a third of the numbers found in vehicle-gavaged *A1*$^{-/-}$ controls (**Figure 5C**). These findings demonstrate that A1 and BCL-2 have critical overlapping functions in the survival of cDCs, revealing a previously undocumented role for BCL-2 both in culture and within the intact animal.

BCL-2 antagonism unmasks the sensitivity of B and T lymphoid cells to the loss of A1

As previously reported [37, 38], there was no significant difference in B or T cell numbers between wt and *A1*$^{-/-}$ mice (**Figure 5D**). Thus, at first glance one could conclude that A1 is not important for the survival of B and T cells. However, we wanted to test our quantitative model, which predicts that when A1 is very low or absent, other anti-apoptotic BCL-2 family members become limiting for safeguarding survival. In line with this hypothesis, when BCL-2 was inhibited, loss of A1 resulted in a halving of B and T cell numbers, compared with ABT-199 treated wt mice (**Figure 5D**). From these findings, we conclude that at physiological levels A1 can promote survival of B and T cells, but is functionally redundant in cells from wt mice as there is sufficient BCL-2 to maintain survival.

Loss of A1 unmasks the sensitivity of cells to reduced levels of MCL-1

Given that loss of A1 could sensitize cells to BCL-2 antagonism, we were interested to examine whether the same was true for MCL-1 antagonism. Indeed, when splenocytes were cultured in the presence of MCL-1 inhibitor, *A1*$^{-/-}$ cDC and T cells displayed significantly more death over the dose range than their wt counterparts (**Figure 6A**). As noted above (**Figure 2C**), B cells were so sensitive to MCL-1

inhibition *in vitro* that under the conditions used it was not possible to reveal any meaningful synergistic or additive effect between A1 loss and MCL-1 inhibition. Notably, the increased death of *A1*$^{-/-}$ cells evinced by BCL-2 inhibition (using ABT-199) could be further exacerbated by genetically reducing levels of *Mcl-1* (haplo-insufficiency). When splenocytes that lacked A1 and were also haplo-insufficient for *Mcl-1* (*A1*$^{-/-}$*;Mcl-1*$^{+/-}$) were treated with ABT-199 *in vitro*, cDCs and T cells were significantly more sensitive to treatment than their wt counterparts or cells possessing either deficiency alone (*A1*$^{-/-}$*;Mcl-1*$^{+/+}$ or *A1*$^{+/+}$*;Mcl-1*$^{+/-}$) (**Figure 6B**). Collectively, these data highlight the collaborative effect BCL-2, MCL-1 and A1 have on maintenance of cell survival, cogently supporting a quantitative model of anti-apoptotic protein function, at least in immune cell subsets.

Discussion

The survival of distinct immune cell subsets is differentially dependent on select anti-apoptotic BCL-2 family proteins [14, 22, 26, 27, 29]. However, the reasons underlying this reliance are not fully elucidated. There are at least two plausible scenarios. Firstly, dependence on a particular anti-apoptotic protein could reflect inimitable characteristics offered by the protein. Thus, the function of a given anti-apoptotic member could not be replaced by another. For example, the difference in affinity between MCL-1 vs BCL-2 for NOXA could support this model. Secondly, dependence could reflect differential expression of anti-apoptotic BCL-2 family members within a cell type and the functional load the collective set of proteins has to bear (Quantitative model). In this scenario, sequentially removing individual anti-apoptotic BCL-2 family members would progressively reduce the total load available to neutralize the pro-apoptotic members present within that cell, eventually tipping

the outcome in favour of death. In cells with a high propensity for death, one could speculate that the total levels of the anti-apoptotic BCL-2 family members might be limiting, such that genetically removing or pharmacologically inhibiting a single member may be sufficient to kill a particular subset. Conversely, more resistant cells may require inactivation of multiple anti-apoptotic BCL-2 family proteins.

Herein, we present evidence that immune cell survival is governed by collaboration between multiple anti-apoptotic BCL-2 family members within a given cell, acting to preserve viability by neutralizing the pro-apoptotic members present. In accordance with a quantitative model, we found that apoptosis could be initiated by individually targeting abundantly expressed anti-apoptotic proteins within a given immune cell population. Cell killing could be enhanced or reduced respectively by sequentially subtracting or adding the function of additional anti-apoptotic BCL-2 family members. For example in pDCs and B cells, both BCL-2 and MCL-1 are limiting [26, 27, 39] and therefore genetic loss or pharmacological inhibition of either results in increased sensitivity to loss or antagonism of the other (**Figure 3B**). Moreover, BCL-2 or MCL-1 overexpression prevents cell death caused by loss or drug mediated inhibition of the other (e.g. *Mcl-1* haplo-insufficiency or BCL-2 inhibition by ABT-199; **Figure 2C, 4B**). For cDCs, whilst A1 and MCL-1 are limiting for survival and loss of either reduces cDC numbers on their own [27, 37], this also sensitizes them to loss or antagonism of the other.

We surmise that the effects we have seen are the result of changes to mature cell survival. Firstly, we and others have previously found that immature cells (eg. immature B cells in BM or immature T cells in thymus) are less sensitive to BCL-2

antagonists [39, 40, 41]. Secondly, as well as spleen, we have simultaneously looked at blood and bone marrow for most *in vivo* drug experiments. As expected, we found that the reduction of cells (total, B cells, pDC) in bone marrow (where the precursors were) was less affected compared with the periphery (blood and spleen) (**supp Figure 2**). Therefore, even though we did not subtype immature populations here, it is highly likely that mature cells were the cells primarily affected. Thirdly, the duration of drug treatment both *in vitro (16h)* and *in vivo* (in most cases 1-2d) was intentionally short to limit the impact of proliferation and/or differentiation. Fourthly, in the experiments using BCL-2 overexpression, BM analysis revealed no substantial elevation in the total number, B cells (B220+ cells the majority of which would be immature) or pDC; therefore, this could not account for the compensatory increase of these populations in the periphery (**supp Figure 1**).

Somewhat surprisingly, the proclivity for functional overlap amongst the anti-apoptotic proteins occurred irrespective of their capacity to bind the different pro-apoptotic BCL-2 family members [8, 10, 11]. For example BCL-2, which does not bind NOXA or BAK, could compensate for a reduction (haplo-insufficiency) in MCL-1 function (which does bind NOXA and BAK). Likewise over-expression of MCL-1, which does not bind BAD or BIK could compensate for deficiency in BCL-2, which avidly binds these two BH3-only proteins [8, 10, 11]. Similarly, the killing of BCL-2 dependent cell types by ABT-199 could be blocked by increased expression of MCL-1. Conversely, killing of cells by an MCL-1 inhibitor could be blocked by BCL-2 over-expression. Whilst it is clear that preferential interactions between pro- and anti-apoptotic BCL-2 family members must exist within a physiological setting, it appears that each of these interactions alone does not solely dictate survival outcome. Rather,

our data suggest that properties common or overlapping between all of the five anti-apoptotic BCL-2 proteins affecting their overall restraint of BAX/BAK is likely to be of greater importance in controlling apoptosis.

Notably, our quantitative model only indicates that the five anti-apoptotic BCL-2 family members have functional overlap with regards to cell survival. It does not suggest identical physiological functions. Clearly their expression and protein turnover and hence level of activity are different. For example, BCL-2, BCL-XL and BCL-W are relatively long-lived proteins (half-life ~20 h), whereas MCL-1 and A1 are short-lived (0.5-3 h) [42, 43, 44, 45]. Accordingly, if transcription or translation is blocked, MCL-1 and A1 functions are more quickly lost [43, 46]. Consequently, MCL-1 and A1 levels can be swiftly modulated in response to a range of extracellular signals, such as those provided by cytokines [29, 47, 48, 49, 50] and growth factors [49, 51, 52]. Moreover, BCL-2 and MCL-1 are constitutively expressed in many cell types, whereas A1 is often only transiently expressed, for example as an early response to antigen receptor ligation, further segregating their roles in cell survival [13, 48].

Our model of anti-apoptotic BCL-2 family members acting as a quantitative collective to maintain immune cell survival has important clinical implications. It warns that analysis of an individual member is not an infallible marker of drug sensitivity and propensity to undergo apoptosis. For example, BCL-2 levels alone may not predict the response of a cell population (e.g. leukemic cells) to BCL-2 antagonist drugs. Indeed, it has been noted that a high BCL-2/low MCL-1 ratio in tumor cells is a superior predictor of ABT-199 sensitivity compared to high BCL-2 expression alone [53]. Our model contends that the profile of anti-apoptotic proteins, rather than any one

individual present within the cell, ultimately determines drug-responsiveness. For example, cells that express BCL-2 and MCL-1 but not A1, such as pDCs, would be expected to be most susceptible to BCL-2 plus MCL-1 inhibition. Conversely, cells that express BCL-2, MCL-1 and A1, such as cDCs, would be predicted to be most susceptible to impairment of all three. Our quantitative model also alerts to the possibility of compensation of one family member by another. Thus, under certain conditions the amount of a particular anti-apoptotic protein may be elevated, for example GM-CSF increases A1 expression [48]. Our model also highlights that if *in vitro* expression of any of the anti-apoptotic BCL-2 members does not reflect that *in vivo*, *in vitro* testing of antagonist drugs may be misleading. This is particularly germane to the shorter-lived MCL-1 and A1 proteins, which are not necessarily maintained at high levels during cell culture.

Materials and Methods

Mice and reagents. C57BL/6 wild-type (B6, wt), B6.Ly5.1, *Mcl-1*$^{+/-}$[54], VavP-*BCL2*(69) transgenic (*BCL-2* tg) and [55], VavP-*Mcl-1*(33) (*Mcl-1* tg) transgenic [56] mice were all on a B6-Ly5.2 background. *A1*$^{-/-}$ mice were generated on a C57BL/6 background by sequential targeting of each A1 gene (*A1a*, *A1b*, *A1d*) as described [37]. All mice were housed under specific pathogen-free (SPF) conditions at the Walter and Eliza Hall Institute of Medical Research (WEHI) and were handled according to guidelines approved by the institutional Animal Ethics Committee. Bone marrow (BM) chimeras (used where specified) were generated by transplantation of 5×10^6 BM cells into lethally γ-irradiated congenic recipient mice (2 × 5.5 Gy, 3 h apart from a cobalt-60 source). ABT-199 (Active Biochem, Hong Kong) and MCL-1 inhibitor (example 185, manufactured as specified in US Patent Application No. 20150175623) were

dissolved in DMSO for *in vitro* use. Preparation and *in vivo* use of ABT-199 has been previously described [57] and this drug was administered at the indicated dosage by oral gavage.

Protein isolation and Western blot analysis

Detection of anti-apoptotic proteins by Western blot analysis was performed as described [27, 58]. Primary antibodies used were mouse anti-human BCL-2 (cat. no. sc-7382, Santa Cruz), rat anti-MCL-1 (clone 19C4-15), rat anti-A1 (clone 6D6, in house; [59] and goat anti-β-actin-HRP (cat. no. sc-1616 HRP, Santa-Cruz, Dallas, TX), the latter used as a protein loading control. Secondary antibodies used were goat anti-rat-Ig-HRP or goat anti-mouse-IgG1-HRP antibodies (SouthernBiotech, Birmingham, AL)

Cell subset analysis, antibodies and flow cytometry. Mice were sacrificed and single-cell suspensions of spleen prepared as described [26, 60]. Red blood cells (RBC) were lysed in 0.168M ammonium chloride. For *in vitro* survival assays spleen cells were enriched for DC using a nycodenz density gradient as previously described [60] before 2×10^5 cells were cultured in 96 well plates overnight in RPMI supplemented with 10% (vol/vol) FCS with or without inhibitors as indicated. For each experiment wt and experimental (knockout or transgenic) splenocytes were isolated from age and sex matched animals. Cells were treated (or left untreated) in at least triplicate within the same plate. Where data displayed as "% survival (relative to untreated)" this is calculated by dividing the total number of live cells isolated from each treated well by the average number of live cells isolated from untreated control wells. Fluorochrome-labelled antibodies against CD11c, CD11b, SIGLEC-H (ebioscience, San Diego, CA),

TCRβ, CD8, CD4, B220, CD25, CD4, FOXP3 (ebioscience, San Diego, CA), NK1.1, NK-p46, CD45.1 and CD45.2 were used to identify leukocyte populations by flow cytometry and were purchased from BD Biosciences unless otherwise stated. Regulatory T cells (Treg) were identified by expression of intracellular FOXP3 in conjunction with CD4 and CD25 surface staining. Absolute cellularity was determined by addition of fluorochrome-conjugated calibration beads (BD Biosciences) directly to samples and staining with propidium iodide was used to exclude dead cells. Samples were run on a FACS-Verse (BD Biosciences) and data analysis was performed using FlowJo 9.3.2 (Tree Star, Ashland, OR).

Statistical analysis

Statistical comparisons were made using a 2-tailed student's t-test with Prism v.5.0 software (GraphPad, San Diego, CA). Data are shown as means ± SE with p values <0.05 considered statistically significant.

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Author contributions

Designed research: EMC, AML, IBV, DMT, JGZ, YZ

Performed research: EMC, JLB, JGZ, RMS

Contributed vital new reagents or analytical tools: NSA, RLS, MJH, RBD, NDH, DS, DCSH, SC, PB, AS

Collected data, analysed and interpreted data: EMC, JLB, JGZ, RMS, AML

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Disclosure of conflict of interest

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Figure Legends

FIGURE 1

BCL-2 family and immune cell subsets. A) Schematic of the BCL-2 family proteins and their functions in the intrinsic mitochondrial pathway of apoptosis. The anti-apoptotic "guardians" (BCL-2, BCL-XL, BCL-W, MCL-1 and A1) promote cell survival by preventing mitochondrial outer membrane permeabilization (MOMP) by the downstream pro-apoptotic "effectors" BAX/BAK. MOMP causes release of cytochrome c and other apoptogenic proteins that promote activation of the caspase cascade that mediates cell demolition. The pro-apoptotic BH3-only "sensors" promote death by selectively antagonizing the function of the anti-apoptotic proteins and/or directly activating BAX/BAK. 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. B) Apparent differential dependency of distinct immune cells subsets on individual anti-apoptotic BCL-2 family proteins for survival.

FIGURE 2

Deficiency of MCL-1 in leukocyte subsets can be compensated for by increased amounts of BCL-2. Haplo-insufficient *Mcl-1*$^{+/-}$ mice were bred to *BCL-2* tg mice to generate B6 (wt), *Mcl-1*$^{+/-}$, *BCL-2* tg and *Mcl-1*$^{+/-}$*;BCL-2* tg. BM chimeras were generated from 8 wk old resultant offspring A) Live B cells, CD4^{+} T cells, CD8^{+} T cells, pDC and cDC were enumerated by PI staining and flow cytometry. Data shown are from a single representative experiment of 3 experiments each with n=4 animals;

Mean ± SEM analyzed using a 2-tailed Student's t test. *P < 0.05, **P <0.01, ***P < 0.001, NS not significant. B) Western blot analysis was performed on sorted B cells, pDCs and cDCs to assess the amounts of MCL-1, BCL-2, A1 and β-actin (loading control) proteins. C) Increased BCL-2 expression can compensate for MCL-1 deficiency *in vitro*. wt, *Mcl-1*$^{+/-}$, *Bcl-2* tg and *Mcl-1*$^{+/-}$*;BCL-2* tg spleen cells were cultured with or without treatment of 1 μM MCL-1 inhibitor. After 16 h, cells were recovered and survival of B cells, pDC and cDC determined by PI staining and flow cytometry. Data are shown as the proportion (%) of the average number of live cells isolated from untreated cultures for each strain (% survival) (top panel) or cells recovered/well (bottom panel) as Mean ± SEM. Data shown is from a single representative experiment of 3 experiments of n=3.

FIGURE 3

Haplo-insufficiency of *Mcl-1* unmasks sensitivity of immune cells to antagonism of BCL-2. A) Reduced amounts of MCL-1 sensitize immune cells to death by Bcl-2 antagonism *in vitro*. Spleen cells from 8 wk old B6 wt (solid line) or *Mcl-1*$^{+/-}$ littermates (dashed line) were cultured with the BCL-2 inhibitor ABT-199 at the indicated doses. After 16 h, viable B cells, CD4^{+} T cells, CD8^{+} T cells, pDCs and cDCs were enumerated by PI staining and flow cytometry. Data are shown as the proportion (%) of the average number of live cells isolated from untreated cultures for each strain (% survival) (top panel) or cells recovered/well (bottom panel) as Mean ± SEM. Data shown is from a single representative experiment of 3 experiments of n=3. B) Reduced amounts of MCL-1 sensitize cells to death induced by BCL-2 inhibition *in vivo.* 8wk old B6 (wt) or *Mcl-1*$^{+/-}$ mice were gavaged with a single 100 mg/kg body weight dose of ABT-199 or vehicle, as a control. After 24 h spleens were recovered

and viable B cells, CD4+ T cells, CD8+ T cells, pDC, cDC, NK cells (C), and Treg cells (D) were enumerated by PI staining and flow cytometry. Data show a single representative experiment of 3 experiments; n=3-4 animals; Mean± SEM. All data analyzed using a 2-tailed Student's t test. *P < 0.05, **P <0.01, ***P < 0.001, ns = not significant.

FIGURE 4

Cells can be rendered resistant to BCL-2 antagonism by increased levels of MCL-1. A) Increased amounts of MCL-1 protect immune cells from death induced by BCL-2 inhibition (ABT-199 treatment) *in vitro*. Spleen cells from 12-14 wk old B6 (wt) (dashed line) or *Mcl-1* tg littermates (solid line) were cultured with the BCL-2 inhibitor ABT-199 at the indicated doses. After 16 h, viable B cells, $CD4^+$ T cells, $CD8^+$ T cells, pDC and cDC were enumerated by PI staining and flow cytometry. Data are shown as the proportion (%) of the average number of live cells isolated from untreated cultures for each strain (% survival) (top panel) or cells recovered/well (bottom panel) as Mean ± SEM. Data shown is from a single representative experiment of 3 experiments of n=3. B) Increased amounts of MCL-1 protect cells from death induced by the BCL-2 inhibitor ABT-199 *in vivo.* 12-14 wk old B6 (wt) or *Mcl-1* tg littermate mice were gavaged with 5 daily doses of 100 mg/kg body weight ABT-199 or vehicle, as a control. After treatment spleens were recovered and viable B cells, CD4+ T cells, CD8+ T cells, pDC and cDC remaining in the organ were assessed by PI staining and flow cytometry. Data show a single representative experiment of 3 experiments;

n=3-4 animals ± SEM. All data were analyzed using a 2-tailed Student's t test. *P < 0.05, **P <0.01, ***P < 0.001.

FIGURE 5

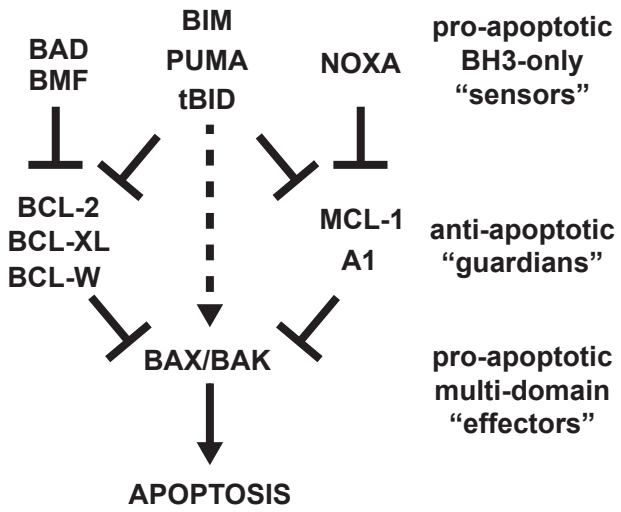
Loss of A1 sensitizes immune cells to BCL-2 antagonism A) Loss of A1 sensitizes cDCs to death induced by the BCL-2 inhibitor ABT-199 both *in vitro* and *in vivo*. Spleens were isolated from 9 wk old B6 and A1$^{-/-}$ littermates. A) Western blot analysis was performed on sorted cDCs to assess the amounts of MCL-1, BCL-2, A1 and β-actin (loading control) proteins confirming loss of A1 protein expression in knockout cells. B) Spleen cells from B6 (wt) (solid line) or *A1*$^{-/-}$ littermates (dashed line) were cultured with the BCL-2 inhibitor ABT-199 at the indicated dose. After 16 h, viable cDCs were enumerated by PI staining and flow cytometry. Data are shown as the proportion (%) of the average number of live cells isolated from untreated cultures for each strain (% survival) (left panel) or cells recovered/well (right panel) as Mean ± SEM. Data shown is from a single representative experiment of 3 experiments of n=3. C) 10 wk old B6 (wt) or *A1*$^{-/-}$ mice were gavaged with 5 daily doses of 50 mg/kg body weight ABT-199 or vehicle, as a control. Following treatment, spleens were recovered and cDCs were enumerated by PI staining and flow cytometry. Representative flow cytometry plots show percentage of cDC per spleen preparation. (D) Loss of A1 sensitizes T and B lymphocytes to death induced by the BCL-2 inhibitor ABT-199. 10 wk old B6 (wt) or A1-/- mice were gavaged with 5 daily doses of 50mg/kg body weight ABT-199 or vehicle, as a control. Following treatment spleens were recovered and total cellularity, B cells and T cells was enumerated by PI staining and flow cytometry. *In vitro* data show a single representative experiment of 3 experiments of n=3. *In vivo* data shows a single representative experiment of 3

experiments; n=3 animals; Mean± SEM. All data were analyzed using a 2-tailed Student's t test. *P < 0.05, **P <0.01, ***P < 0.001.

FIGURE 6

Loss of A1 sensitizes immune cells to reduced levels of MCL-1 A) Loss of A1 sensitizes immune cells to death induced by reduced levels of MCL-1. Spleen cells from 8 wk old B6 (wt) (solid line) or *A1*$^{-/-}$ littermates (dashed line) were cultured with MCL-1 inhibitor at the indicated doses. After 16 h, viable cells were enumerated by PI staining and flow cytometry. Data show a single representative experiment of 3 experiments of n=3 B) Spleen cells from 12-14 wk old B6 (wt), *A1*$^{-/-}$, *Mcl-1*$^{+/-}$ and *A1*$^{-/-}$*;Mcl-1*$^{+/-}$ mice were cultured with the BCL-2 inhibitor ABT-199 at the indicated concentrations. After 16 h, live cells were enumerated by PI staining and flow cytometry. Data show a single representative experiment of 2 experiments of n=3. All data are shown as the proportion (%) of the average number of live cells isolated from untreated cultures for each strain (% survival) (top panel) or cells recovered/well (bottom panel) as Mean ± SEM. P values indicated show a comparison of *A1*$^{-/-}$ and wt (A) and A1$^{-/-}$ and A1$^{-/-}$;Mcl-1$^{+/-}$ (B) Data were analyzed using a 2-tailed Student's t test.. *P < 0.05, **P <0.01, ***P < 0.001.

A



B

Immune cell	Critical molecule	Ref.
Naïve B cell	BCL-2	24,25,26
Naïve T cell		24,25,26
pDC		27
B cell	MCL-1	34,39
GC B cell		14
Plasma cell		28
Naïve T		34
Memory T		34,35
T reg		30
NK		29
pDC		27
Neutrophil		31
HSC		33
cDC	A1	27,37
Erythroid progenitors	BCL-XL	32
Platelets		22,23

FIGURE 1

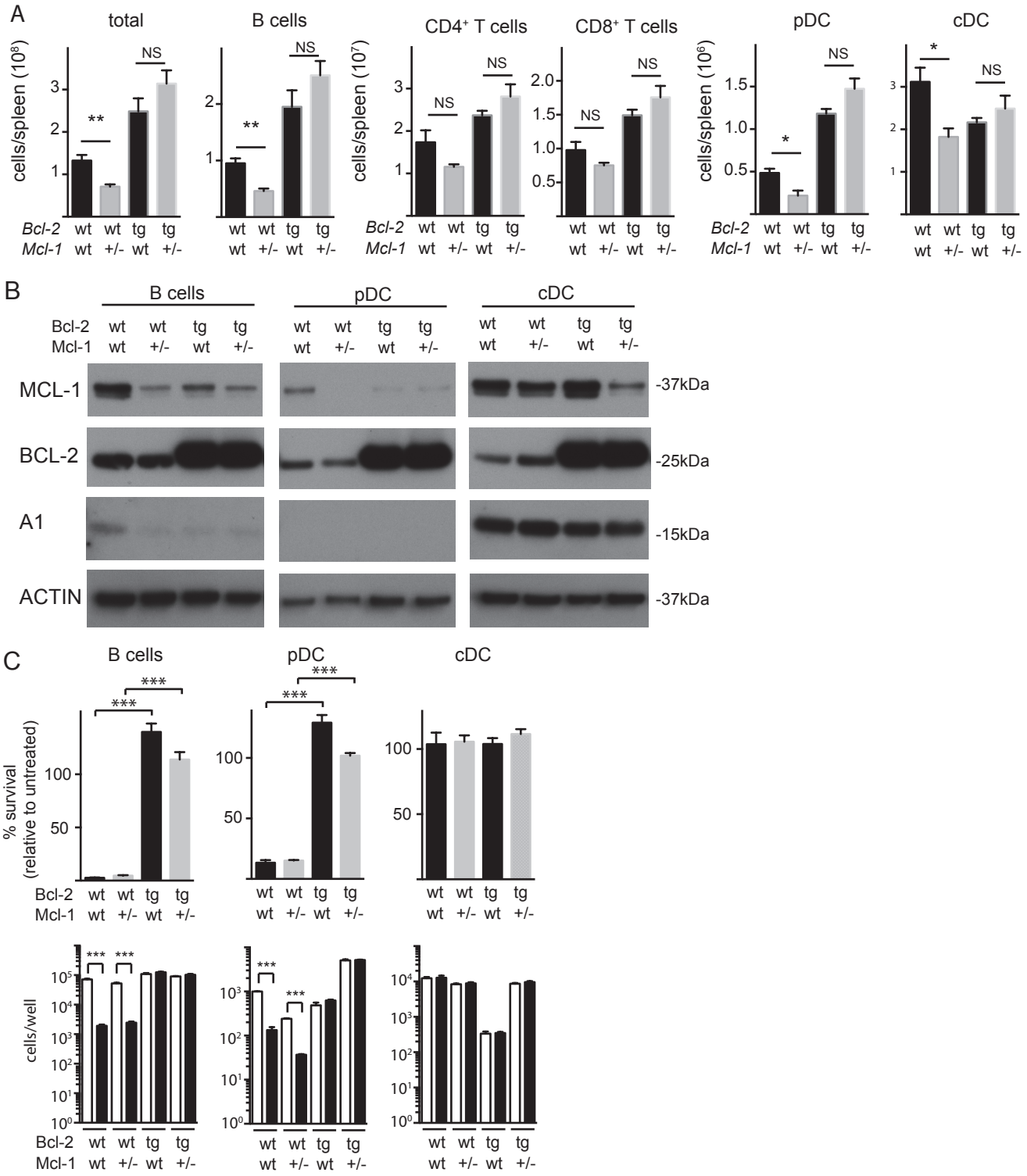

A
total
B cells
CD4+ T cells
CD8+ T cells
pDC
cDC
cells/spleen (10^8)
cells/spleen (10^7)
cells/spleen (10^6)
NS
**
*
Bcl-2 wt wt tg tg
Mcl-1 wt +/- wt +/-
B
B cells
pDC
cDC
MCL-1
BCL-2
A1
ACTIN
-37kDa
-25kDa
-15kDa
C
B cells
pDC
cDC
% survival (relative to untreated)
***
cells/well

FIGURE 2

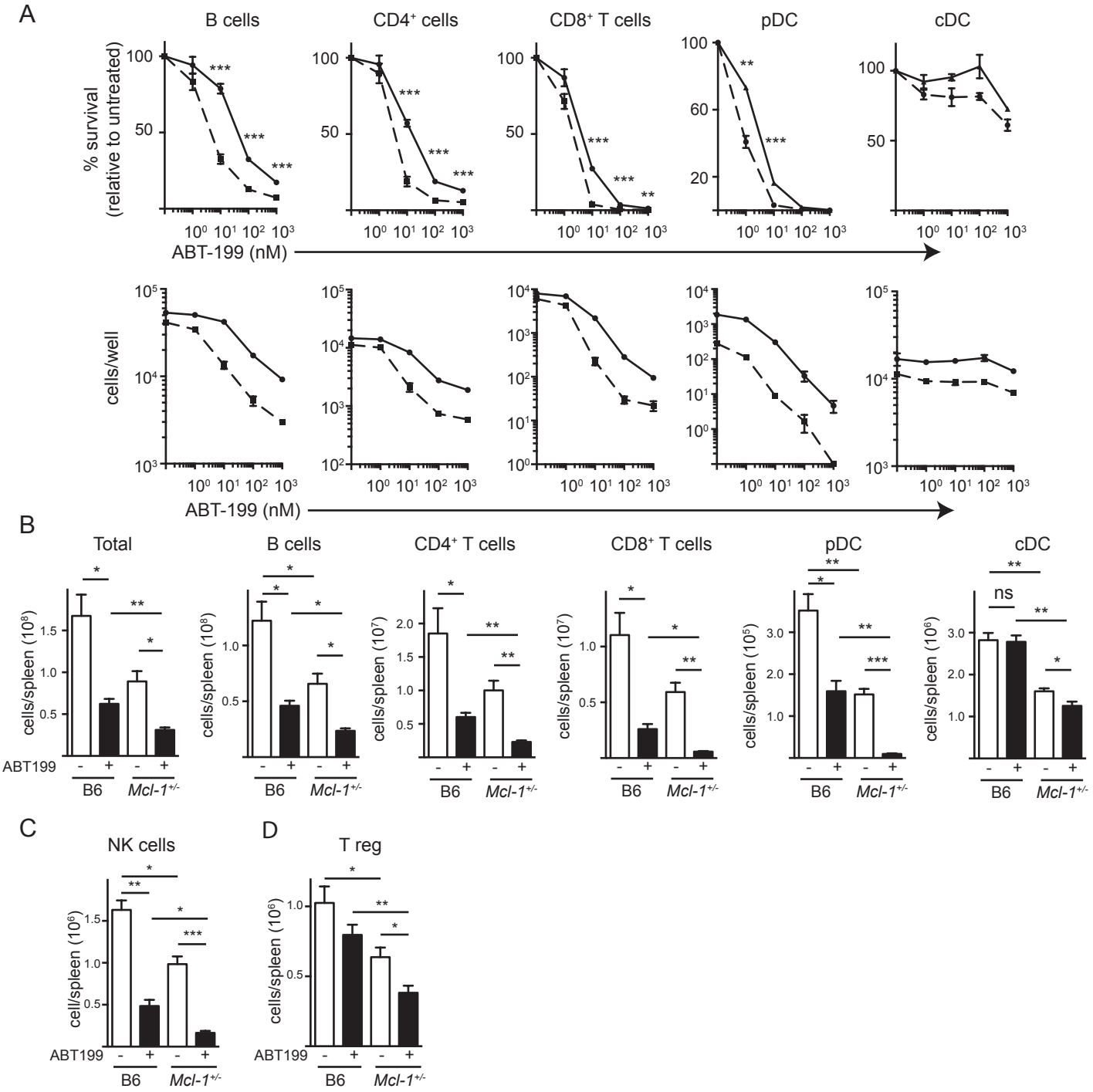
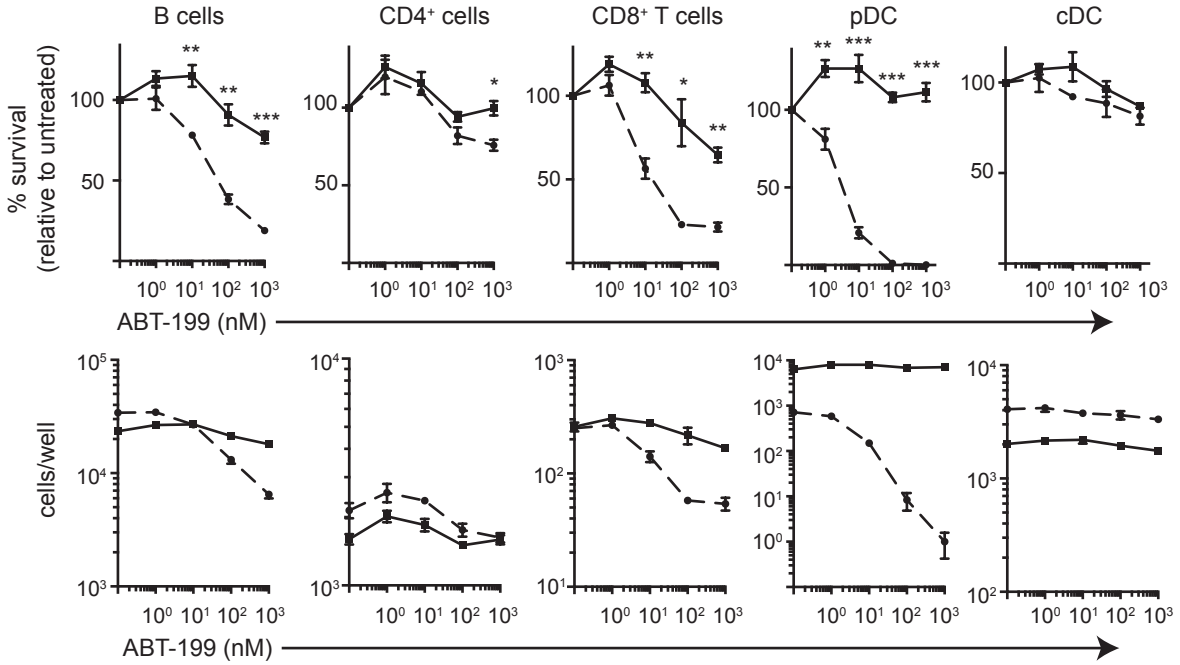

A
B cells
CD4+ cells
CD8+ T cells
pDC
cDC
% survival (relative to untreated)
ABT-199 (nM)
cells/well
B
Total
B cells
CD4+ T cells
CD8+ T cells
pDC
cDC
cells/spleen (10^8)
cells/spleen (10^7)
cells/spleen (10^5)
cells/spleen (10^6)
ABT199
B6
Mcl-1+/-
ns
C
NK cells
cell/spleen (10^6)
D
T reg

FIGURE 3

A


B cells
CD4+ cells
CD8+ T cells
pDC
cDC
% survival
(relative to untreated)
ABT-199 (nM)
cells/well
ABT-199 (nM)

B

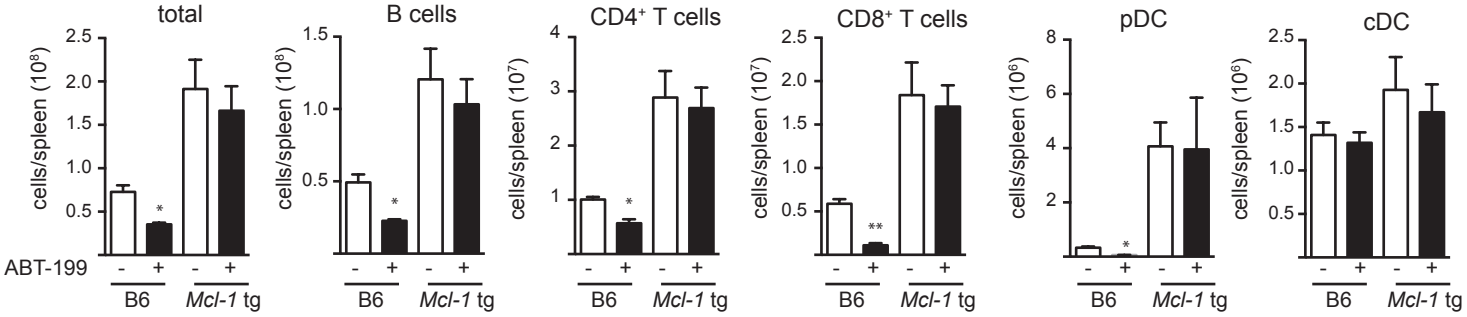

total
B cells
CD4+ T cells
CD8+ T cells
pDC
cDC
cells/spleen (10^8)
cells/spleen (10^8)
cells/spleen (10^7)
cells/spleen (10^7)
cells/spleen (10^6)
cells/spleen (10^6)
ABT-199
B6
Mcl-1 tg

FIGURE 4

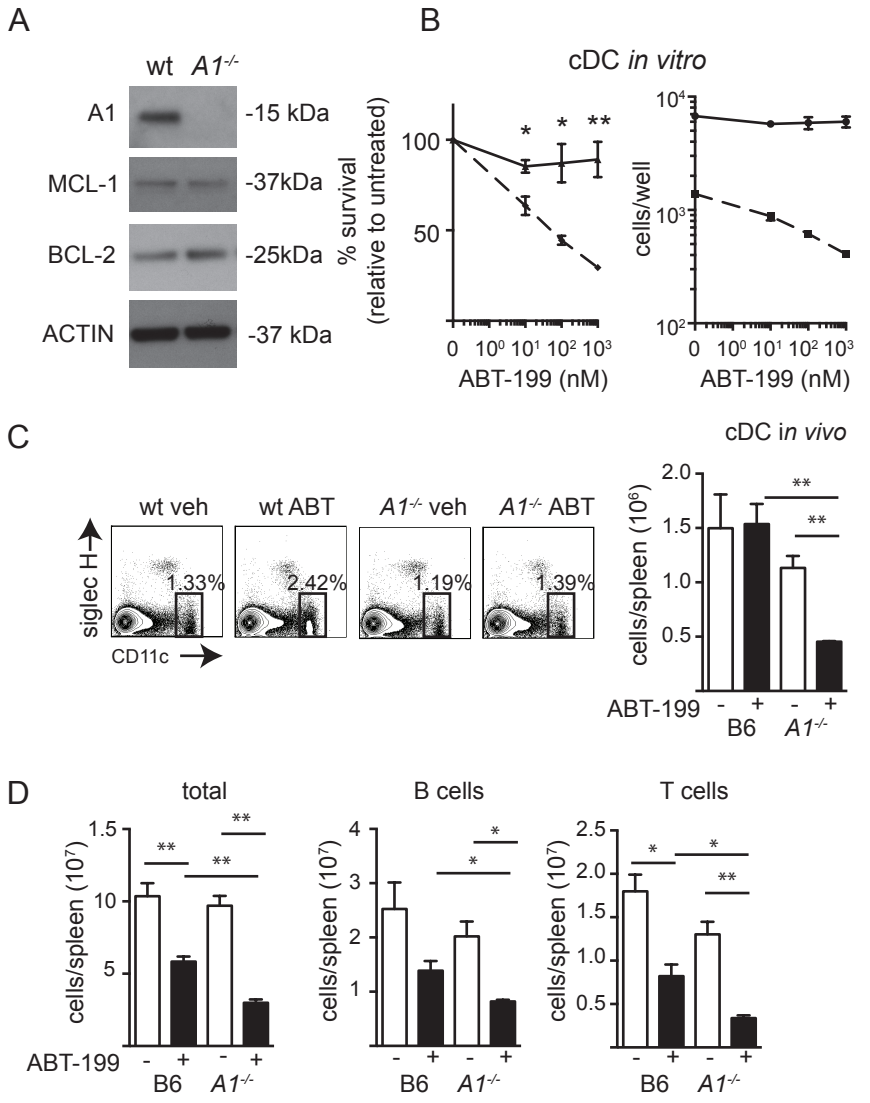

A
wt
A1-/-
A1
-15 kDa
MCL-1
-37kDa
BCL-2
-25kDa
ACTIN
-37 kDa
B
cDC in vitro
% survival (relative to untreated)
100
50
0
10^0 10^1 10^2 10^3
ABT-199 (nM)
cells/well
10^4
10^3
10^2
ABT-199 (nM)
C
cDC in vivo
wt veh
wt ABT
A1-/- veh
A1-/- ABT
siglec H
CD11c
1.33%
2.42%
1.19%
1.39%
cells/spleen (10^6)
2.0
1.5
1.0
0.5
ABT-199
B6
A1-/-
D
total
B cells
T cells
cells/spleen (10^7)
ABT-199
B6
A1-/-

FIGURE 5

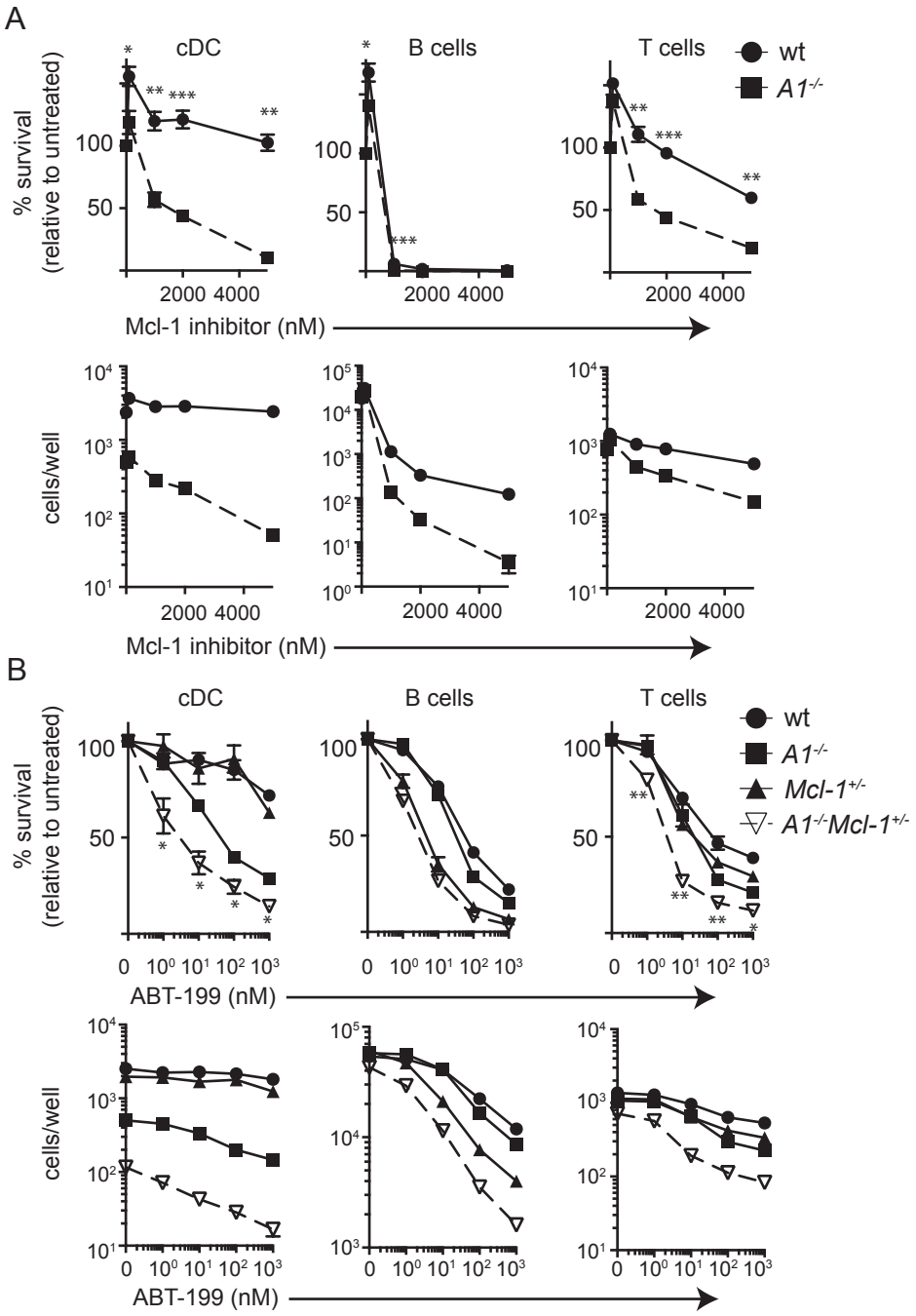

A
cDC
B cells
T cells
wt
A1-/-
% survival
(relative to untreated)
100
50
2000 4000
Mcl-1 inhibitor (nM)
cells/well
B
Mcl-1+/-
A1-/-Mcl-1+/-
ABT-199 (nM)

FIGURE 6