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Loss of PUMA (BBC3) does not prevent thrombocytopenia caused by the loss of BCL-XL (BCL2L1)

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Summary

Apoptosis is required to maintain tissue homeostasis in multicellular organisms. Platelets, the anucleate cells that are essential for blood clotting, are a prime example. Their brief lifespan in the circulation is regulated by the intrinsic apoptosis pathway. Pro-survival BCL-XL (also termed BCL2L1) is essential for platelet viability. It functions to restrain the pro-apoptotic BCL-2 family members BAK (also termed BAK1) and BAX, the essential mediators of intrinsic apoptosis. Genetic deletion or pharmacological inhibition of BCL-XL results in thrombocytopenia. Conversely, deletion of BAK in platelets doubles their circulating life span. However, what triggers platelet apoptosis *in vivo* remains unclear. The pro-apoptotic BH3-only proteins are essential for initiating apoptosis in nucleated cells, and there is some evidence to suggest they also play a role in platelet biology. We investigated whether PUMA (also termed BBC3), a potent BH3-only protein that can inhibit all pro-survival BCL-2 family members as well as directly activate BAX, regulates the death of platelets. Surprisingly, loss of PUMA had no impact on the loss of platelets caused by loss of BCL-XL. It therefore remains to be established whether other BH3-only proteins play a critical role in induction of apoptosis in platelets or whether their death is controlled solely by the interactions between BCL-XL with BAK and BAX.

Keywords: Apoptosis, Platelets, Thrombocytopenia, Therapy, Cell biology

Introduction

Apoptosis is a cellular process critical for tissue homeostasis in multicellular organisms (Hotchkiss*, et al* 2009). In the haematopoietic system aberrant apoptosis activation can compromise blood cell production and function (Delbridge*, et al* 2015, Opferman*, et al*

2005). Conversely, impaired apoptosis can result in the accumulation of functionally expended, damaged or dangerous cells (Bouillet*, et al* 1999, Erlacher*, et al* 2006) and this can result in cancer (Strasser*, et al* 1990, Vaux*, et al* 1988) and autoimmunity (Bouillet*, et al* 2002, Mason*, et al* 2013, Strasser*, et al* 1991).

Apoptosis can be initiated either through the extrinsic pathway, via death receptor ligation, or through the intrinsic pathway, which is regulated by the BCL-2 family of proteins (Adams and Cory 2007, Strasser*, et al* 2000). The BCL-2 proteins consist of 2 major groups of proteins: the pro-survival proteins BCL-2, BCL-XL (also termed BCL2L1), BCL-W (also termed BCL2L2), MCL-1 and A1 (also termed BCL2A1) and the pro-apoptotic proteins, which are further subdivided into the BH3-only proteins, such as PUMA (also termed BBC3) and BIM (also termed BCL2L11), and the multi-BH domain BAX/BAK-like proteins (Czabotar*, et al* 2014, Delbridge and Strasser 2015). In healthy cells, the pro-survival proteins restrain the pro-apoptotic activity of the BAX/BAK-like proteins. Upon stress stimuli, the pro-apoptotic BH3-only proteins are activated transcriptionally or post-transcriptionally (Puthalakath and Strasser 2002, Youle and Strasser 2008). The BH3-only proteins then activate the pro-apoptotic effectors, BAX and BAK, either directly or indirectly by displacing them from their restraint by the pro-survival BCL-2-like proteins. Of particular importance are the BH3-only proteins termed 'activators', such as BID, BIM and PUMA, which not only bind all the pro-survival proteins with high affinity but also have the ability to directly activate BAX and BAK, and hence are extremely potent inducers of apoptosis (Chen*, et al* 2005, Dai*, et al* 2014, Delbridge and Strasser 2015, Desagher*, et al* 1999, Gavathiotis*, et al* 2010, Gavathiotis*, et al* 2008, Kuwana*, et al* 2005, Moldoveanu*, et al* 2014, Robin*, et al* 2015). Different BH3-only proteins are required for initiation of apoptosis by distinct cytotoxic stimuli and they also function in a cell type specific manner (Puthalakath and Strasser 2002, Youle and Strasser 2008). Following activation, BAX and BAK disrupt the mitochondrial outer membrane (MOMP) with consequent cytosolic release of apoptogenic molecules, such as Cytochrome c and Smac/DIABLO (Chipuk and Green 2008). This promotes assembly of the apoptosome and thereby causes activation of caspase-9 and consequently the downstream effector caspases 3, 6 and 7, which facilitate cellular degradation and engulfment (Chipuk and Green 2008).

Previous research has shown that BCL-XL plays a central role in the development and survival of the megakaryocyte lineage (Josefsson*, et al* 2011, Mason*, et al* 2007, Wagner*,*

et al 2000). Deletion of BCL-XL in mice results in severe thrombocytopenia due to truncation of platelet life span, and impaired platelet shedding by dying megakaryocytes. Both cells depend on BCL-XL for their survival. Accordingly, pharmacological inhibition of BCL-XL in mice by treatment with the BH3 mimetic ABT-263 causes thrombocytopenia (Mason*, et al* 2007, Oltersdorf*, et al* 2005, Roberts*, et al* 2012, Zhang*, et al* 2007). Most importantly, the dose limiting toxicity of the investigational drug ABT-263 (Navitoclax), an inhibitor of BCL-XL, BCL-2 and BCL-W, is thrombocytopenia (Roberts*, et al* 2009). In contrast, treatment of mice or humans with ABT-199, which specifically blocks BCL-2, has no impact on platelet counts (Roberts*, et al* 2016, Souers*, et al* 2013), as BCL-2 is dispensable for platelet and megakaryocyte survival (Debrincat*, et al* 2015).

The apoptotic death of platelets and consequent thrombocytopenia that is triggered by genetic deletion or pharmacological blockade of BCL-XL can be completely rescued by combined deletion of BAX and BAK (Josefsson*, et al* 2011, Kodama*, et al* 2011). Thus, it is clear that platelet survival and life span is governed by the interplay between pro-survival BCL-XL, and pro-death BAK and BAX. The mechanism by which platelets enter into apoptosis, however, remains unclear. Mason *et al* (2007) posited the existence of a "molecular clock", with degradation of BCL-XL representing the "sand through the hourglass". However, they later suggested this model was unlikely to explain the tight regulation of platelet life span, given that BCL-XL levels do not appear to decline as platelets age *in vivo*. This suggests that platelet apoptosis must be actively triggered, either by the loss of some pro-survival signal beyond BCL-XL, or alternatively, the accumulation of a pro-death protein. Regarding the latter, BH3-only proteins are the obvious candidate. Loss of BAD results in a modest but significant extension of platelet life span (Kelly*, et al* 2010). However, there are 8 BH3-only proteins, and whether they are truly central to the initiation of platelet apoptosis *in vivo* remains to be established. It has been reported that BIM and BID are dispensable for apoptosis of platelets triggered by loss of BCL-XL (Kodama*, et al* 2011). Thus, we hypothesized that loss of PUMA, the remaining prominent activator BH3-only protein might be the critical regulator. Surprisingly, we found that deletion of PUMA did not prevent BCL-XL-deficient platelets from undergoing apoptotic cell death, and mice lacking both PUMA and BCL-XL developed thrombocytopenia similar to that observed in mice deficient for BCL-XL alone.

Methods and Materials

Mice

All experiments with mice were conducted according to the guidelines of The Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. Mouse strains utilized in this study have been previously published: *Bclx*^{fl} (also known as *Bcl2l1*^{fl}) (Wagner*, et al* 2000), *Bim*$^{-/-}$ (also known as *Bcl2l11*$^{-/-}$) (Bouillet*, et al* 1999), *Puma*$^{-/-}$ (also known as *Bbc3*$^{-/-}$) (Villunger*, et al* 2003), hUbiquitinC-GFP transgenic (Schaefer*, et al* 2001) and *RosaCreERT2* mice (Seibler*, et al* 2003). All mice were maintained on a C57BL/6 background (either generated on this background or backcrossed for >20 generations before commencement of our studies).

Genotyping

Genotyping was performed as previously reported (Delbridge*, et al* 2015). Oligonucleotide sequences for genotyping of these alleles will be provided on request.

Generation of bone marrow chimeras

Experiments were performed as previously described by Delbridge *et al* (2015).

Flow cytometric analysis

Spleen and bone marrow cells were harvested from reconstituted mice and single-cell suspensions prepared. Cells were counted using the CasyCell Counter (Schaefer System GmbH, Germany; discontinued). Retro-orbital bleeds were taken for a haemogram (ADVIA; Siemens Healthcare, East Walpole, MA). The distribution of platelet contribution from either wild-type or test bone marrow from peripheral blood of reconstituted mice was determined by flow cytometric (fluorescence-activated cell sorter [FACS]) analysis after staining for the platelet specific surface marker CD41 (MWReg30; Abcam, Cambridge, MA). GFP^{+} (wild-type bone marrow) and GFP^{-} (test bone marrow) cells were used to determine the chimerism of the bone marrow–reconstituted recipient mice. Samples were analysed in a LSR-II flow cytometer (BD Biosciences; Franklin Lakes, NJ). Dead cells were excluded using light forward and side scatter as well as propidium iodide (PI) staining.

RNA sequencing and analysis

Platelets were purified as previously described (Josefsson*, et al* 2011) from individual wild-type BALB/c males. The purity of each platelet suspension was assessed by flow cytometry and suspensions for which more than 98% of total events were CD41+ platelets were pooled together. RNA extraction and purification was then performed with a Norgen RNA purification kit (Norgen Biotek; Thorold, Canada) and RNA integrity was evaluated on an Agilent Bioanalyser (Agilent Technologies; Lexington, MA). Poly(A) tailed RNA from 3 independent platelet suspensions were used for 100 bp paired-end sequencing on an Illumina HiSeq (Illumina; San Diego, CA) by the Australian Genome Research Facility.

Paired-end sequencing reads were aligned to the mouse reference genome (*mm10*; http://hgdownload.soe.ucsc.edu/goldenPath/mm10/chromosomes/) using the subread algorithm (Liao*, et al* 2013) with default parameters and gene-level counts were obtained using the featureCounts function (Liao*, et al* 2014) using the in-built mm10 annotation from the *Rsubread* package (version 1.18.0; https://bioconductor.org/packages/3.1/bioc/html/Rsubread.html). Reads per kb per million (RPKM) were calculated for each gene using the *edgeR* software (version 3.11.5, (Robinson*, et al* 2010). These data are available under GEO series accession number GSE75896 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75896).

Statistical analysis

Blood parameters and mRNA levels were plotted and analysed with GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA); error bars are presented as standard error of mean (± SEM).

Results

The cell death mechanisms that govern platelet survival and lifespan are of critical importance for the management of thrombocytopenia as a therapeutic side effect, as well as, for the storage and clinical deployment of platelets. Therefore we decided to inducibly delete BCL-XL in the haematopoietic system to further investigate how platelet apoptosis is regulated. To that end, we generated bone marrow chimeras, using as recipients lethally-irradiated mice that carried a GFP-transgene under the control of the human

Ubiquitin C promoter (Schaefer, *et al* 2001). This mouse constitutively expresses GFP in all cells, importantly including platelets. Post γ-irradiation, these animals were transplanted with unfractionated bone marrow cells from either *Bclx*$^{+/+}$;*RosaCreERT2*$^{Ki/+}$ or *Bclx*$^{fl/fl}$;*RosaCreERT2*$^{Ki/+}$ mice (Fig. 1A). 8 weeks later, we analysed peripheral blood cells, determining GFP$^-$ donor derived cell contribution relative to GFP$^+$ host-derived cells. All mice utilized in subsequent experiments exhibited >97% donor engraftment (Fig. 1B).

Reconstituted mice were treated with tamoxifen (3.6 mg/day) for 3 consecutive days, thereby activating Cre recombinase (CreERT2 inducible by tamoxifen administration) to excise the LoxP flanked *Bclx* (*Bcl2l1*) gene. Consistent with previous studies (Kodama, *et al* 2011, Mason, *et al* 2007, Wagner, *et al* 2000), deletion of BCL-XL resulted in severe thrombocytopenia within 30 days of treatment in all *Bclx*$^{fl/fl}$;*RosaCreERT2*$^{Ki/+}$ animals. *Bclx*$^{+/+}$;*RosaCreERT2*$^{Ki/+}$ animals exhibited no change in platelet counts (Fig. 1C), serving as a control for toxicity induced by tamoxifen or the activation of the CreERT2 recombinase.

We conducted a survey of the mRNA expression levels of all BCL-2 family members in resting platelets isolated from wild-type mice. Polyadenylated mRNA was isolated from platelets and subjected to RNA sequencing. Consistent with the critical role of BCL-XL in platelet survival, *Bclx* mRNA transcripts were detected at very high levels relative to the other BCL-2 family members (Fig. 2). Among the pro-apoptotic BCL-2 family members, *Bax* and *Bak* (*Bak1*) mRNA transcripts were expressed highly, as were *Bid, Bad*, *Puma* and *Bim*. Interestingly, the expression levels of *Puma*, exceeded the levels of *Bad* and *Bim*, with levels comparable to *Bak* and *Bax,* both of which are critical regulators of platelet survival (Fig. 2).

The BH3-only proteins BIM, BID and PUMA are the most potent antagonists of the pro-survival BCL-2-like proteins. They can also directly activate BAX and/or BAK (Chen, *et al* 2005, Dai, *et al* 2014, Delbridge and Strasser 2015, Desagher, *et al* 1999, Gavathiotis, *et al* 2010, Gavathiotis, *et al* 2008, Kuwana, *et al* 2005, Moldoveanu, *et al* 2014, Robin, *et al* 2015). Whether direct activation of BAX/BAK is essential for apoptosis induction remains the subject of controversy. Given that concomitant loss of BIM or BID is not able to prevent the thrombocytopenia caused by loss of BCL-XL, and our observation that PUMA is highly

expressed in platelets (Fig. 2), we investigated the role of PUMA in platelet survival using our reconstitution model. *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$ mice were mated with *Puma*$^{-/-}$ mice to produce *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Puma*$^{-/-}$ offspring. *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Bim*$^{-/-}$ mice were generated as controls.

Bone marrow cells harvested from these mice were used to reconstitute lethally-irradiated GFP$^+$ recipient animals as described in Figure 1A. At 8 weeks post-transplant, peripheral blood cells were enumerated. Consistent with the role of BIM in the regulation of blood leucocyte numbers (Bouillet*, et al* 1999), the white blood cell numbers were increased in recipients of *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Bim*$^{-/-}$ bone marrow (due to the lack of BIM-dependent apoptosis). Leucocyte numbers in the *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Puma*$^{-/-}$ reconstituted mice were comparable to those observed in control animals (Fig. 3A). This is consistent with the finding that in the absence of stress PUMA-deficient mice possess normal numbers of these cells (Erlacher*, et al* 2006, Villunger*, et al* 2003). Platelet numbers were comparable across all genotypes (Fig. 3B).

Reconstituted mice were treated with tamoxifen to induce deletion of *Bclx*. As previously reported (Kodama*, et al* 2011), loss of BIM failed to mitigate the deleterious impact of acute loss of BCL-XL on platelet numbers, with all *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Bim*$^{-/-}$ mice developing thrombocytopenia within 30 days of administration of tamoxifen (Fig. 3C). To our surprise, loss of PUMA did not rescue the thrombocytopenia induced by loss of BCL-XL, with tamoxifen-treated *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Puma*$^{-/-}$ reconstituted mice exhibiting platelet numbers comparable to the *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$ controls (Fig. 3C).

To uncover any subtle defects in platelet recovery and obviate the non-thrombopoietic effects of acute loss of BCL-XL, we generated mixed bone marrow chimeras by transplanting lethally-irradiated GFP$^+$ recipients with a 1-to-1 mixture of wild-type (GFP$^+$) plus *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Puma*$^{-/-}$ or *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$ bone marrow cells. Mixed chimeric mice reconstituted with *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Bim*$^{-/-}$ or *RosaCreERT2*$^{Ki/+}$ cells (mixed 1-to-1 with wild-type (GFP$^+$) bone marrow cells) served as controls (Fig. 4). We determined the relative contribution of the wild-type and mutant cells for each reconstituted mouse in the peripheral blood at 8 weeks after reconstitution and then treated all reconstituted mice with tamoxifen as described above. Mice were analysed at 30 days post-tamoxifen treatment to determine the impact of BCL-XL loss on peripheral blood cell

counts. We found that, regardless of whether PUMA or BIM were present or absent, acute loss of BCL-XL precipitated a severe depletion of the BCL-XL-deficient platelets. In *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Puma*$^{-/-}$ mice, platelets were almost wholly derived from the wild-type competitor cells following tamoxifen treatment (Fig. 4A and B). In contrast, the ratios of leucocytes exhibited only minor changes, with a modest underrepresentation of cells that had lost BCL-XL. This shift in cell composition was corrected by the additional loss of either BIM or PUMA (Fig. 4C).

Discussion

PUMA is a major initiator of apoptosis in the haematopoietic stem and progenitor cell compartment, where it functions as the key antagonist of the pro-survival protein MCL-1 (Delbridge*, et al* 2015, Opferman*, et al* 2005). Furthermore, *Puma*/*Bbc3* mRNA is readily detectable in platelets, at a level comparable to known regulators of platelet survival (Fig. 2). Despite this, we have demonstrated that PUMA is dispensable for the induction of apoptosis in platelets following acute deletion of BCL-XL (Fig. 3, 4). Whether apoptosis in platelets is initiated simply by unleashing BAK (from BCL-XL inhibition) or whether another apoptotic signal involving BH3-only proteins is required, remains to be established. BID, BIM and PUMA are among a unique subset of the BH3-only family members that are able to directly activate BAX and BAK to induce apoptosis (Dai*, et al* 2014, Desagher*, et al* 1999, Gavathiotis*, et al* 2010, Gavathiotis*, et al* 2008, Robin*, et al* 2015). Previous reports indicate that BID and BIM are not required for the regulation of steady state platelet life span (Kodama*, et al* 2011). Therefore, further insight into these mechanisms will have to await the generation of mice lacking BCL-XL in addition to deficiency in BID, BIM and PUMA. Alternatively, it remains conceivable that the activation of BAK and BAX in platelets may occur by some as yet to be elucidated mechanism.

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Author contributions

Study was conceived by AD and SG with guidance from AS. Experiments were designed and conducted by AD, SG, SC, BK, MR and AS. Manuscript was prepared by AD, SG, SC, BK, MR and AS.

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Figure Legends

Figure 1: Inducible deletion of *Bclx* causes thrombocytopenia in mice

(A) Schematic of reconstitution strategy. (B) Percentage of donor (test)-derived cells in the platelet compartment determined by flow cytometry on peripheral blood harvested at 8 weeks post-reconstitution. (C) Platelet numbers of reconstituted mice prior to and one month after tamoxifen treatment. n = 6-8 reconstituted mice per genotype (each symbol represents one mouse). Mean ± SEM shown. TAM, tamoxifen.

Figure 2: mRNA expression of BCL-2 family members in platelets

Platelets were purified from wild-type mice and subjected to RNAseq analysis to determine expression levels of the BCL-2 family members. Genes with detectable transcript levels are shown: BH3-only subfamily (orange), BCL-2-like pro-survival subfamily (blue), multi-BH domain pro-apoptotic subfamily (green). ND indicates transcript not detected. n = 3 independent platelet suspensions. RPKM (Reads Per kb of transcript per Million mapped reads) shown. Mean +/- SEM are shown.

Figure 3: Loss of BIM or PUMA does not prevent thrombocytopenia caused by acute loss of BCL-XL

White blood cell (A) and platelet (B) counts from blood analysis 8 weeks post-reconstitution prior to tamoxifen administration (preTAM). (C) Platelet counts 1 month following tamoxifen treatment (postTAM). n = 6-8 reconstituted mice per genotype (each symbol represents one mouse). Mean ± SEM are shown. TAM, tamoxifen.

Figure 4: *Bclx*$^{fl/fl}$*;RosaCreERT2*$^{Ki/+}$*;Puma*$^{-/-}$ platelets were almost entirely lost in 1-to-1 mixed chimeric mice following induced deletion of BCL-XL.

Blood analysis 1 month following tamoxifen administration: (A) representative FACS plots demonstrating platelet contribution, test contribution ratio (%pre-TAM / %post-TAM) in

platelets (B) and in white blood cells (C). n = 6-8 mixed chimeric mice per genotype (each symbol represents data from one mouse). Mean ± SEM shown. TAM, tamoxifen.