Anaemia is a major global health problem arising from diverse causes and for which improved therapeutic strategies are needed. Erythroid cells can undergo apoptotic cell death and loss of pro-survival BCL-XL is known to trigger apoptosis during late-stage erythroid development. However, the mechanism by which loss or pharmacological blockade of BCL-XL leads to erythroid cell apoptosis remains unclear. Here we sought to identify the precise stage of erythropoiesis that depends on BCL-XL. We also tested whether deficiency of BIM or PUMA, the two main pro-apoptotic antagonists of BCL-XL, could prevent reticulocyte death and anaemia caused by BCL-XL loss. Using an in vivo mouse model of tamoxifen-inducible Bclx gene deletion and in vitro assays with a BCL-XL-selective inhibitor, we interrogated each stage of erythroid differentiation for BCL-XL dependency. This revealed that reticulocytes, but not orthochromatic erythroblasts, require BCL-XL for their survival. Surprisingly, concurrent loss of BIM or PUMA had no significant impact on the development of anaemia following acute BCL-XL deletion in vivo. However, analysis of mixed bone marrow chimeraic mice revealed that loss of PUMA, but not loss of BIM, partially alleviated impaired erythropoiesis caused by BCL-XL deficiency. Insight into how the network of pro-survival and pro-apoptotic proteins works will assist the development of strategies to mitigate the effects of abnormal cell death during erythropoiesis and prevent anaemia in patients treated with BCL-XL-specific BH3-mimetic drugs.

Anaemia affects over 1.5 billion people worldwide1 and is a major cause of morbidity that requires improved therapeutic interventions. Underlying causes for anaemia are diverse and include haemoglobinopathies, red cell enzyme disorders, nutritional deficiencies, cytoskeletal abnormalities and autoimmune diseases. Furthermore, anaemia is a frequent toxicity associated with cancer therapy, including both conventional chemotherapy as well as emerging targeted therapies, such as some of the BH3-mimetics.2,3 Understanding the requirements for normal erythroid development and survival will help develop improved treatment strategies to support erythropoietic function in patients.

In the adult, mature red blood cells derive from haematopoietic stem cells. Erythropoiesis proceeds through several differentiation steps whereby the potential for alternate blood cell lineages is progressively lost and immature progenitors expand in number to facilitate production of sufficient mature red blood cells, with half-lives spanning 100–120 days in humans and 8–20 days in mice.4–7 Should bone marrow erythropoiesis become compromised – due to bone marrow failure/dysplasia or infiltration, or excessive loss/destruction of peripheral red blood cells – extramedullary erythropoiesis can occur in the spleen to facilitate enhanced erythrocyte production in mice. Robust pro-survival signalling is essential to prevent excessive apoptotic cell death during erythropoiesis and the maintenance of adequate red cell numbers. Expression analysis has identified the pro-survival gene Bclx as particularly highly expressed in the erythroid lineage,8 suggestive of its critical importance for countering apoptosis during erythropoiesis. Aberrant apoptosis of red blood cells and erythroid progenitors is a common feature of chronic anaemias and is likely to contribute to their severity. For example, thalassaemia patients have elevated numbers of early precursor cells in the bone marrow but abnormally high rates of apoptosis in more mature progenitors,9 whereas in anaemia of chronic disease, elevated interferon-γ levels are associated with increased apoptosis of erythroid progenitors and inversely correlated with reticulocyte numbers and haemoglobin levels.10 Aberrant apoptosis is also a key feature of Diamond-Blackfan Anaemia where ribosomal stress drives abnormal TPS3 activation and cell death.11 TPS3 is known to regulate the expression of several key initiators of apoptosis, including Puma, Noxa and Bax.12–15

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loss of BIM, Tissue-restricted deletion of Bclx results in severe anaemia, splenomegaly due to erythroblast accumulation, and thrombocytopenia. Loss or inhibition of BCL-XL (e.g., using the BH3-mimetics ABT-737 or ABT-263 ref. 27,28), but not of BCL-2 (e.g., using ABT-199 ref. 29), results in thrombocytopenia in patients and mouse models and also anaemia in mice. It has yet to be established whether pharmacological inhibition of BCL-XL will also cause anaemia in patients. Published data demonstrate that both BAX and BAK must be removed to prevent anaemia caused by loss or drug mediated inhibition of BCL-XL, as they have largely overlapping roles in the execution of apoptosis. It is, however, still unknown which BH3-only protein is responsible for the initiation of apoptosis in this context.

We defined the requirement for BCL-XL at various stages of adult erythropoiesis by using a discriminating flow cytometry method and a tamoxifen-inducible, acute Bclx gene deletion mouse model. Given that BIM is essential for the aberrant apoptosis of erythroid progenitors in embryonic mice caused by the absence of BCL-XL, we investigated the role of this pro-apoptotic BH3-only protein in adult erythropoiesis. We found that BCL-XL is critical for the survival of reticulocytes and that BIM is not essential for the anaemia that is caused by acute loss of BCL-XL, whereas pro-apoptotic PUMA has a minor role. These discoveries inform the development of strategies to alleviate anaemia caused, for example, by inherited mutations, infections or treatment with anti-cancer agents, including the new BH3-mimetic drugs.

**Results**

**Acute loss of BCL-XL causes profound anaemia in adult mice owing to failure of erythropoiesis.** To confirm and extend published data characterising the role of BCL-XL in erythroid cell survival, we generated mice bearing floxed Bclx alleles that could be deleted in an inducible manner by tamoxifen-dependent CreERT2-recombinase activity. Bclx floxed mice were crossed with Rosa-CreERT2 mice to generate Bclx floxed;RosaCreERT2 compound mutant mice. In these mice, tamoxifen administration activates the latent CreERT2 recombinase to facilitate recombination of the floxed Bclx alleles, leading to loss of BCL-XL expression.

A cohort of Bclx floxed;RosaCreERT2 mice and Bclx control mice were treated with tamoxifen at 8 weeks of age. At 1 month post treatment these adult mice were analysed to determine the effects of BCL-XL loss. Peripheral blood analysis confirmed previous reports with profound anaemia observed in the Bclx floxed;RosaCreERT2 mice. Haemoglobin (P = 0.0001) levels, haematocrit (P < 0.0001) and reticulocyte as well as mature red blood cell numbers were all significantly reduced (Figure 1a). Conversely, both spleen weight and cellularity were increased in the Bclx floxed;RosaCreERT2 mice (Figure 1b). In contrast, the tamoxifen-treated Bclx control mice retained normal blood and spleen cell counts.

A previous study reported that haemolysis was associated with BCL-XL deletion. We therefore examined the impact of inducible adult loss of BCL-XL on red blood cell turnover.
Consistent with abnormal red blood cell destruction, we observed an increase in serum levels of unconjugated bilirubin in the tamoxifen-treated \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) mice (Supplementary Figure 1A). Consistent with normal hepatic function, no increases in the levels of serum albumin, alkaline phosphatase, aspartate aminotransferase and gamma-glutamyl transferase (\(< 4\) U/l; data not shown) were observed.

There was a marginal increase in alanine aminotransferase; however, the levels observed were below those indicative of significant liver damage (Supplementary Figure 1B). Hence, we conclude that liver dysfunction did not account for the accumulation of serum bilirubin. The serum haptoglobin levels were very low (\(< 0.08\) g/l; data not shown), consistent with abnormal red blood cell breakdown and release of haemoglobin. However, the lactate dehydrogenase levels were not increased, which suggests that red blood cell destruction may occur predominantly within the extra-vascular space (bone marrow and spleen) (Supplementary Figure 1B).

Blood films were prepared from tamoxifen-treated \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) and \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) (control) mice to identify changes in red blood cells caused by loss of BCL-XL. Remarkably, almost complete disappearance of reticulocytes was seen by day 2 (Supplementary Figure 2A). This was followed by morphologic changes in the reticulocytes of coarse basophilic stippling (Supplementary Figure 2B). There was no evidence of spherocytosis, aspargate aminotransferase and gamma-glutamyl transferase (\(< 4\) U/l; data not shown) were observed.

Concentrations of liver enzymes as well as liver function tests were performed and determined in tamoxifen-treated \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) and \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) (control) mice. The serum levels of aspartate aminotransferase and gamma-glutamyl transferase (\(< 4\) U/l; data not shown) were observed. There was a marginal increase in alanine aminotransferase; however, the levels observed were below those indicative of significant liver damage (Supplementary Figure 1B). Hence, we conclude that liver dysfunction did not account for the accumulation of serum bilirubin.

**Table 1** Haematopoietic potential determined by colony formation in semi-solid agar

| Bone marrow | Total | Blast | G | GM | M | Eo | Meg |
|-------------|-------|-------|---|----|---|----|-----|
| \( \text{Bcl}^\text{fl/fl} \) (n = 6) | 106 ± 29 | 14 ± 5 | 24 ± 9 | 23 ± 4 | 12 ± 4 | 7 ± 2 | 21 ± 6 |
| \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) (n = 4) | 114 ± 21 | 21 ± 4 | 26 ± 7 | 24 ± 8 | 14 ± 4 | 7 ± 4 | 23 ± 5 |

| Spleen | Total | Blast | G | GM | M | Eo | Meg |
|--------|-------|-------|---|----|---|----|-----|
| \( \text{Bcl}^\text{fl/fl} \) (n = 6) | 9 ± 1 | 1 ± 1 | 1 ± 1 | 1 ± 1 | 1 ± 1 | 0 | 6 ± 1 |
| \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) (n = 4) | 21 ± 12 | 5 ± 3 | 3 ± 3 | 2 ± 2 | 4 ± 2 | 1 ± 1 | 7 ± 3 |

Bone marrow cells were cultured in semi-solid agar in the presence of SCF+IL-3+EPO for 7 days. The numbers and types of colonies were scored from dried, stained cultures. Means ± S.D.s are shown.

had similar proportions and numbers of stem cells (LT-HSC, ST-HSC), multipotent progenitors (MPP) and committed progenitor cells (MEP, CMP, GMP) (Supplementary Figure 3A). As a functional readout of progenitor cells, we performed colony-formation assays with bone marrow and spleen cells in semi-solid agar. No significant differences were observed in the relative proportions of the various myeloid-committed colony-forming cells in the bone marrow and spleen between tamoxifen-treated \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) and \( \text{Bcl}^\text{fl/fl} \) (control) mice (Table 1). Therefore, we conclude that BCL-XL is dispensable for the survival of haematopoietic stem/progenitor cells in adult mice at steady state.

We next wanted to define the precise stage at which red blood cell production is perturbed to identify the cause of the anaemia caused by loss of BCL-XL. One month after deletion of Bclx, red blood cell maturation was analysed by flow cytometry based on expression of TER119, CD44 and cell size (FSC) (ref. 36; Figure 2a). Following Bclx gene deletion we observed significantly decreased percentages of mature red blood cells, but not reticulocytes in the bone marrow with a corresponding increase in the percentages of the more immature precursors (Figure 2b). This phenomenon was mirrored in the spleen of the tamoxifen-treated \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) mice, where normally only few immature erythroid progenitor cells are found. Colony-formation assays revealed a pronounced increase in the numbers of colony-forming units-erythroid (CFU-e) in the spleen of tamoxifen-treated \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) mice compared with \( \text{Bcl}^\text{fl/fl} \) mice, whereas no differences were observed in the bone marrow (Supplementary Figure 3B). These findings are consistent with erythropoietic insufficiency in the tamoxifen-treated \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) mice, whereupon the spleen has been recruited as a site for extramedullary erythropoiesis in a reactive attempt to overcome the low numbers of mature red blood cells in the circulation.

Next, we harvested bone marrow cells from \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) and \( \text{Bcl}^\text{fl/fl} \) mice one month following tamoxifen treatment and tested their ability to reconstitute the haematopoietic system of lethally irradiated recipient mice. The animals reconstituted with \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) mice had similar proportions and numbers of stem cells (LT-HSC, ST-HSC), multipotent progenitors (MPP) and committed progenitor cells (MEP, CMP, GMP) (Supplementary Figure 3A). As a functional readout of progenitor cells, we performed colony-formation assays with bone marrow and spleen cells in semi-solid agar. No significant differences were observed in the relative proportions of the various myeloid-committed colony-forming cells in the bone marrow and spleen between tamoxifen-treated \( \text{Bcl}^\text{fl/fl};\text{RosaCreERT2}^\text{Ko/+} \) and \( \text{Bcl}^\text{fl/fl} \) (control) mice (Table 1). Therefore, we conclude that BCL-XL is dispensable for the survival of haematopoietic stem/progenitor cells in adult mice at steady state.

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analysis gated on donor, non-transfused cells (GFP−) in the spleen and bone marrow revealed elevated proportions of immature erythroid progenitors with a decrease in the proportions of reticulocytes and mature red blood cells in the tamoxifen-treated Bclxfl/fl;RosaCreERT2Ki/+ mice (Supplementary Figure 4). Although no major architectural changes were observed in the bone marrow of these mice (Figures 2c and d), widespread disruption of splenic architecture was observed, consistent with compensatory erythropoiesis in this organ (Figures 2e and f). Mice reconstituted with Bclxfl/fl bone marrow cells exhibited the expected erythroid subset proportions as well as normal splenic and bone marrow architecture.

**BCL-XL is essential for the survival of reticulocytes.**

Analysis of mitochondrial content revealed that, as expected, 95% of reticulocytes contained mitochondria and only mature red blood cells were devoid of mitochondria (Figures 3a and b). Having confirmed that reticulocytes and erythroblasts contain the organelle required for the mitochondrial apoptotic pathway, we examined whether the loss of reticulocytes resulting from the absence of BCL-XL was caused by apoptosis. Wild-type reticulocytes were FACS-sorted and treated in vitro with BH3-mimetic drugs that specifically inhibit BCL-XL (A-1331852) or BCL-2 (ABT-199; used as a control) (Figure 3c). Treatment with A-1331852 resulted in the rapid death of reticulocytes, and this could be substantially reduced during the first 24 h by blocking caspase activation with QVD-OPH. In contrast, treatment with ABT-199 had no effect on reticulocyte viability (Figure 3c). Interestingly, the marked dependency on BCL-XL for survival was specific to reticulocytes, as orthochromatic erythroblasts were far less sensitive to A-1331852. Furthermore, following Bclx gene deletion a significant increase in the exposure of phosphatidyl-serine (detected by Annexin V staining) – an early apoptotic marker – was observed on reticulocytes, but not mature red blood cells, from the bone marrow and spleen (Figure 3d).

BCL-XL was implicated in mitochondria specific-autophagy (mitophagy) owing to the established role of Nip3-like protein-X (NIX) in the clearance of mitochondria from maturing red blood cells and the ability of BCL-XL to interact with the NIX homologue BNIP3. To investigate the involvement of mitophagy in the loss of reticulocytes caused by BCL-XL deletion, cell viability was measured following treatment of erythroid progenitors with the autophagy inhibitor –
bafilomycin – that prevents fusion between autophagosomes and lysosomes.\textsuperscript{41} Bafilomycin had no impact on erythroid precursor survival (Figure 3e), consistent with the notion that autophagy plays no role in the loss of reticulocytes caused by BCL-XL deletion.

Hematopoietic-specific loss of BCL-XL provokes anaemia in adult mice that cannot be alleviated by concomitant loss of BIM or PUMA. We next investigated which pro-apoptotic BH3-only protein might be critical for reticulocyte apoptosis following loss of BCL-XL. BIM and PUMA are prime candidates because they bind to all pro-survival BCL-2 proteins and are critical for apoptosis induction in many haematopoietic cell types, particularly lymphocytes,\textsuperscript{30,42-44} after exposure to diverse cytotoxic insults.\textsuperscript{22,45,46} To investigate the roles of BIM and PUMA, we reconstituted lethally irradiated mice with bone marrow from Bclx\textsuperscript{fl/fl};RosaCreERT2\textsuperscript{Ki/};Bim\textsuperscript{−/−}, Bclx\textsuperscript{fl/fl};RosaCreERT2\textsuperscript{Ki/};Puma\textsuperscript{−/−} or relevant control mice (Figure 4a). Mice expressing GFP in all cells, importantly including erythroid cells,\textsuperscript{47} were used as recipients to distinguish donor-derived haematopoietic cells (GFP\textsuperscript{+}) from residual recipient-derived cells (GFP\textsuperscript{−}) (Supplementary Figure 5).

Efficient haematopoietic reconstitution was confirmed 8 weeks post-transplantation with donor-derived (GFP\textsuperscript{+}) red blood cells present at > 95\% (Figure 4b). Bclx gene deletion was induced with tamoxifen and after 1 month bone marrow, spleen and peripheral blood cells were analysed. As expected,\textsuperscript{26} tamoxifen-treated Bclx\textsuperscript{fl/fl};RosaCreERT2\textsuperscript{Ki/} reconstituted mice developed severe anaemia evident by low red blood cell numbers and splenomegaly, whereas RosaCreERT2\textsuperscript{Ki/} reconstituted mice presented with normal blood counts and spleen cellularity (Figure 4c). Surprisingly, all tamoxifen-treated Bclx\textsuperscript{fl/fl};RosaCreERT2\textsuperscript{Ki/};Bim\textsuperscript{−/−} reconstituted mice presented with splenomegaly and anaemia (Figure 4c). Hence, in contrast to its importance in embryonic erythropoiesis, loss of BIM failed to mitigate the erythropoietic insufficiency caused by BCL-XL deletion in adult mice. Loss of PUMA also provided no protection. Accordingly, no differences in
Caspase-9 activity were observed between reticulocytes from wild-type, Bim−/− or Puma−/− mice following treatment with the BCL-XL-selective inhibitor A-1331852 (Figure 4d). These findings show that on their own BIM and PUMA are not essential for the loss of reticulocytes and anaemia caused by BCL-XL deletion.

PUMA has a minor role in anaemia induced in adult mice by acute BCL-XL loss. To further investigate whether BIM or PUMA may have a minor role in the apoptosis of erythroid cells caused by BCL-XL loss, we adopted a mixed bone marrow reconstitution approach (Figure 4a). GFP+ mice were reconstituted with a 1:1 mix of bone marrow cells comprising wild-type (GFP+) ‘competitor’ cells and ‘test’ cells of the following genotypes: RosaCreERT2<sup>Ki</sup>/+, Bclx<sup>fl/fl</sup>;RosaCreERT2<sup>Ki</sup>/+, Bclx<sup>fl/fl</sup>;Puma<sup>−/−</sup>;CreERT2 and Bclx<sup>fl/fl</sup>;RosaCreERT2<sup>Ki</sup>/+, Bim<sup>−/−</sup> and Bclx<sup>fl/fl</sup>;RosaCreERT2<sup>Ki</sup>/+, Puma<sup>−/−</sup> (all GFP−). As expected, the presence of the wild-type cells prevented the anaemia caused by BCL-XL deletion (Figure 5a). When the ratio of test to competitor derived cells was determined within the red blood cell compartment, relative to the RosaCreERT2<sup>Ki</sup>/+ control cells, the Bclx<sup>fl/fl</sup>;RosaCreERT2<sup>Ki</sup>/+, Puma<sup>−/−</sup> cells were profoundly depleted (Figure 5b). This deficit was mildly alleviated by concomitant loss of PUMA, whereas loss of BIM had no impact (Figure 5b). These results indicate that PUMA has a minor role in the loss of red blood cells resulting from acute BCL-XL loss.

Figure 4  Concomitant loss of BIM or PUMA fails to rescue anaemia caused by acute deletion of Bclx. (a) Lethally irradiated GFP+ recipient mice were reconstituted with ‘test’ bone marrow of the indicated genotypes (GFP−). At 8 weeks after reconstitution a mandible bleed was taken from each mouse and analysed to confirm successful haematopoietic reconstitution and to establish baseline blood cell counts. Mice were then treated with tamoxifen (TAM, red arrows). Peripheral blood and spleens were collected for analysis 1 month after TAM administration. (b) Test red blood cell contribution at 8 weeks, prior to TAM treatment. n = 6–8 (N = 3–4 bone marrow donors); mean ± S.E.M shown. (c) Red blood cell counts and spleen cellularity after TAM treatment. n = 6–14 (N = 3–7 bone marrow donors); mean ± S.E.M. (d) In vitro Caspase-9 activity in FACS-sorted reticulocytes, control versus A-1331852-treated (1 μM). a.u. denotes arbitrary units. Significant differences as shown, determined by unpaired Student’s Test.
numbers, the authors of that study attributed the anaemia to excessive haemolysis. Conversely, a subsequent study using the same model, but a later time point for analysis, reported reduced reticulocyte numbers. The authors of the second study concluded that an erythrocyte production defect was responsible for the anaemia caused by loss of BCL-XL and proposed that mature erythroblasts rather than reticulocytes depend on BCL-XL for survival.

Using genetic and pharmacological approaches coupled with FACS analysis to delineate discrete stages of erythropoiesis differentiation, we found that BCL-XL is essential for reticulocyte survival but largely dispensable for the survival of orthochromatic erythroblasts and earlier progenitors. This is consistent with reports that the highest levels of BCL-XL are observed during terminal erythrocyte differentiation when haemoglobin production is maximal. Apoptosis of reticulocytes following acute Bclx gene deletion – documented by appearance of apoptosis-specific markers – results in a compensatory increase in erythroblasts in the bone marrow and the spleen. Our findings clarify previously reported observations. Our use of the RosaCreER(T2) allele enabled efficient inducible Bclx gene deletion resulting in anaemia within 1 month, compared with the 3–4 months in the previously described models. This allowed primary effects of BCL-XL loss to be studied in the absence of potentially confounding secondary effects or compensatory processes. Our acute Bclx deletion model closely mimics the scenario of therapeutic BCL-XL inhibition by BH3-mimetic drugs and therefore provides insight into their likely impact on patients.

Pro-apoptotic PUMA and BIM were investigated as potential initiators of reticulocyte death caused by loss of BCL-XL. In contrast to the published role for BIM as a critical initiator of erythrocyte precursor death during embryogenesis in Bclx deficient mice, we found that concomitant loss of BIM did not alleviate the anaemia caused by acute BCL-XL loss in the adult. Loss of PUMA also provided no rescue. However, experiments using mixed bone marrow reconstituted mice provided evidence that PUMA plays a minor role in the reticulocyte death. Hence, we conclude that other BH3-only proteins may be critical (either by themselves or together with PUMA and BIM) for the induction of apoptosis following BCL-XL loss in reticulocytes.

Our findings demonstrate that reticulocytes are the key erythroid precursors that require BCL-XL for survival and that acute loss of BCL-XL results in anaemia owing to increased apoptosis of reticulocytes and thus diminished production of mature red blood cells. In mice, mature red blood cells spontaneously lyse after 8–20 days, unless taken up by macrophages for retrieval of iron. Hence, anaemia will ensue upon loss of BCL-XL-dependent reticulocytes, although in mice this can be delayed by compensatory erythropoiesis in the spleen. This accounts for the relatively slow development of anaemia, the low numbers of reticulocytes as well as the elevated numbers of the more primitive erythroid precursors.

In humans, red blood cells survive in the circulation for up to 120 days, significantly longer than mice. The extended survival of red blood cells in humans may provide a therapeutic window for intervention with BCL-XL inhibitors, unless erythropoiesis is already compromised prior to treatment.
Cell Death and Disease

Materials and Methods

Reagents. The BCL-2 inhibitor ABT-199 (ref. 38) was provided by AbbVie and QVD-OPH was purchased from MP Biomedical (#030PH10903, Santa Ana, CA, USA).38 The BCL-XL inhibitor A-1331852 was prepared according to procedures described in the literature (patent: Wang et al., WO 2013055897).34 Bafilomycin was purchased from Sigma Aldrich (#B1793, St. Louis, MO, USA). Tamsulosin was purchased from Sigma Aldrich.

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 utilised in this study have been previously published (Bckx226 Bmc510;20 Pumaslac−/−,42 UBC-GFP Tg7; and RosaCreERTZ21). All mice were either generated on a C57BL/6 background or crossed onto this background for at least 20 generations before commencement of our studies. Tamsulosin administration was performed by oral gavage, 3.6 μg/day on 3 consecutive days. Blood transfusion was performed with blood isolated from UBC-GFP Tg mice (GFP−), 250 μl blood/recipient.

Genotyping was performed as previously reported.22 Oligonucleotide sequences for genotyping of the mutant alleles will be provided on request.

Generation of bone marrow chimaeric mice. Haematopoietic reconstitutions were performed as previously described.22

Histology and blood film analysis. Sternum and spleen specimens were fixed in 10% formalin. Paraffin tissue sections were prepared at 7.5 μm and stained with haematoxylin and eosin. Images (stereum × 20; spleen × 40) were taken with an Olympus BX43 (Olympus, Shinjuku, Japan) using the acquisition program CellSens Standard (Olympus). Slides for analysis of blood cell morphology were prepared by spreading freshly venulesected blood by hand onto slides, which were allowed to air-dry. Blood films were fixed in methanol and stained using May-Grunwald and Giemsa stains. Blood film images were acquired using a Nikon Eclipse 90i microscope. Images presented were taken using a ×100 oil objective and inset views for reticulocyte features utilised digital zoom.

Serum analysis. Blood from mice was collected 1 month after tamsulosin treatment via cardiac puncture. Bloods were left to coagulate at room temperature for 5 min and centrifuged for 1 min at 13 000 rpm to collect serum. Sera were stored at −20 °C and analysed using Architect c1600 (Abbott Diagnostics, Santa Clara, CA, USA).

Flow cytometric analysis. Spleen and bone marrow cells were harvested and single-cell suspensions prepared. Cells were counted using the EasyCell Counter (Scheall System GmbH, Neunkirchen, Germany). Retroblot-bleeds were collected into EDTA for differential cell counts using an ADVIA 2100 analyser (Siemens Healthcare PTY, Ltd, Bayswater, VIC, Australia). The analysis of red blood cells and leukocytes from either wild-type or test bone marrow in the haematopoietic reconstituted mice was determined by flow cytometric analysis of GFP and staining for haematopoietic subset-specific surface markers (CD44 [IM781], TER119 [TER119], B220 [RA3-6B2] or CD19 [ID3], GR-1 [RB6-8C5], MAC-1 [M1/70], CD45.2 [5.450.15.2], CD3 [K3-1.1]). Antibodies were conjugated to FITC, R-PE or APC. For erythroid precursor analysis, mature cells were gated out prior to CD44 versus FSC analysis using a "DUMP" channel with the following surface markers: B220 or CD19, GR-1, MAC-1, CD45.2, GFP" (wild-type bone marrow derived) and GFP" (test bone marrow derived) cells were used to determine relative contributions in the haematopoietic reconstituted mice. Samples were analysed in a LSR-II flow cytometer (Becton Dickinson, San Jose, CA, USA). Dead cells were excluded using forward and side light scatter and propidium iodide exclusion (Sigma Aldrich).

Flow cytometry with haematoxylin to determine the numbers and type of colonies. Stem and progenitor cell analysis, cells were stained with Alexa Fluor 700 conjugated antibodies against the following surface markers: CD45R (B220), CD19, GR-1, Ly6G, F4/80, TER119, NK1.1, CD2, CD4, and CD8 and CD117 PerCP/Cy5.5 (c-KIT, clone 2B8 BD Biosciences), SCA-1 Alexa Fluor 594, CD135 PE (FL3/FLK2 clone A2F10 Biogend, San Diego, CA, USA), CD34 Alexa Fluor 647 (clone RAM34 BD Biosciences) CD16/32 biotin (FCγ receptor II/III clone 24G2 BD Biosciences) and Streptavidin BV650 (BD Biosciences) and analysed in a Fortessa I flow cytometer (BD Biosciences). Dead cells were excluded using forward and side light scatter and Fluoro-Gold (Fluorochrome LLC, Denver, CO, USA) exclusion. Mature cells were gated out using the Alexa Fluor 700 (Lineage) "DUMP" channel.

Mitochondrial content analysis. Cells were incubated with 500 nM MitoTracker Deep Red for 20 min at 37 °C (Thermo Fisher, #M22426). Cells were then stained for the appropriate lineage markers, TER119 and CD44 to delineate specific erythroid cell populations by flow cytometry.

Caspase-9 activity assay. 5 × 10⁵ bone marrow cells were cultured for 4 or 24 h with or without the BCL-XL inhibitor A-1331852 in a 96-well plate in 100 μl of medium. Plates were allowed to equilibrate at room temperature prior to adding 100 μl Caspase-9 Glo reagent (Promega, Madison, WI, USA). Assays were analysed after 30 min incubation according to the manufacturer’s instructions.

Clonogenic assays. CFU-e were enumerated by culturing single-cell suspensions of bone marrow (25 000) or spleen (50 000) in 1 ml of MethoCult 3234 medium (Ster Cell Technologies, Vancouver, BC, Canada) supplemented with 2 U/ml erythropoietin (EPO, Janssen-Cilag Ltd, Buckinghamshire, UK) with incubation in 5% CO2 in air for 2-3 days. The numbers of myeloid colony-forming cells in single-cell suspensions of bone marrow (25 000) or spleen (50 000) were assessed in 1 ml cultures of 0.3% agar in Dulbecco's modified Eagles medium containing 20% newborn calf serum, stem cell factor (SCF, 100 ng/ml, WEHI), EPO (2 U/ml, Janssen-Cilag Ltd) and interleukin-3 (IL-3, 10 ng/ml). Cultures were incubated at 37 °C for 7 days in 10% CO2 in air. Cultures were fixed, dried onto glass slides and stained for acetylcholinesterase followed by Luxol fast blue and haematoxylin to determine the numbers and type of colonies.

Cell survival assays in vitro. Bone marrow cells were collected and stained with antibodies against TER119 (TER119) and CD44 (IM781) and a lineage marker cocktail (CD3 [K3T], MAC-1 [M170], GR-1 [RB6-8C5], B220 [RA3-6B2], CD45.2 [5.450.15.2], lineage "TER119" cells were sorted using the Aria cell sorter (BD) and further subdivided based on CD44 staining and forward light scatter. Reticulocytes and orthochromatic reticulocytes were resuspended in Dulbecco's medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FCS (Bovogen, East Keilor, VIC, Australia, 100 mM asparagine (Sigma Aldrich) and 50 mM 2-mercaptoethanol (Sigma Aldrich) without addition of cytokines. Cells were treated either with DMSO (control, 1×10−6 M; Sigma Aldrich), 1 μM of A-1331852 (provided by AbbVie, North Chicago, IL, USA), 1 μM A-1331852+25 mM QVD-OHP (MP Biomedical) or 1 μM ABT-199. Cells were collected after 0, 24, 48 and 72 h and viability was determined by FACs analysis using forward by side light scatter profile to gate for live cells or Annexin V staining.

Statistical analysis. Cellularity, weight, blood parameters, flow cytometric results and in vitro cell survival were plotted and analysed with GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA). Statistical comparisons were conducted in a pair-wise manner using unpaired two-tailed Student's Hest assuming equal variance. For Figure 2e we utilised the Sidak-Bonferroni method for multiple comparisons correction. For Figure 4 we performed two-way ANOVA analysis with Tukey's multiple comparisons test. Stars indicate significant values. Error bars are presented as standard error of mean (±S.E.M.).

Conflict of Interest

The authors declare no conflict of interest.

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BIM or PUMA do not counter BCL-XL in reticulocytes
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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)