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Loss of Bak enhances lymphocytosis but does not ameliorate thrombocytopaenia in *BCL-2* transgenic mice

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Bax and Bak are critical effectors of apoptosis. Although both are widely expressed and usually functionally redundant, recent studies suggest that Bak has particular importance in certain cell types. Genetic and biochemical studies indicate that Bak activation is prevented primarily by Mcl-1 and Bcl-x_L, whereas Bax is held in check by all pro-survival Bcl-2 homologues, including Bcl-2 itself. In this study, we have investigated whether loss of Bak or elevated Mcl-1 modulates haemopoietic abnormalities provoked by overexpression of Bcl-2. The *Mcl-1* transgene had little impact, probably because the expression level was insufficient to effectively reduce Bak activation. However, loss of *Bak* enhanced lymphocytosis in vavP-*BCL-2* transgenic mice and increased resistance of their thymocytes to some cytotoxic agents, implying that Bak-specific signals can be triggered in certain lymphoid populations. Nevertheless, lack of Bak had no significant impact on thymic abnormalities in vavP-*BCL-2*tg mice, which kinetic analysis suggested was due to accumulation of self-reactive thymocytes that resist deletion. Intriguingly, although *Bak*$^{-/-}$ mice have elevated platelet counts, *Bak*$^{-/-}$vavP-*BCL-2* mice, like vavP-*BCL-2* littermates, were thrombocytopaenic. To clarify why, the vavP-*BCL-2* platelet phenotype was scrutinised more closely. Platelet life span was found to be elevated in vavP-*BCL-2* mice, which should have provoked thrombocytosis, as in *Bak*$^{-/-}$ mice. Analysis of bone marrow chimaeric mice suggested the low platelet phenotype was due principally to extrinsic factors. Following splenectomy, blood platelets remained lower in vavP-*BCL-2* than wild-type mice. However, in *Rag1*$^{-/-}$ *BCL-2*tg mice, platelet levels were normal, implying that elevated lymphocytes are primarily responsible for *BCL-2*tg-induced thrombocytopaenia.

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Apoptosis has a vital role in regulating cell numbers during haemopoiesis and failure to remove damaged, superfluous or potentially dangerous cells can lead to malignancy or autoimmunity. Many critical life-or-death checkpoints, particularly during lymphopoiesis, are governed by opposing factions of the Bcl-2 protein family, which regulate the 'intrinsic' apoptosis pathway.[1,2] Bcl-2, discovered via the t(14;18) chromosome translocation typical of human follicular lymphoma,[3–5] inhibits apoptosis,[6] as do its closest homologues (Bcl-x_L, Bcl-w, A1/Bfl1, Mcl-1 and, in humans, Bcl-B). Other close homologues (Bax and Bak) instead promote apoptosis, as do distant relatives known as BH3 (Bcl-2 homology region 3)-only proteins because they share only an ~26 amino-acid motif with the wider Bcl-2 family. During cellular stress, BH3-only proteins are induced and bind with high avidity via their amphipathic BH3 α-helix to the hydrophobic groove on the surface of pro-survival Bcl-2-like proteins,[7–9] thereby preventing them from restraining any activated Bax or Bak molecules. Certain BH3-only proteins (particularly Bim and cleaved Bid) can also bind weakly and transiently to Bax and/or Bak, triggering their conformational change and subsequent homo-oligomerisation on the outer mitochondrial membrane. As a consequence, cytochrome *c* is released into the cytoplasm, leading to the activation of the proteases (caspases) that provoke cellular demolition by cleaving vital proteins.

Although Bax and Bak are both widely expressed and functionally redundant,[10] recent studies suggest Bak may have particular importance in certain cell types. Thus, loss of Bak results in thrombocytosis, whereas loss of Bax does not, indicating that Bak has the more important role in regulating platelet life span.[11,12] Furthermore, loss of Bak was able to partially rescue thymic defects caused by conditional deletion of Mcl-1, whereas neither overexpression of Bcl-2 nor loss of Bax was able to do so.[13] Specificity of interactions may account for these observations: Bak binds tightly to Mcl-1 and Bcl-x_L but only poorly to Bcl-2, whereas Bax binds avidly to all the pro-survival proteins.[14–16] Presumably, therefore, Bax

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Abbreviations: APS, anti-platelet serum; ASC, antibody-secreting cell; BH, Bcl-2 homology; DN, double negative; DP, double positive; FACS, fluorescence-activated cell sorting; Ig, immunoglobulin; mAb, monoclonal antibody; PMA, phorbol 12-myristate-13-acetate; SP, single positive; TCR, T-cell receptor; tg, transgenic; WT, wild type
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activation can be thwarted by all pro-survival proteins, including Bcl-2, whereas Bak is kept in check by Mcl-1 and Bcl-x_L.

To further explore the role of Bak-specific cell death during haemopoiesis, we have investigated whether loss of Bak or increased expression of Mcl-1 enhances the impact of pan-haemopoietic overexpression of Bcl-2. To do so, we crossed vavP-*BCL-2* transgenic (hereafter *BCL-2*tg) mice[17] with *Bak*$^{-/-}$[10] or vavP-*Mcl-1* transgenic (hereafter *Mcl-1*tg) mice[18] and compared the phenotypes of single and doubly mutant offspring. This study also enabled us to undertake further analysis of the puzzling thymic and platelet phenotype of *BCL-2*tg mice.

Results

Impact of overexpression of Mcl-1 and loss of Bak on haemopoiesis in *BCL-2tg* mice. Overexpression of Bcl-2 via the haemopoietic cell-specific vavP-driven transgene[19] enhances the survival of T- and B-lymphoid cells, which accumulate in excessive numbers in the periphery.[17,20] In addition, *BCL-2*tg mice have a distinctive thymic phenotype: a reduced proportion of pre-T cells (CD4$^+$CD8$^+$ double positive; hereafter DP) and elevated proportions of the other three major populations (CD4$^-$CD8$^-$ double negative, hereafter DN; CD4$^+$CD8$^-$ single positive, hereafter CD4SP; and CD4$^-$CD8$^+$ single positive, hereafter CD8SP).[17] This is referred to as the 'low DP' thymic phenotype of *BCL-2*tg mice (see further below).

To assess whether overexpression of Mcl-1 exacerbates the *BCL-2*tg phenotype, we compared the composition of blood and haemopoietic tissues in 6-week-old neonates. Despite a minor ($P \leq 0.05$) increase in the total number of DP thymocytes in *Mcl-1/BCL-2* bi-transgenic compared with *BCL-2*tg mice, the *proportion* of DP thymocytes was comparably low (lower left panel in Supplementary Figure 1B) and there was little impact on the *BCL-2*tg-induced lymphocytosis in the other lymphoid organs (Supplementary Figure 1 and Supplementary Table 1).

To assess the impact of loss of Bak, we first analysed haemopoietic tissues in young adult (12- to 14-week-old) mice. Loss of Bak had little impact alone, but did increase lymphocytosis in *BCL-2*tg mice (Figure 1 and Supplementary Table 2). This was most apparent in the spleen where cellularity, already elevated approximately fivefold in *BCL-2*tg mice, increased to nearly sevenfold in *Bak*$^{-/-}$ *BCL-2*tg animals, due primarily to a further elevation in the number of immunoglobulin (Ig) isotype-switched (B220$^+$IgM$^-$IgD$^-$)

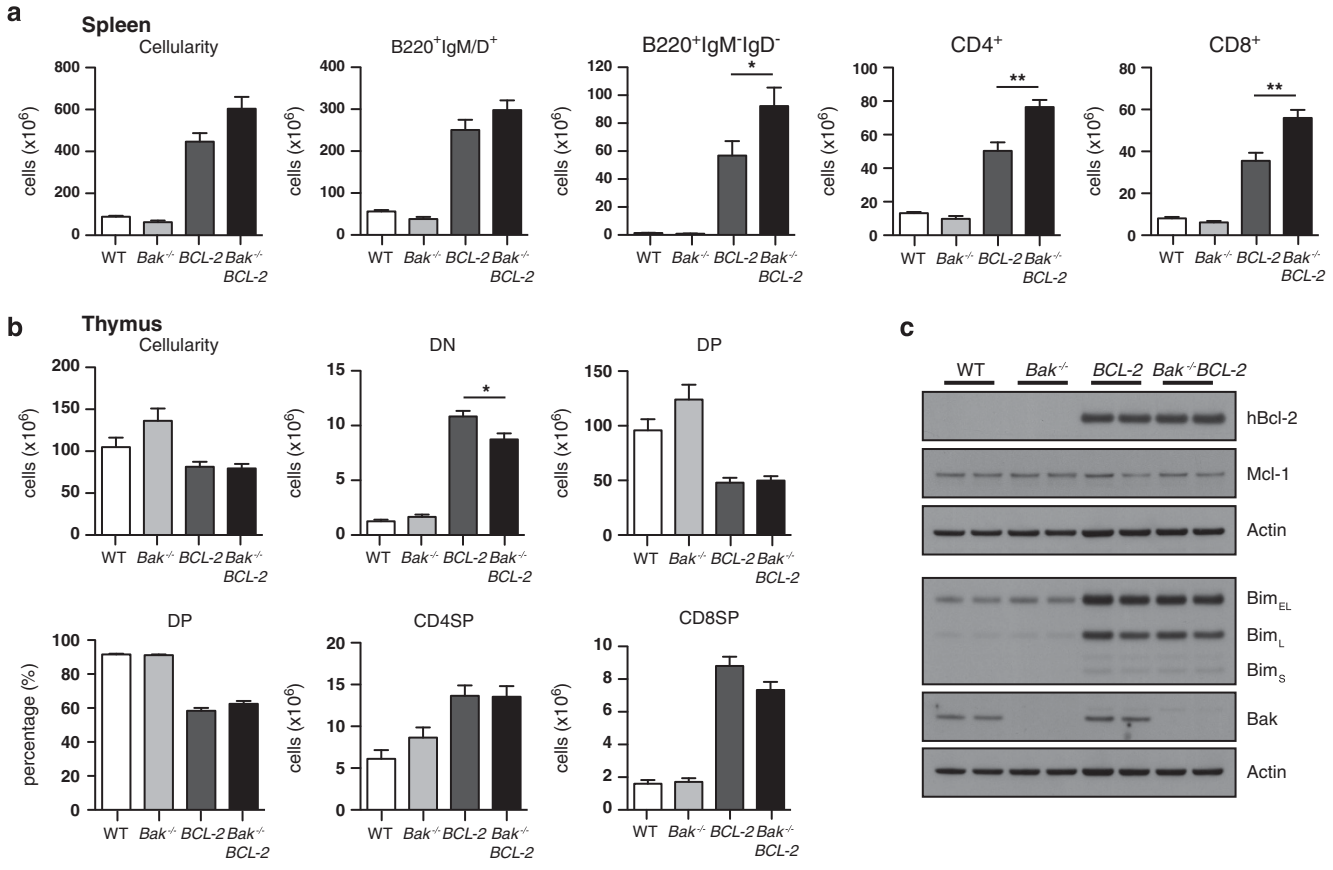

Figure 1 Loss of Bak exacerbates lymphocytosis in *BCL-2*tg mice. Enumeration of total leukocytes and indicated lymphoid populations in the (**a**) spleen and (**b**) thymus of 12- to 14-week-old male mice ($n = 6$–10 per genotype: WT, white; *Bak*$^{-/-}$, light grey; *BCL-2*tg, dark grey; *Bak*$^{-/-}$ *BCL-2*tg, black). B220$^+$IgM/D$^+$ indicates B220$^+$ cells that are IgM$^+$ and/or IgD$^+$. Bars represent mean ± S.E.M.; see also Supplementary Table 2. Statistical significance is shown only for *BCL-2*tg *versus* *Bak*$^{-/-}$ *BCL-2*tg; *$P < 0.05$, **$P < 0.01$, Mann–Whitney test. (**c**) Expression of the indicated Bcl-2 family proteins in sorted DP thymocytes determined by western blot analysis of cells from two independent mice for each genotype

B-lymphoid cells and mature T cells ($CD4^+$ and $CD8^+$; Figure 1a). A comparable cross of $Bax^{-/-}$ and *BCL-2*tg mice revealed no comparable differences between *BCL-2*tg and $Bax^{-/-}$ *BCL-2*tg animals (Supplementary Table 3).

Lack of Bak did not notably alter the low proportion of DP thymocytes in the *BCL-2*tg thymus (Figure 1b and Supplementary Table 2). *BCL-2*tg thymocytes have very high levels of the pro-apoptotic BH3-only protein Bim, most being sequestered by BCL-2,[21] and Bim levels were unchanged in either $Bak^{-/-}$ *BCL-2*tg or *Mcl-1*tg/*BCL-2*tg mice (Figure 1c and Supplementary Figure 1C).

To explore the perturbation of T lymphopoiesis more closely, we compared the thymus and spleen of *BCL-2*tg and $Bak^{-/-}$ *BCL-2*tg mice at three time points: 6–8, 12 and 24 weeks (Figure 2 and Supplementary Table 4). The decrease in the number of DP thymocytes was not as marked at 6–8 weeks (~80 % that in wild-type (WT) littermates) as at 12 and 24 weeks (~46% and ~47%, respectively), although mature thymocytes (CD4SP and CD8SP) were already significantly elevated at 6–8 weeks, as were T cells in the spleen. Strikingly, there was an approximately fourfold increase in the DN population in 6- to 8-week-old *BCL-2*tg and $Bak^{-/-}$ *BCL-2*tg mice, and this was almost entirely due to T-cell receptor β^+ ($TCR\beta^+$) DN cells (elevated 16-fold; Figure 2a). These cells are probably mature T cells ($TCR\beta$ is highly expressed) that have downregulated their co-receptors (CD4 or CD8) because they are autoreactive and have escaped negative selection.[22] The early increase in these 'pseudo DN' cells may inhibit the production of *bona fide* DN thymic progenitor cells (see Discussion).

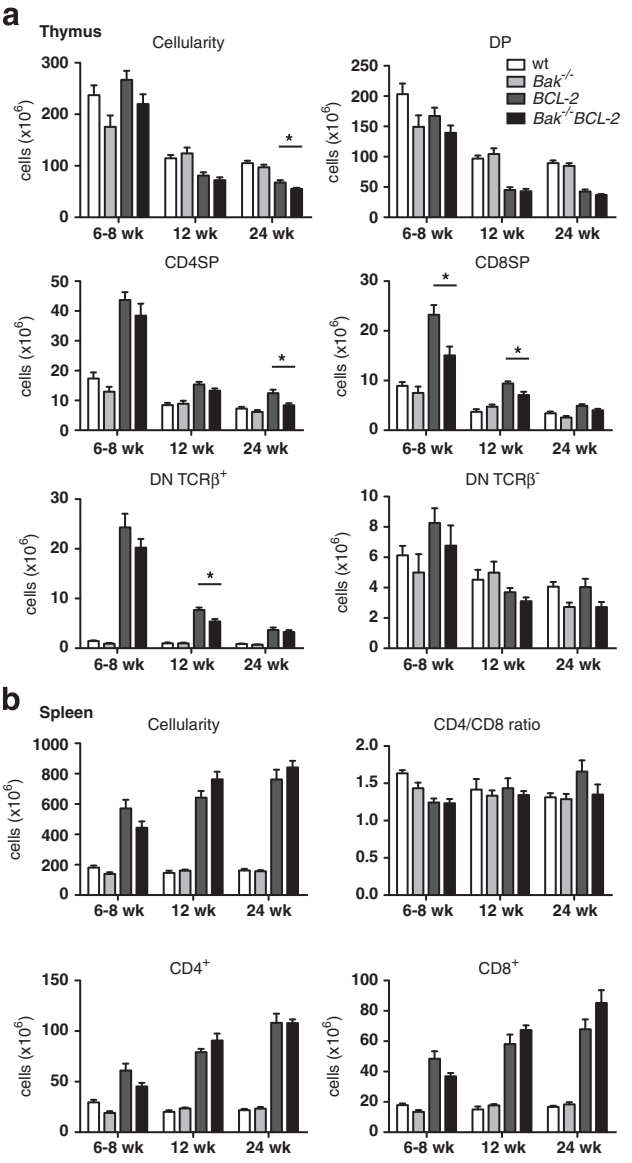

Figure 2 Kinetic analysis of T lymphopoiesis in *BCL-2*tg and $Bak^{-/-}$ *BCL-2*tg mice. Enumeration of total cellularity and indicated T-lymphoid populations in the (**a**) thymus and (**b**) spleen of 6- to 8-, 12- and 24-week-old male mice ($n = 6$–8 per genotype: WT, white; $Bak^{-/-}$, light grey; *BCL-2*tg, dark grey; $Bak^{-/-}$ *BCL-2*tg, black). Bars represent mean ± S.E.M.; see also Supplementary Table 4. Statistical significance is shown only for *BCL-2*tg *versus* $Bak^{-/-}$ *BCL-2*tg; $^*P < 0.05$, Mann–Whitney test

Loss of Bak increases resistance of *BCL-2*tg thymocytes to certain apoptotic stimuli. To compare the resistance of thymocytes of the different genotypes to apoptosis, we first performed *in vitro* tests. In the absence of cytokines, the *Mcl-1* and *BCL-2* transgenes provided comparable protection, but in the presence of cytotoxic agents, the *BCL-2* transgene provided greater protection, consistent with previous observations,[23] and co-expression of both conferred no additional advantage (Supplementary Figure 2).

By itself, loss of Bak did not protect DP thymocytes from spontaneous death in culture or from apoptosis induced by dexamethasone or phorbol 12-myristate-13-acetate (PMA), but it did enhance resistance to ionomycin at low (1 μg/ml) concentration and, at early time points, to DNA damage (γ-irradiation and etoposide; Figure 3a and Supplementary Figure 3). Furthermore, loss of Bak enhanced the resistance of DP thymocytes expressing the *BCL-2* transgene to apoptosis induced by etoposide treatment and, modestly, to γ-irradiation (Figure 3a). DP thymocytes are exquisitely sensitive to CD3 antibody, which triggers apoptosis by aggregating the TCR-CD3 complex.[24,25] As a further test, therefore, we injected mice of all four genotypes with an optimal dose of CD3ε monoclonal antibody (mAb) or with an Ig isotype-matched control antibody, and determined thymus weight and cellularity after 40 h (Figure 3b). Following CD3 antibody treatment, thymus weight dropped substantially in both WT and $Bak^{-/-}$ mice, due primarily to a reduction in DP thymocytes. As reported previously,[26] expression of the *BCL-2* transgene provided DP thymocytes with only partial protection against this insult (65%). Of note, however, protection in the $Bak^{-/-}$ *BCL-2*tg mice was considerably more robust (>90%), suggesting that TCR-activated apoptosis involves a Bak-specific component.

Pathology. *BCL-2*tg mice are predisposed to the development of autoimmune kidney disease and, in later life, follicular lymphoma.[20] To determine whether overexpression of Mcl-1 or loss of Bak increased the risk of morbidity, cohorts of mice were monitored for 12 months. Most mice

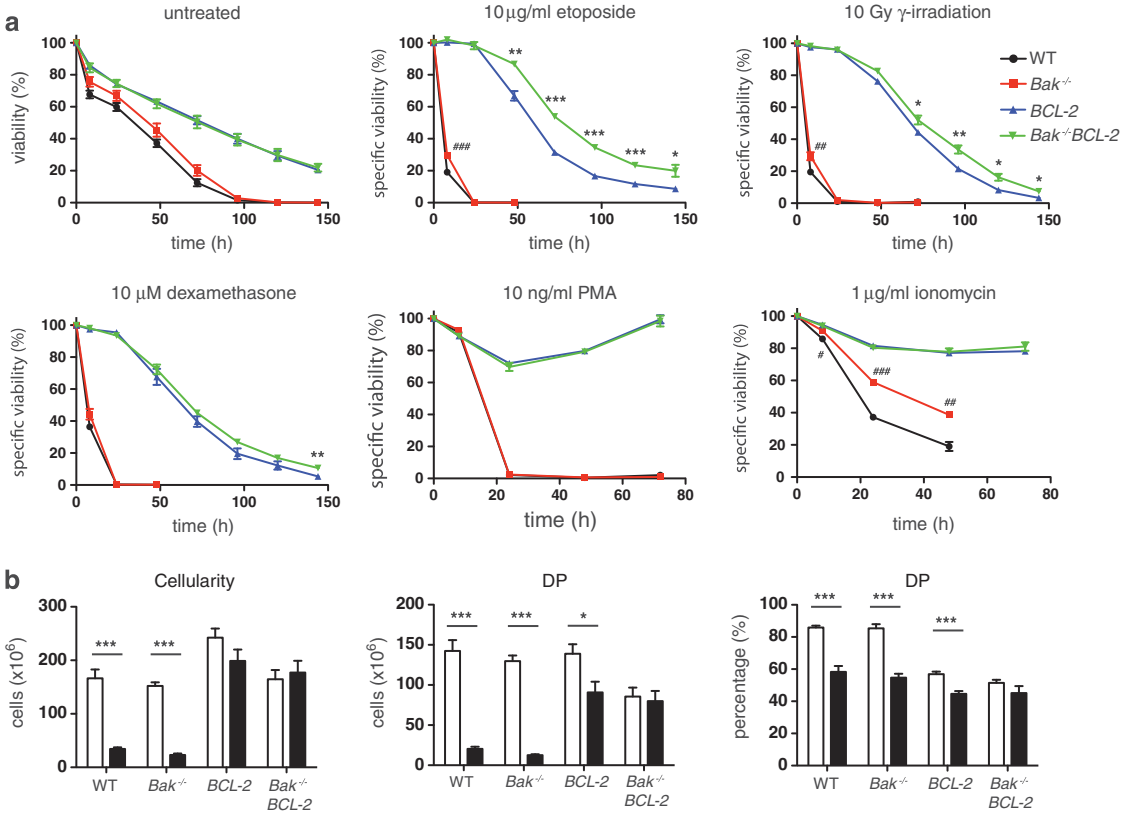

Figure 3 Loss of Bak further enhances survival of thymocytes overexpressing Bcl-2. (**a**) DP thymocytes isolated by FACS were cultured in medium lacking cytokines (untreated), or following exposure to 10 Gy γ-irradiation, or in the presence of the indicated concentrations of etoposide, dexamethasone, PMA or ionomycin. Cell viability was determined by propidium iodide and Annexin V staining followed by flow cytometry. Stimulus-specific viability was calculated relative to viability of untreated cells at each time point (see Materials and Methods). $n=4$ from two independent experiments; values are mean ± S.E.M. Statistical significance (Student's *t*-test) is only indicated for *Bak*$^{-/-}$ *BCL-2*tg *versus BCL-2*tg (*$P<0.05$, **$P<0.01$, ***$P<0.001$) and *Bak*$^{-/-}$ *versus* WT ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$). (**b**) Mice (6- to 8-week-old males) were injected intraperitoneally with 30 μg CD3ε antibody (black bars) or with an Ig isotype-matched control antibody (anti-TCRγ ; white bars) and thymic analysis performed 40 h later. Data are presented as total cellularity (left), number of DP cells (centre) and DP as % of total thymocytes (right). Data represent mean ± S.E.M., $n=5$–8 mice per indicated genotype. *$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's *t*-test

that became ill during this period showed evidence of autoimmune kidney disease: weight loss, blood in the urine and pale, often speckled, kidneys.

The overall survival of *Mcl-1*tg/*BCL-2*tg mice over 12 months was comparable to that of *BCL-2*tg mice and serum Ig levels were similarly elevated in both cohorts (Supplementary Figure 4). The autoimmune kidney disease was most likely due to overexpression of Bcl-2 as *Mcl-1*tg mice do not develop comparable symptoms.[18]

Loss of Bak resulted in a trend towards earlier morbidity in *BCL-2*tg mice, although the difference between *Bak*$^{-/-}$ *BCL-2*tg and *BCL-2*tg animals did not achieve statistical significance (Supplementary Figure 5A). The elevation in IgM, IgG and IgA antibody-secreting cells (ASCs) in the spleen and bone marrow was similar between these cohorts (Supplementary Figures 5B and C).

Although a few lymphomas developed in transgenic mice of each genotype, neither overexpression of Mcl-1 nor loss of Bak enhanced the susceptibility of the *BCL-2*tg mice to follicular lymphoma during the time period analysed. Like *Mcl-1*tg mice,[18] certain *Mcl-1*tg/*BCL-2*tg mice developed other lymphomas, at comparable frequency.

Impact on platelets. Both *Bak*$^{-/-}$ and *BCL-2*tg mice have platelet abnormalities: loss of Bak causes thrombocytosis,[11,12] but Bcl-2 overexpression results, surprisingly, in thrombocytopaenia.[17] We therefore determined platelet levels in the blood of *Bak*$^{-/-}$*BCL-2*tg mice. Unexpectedly, loss of Bak did not elevate platelets in the blood of *BCL-2*tg mice; rather, *Bak*$^{-/-}$ *BCL-2*tg mice, like *BCL-2*tg mice, were thrombocytopaenic (Figure 4a). Thus, Bcl-2 overexpression is dominant over loss of Bak in regard to this phenotype.

At steady state, platelets circulate in the blood for up to 5 days in mice, 10 days in humans[27,28] and the elevated platelet count in *Bak*$^{-/-}$ mice is due to increased platelet life span.[11] To determine the impact of overexpression of BCL-2 on platelet life span, we injected *BCL-2*tg, *Bak*$^{-/-}$*BCL-2*tg and *Bak*$^{-/-}$ mice with NHS-biotin and tracked the disappearance of labelled platelets from the circulation (Figure 4b). Consistent with previous reports,[11] platelet life span was significantly extended in *Bak*$^{-/-}$ mice, with a half-life of 100 h compared with 60 h in WT littermates. Importantly, *BCL-2*tg animals showed an intermediate platelet half-life of 75 h. Thus, overexpression of Bcl-2 enhances the life span of platelets, albeit more modestly than loss of Bak. Underlining the fundamental importance of Bak in regulating

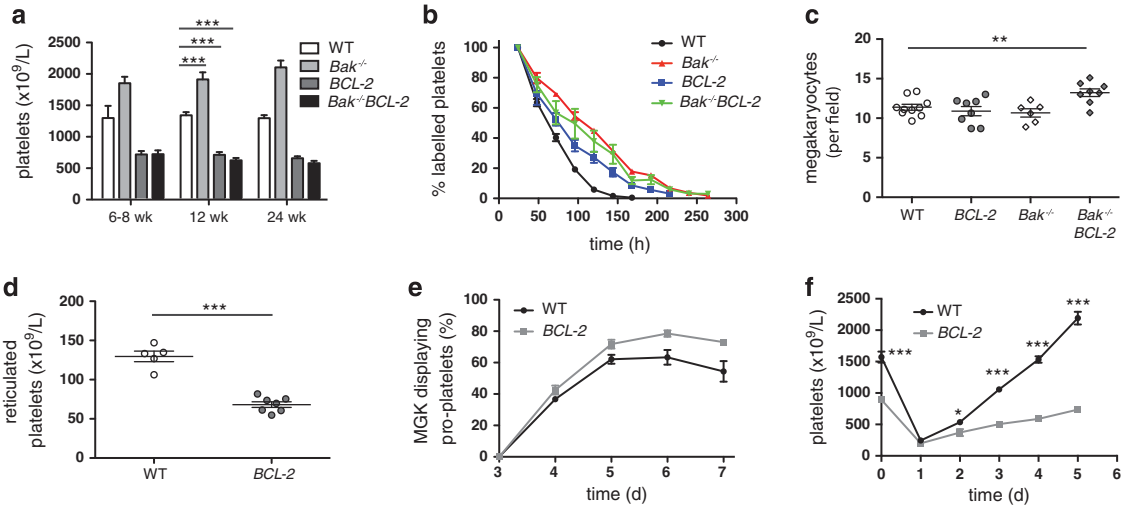

Figure 4 Loss of Bak does not prevent thrombocytopaenia in *BCL-2*tg mice. (**a**) Blood platelet counts in WT (white), $Bak^{-/-}$ (light grey), *BCL-2*tg (dark grey) and $Bak^{-/-}$ *BCL-2*tg (black) mice at 6–8 weeks ($n=4$–7 per genotype), 12 weeks ($n=9$–14) and 24 weeks ($n=15$–20). Bars represent mean ± S.E.M. Statistical significance is shown only for 12-week-old mice; $^{***}P<0.001$, Student's *t*-test. (**b**) Platelet clearance. Peripheral blood samples were taken daily following injection of NHS-biotin to determine the proportion of biotin-labelled platelets. Data represent mean ± S.E.M.; $n=6$. (**c**) Megakaryocyte frequency is normal in the bone marrow of *BCL-2*tg and $Bak^{-/-}$ mice and somewhat increased in $Bak^{-/-}$ *BCL-2*tg mice. Megakaryocytes were counted manually at $\times 200$ magnification in sternum sections stained with haematoxylin and eosin (>10 fields scored per section). Data represent overall mean per field ± S.E.M.; $n=6$–10 male mice; $^{*}P<0.05$, $^{**}P<0.01$, Student's *t*-test. (**d**) Platelet maturation in *BCL-2*tg mice. The percentage of reticulated platelets was determined by flow cytometry following staining with thiazole orange and APC-conjugated CD41 antibody, then converted to absolute platelet numbers using the ADVIA platelet count. Data represent mean ± S.E.M.; $n=5$–7 male mice at 6 weeks. $^{***}P<0.001$, Student's *t*-test. (**e**) Overexpression of Bcl-2 does not impair proplatelet formation *in vitro*. Fetal liver cells were cultured with thrombopoietin and the frequency of large, mature BSA gradient-purified megakaryocytes displaying proplatelet formation assessed. Data are representative of two independent experiments; $n=4$ technical replicates. (**f**) Recovery from anti-platelet serum (APS)-induced thrombocytopaenia is impaired in *BCL-2*tg mice. Mice were treated with a single dose of APS and blood samples taken by tail pricks daily. Platelet counts were determined by flow cytometry. Data represent mean ± S.E.M., $n=6$. $^{*}P<0.05$, $^{***}P<0.001$, Student's *t*-test

platelet life span, there was no additive effect in $Bak^{-/-}$ *BCL-2*tg mice; platelet survival in these animals was indistinguishable from that seen in the $Bak^{-/-}$ cohort.

Megakaryocyte (not shown) and platelet counts were normal in young *Mcl-1*tg mice[18] (Supplementary Figures 6A and B) and platelet life span was also normal (Supplementary Figure 6C), suggesting that the modest increase in platelet Mcl-1 levels in this model (Supplementary Figure 6D), coupled with its short half-life,[29] is not sufficient to have any measurable effect on overall survival times. Like *BCL-2*tg mice, *Mcl-1*tg/*BCL-2*tg mice were thrombocytopaenic, although the deficit in mature platelets was not quite as profound (Supplementary Figures 6A and B).

Why does haemopoietic overexpression of Bcl-2 cause thrombocytopaenia? The thrombocytopaenia in *BCL-2*tg and $Bak^{-/-}$ *BCL-2*tg mice (Figure 4a) is at odds with the enhanced platelet life span in these animals (Figure 4b). To clarify this conundrum, we decided to investigate the *BCL-2*tg platelet phenotype in more detail.

Megakaryocyte frequency was normal in the bone marrow of *BCL-2*tg mice, as in $Bak^{-/-}$ mice, albeit somewhat increased in $Bak^{-/-}$ *BCL-2*tg mice (Figure 4c). Although the percentage of reticulated (newly formed) platelets was unchanged in *BCL-2*tg compared with WT mice, their absolute number was significantly reduced (Figure 4d), implying a defect in platelet production from megakaryocytes. We therefore tested the ability of megakaryocytes from WT and *BCL-2*tg mice to produce pro-platelets *in vitro*. Consistent with a previous report,[30] Bcl-2 overexpression did not decrease proplatelet formation by fetal liver-derived megakaryocytes but, rather, caused a small but significant increase (Figure 4e). These data indicate that, at least in culture, *BCL-2*tg megakaryocytes exhibit no obvious defects in maturation and proplatelet formation. Nevertheless, when we analysed the ability of mice to recover from acute thrombocytopaenia induced by injection of anti-platelet serum (APS), we found that platelet rebound was significantly impaired in *BCL-2 versus* WT mice (Figure 4f).

To further clarify the basis for the platelet defect, we examined whether thrombocytopaenia in *BCL-2*tg mice is platelet-intrinsic or -extrinsic (Figure 5). Bone marrow chimaeric mice were generated by injecting lethally irradiated Ly5.1 mice with 2×10^6 Ly5.2 bone marrow cells from *GFP*tg mice[31] (in which GFP expression is ubiquitous) or *BCL-2*tg mice, or with a 50 : 50 mixture of both. Analysis at 9 weeks post-transplantation showed that the red blood cell count was comparable in all three classes of reconstituted mice but, as expected, the white blood cell count was higher and the platelet count was lower in mice reconstituted with *BCL-2*tg cells than in those reconstituted solely with *GFP*tg cells (Figures 5a–c). In the *GFP*tg/*BCL-2*tg chimaeras, where the proportion of GFP^+ platelets was around 40% (Figure 5d and Supplementary Table 5), total platelet counts were equivalent to those seen in mice reconstituted solely with *BCL-2*tg cells (Figure 5c). By 12 weeks, there were 24% GFP-positive platelets and the proportion of GFP-positive megakaryocytes was similar (22%; Supplementary Table 5). We infer that (i) the *BCL-2*tg-driven thrombocytopaenia derives from bone marrow cells; (ii) WT (*GFP*tg) haemopoietic cells cannot

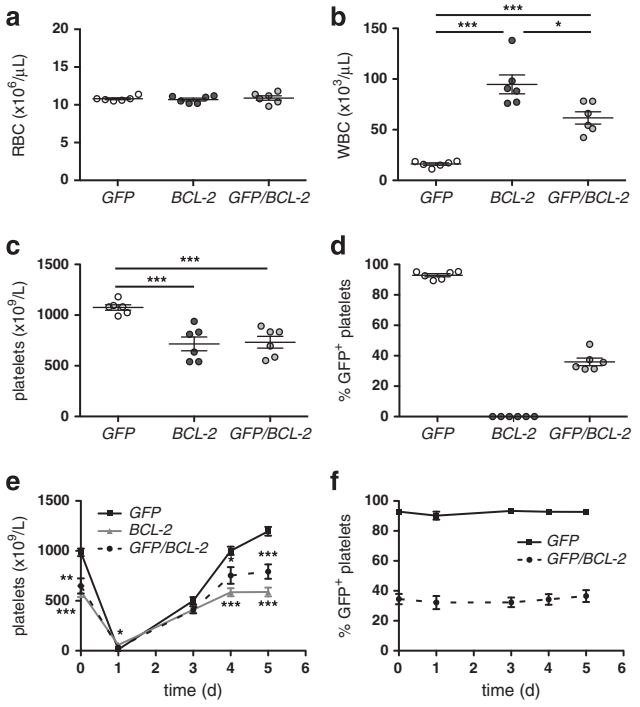

Figure 5 Thrombocytopaenia in *BCL-2*tg mice is platelet extrinsic. (**a**–**d**) Analysis of red blood cell (RBC) (**a**), white blood cell (WBC) (**b**) and platelets (**c**, **d**) in the blood of mice 9 weeks following lethal irradiation and reconstitution with *GFP*tg, *BCL-2*tg or a 50 : 50 mix of *GFP*tg and *BCL-2*tg bone marrow cells. Data represent mean ± S.E.M., $n=6$; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Student's *t*-test. (**e**) Mice reconstituted with *GFP*tg, *BCL-2*tg or a 50 : 50 mix of *GFP*tg and *BCL-2*tg bone marrow cells were treated with a single dose of APS (10 weeks post reconstitution) and peripheral blood samples taken by tail pricks at the indicated times. Platelets were enumerated by flow cytometry and (**f**) the percentage of GFP^+ platelets was determined. Data represent mean ± S.E.M., $n=6$; statistically significant differences from *GFP*tg are shown $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Student's *t*-test

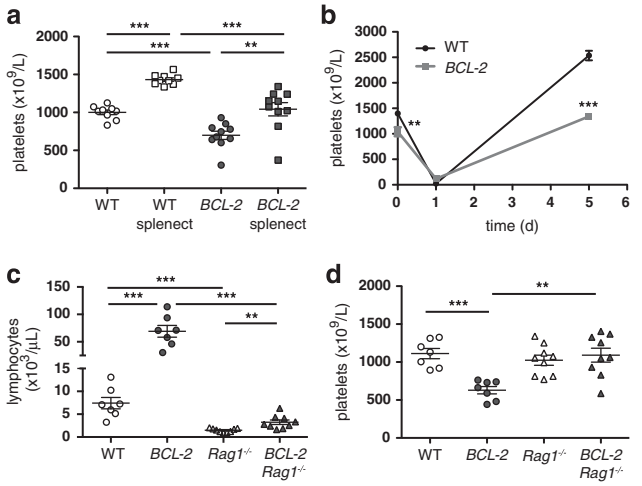

Figure 6 Thrombocytopaenia in *BCL-2tg* mice is provoked by the external milieu. (**a**) Blood platelets increase following splenectomy in WT and *BCL-2*tg mice. Platelet counts were determined before and 4 weeks after surgery on 8-week-old female mice ($n=9$–10). (**b**) Recovery from anti-platelet serum (APS)-induced thrombocytopaenia is impaired in splenectomised *BCL-2*tg mice. Mice from **a** were injected with APS 5 weeks after splenectomy and platelet counts determined on d0 ($n=9$–10), d1 ($n=5$) and d5 ($n=4$–5). (**c**) Lymphocyte counts in *BCL-2*tg mice are significantly reduced on a $Rag1^{-/-}$ background, to around WT numbers. *BCL-2*tg mice were crossed to $Rag1^{-/-}$ mice and blood counts determined in offspring of the indicated genotypes ($n=7$–9). (**d**) Platelet counts are normal in *BCL-2*tg mice that lack mature lymphocytes. Data represent mean ± S.E.M.; $^{**}P<0.01$, $^{***}P<0.001$, Student's *t*-test

compensate for the defect; and (iii) the defect in platelets is cell extrinsic.

Ten weeks post-reconstitution, mice were injected with APS and platelet numbers assessed daily for 5 days (Figure 5e). In all mice, APS reduced circulating platelet numbers to <10% within 24 h. In the chimaeric *GFP*tg/*BCL-2*tg mice, the rebound was more robust than that seen in *BCL-2*tg-reconstituted mice, but still significantly impaired relative to *GFP*tg-reconstituted counterparts (Figure 5e). If the rate of production of platelets had been slower only in cells expressing the *BCL-2* transgene, then the proportion of GFP-positive (i.e., *BCL-2*tg negative) platelets would have been expected to increase over time in the chimaeric *GFP*tg/*BCL-2*tg mice. However, it remained constant over the 5 days (Figure 5f), again suggesting that megakaryocyte- and platelet-extrinsic factors underlie the thrombocytopaenia observed in *BCL-2*tg animals.

Role of the spleen. As the spleen can increase or decrease the pool of platelets that it sequesters,[32] and *BCL-2*tg (and $Bak^{-/-}$*BCL-2*tg) mice have enlarged spleens (Supplementary Table 2), we determined platelet counts before, and 4 weeks post, splenectomy in both WT and *BCL-2*tg mice. Although platelet numbers increased in both cohorts, the relative increment was equivalent: 1.4-fold increase in WT and 1.5-fold in *BCL-2*tg mice (Figure 6a). Furthermore, when acute thrombocytopaenia was induced in splenectomised mice, *BCL-2*tg animals mounted a significantly impaired response, with platelet counts very significantly lower than those seen in WT littermates 5 days post induction (Figure 6b). Although the degree of thrombocytopaenia in the splenectomised *BCL-2*tg animals is modest, when the increase in platelet life span in these animals is taken into account, it indicates a significant residual defect, which is amplified under conditions of emergency thrombopoiesis (Figure 6b).

Role of lymphocytes. Having established that the thrombocytopaenia in *BCL-2*tg mice was due to environmental factors involving bone marrow-derived cells but extrinsic to megakaryocytes and platelets, we reasoned that a non-myeloid cell might be playing a role. We therefore crossed the *BCL-2*tg mice with $Rag1^{-/-}$ mice, which lack mature B and T lymphocytes.[33] Circulating lymphocytes were greatly reduced in *BCL-2*tg $Rag1^{-/-}$ compared with *BCL-2*tg mice (Figure 6c), as expected, but blood platelet counts were elevated 1.7-fold (Figure 6d). Indeed, platelet counts in *BCL-2*tg $Rag1^{-/-}$ mice were comparable to those in WT and $Rag1^{-/-}$ mice. Thus, the absence of mature lymphocytes prevents thrombocytopaenia in *BCL-2*tg mice. Given the increased platelet life span conferred by overexpression of BCL-2, however, the increase is not as much as might be expected, suggesting that additional (unknown) factors might contribute to the *BCL-2*tg phenotype.

Discussion

Bax and Bak are the critical effectors of apoptosis. Although both are widely expressed and functionally redundant, genetic

and biochemical data suggest that Bak is held in check primarily by Mcl-1 and Bcl-x_L, whereas Bax activation is inhibited by all five pro-survival Bcl-2 homologues.[14–16] Recent studies have suggested that Bak activation is the principal trigger for apoptosis in platelets[11] and in certain thymic populations.[13] To test whether Bak is critical at other stages of haemopoietic development, we crossed vavP-*BCL-2*tg mice with mice lacking Bak. We also crossed vavP-*BCL-2*tg mice with vavP-*Mcl-1*tg mice, in which Bak activation in haemopoietic cells should be more difficult to achieve than in WT mice.

Constitutive overexpression of Bcl-2 during haemopoiesis results in many changes: thymic abnormalities, most notably a low proportion of DP thymocytes; striking peripheral lymphocytosis involving both B- and T-lymphoid cells; elevated ASCs and serum Ig; poorly understood thrombocytopaenia; and predisposition to autoimmune kidney disease and late onset follicular lymphoma.[19,20]

Our analysis of young *Mcl-1*tg/*BCL-2*tg mice suggested that overexpression of Mcl-1 did not significantly modulate the *BCL-2tg* phenotype (Supplementary Figure 1), probably because the level of Mcl-1, albeit significantly higher than normal,[18] was insufficient to effectively reduce Bak activation.

In contrast, $Bak^{-/-}$ *BCL-2*tg mice developed a more severe lymphocytosis than *BCL-2*tg littermates, primarily due to a further elevation in the numbers of mature B- and T-lymphoid cells (Figure 1a), changes that were not seen in $Bax^{-/-}$ *BCL-2*tg mice (Supplementary Table 3). The implication is that Bak can have a non-redundant role in maintaining homeostasis within these populations. Bak must be less critical in regulating ASCs, however, because their frequency was comparable in *BCL-2*tg and $Bak^{-/-}$ *BCL-2*tg mice, as was the risk of autoimmune kidney disease (Supplementary Figure 5).

Lack of Bak had no impact on the low proportion of DP thymocytes characteristic of *BCL-2*tg mice (Figure 1b), which kinetic analysis suggested was preceded by an accumulation of DN cells expressing high levels of TCRβ (Figure 2a). Also notable in $Bim^{-/-}$ and $Bim^{-/-}Puma^{-/-}$ mice, these TCR$\beta+$DN cells are thought to be thymocytes that have downregulated their co-receptors[26,34] after escaping negative selection at the DP stage.[22] The 'pseudo' DN cells may inhibit the expansion and maturation of *bona fide* DN progenitor cells to DP (pre-T) cells,[22] perhaps due to competition for niche cells or cytokines.

DP thymocytes, normally highly sensitive to apoptosis, are rendered significantly resistant to most cytotoxic agents by overexpression of Bcl-2.[24] Loss of Bak modestly enhanced the resistance of DP thymocytes from *BCL-2*tg mice to apoptosis induced by DNA-damaging agents *in vitro* (Figure 3a) and significantly increased their resistance to CD3 antibody treatment *in vivo* (Figure 3b). Thus, Bak may be non-redundant for effecting apoptosis of certain lymphoid populations, perhaps because of kinetic differences in the activation of Bak *versus* Bax and/or a Bak-specific component of certain apoptosis-inducing signals. Ca^{+} flux (which is induced by TCR/CD3 ligation) may have an important role in the Bak activation, as, by itself, loss of Bak provided significant protection against apoptosis induced by ionomycin (Figure 3a). We have shown previously that loss of the pro-apoptotic BH3-only protein Bim also provides greater resistance to CD3 antibody than the *BCL-2* transgene and that Bim is the critical downstream apoptosis effector.[26,35] Bim can activate both Bak and Bax as well as inhibit all pro-survival Bcl-2 family members.[36–38]

Our most unexpected finding involved platelets. The observation that *BCL-2*tg mice are thrombocytopaenic[17] led to conjecture that platelet shedding by megakaryocytes involves activation of apoptosis,[39] and a considerable body of subsequent work supported that notion (reviewed in White and Kile[32]). However, counter to this proposal, we and others recently demonstrated that deletion of Bak and Bax does not impair platelet production.[12,40,41] Thus, an explanation for the thrombocytopaenic phenotype of *BCL-2*tg mice has remained elusive.

Platelet survival is primarily dependent on Bcl-x_L, which is required in ageing platelets to restrain Bak, the principal mediator of their apoptosis.[11,12,42] We found that platelet life span is extended in *BCL-2*tg mice (Figure 4b), demonstrating for the first time that overexpression of a Bcl-2 family pro-survival protein can positively influence the survival of platelets in the circulation. BCL-2 may be able to partly restrain Bak at these high, non-physiological concentrations, as well as inhibit normal targets Bax and Bad, which also influence platelet life span,[12,43] albeit not to nearly the same extent as loss of Bak.[12] In any event, the thrombocytopaenia in *BCL-2*tg animals must actually be more severe than suggested by the platelet count alone – were this not the case, the extended platelet life span conferred by the *BCL-2* transgene would have provoked mild thrombocytosis.

Consistent with Kozuma and colleagues,[30] megakaryocyte numbers were normal in *BCL-2*tg mice, and their ability to form pro-platelets *in vitro* was unimpaired. However, contrary to that study, removal of the spleen did not raise their platelet counts to that found in splenectomised WT mice, either at steady state or during emergency thrombopoiesis (Figures 6a and b), ruling out increased splenic sequestration as the principal cause. Instead, we believe the thrombocytopaenia is underpinned by a defect in platelet shedding caused by extrinsic factors in the *BCL-2*tg haemopoietic microenvironment. Analysis of bone marrow chimaeras supported this notion, as WT megakaryocytes in a *BCL-2*tg environment were unable to produce platelets at the same rate as those in a WT environment (Figure 5e). Our data suggest that the lymphocytosis induced by the *BCL-2* transgene is a major contributing factor, as platelet counts increased to WT levels in a $Rag1^{-/-}$ background (Figure 6d). Other as yet unidentified haemopoietic cells must also have a role, however, as platelet counts were not elevated above WT levels, as would be expected from the increased platelet life span conferred by overexpression of BCL-2. As vavP-*BCL-2* transgene expression also elevates levels of bone marrow macrophages and monocytes (Supplementary Table 2), these cells may contribute. However, whether the impairment of platelet production is mediated directly by lymphoid and other haemopoietic cell types or indirectly, via effects on other tissues, remains to be determined. Furthermore, it remains formally possible that, in addition, BCL-2 overexpression does have a subtle intrinsic impact on megakaryocyte function. Specific overexpression of BCL-2 in the megakaryocyte lineage *in vivo* would enable this to be assessed in the absence of extrinsic factors.

Materials and Methods

Mice. All mice used were on a C57BL/6J background and bred at the Walter and Eliza Hall Institute (WEHI). Experimental protocols were approved by Animal Ethics Committee of WEHI. Transgenic mouse lines were vavP-*Mcl-1*(33)[18] and vavP-*BCL-2*(69),[17,20] which, respectively, express FLAG-tagged mouse Mcl-1 protein and human BCL-2 protein in haemopoietic cells.[19] To generate *Mcl-1*tg/*BCL-2*tg mice, *Mcl-1*tg males were mated with *BCL-2*tg females. To generate $Bak^{-/-}$ *BCL-2*tg mice, *BCL-2*tg males were crossed with $Bak^{-/-}$[10] females, then $Bak^{+/-}$ *BCL-2*tg males were mated with $Bak^{+/-}$ females. Crosses were also performed with $Rag1^{-/-}$,[44] $Bax^{+/-}$[45] and Tg(UBC-GFP) 30Scha/J mice (*GFP*tg mice).[31]

Haemopoietic analysis. Single-cell suspensions were prepared from spleen, lymph nodes, bone marrow and thymus and viable leukocytes enumerated using a haemocytometer and trypan blue exclusion or with a CASY Cell Counter (Scharfe System GmbH, Reutlingen, Germany). An ADVIA 2120 haematology analyser (Siemens, Erlangen, Germany) was used for obtaining blood cell counts. The remaining blood was depleted of red cells by treatment with 0.168 M ammonium chloride before fluorescence-activated cell sorting (FACS) analysis. Cell composition was determined by staining with fluorochrome-labelled surface marker-specific monoclonal antibodies followed by FACS analysis using an LSRI (BD Biosciences, Franklin Lakes, NJ, USA). Data were processed using FlowJo Version 9.3.2 (TreeStar, Ashland, OR, USA) and Weasel Version 3.0 software (Walter and Eliza Hall Institute, Melbourne, VIC, Australia). The monoclonal antibodies, produced and labelled with fluorescein isothiocyanate, R-phycoerythrin or allophycocyanin (APC) at WEHI unless otherwise indicated, were: RB6-8C5, anti-Gr1; MI/70, anti-Mac1; H129.19, anti-CD4; YTS169, anti-CD8; Ter119, anti-erythroid marker; ID3, anti-CD19; RA3-6B2, anti-CD45R-B220; 5.1, anti-IgM; 11-26C, anti-IgD; S7, anti-CD43; T24-31, anti-Thy1; H57-59, anti-TCRβ; Jo2, anti-Fas/CD95 (BD Biosciences); anti-PNA (Vector Laboratories, Cambridgeshire, UK).

Blood platelet counts were determined using an ADVIA 2120 analyser (Siemens) or by flow cytometry, as follows: 5 μl of tail vein blood was diluted 40-fold in PBS in an EDTA tube and stained with CD41 antibody (clone MWReg30, BD Biosciences) labelled with APC; 10 μl was then added to PBS (980 μl) containing 10 μl beads (ProSciTech, Townsville, QLD, Australia; 3.5–4 μm beads, 1×10^7/ml) and 1000 events were acquired by flow cytometry; platelet frequency was calculated as dilution factor (4000) $\times$ number of events in platelet gate ($CD41^+$) $\times 1 \times 10^5$. Reticulated platelet numbers were determined by staining with thiazole orange and CD41-APC antibody[11] followed by analysis on a FACSCalibur flow cytometer (BD Biosciences). Platelet life span was investigated by *in vivo* labelling with biotin.[11] Induction of thrombocytopaenia with APS, culture of fetal liver megakaryocytes, proplatelet formation assays and platelet preparation were performed as described.[11,12]

CD3 antibody treatment. Mice were injected intraperitoneally with 30 μg hamster mAb to mouse CD3ε (145-2C11) or, as a control, isotype-matched antibody to TCRγ (GL3) and killed after 40 h for analysis of thymic cellularity and composition by flow cytometry.

Survival assays. Thymocyte populations isolated by flow cytometry were cultured at $0.2–0.5 \times 10^6$ cells/ml in high-glucose Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum (Bovogen, Melbourne, VIC, Australia), 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and 100 μM asparagine (Sigma-Aldrich) without additional cytokines in the presence or absence of 10 μg/ml etoposide (Pfizer, Sydney, NSW, Australia), 10 μM dexamethasone phosphate (Hospira, Lake Forest, IL, USA), 10 ng/ml PMA (Sigma-Aldrich), 10 μg/ml ionomycin (Sigma-Aldrich) or following treatment with 10 Gy γ-irradiation. Cell viability was determined by flow cytometry after staining with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide. Specific viability was calculated at each time point as (viability of treated cells/viability of untreated cells) $\times$ 100%. Alternatively, apoptotic cells were identified by active caspase-3 staining. Cells were fixed and permeabilised using the BD Cytofix/Cytoperm Kit for 20 min, then washed with BD Perm/Wash buffer and stained with phycoerythrin rabbit anti-active caspase-3 antibody (clone C92-605, BD Biosciences), then washed again in BD Perm/Wash buffer before analysing by flow cytometry.

Haemopoietic reconstitutions. The *GFP*tg/*BCL-2*tg haemopoietic chimaeras were generated using bone marrow collected from *GFP*tg and *BCL-2*tg mice. Bone marrow (2×10^6) cells suspended in phosphate-buffered saline were injected into lethally irradiated (2×5.5 Gy spaced by 2 h) C57BL/6-CD45.1 (Ly5.1) mice. To prevent infections, transplanted animals were initially provided with water containing neomycin (Sigma-Aldrich). After stable reconstitution of their haemopoietic system (9 weeks later), blood was collected from the retro-orbital plexus for ADVIA and FACS analysis (see above).

Western blot analysis. Thymocyte lysates were prepared using RIPA buffer (300 mM NaCl, 2% octylphenoxypolyethoxyethanol (IGEPAL CA-630; Sigma-Aldrich), 1% deoxycholic acid, 0.2% SDS, 100 mM Tris-HCl pH 8.0) containing protease inhibitors (Roche, Basel, Switzerland) and platelet lysates with NP40 lysis buffer (1% octylphenoxypolyethoxyethanol, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing protease inhibitors. Proteins were separated on NuPAGE Bis-Tris gels (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. Blots were probed with: anti-Mcl-1 (clone 19C4-15; WEHI mAb facility), anti-human Bcl-2 (clone Bcl-2-100;[46] WEHI mAb facility), anti-Bcl-2 (clone 7; BD Biosciences), anti-Bim (polyclonal; Enzo Lifesciences, Farmingdale, NY, USA), anti-Bcl-x_L (polyclonal; BD Biosciences), anti-Bak (polyclonal; Sigma-Aldrich) and anti-β-actin (clone AC-74; Sigma-Aldrich).

ELISA and enzyme-linked immunospot (ELISPOT). ASCs were enumerated by ELISPOT. MultiScreen-HA filter plates (Merck Millipore, Billerica, MA, USA) were coated with 2 μg/ml anti-mouse total Ig (DA, Silenus Laboratories, Boronia, VIC, Australia) or 10 μg/ml anti-mouse IgA (Southern Biotech, Birmingham, AL, USA). Red cell-depleted spleen or bone marrow cells were added at 1×10^4 or 1×10^5 per well in RPMI/5% FCS/5 μM 2-mercaptoethanol and incubated for 18–19 h. The plates were washed and incubated with secondary antibodies: anti-mouse IgA-biotin, anti-mouse IgG1-HRP/IgG2a-HRP/IgG2b-biotin/IgG3-HRP or anti-mouse IgM-HRP (Southern Biotech). For biotinylated antibodies, plates were washed again and incubated with streptavidin-HRP (Southern Biotech). ELISPOTs were revealed by the addition of substrate solution: 250 μg/ml 3-amino-9-ethylcarbazole (Sigma-Aldrich) in 0.05 M sodium acetate (pH 5.0) and 0.03% H_2O_2. ELISPOTs were counted on an ELISPOT reader (Autoimmun Diagnostika GMBH, Strasburg, Germany).

Serum Ig levels were determined by ELISA. Plates were coated with specific anti-mouse Ig antibodies (Southern Biotech). Purified mouse monoclonal IgMκ (TEPC 183), IgG1κ (MOPC31c), IgG2aκ (UPC 10), IgG2bκ (MOPC 141), IgG3λ (Y5606) and IgAλ (MOPC315; Sigma-Aldrich) were used to quantify Ig concentration. Biotinylated or HRP-conjugated isotype-specific antibodies were as for ELISPOT assay (with streptavidin-HRP, if required). The assay was developed with 0.54 mg/ml diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Sigma-Aldrich) in 0.1 M citric acid (pH 4.4) and 0.03% H_2O_2. Absorbance at 492 nm was measured in a microplate reader.

Statistical analysis. GraphPad Prism (Version 5.0a; GraphPad Software, La Jolla, CA, USA) was used to graph and statistically analyse data. The Mann–Whitney test was used to determine statistical significance when samples had a clear difference in S.D., otherwise, an unpaired two-tailed *t*-test was performed. For analysis of Kaplan–Meier mouse survival curves, significance was determined using the log-rank (Mantel–Cox) test.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

CJV and SC conceived the studies, planned experiments, analysed data. BTK and ECJ provided advice and designed the platelet experiments. CJV, ECJ, KJC, CJ and KEL performed the experiments. CJV, SC, ECJ and BTK wrote the manuscript.

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