

## Foxo-mediated *Bim* transcription is dispensable for the apoptosis of hematopoietic cells that is mediated by this BH3-only protein

Marco J Herold<sup>1,2</sup>, \*Leona Rohrbeck<sup>1,2</sup>, \*Mathias J Lang<sup>1,2,3</sup>, Raelene Grumont<sup>4</sup>, Steve Gerondakis<sup>4</sup>, Lin Tai<sup>1,2</sup>, Philippe Bouillet<sup>1,2</sup>, #Thomas Kaufmann<sup>1,5</sup> and #^Andreas Strasser<sup>1,2</sup>

<sup>1</sup> The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

<sup>2</sup> Department of Medical Biology, The University of Melbourne, Melbourne, Victoria, Australia

<sup>3</sup> current address: Biocentre, University of Basel, Basel, Switzerland

<sup>4</sup> Australian Centre for Blood Diseases and Department of Clinical Hematology, Central Clinical School, Monash University, Melbourne, Victoria, Australia.

<sup>5</sup> current address: Institute of Pharmacology, University of Bern, Bern, Switzerland

\* LR and MJL contributed equally to this work

# TK and AS share senior authorship

^ address for correspondence: Andreas Strasser, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville VIC 3052, Australia, ph: +61-3-9345-2624, FAX: +61-3-9347-0852, email: strasser@wehi.edu.au

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## **Abstract**

The BH3-only protein Bim is a critical initiator of apoptosis in hematopoietic cells. Bim is up-regulated in response to growth factor withdrawal and over-expression studies have implicated the transcription factor Foxo3a as a critical inducer. To test this hypothesis *in vivo*, we generated mice with mutated Foxo binding sites within the *Bim* promoters (*Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup>). Contrary to Bim-deficient mice, *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup> mice had a normal hematopoietic system and, upon cytokine withdrawal, diverse hematopoietic cells from these mice died at a normal rates. These results indicate that regulation of *Bim* by Foxo transcription factors is not critical for the killing of hematopoietic cells.

## **Introduction**

The “Bcl-2-regulated” apoptotic pathway plays a critical role in the hematopoietic system[1]. It is controlled by the complex interactions of three sub-groups of the Bcl-2 protein family: the pro-survival Bcl-2-like proteins, the Bax/Bak multi-BH domain pro-apoptotic proteins and the pro-apoptotic BH3-only proteins[2]. Stress stimuli, such as growth factor deprivation, cause induction of BH3-only proteins[3]. These proteins then activate Bax/Bak, either through direct binding and/or indirectly by binding to the pro-survival Bcl-2-like proteins[4]. Activated Bax/Bak trigger mitochondrial outer membrane permeabilisation (MOMP), which unleashes the caspase cascade that causes cell demolition[2].

The BH3-only proteins are essential for the initiation of apoptosis and different members of this subgroup are critical for cell death triggered by distinct apoptotic stimuli[2]. Bim[5] is a critical regulator of hematopoietic cells[6, 7]: it is, for example, essential for

the deletion of auto-reactive T[8] and B cells[9] and the killing of effector T and B lymphocytes during shut-down of immune responses[10, 11].

Experiments using dominant negative or constitutively active mutants of Foxo3a implicated this transcription factor as a critical inducer of Bim in apoptosis triggered by cytokine deprivation[12-14]. To explore the physiological relevance of this process, we generated mice in which all Foxo binding sites within the *Bim* promoters[15] had been mutated (called *Bim*<sup>ΔFoxo/ΔFoxo</sup> mice). Contrary to Bim-deficient mice, *Bim*<sup>ΔFoxo/ΔFoxo</sup> and *Foxo3a*<sup>-/-</sup> mice had a normal hematopoietic system. Moreover, in contrast to Bim-deficient cells diverse lymphoid cell subsets and mast cells from *Bim*<sup>ΔFoxo/ΔFoxo</sup> mice were normally sensitive to cytokine deprivation. These results indicate that direct transcriptional induction of *Bim* by Foxo is not critical for homeostasis and cytokine deprivation-induced apoptosis of hematopoietic cells.

## **Results**

### ***Identification and validation of Foxo binding sites in the Bim promoters***

Previous over-expression studies showed that Foxo3a can enhance Bim expression by binding to two specific sequence motives (GTAAACAA) within its two promoters[12-14]. Since Bim is critical for cytokine deprivation-induced apoptosis in diverse hematopoietic cell subsets[6, 16], it was concluded that growth factor withdrawal kills cells through transcriptional upregulation of Bim by Foxo3a[12-14].

Our bioinformatics analysis of the *Bim* gene revealed the two published[12-14] and two additional Foxo3a binding sites (-625 and +2509 from Exon1) (FIG 1A). To examine whether Foxo3a can bind to these sites, we performed EMSA with nuclear extracts from

293T cells that transiently expressed a tamoxifen (4-OHT) inducible HA tagged Foxo3a (HA-Foxo3a(A3)ER) (Figure 1B). We only observed reproducible Foxo3a-specific binding to site 4; this band disappeared when nuclear extracts containing HA-Foxo3A(A3)ER were incubated with HA- or Foxo3A-specific antibodies (FIG 1B & C). Moreover, we could efficiently compete Foxo3A binding to site 4 with unlabeled, but not with mutated oligos (supplementary FIG 1). These results reveal that site 4 in the mouse *Bim* promoters is the dominant site bound by Foxo3a.

### ***Bim<sup>ΔFoxo/ΔFoxo</sup> mice have a normal hematopoietic system***

Foxo3a belongs to a transcription factor family with overlapping expression patterns and binding to target sequences in DNAexpression[17]. To examine the physiological role of Foxo driven *Bim* activation, we generated mice in which the invariable core sequence of the common Foxo binding sequences in the *Bim* promoters were exchanged from AACA to TCGA or GTGG for site 1 or sites 2, 3 and 4, respectively (supplementary FIG 2A & B). To verify the functional impact of these mutations, we generated T cell blasts from wt and *Bim<sup>ΔFoxo/ΔFoxo</sup>* mice and ectopically expressed HA-Foxo3a(A3)ER; this elicited *Bim* mRNA upregulation only in wt but not in *Bim<sup>ΔFoxo/ΔFoxo</sup>* T cells treated for 24 h with 4-OHT (supplementary FIG 2C). Finally, we observed no compensatory Foxo3a or Foxo1 protein upregulation in thymocytes from *Bim<sup>ΔFoxo/ΔFoxo</sup>* mice (supplementary FIG 2D).

We examined the hematopoietic system of the *Bim<sup>ΔFoxo/ΔFoxo</sup>* mice using wt, *Foxo3a<sup>-/-</sup>* and *Bim<sup>-/-</sup>* mice as controls. As previously reported[6], *Bim<sup>-/-</sup>* mice had a marked increase of mature CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> thymocytes compared to wt controls (FIG 2A). In contrast, *Bim<sup>ΔFoxo/ΔFoxo</sup>* and *Foxo3a<sup>-/-</sup>* mice had normal thymic cell subset distribution

(FIG 2A). *Bim* mRNA and Bim protein levels in thymocytes from *Bim*<sup>ΔFoxo/ΔFoxo</sup> and *Foxo3a*<sup>-/-</sup> mice were comparable to those seen in wt thymocytes (FIG 2B, C).

Similarly, only *Bim*<sup>-/-</sup> mice but not the *Bim*<sup>ΔFoxo/ΔFoxo</sup> or *Foxo3a*<sup>-/-</sup> animals had abnormally increased numbers of T as well as B cells and granulocytes in the spleen (FIG 3A). Accordingly, spleen cells from *Bim*<sup>ΔFoxo/ΔFoxo</sup> and *Foxo3a*<sup>-/-</sup> mice expressed normal levels of *Bim* mRNA and Bim protein (Figures 3B, C). Based on these results, we conclude that Foxo-mediated transcriptional activation of Bim is not essential for the normal developmentally programmed death of hematopoietic cells.

### ***Foxo-mediated transcriptional activation of Bim is dispensable for killing of thymocytes***

Bim is critical for thymocyte apoptosis triggered by diverse cytotoxic insults[6]. To examine the contribution of Foxo-mediated transcriptional induction of *Bim* in this scenario, we isolated thymocytes from *Bim*<sup>ΔFoxo/ΔFoxo</sup>, *Foxo3a*<sup>-/-</sup>, *Bim*<sup>-/-</sup> and wt animals, and cultured them in the absence of cytokines or treated them with ionomycin and dexamethasone. Phorbol ester (PMA) an apoptotic stimulus that does not depend on Bim[6] served as a control. The *Bim*<sup>-/-</sup> and to a lesser extent the *Foxo3a*<sup>-/-</sup> thymocytes showed abnormally increased survival in simple medium, but the *Bim*<sup>ΔFoxo/ΔFoxo</sup> thymocytes died at the same rate as their wt counterparts (FIG 4). As previously reported[6], loss of Bim substantially reduced ionomycin induced killing of thymocytes, but cells from the *Bim*<sup>ΔFoxo/ΔFoxo</sup> and *Foxo3a*<sup>-/-</sup> mice behaved like wt thymocytes (FIG 4). Finally, thymocytes from *Bim*<sup>-/-</sup> and to a lesser extent those from *Foxo3a*<sup>-/-</sup> mice survived treatment with dexamethasone better than cells from the wt or *Bim*<sup>ΔFoxo/ΔFoxo</sup> mice (FIG 4).

***Foxo-mediated transcriptional activation of Bim is dispensable for cytokine withdrawal induced apoptosis of hematopoietic cells***

Previous studies showed that cytokine deprivation activates Foxo3a and that the resulting apoptosis can be inhibited by expression of a dominant negative Foxo3a[12-14]. It was therefore concluded that Foxo3a-mediated transcriptional induction of *Bim* is essential for cytokine deprivation induced apoptosis.

Mature T cells require IL-7 for survival[18] and the cytokine deprivation-induced death of these cells requires Bim[6]. To examine the role of Foxo-mediated transcriptional upregulation of *Bim* in this setting, we isolated CD4<sup>+</sup> and CD8<sup>+</sup> T cells from the spleens of *Bim*<sup>ΔFoxo/ΔFoxo</sup>, *Foxo3a*<sup>-/-</sup>, *Bim*<sup>-/-</sup> and wt mice and cultured them in the presence or absence of IL-7. CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells of all genotypes survived well in the presence of IL-7, but only the *Bim*<sup>-/-</sup> T cells showed extended survival in the absence of this cytokine, whereas the *Foxo3a*<sup>-/-</sup> and *Bim*<sup>ΔFoxo/ΔFoxo</sup> T cells died at similar rates as the wt cells (FIG 5A, upper panel).

Bim is also critical for cytokine deprivation-induced apoptosis in mature B cells[6]. When FACS-sorted transitional (IgM<sup>+</sup>IgD<sup>+</sup>) and follicular (IgM<sup>low</sup>IgD<sup>+</sup>) B cells from the spleens were cultured in simple medium, only the *Bim*<sup>-/-</sup> cells showed extended survival, whereas *Foxo3a*<sup>-/-</sup> and *Bim*<sup>ΔFoxo/ΔFoxo</sup> B cells died at similar rates as the wt B cells (FIG 5A, lower panel).

During shutdown of immune responses, a substantial fraction of activated T cells are killed to minimise the risk of immunopathology and to make space for subsequent immune responses against different pathogens[19]. Since Bim triggered by a reduction in

cytokine levels is essential for this death of activated T cells[10], Foxo transcription factors have been implicated as the critical inducers of *Bim* in this process[14]. We examined the importance of Foxo-mediated transcriptional induction of *Bim* in cytokine deprivation-induced killing of activated T cells. In the presence of IL-2, mitogen stimulated T cell blasts of all genotypes survived comparably. *Bim*<sup>-/-</sup> T cell blasts were almost completely protected from cytokine deprivation and those from the *Foxo3a*<sup>-/-</sup> mice showed modest (albeit significant) protection (FIG 5B). In contrast, the *Bim*<sup>ΔFoxo/ΔFoxo</sup> T cell blasts died at the same rate as wt cells (Figure 5B). Upon IL-2 withdrawal, *Bim* mRNA and Bim protein levels increased to similar extents in *Bim*<sup>ΔFoxo/ΔFoxo</sup>, *Foxo3a*<sup>-/-</sup> and wt T cell blasts (FIG 5C).

In order to examine whether direct transcriptional induction of Bim by Foxo3a or related Foxo family members is critical for growth factor deprivation induced apoptosis of myeloid cells, we generated IL-3+SCF-dependent mast cells from the bone marrow of *Bim*<sup>ΔFoxo/ΔFoxo</sup>, *Bim*<sup>-/-</sup>, *Foxo3a*<sup>-/-</sup> and wt mice. As reported[20], mast cells from *Bim*<sup>-/-</sup> and *Foxo3a*<sup>-/-</sup> mice survived cytokine deprivation significantly better than their wt counterparts, but the *Bim*<sup>ΔFoxo/ΔFoxo</sup> mast cells died at a normal rate (FIG 5D). Interestingly, cytokine deprivation caused comparable levels of Bim protein upregulation in wt, *Foxo3a*<sup>-/-</sup> and *Bim*<sup>ΔFoxo/ΔFoxo</sup> mast cells (FIG 5E).

Collectively, these results show that direct induction of *Bim* mRNA expression by Foxo transcription factors is not essential for cytokine deprivation-induced apoptosis in diverse hematopoietic cell types.

***Myc-driven lymphoma development is not affected by the *Bim*<sup>ΔFoxo</sup> mutation***

Both, the pro-apoptotic BH3-only protein Bim and Foxo transcription factors have been shown to act as tumor suppressors[17, 21]. Loss of both or even one allele of *Bim* substantially accelerates pre-B/B lymphoma development in *Eμ-myc* transgenic mice[22]. To test whether Foxo-mediated transcriptional induction of *Bim* is critical for its tumour suppressive function, we crossed the *Bim*<sup>ΔFoxo/ΔFoxo</sup> mice with the *Eμ-myc* transgenic mice[23] and monitored tumour development. There was no significant difference in the rate of lymphoma development or tumour immunophenotype (~60% B220<sup>+</sup>sIg<sup>-</sup> pre-B and ~40% B220<sup>+</sup>sIg<sup>+</sup> B) between *Eμ-myc* (control), *Eμ-myc;Bim*<sup>ΔFoxo/wt</sup> and *Eμ-myc;Bim*<sup>ΔFoxo/ΔFoxo</sup> mice (supplementary FIG 3A & B). We therefore conclude that direct transcriptional induction of *Bim* by Foxo transcription factors is not critical for Bim's tumour suppressive function.

## Discussion

The pro-apoptotic BH3 only Bcl-2 family member Bim is critical for several developmentally programmed pathways to cell death in the hematopoietic system [8][9][6]. Studies *in vitro* have shown that Bim is essential for the death of diverse cell types triggered by a broad range of apoptotic stimuli[6].

Despite the importance of Bim in so many processes, no transcriptional and/or post-transcriptional regulator has been shown to be critical in physiological settings *in vivo*. This is particularly the case for cytokine deprivation-induced apoptosis, a process that is critical for at least some of the functions of Bim within the whole animal[24]. Experiments using immortalised cytokine-dependent cell lines and enforced expression of dominant negative or constitutively active Foxo3a mutants have indicated that this



transcription factor binds within the two *Bim* promoters and is essential for the activation of *Bim* in cytokine deprivation-induced apoptosis[12-14]. It appears likely that the dominant negative mutant of Foxo3a can also inhibit transcriptional induction of target genes by the other Foxo family members, Foxo1, Foxo4 and Foxo6[17]. Therefore several of these transcription factors may be able to drive *Bim* induction in response to growth factor withdrawal.

To determine the impact of loss of transcriptional induction of *Bim* by all Foxo family members, we generated mice in which all four potential Foxo binding sites in the two *Bim* promoters had been mutated. In contrast to *Bim*-deficient cells[6], T as well as B lymphoid cells and mast cells from these animals were normally sensitive to cytokine deprivation. How can these results be reconciled with previous studies demonstrating a role for Foxo transcription factors in *Bim* activation in response to cytokine deprivation[12-14]? It remains theoretically possible that Foxo transcription factors can directly activate *Bim* expression by binding to sites other than the four that we have mutated. However, our sequence analysis and overexpression of Foxo3a in T cell blasts from *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup> mice (supplementary FIG 2C) did not reveal any additional candidate Foxo binding sites in the *Bim* promoter region. It also remains possible that Foxo proteins regulate *Bim* expression indirectly, for example by inducing another transcription factor that does bind to the *Bim* promoter or by controlling the expression of microRNAs that regulate *Bim* expression levels. In this regard it is noteworthy that the mir17-92 cluster has been shown to regulate apoptosis by repressing *Bim* expression[25]. It will therefore be interesting to examine whether the mir17-92 cluster can be regulated by Foxo transcription factors. Alternatively, cytokine deprivation may activate *Bim* by a

mechanism that is entirely independent of the Foxo transcription factors. This question might be resolved by generating cells that lack all Foxo family members, although such cells may be non-viable due to the loss of essential functions of Foxo transcription factors that are not related to transcriptional induction of *Bim*. It must also be noted that our studies were conducted exclusively with hematopoietic cells. *Bim* expression was reported to be regulated by Foxo transcription factors in neurons[26]; hence it will be interesting to use our *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup> mice to examine the physiological relevance of this finding. However, the mechanisms that control Bim expression in response to growth factor withdrawal in hematopoietic cells remain to be defined.

It is interesting that loss of Foxo3a could significantly delay spontaneous and dexamethasone induced thymocyte apoptosis and cytokine deprivation-induced apoptosis of T cell blasts and mast cells, although it did not reduce Bim expression. This indicates that Foxo3a must regulate a critical pro-apoptotic gene other than *Bim* under these stress conditions. Notably, Foxo3a was shown to up-regulate *Puma* expression in response to cytokine deprivation[27] and to cooperate with Bim in spontaneous and dexamethasone induced thymocyte killing, and IL-2 or IL-3+SCF withdrawal induced killing of T cell blasts or mast cells, respectively[7, 28]. This indicates that Foxo3a may play a critical role in these pathways to apoptosis by transcriptional induction of *Puma* (rather than *Bim*).

Finally, we showed that although Bim suppresses Myc-induced lymphomagenesis[22], mutation of all Foxo binding sites in the two *Bim* promoters did not alter the incidence or rate of pre-B/B lymphoma development in *E $\mu$ -myc* transgenic mice. It remains possible that Foxo proteins regulate the expression and thereby the tumour suppressive action of

Bim indirectly (see above for possible processes). Alternatively, the activation of Bim during tumour suppression is mediated by a yet to be identified process that is independent of Foxo transcription factors.

## **Methods**

see supplementary material section

## **Acknowledgements**

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### **Author contributions**

MJH planned and conducted most experiments, interpreted results and wrote the manuscript. LR and MJL performed some experiments. LT performed gene-targeting in ES cells. TK, RG and SG performed the EMSA experiments. PB, TK and AS conceived study, planned experiments, interpreted data and wrote the manuscript.

### **Conflict of interest**

All authors declare that they have no conflicts of interest in regards to this manuscript.

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## Figure Legends

**Figure 1** Identification of candidate Foxo binding sites in the *Bim* promoters. **(A)** Map showing four Foxo binding sites within the two *Bim* promoters. **(B)** Electrophoretic mobility shift assay (EMSA) to test for binding of Foxo3a to the four Foxo binding sites in the *Bim* promoters.  $^{32}$ P-labelled oligos corresponding to sites 1, 2, 3 or 4 were incubated with nuclear extracts from 293T cells transiently transfected with a control plasmid or a plasmid encoding tamoxifen inducible active HA-tagged Foxo3a (HA-Foxo3A(A3)ER) that had been treated for 24 h with tamoxifen (to activate Foxo3a). **(C)** Control and HA-Foxo3a(A3)ER nuclear extracts were left untreated or incubated with either HA or Foxo3A specific antibodies to verify the specificity of Foxo3A binding to site 4 in the *Bim* promoters. Arrows indicate specific bands in **(B)** and **(C)**.

**Figure 2** *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup> mice have normal T lymphoid cell subset composition in the thymus. **(A)** Cell numbers of CD4<sup>-</sup>8<sup>-</sup> progenitors, CD4<sup>+</sup>8<sup>+</sup> immature, CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> mature thymocytes were determined by flow cytometry in wt, *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup>, *Foxo3a*<sup>-/-</sup> and *Bim*<sup>-/-</sup> mice (n indicates the numbers of mice analysed for each genotype). The data represent means +/-SD; \* p<0.05. **(B)** Western blot analysis to determine the levels of Bim protein expression in extracts from thymocytes of wt, *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup>, *Foxo3a*<sup>-/-</sup> and *Bim*<sup>-/-</sup> mice. Probing for  $\beta$ -actin served as a loading control. **(C)** qRT-PCR analysis to determine the levels of *Bim* mRNA expression in extracts from thymocytes of wt, *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup> and *Foxo3a*<sup>-/-</sup> mice. The expression of *Bim* mRNA was normalised to HMBS mRNA. The data represent means +/- SD; \*\* p<0.01.



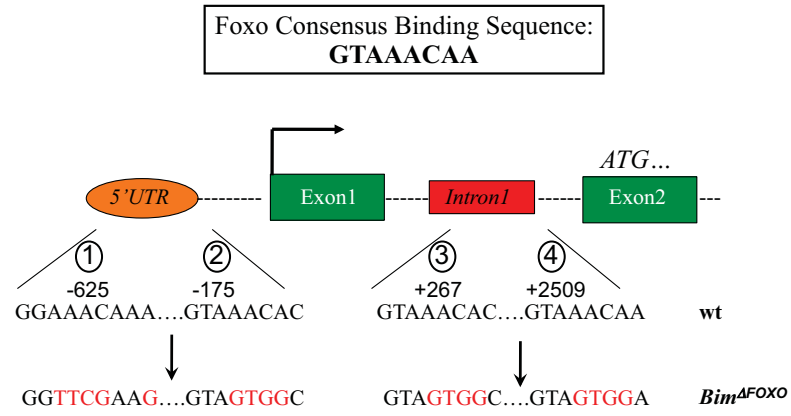
**Figure 3** *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup> mice have normal hematopoietic cell subset composition in the spleen. (A) The numbers of B cells, CD4<sup>+</sup>8<sup>-</sup> as well as CD4<sup>-</sup>8<sup>+</sup> mature T cells, macrophages (Mac-1<sup>+</sup>Gr-1<sup>-</sup>) and granulocytes (Mac-1<sup>+</sup>Gr-1<sup>+</sup>) in the spleens of wt, *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup>, *Foxo3a*<sup>-/-</sup> and *Bim*<sup>-/-</sup> mice were determined by flow cytometry (n indicates the numbers of mice for each genotype analysed). The data represent means +/-SD; \* p<0.05, \*\*\* p<0.0005, \*\*\*\* p<0.0001. (B) Western blot analysis to determine the levels of Bim protein expression in extracts from spleen cells of wt, *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup>, *Foxo3a*<sup>-/-</sup> and *Bim*<sup>-/-</sup> mice. Probing for HSP70 served as a loading control. (C) qRT-PCR analysis to determine the levels of *Bim* mRNA expression in extracts from spleen cells of wt, *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup> and *Foxo3a*<sup>-/-</sup> mice. The expression of *Bim* mRNA was normalised to HMBS mRNA.

**Figure 4** Thymocytes from *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup> mice are normally sensitive to a broad range of apoptotic stimuli that kill these cells in a Bim-dependent manner. Survival of thymocytes from wt, *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup>, *Foxo3a*<sup>-/-</sup> and *Bim*<sup>-/-</sup> mice in culture, either in simple medium (no added growth factors, to simulate growth factor withdrawal) or after treatment with dexamethasone, ionomycin or phorbol ester (PMA), was determined at the indicated time points by flow cytometry (n indicates numbers of mice from each genotype). The data represent means +/-SD; \* p<0.05, \*\* p<0.01, \*\*\* p<0.0005, \*\*\*\* p<0.0001.

**Figure 5** Diverse lymphoid as well as mast cells from *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup> mice are normally sensitive to cytokine deprivation. (A) Survival of FACS-sorted CD4<sup>+</sup>8<sup>-</sup> or CD4<sup>-</sup>8<sup>+</sup> mature T cells and transitional or follicular B cells from the spleens of wt, *Bim* <sup>$\Delta$ Foxo/ $\Delta$ Foxo</sup>, *Foxo3a*<sup>-/-</sup> and *Bim*<sup>-/-</sup> mice in culture in simple medium (no added growth factors) was

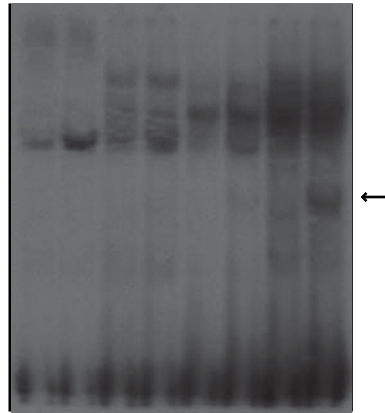
determined at the indicated time points by flow cytometry (n indicates numbers of mice from each genotype). The data represent means +/-SD; \* p<0.05, \*\* p<0.01, \*\*\* p<0.0005, \*\*\*\* p<0.0001. **(B)** Survival of mitogen-stimulated T cell blasts from wt, *Bim*<sup>ΔFoxo/ΔFoxo</sup>, *Foxo3a*<sup>-/-</sup> and *Bim*<sup>-/-</sup> mice in culture in simple medium (after removal of IL-2) was determined at the indicated time points by flow cytometry (n indicates numbers of mice from each genotype). The data represent means +/-SD; \* p<0.05, \*\* p<0.01, \*\*\* p<0.0005, \*\*\*\* p<0.0001. **(C)** qRT-PCR and Western blot analyses to determine the levels of *Bim* mRNA and Bim protein expression, respectively, in the T cell blasts described in **B**, in the presence of IL-2 or 8 h after removal of IL-2 (in the presence of the pan-caspase inhibitor QVD-OPH (20 μM) to prevent RNA and protein degradation). For qRT-PCR analysis *Bim* mRNA levels were normalised to HMBS. Probing for HSP70 served as a loading control. **(D)** Survival of IL-3+SCF-dependent mast cells from wt, *Bim*<sup>ΔFoxo/ΔFoxo</sup>, *Foxo3a*<sup>-/-</sup> and *Bim*<sup>-/-</sup> mice in simple medium (after removal of IL-3+SCF) was determined at the indicated time points by flow cytometry (n indicates numbers of mice from each genotype). The data represent means +/-SD; \*\* p<0.01, \*\*\* p<0.0005, \*\*\*\* p<0.0001. **(E)** Western blot analysis to determine the levels of Bim protein expression in the mast cells described in **D**, in the presence of IL-3+SCF or 8 h after removal of these growth factors (in the presence of the pan-caspase inhibitor QVD-OPH (20 μM) to prevent RNA and protein degradation). Probing for HSP70 served as a loading control.

A



B

FBS ( <sup>32</sup> P) wt:	1		2		3		4	
+control:	+	-	+	-	+	-	+	-
+HA-Foxo3a(A3)ER:	-	+	-	+	-	+	-	+



C

FBS ( <sup>32</sup> P) wt: 4						
+control			+HA-Foxo3a (A3)ER			
+anti-HA:	-	+	-	-	+	-
+anti-Foxo3A:	-	-	+	-	-	+

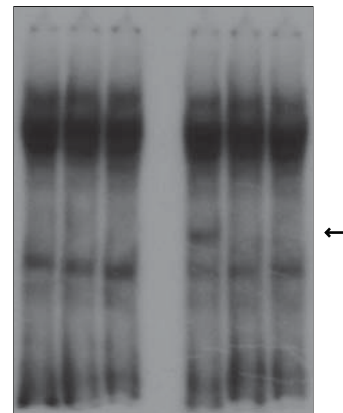


Figure 1

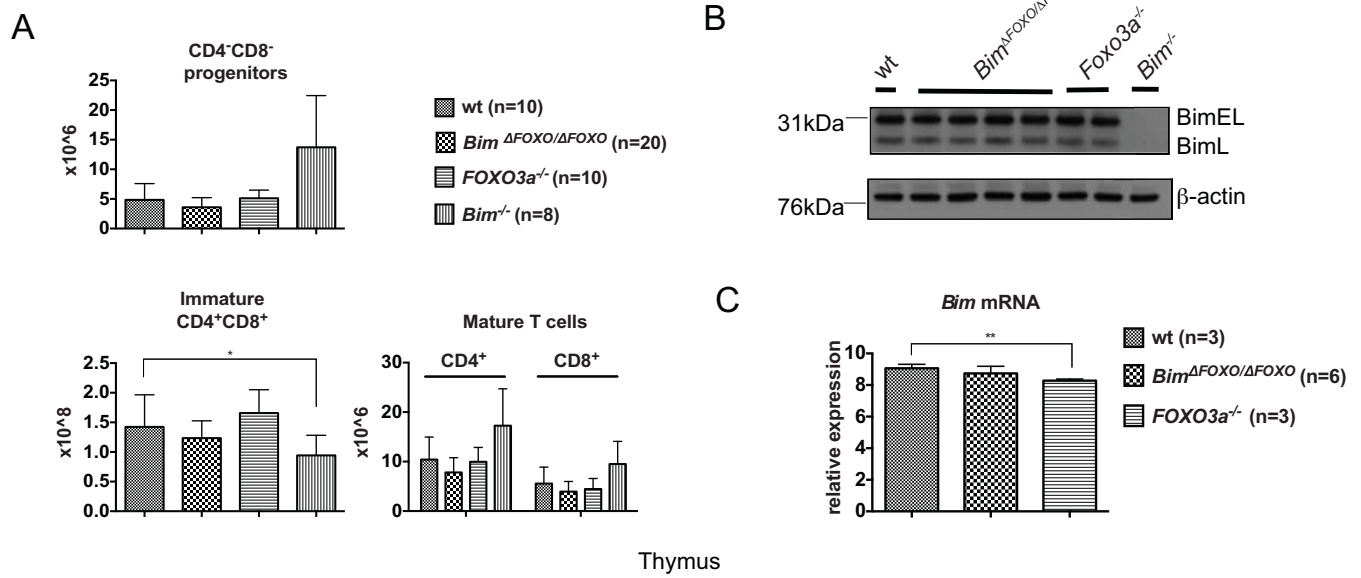


Figure 2

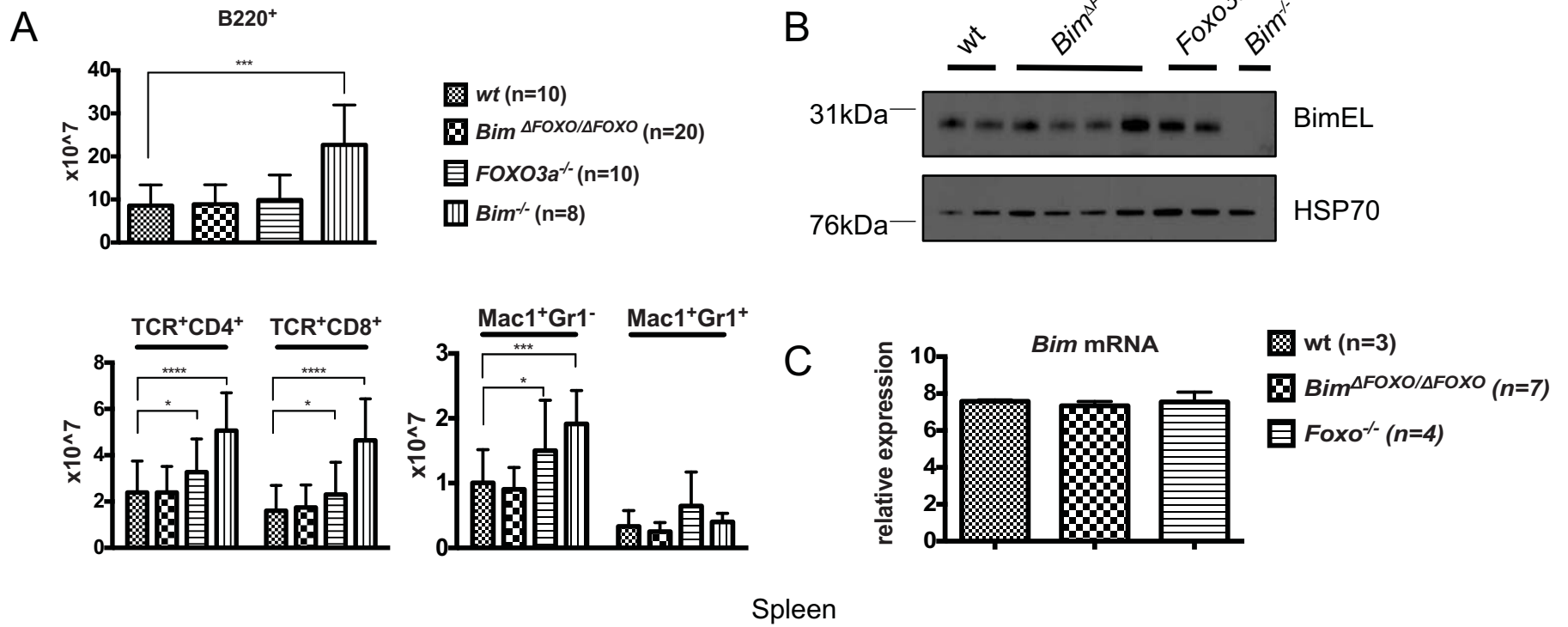


Figure 3

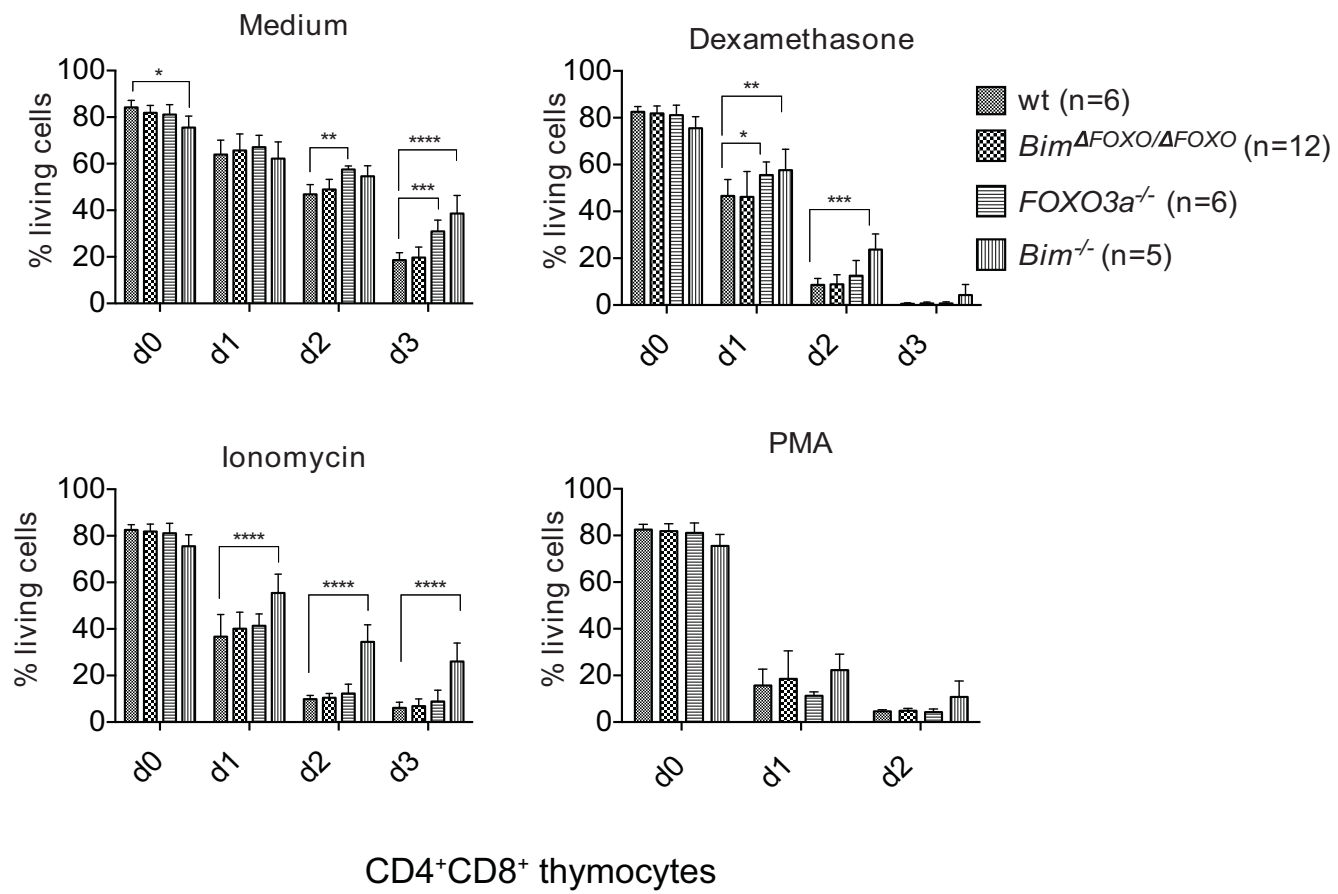


Figure 4

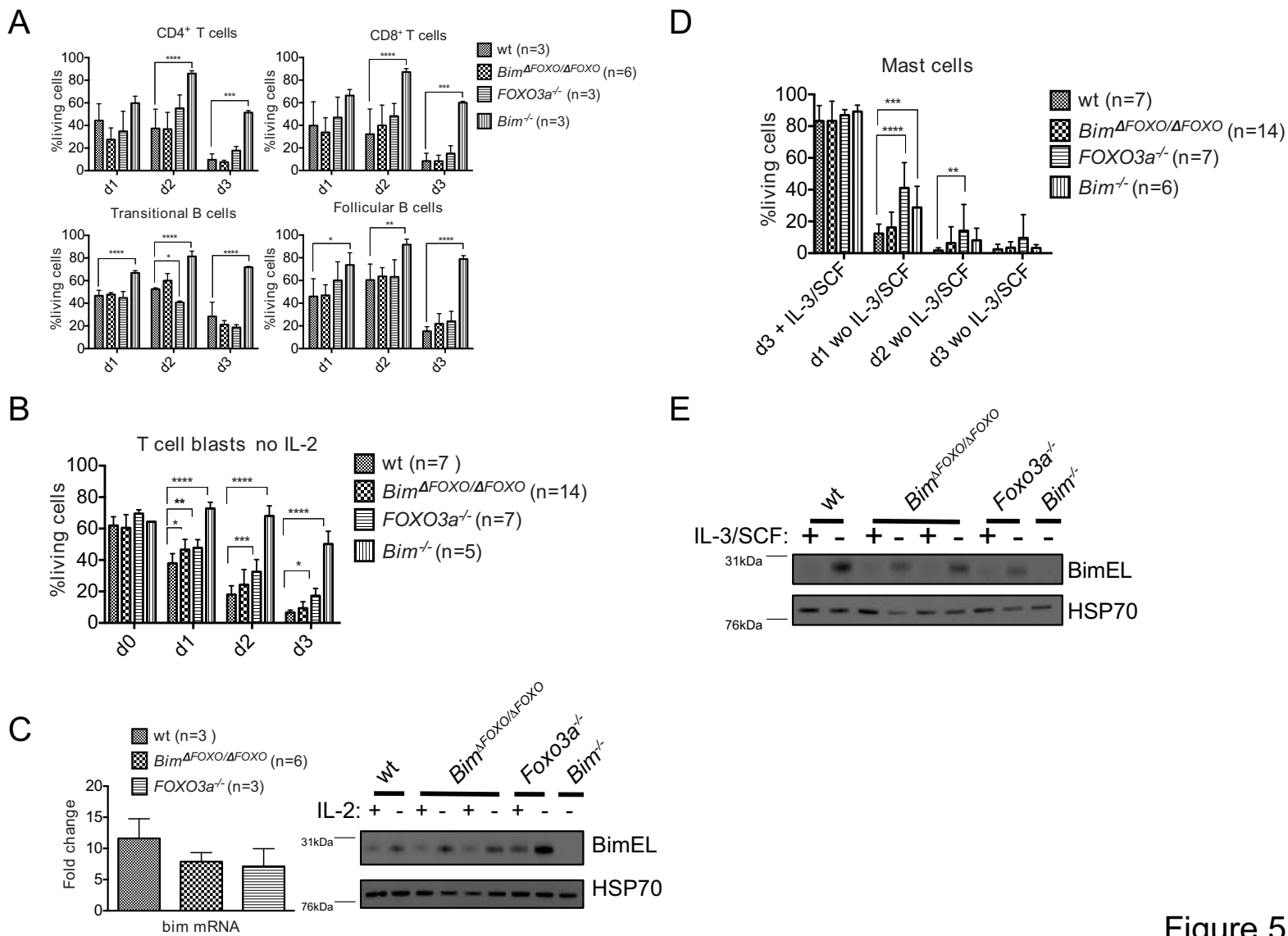


Figure 5