**Foxo3**-/- mice demonstrate reduced numbers of pre-B and recirculating B cells but normal splenic B cell sub-population distribution

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Abstract

B cell antigen receptor (BCR) cross-linking promotes proliferation and survival of mature B cells. Phosphoinositide-3-kinase-mediated down-regulation of pro-apoptotic and anti-mitogenic genes such as the Foxo family of transcription factors is an important component of this process. Previously, we demonstrated that BCR signaling decreases expression of transcripts for Foxo1, Foxo3 and Foxo4. We now show that BCR-induced down-regulation of Foxo3 and Foxo4 mRNA expression occurs via distinct mechanisms from those established for Foxo1. While Foxo1, Foxo3 and Foxo4 bind the same DNA sequence, the differential control of their expression upon B cell activation suggests that they may have unique functions in the B lineage. To begin to address this issue, we evaluated B cell development and function in Foxo3-/- mice. No effect of Foxo3 deficiency was observed with respect to the following parameters in the splenic B cell compartment: sub-population distribution, proliferation, *in vitro* differentiation and expression of the Foxo target genes cyclin G2 and *B cell translocation gene 1*. However, Foxo3-/- mice demonstrated increased basal levels of IgG2a, IgG3 and IgA. A significant reduction in pre-B cell numbers was also observed in Foxo3-/- bone marrow. Finally, recirculating B cells in the bone marrow and peripheral blood were decreased in Foxo3-/- mice, perhaps due to lower than normal expression of receptor for sphingosine-1 phosphate, which mediates egress from lymphoid organs. Thus, Foxo3 makes a unique contribution to B cell development, B cell localization and control of Ig levels.

Introduction

Within the immune system, the maintenance of homeostasis relies on the interplay between survival, proliferation and death-inducing factors. The number of T and B cells that enter the periphery is only a fraction of the total initially generated. Programmed cell death eliminates lymphocytes with non-functional or autoreactive antigen receptors. T and B cells circulating in the periphery must be kept quiescent, only to divide and differentiate if/when they encounter their cognate antigen. These activated cells later undergo apoptosis at the termination of the immune response.

The Foxo family of transcription factors has been the subject of much interest with regard to the control of cellular homeostasis. These pro-apoptotic and anti-mitogenic genes were initially isolated in humans at chromosomal break points in rhabdomyosarcomas (1) and leukemias (2). These transcription factors play a role in a diverse range of physiological processes, including cell survival, proliferation, metabolism and stress resistance (3–5). The three principal family members, Foxo1, Foxo3 and Foxo4, are post-translationally controlled via Akt (also known as protein kinase B) in the Phosphoinositide-3-kinase (PI3K) signaling pathway. In numerous cell types, including T and B lymphocytes, Akt-mediated phosphorylation of Foxos in response to cytokines, growth factors and other stimuli results in their exclusion from the nucleus and subsequent degradation, leading to cell cycle entry (6–13). In B cells pre-stimulated with LPS, over-expression of a constitutively active Foxo protein has been shown to result in cell cycle arrest and apoptosis (13).
Foxo1, Foxo3 and Foxo4 share largely overlapping patterns of tissue expression and interact preferentially with the same DNA-binding motif 5′-TTGTTCAC-3′ (14). This raises the question of functional redundancy. Yet, individual disruption of each gene results in a distinct phenotype (15). Murine knockouts of Foxo1 are embryonic lethal due to defects in angiogenesis (16). Knockouts of both Foxo3 and Foxo4 are viable, however. Foxo3-deficient mice exhibit age-dependent infertility in females (17, 18) as well as spontaneous T cell activation and lymphoproliferation over time (19). Mice deficient in Foxo4 display decreased migration of vascular smooth muscle cells (20).

Previous work in our laboratory has established that in mature B cells, there is an additional means of control for Foxos beyond that established at the post-translational level. Upon engagement of the B cell antigen receptor (BCR) in resting B cells, Foxo mRNA expression is down-regulated via PI3K (21). We now extend our findings to reveal that the mRNA expression of each of the principal Foxo family members is controlled by a unique mechanism downstream of PI3K. This suggests that Foxo family members may have unique functions during B cell development and activation. Indeed, we show here that Foxo3−/− mice have reduced frequencies of pre-B cells in the bone marrow and recirculating B cells in the blood and bone marrow. They also demonstrate increased basal levels of IgG2a, IgG3 and IgA. However, splenic B cell sub-populations are normal and the activation of mature resting B cells in vitro is unimpaired in the absence of Foxo3, suggesting that other Foxo family members may play a role in these processes.

Materials and methods

Mice

Animals were housed and studied in accordance with protocols approved by the institutional animal care and use committee. Wild-type mice used in the inhibitor studies were of the C57BL/6 genetic background. Phospholipase C (PLC) and PDK4 (22) were of mixed genetic background (C57BL/6 × 129). Littermates were therefore compared directly when possible and experiments repeated with multiple litters. Foxo3−/− (17) mice were of the FVB background. Experiments with these mice were completed using wild-type littermates as controls.

Flow cytometry

Splenic, bone marrow, peritoneal wash and peripheral blood were depleted of RBCs and stained with combinations of anti-CD21–FITC, anti-CD43–FITC, anti-IgM–PE, anti-CD23–PE, anti-CD5–PE, anti-B220–PerCP, anti-CD23–biotin, anti-CD93–biotin, anti-CD138–biotin, anti-CD22–biotin (eBioscience, San Diego, CA, USA) and anti-CD127–biotin (eBioscience). Antibodies were purchased from BD PharMingen (San Diego, CA, USA) unless otherwise indicated. Biotinylated antibodies were detected with streptavidin–allophycocyanin (Caltag, Burlingame, CA, USA). Samples were run on a FACS Calibur (Becton Dickinson, San Jose, CA, USA). Data were analyzed with CellQuest (Becton Dickinson) software. Live cells were gated based on forward and side scatter.

B cell purification

Splenic B cells were harvested at time 0 or stimulated with 10 μg/ml goat anti-mouse IgM F(ab′)2 (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 6 h. For inhibitor studies, cells were pre-treated with either 10 μM LY294002, 50 μM Go6983, 10 μM U0126 or 100 ng/ml Cyclosporin A (CsA) for 15 min prior to anti-IgM treatment or 10 μM 11R-VIVIT for 2 h prior to anti-IgM treatment. All inhibitors were obtained from Calbiochem (San Diego, CA, USA).

Quantitative real-time PCR

Total RNA was prepared using the RNeasy Kit (Qiagen, Valencia, CA, USA). cDNA was generated with a cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed in an Applied Biosystems 7300 Real-Time PCR system using TaqMan reagents specific for mouse B cell translocation gene 1 (Btg-1), cyclin D2, cyclin G2, Foxo1, Foxo3, Foxo4, Edg1 [receptor for sphingosine-1-phosphate (S1P1)], Rag2 and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems). Data were normalized to GAPDH using the delta comparative threshold cycle (Ct) method.

B cell proliferation, survival and differentiation

Splenic B cells were plated at 10^6/ml in RPMI + 10% fetal bovine serum (FBS) with media alone, 2 or 20 μg/ml anti-IgM F(ab′)2 fragments (Jackson Immunoresearch Laboratories), 0.1, 1 or 10 μg/ml LPS (Sigma, St. Louis, MO, USA), 10 ng/ml phorbol myristate acetate (Sigma) + 1 μg/ml ionomycin (Calbiochem) or 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN, USA) + 1 μg/ml or 10 μg/ml anti-CD40 (BD PharMingen) for 48 h. Proliferation was measured by labeling cells with [3H]thymidine ([3H]dUrd) (Amersham, Piscataway, NJ, USA) for the final 8 h. Apoptosis was measured by staining with Annexin V–PE and 7-AAD (BD PharMingen). For differentiation studies, splenic B cells were plated at 5 × 10^5/ml in RPMI + 10% FBS with media alone, 20 μg/ml LPS (Sigma) or 1 μg/ml anti-CD40 (BD PharMingen) + 10 ng/ml IL-4 (R&D Systems) for 72 h and subsequently stained with antibodies against B220 and CD138.

Measurement of Ig Levels by ELISA

Mice were bled prior to and 1 and 2 weeks after immunization with either 10 μg trinitrophenol (TNP)–Ficoll (Biosearch Technologies, Novato, CA, USA) in PBS or 100 μg NP–CGG (Biosearch Technologies) in In ject Alum (Pierce, Rockford,
Proliferation was measured by [3H]TdR (Amersham) for varying concentrations of IL-7 (R&D Systems) for 5 days.

Bone marrow was cultured at 10^6 ml in RPMI + 10% FBS + varying concentrations of IL-7 (R&D Systems) for 5 days. Proliferation was measured by [3H]Thymidine incorporation. Additional analysis was performed on cells expanded for 5 days in 10 ng ml^{-1} IL-7. B220+ cells were enumerated by flow cytometry. cDNA was prepared for quantitative real-time PCR (Q-PCR) analysis of Rag2 expression and cell cycle status was assessed by fixing cells in 70% ethanol for >24 h and then incubating with propidium iodide (Roche, Indianapolis, IN, USA) and RNase A (Qiagen) immediately (time 0) or incubated for 6 h in the presence 10 µg ml^{-1} anti-IgM F(ab')_2. (A) Purified wild-type B cells were harvested immediately (time 0) or stimulated for 6 h with 10 µg ml^{-1} anti-IgM F(ab')_2 in the presence or absence of the PI3K inhibitor LY294002 (10 µM), the calcineurin inhibitor CsA (100 ng ml^{-1}), the NFAT inhibitor 11R-VIVIT (10 µM), the PKCβ inhibitor Gö6980 (50 µM) or the ERK inhibitor U0126 (10 µM). (A and B) Independent RNA samples derived from at least two separate B cell preparations were analyzed by Q-PCR for expression of Foxo family members. Expression levels were normalized to GAPDH using the delta Ct method. Results from stimulated cells were then plotted as an average (%± SD) percentage of the expression level in fresh unstimulated B cells of the appropriate genotype (time 0).

CsA, an inhibitor of calcineurin, blocked the down-regulation of Foxo1, Foxo3 and Foxo4 to a similar degree as the PI3K inhibitor LY294002 (10 µM) (Fig. 1b). However, the effects of 11R-VIVIT, which specifically prevents the interaction of calcineurin with the transcription factor NFAT (29), were unique to each Foxo family member (Fig. 1b). 11R-VIVIT did not affect Foxo1 mRNA expression. It inhibited BCR-induced Foxo4 mRNA down-regulation and induced the up-regulation of Foxo3 mRNA. mRNA for all three Foxo family members was down-regulated normally in the presence of the ERK inhibitor U0126 (10 µM). (A and B) Independent RNA samples derived from at least two separate B cell preparations were analyzed by Q-PCR for expression of Foxo family members. Expression levels were normalized to GAPDH using the delta Ct method. Results from stimulated cells were then plotted as an average (%± SD) percentage of the expression level in fresh unstimulated B cells of the appropriate genotype (time 0).

Thus, down-regulation of Foxo1, Foxo3 and Foxo4 mRNA expression following BCR cross-linking is influenced by a distinct set of mechanisms in the PI3K/PLCγ2 signaling cascade. This observation suggests that Foxo family members may have unique functions during B cell development or activation.

We chose to address this issue by characterizing the B cell compartment in Foxo3/−/− mice based on the following observations. First, the pattern of Foxo3 expression in our inhibitor studies was the most distinct, while Foxo1 and Foxo4 expression...
differed only with respect to dependence on NFAT (Fig. 1). Second, Foxo3 has been shown to have an effect on T cell activation, suggesting that it may also play a role in other lymphocytes (19). Finally, Foxo1−/− mice are embryonic lethal (16), while Foxo3-deficient mice are viable and readily examined for B cell phenotypes (17–19).

Fig. 2. Splenic B cell sub-populations are normal in Foxo3−/− mice. Splenocytes of the indicated mouse strain were stained with antibodies against B220, CD21, CD23, and IgM. Plots are representative of four mice. (A) The percentage of total cells in each population is shown. (B) The percentage of B220+CD23low cells in each population (T1 = CD21−/−IgM+, MZ = CD21+/IgM+) is shown. (C) The percentage of B220+CD23high cells in each population (T2 = CD21high/IgMhigh, FO = CD21+IgM+) is shown. (D) The total number of cells in each population is shown as mean ± SD, n = 4. There is no significant difference between wild-type and Foxo3−/− mice.
Foxo3 is dispensable for splenic B cell development and response to BCR engagement

Similar frequencies and total numbers of follicular, marginal zone and transitional B cells were found in the spleens of wild-type and Foxo3−/− mice (Fig. 2). Despite the anti-mitogenic and pro-apoptotic functions of Foxo3, splenic B cells lacking Foxo3 did not demonstrate altered proliferation (Fig. 3a) or survival (Fig. 3b) either basally or in response to anti-IgM. The lack of an effect of Foxo3 deficiency was not due to compensatory changes in the expression levels of Foxo1 or Foxo4 (Fig. 3c). mRNA expression of cyclin G2 and Btg-1, cell cycle inhibitory genes which are known targets of Foxo3 (30–34), was comparable in wild-type and Foxo3−/− B cells both basally and upon BCR engagement (Fig. 3d).

Increased basal IgG2a, IgG3 and IgA levels in Foxo3−/− mice

Forced expression of a constitutively active form of Foxo1 in LPS-stimulated B cells inhibits the ability of these cells to differentiate into plasma cells (35). This suggested that plasma cell differentiation may be increased in the absence of Foxo3. However, up-regulation of the plasma cell marker CD138 was normal in cultures of Foxo3−/− B cells incubated with either LPS or CD40 plus IL-4 (Fig. 4a). Wild-type and Foxo3−/− B cells also had a similar proliferative response to these stimuli (Fig. 4b and c).

Although there was no general enhancement of plasma cell differentiation in vitro in the absence of Foxo3, it is possible that Foxo3 regulates B cell differentiation and antibody production in vivo. We therefore measured serum Ig levels, both basally and in response to immunization. Unimmunized Foxo3−/− mice had significantly increased amounts of total IgG2a, IgG3 and IgA relative to wild-type controls, although levels of total IgM, IgG1 and IgG2b were unchanged (Fig. 5a).

The ability of Foxo3−/− mice to mount a humoral immune response to specific antigens was also assessed. Responses to the T-independent antigen TNP–Ficolll (Fig. 5b) and the T-dependent antigen NP–CGG (Fig. 5c) were not significantly different between wild-type and Foxo3−/− mice, although there was a trend toward a slightly increased response to NP–CGG in the absence of Foxo3 2 weeks after immunization.

Decreased pre-B and recirculating B cells in Foxo3−/− mice

To determine whether Foxo3 uniquely contributes to other stages of B cell development, we examined the bone marrow of Foxo3−/− mice. Intriguingly, while both B220+CD43+ pre-B cells and B220+CD43+ IgM− immature B cells were present at normal numbers, there was a significant reduction in both the percentage and total number of B220+CD43−CD93+IgM− pre-B cells in the absence of Foxo3 (Fig. 6). IL-7 is an important growth and differentiation factor for B cell progenitors. In its absence, B cell development is...
blocked at the transition between pro- and pre-B cells (36). However, we did not observe an impaired response to IL-7 in the absence of Foxo3. The IL-7R alpha chain (CD127) was expressed at similar levels on pro- and pre-B cells from wild-type and Foxo3−/− mice (Fig. 7a). B220+ cells from both wild-type and Foxo3-deficient bone marrow expanded

Fig. 4. Normal in vitro proliferation and differentiation of Foxo3−/− B cells in response to LPS and anti-CD40+IL-4. (A) Purified B cells were stimulated with 20 μg ml⁻¹ LPS or 1 μg ml⁻¹ anti-CD40 plus 10 ng ml⁻¹ IL-4 for 72 h and stained with anti-B220 and anti-CD138 antibodies. The frequency of CD138+ plasma cells is indicated for one of two to four representative mice per genotype. (B) Purified B cells were stimulated with the indicated concentrations of LPS for 48 h. Cells were labeled with [³H]TdR for the final 6 h of culture to measure proliferation. Data represent mean ± SD of triplicate cells and are representative of two independent experiments. (C) Purified B cells were stimulated with the indicated concentrations of CD40 + 10 ng ml⁻¹ IL-4 for 48 h. Cells were labeled with [³H]TdR for the final 6 h of culture to measure proliferation. Data represent mean ± SD, n = 4.
to a similar degree (2- to 3-fold) upon culture with IL-7 for 5 days. IL-7-expanded cells from Foxo3−/− bone marrow pro-liferated normally (Fig. 7b) and had a cell cycle profile nearly identical to that of wild-type cells (Fig. 7c). Rag2 is required for both heavy and light chain rearrangement and thus the development of B cells beyond the pro-B cell stage (37). However, IL-7-stimulated pre-B cultures from wild-type and Foxo3−/− bone marrow expressed similar levels of Rag2 mRNA (Fig. 7d).

Analysis of Foxo3−/− bone marrow also revealed decreased frequencies and numbers of B220hiIgM−CD93− recirculating B cells (Fig. 6). Since homing of mature B cells to the bone marrow is controlled in part by the interaction of CD22 on B cells with a sialic acid-containing ligand in the bone marrow microenvironment (38, 39), we examined expression of CD22 on Foxo3−/− B cells (Fig. 8a). No difference was observed.

An alternative explanation for the decreased recirculating B cells in Foxo3−/− bone marrow is that these mice demonstrate a general impairment of B cell migration rather than a defect in homing specifically to the bone marrow. To address this issue, we analyzed B cells in the peripheral blood and peritoneal cavity. Foxo3−/− mice had a reduced frequency of B cells in the peripheral blood and fewer mature B-2 cells in the peritoneal cavity than wild-type mice (Fig. 8b–d).

Sphingosine-1 phosphate (S1P) contributes to the egress of lymphocytes from the peripheral lymphoid organs and their subsequent circulation (40). Mice deficient in S1P1, a receptor for S1P, have only a small reduction in splenic B cells but a dramatic decrease in recirculating B cells in the blood and bone marrow (40). Inhibition of S1P signaling with FTY720 also reduces peritoneal B cell numbers (41). PI3K has recently been shown to down-regulate S1P1 mRNA expression in T cells (42). Taken together, these results suggested that Foxo3 may control expression of S1P1. Indeed, there was a small, but consistent, reduction in S1P1 mRNA in Foxo3−/− B cells to ~75% of wild-type levels (Fig. 8e).

**Discussion**

Here, we provide evidence that the expression of Foxo3 is controlled distinctly from other Foxo family members during B cell activation. We also show that it plays a unique role in regulating pre-B cell numbers, B cell recirculation and levels of total serum IgG2a, IgG3 and IgA. Foxo3 is dispensable, however, for the normal distribution of splenic B cell subpopulations and the response of splenic B cells to BCR cross-linking, CD40 plus IL-4, and LPS in vitro.

In B cells, activation of PI3K leads to phosphorylation and nuclear exclusion/degradation of Foxo protein (13) and the down-regulation of Foxo mRNA (21). In this study, we describe how the mRNA expression of each Foxo family member is influenced by a distinct set of mechanisms in the PI3K/PLCγ2 signaling cascade. BCR signaling induces down-regulation of Foxo1 mRNA via calcineurin but not NFAT, Foxo3 mRNA via PKCβ and calcineurin/NFAT and Foxo4 mRNA via calcineurin/NFAT. Calcineurin is known to have functions independent of NFAT (28, 29, 43). For example, BCR-induced activation of serum response factor (SRF) is inhibited by CsA (28). However, SRF is unlikely to be the downstream target of calcineurin responsible for the regulation of Foxo1 at the mRNA level. Whereas SRF activity is influenced by both PKCβ and ERK (28), we have shown that the mRNA expression of Foxo1 is unaffected by inhibitors of these molecules (21).

Promoter studies in a mature B cell line would permit the identification of the specific cis-acting elements and transcription factors that control expression of each Foxo family member. However, in our hands, basal levels of Foxo mRNA expression in the A20 cell line are low and remain
unchanged in response to BCR stimulation (data not shown). Consistent with this observation, in studies of the promoter region of cyclin G2 in this cell line by Chen et al. (32), it was only after co-transfection of Foxos with the reporter construct that significant luciferase activity was observed. These findings are not surprising given the known anti-mitogenic properties of Foxo family members and the difference in cell cycle status between continuously proliferating cell lines and resting mature primary B cells.

Foxo3 has been shown to transactivate the cyclin G2 and Btg-1 promoters in reporter assays in A20 B cells and NIH 3T3 cells, respectively (32, 34). However, we show that Foxo3 is not required for expression of these target genes in primary mature B cells. Nor is it necessary for the development or in vitro activation of normal splenic B cell sub-populations. This suggests one of two possibilities. First, Foxo family members may be redundant for the control of target genes in splenic B cells. Addressing this question will likely require elimination of all three Foxo family members specifically in mature B cells. When deletion of Foxo1, Foxo3 and Foxo4 is induced simultaneously in all cells of adult animals, an extensive developmental block in the B cell lineage as well as a reduction in hematopoietic stem cells occurs (44).

Alternatively, individual Foxo family members may have unique targets in B cells. In support of this model, the mRNA expression patterns for both cyclin G2 and Btg-1 closely correlate with that of Foxo1, but not Foxo3 or Foxo4, in B cells treated with anti-IgM and various signaling inhibitors (data not shown). This suggests that Foxo1 may play a prominent role in regulating these and other Foxo targets controlled by BCR engagement in splenic B cells. Indeed, a study published while the manuscript was in revision demonstrates that Foxo1-deficient B cells have altered responses to BCR cross-linking (45).

Although Foxo3 is dispensable for the normal sub-population distribution of splenic B cells, we show that it
has a unique role in regulating pre-B cell numbers. Despite this observation, no difference in IL-7R expression or expansion of B220+ cells in IL-7 cultures was observed between wild-type and Foxo3−/− bone marrow. Rag2 expression was also normal in IL-7-expanded pre-B cells from Foxo3−/− mice. Impairment of earlier stages of B lymphopoiesis may...

Fig. 7. Foxo3−/− bone marrow cells respond normally to IL-7. (A) Bone marrow was stained with antibodies against IgM, B220, CD43 and IL-7Rα (CD127). The level of IL-7Rα on pro- and pre-B cells (B220+IgM−) is shown for wild-type (shaded histogram) and Foxo3−/− (open histogram, dark line) mice. (B) Bone marrow cells were plated in media alone or varying concentrations of IL-7 at 10^6 ml^{-1} for 5 days. Proliferation was measured by [3H]TdR incorporation for an additional 24 h. Data represent mean ± SD, n = 3. (C) Bone marrow cells were plated in 10 ng ml^{-1} IL-7 at 10^6 ml^{-1} for 5 days and stained with propidium iodide (PI) to assess cell cycle status. The frequency of apoptotic (sub 2n) and S/G2/M cells (>2n) is indicated. Plots are representative of n = 3–4. (E) cDNA was prepared from day 5 IL-7 bone marrow cultures and subjected to Q-PCR for Rag2. Expression levels were normalized to GAPDH using the delta Ct method and plotted as a percentage of the average expression level in wild-type B cells. Data represent mean ± SD, n = 2.

Fig. 8. Reduced recirculating B cells in Foxo3−/− mice. (A) Splenocytes were stained with antibodies against CD22, B220, CD21 and CD23. The level of CD22 expression on follicular B cells (B220+CD23+CD21+) from wild-type (shaded histogram) and Foxo3−/− (open histogram, dark line) mice is shown. Data are representative of three mice. (B) B220 expression is shown for cells in the lymphocyte gate of peripheral blood from wild-type (shaded histogram) and Foxo3−/− (open histogram, dark line) mice. Data are representative of three mice. (C) Peritoneal cells were stained with antibodies against B220 and CD5. The frequency of B-2 (B220+CD5−) and B-1 (B220−/CD5+) cells in the lymphocyte gate is indicated. Data are representative of three mice. (D) The mean ± SD of three mice analyzed as in (B and C) is shown. *P < 0.05 by paired Student’s t-test. (E) Purified B cells were analyzed by Q-PCR for expression of S1P1 (Edg1). Expression levels were normalized to GAPDH using the delta Ct method and plotted as a percentage of the average expression level in wild-type B cells. Each symbol represents an independent experiment with one mouse of each genotype. *P < 0.05 by paired Student’s t-test.
play a role, however. While the frequency and number of pre-B cells was not significantly different between wild-type and Foxo3−/− mice, there was a trend toward a slight reduction in this population in the absence of Foxo3. In addition, fewer cells fell into the lymphoid gate in Foxo3−/− bone marrow. Thus, some of the effect of loss of all three Foxo family members on hematopoietic stem cells and pro-B cells (44) may be due specifically to Foxo3. Foxo3 may also act in the bone marrow microenvironment to support the development or survival of pre-B cells. Intriguingly, mice lacking Foxo1 specifically in the B lineage were recently shown to also have a block in development at the pre-B stage (45). However, unlike the case in Foxo3−/− mice, the absence of Foxo1 results in decreased expression of both Rag2 and IL-7R in early B lineage cells (45, 46). Thus, Foxo3 and Foxo1 maintain pre-B cell numbers via distinct mechanisms.

Foxo3 deficiency also results in a decreased frequency of recirculating B cells in the bone marrow and blood as well as a reduction in B-2 cells in the peritoneum. This function of Foxo3 is B cell intrinsic, as a similar result was observed in a recent study examining mice lacking Foxo3 specifically in B cells (47). B cell expression of CD22, which mediates B cell homing to the bone marrow (38, 39), was normal in the absence of Foxo3. However, mRNA encoding S1P1+, which mediates S1P-induced egress of B and T cells from lymphoid organs (40), was reduced in B cells from Foxo3−/− mice. This is consistent with recent observations indicating that PI3K signaling down-regulates S1P1 expression via Foxo transcription factors in T cells (42, 48). B cells from S1P1+/− mice demonstrate impaired exit from peripheral lymphoid organs, suggesting that degree of change in S1P1 expression seen in Foxo3−/− B cells is physiologically relevant (49). S1P has recently been shown to promote egress by counteracting other chemokines that retain lymphocytes in the spleen and lymph nodes (50). Foxo3 deficiency may disrupt the balance of these signals in favor of retention by altering responses to either S1P or other chemokines. It is possible that Foxo3 targets in addition to S1P1 also promote B cell egress, migration or homing. It should be noted that the block in B cell recirculation is incomplete in Foxo3−/− mice, indicating that other Foxo family members, or Foxo-independent mechanisms, likely contribute this process as well.

Finally, Foxo3 has a unique role in regulating basal levels of serum IgG2a, IgG3 and IgA. This could result from either a heightened ability of B cells to respond to factors that induce switching to these isotypes (i.e. IFNγ and transforming growth factor β) or an increased production of these factors by other cell types. The latter is a likely possibility given that generalized CD4+ T cell hyperactivity has been reported in an independently generated line of Foxo3−/− mice (19). Mice were able to respond and class switch upon immunization with TNP-Ficoll and NP-CGG in the absence of Foxo3. This is in contrast to mice lacking Foxo1 in mature B cells, which fail to class switch in response to either T-independent or T-dependent antigens (45). Taken together, these results indicate that Foxo1 and Foxo3 regulate Ig production via distinct mechanisms.

Foxos and their targets play important roles in diseases associated with altered proliferation and/or activation of B cells. Resistance to cell death in B-chronic lymphocytic leukemia has been linked to inactivation of Foxo3 via protein phosphorylation (51). Similarly, BCR-ABL, which causes both chronic myelogenous leukemia and acute lymphocytic leukemia, acts in part by down-regulating Foxo3 (52, 53). It has also been proposed that increased PKCβ signaling, which we show here down-regulates Foxo3 mRNA levels in normal B cells stimulated through the BCR, could contribute to lymphoma (26). These findings are interesting in that they support the theory that both transcriptional and post-translational control of Foxo family members could have consequences for B cell malignancy. Btg-1, like the Foxo transcription factors themselves (2), has been associated with chromosomal translocations in leukemia (54). mRNA expression of this target gene is also up-regulated in EBV-transformed B cells from X-linked agammaglobulinemia patients (55), suggesting that aberrant expression of Btg-1 has consequences not only in cancer but immunodeficiency as well. Foxo family members may also play a role in immune tolerance. Foxo-induced Rag expression in immature B cells is likely to promote elimination of autoreactive B cells via receptor editing (46, 47). Preliminary studies have also shown altered Foxo1 transcript levels in the PBMC of both systemic lupus erythematosus and rheumatoid arthritis patients (56). Thus, teasing out the roles for each Foxo family member and its associated target genes in the maintenance of B cell homeostasis has implications for prevention and/or treatment of cancer, immunodeficiency and autoimmunity.

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Abbreviations
BCR B cell antigen receptor
Btg-1 B cell translocation gene 1
CsA cyclosporin A
Ct comparative threshold cycle
FBS fetal bovine serum
GAPDH glyceraldehyde-3-phosphate dehydrogenase
HITTdr [3H]thymidine
PKC phosphoinositide-3-kinase
PKC protein kinase C
PLC phospholipase C
Q-PCR quantitative real-time PCR
S1P sphingosine-1-phosphate
SIP receptor for sphingosine-1-phosphate
SRF serum response factor
TNP trinitrophenol

References
Foxo3 in B cell development and activation


