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FOXO1 REGULATES MARGINAL ZONE B CELL DEVELOPMENT

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Summary

A fundamental component of signaling initiated by the BCR and CD19 is the activation of phosphoinositide 3-kinase (PI3K). Downstream of PI3K, the protein kinase AKT phosphorylates several substrates, including members of the Forkhead Box Subgroup O (Foxo) transcription factor family. Among the Foxo proteins, Foxo1 has unique functions in bone marrow B-cell development and peripheral B-cell function. Here we report a previously unrecognized role for Foxo1 in controlling the ratio of mature B-cell subsets in the spleen. Conditional deletion of Foxo1 in B cells resulted in an increased percentage of marginal zone B cells and a decrease in follicular B cells. In addition, Foxo1 deficiency corrected the absence of marginal zone B cells that occurs in CD19-deficient mice. These findings show that Foxo1 regulates the balance of mature B cell subsets and is required for the marginal zone B-cell deficiency phenotype of mice lacking CD19.

Keywords

B cell development; B cells; signal transduction; transcription factors

Introduction

BCR crosslinking activates PI3K, whose lipid products orchestrate the assembly of membrane-associated signaling complexes [1]. One group of proteins, termed the BCR signalosome, is responsible for maximal activation of phospholipase C-γ and subsequent phosphoinositide hydrolysis and Ca^{2+} mobilization. Another outcome of PI3K signaling is the activation of AKT. The AKT the serine/threonine kinases have numerous substrates, whose phosphorylation state controls diverse processes including proliferation, survival, metabolism and differentiation. The roles of most AKT substrates in B cell biology have not been defined.

CD19 is a transmembrane protein that enhances BCR signaling by multiple mechanisms [2,3]. CD19 interacts with CD21 and CD81 to form the B cell co-receptor, which clusters with the BCR in response to complement-tagged antigens to augment signaling. CD19 can also associate with the BCR in the absence of CD21 to promote BCR signalosome assembly upon recognition of membrane-associated antigens [4]. The cytoplasmic tail of CD19 contains two canonical motifs for recruitment of PI3K (YXXM), and these are required for

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Conflict of interest
The authors declare no financial or commercial conflict of interest.

CD19 function [5]. Genetic evidence supports a functional role for AKT downstream of CD19, in that combined deletion of two AKT genes (*Akt1* and *Akt2*) in mouse B cells confers a defect in marginal zone (MZ) B cell development [6] similar to the phenotype of CD19-deficient mice [5,7]. However, it is not yet clear which AKT substrates regulate MZ-cell development.

Foxo transcription factors activate or suppress target genes in a cell type-specific and context-dependent manner [8,9]. In resting lymphocytes, Foxo proteins are localized to the nucleus and activate genes that maintain quiescence as well as proper homing and recirculation [1]. Phosphorylation by AKT causes cytoplasmic sequestration and degradation of Foxo factors, inhibiting the Foxo gene expression program. The Foxo1 family member has been studied in lymphocytes by conditional deletion using Cre-lox systems. This work has identified unique roles for Foxo1 in several aspects of B cell function [10]. Deletion of the *Foxo1* gene in early B cell progenitors using $Mb1^{Cre}$ caused a block at the pro-B cell stage. Deletion at a later stage with $Cd19^{Cre}$ caused a partial block at the pre-B cell stage. Deletion of *Foxo1* in late transitional B cells with $Cd21^{Cre}$ blocked class-switch recombination. We have examined in more detail the phenotype of mature B cells in mice with $Cd19^{Cre}$-mediated deletion of *Foxo1*. We find that these mice have fewer follicular (FO) B cells and a higher percentage of MZ cells. In mice homozygous for the $Cd19^{Cre}$ knock-in allele, which lack CD19 protein, MZ cells are absent as reported previously [5,7] but this defect is reversed by the concomitant deletion of *Foxo1*. This genetic epitasis analysis suggests the possibility that CD19 negatively regulates Foxo1 to promote MZ B cell development.

Results and Discussion

Deletion of Foxo1 affects B cell development

We generated a conditional *Foxo1* allele by inserting LoxP sites flanking the first exon of Foxo1 [11]. Mice homozygous for the *Foxo1*-flox allele are denoted $Foxo1^{f/f}$ herein. We bred $Foxo1^{f/f}$ mice with $Cd19^{Cre}$ mice in which the Cre recombinase is knocked into the *Cd19* locus [12]. Splenic B cells from $Foxo1^{f/f}Cd19^{Cre}$ mice expressed no detectable Foxo1 protein as determined by immunoblot, while Foxo3a expression was unchanged (Supporting Information Fig. 1A). Several aspects of B cell development in these mice were altered in a manner similar to the phenotype of another strain of $Foxo1^{f/f}Cd19^{Cre}$ mice reported by Dengler and colleagues [10]. In particular, our $Foxo1^{f/f}Cd19^{Cre}$ mice had fewer IgM^+ bone marrow B cells (Supporting Information Fig. 1B), and a population of peripheral $B220^+$ cells lacking surface expression of IgM or IgD (Supporting Information Fig. 1C). Furthermore, $Foxo1^{f/f}Cd19^{Cre}$ mice had markedly fewer lymph node (LN) B cells and an increase in peripheral blood B cells (Supporting Information Fig. 1D). The paucity of LN B cells correlated with reduced surface expression of CD62L (L-selectin), the LN homing receptor (Supporting Information Fig. 1E). The mice also had a reduced percentage of $CD5^+$ B cells in the peritoneal cavity (Supporting Information Fig. 1F).

The report from Dengler et al. did not examine the developmental status or function of peripheral $B220^+IgM^+$ cells in $Foxo1^{f/f}Cd19^{Cre}$ mice [10]. We stained splenocytes from our $Foxo1^{f/f}Cd19^{Cre}$ mice and controls with antibody combinations that distinguish two mature subsets (FO, MZ) and four transitional B cell subsets (T1, T2, T3 and marginal zone precursor (MZP)) [13]. When compared to control $Foxo1^{f/+}Cd19^{Cre}$ mice, $Foxo1^{f/f}Cd19^{Cre}$ mice displayed a consistent and statistically significant increase in the percentage of MZ cells, defined as $B220^+AA4.1^-IgM^{hi}CD21^{hi}CD23^{lo}$ (Fig. 1A). In contrast, the percentage of FO cells ($B220^+AA4.1^-IgM^{lo}CD21^{int}CD23^{hi}$) was reduced (Fig. 1A). A normal percentage of MZP cells was present in $Foxo1^{f/f}Cd19^{Cre}$ mice, despite reduced percentages of T1 and T2 cells; this suggests that immature transitional cells might commit preferentially to the

MZP stage. The absolute numbers of splenocytes were equivalent between *Foxo1*$^{f/f}$*Cd19*^{Cre} mice and controlmice (data not shown). Increased abundance of B220+ cells in the splenic marginal zone and other extrafollicular regions was also apparent by immunofluorescent staining of spleen sections (Fig. 1B). The percentages of mature FO and MZ cells were comparable in the two control groups (*Foxo1*$^{f/+}$*Cd19*^{Cre} and *Foxo1*$^{f/f}$) (Fig. 1A), and other experiments showed a consistently greater population of MZ cells (B220^{+}CD21^{hi}CD23^{lo}) in *Foxo1*$^{f/f}$*Cd19*^{Cre} compared to *Foxo1*$^{f/f}$ mice (data not shown). Therefore, we used *Foxo1*$^{f/f}$ mice as controls in Figure 1B and in other experiments to simplify breeding schemes.

The altered balance of FO and MZ cells in *Foxo1*$^{f/f}$*Cd19*^{Cre} mice was not observed in analyses of mice with Foxo1-deficient B cells generated using *Cd21*^{Cre} [10]. A likely explanation is that *Cd21*^{Cre} drives deletion of *Foxo1* at a time point after transitional B cells commit to either the FO or MZ lineage, whereas *Cd19*^{Cre} deletion is complete by this stage. Interestingly, *Foxo1*$^{f/f}$*Cd21*^{Cre} mice [10] shared the reduced LN B cell population and CD62L expression observed here in *Foxo1*$^{f/f}$*Cd19*^{Cre} mice. This could be explained by a requirement for Foxo1 in CD62L gene expression in mature B cells, after *Cd21*^{Cre}-mediated deletion is completed.

Altered proliferative responses in Foxo1-deficient B cells

We purified splenic B cells and activated them in vitro with titrated doses of either a BCR stimulus (anti-IgM) or a TLR stimulus (LPS). We measured cell proliferation and survival by cell division tracking using CFSE. B cells from *Foxo1*$^{f/f}$*Cd19*^{Cre} proliferated more weakly to anti-IgM, compared to B cells from *Foxo1*$^{f/f}$ mice (Fig. 2A). There was no significant difference in apoptosis in *Foxo1*$^{f/f}$*Cd19*^{Cre} B cells stimulated with anti-IgM or LPS (Fig. 2B). The altered response to anti-IgM could arise from a decreased FO/MZ ratio, since BCR engagement causes proliferation of FO cells but apoptosis of MZ cells [14,15]. However, reduced anti-IgM-mediated proliferation was also observed in B cells from *Foxo1*$^{f/f}$*Cd21*^{Cre} mice in which no changes in FO/MZ ratios were reported [10], and is consistent with the presence of a prominent IgM^{-} B cell population (Supporting Information Fig. 1C). Measuring live cell number using a metabolic dye conversion (MTS) assay confirmed the finding of impaired anti-IgM response in *Foxo1*$^{f/f}$*Cd19*^{Cre} B cells (Supporting Information Fig. 2A). The LPS response in *Foxo1*$^{f/f}$*Cd19*^{Cre} B cells was increased when measured using MTS assays (Supporting Information Fig. 2A), but not using the CFSE assay (Fig. 2A). This might indicate that LPS-stimulated B cells have altered metabolism when Foxo1 is absent, leading to increased MTS conversion despite equivalent cell number.

Transforming growth factor-beta (TGF-β) is a cytokine with potent anti-proliferative effects in lymphocytes [16]. TGF-β signaling activates Smad transcription factors, which in several cellular systems cooperate with Foxo proteins to activate target promoters [17,18]. Furthermore, the TGF-β/Smad signaling axis regulates marginal zone B cell development [19]. Although we obtained evidence for functional cooperation of Foxo1 and Smad transcription factors in B cells (Supporting Information Fig. 2B, C), Foxo1 was not required for TGF-β-mediated suppression of B cell proliferation triggered by anti-IgM or LPS (Supporting Information Fig. 2A).

Gene expression changes in Foxo1-deficient B cells

CD62L mRNA was consistently reduced about 3-fold in *Foxo1*$^{f/f}$*Cd19*^{Cre} B cells (Fig. 2C), indicating that lower CD62L protein expression on the surface of these cells is at least partly due to reduced steady state mRNA levels, resulting from altered transcription and/or RNA processing. Foxo1 also controls expression of the *Sell* gene encoding CD62L in T cells [20–22]. Another Foxo target gene, *Klf2*, regulates CD62L expression in T cells and might be a link between Foxo1 and CD62L [20,21,23–25]. *Klf2* mRNA expression was also

significantly reduced in Foxo1-deficient B cells, though less prominently than the reduction in *Sell* mRNA (Fig. 2C). Previously, we identified *Ccng2*, *Rbl2* and *Klf4* as Foxo target genes in B cells [26,27]. By various criteria, including reporter assays, electrophoretic mobility shift assays and chromatin immunoprecipitation, these genes were regulated similarly by Foxo1 and Foxo3a [26,27]. RNA measurements using quantitative real-time PCR showed that none of these genes were differentially expressed in Foxo1-deficient B cells (Fig. 2C), further suggesting that Foxo1 and Foxo3a have redundant functions at these target promoters.

Foxo1 deficient complements MZ phenotype in CD19 knockout mice

The increased population of MZ B cells in $Foxo1^{f/f}Cd19^{Cre}$ mice was intriguing, since Foxo factors are turned off by the PI3K/AKT pathway and the opposite phenotype occurs in mice lacking PI3K genes [28–30]. CD19-deficient mice, which display reduced PI3K/AKT signaling in B cells, also have a greatly reduced MZ population [5,7], as do chimeric mice generated with *Akt1*/*Akt2*-deficient progenitors [6]. Conversely, loss of the PTEN phosphatase that opposes PI3K signaling expands the MZ subset and overcomes the loss of CD19 [31]. Like the MZ cell increase in $Foxo1^{f/f}Cd19^{Cre}$ mice, the MZ cell decreases in mice lacking PI3K, *Akt1*/*Akt2* or CD19 are B cell-intrinsic [6,7,32]. We therefore considered the possibility that Foxo1 inactivation is central to MZ lineage choice promoted by CD19/PI3K. It was convenient to test this possibility for CD19 in our system, since breeding of the $Cd19^{Cre}$ knock-in allele to homozygosity generates mice lacking CD19 expression. As expected, homozygous $Cd19^{Cre/Cre}$ mice had a profound reduction in the MZ population as determined by CD21/CD23 staining (Fig. 3A, B) and immunofluorescent staining of spleen sections (Fig. 3C). In CD19/Foxo1 double-deficient mice (genotype = $Foxo1^{f/f}Cd19^{Cre/Cre}$), the frequency of MZ B cells was restored to the levels seen in $Foxo1^{f/f}Cd19^{Cre}$ mice, again elevated relative to $Foxo1^{f/f}$ mice (Fig. 3A, B). Therefore, loss of Foxo1 has a dominant effect on MZ lineage choice and is sufficient to complement the MZ B cell defect arising in CD19-deficient mice. Interestingly, CD19/Foxo1 double-deficient mice had a greater reduction of FO B cells than either $Foxo1^{f/f}Cd19^{Cre}$ or $Cd19^{Cre/Cre}$ mice (Fig. 3A, B). Further work is required to investigate whether this phenomenon results from impaired development or survival of CD19/Foxo1 double-deficient FO cells.

Concluding Remarks

CD19 is essential for proper B cell development and activation, and most of these functions require the PI3K binding sites in the cytoplasmic tail of CD19 [5] and are opposed by PTEN [31]. One phenotype shared by mice lacking CD19 or PI3K/AKT signaling components is a near absence of MZ B cells. Other work has shown that the MZ lineage choice is promoted by a low level of self-antigen [33] and that CD19 associates with BCR signaling clusters and promotes activation even in the absence of complement fragments and co-receptor action [4]. Together these observations suggest a model in which CD19 promotes MZ development by enhancing self antigen-triggered BCR signaling and PI3K activation. CD19 and PI3K augment Ca2+ mobilization, in part through membrane recruitment and activation of the tyrosine kinase BTK [34]. However, mice lacking BTK have a normal MZ B cell compartment [29,35]. Recent findings indicate that AKT, a well known downstream target of PI3K, is a relevant effector for MZ B cell lineage choice [6]. The results presented here suggest that of the many downstream sequelae of AKT activation, the inactivation of Foxo1 is integral to the developmental choice between FO and MZ B cell lineages. Along with previous findings demonstrating stage-specific functions of Foxo1 in B cells and T cells [10,21,22], these findings further illustrate the complex and context-specific role of forkhead transcription factors such as Foxo1 in lymphocyte development, differentiation, and activation.

Materials and methods

Mice

Foxo1$^{f/f}$ mice were reported previously [11] and were used here in a mixed genetic background. CD19-Cre C57Bl/6 mice were purchased from the Jackson Laboratory. CD19-Cre C57Bl/6 mice were bred to *Foxo1*$^{f/f}$ mice and the progeny were intercrossed to generate mice of the different genotypes used in this study. Control mice were littermates or relatives in a similar mixed background. All animal protocols were approved by the Institutional Animal Care and Use Committee of University of California, Irvine.

Flow cytometry

Single cell suspensions were obtained from the spleens, lymph nodes, bone marrow and peritoneal lavages of 6–8 week-old mice. Cell suspensions from spleen and bone marrow were depleted of RBCs by hypotonic lysis. Approximately 1 million cells were used for antibody staining. All antibodies were purchased from eBioscience. Data from at least 20, 000 total events were acquired and analyzed (FACSCalibur and CellQuest software, BD Biosciences; FlowJo software, Treestar).

Immunohistochemistry

Immunohistochemistry was carried out as previously described [3] with slight modification. Mouse spleens were harvested, embedded in OCT medium (Sakura, Torrance, CA) and frozen in 2-methylbutane precooled by liquid nitrogen. Eight-micrometer sections were cut and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Slides were cleared with CitriSolv (Fisher), fixed with Acetone in −20°C for 20 min, and blocked with 10% goat serum (Vector Laboratories, Burlingame, CA) in PBS for 30 min at room temperature (25°C). Immunohistochemical staining was done sequentially with rat anti-mouse B220 (BD Biosciences), goat anti-rat IgG Alexa Fluor 594 (Molecular Probes) and FITC-conjugated rat anti-mouse metallophilic macrophage (MOMA-1, MCA947F; Serotec) each diluted in PBS, for 1 h at room temperature, and followed by 3×5 min wash in PBS after each staining. All images shown were acquired at 10× magnification using Olympus Fluoview FV1000 Laser Scanning Confocal Microscope.

B cell proliferation and survival assays

Purified B cells were cultured in RPMI 1640 supplemented with 10% heatinactivated FBS, 5 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-ME at 37°C incubator with 5% CO_2. For cell division tracking, B cells were labeled with carboxyfluorescein succinimidylester (CFSE) as described previously [2,4,5]. Labeled cells were stimulated in 48-well dishes with goat anti-mouse IgM ($F(ab')_2$,; Jackson ImmunoResearch Laboratories, West Grove, PA) or LPS (serotype 0127:B8; Sigma-Aldrich, St Louis, MO). After 66 hr, cells were harvested, stained with Annexin-V-PE and analyzed by FACS. For the MTS assay format, B cells were first treated with or without TGF-β (Sigma) at 5ng/ml for 15 minutes, and were stimulated in triplicate in 100 μl of total volume in 96-well flat bottom dishes, using mitogens as described for the CFSE assays. After an incubation period of 66 hr, twenty microliters of CellTiter 96 Aqueous One Solution Reagent (Promega) were added to each well, incubated for 3-4 hours at 37°C, and then absorbance at 490 nm was determine in a plate reader.

qRT-PCR

RNA was prepared from purified B cells of *Foxo1*$_{f/f}$*Cd19*$_{Cre}$ and *Foxo1*$_{f/f}$ mice using Trizol reagent (Invitrogen) as previously described [1,2]. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Primers for genes of interest (*Klf2, Klf4,*

Ccng2, *Rbl2*, *Sell* and *Ltb*) and housekeeping gene (β-actin) were optimized to amplify products between 75 and 200 nucleotides. Primer sequences are available on request. Q-PCR was performed with SyBr green as previously described [1,2].

Statistics

A two-tailed student's t-test was used for all comparisons. The specifics of each test (one vs. two-tailed) are indicated in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PI3K	phosphoinositide 3-kinase
Foxo	Forkhead Box Subgroup O
FO	follicular
MZ	marginal zone
Tr	transitional
TGF-β	Transforming growth factor-beta

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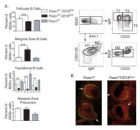

Figure 1.

Comparison of splenic B cell subsets by FACS and histology. (A) Flow cytometry was used to distinguish FO, MZ and immature (T1, T2, T3 and MZP) subsets. Spleen cells were stained with antibodies to B220, AA4.1, IgM, CD21 and CD23. The gating strategy is shown on the right for a representative control spleen. Graphs on the left depict mean cell percentages + SD. $n = 4$ *Foxo1*$^{f/+}$*Cd19*^{Cre} and *Foxo1*$^{f/f}$*Cd19*^{Cre}; $n = 2$ *Foxo1*$^{f/f}$. *$p<0.05$; ***$p<0.001$, unpaired two-tailed t-test. (B) Immunofluorescent staining of spleen sections with antibodies to B cells (anti-B220; red) and macrophages lining the marginal zone (anti-MOMA-1, green) shows increased population of MZ B cells (white arrows) in *Foxo1*$^{f/f}$*Cd19*^{Cre} mice. Confocal images (10X magnification; scale bar = 100μM) are representative of at least 10 follicles from 2 mice of each genotype.

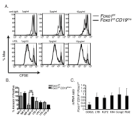

Figure 2.

Comparison of proliferation, apoptosis and gene expression. (A) Purified B cells from *Foxo1*$^{f/f}$*CD19*^{Cre} and control *Foxo1*$^{f/f}$ mice were labeled with CFSE, then stimulated with anti-IgM or LPS at the indicated concentrations for 66 h at 37 °C. Filled gray histogram shows unstimulated control cells. Similar results were obtained in 4 separate experiments. (B) In 3 of the CFSE labeling experiments, cells were stained at 66 hr with Annexin V to quantitate apoptotic cells among the divided population. Data show mean + SD. Unpaired two-tailed t-tests showed no significant differences between genotypes (p>0.05 for all comparisons; p = 0.055 for comparison labeled “NS”). (C) Quantitative real-time PCR (qRT-PCR) data using RNA extracted from B cells of *Foxo1*$^{f/f}$*CD19*^{Cre} and control *Foxo1*$^{f/f}$ mice. The numbers indicate the ratio of each gene in *Foxo1*$^{f/f}$*CD19*^{Cre} as compared to *Foxo1*$^{f/f}$ mice, after normalization to βactin. Data show mean + SD of three separate experiments. *p<0.05; * p<0.01; comparing ratio of each gene to 1.0 using a one-tailed t-test.

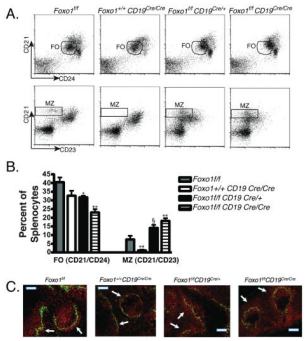

Figure 3.

Assessment of complementation of the MZ B cell defect in CD19-deficient mice. (A) Flow cytometry was used to compare FO and MZ B cell percentages from representative mice. B220-gated cells were analyzed for CD21 and CD24 to quantitate FO cells ($CD21^{int}CD24^{int}$), or CD21 and CD23 to quantitate MZ cells ($CD21^{hi}CD23^{lo}$). (B) The percentages of FO and MZ cells in the spleens from mice of each genotype are shown. Data represent mean ± SD, n = 3-5 mice. *p<0.05; **p<0.01 vs. $Foxo1^{f/f}$, using unpaired, two-tailed t-test. §p = 0.051 vs. $Foxo1^{f/f}$. (C) Immunofluorescent staining of spleen sections was done as in Figure 1B.