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Impaired B Cell Development in the Absence of Krüppel-like Factor 3

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Krüppel-like factor 3 (Klf3) is a member of the Klf family of transcription factors. Klfs are widely expressed and have diverse roles in development and differentiation. In this study, we examine the function of Klf3 in B cell development by studying B lymphopoiesis in a Klf3 knockout mouse model. We show that B cell differentiation is significantly impaired in the bone marrow, spleen, and peritoneal cavity of Klf3 null mice and confirm that the defects are cell autonomous. In the bone marrow, there is a reduction in immature B cells, whereas recirculating mature cells are noticeably increased. Immunohistology of the spleen reveals a poorly structured marginal zone (MZ) that may in part be caused by deregulation of adhesion molecules on MZ B cells. In the peritoneal cavity, there are significant defects in B1 B cell development. We also report that the loss of Klf3 in MZ B cells is associated with reduced BCR signaling strength and an impaired ability to respond to LPS stimulation. Finally, we show increased expression of a number of *Klf* genes in Klf3 null B cells, suggesting that a Klf regulatory network may exist in B cells. *The Journal of Immunology*, 2011, 187: 5032–5042.

Krüppel-like factors (Klfs) are a family of 17 transcription factors that are characterized by three C-terminal classical C2H2 zinc fingers (1). They have multiple roles in regulating cellular differentiation in many tissues (2), and a number of Klfs play important roles in hematopoiesis (3). Klf1 is essential for erythropoiesis (4); Klf2 is required for T cell migration and survival (5), and has recently also been shown to be involved in B cell differentiation, migration, and homing (6–8). Klf4 has roles in both monocyte and B cell differentiation and activation (9, 10). Accordingly, their deregulation underlies many human blood diseases, including thalassemia, lymphoma, and leukemia (3, 11, 12).

Klf3 was first identified in an erythroid screen for factors related to Klf1 (13). Klf3 is generally accepted to act as a transcriptional repressor in combination with its corepressor Ctbp (14), although in certain contexts it can function as an activator (15, 16). Its hematopoietic role has not been fully characterized, although it is known to be a direct target gene of the erythroid factor Klf1 (17). Initial studies of the Klf3 knockout mouse revealed a role in adipogenesis (18); however, Klf3 is broadly expressed, and its functions in other tissues are beginning to be described (19).

A number of studies have revealed a role for Klf3 in B cell function. The immortalized pre-B cell line 18-81 is notable in having a single Abelson-murine leukemia proviral integration within the *Klf3* locus, resulting in increased expression of Klf3 (20). This cell line undergoes premature somatic hypermutation and class switching, suggesting a role for Klf3 in B cell activation and potentially tumorigenesis. In support of a role in B cell activation, it has been observed that Klf3 expression levels are altered in response to Ag stimulation of naive B cells (21). Klf3's role in B cell cancer is further supported by inspection of the Retroviral Tagged Cancer Gene Database developed by Akagi et al. (22), which reveals that viral integration into the *Klf3* locus is associated with lymphoma and marginal zone (MZ) B cell cancer. Finally, we have recently shown that B cell-specific transgenic overexpression of Klf3 results in increased MZ B cells in vivo, thereby confirming a role for Klf3 in B lymphopoiesis (23).

To further investigate the in vivo roles of Klf3 in B cell development and function, we have carried out an analysis of B lymphopoiesis in Klf3 null mice (18). We have found that Klf3 has a role in ensuring normal B cell development in the bone marrow, spleen, and peritoneal cavity. Specifically in the bone marrow, there is a marked reduction in pre-B cells and an overrepresentation of recirculating mature B cells. In the spleen, we see defects in MZ B cell positioning, leading to the formation of a poorly defined MZ. As a possible explanation for this, MZ B cells from the spleen of Klf3 knockout mice show increased cell surface expression of the trafficking molecules CD62L and β7 integrin. Klf3 null MZ B cells also have an impaired response to both LPS stimulation and BCR crosslinking. In the peritoneal cavity of Klf3 knockout mice, there is a significant reduction in the percentage and number of B1 B cells. Finally, we observed

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Abbreviations used in this article: FL, fetal liver; FO, follicular; Klf, Krüppel-like factor; LT-α, lymphotoxin α; MZ, marginal zone.

derepression of *Klf8* and *Klf12* genes in sorted B cell populations in the absence of Klf3 protein. This suggests that a network of Klfs may exist in B cells, and that the related factors Klf8 and Klf12 may be able to partially compensate for the lack of Klf3 in Klf3 knockout mice.

Materials and Methods

Mice

The generation and genotyping of the Klf3 knockout mouse is described elsewhere (18). Mice were maintained on either FVB/NJ or C57BL/6 backgrounds. All procedures were approved by the Animal Care and Ethics Committee, University of Sydney (Approval no. L02/6-2009/3/5097). Age-matched littermates were sacrificed between 10 and 12 wk of age.

B cell isolation and flow cytometry

Single-cell suspensions in RPMI 1640/0.5% FCS were obtained by passing cells through 70-μm nylon filters. RBCs were lysed in ACK buffer containing 10 mM potassium bicarbonate, 150 mM ammonium chloride, and 0.1 mM EDTA, pH 7.3. B cells were purified using either the B Cell Isolation kit from Miltenyi Biotec or by FACS. Follicular (FO) and MZ B cells were identified by including Ab stains for B220, CD21, and CD23. Abs were used at optimally titrated concentrations and cells incubated for 1 h in the dark at 4°C. Live/dead gating was performed by inclusion of either 7-aminoactinomycin D (Sigma-Aldrich) or Topro-3 (Invitrogen). Flow cytometry was performed using FACSCalibur and FACSAria instruments (BD Bioscience). Analysis was done using FlowJo Software (TreeStar).

Abs

All Abs were supplied by BD Biosciences, unless otherwise indicated. The following Abs were used for flow cytometry: PE rat anti-mouse CD45R/B220 (RA3-6B2); FITC anti-mouse BP-1 (6C3; eBioscience); PE anti-mouse integrin β7 (M293), FITC anti-mouse CD11b (MI/70); FITC anti-mouse CD21/CD35 (7G6); PE-Cy7 anti-mouse CD23 (FcεRII; B3B4; eBioscience); PE anti-mouse CD43 (S7); PE anti-mouse CD5 (53-7.3); PE anti-mouse CD62L (MEL-14; eBioscience); allophycocyanin anti-mouse CD93 (AA4.1; eBioscience), Alexa Fluor 647 anti-mouse IgD (11-26c.a; BioLegend); and FITC anti-mouse IgM (II/41).

Fetal liver chimeras

Embryonic day 14.5 Klf3$^{-/-}$ or Klf3$^{+/+}$ (C57BL/6, CD45.2^{+}) fetal liver (FL) cells were mixed 1:1 with wild-type E14.5 FL cells (C57BL/6.SJL-Ptprca, CD45.1^{+}). C57BL/6 recipient mice were whole-body irradiated with two doses of 425 cGy (X-RAD 320 Biological Irradiator; PXI) 24 h before reconstitution. A total of 2×10^6 FL cells was injected into the tail vein of each recipient. Mice were analyzed 8 wk after reconstitution.

Immunohistology

Snap-frozen spleens were sectioned at a thickness of 5–7 μm. Sections were fixed with ice-cold acetone for 10 min and stored at −80°C until used. After rehydration with PBS, sections were blocked with 30% horse serum for 15 min and then incubated with anti–CD3-biotin (500A2; eBioscience), Alexa Fluor 555-streptavidin (Invitrogen), anti–IgD-Alexa Fluor 647 (11-26c.2a), and anti–IgM-FITC (R6-60.2; BD Biosciences). Slides were mounted with Fluoromount G (Southern Biotech). Images were acquired on a Zeiss Inverted microscope at 10× objective magnification.

In vitro CFSE B cell stimulation assays

Wild-type and Klf3 knockout FO and MZ B cells were isolated from mouse spleens using the B Cell Isolation kit from Miltenyi Biotec, followed by FACS after Ab staining for expression of B220, CD21, and CD23. Sorted cells were resuspended in RPMI 1640/0.5% FCS at 1×10^7 cells/ml at room temperature. Cells were then incubated with 5 μM CFSE at 37°C for 15 min in the dark and washed with RPMI 1640/0.5% FCS. After this, cells were seeded in complete RPMI 1640 at 5×10^5 cells/ml and incubated with 10 μg/ml bacterial LPS for 72 h. Cells were then harvested and retained CFSE analyzed by flow cytometry. The experiment was repeated three times.

Calcium mobilization assays

Wild-type and Klf3 knockout splenic B cells were isolated from mouse spleens using the B Cell Isolation kit from Miltenyi Biotec. A total of 3×10^6 B cells was then pelleted in polypropylene tubes at room temperature. Cells were resuspended at a concentration of 10^6–10^7 cells/ml in cell loading medium, containing 4 mM probenecid, 4 μg/ml Fluo-4 AM, and 10 μg/ml Fura Red AM (Molecular Probes), and incubated for 30 min at 37°C. Baseline fluorescence signals were collected for 60 s. Cells were then stimulated with 26 μg/ml anti-mouse IgM F(ab′)$_2$ fragments (Jackson Immunoresearch), and calcium flux was determined by measuring dye fluorescences for 300 s with samples maintained at 37°C. FO and MZ B cells were identified by including Ab stains for B220, CD21, and CD23. The experiment was repeated three times, with at least 500 events/s being counted for each stimulated cell population.

RNA extraction and cDNA synthesis

RNA extraction was performed using TRI-Reagent, according to manufacturer's guidelines (Sigma-Aldrich). RNA samples were further purified using RNeasy columns (Qiagen) and by treating with DNase I (Ambion). cDNA was prepared using Superscript VILO cDNA synthesis kit (Invitrogen), according to instructions.

Primers and real-time PCR

Primers were designed using Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and synthesized by Sigma-Aldrich. Primer sequences are available on request. Real-time PCRs were prepared using SYBR Green PCR master mix kit (Roche) and performed using the ABI PRISM 7700 Sequence Detection System. Samples were run in triplicate with a minimum of three animals per genotype.

Microarray analysis

For global gene expression analysis, total CD19^{+} splenic B cells were positively sorted using MACS (Miltenyi Biotec). RNA was then labeled using the Affymetrix IVT Express kit and hybridized in quadruplicate to Affymetrix Mouse Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA). Robust multichip average normalization of microarray data was carried out using Affymetrix Power Tools (v1.8.6), and normalized data were analyzed by Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea). Microarray data are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) via accession number GSE31622.

Statistical analysis

Statistical analysis was done using Prism 5.0 (GraphPad). Statistical significance was determined using the two-tailed Student *t* test.

Results

Klf3 is required for normal B cell development in the bone marrow

To study the role of Klf3 in B cell development in the bone marrow, we used flow cytometry to assess the pro-B, pre-B, and immature B cell populations. Bone marrow B cells were stained for B220 and CD43 to distinguish pro-B cells (B220^{int}/CD43^{+}), immature B cells (B220^{int}/CD43^{-}), and mature recirculating B cells (B220^{hi}/CD43^{-}). This revealed that, in Klf3 null bone marrow, there is a significant increase in the percentage of recirculating mature B cells, with a concomitant reduction in pro-B and immature B cells (Fig. 1*A*, *top panel*). Additional staining for BP-1 and CD24 allowed identification of the Hardy B cell subsets, fractions A–C′ (Fig. 1*A*, *middle panel*). Staining for IgM and IgD divided the more mature CD43^{-} B cells into pre-B (fraction D), immature (fraction E), and mature recirculating B cells (fraction F) (24) (Fig. 1*A*, *lower panel*). This analysis revealed that in the absence of Klf3, there is an equivalent number of cells in fraction A (Fig. 1*C*). However, in Klf3 knockout mice, there are significantly fewer cells in subsequent fractions (B–D), suggesting that Klf3 may play a role in the initial transition of B cells from fraction A to fraction B.

We also observed a noticeable difference in B cell differentiation when comparing the early (fractions A–E) and mature recirculating B cell populations (fraction F) in the absence of Klf3 (Fig. 1*A*). There is a significant increase in the percentage and number of recirculating mature B cells, at the apparent expense of the earlier

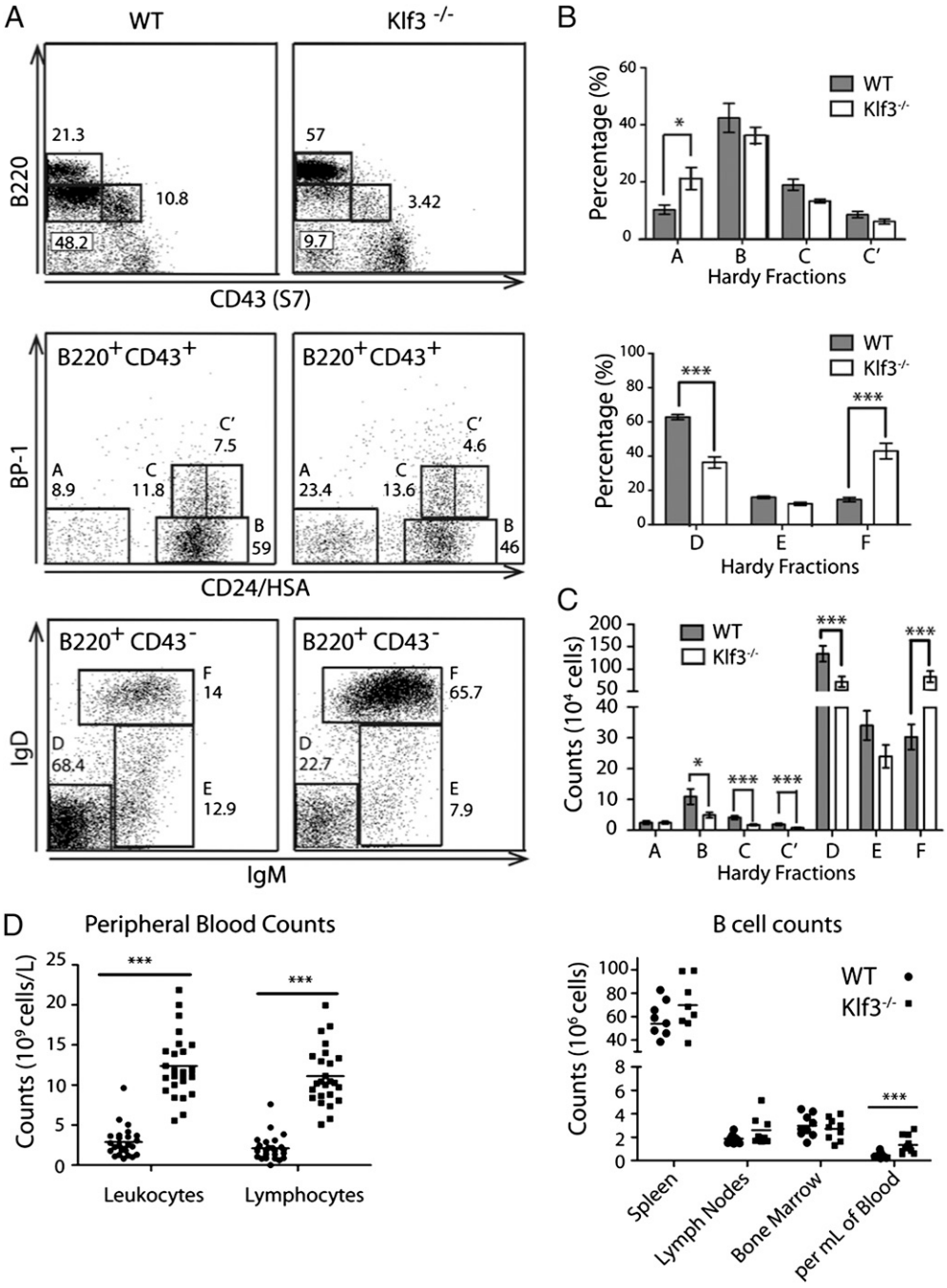

FIGURE 1. B cell development is disrupted in the bone marrow of Klf3 knockout mice. Bone marrow cells from wild-type and Klf3$^{-/-}$ mice were collected at 12 wk of age. RBCs were lysed and single-cell suspensions analyzed by flow cytometry to identify Hardy fractions A–F. *A*, Representative plot of fractions A–F, comparing wild-type and Klf3 knockout mice. *B*, Statistical analysis of fractions A–F, comparing wild-type ($n = 9$) and Klf3 knockout mice ($n = 9$). *C*, B cell counts for fractions A–F, comparing wild-type and Klf3 knockout mice. *D*, Peripheral WBC and secondary lymphoid tissue B cell counts comparing wild-type ($n = 9$) and Klf3 knockout mice ($n = 13$) at 12 wk of age. *$p < 0.05$, ***$p < 0.001$.

B cell populations in Klf3 null bone marrow (Fig. 1*B*, 1*C*). However, there is no difference in the total bone marrow B cell count comparing wild-type and Klf3 null mice (Fig. 1*D*).

Given the dramatic increase in fraction F cells in the Klf3 null bone marrow, we decided to examine the number of B cells in the peripheral blood, spleen, and lymph nodes of Klf3 knockout mice. We also observed a significant increase in the number of B cells in the peripheral blood in the absence of Klf3 (Fig. 1*D*). However, we did not see a difference in B cell counts in the spleen and lymph nodes when comparing wild-type and Klf3 knockout mice (Fig. 1*D*).

Klf3 is required for MZ B cell positioning in the spleen

On exiting the bone marrow, maturing B cells migrate to the spleen, where they undergo further differentiation through a number of transitional stages to become either MZ or FO B cells. We have previously determined that both the percentage and number of MZ B cells are significantly decreased in the spleen of Klf3 null mice (23), demonstrating that Klf3 plays a role in normal MZ B cell development.

To further investigate the role of Klf3 in MZ B cell development, we carried out an immunohistological analysis of the spleen of Klf3 knockout mice (Fig. 2*A*). Staining of FO (IgD^{hi}) and MZ (IgM^{hi})

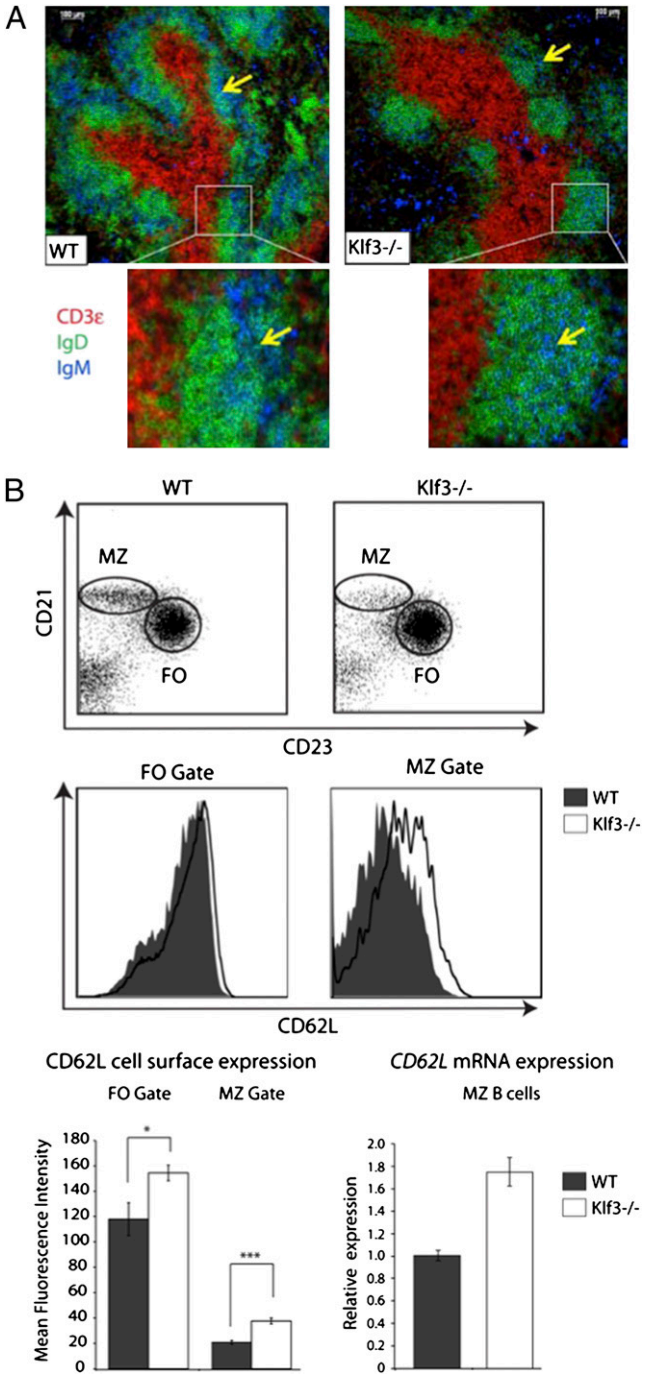

FIGURE 2. MZ B cell development is affected in the spleen of Klf3 knockout mice. *A*, Immunofluorescence analysis of wild-type and Klf3 knockout spleens at 12 wk of age. Spleen sections were stained for CD3 (T cells, red), IgD (FO B cells, green), and IgM (MZ B cells, blue). Arrows compare the normal wild-type MZ with the poorly defined structure seen in the Klf3 knockout section. Images (10×) were obtained on a Zeiss inverted microscope. *B*, Expression of CD62L in Klf3 null MZ B cells. For analysis of cell surface expression, splenic B cells were isolated from eight wild-type and six knockout mice by magnetic bead labeling and column depletion of non-B cells. For each mouse, FO ($CD21^{+}/CD23^{+}$) and MZ ($CD21^{hi}/CD23^{med/lo}$) B cells were gated and analyzed for surface expression of CD62L. Shown are representative flow cytometry plots indicating gating of wild-type and Klf3 knockout FO and MZ B cells (*upper panel*), surface expression of CD62L in wild-type and Klf3 knockout FO and MZ B cells (*middle panel*), and statistical analysis of the mean fluorescence intensity of CD62L surface expression in wild-type and Klf3 knockout gated FO and MZ B cells (*lower panel*). Mice were individually analyzed and error bars represent SEM. $*p < 0.05$, $***p < 0.001$. For analysis of wild-type and Klf3 knockout *CD62L* mRNA levels, MZ B cells were purified by FACS of material pooled from four mice and RNA isolated for real-time RT-PCR analysis (*lower panel*). The relative gene expression was normalized against 18S and the lowest expression set to 1. Real-time runs were performed in triplicate and error bars show SEM.

B cells revealed that the well-defined positioning of MZ B cells around the splenic follicles of the wild-type mouse is absent in the Klf3 null spleen. In knockout mice, the MZ B cell number is reduced, and those MZ cells that are present appear diffusely and aberrantly located, suggesting that Klf3 may have direct or indirect roles in regulating the expression of genes that control B cell migration. To explore this possibility, we first examined global expression changes in cell adhesion and homing genes by sorting $CD19^{+}$ B cells from wild-type and Klf3 null spleens for microarray analysis. We found significant changes in a number of key genes, including those with roles in egress from lymphoid organs (*CD69* and *S1pr3*) (25, 26) and endothelial cell interaction (*CD62L*) (Supplemental Table I).

To determine whether changes in the expression of certain cell surface adhesion molecules in Klf3 null MZ B cells might explain their mislocalization, we sorted MZ B cells from Klf3 knockout mice and examined expression of the trafficking molecules *CD62L* (*L-selectin/Sell*) (27) and *β7 integrin* (*Itgb7*) (28) by real-time PCR. Because MZ B cell numbers are severely reduced in Klf3 knockout mice, cells were pooled from a number of animals to obtain sufficient material for this analysis. We found increased expression of both *CD62L* (Fig. 2*B*) and *β7 integrin* (Supplemental Fig. 1*A*) mRNA in the sorted Klf3 null MZ B cells. We also examined cell surface expression of both of these molecules in gated FO and MZ B cells purified from several mice and found that they are significantly upregulated in Klf3 knockout MZ B cells (Fig. 2*B*, Supplemental Fig. 1*A*). Finally, we examined expression of a number of homing genes in $CD19^{+}$ B cells purified from lymph nodes of Klf3 knockout mice. In these cells, we did not see any significant changes in expression in the absence of Klf3, suggesting that the effects on B cell homing are more pronounced in the MZ lineage (Supplemental Fig. 1*B*).

Defects in B cell development in the bone marrow and spleen are cell autonomous

Our analysis of Klf3 knockout mice revealed an accumulation of recirculating mature B cells in the bone marrow and a decrease in the frequency and absolute number of MZ B cells in the spleen. To confirm that these defects in B cell differentiation result directly from the loss of Klf3 expression in B cells, we performed competitive reconstitution experiments by injecting a mix of Klf3 null and wild-type hematopoietic precursor cells into congenic irradiated recipient mice. Because homozygous *Klf3* knockout mice on a C57BL/6 background are embryonic lethal, E14.5 FL cells were used for this experiment. Wild-type C57BL/6.SJL-Ptprca (CD45.1) control FL cells were cotransferred together with either $Klf3^{-/-}$ or $Klf3^{+/+}$ C57BL/6 (CD45.2) FL cells.

An analysis of bone marrow B cells derived from the wild-type reconstitution mix revealed a similar proportion of cells in fractions D–F, irrespective of the CD45.2 gate (Fig. 3*A*). This shows that B cells from the wild-type littermates of Klf3 knockout mice are able to progress normally through this stage of differentiation in the bone marrow. Inspection of the CD45.2 histograms (Fig. 3*A*) and cell counts (Fig. 3*C*) reveals that $Klf3^{+/+}$ CD45.2 B cells appear to have a modest competitive advantage over the C57BL/6 CD45.1 wild-type cells in the repopulation of the bone marrow,

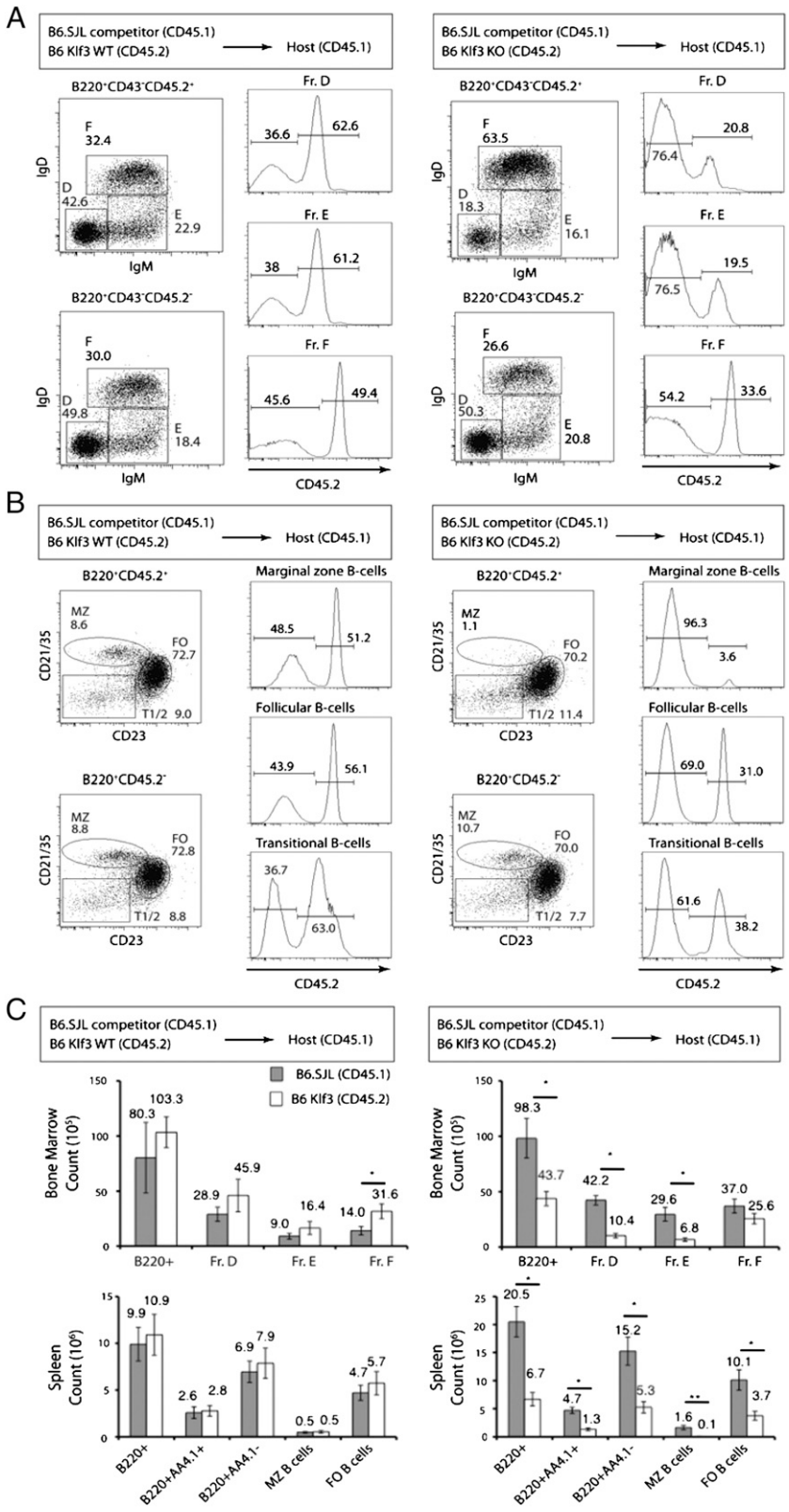

FIGURE 3. Defects in B cell development in the bone marrow and spleen of Klf3 knockout mice are cell autonomous. B cell development in the bone marrow and spleen was analyzed in irradiated B6.SJL-Ptprca mice after reconstitution with normal control B6.SJL-Ptprca (CD45.1) FL cells, mixed with FL cells from either C57BL/6 Klf3 (CD45.2) wild-type or knockout littermates. Samples were analyzed 8 wk after reconstitution. *A*, Representative FACS analysis of bone marrow B cells comparing wild-type ($n = 3$) and Klf3 knockout ($n = 3$) reconstitutions. Shown are Hardy fractions D ($B220^+/CD43^-/IgD^-/IgM^-$), E ($B220^+/CD43^-/IgD^{lo-int}/IgM^{hi}$), and F ($B220^+/CD43^-/IgD^{hi}/IgM^{hi}$). *B*, Representative FACS analysis of splenic B cells comparing wild-type ($n = 5$) and Klf3 knockout ($n = 5$) reconstitutions. Shown are transitional ($B220^+/CD21^-/CD23^-$), FO ($B220^+/CD21^+/CD23^+$), and MZ B cells ($B220^+/CD21^{hi}/CD23^{med/lo}$). *C*, Mean B cell bone marrow ($n = 3$) and spleen counts ($n = 5$) for subsets derived from either B6.SJL-Ptprca (CD45.1) or C57BL/6 Klf3 (CD45.2) FL cells after reconstitution of irradiated B6.SJL-Ptprca recipient mice. $*p < 0.05$, $**p < 0.01$.

providing a higher proportion of cells within the analyzed fractions.

When the recipient bone marrow is reconstituted with a mix of CD45.1 wild-type and CD45.2 $Klf3^{-/-}$ cells, we observed an increase in the percentage of fraction F cells and a decrease in the percentage of fraction D cells, when gating on $CD45.2^+$ cells (Fig. 3*A*). This reduction in the $Klf3^{-/-}$–derived immature B cell population (fraction D) and the significant increase in recirculating $Klf3^{-/-}$ B cells (fraction F) are very well aligned to the observations made in the bone marrow of Klf3 null mice (Fig. 1). It is also apparent that the CD45.2 $Klf3^{-/-}$–derived cells are at a competitive disadvantage to the CD45.1 wild-type cells in their ability to reconstitute fractions D and E in the bone marrow (Fig. 3*C*).

We also examined B cell development in the spleen of the recipient mice. Analysis of B cells derived from the wild-type reconstitution mix revealed a similar proportion of transitional, MZ, and FO B cells, irrespective of the CD45.2 gate (Fig. 3*B*), indicating that transplanted Klf3$^{+/+}$ CD45.2 precursors cells are able to repopulate the splenic B cell compartment. The Klf3$^{+/+}$ CD45.2 B cells appear to compete well with the CD45.1 C57BL/6 wild-type cells in the repopulation of transitional, MZ, and FO B cells in the spleen (Fig. 3*C*). However, when recipient mice are reconstituted with a mix of CD45.1 wild-type and CD45.2 Klf3$^{-/-}$ cells, Klf3 null B cells show a reduced ability to repopulate the splenic B cell compartment, with Klf3 null B220^{+} cells being present at one third the number of wild-type B220^{+} cells. Even more noticeable is the dramatic failure of the Klf3$^{-/-}$–derived B cells to repopulate the MZ B cell compartment, with <5% of Klf3 null cells, compared with competitor wild-type cells, accounting for the MZ B cell niche (Fig. 3*B*). Klf3$^{-/-}$ CD45.2 cells are also less able to compete in the repopulation of the transitional and FO B cell populations (Fig. 3*C*). The observation that Klf3 null precursors are impaired in their ability to reconstitute the MZ B cell population agrees with our previous analysis of splenic B cell development in Klf3 knockout mice (23) and demonstrates that these defects are directly due to the absence of Klf3 expression in B cells.

Taken together, our reconstitution experiments using Klf3 null FL cells support a significant B cell autonomous role for Klf3 in early B cell differentiation in the bone marrow and also in specifying MZ B cell fate in the spleen.

Klf3 is required for B1 B cell development in the peritoneal cavity and spleen

Given that MZ B cell development is disrupted in the Klf3 null mouse (23), we decided to examine the differentiation of the functionally related B1 B cells. Although the number and proportion of B2 cells in the peritoneal cavity is not significantly different in Klf3 knockout mice, we noticed a considerable

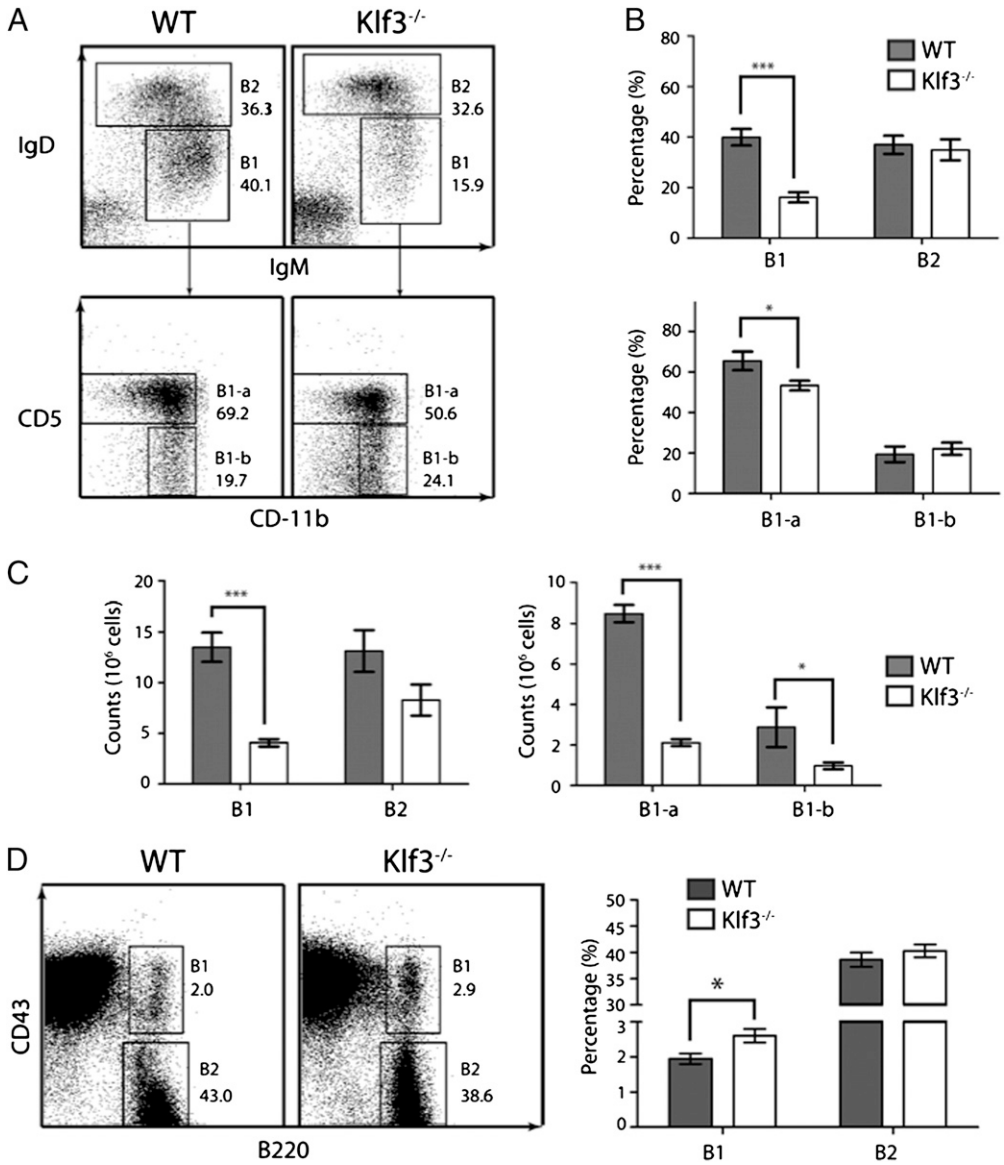

FIGURE 4. Abnormal B1 B cell development in the peritoneal cavity of Klf3 knockout mice. *A*, Peritoneal cavity B cells were analyzed by flow cytometry to identify B2, B1a, and Blb B cell populations. Shown is a representative plot comparing wild-type and Klf3 knockout mice. *B*, Statistical analysis of peritoneal B1 and B2 cells, comparing wild-type ($n = 6$) and Klf3 knockout mice ($n = 8$). *C*, Total peritoneal B1 and B2 cell counts, comparing wild-type ($n = 6$) and Klf3 knockout ($n = 8$) mice. *D*, Representative flow cytometry plot and statistical analysis comparing wild-type ($n = 8$) and Klf3 knockout ($n = 8$) splenic B1 and B2 cells. Mice were analyzed at 12 wk of age. $*p < 0.05$, $***p < 0.001$.

reduction in the percentage and count of B1 B cells (Fig. 4). Within the B1 compartment, we found that the number of both B1a and B1b cells were significantly lower in the knockout mice (Fig. 4*C*). B1 cells are also present in the spleen at low numbers but are believed to be functionally distinct from peritoneal B1 cells (29). In the spleen, we observed a modest but significant increase in the percentage of B1 cells (Fig. 4*D*).

Klf3 is differentially expressed in B cell subsets in the bone marrow and spleen

Having determined that both the proportion and number of B cells is altered in certain populations in the spleen and bone marrow, we next examined the expression levels of *Klf3* in these cells in wild-type mice. To do this, we first sorted pre-B cells, immature B cells, and recirculating mature B cells (fractions D, E, and F, respectively) from the bone marrow and examined the relative expression of *Klf3* in the cells by real-time RT-PCR. We observed a progressive increase in *Klf3* mRNA levels as B cells mature in the bone marrow, with an approximate 8-fold increase in the recirculating B cell population compared with pre-B cells (Fig. 5*A*). We also examined Klf3 expression in MZ and FO sorted B cell populations in the spleen, again by real-time PCR. In the spleen, we found that *Klf3* expression is ~2-fold lower in MZ B cells compared with FO cells (Fig. 5*B*).

Klf3 null MZ B cells show an impaired LPS response

To further investigate the disruption to B cell function in the spleen of Klf3 knockout mice, we used microarray analysis to examine gene expression changes in sorted CD19$^+$ B cells. Microarray data were subject to Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea) to identify pathways disrupted in Klf3 knockout B cells. One of the pathways most significantly affected was the TLR signaling pathway with the downregulation of a number of TLR pathway genes (Supplemental Table II and data not shown). These genes were ranked in the top 500 most downregulated genes, with Tlr6 being the third most downregulated gene, with a 3.7-fold reduction in expression. TLRs 2, 3, 7 and 8 were also downregulated. In addition, the expression of several coreceptors and TLR pathway effector molecules was also altered in B cells lacking Klf3. The full interpretation of these results, however, is complicated by the general reduction in the number of MZ B cells in the spleens of Klf3 knockout animals. To address this, we purified FO and MZ B cells from wild-type and Klf3 knockout mice (Supplemental Fig. 2) and assessed the ability of these sorted populations to respond to LPS stimulation. We found that although wild-type and Klf3 null FO B cell populations showed an equivalent low-level response to LPS, MZ cells lacking Klf3 had an approximate 50% reduction in their proliferative capacity after LPS activation (Fig. 6). We also analyzed the expression of selected TLR pathway genes in sorted CD19$^+$ lymph node B cells using real-time PCR. We observed a significant decrease in expression of *Tlr-6* (~13-fold) and in *CD36* (2.5-fold) in lymph node B cells lacking Klf3, whereas other genes such as *CD14* and *MD-2* were already expressed at low levels and changes in their expression were not evident (data not shown). Taken together, these results suggest that B cell activation pathways regulated by TLR signaling are impaired in Klf3 knockout animals and argue that TLR-pathway genes may be regulated by Klf3.

Loss of Klf3 affects BCR signaling

Given that BCR signaling strength influences B cell fate (30, 31) and that loss of Klf3 disrupts B cell differentiation, resulting in a reduction of both MZ and B1 B cells, we examined the effect of Klf3 deficiency on BCR signaling. We assessed levels of calcium mobilization in gated FO and MZ Klf3 null B cells after anti-IgM–induced BCR activation. In Klf3 null MZ B cells, we observed both a delay in signaling and a reduced signal peak, whereas wild-type and Klf3 knockout FO B cells showed an equivalent response (Fig. 7).

Klf3 may function within a regulatory network of Klfs in B cells

Klf3 is known to repress the expression of *Klf8* in erythroid tissue (32); Klf8 is closely related to Klf3 and is also able to bind the corepressor Ctbp and function as a transcriptional repressor (33). Examination of our microarray data revealed that both *Klf8* and *Klf12* expression is increased in Klf3 knockout CD19$^+$ splenic B cells. Klf12 is also closely related to Klf3 and again functions primarily as a transcriptional repressor in partnership with Ctbp (34). We confirmed by real-time PCR that *Klf8* and *Klf12* expression is derepressed in Klf3 null CD19$^+$ splenic B cells (Fig. 8*A*). Given that B cell populations are disrupted in Klf3 knockout mice, complicating direct comparison with wild-type mice, we also examined *Klf* gene expression in pure B cell populations. Again, in both splenic B220$^+$CD23$^+$CD21$^+$ FO B cells and lymph node CD19$^+$ B cells sorted from Klf3 knockout mice, we observed derepression of *Klf8* and *Klf12* expression (Fig. 8, Supplemental Fig. 2). Our analysis of the sorted B cell populations also indicated

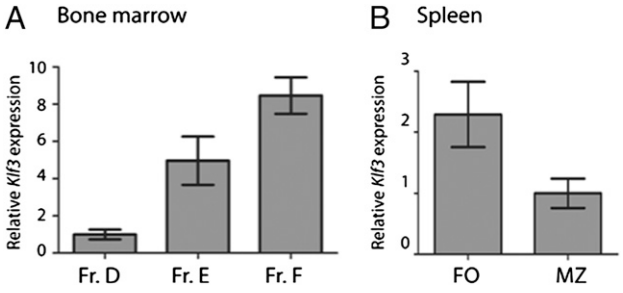

FIGURE 5. *Klf3* is differentially expressed in B cell subsets. *A*, Hardy fractions D, E, and F were FACS sorted from pooled bone marrow from five to seven wild-type mice at 12 wk of age. *Klf3* expression was assessed by real-time RT-PCR. Shown are mean data for three independent sorts. *B*, MZ and FO B cells were FACS sorted from wild-type spleens at 12 wk of age, and *Klf3* expression was assessed by real-time RT-PCR. Shown are mean data for three independent sorts. The relative expression of each gene was normalized against 18S, and the lowest expression in each tissue set to 1.

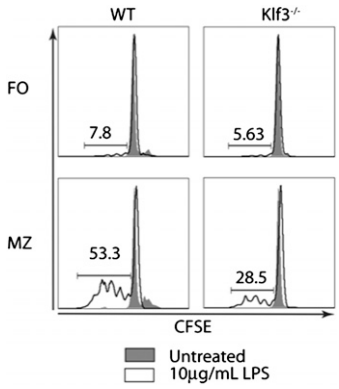

FIGURE 6. Klf3 knockout MZ B cells show a reduced LPS response. Total splenic B cells were isolated by magnetic bead labeling and column depletion of non-B cells. After staining for B220, CD21, and CD23, FO and MZ B cell populations were isolated by FACS (see Supplemental Fig. 2). The sorted populations were then stained with the cell division tracking dye CFSE, stimulated with LPS, and cultured for 72 h. Shown is a representative plot of three repeat experiments, giving the percentage of proliferating wild-type and Klf3 knockout FO and MZ B cells.

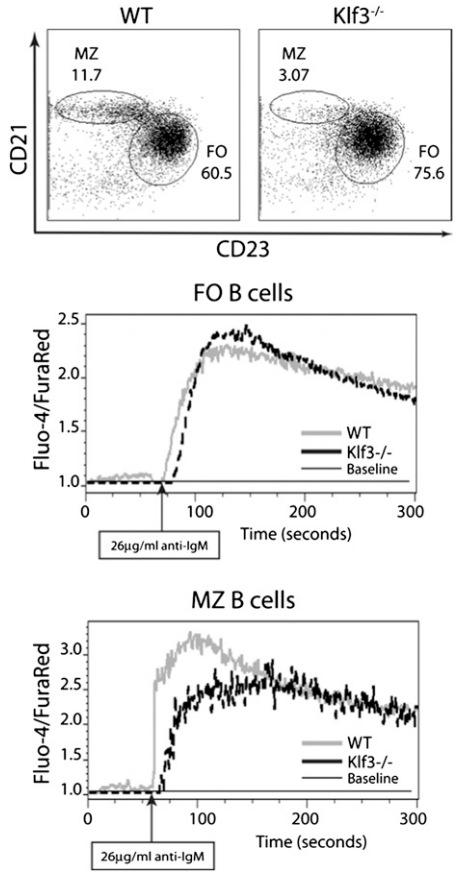

FIGURE 7. Klf3 null MZ B cells show reduced BCR signaling. Total splenic B cells were isolated by magnetic bead labeling and column depletion of non-B cells. Purified B cells were stained for B220, CD21, and CD23 to distinguish FO and MZ populations, and then stimulated with the indicated concentration of anti-mouse IgM $F(ab')_2$ fragments. Calcium mobilization was determined by a ratiometric measurement of Fluor-4 and Fura Red fluorescent dyes. Shown is a representative plot of three repeat experiments.

that *Klf10* is modestly upregulated in Klf3 null FO B cells, whereas expression of *Klf9* and *Klf13*, two factors that play a role in B cell biology (10, 35), was unaltered (Fig. 8). Furthermore, the defects in MZ B cell development seen in mice lacking either Klf3 or Klf2 (7) also suggest that these two Klfs may function in a regulatory network during B cell development (Fig. 9).

Discussion

Klf3 is required for normal B cell development

The absence of Klf3 leads to significant defects in B cell development in the bone marrow, spleen, and peritoneal cavity. In particular, our analysis has revealed a reduction in immature B cells and a dramatic increase in recirculating mature B cells in the bone marrow; in the spleen, there is incorrect positioning of MZ B cells, and in the peritoneal cavity, there is a significant loss of B1 B cells. Importantly, both the proportion and actual number of B cells are altered in all cases, and we have demonstrated that this is a cell autonomous trait. Studies in mouse models have suggested that signaling strength from the BCR influences maturing B cell fate during differentiation. However, interpretation of the data is complex, with some reports suggesting that lower signaling strengths promote MZ cell development (36), whereas others find that stronger signals are important (37). High strength signals have been reported to support B1 B cell pathways (30).

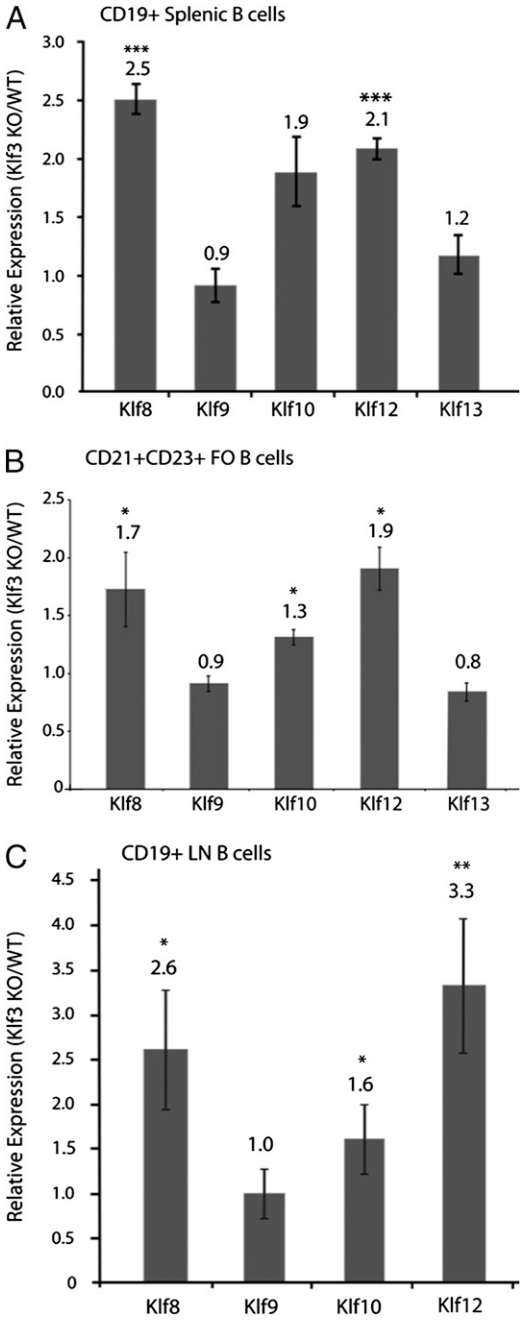

FIGURE 8. Klf3 may regulate the expression of other Klfs in B cells. *A*, $CD19^+$ B cells were isolated by magnetic bead labeling and column purification from wild-type and Klf3 knockout spleens, and analyzed by real-time RT-PCR for expression of various *Klfs*, including *Klf8* and *Klf12*. The relative expression of each gene was normalized against 18S and wild-type expression set to 1. Shown are mean data for independent sorts from five wild-type and six Klf3 knockout mice. $***p < 0.001$. *B*, FO B cells were isolated from wild-type and Klf3 knockout spleens by magnetic bead labeling and column depletion of non-B cells, followed by FACS of $B220^+$ $CD21^+CD23^+$ cells, and then analyzed by real-time RT-PCR for expression of various Klfs, as described in *A*. Shown are mean data for independent sorts from three wild-type and three Klf3 knockout mice. $*p < 0.05$. *C*, $CD19^+$ B cells were isolated by magnetic bead labeling and column purification from wild-type and Klf3 knockout lymph nodes, and analyzed by real-time RT-PCR for expression of various *Klfs* as described in *A*. Shown are mean data for independent sorts from three wild-type and three Klf3 knockout mice. $*p < 0.05$, $**p < 0.01$.

Line	Klf3 expression	Klf2 expression	MZ B cell phenotype	References
Klf3 KO	Null	unchanged	↓↓	
Klf3 -CD19 Transgenic	↓↓ (endogenous)	unchanged	↑↑↑	22
Klf2 KO	↓↓	Null	↑↑	6-8

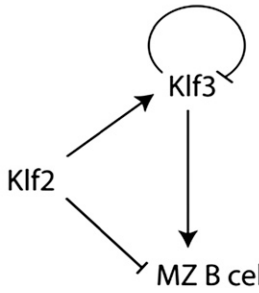

FIGURE 9. Klf2 and Klf3 may function in a regulatory network during B cell development, with the ratio of these two factors influencing B cell fate. The data presented in this article and a number of recently published mouse models (6–8) have revealed that both Klf2 and Klf3 are required for normal MZ B cell differentiation and suggest that expression of *Klf3* may be dependent on levels of Klf2 and Klf3.

The reduction in MZ and peritoneal cavity B1 B cell numbers in the absence of Klf3 suggests that it may influence cell fate decisions downstream of the BCR by affecting signaling strength. It is possible that loss of Klf3 reduces BCR signaling strength, leading to impaired MZ and B1 B cell development in the spleen of Klf3 null mice. This interpretation is consistent with the relatively minor effects on FO B cell development in the Klf3 null spleen and the increase in recirculating mature B cells in the Klf3 knockout bone marrow. Indeed, our calcium mobilization assays confirm that, in the absence of Klf3, MZ B cells do show a moderately impaired BCR response. The reduction in B1 B cell numbers in the peritoneal cavity also suggests that the absence of Klf3 may restrict BCR signaling strength, because B1 B cell differentiation is believed to be dependent on higher strengths.

Several proteins have been implicated in BCR signaling and B cell lineage decisions. Loss of regulators of BCR signaling such as Lyn and CD22 have been reported to lead to an increase in B1 B cells and a reduction of MZ B cells (36, 38–44). Similarly, positive regulators of BCR signaling such as Btk appear to direct B cell differentiation away from the MZ lineage, while promoting the accumulation of B1 B cells (36, 45). Deletion of CD19 and Aiolos leads to a severe reduction in both peritoneal B1 and MZ B cells (36, 46–48), a similar phenotype to that seen in Klf3-deficient mice. In addition to these factors, MZ B cell development is promoted by the cytokine BAFF, a TNF family member with roles in B cell survival and differentiation (49). The notch signaling pathway has also been implicated in MZ B cell development, with a loss of notch function resulting in defective MZ cell differentiation (50). However, inspection of our microarray data from sorted CD19$^+$ splenic B cells did not reveal any significant changes in expression for these factors (data not shown).

In the bone marrow, we observed an equal number of Hardy fraction A cells in wild-type and Klf3 knockout mice. However, in the Klf3 knockout mice, there is a noticeable reduction in both the proportion and number of B cells in fractions B–D. This suggests that the absence of Klf3 may partially block the transition of B cells from fraction A (prepro-B cells) to fraction B (early pro-B cells). Once Klf3 null B cells have differentiated into early pro-B cells, they appear able to progress normally through fractions B–D. Indeed, a comparison of the ratio of fraction D (pre-B cells) with fraction E (immature B cells) cells in wild-type and Klf3 knockout mice suggests that the absence of Klf3 may slightly promote the fraction D-to-E transition. In contrast with earlier fractions, the number of cells in fraction F is significantly increased in the bone marrow of Klf3 knockout mice. This may be because of effects on differentiation of fraction E cells and/or changes in the retention of recirculating mature B cells in the bone marrow (see later). We have previously observed that Klf3 knockout mice are leaner than their wild-type littermates (18). However, a comparison of various wild-type and knockout tissues indicated that this phenotype is predominantly due to a reduction in fat pad size, and is therefore unlikely to explain the differences in B cell numbers seen in Klf3 null mice.

The roles of Klf3 in early B cell, MZ B cell, and B1 B cell differentiation are currently unclear, but the signaling pathways and downstream targets common to both the pre-BCR and BCR offer potential candidates for further investigation.

Klf3 and MZ B cell activation

Analysis of sorted CD19$^+$ splenic B cells revealed that several components of the TLR signaling pathway are significantly downregulated in the Klf3 knockout mouse. These include a number of TLRs (Tlr6, Tlr3, Tlr7, and Tlr8) and coreceptor molecules (CD14 and CD36), implying that several distinct TLR pathways are impaired. The downregulation of CD14 in Klf3 null splenic B cells, together with the LPS-responsive TLR CD180 (RP105) (51) and the Tlr4 cofactor MD-2 (52), suggests a compromised LPS response. To investigate this, we purified FO and MZ B cells from wild-type and Klf3 null animals, and examined the ability of these sorted populations to respond to LPS stimulation. We found that although FO B cells are largely unaffected by the loss of Klf3, its absence from MZ B cells led to a significant reduction in the LPS response. We also investigated the expression of a number of TLR pathway genes in Klf3 null lymph node B cells. In support of a role for Klf3 in TLR pathway regulation, we observed a significant decrease in expression of *Tlr6* and *CD36* in these cells. Other TLR pathway genes, including *CD14* and *MD-2*, were found to be expressed at background levels in wild-type lymph node B cells, and it was hence not possible to assess the effect of the loss of Klf3 on their expression. In addition to defects in the LPS response in Klf3 null MZ B cells, we also observed impaired BCR signaling in this population. This suggests that in vivo Klf3 knockout mice are likely to show compromised innate and humoral immune responses, with increased susceptibility to infection and disease.

Trafficking and homing of B cells in Klf3 null mice

We observed a significant increase in the proportion and number of recirculating mature B cells (Hardy fraction F) in the bone marrow of Klf3 knockout mice. In addition, there is also a large increase in the number of B cells in the peripheral circulation and a loss of normal positioning of MZ B cells. All of these observations are consistent with Klf3 having a role in the trafficking and homing of B cells. In further support of this, our microarray analysis of Klf3 null B cells revealed deregulation of a number of genes that play a role in cell signaling, adhesion, and migration.

Recently, the role of Klf2 in B cell development has been examined in some detail (6–8). In the absence of Klf2, there is a reduction in recirculating B cells in the bone marrow and a significant increase in the number of MZ B cells in the spleen. Furthermore, Klf2 null MZ B cells have decreased expression of

the trafficking molecules CD62L and β7 integrin (7, 8). It was also noted that, in the absence of Klf2, FO B cells showed some characteristics of MZ B cells. Interestingly, our analysis of Klf3 null splenic B cells reveals that in certain respects, the absence of Klf3 leads to an opposite phenotype. Klf3 null MZ B cells are dramatically decreased in number and show increased expression of *CD62L* and *β7 integrin* mRNA and cell surface molecules. This suggests that although the absence of Klf2 causes FO B cells to adopt some MZ B cell characteristics, loss of Klf3 may influence MZ B cells to become more FO-like. However, a reciprocal phenotype is not always seen; both Klf2 and Klf3 knockout mice have significantly less peritoneal B1 B cells.

Gene expression analysis of Klf2 null B cells revealed that Klf3 expression is noticeably reduced in the absence of Klf2, suggesting that Klf2 may drive Klf3 expression in B cells (7). Although Klf2 usually functions as an activator of transcription, Klf3 is generally a transcriptional repressor. This implies that Klf2 may regulate Klf3 expression in B cells to indirectly silence genes that oppose differentiation pathways driven by Klf2. Differences in the phenotypes of the Klf2 and Klf3 knockout mice may be explained by differential gene regulation or where targets are shared (directly or indirectly) by the residual expression of Klf3 in Klf2 null B cells.

Despite B cell counts being significantly elevated in the peripheral blood of Klf3 knockout mice, we did not see a noticeable increase in B cell numbers in the spleen or lymph nodes in the absence of Klf3. High peripheral B cell counts have also been seen in lymphotoxin α (LT-α) knockout mice (53, 54). In a further similarity to Klf3 knockout mice, the MZ is also disrupted in the absence of LT-α. However, LT-α mice also lack lymph nodes, whereas lymph node development appears normal in Klf3 knockout mice. Given this difference and the normal levels of *LT-α* expression in Klf3 null B cells (data not shown), it is unlikely that impaired LT signaling contributes to the defects seen in the absence of Klf3. Because we did not observe any changes in expression of homing genes, such as *CD62L* (55) in Klf3-deficient lymph node B cells, the mechanism underlying the increase in peripheral B cells remains unclear.

Klf3 is differentially expressed during B cell development and may function in a Klf regulatory network in B cells

We examined *Klf3* expression in wild-type B cell populations purified from the bone marrow and spleen. We observed a progressive increase in expression as B cells mature in the bone marrow. In the spleen, we found *Klf3* levels to be lowest in MZ cells, in agreement with previous data comparing gene expression in purified FO and MZ B cell populations (56). This might imply that low levels of *Klf3* promote MZ cell development. However, in Klf3 knockout mice, we see fewer MZ cells, and in transgenic mice overexpressing Klf3, MZ B cells numbers are increased (23). This suggests that it is the expression of *Klf3* in precursor transitional B cells that is important in the FO/MZ lineage decision. Clearly, Klf3 is not essential for MZ B cell development as mature MZ cells still develop, albeit at a significantly reduced number, in the Klf3 knockout spleen.

Evidence of Klf regulatory networks is beginning to emerge in various tissues. In erythroid cells, Klf1 promotes expression of *Klf3*, *Klf8*, and *Klf10*, with Klf3 also able to repress expression of *Klf8* (17, 32). In the intestinal epithelium, Klf4 promotes its own expression, whereas Klf5 acts as a negative regulator of *Klf4* expression (57). Reduced expression of *Klf3* is seen in the skin of Klf4 knockout mice (58). Given that Klf4 has a number of roles in B cell differentiation (10, 59), it is possible that Klf4, or the related activator Klf2 (2), might function with other Klfs in a regulatory network in B cells. Indeed, reduced expression of Klf3 has recently been demonstrated in Klf2 null B cells (7), and it has been shown that *Klf2*, *Klf3*, and *Klf4* all show similar expression patterns on activation of naive B cells (21). Increased repression of the endogenous *Klf3* locus has been reported in transgenic mice overexpressing Klf3 in B cells, suggesting that in addition to regulation by Klf2, Klf3 levels may be autoregulated in B cells (23).

In support of the hypothesis of a Klf network in B cells, we find that *Klf10, Klf8*, and *Klf12* expression are all elevated in Klf3 null B cells. Furthermore, the regulation of *Klf3* by Klf2 (7) and their functional overlap in the differentiation of a number of B cell subsets, in particular, MZ B cells, suggest a biological significance for such a network, with the possibility that the ratio of these factors is important in determining B cell fate (Fig. 9).

Disclosures

The authors have no financial conflicts of interest.

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