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Cutting Edge: Krüppel-like Factor 2 Is Required for Phenotypic Maintenance but Not Development of B1 B Cells

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Several recent studies reported that Krüppel-like factor (KLF)2 controls trafficking, development, and function of B cells. Conditional B cell KLF2 knockout mice have increased numbers of marginal zone B cells and decreased numbers of B1 phenotype cells. However, it was unclear whether KLF2 is required for B1 B cell development, survival, or phenotypic maintenance. We show that B1 phenotype B cells are present in neonatal mice with B cell-specific KLF2 deficiency, suggesting that B1 differentiation can occur even in the absence of KLF2. Furthermore, by use of an inducible knockout strategy, we show that deletion of KLF2 in mature B1 cells causes loss of phenotypic markers associated with B1 cell identity, but it has a minimal effect on short-term cell survival. Taken together, our findings suggest that KLF2 is necessary for the maintenance of B1 cell identity rather than differentiation or survival of the population. *The Journal of Immunology*, 2012, 189: 000–000.

The B1 subset of B cells shows unique developmental, phenotypic, functional, and tissue distribution characteristics (1–5). This population arises during fetal development, and hence precedes the B2 B cells (which include follicular and marginal zone [MZ] B cells). B1 cells are enriched in certain tissue locations, such as the peritoneal and pleural cavities, but they are also present as a minority population in the spleen and blood (5). Studies in mice suggest B1 cells often bear polyspecific B cell receptors and are the major source of “natural” IgM Abs, which offer initial protective immunity against various infections. The B1 pool mediates rapid responses to viral and bacterial pathogens, especially following infection at mucosal surfaces (2, 5, 6). B1 B cells are also associated with self reactivity and may contribute to certain autoimmune conditions (1, 5, 7). At the same time, the IL-10–producing “regulatory B cell” subset may comprise a subset of the B1 pool (8). Recent studies suggest that cells with characteristics of B1 cells are present in humans and are

expanded in patients with lupus (9, 10), although this issue remains controversial.

Although first identified >35 y ago (1), considerable controversy surrounds the basis for B1 differentiation (3, 4, 11). Whereas some studies indicate that BCR specificity dictates the differentiation of B cell precursors into B1 versus B2 fates, there is also considerable evidence that distinct precursor populations may exist for B1 and B2 lineage cells (4). Present models suggest that most B1 B cells (especially the prototypical CD5^{high} B1a B cell pool) (4) differentiate before or just after birth, and that these cells are maintained throughout adult life by self renewal, rather than the continuous generation characteristic of B2 B cells (3–5). Several studies have focused on the role of signals through the BCR and survival receptors in their generation (12), and they have suggested that this pool may be induced by exposure to strong BCR signals with self ligands (5, 7, 13). This is in keeping with the fact that B1 cells display an activated phenotype (including elevated CD43 and CD5 expression) at steady-state. Alternatively, in contrast to B2 B cells, the development and survival of B1 cells are minimally affected by disruption of BAFF receptor signaling (4, 14). Hence, the signals and timing of B1 versus B2 development appear quite distinct.

However, the role of transcription factors in mediating the distinct development of B1 and B2 B cells is unclear. Recently, we and others showed that members of the Krüppel-like family, Krüppel-like factor (KLF)2 and KLF3, dramatically affect B cell subset development (15–19). Intriguingly, whereas KLF2 and KLF3 appear to play opposing roles in generation of MZ B cells, deficiency in either factor leads to profound loss of the peritoneal B1 population (15–17, 19). Whether the B1 pool in other tissues (such as the spleen) is similarly affected was less clear, although the decreased levels of serum IgA, IgG1, and IgE in B cell-specific KLF2-deficient mice (15) are consistent with a global loss of B1 cells, as the B1 population is responsible for a large fraction of these Abs in unimmunized mice (20–22).

However, existing studies have not addressed the stage or mechanism by which KLF2 influences the B1 subset: KLF2

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Abbreviations used in this article: KLF, Krüppel-like factor; KO, knockout; MZ, marginal zone; Tg, transgenic; WT, wild-type.

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may be needed for initial B1 differentiation, maintenance of the mature B1 pool, or preservation of the unique B1 phenotype. The fact that B1 differentiation peaks at or before birth makes these issues difficult to address in adult animals. Hence, in this study we explore the requirement for KLF2 in development of B1 cells in neonatal mice, and we use an inducible *Klf2* deletion model to test the function of this factor in mature B1 B cell maintenance. Our studies demonstrate that KLF2 is, unexpectedly, not required for B1 B cell generation but plays a key role in maintenance of mature B1 phenotype, as indicated by loss of typical B1 markers with KLF2 deletion. Hence, our data indicate that KLF2 is required for preservation of B1 B cell identity.

Materials and Methods

Mice

Klf2^{fl/fl} and *CD19-Cre Klf2^{fl/fl}* mice were described earlier (15). Mice carrying the *ROSA26-(floxed STOP)-YFP* reporter (23) were provided by Frank Costantini at Columbia University. Other animals were purchased from the National Cancer Institute. All animal studies were conducted under approval from the University of Minnesota Institutional Animal Care and Use Committee.

Flow cytometry

All fluorochrome- and biotin-conjugated Abs were purchased from eBioscience, BD Biosciences, or BioLegend. Flow cytometry samples were prepared by staining single-cell suspensions with Abs in FACS buffer (PBS, 1% FCS, 0.02% sodium azide) for 30 min at 4°C and washing cells twice with FACS buffer. Flow cytometry data were collected using an LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Peripheral B1 B cells were defined as CD19⁺, B220^{int/low}, CD43⁺, IgM⁺, CD21⁻, and CD23⁻ (24), whereas B2 B cells were identified as CD19⁺, B220^{high}, and CD93⁻. The typical gating scheme is illustrated in Supplemental Fig. 1.

Tat-Cre transduction

Tat-Cre (HTNC) was generated as previously described and either purchased from Excellgen (Rockville, MD) or provided by Kevin Otipoby and Klaus Rajewsky (Harvard University) (25). Single-cell suspensions were washed three times in ADCF-MAb serum-free media and resuspended in HyClone serum-free media. Cells (5×10^6 cells/ml, final density) were then incubated with Tat-Cre (50 μ g/ml or 1.25 μ M) in the presence of polymyxin B sulfate (50 μ g/ml final concentration) in HyClone media for 45 min at 37°C. Cells were then washed in HyClone media containing 10% serum prior to *in vivo* transfer.

Cell isolation, sorting, and adoptive transfers

CD19-Cre transgenic (Tg)⁺, *Klf2^{fl/fl}* (B cell-specific, KLF2-deficient) and *CD19-Cre* Tg⁺, *Klf2^{+/+}* (wild-type [WT] controls) of indicated ages were sacrificed and cells were isolated from the spleen, peritoneal cavity, and pleural cavity. For studies involving Tat-Cre treatment, peritoneal cells were isolated from *Klf2^{+/+}* CD45.2/CD45.1 *ROSA26-(floxed STOP)-YFP* Tg mice (as controls) and *Klf2^{fl/fl}* CD45.2/CD45.2 *ROSA26-(floxed STOP)-YFP* Tg mice (as the experimental group). Peritoneal lavages (from at least 10 mice) were obtained and mixed, and the cells were cultured with or without Tat-Cre. An aliquot was subjected to FACS analysis to assess the input knockout (KO)/WT ratio. Approximately 10^6 cells were transferred interperitoneally into B6.SJL (CD45.1/CD45.1) mice. Peritoneal and splenic cells were assessed via flow cytometry 3 and 10 d later. In some experiments, B1 phenotype B cells were sorted (on a FACS Aria; BD Biosciences) for lack of "dump" markers (CD4, CD8, Gr-1, and F4/80) and for expression of B1 characteristics (CD19⁺, B220^{low}, CD43⁺, CD23⁻) prior to Tat-Cre treatment. Postsort analysis showed >99% efficiency of sorting. Approximately 5×10^4 cells were transferred interperitoneally into B6.SJL mice and assessed by flow cytometry 10 d later. From compiled Tat-Cre transduction experiments, YFP⁺ cells were $32 \pm 5\%$ for the *Klf2^{+/+}* controls and $41 \pm 7\%$ for the *Klf2^{fl/fl}* groups.

Statistical methods

The *p* values were calculated by a Student unpaired *t* test using Prism software (GraphPad Software). Where values varied by 10-fold or more, they were converted to log₁₀ prior to the *t* test.

Results and Discussion

B1 B cells develop in the absence of KLF2

We and others have reported that conditional KLF2 deficiency in B cells leads to dramatic reduction of peritoneal cavity B1 B cells (15–17). It was less clear whether a residual population of B1 cells exists in the spleen of adult *CD19-Cre/Klf2^{fl/fl}* mice, with reports differing on whether this pool was reduced (15, 17) or elevated (16). To address this issue we used a rigorous gating strategy to identify mature B cell subsets (Supplemental Fig. 1A) and observed a dramatic reduction in the number of cells expressing typical B1 B cell markers (CD19⁺, B220^{int}, CD43⁺, IgM⁺, CD21⁻, CD23⁻) in the spleen of adult *CD19-Cre/Klf2^{fl/fl}* mice (Fig. 1A). As reported previously (15–17), *CD19-Cre/Klf2^{fl/fl}* animals also show a substantially lower percentage of peritoneal B1 B cells (Fig. 1A) and correspondingly low numbers of B1 cells in both peritoneal and pleural cavities (Supplemental Fig. 1B, 1C). Accordingly, these data confirm and extend our previous observations that KLF2 deficiency in B cells leads to a severe deficiency of B1 phenotype cells in adult mice.

Such findings might suggest that KLF2 is important for B1 B cell differentiation, maintenance, or expression of characteristic B1 phenotypic markers (or a combination of these roles). This issue is compounded by the fact that the B1 B cell differentiation is largely limited to fetal and neonatal life (4, 5,

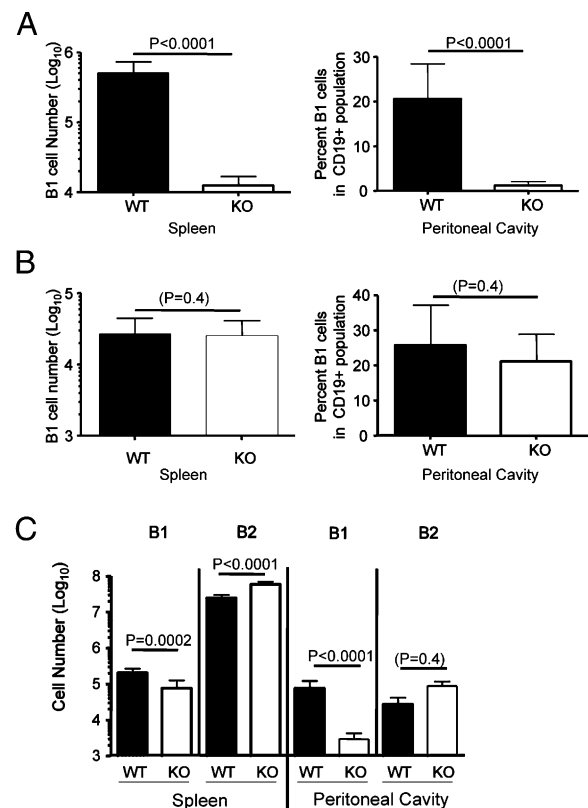


FIGURE 1. B1 B cells are present in young, but not adult, KLF2-deficient mice. B1 B cells were analyzed in the spleen and peritoneal cavity of *CD19-Cre* Tg⁺, *Klf2^{+/+}* mice (WT) or *CD19-Cre* Tg⁺, *Klf2^{fl/fl}* (KO) mice that were (A) >10 wk old, (B) 8–11 d old, or (C) 30 d old. The number or percentage of B1 B cells is indicated for each tissue. In (C), the number of B2 B cells is also indicated. These data are compiled from at least three experiments [the number of animals represented are 9 WT and 8 KO in (A), 8 WT and 12 KO in (B), and 9 WT and 7 KO in (C)].

13, 26); hence, analysis of adult *CD19-Cre/Klf2^{fl/fl}* mice cannot distinguish between defects in generation versus maintenance of B1 B cells.

To begin addressing this issue, we assessed whether B1 B cells were generated in young *CD19-Cre/Klf2^{fl/fl}* mice. Surprisingly, a population of B1 B cells was readily detected in the spleen of neonatal *CD19-Cre/Klf2^{fl/fl}* mice, similar in numbers to those found in control mice of the same age (Fig. 1B). Furthermore, B1 B cell phenotype B cells were found at normal frequencies in the peritoneal cavity of young *CD19-Cre/Klf2^{fl/fl}* mice (Fig. 1B). More than 50% of the peritoneal B1 B cells from both *CD19-Cre/Klf2^{fl/fl}* and control (*CD19-Cre/Klf2^{+/+}*) neonatal mice were CD5⁺ (data not shown), indicating similar generation of B1a and B1b populations. By 30 d after birth, the number of B1 cells was significantly reduced in both the spleen and peritoneal cavity of *CD19-Cre/Klf2^{fl/fl}* mice, yet this population was still clearly detectable, especially in the spleen (where the average number of *CD19-Cre/Klf2^{fl/fl}* B1 B cells was <3-fold fewer than that in the *CD19-Cre/Klf2^{+/+}* controls) (Fig. 1C). At this time point, the increase in splenic B2 B cells reported previously (15–17) was already observed (Fig. 1C). Hence, our data suggested that KLF2 is not essential for B1 development, but rather may be important for maintenance of the B1 pool.

Inducible KLF2 deficiency leads to loss of the mature B1 B cell pool

Although these findings suggested that KLF2 was dispensable for B1 B cell differentiation, it was possible that KLF2 was important for a step in final maturation of B1 cells (e.g., acquiring the capacity for self renewal) rather than playing a role in maintenance of fully mature B1 cells. To test this hypothesis further, we sought a model by which KLF2 could be deleted after maturation of B1 B cells. To achieve this, we used the documented ability of the fusion protein Tat-Cre to induce deletion of floxed genes following protein transduction (25). In this system, the HIV Tat peptide induces entry of the Cre cargo into the cell cytosol, and the presence of a nuclear localization signal directs the active Cre enzyme to the nucleus, prompting floxed gene excision (25, 27). To track Cre activity, we also employed the *ROSA26-(floxed-STOP)-YFP* allele, which offers the ability to use flow cytometry to identify cells that have been successfully transduced by Tat-Cre protein (see *Materials and Methods*).

We applied this system to induce KLF2 deletion in mature B1 B cells. Peritoneal cavity cells were isolated from congenically distinct *Klf2^{+/+}* and *Klf2^{fl/fl}* mice, and the cells were mixed, transduced with Tat-Cre (or incubated with PBS as control), and injected interperitoneally into congenic recipient mice. By using this approach, we could assess the impact of inducible KLF2 deletion by measuring the ratio of *Klf2^{fl/fl}* to *Klf2^{+/+}* donor cells within distinct B cell populations. Because the populations are mixed prior to Tat-Cre transduction and transfer, this approach minimizes nonspecific effects (e.g., potential toxicity of Tat-Cre treatment). The data were further normalized to the input ratio of *Klf2^{fl/fl}* to *Klf2^{+/+}* cells to allow compilation of distinct experiments.

Donor cells were analyzed in the peritoneum at both 3 and 10 d following adoptive transfer (very few donor cells appeared in the spleen at either time point; data not shown). Treatment with Tat-Cre induced substantial changes in the *Klf2^{fl/fl}/Klf2^{+/+}* ratio. At day 3, Cre treatment led to a significant reduction in

the proportion of *Klf2^{fl/fl}* cells in the B1 phenotype population, and this effect was even more notable by day 10 (Fig. 2A). This effect was further magnified because (for unknown reasons) untreated *Klf2^{fl/fl}* donor B1 cells displayed a slight advantage over *Klf2^{+/+}* control cells at both time points after adoptive transfer (open bars for B1 cells in Fig. 2A). In contrast, the proportion of *Klf2^{fl/fl}* donor cells in the B2 phenotype pool (CD19⁺, B220^{high}, CD93⁻, CD23⁺, CD43⁻) cells was not changed by Tat-Cre treatment (Fig. 2A). Although these data would appear consistent with a requirement for KLF2 in maintenance of B1 B cells, closer inspection revealed that Tat-Cre treatment of *Klf2^{fl/fl}* cells led to the appearance of a novel CD43^{int} pool, as the canonical CD43^{high} B1 phenotype population declined (Fig. 2B). Such findings raised

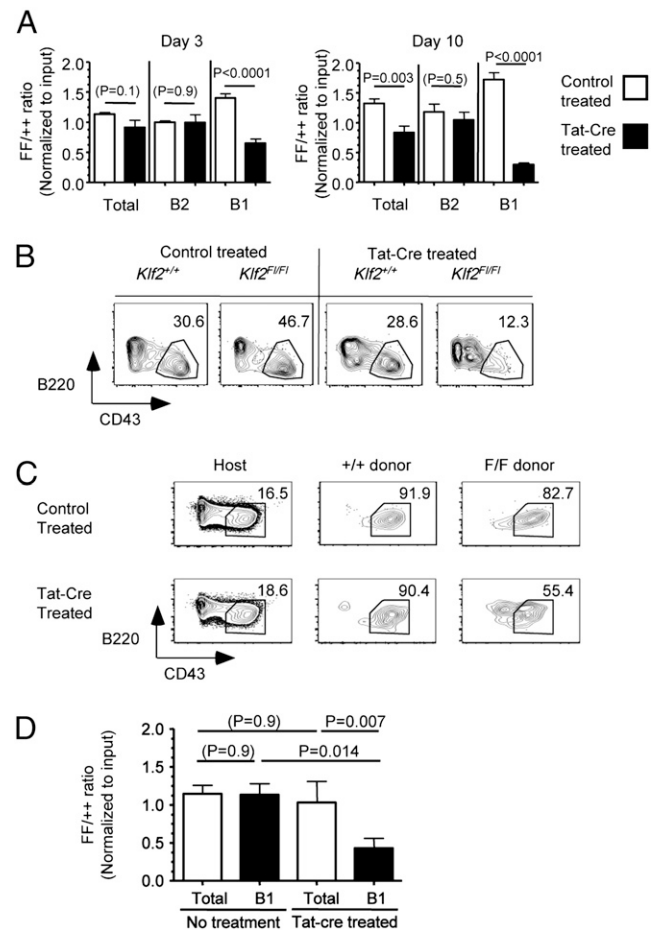


FIGURE 2. Induced KLF2 deletion in mature peritoneal B1 B cells leads to loss of B1 B cell phenotype. (A and B) Peritoneal cells were isolated from congenic mice of either *Klf2^{+/+}* (++) or *Klf2^{fl/fl}* (FF) genotype and treated with Tat-Cre prior to adoptive transfer. The data in (A) represent the ratio of *Klf2^{fl/fl}* to *Klf2^{+/+}* donor cells, for the B cell subsets indicated, among the peritoneal lavage population at the time point indicated. These data are compiled from three experiments. (B) Representative B220/CD43 staining of the indicated donor populations from day 10 posttransfer. (C and D) Peritoneal B1 B cells were sorted from *Klf2^{+/+}* (++) or *Klf2^{fl/fl}* (FF) mice and treated with Tat-Cre or control prior to adoptive transfer. At day 10, cells were isolated from the peritoneum of the host. (C) Representative data for B220/CD43 expression on the *Klf2^{+/+}* and *Klf2^{fl/fl}* donor populations (as well as host cells, for reference). (D) The ratio of *Klf2^{fl/fl}* to *Klf2^{+/+}* donor cells among the total B cell and B1 phenotype population is shown for control and Tat-Cre–treated populations. Data were compiled from three experiments [the number of animals represented are nine mice per group in (A) and three untreated and eight Tat-Cre–treated in (D)].

the possibility that the B1 pool is not eliminated but rather alters its phenotype after KLF2 loss.

Sustained KLF2 expression is needed for maintenance of the B1 B cell phenotype

The origin of the CD43^{int} population induced by KLF2 deletion was impossible to determine from studies using transfer of bulk peritoneal B cells, because these cells could arise from either B1 or B2 cells in the inoculum. Hence, we performed similar Tat-Cre treatment experiments, but we used sorted peritoneal B1 B cells. Postsort analysis indicated highly efficient sorting of CD19⁺, B220^{low}, CD43^{high}, and CD23⁻ B1 cells (see *Materials and Methods*). In the absence of Tat-Cre transduction, adoptively transferred B1 cells (whether *Klf2*^{fl/fl} or *Klf2*^{+/+}) maintained their phenotype for at least 10 d (Fig. 2C). However, treatment with Tat-Cre led to a dramatic change in the *Klf2*^{fl/fl} donor cells, as indicated by a large fraction of this population showing reduced expression of CD43 and elevated B220 staining, suggesting loss of two standard phenotypic traits defined for murine B1 B cells (Fig. 2C). This effect was observed for the bulk donor B cell population (Fig. 2C), but gating on YFP reporter-expressing cells confirmed that this change in phenotype was a feature of *Klf2*^{fl/fl} (but not *Klf2*^{+/+}) donor B cells in which Cre had been active (Supplemental Fig. 2A). Predictably, these effects led to a reduction in the frequency of B1 phenotype-gated cells in the Tat-Cre-treated *Klf2*^{fl/fl} population, compared with their WT counterparts (Fig. 2D, filled bars). Interestingly, however, the ratio of *Klf2*^{fl/fl} to *Klf2*^{+/+} cells in the total donor B cell pool was not affected by Tat-Cre treatment, and it matched the input ratio (Fig. 2D, open bars), suggesting similar maintenance of the Tat-Cre-transduced *Klf2*^{fl/fl} donor B1 cells (at least for the 10-d period studied). It is very unlikely that these findings represent contamination with B2 B cells in the B1 sorted population, as very few of the Tat-Cre-treated *Klf2*^{fl/fl} cells gained expression of CD23 (Supplemental Fig. 2B). Furthermore, although induced *Klf2* deletion led to some loss of CD5, this marker was retained on the majority of cells (Supplemental Fig. 2C). Hence, these data support a model in which KLF2 is important primarily for maintenance of some (but not all) phenotypic characteristics of B1 B cells.

Previous studies indicated that B cell-specific KLF2 deficiency leads to a substantial loss of B1 B cells, yet there was considerable discordance about whether this effect was uniform in diverse tissues (15–17). Our data help resolve this issue by demonstrating that the B1 B cell pool is significantly reduced in adult, but not very young, KLF2-deficient mice. Furthermore, in mice of intermediate ages (e.g., 1 mo of age; Fig. 1C) the loss of the B1 pool is much more profound in the peritoneal cavity than in the spleen. Hence, depending on the timing of analysis (and possibly other aspects of the conditional KLF2 KO system), the extent of apparent B1 B cell deficiency would vary widely in different tissues. The fact that B1 generation in the neonate (and presumably the fetus) is independent of KLF2 may also address another unexpected feature of the described KLF2-deficient models. Previous studies have argued that >80% of serum IgM derives from B1 B cells (5), yet we and others noted minimal impact of KLF2 deficiency on IgM levels (15–17). Although this result could be explained in other ways (e.g., as a consequence of the increased MZ B cell pool in KLF2-deficient mice), our

present findings show that KLF2-deficient B1 cells are present (at least through the neonatal phase) and so may contribute to the levels of “natural” circulating IgM Abs found in adults.

Rather than having a role in initial B1 B cell development, our data suggest that KLF2 is essential for preservation of the B1 phenotype. By using an inducible gene ablation system, we could show that KLF2 deletion induces loss of typical B1 markers without affecting short-term survival. This raises the possibility that cells selected into the B1 lineage are actually present in adult *CD19-Cre/Klf2*^{fl/fl} mice but that these cells lack typical B1 phenotypic markers. We were unable to identify a distinct pool of CD43^{int}B220^{high}CD23^{low} cells in such mice (data not shown), suggesting that the “ex-B1” population is not stable. It is possible that KLF2-deficient B1 lineage cells accumulate further phenotypic changes that make them difficult to distinguish from the B2 cell subsets (e.g., upregulation of CD23 and further decline of CD43 expression would cause this population to be included in the follicular B cell phenotype pool). In contrast, it is possible that KLF2 ablation causes a loss of B1 B cell survival not detected within our 10-d time course. In either case, it is apparent that KLF2 is not simply required for acute B1 B cell survival but plays a role in sustaining characteristic phenotypic features of B1 cells.

The concept that KLF2 is important for preservation of normal B1 B cell “identity” resonates with previous reports in which we and others concluded that KLF2 deficiency caused follicular B cells to exhibit some gene expression characteristics of MZ B cells (15, 16). Hence, KLF2 may play analogous roles in maintaining the identity of B1 B cells and follicular B cells. Interestingly, KLF2 protein expression in mature B cell subsets follows the hierarchy B1 > follicular > MZ (15, 16), which would conform with the idea that loss of the KLF2 gene might provoke cells to acquire characteristics of cells that naturally express lower levels of KLF2. Thus, understanding how the levels of KLF2 are dictated by signals (via the BCR or other receptors) during B cell differentiation will be important for determining how stable B cell subsets are generated.

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Disclosures

The authors have no financial conflicts of interest.

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