Ikaros is a negative regulator of B1 cell development and function

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ABSTRACT

B1 B cells secrete most of the circulating natural antibodies and are considered key effector cells of the innate immune response. However, B1 cell-associated antibodies often cross-react with self-antigens, which leads to autoimmunity, and B1 cells have been implicated in cancer. How B1 cell activity is regulated remains unclear. We show that the Ikaros transcription factor is a major negative regulator of B1 cell development and function. Using conditional knockout mouse models to delete Ikaros at different locations, we show that Ikaros deficient mice exhibit specific and significant increases in splenic and bone marrow B1 cell numbers, and that the B1 progenitor cell pool is increased ~10-fold in the bone marrow. Ikaros null B1 cells resemble WT B1 cells at the molecular and cellular levels, but show a downregulation of signaling components important for inhibiting proliferation and immunoglobulin production. Ikaros null B1 cells hyper-react to TLR4 stimulation and secrete high amounts of IgM autoantibodies. These results indicate that Ikaros is required to limit B1 cell homeostasis in the adult.

INTRODUCTION

B1 B cells are an important part of the innate immune response because they spontaneously secrete natural, polyreactive IgM antibodies in the absence of infection (1,2). In the mouse, they differ from conventional B2 B cells (follicular and marginal zone) in that they do not express CD21 and CD23, and express only low levels of B220 and
Ikaros but high levels of IgM. B1 cells are abundant in the peritoneal and pleural cavities where they can be further divided into B1a (CD19+CD5−CD11b+) and B1b (CD19+CD5−CD11b+) cells. Splenic B1 cells are CD19+CD43+CD5+. The majority of B1 cells develop early in utero from fetal liver progenitor cells, and they are maintained over time in the adult through self-renewal. However, Lin−CD93+CD19+B220+ bone marrow B1 progenitor cells have been identified (3). In addition, B cell receptor (BCR) signal strength appears to be important for B1 cell generation, as strong signals increase B1 cell numbers and weak signals decrease their numbers (4,5).

Because natural antibodies are polyreactive, they also bind to self-antigens and contribute to autoimmunity, suggesting that B1 cells must be tightly regulated during homeostasis. In addition, because they comprise the first wave of B cell development, B1 cells may be linked to childhood leukemias. Work in recent years have begun to reveal a network of transcriptional regulators important for B1 cell development and function. Among them, members of the classical NFκB pathway (eg. p50, Malt1, Carma1, Ikκ complex), downstream of the BCR, have been shown to be essential for B1 cell development (6). The RNA-binding protein Lin28b, and its downstream effectors Let-7 and Arid3a, were revealed to promote fetal B1 cell lymphopoiesis (7,8). Similarly, Ebf1 is required, and its overexpression induces B1 cell development at the expense of B2 cells (9) (10). In contrast, PU.1 (encoded by Prmd1) and Xbp1 are required for B1 (and B2) cell immunoglobulin secretion (14), while Irf4 is required for IgM secretion by splenic B1 cells (15).

Interestingly, NFκB, Lin28b/Let-7/Arid3a, Ebf1, Blimp1 and Xbp1 all work to positively regulate B1 cell development and function. Regulators that limit B1 cell activity are still poorly understood.

Ikaros, encoded by the Ikaros gene, is a zinc finger DNA-binding protein, that is a key transcriptional regulator and tumor suppressor in B cells. It is required for the specification and development of all B cell lineages (16,17), and plays specific roles in pre-pro-B and pre-B cells to activate Rag1,2 expression, mediate chromatin accessibility during immunoglobulin gene rearrangement and allelic exclusion at the Igk locus (18-23). In mature B2 cells, Ikaros directs Ig class switch recombination (24). It functions both as a transcriptional repressor and activator, and acts at least in part through its association with Polycomb repressive complex 2 (25), NuRD and SWI/SNF complexes (26,27). In pre-B cells, Ikaros activates the transcription of genes important for pre-BCR and BCR signaling, cell survival and cell migration, as well as that of B cell regulators like Fox5, Foxo1 and Ebf1 (22,28). Thus Ikaros modulates B cell function at multiple stages.

Here, we reveal a novel function for Ikaros as a major negative regulator of B1 cell development and function in the adult bone marrow and spleen.

**EXPERIMENTAL PROCEDURES**

*Mice.* The IkL/L and IkF/F mouse lines have been described (18,22). IkL/L mice were backcrossed 10 generations onto the C57BL/6 background and analyzed at 6-8 weeks of age. IkF/F mice were crossed with CD21-Cre, CD19-Cre or R26-CreERT2 tg animals (29-31). Ikaros was deleted in adult IkF/F R26-CreERT2+ mice after daily intraperitoneal injections of tamoxifen (50 mg/kg weight of mouse, dissolved in sunflower oil) for 3d. Female MRL/lpr mice were purchased from Harlan.

*Cell culture.* FO B cells were sorted (B220−CD23−CD21+; >98% purity) on a FACSVantage SE option DiVa (BD Biosciences, San Jose, CA) or a FACSARia II SORP (BD Biosciences), or enriched by depletion of CD43− cells followed by positive selection of CD23+ cells with MACS beads (>90% purity; Miltenyi Biotech, Bergisch Gladbach, Germany). Both methods gave similar results. B1 B cells were sorted (CD19+CD43+) on a FACSARia II SORP (BD Biosciences). For BM cultures, 1x10⁶ CD19+ BM B cells were co-cultured on S17 stromal cells in Iscove’s medium supplemented with 10% FCS, 2 mM L-glutamine, 1x non-essential amino acids, 50µM 2-mercaptoethanol (2-ΜΕ), 1% antibiotics plus cytokines IL-7 (7% of supernatant from mIL-7 cells). For proliferation assays, cells were labeled with CFSE (5 µg/ml; Sigma), Sigma, St. Louis, MO) and 2.5x10⁵ cells were co-cultured in complete medium (RPMI 1640, 10% FCS, 25 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 1x non-essential amino acids, 50µM 2-ΜΕ, 1% antibiotics). Cells were stimulated with 10 µg/ml goat anti-mouse IgG F(ab')2 (Jackson ImmunoResearch, West Grove, PA), 2-10 µg/ml mouse anti-CD40 (e Bioscience, San Diego, CA), 10 ng/ml IL-4 (Peprotech), and 25 µg/ml LPS (E. coli 0111:B4; Sigma). For intracellular staining cells were fixed with 2% paraformaldehyde (PFA) and permeabilized with methanol. For cell viability assays, DiOC₆ (Molecular Probes, Eugene, OR) was
added at 50 nM. For cell cycle staining, PFA fixed cells were permeabilized with 0.1% saponin, and stained with anti-Ki67-AF700 (BD Bioscience) and DAPI (2 µg/ml).

**Flow cytometry.** The following reagents were used: anti-B220-PE-Cy7, anti-CD19-PerCP-Cy5.5, anti-CD21-APC or -FITC, anti-CD23-PE-Cy7, anti-CD43-FITC, -PECy7 or -PE, anti-IgD-FITC, anti-CD11b-APC, anti-TCRε-biotin, anti-TCRδ-biotin and streptavidin (SA)-APC-Cy7 from BD Biosciences; anti-CD16/32, anti-CD23-biotin, anti-CD3ε-biotin, anti-CD71-biotin, anti-Gr-1-biotin, anti-NK1.1-biotin, anti-CD8-biotin, anti-CD5-biotin, anti-CD5-FITC and anti-CD93-APC from eBioscience; anti-CD59-biotin from BMA Biomedica (Augst, Switzerland); anti-IgM-PE, -FITC or -APC and SA-PE from Jackson ImmunoResearch; anti-rabbit IgG (H+L)-biotin from Vector Laboratories (Burlingame, CA); SA-Alexa Fluor 405 and anti-rat IgG-Qdot 605 from Invitrogen (Carlsbad, CA). Lin− cells were defined by staining with a cocktail of antibodies for mature hematopoietic lineage marker including CD3ε, CD11b, CD59, CD71, Gr-1, NK1.1, TCRε, TCRδδ, CD8 and IgM. Cells were analyzed with a FACSCalibur and FACS LSR II (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

**ELISA.** ELISAs of sera were performed as described (33). Briefly, polystyrene plates (MaxiSorb; Nunc, Rochester, NY) were coated ON at 37°C with the following antigens: salmon dsDNA (100 ng/ml in 25 mM citrate buffer, pH 5.4; Sigma) and mouse chromatin extracted and purified from mouse lymphocytic leukemia cells L1210 [200 ng/ml expressed as dsDNA concentration in PBS, pH 7.2]. Mouse sera [diluted 1:250 in PBS containing 0.05% Tween-20 (T) and 1% BSA] were added for 1h, followed by goat anti-mouse IgG (diluted 1:2x10^4 in PBS-T; Fc gamma specific; Jackson ImmunoResearch) supplemented with goat anti-mouse IgG3 (diluted 1:7,500; Fc gamma specific; Nordic Immunology, Tilburg, Netherlands) or by goat anti-mouse IgM (diluted 1:2x10^4; µ chain specific F(ab’)2; Jackson ImmunoResearch) conjugated to horseradish peroxidase. After a 30 min incubation at 37°C, H2O2 and 3,3’,5,5’-tetramethyl benzidine were added as substrate and chromogen, respectively, for 15 min. The reaction was stopped and the absorbance was measured at 450 nm.

For ELISAs of cell culture supernatants, polystyrene plates (MaxiSorb; Nunc) were coated ON at 4°C with the following antigens: salmon dsDNA (100 µg/ml in PBS, pH 7.2; Sigma) or mouse chromatin (15 µg/ml) extracted and purified from total mouse splenocytes as described (34). Plates were blocked with 3% BSA in PBS containing 0.05% Tween-20 1h at 37°C. Supernatants (undiluted) or mouse sera (diluted 1:250 in PBS containing 0.05% Tween-20 and 1% BSA) were added for 2h at RT followed by goat anti-mouse IgM (diluted 1:2x10^5; µ chain specific F(ab’)2; Jackson ImmunoResearch) conjugated to alkaline phosphatase. After 2h incubation at RT, p-nitrophenyl phosphate was added as substrate for 20 min and the absorbance measured at 405 nm.

**Detection of antinuclear antibodies (ANAs).** Assays were performed by indirect immunofluorescence with HEp-2 cells (HEp-2 substrate slides; Zeus Scientific, Raritan, NJ). HEp-2 cell coated slides were incubated for 30 min at RT with diluted sera, washed 2x in PBS and visualized by fluorescence microscopy with FITC-labeled anti-mouse IgG or IgM (1:100). Controls included negative and positive sera from BALB/c and MRL/lpr mice, respectively.

**Western blot.** Whole cell lysates were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). The following antibodies were used: anti-Ikaros (rabbit polyclonal antibody generated against the C-terminus of Ikaros; in-house), anti-β-actin (Sigma), anti-rabbit and anti-mouse HRP (Jackson ImmunoResearch). Blots were revealed with Immobilon Western Chemiluminescent HRP substrate (Millipore).

**RT-qPCR and primers.** RNA was extracted using RNeasy kit (Qiagen, Hombrechtikon, Switzerland). RNA was reverse described with Superscript Reverse Transcriptase (Invitrogen). RT-qPCR was performed with 1 cycle at 95°C for 15 min, then 50 cycles of 95°C for 10s, 60°C or 63°C for 10s and 72°C for 15s with SYBR Green PCR MasterMix (Sigma or Roche) on an LC480 light cycler (Roche, Basel, Switzerland). The following primers were used: Irf4 (5’-AAGGCAAGTT CCGAGAAGGG, 5’-TTATGAACCTGCTGGGCT GG), Pax5 (5’-CAGATGTAGTCCGCAACAGGATAG, 5’-ATGCCTGATTAAGCTGTTCACAC), Gna15 (5’-AAGGCAAGTT CCGAGAAGGG, 5’-TTATGAACCTGCTGGGCT GG), Ikaros and B1 cells
Chromatin immunoprecipitation and ChIP-Sequencing. The ChIP protocol was adapted from the Millipore ChIP Assay Kit (17-295) with minor modifications and described (25). Shortly, BH1-Ik1-ER-Bcl2 pre-B cells (22) were treated with 4OHT for 24h in the absence of IL-7 to induce Ikaros function. After crosslinking, the chromatin was sonicated to 300-500 bp using a Bioruptor 200 (Diagenode, Denville, NJ) and cleared by centrifugation. After pre-cleaning with protein A- magnetic beads, the lysate was incubated ON with the anti-Ikaros antibody. Precipitation was carried out using protein A-magnetic beads. De-crosslinked DNA was purified using the iPure Kit (Diagenode). Microarray data are available in the GEO databank (GSE 75827).

RESULTS

Ikaros deficiency alters B cell homeostasis in the spleen. To evaluate mature B cell populations in vivo, we first analyzed IkL/L mice carrying a germline hypomorphic Ikaros mutation. IkL/L cells express functional Ikaros proteins at ~10% of levels detected in WT cells (18,39). Although IkL/L mice die from T cell-acute lymphoblastic leukemia (T-ALL) at 4-6 months of age, the animals used here (6-8 weeks old) showed no signs of transformation in the thymus, as defined by CD4 and CD8 profiling, T cell receptor α and β chain usage, and the absence of a deregulated Notch pathway (39,40). In addition, mice with B cell-specific null mutations for Ikaros were analyzed (22). IkE mice were crossed with CD19-Cre tg animals to activate the Cre recombinase from the bone marrow (BM) pro-B cell stage, and with CD21-Cre tg mice to activate Cre activity from the splenic T2 B cell stage (29,30,32). Deletion of the floxed Ikaros alleles at these stages did not grossly affect B cell differentiation in the BM, although IkE CD19-Cre+ (CD19 cKO) mice showed an increase in pro- and pre-B cells and a corresponding decrease in B220+CD43+ cells, suggesting a bottleneck in differentiation, in line with the requirement for Ikaros in pre-B cell development (Fig. 1A) (22). In the spleen, Ikaros proteins were detected in WT B cell populations (Fig. 1B) (22), but they were selectively absent in splenic B cells of both CD19 cKO and IkL/L CD21-Cre+ (CD21 cKO) mice (Fig. 1C).

Splenic B cell populations were analyzed in the different mutant mice by flow cytometry. While total splenocyte numbers were reduced in IkL/L mice, as previously described (18), they were slightly increased or similar in CD19 and CD21 cKO animals, respectively, compared with control mice (Fig. 1D). CD19+ B cells were also reduced in the IkL/L spleens but not in the cKO organs, suggesting that mature B cell numbers in general are not directly affected by Ikaros loss (Fig. 1D). Nonetheless, some B cell subsets were altered (Figs. 1, E and F). IkL/L spleens contained less CD19+CD21hiCD23lo marginal zone (MZ) B cells, while cKO spleens contained variable numbers of FO but no detectable MZ B cells, when compared with control organs. These results suggested that Ikaros loss in late B cell development does not significantly affect FO B cell homeostasis, whereas MZ B cell homeostasis depends on Ikaros function.

Unexpectedly, CD19+B220loCD43−CD5+IgMhi IgDlo B1 cells were significantly increased in frequency and absolute number in all mutant spleens (Fig. 2A and not shown). While control spleens contained 1-2% of B1 cells, as reported (41), IkL/L and cKO spleens showed 2-4 fold more B1 cells. Similar increases were not observed in the peritoneal cavity (PEC), as both CD19+CD5+CD11b+ B1a and CD19+CD5+CD11b− B1b cells were detected in similar or slightly reduced numbers in the mutant mice compared with control littermates (Fig. 2B). These results suggested that the splenic B1 cell compartment is consistently and selectively enhanced in the absence of Ikaros.

Ikaros null B1 cells develop rapidly from adult progenitor cells. Because B1 cells differentiate from fetal liver and BM progenitors (42), we asked if loss of Ikaros could affect B1 cell homeostasis in the adult bone marrow. The percentage and number of phenotypically mature CD19 IgMhiCD43+CD5− B1 cells were therefore assessed in the BM of IkL/L, CD19 cKO and CD21 cKO mice (Fig. 3A). B1 cells were detected in significantly higher numbers in the IkL/L and CD19, but not CD21, cKO BM compared with control samples, suggesting that Ikaros already plays a role in expanding BM B1 progenitor cells.

To determine if B1 progenitor cells are affected by
Ikaros deficiency, we evaluated the number of Lin<sup>-</sup>CD93<sup>-</sup>CD19<sup>-</sup>B220<sup>lo</sup> BM cells, previously shown to contain B1 progenitors (3), in Ikzf1<sup>–/–</sup> and WT mice (Fig. 3B). This showed that the Ikzf1<sup>–/–</sup> BM had significantly more Lin<sup>-</sup>CD93<sup>-</sup>CD19<sup>-</sup>B220<sup>lo</sup> cells than the WT BM. These results thus suggested that the BM contains more B1 progenitor and mature cells when Ikaros function is impaired.

To determine if loss of Ikaros enhances B1 cell development throughout adult life, Ikzf1<sup>–/–</sup> mice were crossed with R26-CreERT2 tg animals (31), and Ikzf1<sup>–/–</sup> littermates (Ikzf1<sup>–/–</sup> R26-CreERT2<sup>–/–</sup>) were analyzed after CD19<sup>+</sup>CD5<sup>-</sup> non-B1 B cells in cell cycle status (Fig. not shown). Further, these cells were similar to cultures, but not in the control wells (Fig. 3E and significant numbers in the 4OHT- treated R26 cKO and loss of Ikaros proteins in splenocytes was in all tissues. Mice were treated with TAM for 3d, and loss of Ikaros proteins in splenocytes was confirmed 1 month later by Western blot (Fig. 3C). TAM treatment increased splenic CD19<sup>+</sup>CD43<sup>+</sup>B1 cell numbers by approximately 3-fold in R26 cKO mice (Fig. 3D), while the numbers of CD19<sup>+</sup>CD43<sup>-</sup>B cells (which includes transitional, FO and MZ B cells) decreased. These results indicated that splenic B1 cell numbers are rapidly increased in adult mice when Ikaros function is lost.

To demonstrate that B1 cells develop from BM progenitor cells in the absence of Ikaros, we co-cultured R26 cKO or control CD19<sup>+</sup> BM cells with S17 stromal cells and cytokines for 6d under B lymphopoietic conditions (11), in the presence or absence of 4-hydroxytamoxifen (4OHT) to induce Ikzf1 deletion. These experiments showed that CD19<sup>+</sup>CD43<sup>+</sup>CD5<sup>-</sup>CD11b<sup>-</sup> cells were present in significant numbers in the 4OHT-treated R26 cKO cultures, but not in the control wells (Fig. 3E and not shown). Further, these cells were similar to CD19<sup>+</sup>CD5<sup>-</sup> non-B1 B cells in cell cycle status (Fig. 3F), suggesting that they appeared as a result of differentiation and not increased proliferation. Altogether, our results indicated that Ikaros limits splenic B1 cell numbers in the adult in a cell-intrinsic manner, in part by inhibiting their differentiation from bone marrow progenitor cells.

**Ikaros null B1 cells are bona fide B1 lineage cells.** To determine if the B1 cells in Ikaros null mice are bona fide B1 cells, we analyzed their transcriptomes. R26 cKO and control littermates were TAM injected and their splenic B1 cell transcriptomes were analyzed 10d afterwards by Affymetrix arrays. Comparison of the mutant and WT B1 cell transcriptomes clearly showed that they were alike in global gene expression (Fig. 4A, clusters a and c, and data not shown). Most of the genes, whose expression levels were previously shown to be increased or decreased in B1a cells compared with FO B cells (43,44), were similarly increased or decreased in control and R26 cKO B1 cells. Importantly, genes encoding transcription factors essential for B2 cell development and function (eg. SpiB, Pax5, Ebf1, Mef2c) were downregulated in R26 cKO B1 cells, like control B1 cells. Interestingly, R26 cKO B1 cells expressed intermediate levels of some B2-associated genes (Fig. 4A, clusters b and d), suggesting that Ikaros plays a role in defining the B1 cell signature.

Ikaros deletion resulted in the increase or decrease (fold-change>2) of 96 and 36 genes, respectively, in B1 cells (Fig. 4B). Many of the upregulated genes belonged to the interferon response signature (not shown), which was probably non-specific and Cre-dependent, as these genes were also upregulated in R26 cKO thymocytes after TAM treatment, but not in germline deficient cells (45). In addition, 10 other upregulated genes corresponded to genes normally expressed in hematopoietic stem cells, which are Ikaros-dependent but also upregulated in other Ikaros deficient hematopoietic cells (25). Thus few upregulated genes appeared to be B cell-specific. Among the B-cell specific genes, some have been implicated in lymphocyte activation, like: Gna15 (up 2.1x) and Slampf (up 3.5x) (46); Tmem176b (up 7.3x) is highly related to the B cell co-signaling molecule CD20 (47); Nrp2 (up 2x), homologous to Nrp1, which is induced in activated T cells (48). In contrast, some of the downregulated genes are known negative regulators of B cell signaling. These include: Slpi, a secretory leukoprotease inhibitor that negatively regulates B cell proliferation and IgM production following LPS activation (down 2.8x) (35); Tyrobp, which encodes the Dap12 signaling adaptor, and is a negative regulator of B cell-dependent responses including autoantibody production (down 3x) (49); Pirb, which encodes the paired Ig receptor PIR-B, and has been found to negatively regulate BCR-mediated activation and inhibit TLR9-induced autoantibody production (down 2.1x) (50,51); Rgs13, which attenuates G protein-mediated signal transduction and has been shown to limit extra-follicular plasma cell generation (down 2.2x) (52,53); Rapgef4, which encodes Epac, a downstream target of cAMP, and which is implicated in BCR-induced growth arrest and apoptosis (down 2.2x) (54). Many of these changes were validated by RT-qPCR (Fig. 4C). The activation of these genes was likely to be Ikaros dependent, as the expression of other transcription factors (Spi1, Pax5, Irf4, Prdm1, Xbp1, Tcf1, Ebf1) important for B cell function were similar between Ikaros null and control B1 cells (Fig. 4D). To determine if the identified genes were direct Ikaros targets, we analyzed Ikaros binding by ChIP-sequencing on chromatin from a pre-B cell line (22),
as primary B1 cell numbers were too limiting for these studies and pre-B cells may contain B1 cell precursors (22) (Fig. 4E and data not shown). This showed that Ikaros bound to the loci of Tyrobp, Nrp2, Tmem176b, Gna15, and Slamf9, suggesting that Ikaros directly regulates the transcription of some of the positive and negative regulator genes. Collectively, these results suggested that Ikaros is required for maintaining the correct expression of regulator molecules of B1 cell activation and signaling.

_Ikaros deficient B1 cells hyper-proliferate in response to TLR4 stimulation._ Because Ikaros has been shown to negatively regulate the activation response in B2 cells (18), we asked if Ikaros also sets the activation threshold in B1 cells, and whether common pathways are regulated by Ikaros in these cell types.

We first compared the proliferative response of splenic B2 FO and B1 cells to stimulation using a variety of stimuli. IkL/L and WT FO B cells (CD19^+CD23^hiCD21^-) were stimulated with anti-IgM antibodies, anti-IgM + anti-CD40, anti-CD40 + interleukin-4 (IL-4), or LPS, and cell division was measured by CFSE dilution after 3d of culture. These experiments showed that IkL/L cells proliferated more (loss of CFSE intensity), and more IkL/L cells proliferated (sum of the lower CFSE peaks), in response to anti-IgM, anti-IgM + anti-CD40, and anti-CD40 + IL-4 stimulation, when compared with WT cells (Fig. 5A), even though cells of both genotypes exhibited similar surface levels of IgM and TLR4 (Fig. 5B). However, increased cell division was not detected after LPS stimulation, suggesting that LPS alone saturated the proliferative response in Ikaros deficient B2 cells. In addition, CD19, CD21 and R26 cKO FO B cells also hyper-proliferated in response to anti-IgM stimulation (Fig. 5C). These data extend previous findings, and indicate that FO B2 cells require Ikaros to limit their proliferative response in a B cell-specific and cell-intrinsic manner, especially when stimulation is sub-optimal (i.e. anti-IgM stimulation alone). To test if B1 cells also require Ikaros for proliferation, splenic IkL/L and WT B1 cells (CD19^-CD43^-) were stimulated with anti-IgM antibodies or LPS. Although these cells expressed comparable levels of surface IgM and TLR4 (Fig. 5D), IkL/L B1 cells did not respond to anti-IgM stimulation, like WT B1 cells (Fig. 5E) (55). However, IkL/L B1 cells hyper-proliferated to LPS stimulation. Thus, Ikaros sets the activation threshold in B1 cells in response to TLR4 stimulation.

To determine if Ikaros deficiency affects cell survival, we first stimulated IkL/L and WT FO B cells with anti-IgM ± anti-CD40 antibodies for 18h, and evaluated them with DiOC6, a mitochondria dye that stains live cells (56,57). This showed that the WT samples contained on average 20% fewer live cells after anti-IgM stimulation (Fig. 5F), compared with no stimulation, as expected (58). In contrast, IkL/L samples stimulated with anti-IgM contained as many live cells as cultures without stimulation, suggesting less BCR-induced apoptosis. Cells of both genotypes showed enhanced viability when stimulated with anti-IgM + anti-CD40, compared with unstimulated cells, and there was little difference between WT and mutant cultures. Thus Ikaros deficient FO B cells survive better than WT cells in response to anti-IgM stimulation. To determine if Ikaros deficient B1 cells also survive better upon stimulation, we stimulated IkL/L and WT CD19^-CD43^- B1 cells with LPS and evaluated them after 18h (Fig. 5G). Interestingly, in the absence of stimulation, the percentage of live IkL/L B1 cells was always superior to that of WT cells, even though LPS stimulation decreased IkL/L cell survival compared with WT B1 cells. Unexpectedly, however, the expression levels of the anti-apoptotic Bcl2 family member proteins (Bcl2, Bcl2L1, Mcl1) were decreased in the mutant B1 cells, suggesting that other mechanisms are behind the increased survival of Ikaros deficient cells (Fig. 5H). Altogether, our results indicated that Ikaros negatively regulates B1 cell survival under non-stimulating conditions.

_Ikaros is a negative regulator of autoantibody production in B1 cells._ Because B1 cells constitute a major source of self-reactive IgM antibodies in vivo, we asked if loss of Ikaros affects the serum autoantibody pool. We previously showed that total IgM antibodies were slightly increased in IkL/L mice compared with control animals (18). Here we tested the serum of IkL/L and WT mice (6-10 weeks of age) for IgM anti-dsDNA, anti-chromatin, antithyroglobulin and rheumatoid factor, by ELISA (Figs. 6, A and B). As control, the serum of lupus-prone MRL/lpr mice was also analyzed. Strikingly, the levels of all IgM autoantibodies tested were increased in the IkL/L serum. The IkL/L serum also exhibited increased antinuclear antibody (ANA) activity, as detected on HEP-2 cells (Fig. 6C). Indeed, the serum of young (5-9 weeks) and mature (20-41 weeks) CD19 and CD21 cKO mice contained significantly more anti-chromatin and anti-dsDNA IgM antibodies than control mice (Figs. 6, D and E), and this was particularly striking in the CD19 cKO serum, where autoantibody levels reached those found in the serum of sick MRL/lpr mice. However, the cKO mice did not produce IgG autoantibodies, even at an older age (Fig. 6E). These
results indicated that Ikaros deficiency in B cells triggers an overproduction of IgM autoantibodies to self-antigens.

We then asked which B cell type was responsible for the autoantibody production. As the CD19 and CD21 cKO mice do not have MZ B cells, and peritoneal B1 cells have not been shown to secrete significant amounts of IgM antibodies (59), we focused our analysis on splenic FO B2 and B1 cells. These cells were sorted from Ikl/L and CD19 cKO (and control) mice, and stimulated with anti-IgM + anti-CD40 + LPS for 6d, and their supernatants were evaluated for anti-chromatin and anti-dsDNA antibodies by ELISA (Fig. 7A). Serum from CD19 cKO and control littermates was used as controls. FO B cells did not secrete detectable autoantibodies, regardless of genotype or stimulation. Likewise, control B1 cells made little or no autoantibodies, even upon stimulation. However, both mutant B1 cells secreted significant quantities of IgM anti-chromatin and anti-dsDNA antibodies upon stimulation. Increased antibody secretion was found even after normalizing for cell numbers (Fig. 7B). These results thus identify splenic B1 cells as a major source of autoantibody production in Ikaros deficient mice.

DISCUSSION

Our results indicate that Ikaros is a major negative regulator of B1 cell development and function. Upon loss of Ikaros, B1 cell numbers are increased in the spleen and bone marrow, and adult B1 progenitors are enhanced in the bone marrow. Ikaros null B1 cells resemble WT B1 cells at the transcriptome level and in their ability to respond to TLR4 stimulation but not anti-IgM activation. However, mutant B1 cells are hyper-reactive, and only Ikaros null B1 cells can be induced to secrete high amounts of IgM autoantibodies. These results provide a possible explanation for why dominant-negative Ikaros proteins were previously associated with autoantibody production (60).

At least part of the Ikaros null B1 cell population develops from the bone marrow, as Ikaros deficiency in the germline (Ikl/L, R26 cKO) or bone marrow (CD19 cKO) leads to an increase in BM B1 cell numbers, while loss of Ikaros in the transitional B cells of the spleen (CD21 cKO) does not. Why CD21 cKO mice also have increased splenic B1 cell numbers remains unknown, although this may be connected to the increase in cell survival of Ikaros deficient cells that we observed in culture (see Fig. 4). Our results thus support the concept that B1 cells can develop from the bone marrow in addition to the fetal liver, and suggest that WT BM B1 cell development is normally kept in check by Ikaros. This is in line with our previous observations that Ikl/L fetuses show a near-complete loss of B lineage cells (18). Interestingly, PEC B1 cells do not appear to be negatively regulated by Ikaros in the mouse lines studied here, suggesting different or additional mechanisms of regulation.

Several lines of evidence suggest that Ikaros null B1 cells develop from B1 progenitor cells and not from committed B2 cells. At the transcriptional level, the mutant B1 cells exhibit a gene expression signature most resembling WT B1 cells. At the functional level, Ikaros deficient B1 cells behave like WT B1 cells, as they do not proliferate in response to anti-IgM stimulation and they do not undergo class switch recombination to secrete IgG autoantibodies. Further, we were unable to differentiate B1 cells from 4OHT-treated R26 cKO spleen cells (BH, unpublished results) like we did from the BM, arguing against a possible switch from B2 to B1 cells in the spleen, as has been reported for PU.1 null B cells (11). Thus, although Ikaros sets the activation threshold for both B2 and B1 cells, this does not appear to influence the phenotypic and functional phenotype of B1 cells.

Nonetheless, Ikaros deficient B1 cells show downregulated mRNA levels for important negative regulators of B cell signaling and autoantibody production. These genes may be direct Ikaros targets, as Ikaros binds to the loci of several of these genes in pre-B cells. Further, we found that many transcriptional regulators essential for B2 and B1 cell biology remain relatively unchanged between control and Ikaros null B1 cells, suggesting that they do not play a major role in regulating the activation threshold or state of Ikaros null B1 cells. This is in contrast to a recent study in peritoneal B1 cells in which the knockdown of Ikaros by siRNA induced an upregulation of Spi1 mRNA levels (61), suggesting perhaps that peritoneal and splenic B1 cells respond differently to Ikaros.

Our results indicate that Ikaros is an important transcriptional activator in B1 cells, similar to what we and others have observed in pre-B cells (22,23). This is in contrast to T cells where Ikaros deficiency leads to the activation of the Notch pathway and hematopoietic stem cell-associated genes (25,45), indicating a key role as a transcriptional repressor. Previous studies of transcriptional regulation in B1 cells have mainly identified proteins important for promoting B1 cell function. Ikaros, on the other hand, appears to be unique in that it is required to limit B1 cell development, survival and expansion.

Finally, our results have implications for Ikaros and B1 cells in autoimmunity and cancer. B1 cells and polyreactive antibodies are elevated in several autoimmune diseases, and the IKZF1 gene was identified as a susceptibility locus for systemic lupus erythematosus (62,63). Further investigation
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into the development and function of B1-like cells from patients with \textit{IKZF1} polymorphisms may reveal a similar deregulation of B1 cell function in the human system. Similarly, Ikaros is a tumor suppressor in B cell acute lymphoblastic leukemias (B-ALL) (64), particularly those with oncogenic mutations or amplifications that activate the JAK-STAT pathway (eg. BCR-ABL, JAK2, CRLF2). Many cases debut in early childhood, a time when human B1 cells have been speculated to be abundant (65). Indeed, BCR-ABL transformed murine B1 progenitor cells initiate disease more rapidly than B2 progenitors, suggesting that B1 cells may be the cause of high risk cases (66). Our results may therefore suggest a crucial link between Ikaros loss and B1 cell-associated ALL. Finally, as CD5+ B1 cells have been implicated in chronic lymphoid leukemia (CLL) (67), Ikaros may also act as a tumor suppressor in B1 cell-related CLL.

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Abbreviations: BCR, B cell receptor; T-ALL, T cell acute lymphoblastic lymphomas/leukemias; BM, bone marrow; FO, follicular; MZ, marginal zone; PEC, peritoneal cavity; TAM, Tamoxifen; 4OHT, 4-hydroxytamoxifen; B-ALL, B cell acute lymphoblastic leukemias.

FIGURE LEGENDS

FIGURE 1. Consequence of Ikaros deletion on bone marrow and splenic B cell populations. (A) BM B cell populations of the indicated mice. Gated populations in the left panels: pro- and pre-B (B220⁺CD43⁺); small pre-B and immature B (B220⁺CD43⁺); recirculating B (B220⁺CD43⁺). B220⁺CD43⁺ cells were further analyzed for CD24 and BP-1 in the right panels: pro-pre-B (CD24⁺BP-1⁻), early pre-B (CD24⁺BP-1⁻), late pre-B and large pre-B (CD24⁺BP-1⁺). Numbers correspond to percentages. (B) Ikaros expression in FO and B1 cells was assessed by intracellular staining. As a control, the Ikaros deficient cell line BH1 was used (gray histogram). (C) Western blot of Ikaros proteins in CD43⁺ non-B cells and FO B cells from spleens of the indicated mice. β-actin was used as a loading control. The different Ikaros bands correspond to various isoforms. All data are representative of 3 independent experiments. (D) Graphs show cellularity (top), and absolute numbers of CD19⁺ B cells (bottom) from the indicated mice. Lines denote median. Each circle represents one mouse, cumulative data from >5 independent experiments. (E) Graphs show percentages and absolute numbers of MZ and FO B cells from the indicated mice. Lines denote median. Each circle represents one mouse, cumulative data from >5 independent experiments. (F) Representative staining of splenic B cell populations of the indicated mice. B220⁺CD93⁺ mature B cells were analyzed for CD21 and CD23 expression to delineate FO (CD23hiCD21lo) and MZ (CD23loCD21hi) B cells. *p<0.01, **p<0.005, ***p<0.001 (unpaired Student's t-test).

FIGURE 2. Loss of Ikaros leads to accumulation of splenic B1 cells. (A) (top) CD19⁺ splenic B cells from the Ik⁻/⁻, CD19 cKO and CD21cKO mice were analyzed for CD43 expression by flow cytometry to define B1 cells (CD19⁺CD43⁺). Numbers correspond to percentages. CD19⁺CD43⁺ and CD19⁺CD43⁻ cells were further analyzed for CD5 expression. (bottom) Graphs show percentages and absolute numbers of splenic B1 cells in the indicated mice from >5 independent experiments. Each circle represents one mouse. Lines denote median. (B) (top) PEC CD19⁺ cells were analyzed for CD11b and CD5 expression to delineate B1a (CD11b⁺CD5⁻) and B1b (CD11b⁺CD5⁺) cells. (bottom) Graphs show percentages and absolute numbers of PEC B1 cells from >5 independent experiments. Each circle represents one mouse. Lines denote median. *p<0.003, **p<0.001, ***p<0.0001 (unpaired Student's t-test).

FIGURE 3. Ikaros controls B1 cell differentiation in the bone marrow. (A) B1 cells (IgM⁺CD19⁺CD5⁺CD43⁺) in the WT and Ik⁻/⁻ BM. Graphs show percentages and absolute numbers (one tibia and one femur) of BM B1 cells in the indicated mice from >5 independent experiments. Each circle represents one mouse. Lines denote median. *p=0.01, **p>0.001 (unpaired Student's t-test). (B) Lin⁻ CD93⁺CD19⁺B220⁺ B1 cell progenitors in the WT and Ik⁻/⁻ BM. Graphs show percentages and absolute numbers (one tibia and one femur) of BM B1 cell progenitors from >5 independent experiments. Each circle represents one mouse. Lines denote median. **p<0.005, ***p<0.0001 (unpaired Student's t-test). (C) Western blot of Ikaros proteins in splenocytes of R26 cKO and control mice treated with TAM for 3d and analyzed after 4 weeks. β-actin was used as a loading control. The different Ikaros bands correspond to
FIGURE 4. Transcriptome analysis of Ikaros-regulated genes in B1 cells. (A and B) R26 cKO or control mice were treated with TAM for 3 days. CD19+CD43+B1 cells, as well as non-B1 B cells (CD19+CD43-) were isolated 10d after initiation of TAM treatment, and their transcriptomes were analyzed (2 independent samples in each case). (A) Genes up- or downregulated in the B1 vs non-B1 B cells from control mice were identified (log2 FC up or down >0.5 on average, and >0.4 for each pairwise comparison), and the expression of these genes in R26 cKO B1 cells was visualized by K-means clustering. Genes, previously found to be up- or downregulated in B1 cells (43,44), are listed next to their respective clusters. (B) Genes up- or downregulated in R26 cKO B1 cells (log2 FC >1, up or down, and >0.8 for each pairwise comparison) were selected and visualized by hierarchical clustering. Upregulated hematopoietic stem cell genes that were also detected in Ikaros deficient T cells are listed on the left. Genes that encode positive or negative regulators of signaling are indicated. In (A) and (B), green and red colors correspond to low and high values, respectively, of the normalized expression. (C) RT-qPCR analysis of the indicated genes in WT and CD21 cKO B1 cells relative to HPRT. Graphs represent mean ± SD of duplicate samples from 3 mice each. *p<0.01, **p<0.001, ***p<0.0001 (unpaired Student's t-test). (D) RT-qPCR analysis of the indicated genes in WT and CD21 cKO B1 cells relative to HPRT. Graphs represent mean ± SD of duplicate samples from 3 mice each. (E) Genome browser tracks of Ikaros binding at the loci of the indicated genes in the BH1-Ik1-ER-Bcl2 pre-B cell line (22). Sequencing of input DNA is shown in green.

FIGURE 5. Increased proliferation and survival by Ikaros deficient B1 cells. (A) CFSE-labeled WT and IkL/L splenic FO B cells were stimulated for 72h with anti-IgM, anti-IgM + anti-CD40, anti-CD40 + IL-4, or LPS. Gray histograms indicate unstimulated WT cells. The number of divisions completed is indicated. Representative of >3 independent experiments. (B) Expression of surface IgM and TLR4 on the indicated FO B cells before stimulation. For IgM, the gray histogram denotes non-B cells; for TLR4, the corresponding isotype control. Representative of 2 independent experiments. (C) CFSE-labeled splenic FO B cells from the indicated mice were stimulated for 72h with anti-IgM antibodies. R26 cKO mice were first treated in vivo with TAM for 4d. Ctrl, control cells for the corresponding cKO. Gray histograms indicate unstimulated cells. Representative of >3 independent experiments for each genotype. (D) Expression of surface IgM and TLR4 on splenic B1 cells before stimulation. For IgM, the gray histogram denotes non-B cells; for TLR4, the corresponding isotype control. Representative of 2 independent experiments. (E) CFSE-labeled WT and IkL/L B1 cells were stimulated for 72h in the presence of anti-IgM or LPS, and analyzed. The number of divisions completed is indicated. Gray histograms denote the CFSE labeling for unstimulated controls. Representative of 3 independent experiments. (F) WT and IkL/L FO B cells were stimulated for 18h with anti-IgM, or anti-IgM + anti-CD40, and analyzed for survival using DiOC6. (left) The percentages of the live cells are indicated. (right) Statistical analysis of 3 independent experiments was performed with the indicated samples. *p<0.05, **p<0.02 (paired Student's t-test). (G) WT and IkL/L B1 cells were stimulated for 18h with LPS, then analyzed for survival with DiOC6 to stain active mitochondria (live cells). (left) The percentage of live cells is indicated. (right) Graph shows quantification of 2 independent experiments. (H) Relative fold expression of the indicated genes in unstimulated IkL/L B1 cells compared with WT (mean ± SD of duplicate samples from 3 mice each.). *p<0.01, **p<0.001 (unpaired Student's t-test).

FIGURE 6. Ikaros deletion enhances anti-chromatin and anti-dsDNA autoantibody secretion in vivo. (A) Sera of IkL/L (n=32, 6-8 weeks of age) and WT littermates (n=18, 6-9 weeks of age) were tested for IgM antibodies to the indicated antigens by ELISA. MRL/lpr sera (n=4, ~20 weeks of age, diseased) were used as a positive control. Bars show the mean of the indicated values. Each circle represents one mouse. ***p<0.0001 (Wilcoxon signed rank test). (B) Sera of IkL/L (n=41, 6-10 weeks) and WT (n=26, 32-52 weeks) mice were tested for rheumatoid factors (IgM anti-IgG) and anti-Thyroglobulin (IgM) antibodies by ELISA. MRL/lpr serum (n=10, 20 weeks) was used as a positive control. Bars denote the mean values. ***p<0.0001 (two-tailed Student's t-test assuming unequal variance). (C) (left) Representative ANA levels

Various isoforms. Representative of 3 independent experiments. (D) Mice were treated with TAM for 3 days. CD19+ splenic cells were analyzed 1 month later for B1 (CD19+CD43+) and B2 (CD19+CD43-) cells. Graphs show absolute numbers from 5 independent experiments. Lines denote median. Ctl, control. *p<0.01, **p<0.003 (paired Student's t-test). (E) CD19+ BM B cells were cultured on stromal cells and cytokines ± 4OHT. After 6 days, CD19+ cells were analyzed for CD5 and CD43 expression. Ctl, control. The relative fold-increase in cell numbers of CD19+CD5+CD43+ cells is shown where control values were set at 1.0, from 3 independent experiments. (F) CD19+CD5+ or CD19+CD5- cells from the R26 cKO + 4OHT cell cultures (see E) were analyzed by Ki-67 and DAPI staining. Representative staining from 3 independent experiments.
of IκL/L serum. Numbers represent the scores. (right) Relative ANA levels of IκL/L vs WT sera. Scores were blindly assigned to samples on a scale of 0-5 by four individuals, where 5 is the highest intensity. Bars indicate the mean of the scores. *p<0.03 (Wilcoxon signed rank test). (D) Sera of CD19 cKO (n=7) and control (n=5), and of CD21 cKO (n=14) and control (n=10), mice were tested for IgM antibodies to the indicated antigens by ELISA. Mice were between 7-12 weeks of age. MRL/lpr sera from mice of similar ages (n=12, 10 weeks, non-diseased) were used as a positive control. The inset in the left panel indicates the same values presented on a smaller scale to highlight the statistical significance. Each value represents the average of 2 independent ELISA tests. Bars show the mean of the indicated values. Each circle represents one mouse. **p<0.001 (Wilcoxon signed rank test). (E) Sera of young and mature CD19 cKO (young, n=11; mature, n=12) and control (Ctl) littermates (young, n=6; mature, n=10) mice were tested for IgM (young, Y and mature, M) and IgG (mature, n=5) antibodies to the indicated antigens by ELISA. Young mice were defined as 5-9 weeks; mature mice as 20-41 weeks of age. MRL/lpr sera (non-diseased: n=12, 10 weeks; diseased: n=5, 20 weeks) were used as positive controls. Each value represents the average of 2 independent ELISA tests. Bars denote the mean values. *p<0.01, **p<0.001 (Wilcoxon signed rank test).

FIGURE 7. Ikaros deficient B1 cells secret more anti-chromatin and anti-dsDNA autoantibody in vitro. (A) Splenic FO and B1 cells from IκL/L and WT mice (left), or CD19 cKO (right) and control mice were cultured in the absence or presence of anti-IgM + anti-CD40 + LPS for 6d. Supernatants from the cultures were tested for the indicated antibodies by ELISA. Sera of CD19 cKO (+) and control (-) mice were used as controls. Each circle represents data from one mouse. Graphs represent data from ≥3 independent experiments. *p<0.01 (unpaired Student's t-test). (B) Assessment of anti-dsDNA autoantibodies by ELISA in the supernatants of CD19 cKO splenic FO and B1 B cells cultured for 6d in the absence or presence (stim) of anti-IgM + anti-CD40 + LPS. Data are normalized to cell numbers. Cumulative data from 2 independent experiments.
Ikaros and B1 cells

FIGURE 1

A

CD19 Cre

CD21 Cre

WT

cKO

B

Ikaros

non-B FO B

Cre

Cre

non-B FO B

kD

C

Ikaros

β-actin

CD21 Cre

CD19 Cre

D

total splenocytes

# cells (x10^6)

CD19+ splenocytes

# cells (x10^5)

WT

IkL/L

IkL/L

CD19 Cre

CD21 Cre

E

MZ B cells

% total

# cells (x10^6)

FO B cells

% total

# cells (x10^6)

WT

IkL/L

IkL/L

CD19 Cre

CD21 Cre

F

IkL/L

CD19 Cre

CD21 Cre

WT

Mutant

CD21

CD23

B220

CD43

CD24

B220

B220

CD23

CD23

CD23

CD23
Ikaros and B1 cells

FIGURE 2

A

WT

Mutant

CD43

CD19 Cre CD21 Cre

Ik^L/L

# cells (x10^6)

% total

WT Ik^L/L - + - + Cre

CD19 Cre

CD21 Cre

CD43

CD19

CD5

CD5

CD5

2.7

15.8

2.3

7

3.7

6.5

CD19 Cre CD21 Cre

Ik^L/L

% total

WT Ik^L/L - + - + Cre

CD19 Cre

CD21 Cre

CD43

CD19

CD5

CD5

CD5

2.7

15.8

2.3

7

3.7

6.5

WT

Mutant

CD43

CD19 Cre CD21 Cre

Ik^L/L

# cells (x10^6)

% total

WT Ik^L/L - + - + Cre

CD19 Cre

CD21 Cre

CD43

CD19

CD5

CD5

CD5

2.7

15.8

2.3

7

3.7

6.5

WT

Mutant

CD43

CD19 Cre CD21 Cre

Ik^L/L

% total

WT Ik^L/L - + - + Cre

CD19 Cre

CD21 Cre

CD43

CD19

CD5

CD5

CD5

2.7

15.8

2.3

7

3.7

6.5

WT

Mutant

CD43

CD19 Cre CD21 Cre

Ik^L/L
**FIGURE 3**

A

WT

Ik^L/L

IgM

CD19

CD5

CD43

B

WT

Ik^L/L

CD93

Lin

B220

C

Spleen

Cre

TAM

IkAROS

β-actin

R26 Cre

D

Ctl

R26 cKO

CD43

CD19

3

7

91

86

F

CD5^−

CD5^+

4OHT

DAPI

CD19^+CD5^+CD43^+

fold increase

- + - + 4OHT

Ctl R26 cKO

8 24

76 20

1.5

10

86

3.8

11

0.8

0.5

0.1

0.4

0.6

0.8

1.0

0

0.01

0.02

0.03

0

0.02

0.04

0.06

0.08

0.10

0

10

20

30

40
**FIGURE 4**

**A** non-B1 B1

| Cre | - | - | + |
|-----|---|---|---|

**B1 signature**

(cluster a (n=314)

(cluster b (n=58)

(cluster c (n=323)

(cluster d (n=196)

**C**

WT CD21 cKO

| mRNA | mRNA |
|------|------|
| Nr2  | Tmem176b |
| Gna15| Slamf9 |

**D**

WT CD21 cKO

| mRNA | mRNA |
|------|------|
| Sw1  | Par5  |
| If4  | Prdm1 |
| Xbp1 | Tcf3  |

**E**

pre-B Nrp2

| mRNA | mRNA |
|------|------|
| Nrp2 | Tmem176b |

**pre-B Gna15**

| mRNA | mRNA |
|------|------|
| Gna15| Slamf9 |

**pre-B Tyrobp**

| mRNA | mRNA |
|------|------|
| Tyrobp | Rgs17 |

Ikaros and B1 cells
FIGURE 5

A. IgM, IgM + CD40, CD40 + IL-4, LPS

B. IgM, TLR4

C. CD19 Cre, CD21 Cre, R26 Cre

D. IgM, TLR4

E. IgM, LPS

F. WT, IkBα/−/−

G. WT, IkBα/−/−

H. mRNA expression
**FIGURE 7**

A  
Ik\(^{L/L}\)  

|          | WT  | L/L | serum | B1  |
|----------|-----|-----|-------|-----|
| Cre      | Ø   | +   | -     | *   |
| stim     | Ø   | +   | -     | +   |

OD 405nm  

B  
anti-dsDNA  

|          | WT  | L/L | serum | B1  |
|----------|-----|-----|-------|-----|
| Cre      | Ø   | +   | -     | +   |
| stim     | Ø   | +   | -     | +   |

OD 405nm / 1.05 cells  

CD19 Cre
Ikaros is a negative regulator of B1 cell development and function
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