

RESEARCH ARTICLE

Arid3b Is Critical for B Lymphocyte Development

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Abstract

Arid3a and Arid3b belong to a subfamily of ARID (AT-rich interaction domain) transcription factors. The Arid family is involved in regulating chromatin accessibility, proliferation, and differentiation. Arid3a and Arid3b are closely related and share a unique REKLES domain that mediates their homo- and hetero-multimerization. Arid3a was originally isolated as a B cell transcription factor binding to the AT rich matrix attachment regions (MARS) of the immunoglobulin heavy chain intronic enhancer. Deletion of Arid3a results in a highly penetrant embryonic lethality with severe defects in erythropoiesis and hematopoietic stem cells (HSCs). The few surviving *Arid3a*^{-/-} (<1%) animals have decreased HSCs and early progenitors in the bone marrow, but all mature lineages are normally represented in the bone marrow and periphery except for B cells. *Arid3b*^{-/-} animals die around E7.5 precluding examination of hematopoietic development. So it is unclear whether the phenotype of Arid3a loss on hematopoiesis is dependent or independent of Arid3b. In this study we circumvented this limitation by also examining hematopoiesis in mice with a conditional allele of Arid3b. Bone marrow lacking Arid3b shows decreased common lymphoid progenitors (CLPs) and downstream B cell populations while the T cell and myeloid lineages are unchanged, reminiscent of the adult hematopoietic defect in Arid3a mice. Unlike *Arid3a*^{-/-} mice, HSC populations are unperturbed in *Arid3b*^{-/-} mice. This study demonstrates that HSC development is independent of Arid3b, whereas B cell development requires both Arid3a and Arid3b transcription factors.

Introduction

The Arid family of proteins is defined by a conserved ARID (AT-rich interaction domain) that mediates DNA binding and is contained within all family members [1]. The Arid family has

been divided into 7 subfamilies based on shared sequence homologies. Family members act as transcription regulators and have been implicated in the control of cell growth and differentiation as well as cancer development. The Arid3 subfamily consists of 3 members Arid3a, b and c, which are expressed throughout most of hematopoietic development [2]. They share a common REKLES domain along with the ARID DNA binding domain [3]. The founding member of the subfamily is Arid3a/Bright. It was originally isolated as a protein bound to the AT rich nuclear matrix attachment regions (MARS) of the immunoglobulin heavy chain intronic enhancer [4,5]. Arid3a expression is tightly regulated during B cell differentiation [2,6]. Low levels are detectable in the HSC and CLP. Arid3a mRNA then rises during commitment to B cells with levels rising through the pro-B cell stage to the mature recirculating B cell stage in the bone marrow.

Deletion of murine Arid3a results in >99% lethality [7]. Embryos die between E11.5 and E13.5 of gestation due to erythropoietic failure with no obvious cardiac or vascular defects. However the fetal liver is hypocellular, and there is a dramatic decrease in hematopoietic colony activity. The few surviving mice have normal levels of erythroid cells in the peripheral blood. Consistent with the observations from the fetal liver, there were decreases in the HSC, CLP, and myeloid progenitor populations in the bone marrow; however, subsequent hematopoietic development is selectively inhibited in the B cell lineage. Pro-B cells, Pre-B cells, and immature B cells are each decreased approximately 3 fold in surviving mutants compared to wildtype animals. However, mature/ recirculating bone marrow B cells are not significantly reduced.

Arid3a has been shown to bind to its family member Arid3b through their REKLES domains [3]. It also has been observed that, in some cases, association with Arid3b is required for import of Arid3a into the nucleus. Deletion of Arid3b results in a more severe embryonic lethality in mice, with homozygous mutant animals dying at ~E10.5 due to multiple cardiovascular defects [8–10]. The distinct knockout phenotypes suggest that Arid3b has functions during embryogenesis not shared with Arid3a. Arid3a and Arid3b share nearly identical expression patterns during early adult hematopoiesis, including B cell commitment and maturation [2]. Since *Arid3b*^{-/-} animals die before definitive hematopoiesis can be assessed, it was unclear whether hematopoiesis required Arid3a alone or both family members.

To investigate the contribution of Arid3b to hematopoiesis we generated mice that harbored a conditional allele of Arid3b flanked by LoxP sites. These mice were crossed to Mx1-cre transgenic mice so that the Arid3b allele could be inducibly deleted in adult mice. Unlike with Arid3a deletion, we observed no effect on HSC production. However, the phenotype in the B cell lineage is nearly identical between the Arid3a knockout and the conditional Arid3b deletion. CLPs are decreased along with pro-, pre-, and immature B cell populations in the bone marrow, whereas the mature /recirculating population is unchanged. Our data support the conclusion that Arid3a and Arid3b cooperate during bone marrow B lymphopoiesis, but Arid3b is not required for Arid3a regulation of hematopoietic stem cells.

Materials and Methods

Generation of Conditional Arid3b Knockout Mouse

All animal research was approved by the University of Notre Dame IACUC under protocol 16-026. JM8.N4 embryonic stem cells (ESCs, C57BL/6N strain) heterozygous for a targeted “knockout first” allele of Arid3b (*Arid3b*^{tm1a(KOMP)Wtsi/+}) were obtained from the KOMP repository (S1 Fig). Chimeric mice were generated by the IUSM Transgenic and Knock-out mouse core facility by injecting heterozygous ESCs into C57BL/6N blastocysts. Blastocysts were implanted into foster mothers to generate chimeric mice. Male chimeras were mated to female

C57BL/6N mice to generate mice with germline transmission of the “knockout first” allele. To generate mice harboring a conditional allele of Arid3b (Exon 5 flanked by LoxP sites), Arid3b^{tm1a(KOMP)Wtsi/+} heterozygous mice were mated to B6;SJL-Tg(ACTFLPe)9205Dym/J (Stock Number: 003800, Jackson Laboratories, Bar Harbor, ME). The resultant Arid3b^{f/f} mice were mated to B6.Cg-Tg(Mx1-cre)1Cgn/J (Stock Number: 003556, Jackson Laboratories, Bar Harbor, ME) to generate Arid3b^{f/f}: Mx1-cre mice. Knockout of the Arid3b allele was induced by intraperitoneal injection of 3–4 week old mice with 3 doses of poly-pIpC that were administered every 2 days apart. Deletion of the targeted Arid3b exon 4 was evaluated by PCR with the primers CTCGAAAGAGCACATATTGCAG and TTCCCTGGGAGACCTTATG.

Cell Culture

70Z/3 cells (ATCC, Manassas, VA) were grown in RPMI supplemented with 10% FBS, 0.1 mM glutamax, 10 mM HEPES, and 1 mM sodium pyruvate. 293FT cells (Invitrogen, Carlsbad, CA) were grown in OptiMEM supplemented with 5% FBS. OP9 cells (ATCC, Manassas, VA) were cultured in alpha MEM supplemented with 20% FBS, 1mM sodium pyruvate, and 55uM BME. BCL1, a Balb/c derived B cell lymphoma cell line [11,12], was cultured in RPMI supplemented with 10% FBS. Primary mouse cells were cocultured on OP9 cells in IMDM supplemented with 10% defined FBS, 55uM BME, 0.1mM Glutamax, 50ng/mL SCF, 10ng/mL IL-3, and 10ng/mL IL-6. Recombinant mouse cytokines were obtained from R&D Systems (Minneapolis, MN) or Invitrogen (Carlsbad, CA). All media contained 50 U/ml penicillin and 50mg/ml streptomycin. 70Z/3 RFP and 70Z/3 RFP Arid3b overexpressing cells were generated as described previously [13].

Polyinosinic:polycytidylic acid (pIpC) Injections

Mice were given an intraperitoneal injection of 250ug pIpC in 200uL of sterile phosphate buffered saline (PBS) every other day for 5 days (3 total injections). DNA was harvested from the bone marrow and spleen of mice 3 or 12 weeks post final injection by DNAzol extraction (ThermoFisher Scientific, Waltham, MA). Confirmation of Arid3b deletion was done by genomic DNA PCR and quantitative real-time PCR.

Bone marrow B cell isolation

Bone marrow cells were collected from the femurs and tibias of conditionally deleted Arid3b null mice 3 weeks post-pIpC injection. Erythroid cells were removed by ammonium chloride lysis for 15 minutes on ice. Fc γ receptors were blocked by incubating cells with anti-Fc γ RIII/II (CD16/32) for 15 minutes on ice. Following Fc block, cells were stained with a Biolegend B220-biotin antibody (San Diego, CA) for 30 minutes at room temperature. Cells were then incubated with magnetic streptavidin beads for 20 minutes at 4 degrees according to Miltenyi MACS separation protocol (Miltenyi Biotec, Auburn, CA). The B220 positive fraction was then eluted from MACS columns and placed into TRIzol for RNA extraction.

Quantitative reverse-transcriptase PCR (qRT-PCR)

Total RNA was isolated from in vitro cell lines using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. RNA from primary B220+ primary cells was isolated using a RNeasy mini kit from Qiagen (Valencia, CA). Complementary DNA (cDNA) was reverse transcribed from 1ug of RNA using Taqman reverse transcription kit according to manufacturer’s protocol (Applied Biosystems, Foster City, CA). Quantitative analysis was performed using gene specific Taqman (Applied Biosystems, Foster City, CA) or SYBR Green (Applied

Biosystems) reagents. All experiments were performed in triplicate using BioRad CFX96 C1000 System (BioRad, Hercules, CA). Relative gene expression was calculated using the $\Delta\Delta CT$ method. GAPDH was used to normalize expression across different RNA preparations. Relative values are presented as the average of three independent experiments.

Flow Cytometry

Bone marrow cells were collected from the femurs and tibias of conditionally deleted Arid3b null mice 12 weeks post injection. Splenic cells were isolated by passing cells through a 70 μ m cell strainer (ThermoFisher Scientific, Waltham, PA). Mature erythroid cells were removed by ammonium chloride lysis for 15 min on ice. Bone marrow and spleen cells were stained to characterize B cell, myeloid, erythroid, CLP, CMP, and HSC cell populations. Unless stated otherwise, all antibodies were obtained from BioLegend (San Diego, CA, USA). Prior to staining, Fc γ receptors were blocked by incubating cells with anti-Fc γ RIII/II (CD16/32) for 15 minutes on ice. Single cell suspensions were incubated with the following combinations of antibodies: B cell/myeloid- GR1 (fluorescein isothiocyanate (FITC), CD19 (6D5)-phycoerythrin (PE), B220 (RA3-6B2)-allophycocyanin (APC), CD11b (M1-70)-allophycocyanin-Cy7 (APC/Cy7). Bone marrow B cell development- IgM (RMM-1)-FITC, CD19 (6D5)-PE, B220 (RA3-6B2)-APC, IgD (11-26c.2A)-APC/Cy7. Bone marrow Hardy A-C'-HSA-FITC, BP-1-PE, B220-APC, CD43 -Biotin, avidin -APC/cy7. Bone marrow Hardy D-F—IgM-FITC, B220-PE, CD43-APC, IgM-APC/cy7. CLP—Ly6D (49-H4)-FITC, Flt3 (A2F10)-PE, lineage cocktail—CD11b (RM 2801)-biotin, B220 (RA3-6B2)-biotin, CD19 (6D5)-biotin, GR1 (RB6-8C5)- biotin, Terr119 (Ter-119)-biotin, CD3e (145-2c11)-biotin), Texas red-streptavidin (TR), IL7R (A7R34)-APC, cKit (2B8)-APC/Cy7. CMP- CD34 (RAM34)-FITC, Fc γ RIII/II (93)-PE, cKit (ACK2)-APC, lineage cocktail-biotin, Sca-1 (D7)-biotin, IL7R (A7R34)-biotin, and APC/Cy7-streptavidin. HSC/MPP—Sca1 (D7)-FITC, CD48 (HM48-1)-PE, lineage cocktail-biotin, TR-streptavidin, CD150 (TC15-12F12.2)-APC, cKit (2B8)-APC/cy7. Spleen Fr.1-Fr.3 – CD21-FITC, IgM-APC/cy7. Peritoneal cavity—CD5-FITC, B220-APC. Stained cells were subsequently assessed using Beckman Coulter FC500 Flow Cytometer (Brea, CA) and data was analyzed using Flowjo software (Tree Star, Ashland, OR). Dead cells were removed from analysis by the use of FSC/SSC gating and/or exclusion of propidium iodide (Sigma-Aldrich, St. Louis, MO). Basis of gates was determined with the use of fluorescence minus one (FMO) controls when necessary. Results are presented as standard error of the mean (SEM) for averages of each mouse genotype.

Hematopoietic OP9 cultures

Nucleated cells from the femur and tibia of 3-week post-pIpC mice were lineage depleted with a MACS lineage cell separation kit according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Lineage depleted cells were cultured onto 30,000 OP9 cells (plated night before) in IMDM supplemented with 10% defined FBS, 55mM BME, 50 U/ml penicillin, 50mg/mL streptomycin, 0.1mM Glutamax, 10ng/mL Flt3L, and 5ng/mL IL-7. Recombinant mouse cytokines were obtained from R&D Systems (Minneapolis, MN) or Invitrogen (Carlsbad, CA). Cells were transferred onto fresh OP9 cells every 3 days. To evaluate myeloid and B cell differentiation, cells were analyzed 6 days after culture with B220-APC and CD11b-APC/cy7 antibodies (BioLegend, San Diego, CA).

B cell Functional Assays

Whole blood was collected via cheek bleed and allowed to clot for 30 minutes at room temperature. Serum was obtained by centrifugation at 1,500xg for 10 minutes at 4°C. IgG serum

concentration was measured using the Easy-Titer Mouse IgG Assay Kit according to manufacturer protocol (Thermo Fisher Scientific, Waltham, MA, USA). For proliferation and differentiation assays, splenic B cells were isolated from wildtype and *Arid3b*^{-/-} mice by negative selection with anti-CD43 (Ly-48) microbeads according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). Cells were stained with 1uM CFSE (in DMSO) in 1X PBS at a concentration of 20x10⁶ cells/mL for 8 minutes at RT using CellTrace CFSE Cell Proliferation Kit for Flow Cytometry (Thermo Fisher Scientific, Waltham, MA, USA). 2x10⁵ labeled B cells were plated into a 48 well plate at a concentration of 1x10⁶ cells/mL with or without 5ug/mL LPS for 72 hours. After 72 hours, cells were assayed for CFSE expression or stained with cell surface markers B220 and CD138 to evaluate plasma cell differentiation.

Co-immunoprecipitation and Immunoblotting

Bcl1 B cell lysates were incubated with 10 ml of 50% slurry of protein-A/G sepharose beads (Biorad, Hercules, CA) for 1 h to preclear. One microgram of affinity purified polyclonal Arid3a antibody was added to the supernatant for 4 hours at 4°C, followed by a 1 hour incubation with 20 ml protein-A/G sepharose beads (Sigma-Aldrich, St. Louis, MO) [4]. After four washes with lysis buffer, the precipitated proteins were analyzed by SDS-PAGE and transferred to PVDF membrane. Membranes were probed with monoclonal anti-Arid3b mouse monoclonal antibody (Abnova, Taiwan, H00010620-M). Western blots were developed with HRP-conjugated Goat anti-Mouse IgG (Li-COR, Lincoln, NE, P/N 926-80010)

Dual luciferase Assays

Bcl1 cells were stably transfected with firefly luciferase fused in frame to the upstream promoter region of S107 that contains the TX125 and BF150 matrix association regions [12]. These cells were transiently transfected under conditions previously described [12] with pcDNA™3.3-TOPO® TA mammalian expression vector plasmid (ThermoFisher Scientific, Waltham, MA) containing either full length Arid3a, full length Arid3b or both, along with uniform levels of *Renilla* luciferase. Transfected cells were plated at 4 × 10⁴ per well in 24-well plates. Twenty-four hours later, luciferase activity was measured within 10 µg of whole-cell lysate using the luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions. Firefly luciferase reporter gene activity was quantified relative to the activities of vector alone normalized to values of *Renilla* luciferase. The relative activities were obtained as averages from four independent experiments.

Electrophoretic Mobility Shift Assays (EMSA)

Details of nuclear extract isolation and EMSA assays in the Bcl1 murine mature B cell line have been described. Briefly, binding reactions consisted of 0.5–2 ~g of nuclear extract, 1 ~g of poly dI-DC), and DNA probes end-labeled to a specific activity of ~120 cpm/ng with [³²P] dATP (ICN, Costa Mesa, CA), with modifications to buffer D [20 mM HEPES (pH 7.9), 20% glycerol [vol/vol], 50 mM KC1, 1% Tween 20 [vol/vol], 0.2 mM EDTA, 5 mM phenylmethylsulfonyl fluoride) [4,14]. Gel shifts were performed by preincubation (5 min at 37°) with rabbit polyclonal anti-Arid3a [4] anti-Arid3b [12] or both before addition of labeled probes, either 150bp (Bf150) or 125bp (Tx125) gel purified MAR regions upstream of the transcriptional start site of the S107 heavy chain variable region. Probes and shifted bands were identified by autoradiography.

Statistical analysis

Statistical data are presented as the mean +/- standard error of the mean (SEM). For qRT-PCR, error bars are presented as the SEM of all qRT-PCR replicates, while p-values are calculated from the average of the experimental biological replicate. Differences between sample groups were determined by performing an unpaired student t-test. Analysis was performed using PRISM software version 6.0 (Graphpad software, La Jolla, CA).

Results

Loss of Arid3b in Bone Marrow Results in Decreased B cells

To investigate the role of Arid3b in adult mice, we obtained mice from the KOMP repository with an allele of Arid3b floxed for exon 4. These mice were then crossed with Mx1-Cre transgenic mice to generate an *Arid3b^{f/f}:Mx1-cre* inducible knockout mouse. To examine the effect of Arid3b loss on hematopoietic cell populations, we isolated nucleated bone marrow (BM) from 12-week pIpC treated control (*Arid3b^{f/f}*) or mutant (*Arid3b^{f/f}:Mx1-cre*) mice and stained them for the pan myeloid marker CD11b and pan B lymphocytic marker B220. Efficient deletion of the Arid3b allele was observed in the BM as evaluated by genomic DNA PCR ([S1 Fig](#)). Loss of Arid3b protein was confirmed by Western blot ([S1C Fig](#)). In *Arid3b^{f/f}:K14-Cre*, we have observed that Cre-mediated excision of Arid3b exon 4 results in no detectable protein expression in epithelial cells as evaluated by immunohistochemistry confirming that Cre mediated deletion results in a null allele (Manuscript in preparation). Henceforth, the pIpC treated *Arid3b^{f/f}:Mx1-cre* mice will be referred to as knockout or *Arid3b^{-/-}*. Analysis of control and knockout populations by flow cytometry revealed a significant 2-fold decrease in the B220⁺ population of *Arid3b^{-/-}* mice ([Fig 1A and 1B](#)). While B cells were significantly decreased in *Arid3b^{-/-}* mice, the CD11b⁺ myeloid population remained largely unchanged ([Fig 1A and 1C](#)). Since CD11b⁺ cells include both monocytes and granulocytes, we stained CD11b⁺ myeloid populations with the granulocyte-specific marker GR1 (Ly6C/G) to ensure *Arid3b^{-/-}* cells were not preferentially skewed towards one lineage of the innate immune system ([Fig 1D](#)). This analysis revealed no difference in granulocyte (CD11b⁺GR1⁺) or monocyte (CD11b⁺GR1⁻) populations in *Arid3b^{-/-}* mice ([Fig 1E and 1F](#)). To investigate whether Arid3b loss affected erythroid development, we isolated BM cells from 12-week pIpC control or *Arid3b^{-/-}* mice and stained them for erythroid markers CD71 and Terr119 without ACK lysis. This analysis revealed that erythroid development is unchanged in the BM of Arid3b knockout mice ([S2A Fig](#)). Overall, this data suggests that loss of Arid3b results in decreased B cell development, while erythroid and myeloid development are unperturbed.

De Novo B cell production is critically impaired in Arid3b null mice

Since B220 is a pan B lymphocytic marker, we sought to determine which developmental B cell populations were aberrant in *Arid3b^{-/-}* mice. To distinguish pro B and pre B, immature B, and recirculating B cell populations, we stained nucleated BM cells from 12-week post pIpC injected control or *Arid3b^{-/-}* mice with B220, CD19, IgM, and IgD ([Fig 2A](#)) [15]. This analysis revealed that *Arid3b^{-/-}* mice have decreased pro B/pre B and immature B cell populations, while mature recirculating B cells were unchanged ([Fig 2B](#)). To better define the stages of B cell development affected by Arid3b loss, we stained nucleated bone marrow with cell surface markers B220, CD43, BP-1, HSA, IgM, and IgD, which can be used to identify Hardy B cell maturation stages A-F ([Fig 2C](#)) [16]. This analysis revealed that all populations were significantly decreased in *Arid3b^{-/-}* mice except for the C' population, and mature/recirculating B

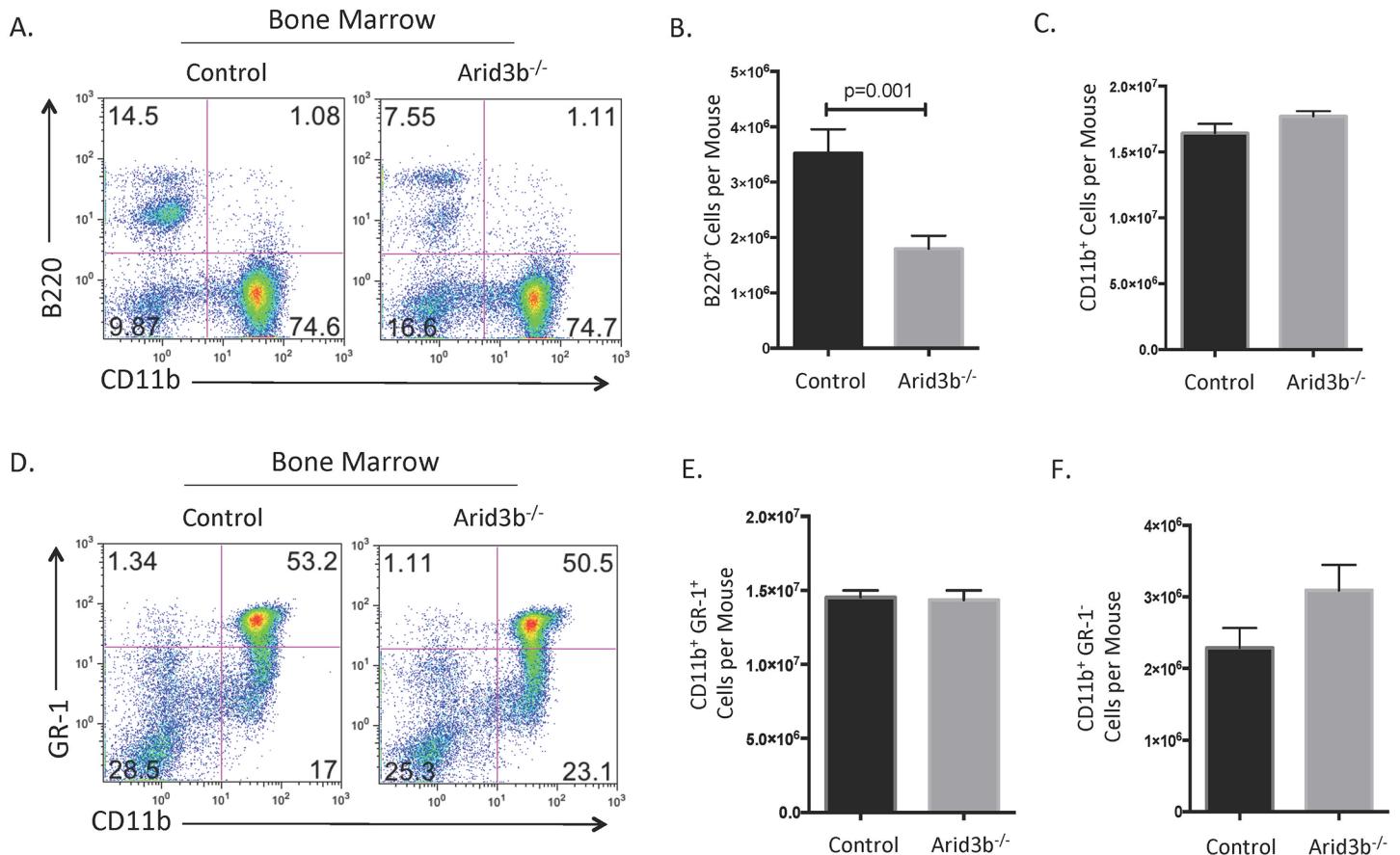


Fig 1. Loss of Arid3b in Bone Marrow Results in Decreased B cells. For analysis of hematopoietic populations by flow cytometry, bone marrow was harvested from *Arid3b^{fl/fl}: Mx1-Cre* (denoted *Arid3b^{-/-}*) mice 12 weeks after final pIpC injection. **A**) Representative FACS plot of B cell (B220+) and myeloid populations (CD11b+) in control or *Arid3b^{-/-}* mice. Control (N = 21) and *Arid3b^{-/-}* (N = 18) were examined. **B**) The decrease in B cells in *Arid3b^{-/-}* bone marrow was statistically significant. **C**) No change was observed in the myeloid populations between control and *Arid3b^{-/-}* mice. **D**) Myeloid populations were further subdivided into granulocyte (CD11b+ GR1+) and monocyte (CD11b+ GR1-) populations to assess any potential differences. Control (N = 16) and *Arid3b^{-/-}* (N = 18) were examined. **E**) No difference was observed in the granulocyte population. **F**) No difference was observed in the monocyte population. Wildtype (N = 21) and *Arid3b^{-/-}* (N = 18) were examined. P values determined by unpaired students t-test.

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cells (fraction F) only being modestly reduced compared to the others (Fig 2D.) Taken together, these data show that Arid3b is critical for the formation of early B cell progenitors.

The CLP Population is Significantly Decreased in Arid3b Null Mice

Since progenitor B cell populations were significantly impaired in *Arid3b^{-/-}* mice, we hypothesized that a defect in the common lymphoid progenitor (CLP), a downstream progenitor of the multipotent progenitor (MPP) that can give rise to B cells, T cells, and NK cells, could be responsible for this decreased downstream B cell production [17]. To investigate the changes in the CLP population, we collected nucleated BM from control and *Arid3b^{-/-}* mice 12 weeks post pIpC injection and stained for cell surface markers lineage positive (Lin+), IL7R, cKit, Ly6D, and Flt3 (Fig 3A). Ly6D has been shown to divide the CLP population into the all lymphoid progenitor (ALP) and b cell biased lymphoid progenitor (BLP) pools [18]. The ALP can give rise to all lymphoid cells while the BLP is restricted mainly to serve as a B cell progenitor pool. This analysis revealed a ~2 fold reduction in the CLP population of *Arid3b^{-/-}* mice (Fig 3B). This majority of this reduction came from reduced BLPs, as ALPs were only modestly

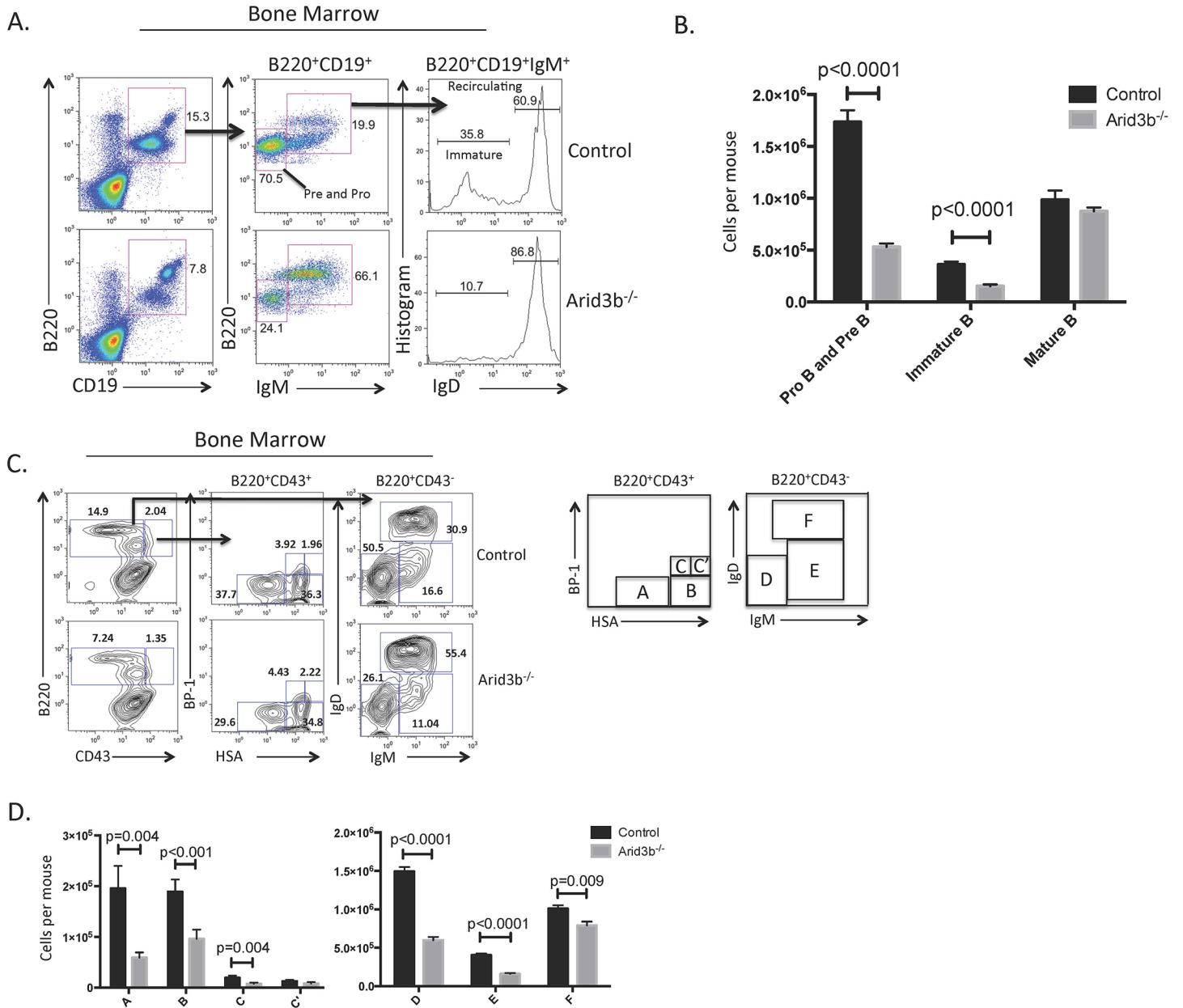


Fig 2. De Novo B cell production is critically impaired in Arid3b null mice. For analysis of hematopoietic populations by flow cytometry, bone marrow was harvested from *Arid3b*^{fl/fl}; *Mx1-Cre* (denoted *Arid3b*^{-/-}) mice 12 weeks after final plpC injection. **A)** Representative FACS plots in control and *Arid3b*^{-/-} mice for analysis of pro and pre B, immature B, and recirculating B cell populations. Control (N = 16) and *Arid3b*^{-/-} (N = 9) were examined. **B)** Pro and Pre B cells, as well as immature B cells, were significantly decreased in *Arid3b*^{-/-} mice. Recirculating B cells were unchanged between control and *Arid3b*^{-/-} mice. **C)** B cell populations were further analyzed using Hardy fraction cell surface markers to identify B cell populations A-F. Control (N = 6) and *Arid3b*^{-/-} (N = 7) mice were examined. **D)** Significant decreases were observed in all B cell populations of *Arid3b*^{-/-} mice, except for the C' population. P values determined by unpaired students t-test.

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reduced (Fig 3B). To investigate whether the hematopoietic stem cell (HSC) population was perturbed in *Arid3b*^{-/-} mice, we stained nucleated cells with Lin cocktail, cKit, Sca1, CD48, and CD150 (Fig 3C). This revealed no significant difference in the HSC population between control and *Arid3b*^{-/-} mice (Fig 3D). We then evaluated whether the common myeloid progenitor population (CMP) was decreased in addition to the CLP, which would suggest a general MPP

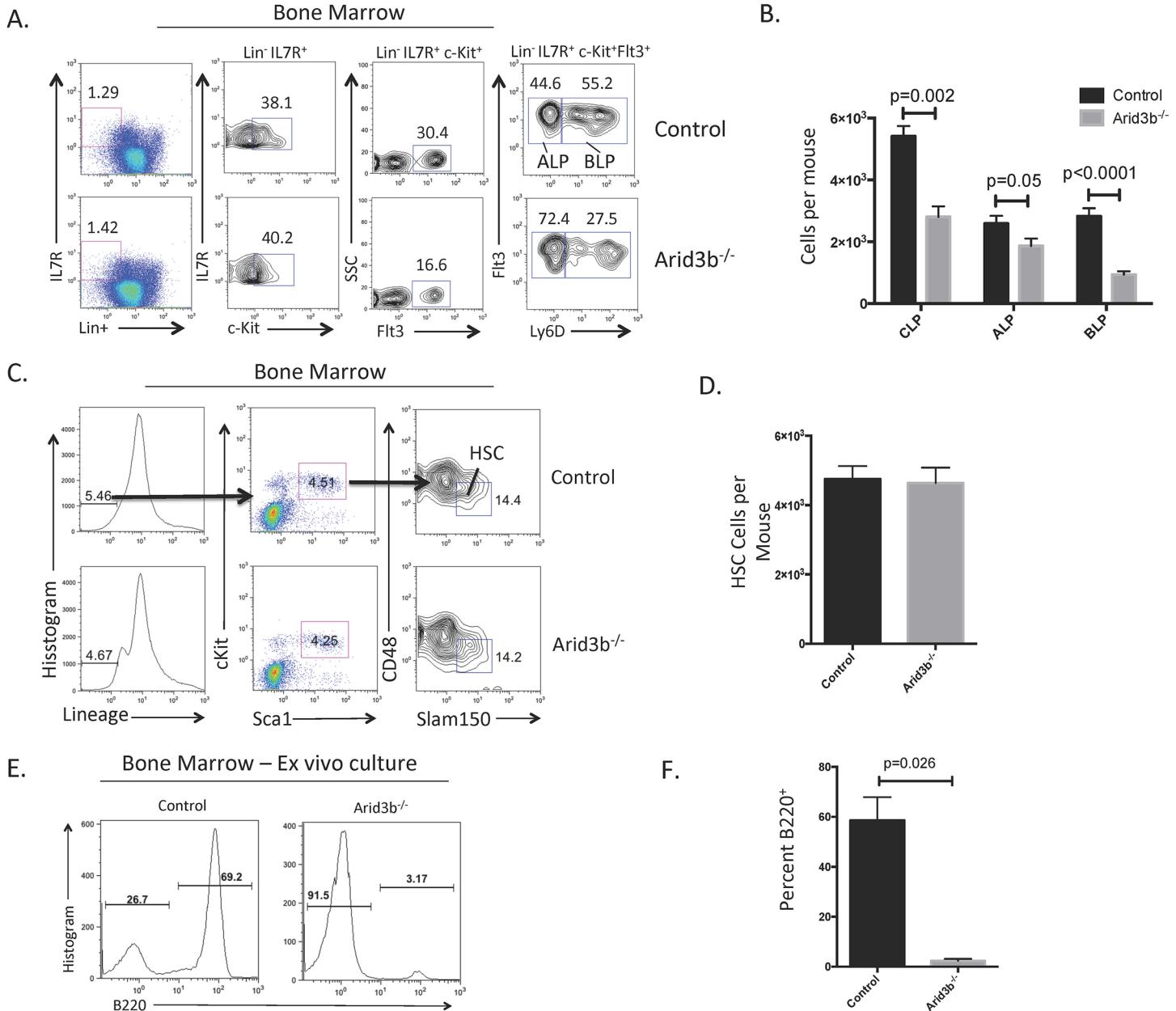


Fig 3. The CLP Population is Significantly Decreased in Arid3b null mice. For analysis of hematopoietic populations by flow cytometry, bone marrow was harvested from *Arid3b*^{fl/fl}; *Mx1-Cre* (denoted Arid3b^{-/-}) mice 12 weeks after final plpC injection. **A)** Representative FACS plots in control and Arid3b^{-/-} mice for analysis of CLP, ALP, and BLP populations. Control (N = 14) and Arid3b^{-/-} (N = 17) were examined. **B)** The CLP population was significantly decreased in Arid3b^{-/-} mice, and the majority of the decrease was due to decreased BLPs. **C)** Representative FACS plots in control and Arid3b^{-/-} mice for analysis of HSC populations. Control (N = 14) and Arid3b^{-/-} (N = 18) were examined. **D)** HSC populations were unchanged between control and Arid3b^{-/-} mice. **E)** 3 weeks after final plpC injection, bone marrow was harvested from *Arid3b*^{fl/fl}; *Mx1-Cre* mice and lineage depleted to enrich for hematopoietic stem and progenitor cells. Cells were then cocultured with OP9 cells in the presence of IL7 and Flt3L to promote B cell development for 6 days and then analyzed by flow cytometry for B cell marker B220. Representative FACS plot of control and Arid3b^{-/-} cells after 6 days of coculture. **F)** OP9 experiments were run in triplicate and the decrease in B cells was statistically significant. P values determined by unpaired students t-test.

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differentiation defect ([S2B Fig](#)). This analysis revealed no differences in the CMP or the downstream granulocyte/monocyte progenitor (GMP) and megakaryocyte/erythroid progenitor (MEP) populations ([S2C Fig](#)). Overall, this data suggests that loss of Arid3b results in decreased CLPs, while other stem and progenitor populations remain unchanged.

Arid3b null stem and progenitor cells fail to differentiate into B cells ex vivo

Decreased CLPs and downstream B cell progenitors in *Arid3b*^{-/-} mice suggested that Arid3b null hematopoietic stem and progenitor cells (HSPCs) fail to differentiate into functional CLPs that can give rise to committed progenitor B cells. To validate this, we isolated Lin⁻ cells from the BM of 3-week post pIpC injected control and *Arid3b*^{-/-} mice by magnetic column separation and co-cultured them on OP9 stromal cells in the presence of B cell promoting cytokines IL-7 and Flt3L for 6 days. These conditions normally promote the growth of pro B cells from hematopoietic progenitors [19]. After 6 days of OP9 coculture with the control sample, B220⁺ B cells make up greater than 60% of the culture population (Fig 3E). In contrast, after 6 days of OP9 coculture with the *Arid3b*^{-/-} samples, almost no B220⁺ B cell growth is observed (Fig 3E). The inability of *Arid3b*^{-/-} cultures to differentiate into B220⁺ B cells is statistically significant (Fig 3F). This data shows that Arid3b null stem cells fail to differentiate into committed progenitor B cells during ex vivo culture.

Splenic B and T cells populations are unchanged in Arid3b null mice. Since we observed decreased *de novo* B cell production in the BM of *Arid3b*^{-/-} mice, but normal numbers of recirculating mature B cells, we next investigated whether B cell populations were aberrant or normal in the peripheral organs of *Arid3b*^{-/-} mice. Spleens were isolated from control and *Arid3b*^{-/-} mice 12-weeks post pIpC injection and evaluated for B cell population numbers by cell surface marker B220. This analysis revealed a slight decrease in B220⁺ B cells in *Arid3b*^{-/-} spleens, but this was not statistically significant (Fig 4A). We then stained with cell surface markers CD21 and IgM to identify different B cell populations in the spleen (Fig 4B). IgM and CD21 staining can differentiate splenic B cell populations into fractions 1 (comprising T1-T2 transitional cells), 2 (comprising follicular and T3 cells), and 3 (containing MZ cells) [20]. In *Arid3b*^{-/-} mice, we observed a significant decrease in the transitional B cell containing fraction 1 population, while the follicular zone dominated fraction 2 population is significantly increased (Fig 4C). Since we observed decreased CLPs in *Arid3b*^{-/-} BM, we reasoned that downstream T cell progenitors may also be affected by Arid3b loss. To investigate this possibility, we isolated the thymus from control and *Arid3b*^{-/-} mice and analyzed T cell development by cell surface markers CD4 and CD8 (S3A Fig). This analysis revealed a modest decrease in CD4⁺ T cells that was statistically significant, but no difference in the double positive or CD8⁺ T cell populations (S3B–3D Fig). Mature T cells were then evaluated in the spleens of control and *Arid3b*^{-/-} mice by cell surface expression of CD4 and CD8 (Fig 4D). This analysis revealed no significant differences in splenic CD4 or CD8 T cell populations (Fig 4E and 4F). To observe if B1 or B2 B cell development was perturbed in the peritoneal cavity, we performed peritoneal lavages on control and *Arid3b*^{-/-} mice and stained cells with cell surface markers CD5 and B220 (Fig 4G) [21]. This analysis revealed a modest increase in B1a cells with a concomitant decrease in B1b cells, while B2 cells were unchanged (Fig 4H). Taken together, these data suggest that *Arid3b*^{-/-} mice can compensate for decreased B cell production in the BM.

Arid3b^{-/-} B cells secrete normal levels of serum IgG and respond to antigen in culture

We next wanted to evaluate whether the developmental defect in the BM affected the peripheral function of B cells in *Arid3b*^{-/-} mice. Peripheral blood from wildtype and *Arid3b*^{-/-} mice was collected and analyzed for total IgG levels to determine if Arid3b loss resulted in defects in steady state production of IgG antibodies (Fig 5A). To investigate changes in B cell proliferation, we isolated CD43 (Ly-48) negative B cells from the spleens of wildtype and Arid3b null mice and labeled them with the cell surface dye CFSE. We then cultured cells with or without

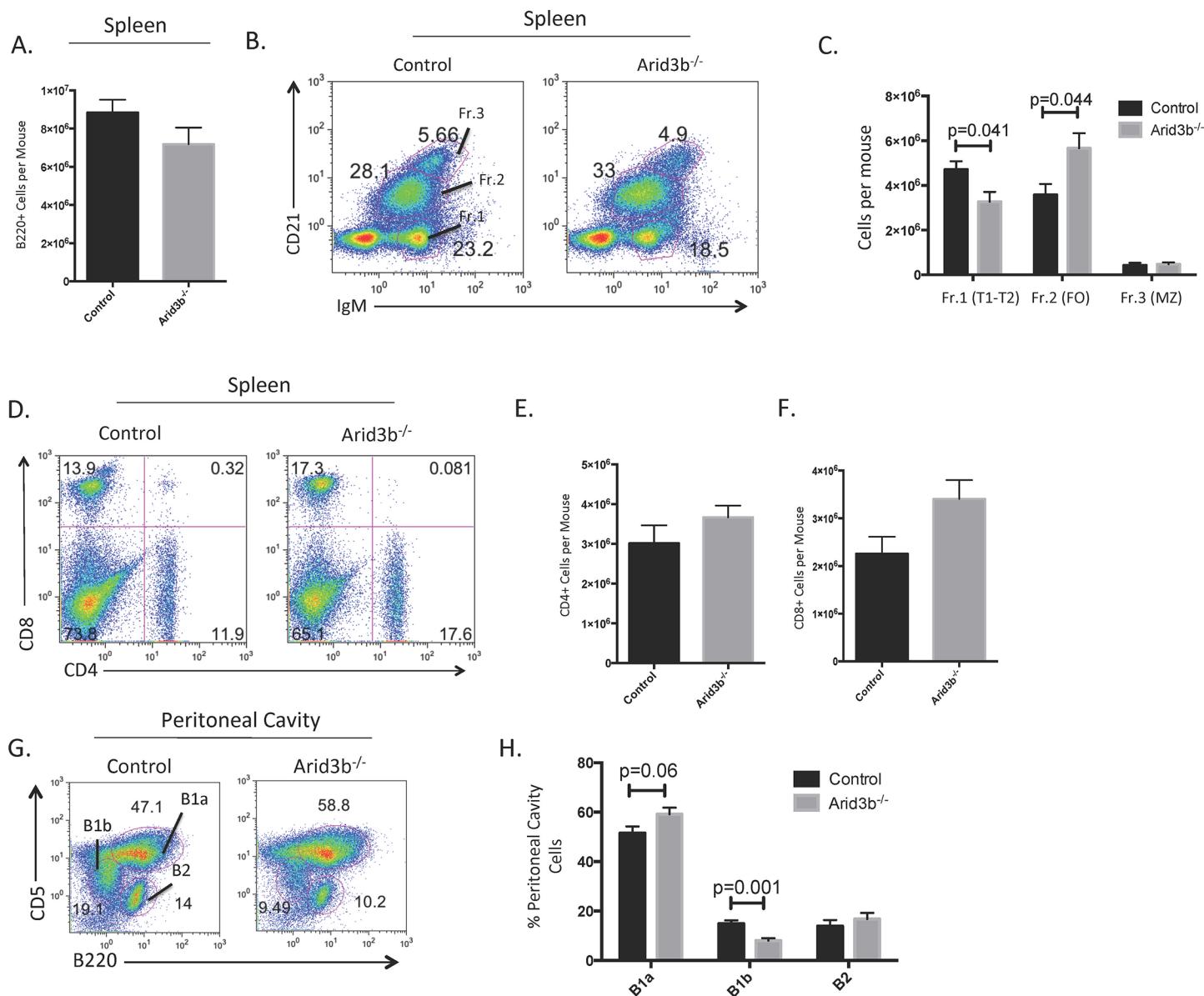


Fig 4. Splenic B and T cell populations are unchanged in ARID3B null mice. Spleens were harvested from *Arid3b*^{fl/fl}:*Mx1-Cre* (denoted *Arid3b*^{-/-}) mice for analysis of B cell and T cell populations by flow cytometry, 12 weeks after final plpC injection. **A)** B220+ B cells were analyzed in control (N = 11) and *Arid3b*^{-/-} (N = 10) mice. No statistically significant differences were observed in B220+ splenic B cell populations. **B)** Splenic follicular zone, marginal zone, and transitional zone B cells were evaluated using cell surface markers CD21 and IgM. Representative plots are shown. Control (N = 5) and *Arid3b*^{-/-} (N = 7) mice were analyzed. **C)** *Arid3b*^{-/-} mice had significantly decreased T1-T2 cell population with significantly increased follicular zone population. No difference was observed in marginal zone B cells. **D)** T cell populations were analyzed in spleens of control (N = 6) and *Arid3b*^{-/-} (N = 9) mice. **E)** No significant differences were observed in the CD4+ T cell populations. **F)** No significant differences were observed in CD8+ T cell populations. **G)** Control (N = 7) and *Arid3b*^{-/-} (N = 7) peritoneal cells were collected by peritoneal lavage and analyzed for cell surface expression of CD5 and B220 to identify B1 and B2 B cell subsets. **H)** A modest but significant increase was observed in the B1a population with a concomitant decrease in the B1b population, while the B2 population was unchanged. P values determined by unpaired students t-test.

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LPS for 72 hours. After 72 hours, cultures were analyzed for dilution of the CFSE by flow cytometry (Fig 5B). No difference was observed in the percentage of proliferating cells between control and *Arid3b*^{-/-} B cells (Fig 5C). These cultures were then stained with cell surface markers B220 and CD138 to investigate whether the *Arid3b*^{-/-} B cells could differentiate into short

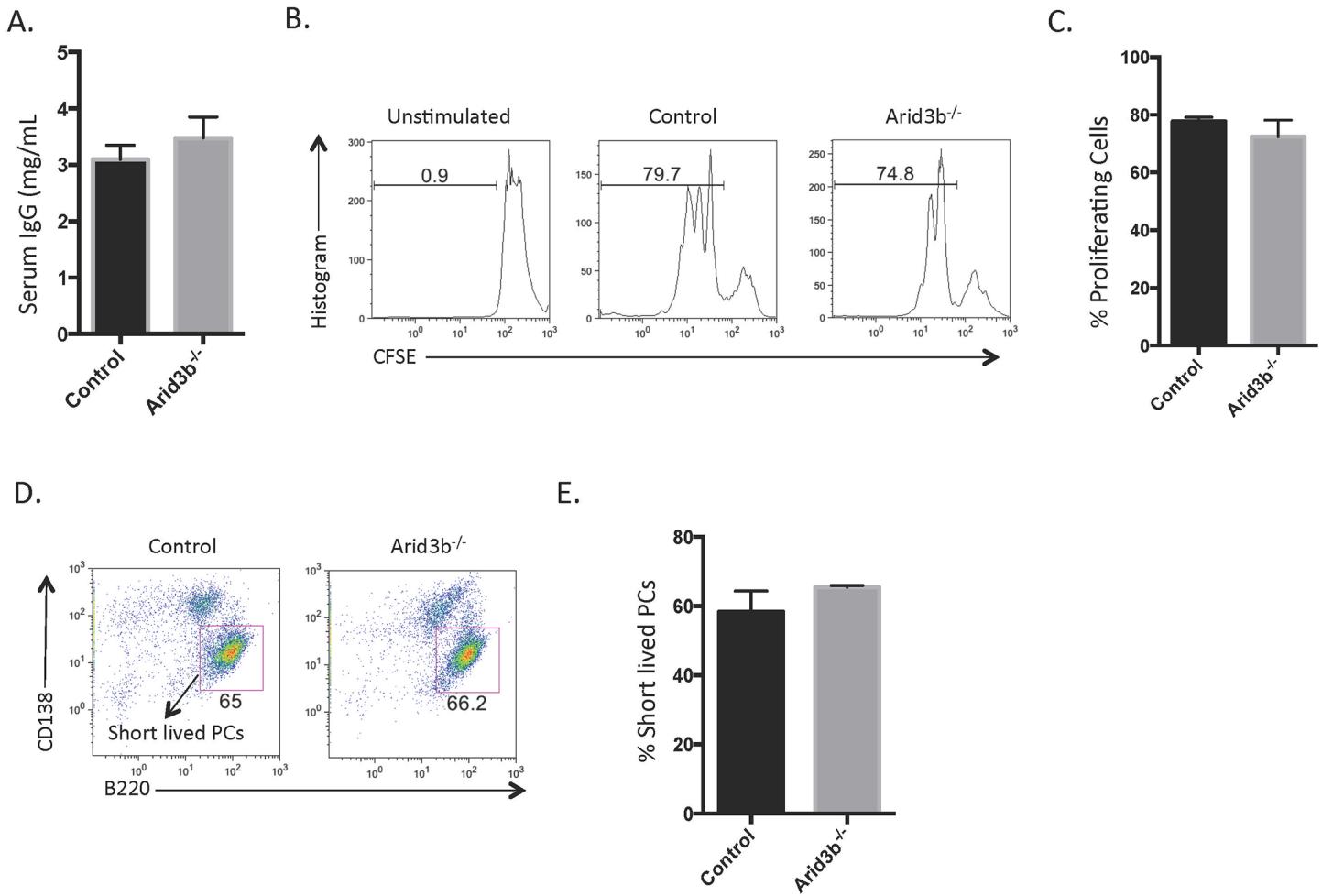


Fig 5. Arid3b^{-/-} B cells produce normal serum IgG and have no defect in response to LPS during ex vivo culture. **A)** Peripheral blood was collected from control (N = 4) and Arid3b^{-/-} (N = 5) mice via cheek bleed and analyzed for total serum IgG levels. No difference was observed between control and Arid3b^{-/-} mice. **B)** Splenic B cells were isolated from control (N = 3) or Arid3b^{-/-} (N = 3) cohorts by negative selection of CD43 (Ly-48). Cells were stained with CFSE and cultured in LPS for 72 hours before analyzing by flow cytometry. Representative plots are shown. P values determined by unpaired students t-test. **C)** No difference was observed in the percentage of proliferating cells between control and Arid3b^{-/-} mice. **D)** Cultures were also analyzed for their ability to differentiate into plasma cells by cell surface markers B220 and CD138. **E)** No difference was observed in the percent of short lived plasma cells between control and Arid3b^{-/-} cultures.

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lived effector plasma cells (Fig 5D). This analysis revealed no difference in the ability of Arid3b^{-/-} cells to differentiate into plasma cells (Fig 5E). Overall, this data shows that loss of Arid3b does not result in major defects in B cell function.

Interaction of Arid3a and Arid3b promotes higher transcriptional activity than either factor alone

The similar B cell lineage phenotypes of Arid3a and Arid3b knockout mice suggested that in B cells, these two family members might physically interact to regulate B cell gene expression. Arid3a and Arid3b are coexpressed in the Bcl1 B cell lymphoma cell line. To determine if these two proteins interacted, whole cell extracts were prepared from unmodified Bcl1 cells. Extracts were subjected to immunoprecipitation with control IgG or antibody specific for Arid3b. The immunoprecipitation products were then probed for the presence of Arid3a by immunoblot.

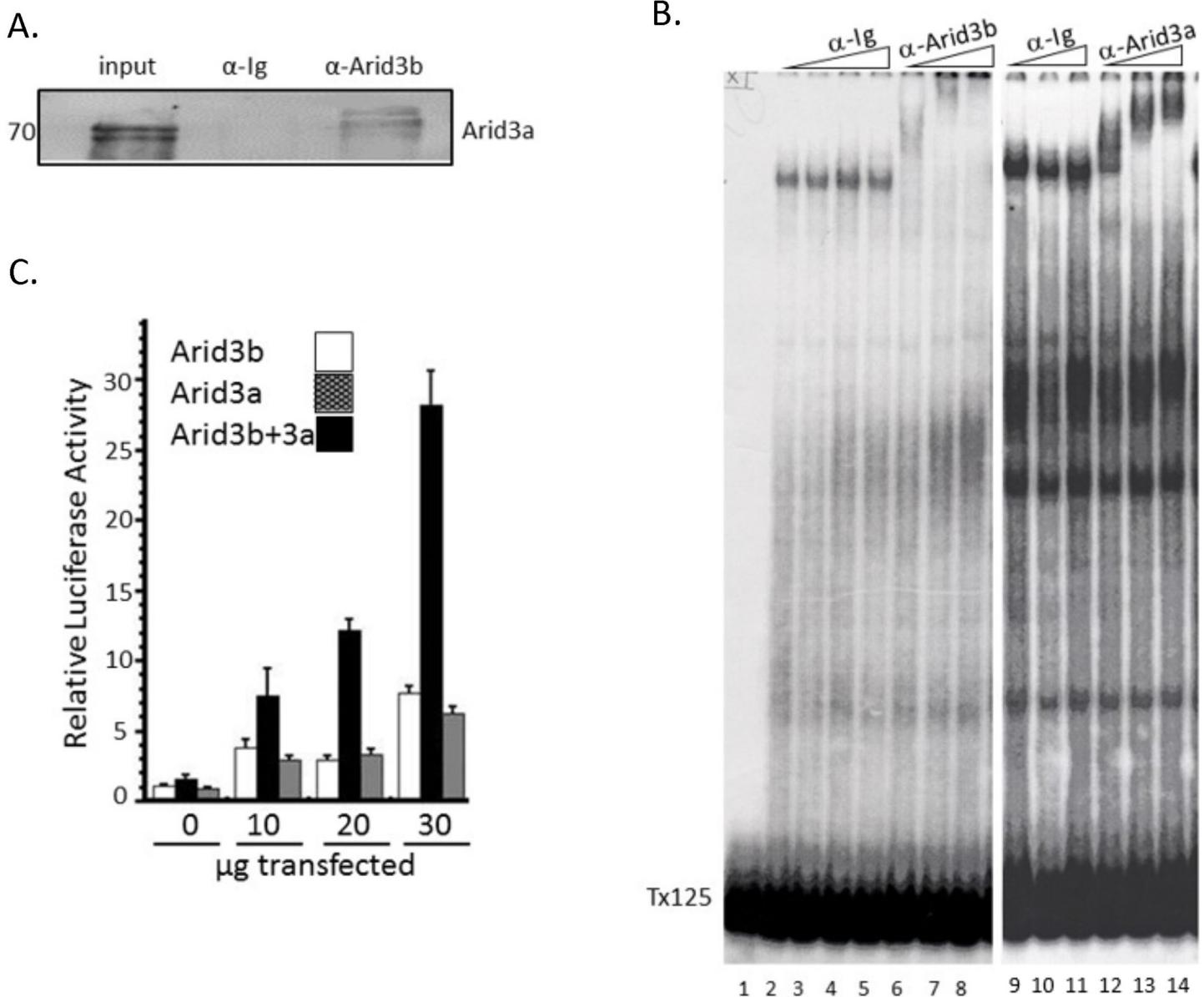


Fig 6. DNA binding and transcriptional consequence of Arid3a-Arid3b interaction. **A)** Co-IP of endogenous Arid3a and Arid3b in the Bcl1 murine B cell lymphoma cell line. Input, whole cell lysate; αlg, pre-immune serum; anti (α)-Arid3b mouse monoclonal antibody; detection: anti-Arid3a polyclonal antibody (details in M&M). **B)** EMSA analysis reveals Arid3a:Arid3b complex on DNA. EMSA employed anti-Arid3a and anti-Arid3b supershifts (performed at increasing concentrations; triangles) of 32 P-labeled Tx125 V_HS107 probe binding to Bcl1 nuclear proteins (details in M&M). **C)** Arid3a:Arid3b interaction results in enhanced transcription (as measured by luciferase assays) in Bcl1 mature B cells stably transfected with a MAR (Tx125 and Bf150)-containing promoter fused in frame to luciferase. Increasing amounts (0–30ug; x axis) of Arid3a and Arid3b each cloned into pcDNA3.3-TOPO were delivered by transient transfection 24 hr prior to harvest and dual luciferase activity measurement (details in M&M).

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Arid3a was specifically detected in the anti-Arid3b immunoprecipitations indicating that endogenous Arid3a and Arid3b associate in a B cell line (Fig 6A). Next we employed electrophoretic mobility shift assays (EMSA) to test whether their interaction in solution was retained on a common DNA binding site—the nuclear matrix attachment region (MAR, Tx125) positioned upstream of the promoter of the VH1 S107 variable region [4]. When nuclear extracts isolated from Bcl1 were incubated with labeled Tx125, a prominent, specific

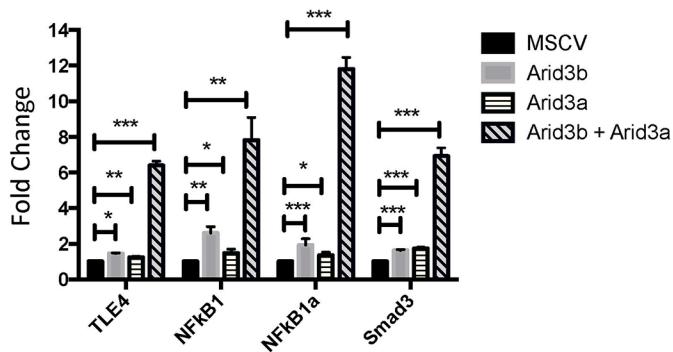
complex was super-shifted with antibodies that recognize either Arid3a or Arid3b (Fig 6B). Given the Co-IP results, these results strongly suggest that both proteins bind simultaneously to the S107-associated MAR as a heteromultimer. To determine the functional consequence of this interaction, transcription assays were performed using as a target the MARs adjacent to the native promoter and 5'UTR of S107 fused in frame to luciferase. When equal, increasing amounts of Arid3a and Arid3b were co-transfected with this reporter plasmid, higher luciferase activity was observed relative to either Arid protein alone, but significantly higher when both Arids were transfected together (Fig 6C). Collectively the data suggest that the direct interaction of Arid3a and Arid3b on target DNA achieves significantly higher B cell gene expression.

Altered Gene Expression in the absence of Arid3b

Based on the interaction between Arid3a and Arid3b, and the similar phenotypes of Arid3a and Arid3b null mice, we reasoned that the Arid3a/Arid3b expression may coregulate B cell specific gene expression programs. To identify potential genes regulated by Arid3b that are implicated in B cell development, we examined our previous genome chromatin precipitation and gene expression data for putative Arid3b regulated genes that have been implicated in B cell differentiation [22,23]. This analysis revealed several potential critical B cell genes, including *NfkB1*, *Tle4*, and *Smad3*. To investigate whether Arid3b, Arid3a, or a combination of Arid3a/Arid3b was capable of regulating these genes in B cells, we generated 70Z/3 Pre B cells stably expressing lentiviral control vector pLenti, pLenti-Arid3a, pLenti-Arid3b, or pLenti-Arid3a+Arid3b (S4 Fig). We isolated RNA from control or overexpressing cultures and analyzed for gene expression using qRT-PCR. Analysis for potential target genes showed modest but statistically significant increases in *Tle4*, *Smad3*, *NfkB1*, and *Nfkbia* in both Arid3a and Arid3b single overexpressing lines, while combinational Arid3a/Arid3b expression resulted in significantly enhanced expression of all genes (Fig 7A).

Loss of *Tle4* could explain the decreased CLPs and B cell progenitors in *Arid3b*^{-/-} mice. *Tle4* deficient mice exhibit a marked leukopenia that is primarily due to decreased B lymphocytes with no effect on myeloid or T cell populations [24]. Furthermore, these mice have significantly decreased CLPs, Pre B, Pro B, and immature B cell populations, while recirculating B cells remain unchanged, strikingly similar to the phenotype observed in Arid3b null mice.

A.



B.

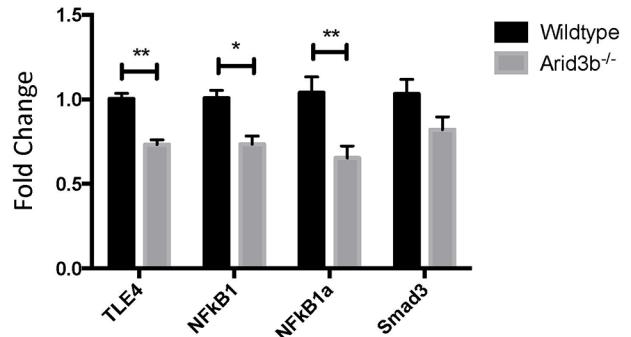


Fig 7. Arid3B regulates essential B cell developmental genes. A) 70Z/3 Pre B cells were transduced with control (pLenti), Arid3b, (pLenti-Arid3b), Arid3a (pLenti-Arid3a), or Arid3a+Arid3b (pLenti-Arid3a/Arid3b) lentivirus. RNA was collected and qRT-PCR was A) Control, Arid3a, Arid3b, or Arid3a+Arid3b overexpressing cells were analyzed by qRT-PCR for expression of B cell genes *Tle4*, *Smad3*, *NfkB1*, and *Nfkbia*. C) Primary B220+ B cells were isolated from control or *Arid3b*^{-/-} mice 3-weeks post final plpC injection and analyzed for qRT-PCR for expression of B cell genes *Tle4*, *Smad3*, *NfkB1*, and *Nfkbia*. P values determined by unpaired students t-test (* p < .05, ** p<0.01, *** p<0.0001).

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To investigate whether these genes were misregulated in *Arid3b*^{-/-} mice, we isolated B220⁺ B cells from the BM of 3-week post injected pIpC control or *Arid3b*^{-/-} mice by magnetic column separation. Analysis with qRT-PCR showed decreased expression of *Tle4*, *Smad3*, *NFkB1*, and *NFkBIA* (Fig 7B). Overall, this data supports the conclusion that Arid3b regulates *Tle4*, *Smad3*, *Nfkbia*, and *Nfkb1* in B cells.

Discussion

Previously it was reported that deletion of the Arid transcription factor family member, Arid3a, resulted in a highly penetrant embryonic lethality due to hematopoietic stem cell and erythropoietic deficiencies with a few (<1%) *Arid3a*^{-/-} animals surviving into adulthood. Loss of Arid3a results in decreased fetal liver and adult bone marrow HSCs. Interestingly though, in the adult, the only hematopoietic lineage that is affected is the B cell lineage with decreased pro-, pre-, and immature B cells. However B cell numbers in the periphery and the recirculating/ mature bone marrow B cell populations are not significantly affected. Since the closely related Arid3b has been shown to associate with Arid3a in some cell types and shares a similar expression pattern with Arid3a during hematopoiesis, we were interested in determining whether Arid3b is required along with Arid3a to regulate hematopoietic development. Due to the fact that germline knockout of Arid3b results in early embryonic lethality before hematopoiesis can be assessed, we developed an *Arid3b*^{fl/fl} MX-Cre inducible knockout model to study the role of Arid3b in blood development. Analysis of bone marrow lacking Arid3b revealed no defects in the HSC populations. However, similar to Arid3a knockout mice, Arid3b knockouts show gross defects in bone marrow B cell development starting at the CLP stage and persisting through the immature BM B cell population. Additionally, and also similar to Arid3a null mice, Arid3b deficiency does not result in significantly decreased B cells in the periphery.

Despite the deficit in bone marrow B cell production, we did not observe any gross functional defects in *Arid3b*^{-/-} B cells. In preliminary functional analyses we observed no difference in serum IgG concentrations of Arid3b null mice following peripheral cheek bleed. Furthermore, splenic *Arid3b*^{-/-} B cells were able to proliferate normally and differentiate into plasma cells during ex vivo culture with LPS stimulation. However, whether these mice can mount an effective immune response in vivo remains unknown. Both murine and human studies demonstrate a role for Arid3a in regulating the function of mature B cell implicating Arid3a in the regulation of B cell autoimmunity [25,26]. The cooperation of Arid3b with Arid3a in bone marrow lymphopoiesis suggests they could cooperate in later B cell biology. Future studies will be aimed at addressing Arid3b's role in regulating mature B cell function.

Based on the strikingly similar B cell phenotypes between Arid3a and Arid3b knockout mice, our current model posits that Arid3a/Arid3b association is critical for DNA binding and regulation of B lymphocyte gene expression. Our in vitro data supports the conclusion that heterodimerization/ multimerization is important for the regulation of B cell genes. Interestingly, despite the similarities in B cell development, we observed no change in the HSC pool of *Arid3b*^{-/-} mice, in contrast to *Arid3a*^{-/-} mice, which show a decreased HSC population. This raises the question why both Arid3a and Arid3b are required for B cell development but only Arid3a expression is necessary for normal HSC function. One possibility is that the target genes of Arid3a/Arid3b heterodimers are only critical upon commitment to the lymphoid lineage, and that expression of these target genes is not necessary in the HSC compartment. In this context, Arid3a homodimers or heterotetramers [27] would regulate a separate set of indispensable genes required for normal HSC function.

Tle4, *Nfkb1*, and *Smad3* were previously identified by us as putative target genes of Arid3b in ovarian cancer cells by genomic gene expression arrays and chromatin immunoprecipitation

[22]. We observed that transcription of these same genes is elevated in response to Arid3b overexpression in pre-B cells and decreased in the bone marrow of our *Arid3b* conditional knockout mice. *Tle4*^{-/-} mice show decreased pre-pro B, pro B, pre B, and immature B cell populations in the BM, while recirculating B cells are unchanged [24]. They also show decreased CLP with no changes in their CMP/GMP/MEP populations. Additionally, T cell and myeloid development is not perturbed in *Tle4*^{-/-} mice. All of these phenotypes are consistent with what we observed in Arid3b conditional knockout mice. NF-kb has also been implicated in B cell development. Pan inhibition of NF-kb using a retroviral transduction of *IkBα* into hematopoietic cells resulted in decreased pre-B and immature B cells, while T cells and myeloid cells were unaffected [28]. However specific deletion of *NfkB1* does not result in any defects in bone marrow B cell development [29,30]. Combined deletion of *NfkB1* and *Ocab* does result in decrease bone marrow B220+IgM+, so potentially reduction in *NfkB1* could cooperate with reduction in other Arid3b target genes to lead to lymphopoiesis defects [30]. Future work using bioinformatic approaches will be aimed at identifying whole genome effects of *Arid3b* to help elucidate more detailed molecular mechanisms regulated by *Arid3b* in B cells.

Overall, this study shows that Arid3b is indispensable for normal B cell development. Conditional deletion of Arid3b results in decreased CLP and downstream B cell progenitors, while T cell and myeloid cell lineages and the HSC compartment from which each of these lineages derive, is unchanged. Future studies will be aimed at elucidating the molecular mechanisms governing these phenotypes.

Supporting Information

S1 Fig. Generation of Arid3b conditional knockout system. A) Schematic diagram of the Arid3b targeting strategy used to generate a conditional knockout. Knockout of the Arid3b allele was induced by intraperitoneal injection of 3–4 week old mice with 3 doses of poly-pIpC that were administered every 3 days apart. B) Confirmation of *Arid3b* deletion was confirmed by genomic DNA PCR. C) Confirmation of Arid3b deletion at the protein level was done by immunoblot using whole cell extracts prepared from control or *Arid3b*^{-/-} nucleated bone marrow.

(TIFF)

S2 Fig. Erythroid and myeloid progenitor lineages are unperturbed in *Arid3b*^{-/-} mice. Bone marrow was harvested from *Arid3b*^{fl/fl}: *Mx1-Cre* (denoted Arid3b^{-/-}) mice treated with pIpC 12 weeks after final injection for analysis of hematopoietic populations by flow cytometry. A) Representative FACS plots in control and *Arid3b*^{-/-} mice for analysis of erythroid populations, no differences were observed. B) Representative FACS plots in control and *Arid3b*^{-/-} mice for analysis of myeloid progenitor populations. C) No difference was observed between control (n = 6) and *Arid3b*^{-/-} (n = 11) mice. P values determined by unpaired students t-test.

(TIFF)

S3 Fig. Analysis of T cell population in thymus of *Arid3b*^{-/-} mice. Thymus was harvested from *Arid3b*^{fl/fl}: *Mx1-Cre* (denoted Arid3b^{-/-}) mice treated with pIpC 12 weeks after final injection for analysis of hematopoietic populations by flow cytometry. A) Representative FACS plots in control and *Arid3b*^{-/-} mice for analysis of T cell populations by CD4 and CD8 expression. B) CD4+ cells were decreased in *Arid3b*^{-/-} mice when compared to control. C) CD8+ cells were unchanged between *Arid3b*^{-/-} and control mice. D) CD4+CD8+ cells were unchanged between *Arid3b*^{-/-} and control mice. Control (n = 6) and *Arid3b*^{-/-} (n = 6) mice were examined. P values determined by unpaired students t-test.

(TIFF)

S4 Fig. Generation of 70Z/3 overexpressing cell lines. 70Z/3 Pre B cells were transduced with control (pLenti), Arid3b, (pLenti-Arid3b), Arid3a (pLenti-Arid3a), or Arid3a+Arid3b (pLenti-Arida/Arid3b) lentivirus. **A**) RNA was collected from control or Arid3b overexpressing cell lines and confirmation of Arid3b overexpression was done using qRT-PCR. **B**) Similarly, confirmation of Arid3a overexpression in the Arid3a overexpressing cell line was done. **C**) Confirmation of Arid3a and Arid3b overexpression in 70Z/3 cells overexpressing both vectors. (TIFF)

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