

# The double bromodomain protein Brd2 promotes B cell expansion and mitogenesis

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## ABSTRACT

Bromodomain-containing transcriptional regulators represent new epigenetic targets in different hematologic malignancies. However, bromodomain-mediated mechanisms that couple histone acetylation to transcription in lymphopoiesis and govern mature lymphocyte mitogenesis are poorly understood. Brd2, a transcriptional coregulator that contains dual bromodomains and an extraterminal domain (the BET family), couples chromatin to cell-cycle progression. We reported previously the first functional characterization of a BET protein as an effector of mammalian mitogenic signal transduction:  $E\mu$ -Brd2 Tg mice develop “activated B cell” diffuse large B cell lymphoma. No other animal models exist for genetic or lentiviral expression of BET proteins, hampering testing of novel anti-BET anticancer drugs, such as JQ1. We transduced HSCs with Brd2 lentivirus and reconstituted recipient mice to test the hypothesis that Brd2 regulates hematopoiesis in BM and mitogenesis in the periphery. Forced expression of Brd2 provides an expansion advantage to the donor-derived B cell compartment in BM and increases mature B cell mitogenic responsiveness *in vitro*. Brd2 binds the cyclin A promoter in B cells, shown by ChIP, and increases cyclin A mRNA and protein levels, and S-phase progression *in vitro* in mitogen-stimulated primary B cells, but not T cells, reinforcing results from  $E\mu$ -Brd2 mice. The small molecule BET inhibitor JQ1 reduces B cell mitogenesis, consistent with the interpretation that BET inhibitors are antiproliferative. Brd2-specific knockdown experiments show that Brd2 is also required for hematopoiesis. We conclude that Brd2 plays a critical, independent role in regulation of mito-

genic response genes, particularly cyclin A, in B cells. *J. Leukoc. Biol.* **95**: 000–000; 2014.

## Introduction

The DNA-binding transcription factors that regulate gene-expression programs during mammalian hematopoiesis are well studied, as are the perturbations in these programs that lead to hematologic malignancy. However, these transcription factors regulate cell-specific promoters and enhancers in a chromatin context, which includes critical activating or silencing histone modifications that also regulate the genes targeted by these transcription factors. The mechanisms by which epigenetic “reader”, “writer”, and “eraser” proteins interact with and modify chromatin to regulate blood cell development and hematologic malignancy are not well understood [1]. One type of chromatin-interacting motif, the bromodomain [2], is the only protein structural motif able to “read” acetylated lysine groups of histones in nucleosomal chromatin [3]. Double-bromodomain proteins play a critical role in several transcriptional programs in hematopoietic cells. This report focuses on one of these proteins—Brd2—and its role in B cell function.

Bromodomain-containing proteins have been implicated in aberrant transcriptional events that are responsible for several types of hematologic cancers, including AML [4, 5], multiple myeloma [6], MLL [7, 8], B cell lymphoma [9], and certain virus-associated lymphomas [1, 10]. The highly conserved, 110-aa bromodomain, comprised of four antiparallel  $\alpha$ -helices and two connecting loops, binds to acetylated lysine groups in nucleosomal histones [11] and is found in a number of eukaryotic transcription factors, coregulators of transcription, histone acetylases [3], DNA helicases, and chromatin-remodeling complexes [2]. In one class of closely related oncoproteins, two bromodomains are found together in tandem next to a protein association motif called the ET domain [12] to define a family of homologous “BET” proteins comprised of Brd2, Brd3, Brd4, and Brdt. Deregulated expression of Brd2 and Brd4 is potentially oncogenic in humans and mouse models;

Abbreviations: 7-AAD=7-aminoactinomycin D, APC=allophycocyanin, BET=bromodomain and extraterminal domain, BM=bone marrow, BUMC=Boston University Medical Center, ChIP=chromatin immunoprecipitation, Ct=comparative threshold, ET=extraterminal, HSC=hematopoietic stem cell, MLL=mixed lineage leukemia, NUT=nuclear protein in testis, P-TEFb=positive transcription elongation factor b, PB=Pacific Blue, PcG=polycomb group, qPCR=quantitative PCR, RBC=red blood cell, sh=short hairpin, SP=side population, TAF=TATA box binding protein-associated factor, Tg=transgenic

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reciprocal chromosomal translocations between human *BRD3* (9q34.2) [13] or *BRD4* (19p13.1) [14] genes and the *NUT* gene (15q14) create an oncoprotein fusion associated with a rare, aggressive carcinoma of the midline that is correlated with high mortality in young people.

Tg models that involve BET proteins are scarce. In mice, *Brd4*<sup>(+/-)</sup> haploinsufficiency is associated with severe organ deficiencies, and *Brd4*<sup>(-/-)</sup> is embryonic lethal [15] as a result of widespread mitotic failure [16, 17]. *Brd2*<sup>(-/-)</sup> is also lethal [18–20]. Our group showed that constitutive expression of the BET protein Brd2 produces malignancy in an animal model [9]. Little is known about Brd3 or Brdt involvement in human cancer or hematopoiesis, but Brd4 is well studied. The extended carboxyl terminal domain of Brd4 differs from other BET proteins and permits association of Brd4 with P-TEFb, a complex of cyclin-dependent kinase 9 and cyclin T that is critical for transcription elongation [21]. The Brd4 ET domain recruits chromatin-modifying enzymes and transcription factors that confer specificity to target loci [22]. Although Brd2 does not recruit P-TEFb to chromatin, Brd2 and Brd4 associate with mitotic chromatin and are important for postmitotic memory [16, 23, 24]. Furthermore, Brd2 and Brd4 bind acetylated histones [16, 23, 25] and mobilize chromatin modification to control cell cycle [17, 26, 27]. However, little is known about BET protein control of transcriptional programs during hematopoiesis in the BM and in the periphery.

BET proteins have been receiving significant attention, as interactions of bromodomains with chromatin are now established to be “druggable” [28, 29]. New small-molecule inhibitors of BET proteins that displace bromodomain-containing oncoproteins and their associated transcriptional coactivator and corepressor proteins from promoter chromatin [1] were found to induce cell-cycle exit and differentiation of malignant cells [4, 6, 8]. BET inhibitor drugs potentially represent a significant, innovative approach to treat several currently difficult-to-treat cancers, including AML [4], MLL [8], and the orphan cancer NUT midline carcinoma [28]. Additional investigation is needed to understand the targets of BET protein function that are critical to proliferation. In particular, current knowledge of the mechanisms of action of small molecule BET protein inhibitors has outpaced understanding of BET protein function as coactivators or corepressors of transcriptional networks in normal cells [1]. The complete lack of Brd3 and Brd4 expression systems in animal models hampers progress in understanding how such inhibitors work. Our studies of Brd2 expression in normal cells provide a much-needed basis to begin to understand the phenotypes and functions of BET proteins.

In this report, we focus on the proliferative mechanisms of the BET protein Brd2, a ubiquitously expressed transcriptional coregulator with increased activity in human primary leukemia [30]. Brd2 is homologous to TAF1 [31], which is required for *cyclin A2* transcription (hereafter “cyclin A”) in mammalian somatic cells [32]. Brd2 and Brd4 promote S-phase progression [9, 27, 33] in association with TAFs [34]. Brd2 provides a scaffold on chromatin that recruits E2F proteins, histone acetylase, and chromatin-remodeling activities to the cyclin A promoter [27, 34, 35]. Constitutive Brd2 expression in B cells

leads to a malignancy [9] that is transcriptionally most similar to the activated B cell form of human diffuse large B cell lymphoma [36]. To test the role of Brd2 in repopulation of the B cell niche and in vitro B cell proliferation, we reconstituted recipient mice with Brd2-overexpressing HSCs. In agreement with our Tg model published previously, we found that forced overexpression of Brd2 increased the response of mature B cells to mitogenic challenge through Brd2 interaction with the cyclin A promoter in B cells.

## MATERIALS AND METHODS

### Mice

C57BL/6j (CD45.2<sup>+/+</sup>; “recipient”) and B6.SJL<sup>*l<sup>tr</sup>(a)Pep3(b)BoyJ*</sup> (B6.SJL; CD45.1<sup>+/+</sup>; “donor”) male mice (The Jackson Laboratory, Bar Harbor, ME, USA) were 6 weeks old for all experiments, which were conducted with BUMC Institutional Animal Care and Use Committee oversight and approval.

### Sorting of SP cells

Whole BM was isolated under sterile conditions, and HSCs were enriched from BM by FACS isolation of SP cells, using MoFlo (Dako Cytomation, Carpinteria, CA, USA) techniques. We modified the methods of Goodell and colleagues [37], who identified the SP of Hoechst 33342 dye-excluding cells that are enriched for long-term, repopulating HSCs. CD45.1 donor mice were killed; long bones and sterna were isolated surgically (Fig. 1A) and then crushed to liberate BM cells into RPMI-1640 medium, buffered with 20 mM HEPES, pH 7.4, and supplemented with 10% FBS, penicillin, streptomycin, and fungizone antimycotic (all obtained from Invitrogen Life Technologies, Carlsbad, CA, USA) and 50  $\mu$ M 2-ME (C-10 medium; Fisher Scientific, Pittsburgh, PA, USA). Unless specified, chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and fluorescent antibodies from eBioscience (San Diego, CA, USA). Bone fragments were removed by sterile filtration with 70  $\mu$ m strainers (BD Biosciences, San Jose, CA, USA), and erythrocytes were removed with RBC lysis buffer (eBioscience). Viability, determined by trypan blue (Fisher Scientific) was always >98%. SP cells were isolated by incubation of BM suspension in PBS with Hoechst 33342 (BD Biosciences) at 10  $\mu$ g/ml for 90 min at 37°C and 5% CO<sub>2</sub>. Dye was always titrated and conditions optimized in preliminary experiments. After staining, BM cells were washed in PBS, counterstained with PI (2  $\mu$ g/ml), and kept on ice for sorting; gates excluded PI-positive cells and included SP cells to yield ~0.025% of total PI-negative cells (Supplemental Fig. 1A–C). On average, we obtained 30,000 SP cells from BM, pooled from five CD45.1 donors/experiment. Sorting gates and other details are shown in Supplemental Fig. 1A–C.

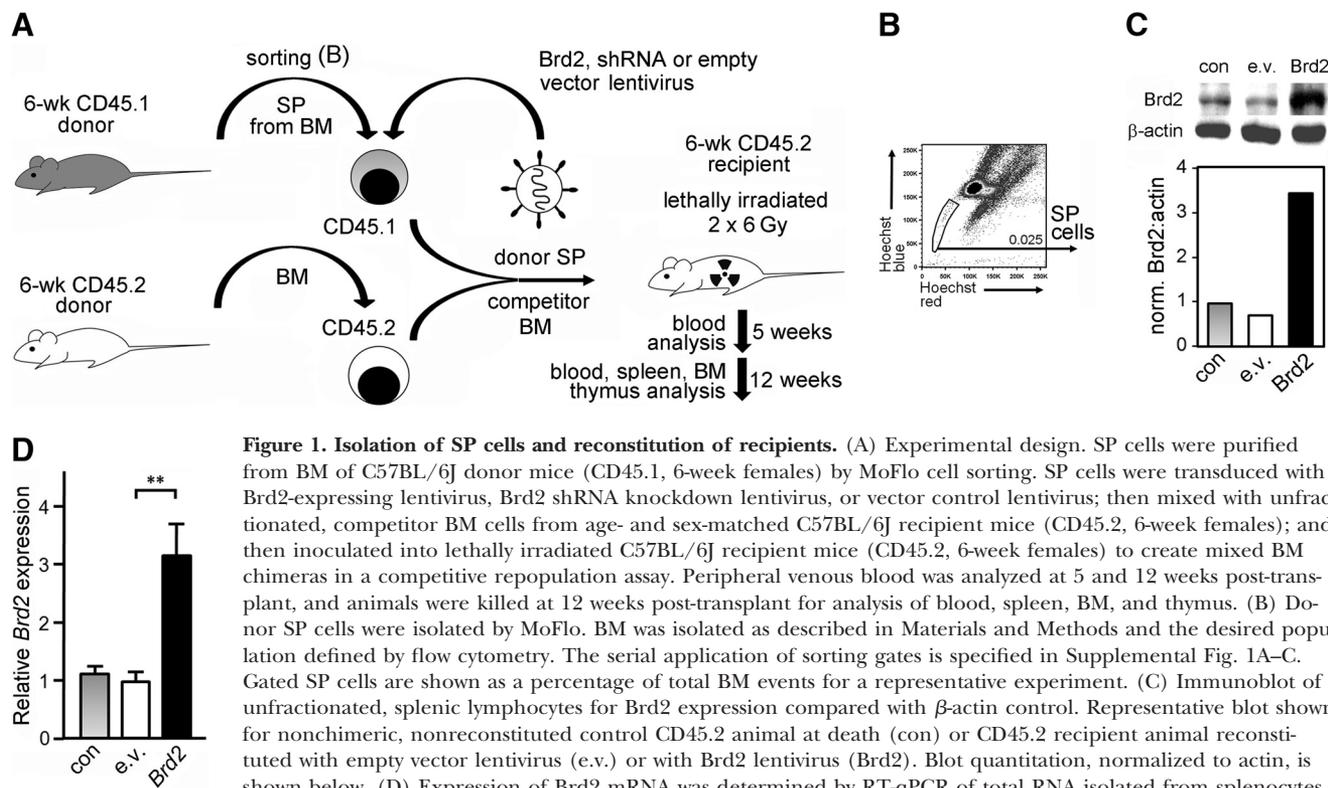
### Lentivirus transduction

Lentiviruses that express the short form of Brd2 under control of the 584-bp CMV promoter fragment or shRNA against Brd2 were molecularly cloned [20] and prepared by transfection of host plasmids into 293T cells with cotransfected helper plasmids to generate vesicular stomatitis virus pseudotype products that were purified for use. The source viruses were third-generation design, self-inactivating, and replication-incompetent; the vector backbones also contained the IRES from the encephalomyocarditis virus for eGFP expression and were generous gifts from Drs. Gustavo Mostoslavsky and Darrell Kotton (Boston University School of Medicine, Boston, MA, USA; www.kottonlab.com). The bicistronic lentivirus contained a CMV promoter that drove Brd2, and 3' to Brd2, an IRES that drove expression of eGFP. SPs were exposed to lentiviruses at 100 MOI in serum-free StemPro medium (Invitrogen Life Technologies), supplemented with stem cell factor (10 ng/ml) and thrombopoietin (100 ng/ml), overnight at 37°C, and 5% CO<sub>2</sub> in the presence of 5  $\mu$ g/ml polybrene. Reactions were quenched with C-10 medium, and cells were combined with unfractionated

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CD45.2 competitor BM to give  $\sim 1400$  CD45.1 donor SPs plus  $2 \times 10^5$  CD45.2 competitor BM cells/recipient animal.

### Protein immunoblotting

Immunoblotting was performed according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and as described previously [27]. Brd2 protein was detected with a rabbit polyclonal antibody. Immunoblot scanning densitometry was performed with ImageJ 1.47r (U.S. National Institutes of Health, Bethesda, MD, USA).

### Competitive BM reconstitution

Mixed BM cells from CD45.1 donor and CD45.2 competitor mice were injected retro-orbitally into anesthetized, lethally irradiated ( $2 \times 6$  Gy) CD45.2 hosts to create BM chimeras. Drinking water contained sulfamethoxazole/trimethoprim oral suspension (BUMC Laboratory Animal Science Center Pharmacy, Boston, MA, USA) for the first 10 days. Survival was 100% in mixed BM chimeras. Chimerism in tail-vein blood was monitored throughout the experiment. Animals were killed and exsanguinated 8–12 weeks after reconstitution, whereupon spleen, thymus, and BM were obtained surgically and blood by cardiac puncture; RBCs were lysed and nucleated cells prepared for analysis.

### Flow cytometry

Nucleated cells (100,000–200,000 gated events) were analyzed by flow cytometry on BD LSR II or FACScan flow cytometers (BD Biosciences). All stains were performed with FcR-blocking antibody (CD16/32; eBioscience), as described [9] and also reported in Supplemental Methods (Supplemental Fig. 1), including isotype controls (Supplemental Fig. 1I). Data analysis was performed using FlowJo 8.7 (TreeStar, Ashland, OR, USA).

### Proliferation of primary lymphocytes

To measure mitogen-responsive proliferation of mature cells in the periphery, lymphoid cells were isolated from spleens of killed mice and stimulated with *Escherichia coli* 0111:B4 LPS (10  $\mu$ g/ml; Sigma-Aldrich) for B cell mitogenesis for 24 h and with anti-CD3 (1  $\mu$ g/ml; BD) and anti-CD28 (1  $\mu$ g/ml; BD) antibodies for 60 h for T cell mitogenesis. Cells were plated at  $10^6$ /ml in a total of  $0.5 \times 10^6$  cells/well of a 24-well plate. For JQ1 experiments, JQ1 (+) and (–) enantiomers (generous gifts of James E. Bradner, Harvard Medical School, Boston, MA, USA), in 10 mM stock solutions in DMSO, were diluted in PBS and added with LPS to a final concentration of 400 nM JQ1. Incorporated BrdU was detected with a kit (BD Biosciences) that included BrdU-FITC mAb. The ratio of CD45.1 and CD45.2 staining was determined by CD45.1-APC (Clone A20) and CD45.2-PE (Clone 104); B or CD4<sup>+</sup> T cells were identified with B220-eFluor 450 or CD4-eFluor 450 (Clone GK1.5). Where indicated, B cells were purified from a mixed splenocyte population by negative selection with anti-CD43-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA) [9].

### ChIP

B cells were isolated from the spleens of WT C57BL/6J mice using a Pan B cell isolation kit (Miltenyi Biotec) and cultured with or without *E. coli* 0111:B4 LPS (10  $\mu$ g/ml) for 24 h. Cellular purity of B cells was confirmed by flow cytometry and was between 93% and 96%. The activated B cells were then subjected to 1 h of JQ1 or vehicle (DMSO) treatment, then fixed in 1% formaldehyde at 37°C for 10 min, and then subjected to ChIP. Chromatin was precipitated with 2  $\mu$ g  $\alpha$ -Brd2 (Bethyl Laboratories, Montgomery, TX, USA) or  $\alpha$ -GST (Upstate Biotechnology, Lake Placid, NY, USA) antibody. Two nanograms of each sample was then analyzed in duplicate or triplicate by qPCR. Fold difference was calculated as  $2^{\Delta\Delta Ct}$  (in-

put)–Ct(ChIP)]; then fold enrichment over an unrelated antibody ( $\alpha$ -GST) was assessed. For qPCR, EpiTect ChIP qPCR primers for the murine cyclin A2 (*Cna2*) gene (Qiagen, Valencia, CA, USA) were used according to the manufacturer's guidelines.

### RT-qPCR

RNA was isolated from splenocytes lysed in RLT buffer (Qiagen), supplemented with 1% 2-ME, using an RNeasy minicolumn kit. DNA was removed with DNase I (Roche Diagnostics, Indianapolis, IN, USA); RNA was quantified by spectrophotometry (BioPhotometer Plus; Eppendorf, Hauppauge, NY, USA). Equal amounts of RNA were used for cDNA synthesis with random hexamer primers (Roche Diagnostics) using Avian myeloblastosis virus RT (Promega, Madison, WI, USA). qPCR was performed with a 7500 Fast Real-Time PCR system (Applied Biosystems/Life Technologies, Foster City, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems/Life Technologies) and QuantiTect primers for mouse Brd2, cyclin A2, and GAPDH (Qiagen).

### Statistical analysis

For comparison of differences between two groups, we used unpaired two-tailed Student's *t*-tests. For flow cytometry analysis, a minimum of 50,000 events were analyzed with Statview (SAS Institute, San Francisco, CA, USA) or Prism 5 (GraphPad Software, San Diego, CA, USA). Data are presented as means  $\pm$  SE, and *P* values indicated with asterisks are reported as significant at *P* < 0.05 or 0.01 as indicated. A one-factor ANOVA, in conjunction with Dunnett's multiple comparisons test, is reported where appropriate.

## RESULTS

### Increased Brd2 expression in hematopoietic progenitors expands the peripheral B cell compartment

Previous work showed that constitutive expression of Brd2 in  $E\mu$ -Brd2 Tg mice promotes B cell expansion and development of an aggressive B cell lymphoma comprised of mature B cells, thus providing a model for elevated Brd2 activity in human lymphoid malignancies [9, 30]. These results provoked two questions: (1) does perturbation of Brd2 expression impact the lymphoid compartment and alter a final balance of mature lymphocytes in the periphery? and (2) does increased Brd2 expression increase the mitogenic sensitivity of mature cells in an acute model? Lentivirus transduction of HSCs and establishment of BM chimeras allowed us to determine whether Brd2 offered a selective advantage to transduced cells during repopulation of the BM during reconstitution. To determine the role of Brd2 in hematopoiesis, we transduced HSCs from CD45.1 donor mice with lentivirus that expressed Brd2, Brd2 shRNA, or the empty vector. We then reconstituted lethally irradiated CD45.2 recipient mice with a mixture of virus-transduced donor HSCs and nontransduced recipient HSC equivalents. In this competitive repopulation model, donor and recipient HSCs engraft in BM and over several weeks, are capable of reconstituting all hematopoietic lineages. The relative contributions of CD45.1 and CD45.2 HSCs to chimerism were assessed by flow cytometry of cells that appear in the peripheral blood over time, as well as in BM, thymus, and peripheral lymphoid organs at death. In this design (Fig. 1A), only CD45.1<sup>+</sup> cells were transduced with lentiviruses so that the effect of Brd2 expression could be resolved by flow cytometry from the CD45.2<sup>+</sup> background in each chimeric recipient ani-

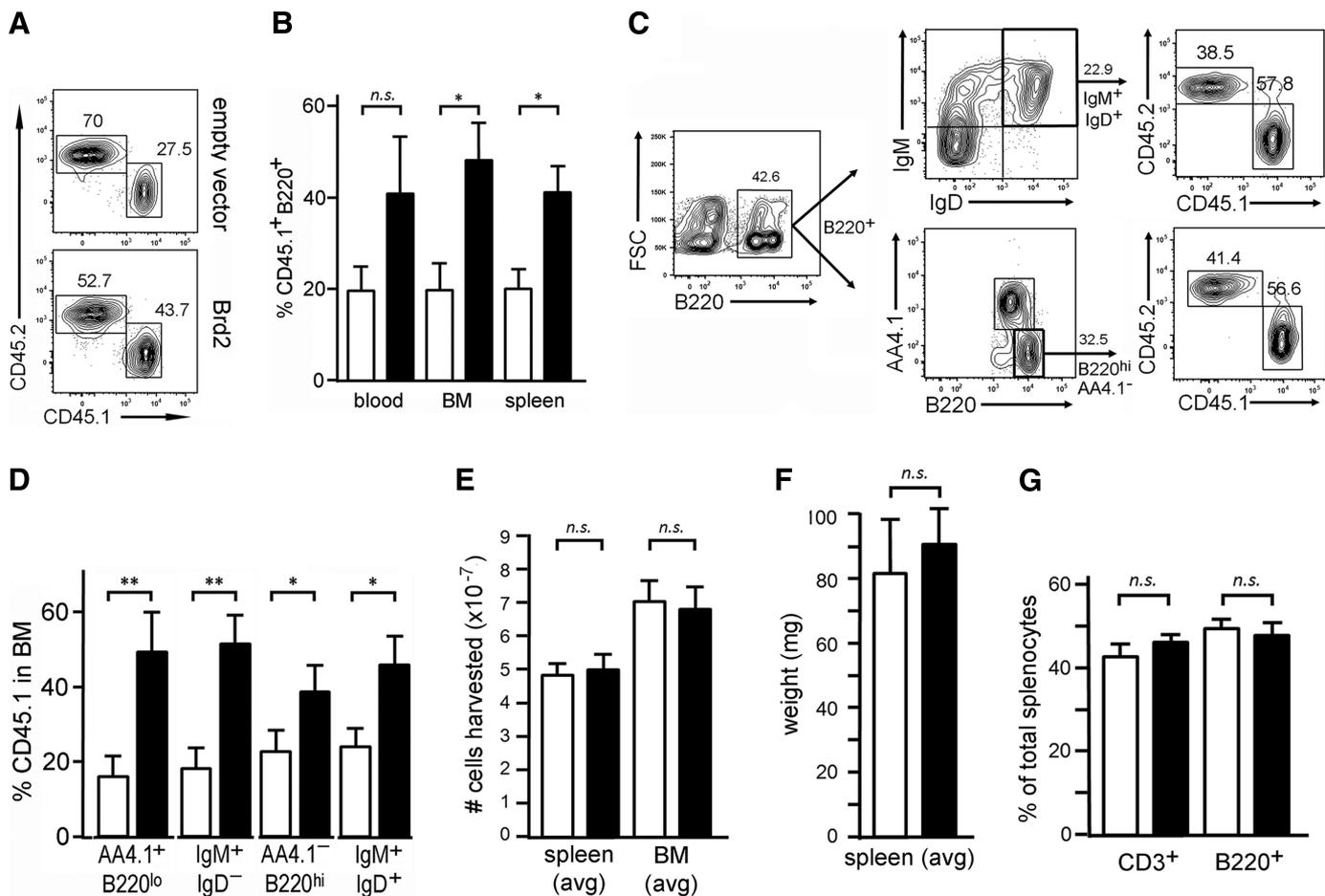
mal. Competitive repopulation, therefore, permitted direct comparison of mature, peripheral cells arising from transduced HSCs versus control HSCs in the same animal. Donor HSCs were enriched by the SP method [37] (Fig. 1B). We first confirmed that Brd2 expression was elevated in splenocytes of mice reconstituted from SP cells, transduced with Brd2 lentivirus; we observed an approximate threefold increase in Brd2 mRNA and protein (Fig. 1C and D). Five weeks post-transplant of lentivirus-transduced SPs, we confirmed engraftment/chimerism from peripheral blood (Supplemental Fig. 1D) by flow cytometry.

To investigate how Brd2 expression alters hematopoiesis and mitogenic responsiveness in the periphery, we characterized the mice in detail by flow cytometry. Lymphoid and myeloid compartments engrafted, and we confirmed their progeny in the periphery. The frequency was independent of lentivirus type (Supplemental Fig. 1F). Although eGFP was useful to validate reagents (Supplemental Fig. 1E), IRES, positioned 3' to a CMV element in bicistronic vectors, functions poorly in vivo [38, 39] and in practice, proved unsuitable to track eGFP<sup>+</sup> cells in the chimeras. Among PBLs, the type of lentivirus did not affect the proportion of B cells and T cells at 5 weeks (Supplemental Fig. 1F). As we used unfractionated, recipient BM to make the chimeras, peripheral blood showed significant recipient bias in the lymphoid compartment at 5 weeks compared with the myeloid compartment (Supplemental Fig. 1G). Differences in T cells were the primary sources of this bias (Supplemental Fig. 1H); donor-derived T cell repopulation is known to lag behind B cell repopulation in this model [40, 41].

Analysis of BM and peripheral lymphoid organs at 12 weeks postreconstitution showed expansion of CD45.1<sup>+</sup> cells that had received Brd2 lentivirus compared with control lentivirus (Fig. 2A). Specifically, percentages of donor B220<sup>+</sup> cells were increased in the hematopoietic organs tested (blood, BM, and spleen) if the HSCs originated from CD45.1<sup>+</sup> donor SPs transduced with Brd2 lentivirus compared with control lentivirus (Fig. 2B). Thymi also showed normal engraftment (Supplemental Fig. 2A), and Brd2 expression had no effect on numbers or proportions of CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup> (double-positive) or CD4<sup>-</sup>CD8<sup>-</sup> (double-negative) T cells in the thymus (Supplemental Fig. 2B) nor on the myeloid compartment in BM (Supplemental Fig. 2C–F).

We investigated the Brd2-driven donor B cell phenotype more deeply. We observed that Brd2 overexpression skewed chimerism significantly in BM, toward CD45.1<sup>+</sup> expansion in mature B220<sup>hi</sup>AA4.1<sup>-</sup> and IgM<sup>+</sup> IgD<sup>+</sup> compartments and immature B220<sup>lo</sup>AA4.1<sup>+</sup> and IgM<sup>+</sup> IgD<sup>-</sup> compartments (Fig. 2C and D). The results confirm that Brd2 overexpression in hematopoietic progenitors confers an advantage to B-lineage cells. To control for putative, intrinsic bias of SP cells, we used an inclusive gate for Hoechst-stained SP cells that yielded 0.025% of total live-cell events (Fig. 1B), which maximized long-term repopulating characteristics [37] while pooling stem cell subtypes that have different long-term functional potential [40, 42]. Results confirmed that the donor bias that we observe in the B lineage is a result of Brd2 rather than the methods of SP isolation or lentivirus transduction. It is noteworthy that in contrast to  $E\mu$ -Brd2-constitutive expression that drives

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**Figure 2. Brd2 skews chimerism to donor origin upon reconstitution.** (A) Chimerism in spleen, 12 weeks post-transplant of SP cells. Percentages of 100,000 total gated events are shown for donor or recipient marker; one representative experiment of 10 is shown. (B) Chimerism expressed as a percentage of donor-derived (CD45.1<sup>+</sup>) B-lineage cells (B220<sup>+</sup>) in three hematopoietic tissues at death (blood, *n*=5; *P*=0.18; BM and spleen, *n*=10; \**P*<0.05; open bars, empty vector lentivirus; solid bars, Brd2 lentivirus). (C) Gating strategy for identification of donor-derived, immature/transitional (AA4.1<sup>+</sup>, B220<sup>lo</sup>, IgD<sup>-</sup>) and mature B cells (IgM<sup>+</sup> IgD<sup>+</sup>) in BM in two staining panels: (1) mature B cells were identified by anti-B220-eFluor 450, anti-IgM-PE-Cy7, and anti-IgD-PE antibodies, and donor origin was identified by anti-CD45.1-PE-Cy5.5 antibody; (2) immature B cells were additionally identified by anti-AA4.1-PE antibody with donor origin identified by anti-CD45.1-PE-Cy7 antibody. Recipient cells were identified in both panels by anti-CD45.2-APC antibody. FSC, Forward-scatter. (D) Quantitation of chimerism in BM. (E) Numbers of splenocytes and BM cells harvested (*n*=10). (F) Spleen weight upon harvest (*n*=10). (G) T and B splenocytes upon harvest (*n*=6). (Open bars, empty vector lentivirus; solid bars, Brd2 lentivirus; *n*=10; \**P*<0.05; \*\**P*<0.01).

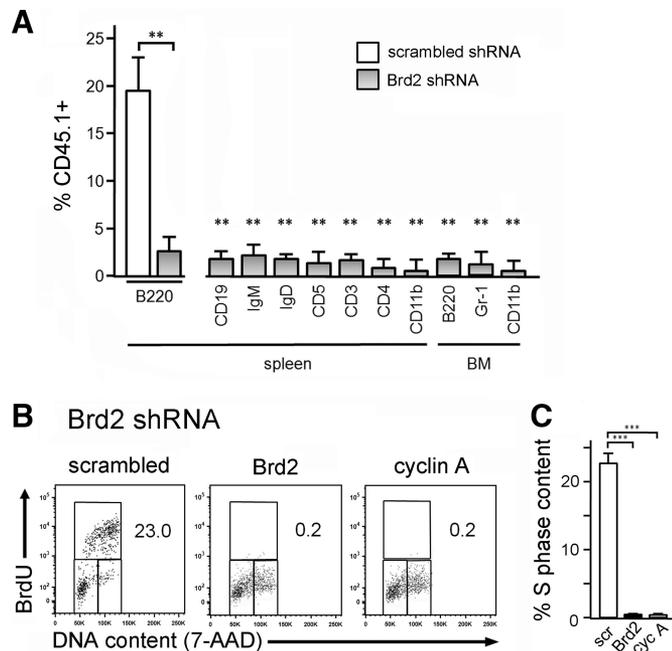
an increase in B cell numbers and B cell lymphoma [9], we did not observe spleen enlargement nor an increase in the absolute numbers of splenic B cells in the reconstituted mice. We also did not observe a change in the B:T cell ratio in the spleen post-reconstitution (Fig. 2E–G). These data mark the observed phenotype as relatively mild compared with the Tg lymphoma model.

**Brd2 expression is critical for hematopoiesis**

We [20, 27] and others [18, 19, 35, 43, 44] reported previously that total absence of Brd2 is lethal as a result of its critical/nonredundant role in mitosis. We tested the effect of Brd2 knockdown in reconstituted mice in the expectation that knockdown would eliminate donor-derived cells in the periphery. We used lentivirus that encoded a shRNA against Brd2

published previously [20] and confirmed this expectation (Fig. 3A). It is likely that shRNA against Brd2 prevents expansion from the common lymphoid progenitor stage, or earlier, consistent with the requirement for Brd2 for S-phase progression.

We could not obtain shBrd2 hematopoietic cells; thus, we switched to the NIH 3T3 model to study cell cycle kinetics. We transduced NIH 3T3 fibroblasts with the Brd2 shRNA lentivirus and then analyzed cell-cycle kinetics in vitro by flow cytometry [27]. Brd2 shRNA caused the vast majority of cells to exit S-phase, very similar to the effect of shRNA directed against cyclin A, the principal cyclin that governs S-phase (Fig. 3B and C). This result reinforces the interpretation that Brd2 is a critical regulator of S-phase in mammalian cells [27, 35]. Insufficient Brd2 is lethal, as proliferation and expansion from



**Figure 3. Knockdown of Brd2 ablates hematopoietic lineages through cyclin A failure.** (A) Percentage of donor-derived (CD45.1<sup>+</sup>) cells in spleen and BM of mice, 12 weeks after transplant. Donor SP cells were transduced with Brd2 shRNA knockdown lentivirus versus scrambled lentivirus control. The chimerism of each lineage marker in Brd2 shRNA lentivirus-reconstituted recipients was evaluated with respect to the corresponding lineage marker of scrambled shRNA lentivirus-reconstituted recipients; significant differences in means were evaluated by *t*-test. B220<sup>+</sup> cells for Brd2 and control shRNAs are shown first; to save space, only the Brd2 shRNA means and statistical significance are shown for each remaining pair. (Open bar, scrambled shRNA lentivirus; gray bars, Brd2 shRNA lentivirus; *n*=4; \*\**P*<0.01). (B) Cell-cycle kinetic analysis of cycling NIH 3T3 fibroblasts transduced with shRNA lentiviruses (against Brd2 or cyclin A or scrambled). After 24 h, cells were pulse-labeled with BrdU for 60 min and then fixed and permeabilized. BrdU epitopes were detected with anti-BrdU FITC antibody (BrdU). DNA content was detected with 7-AAD. Inset, upper boxes, S-phase cells; percentages of total gated events are shown. (C) Quantitation of B (*n*=3; \*\*\**P*<0.005). scr, Scrambled.

progenitors are required to establish and maintain any lineage. We conclude that the functional role of Brd2 in mature blood cells cannot be studied in hematopoiesis *in vivo* with methods that achieve constitutive knockdown in progenitors.

### Elevated Brd2 expression increases mitogenic responsiveness of mature B cells

Brd2-dependent proliferation [9, 27] suggested that mature, peripheral B cells with elevated expression of Brd2 would show increased proliferation in response to mitogenic stimulation compared with peripheral B cells with WT Brd2 expression. To test this possibility, we isolated splenocytes from chimeric recipient mice at 12 weeks postreconstitution, stimulated them with LPS, and then assayed cell-cycle kinetics and S-phase content of B220<sup>+</sup> B cells. B cells derived from Brd2-transduced donor SPs incorporated more BrdU than control B cells derived from lentiviral vector-transduced donor SPs (Fig.

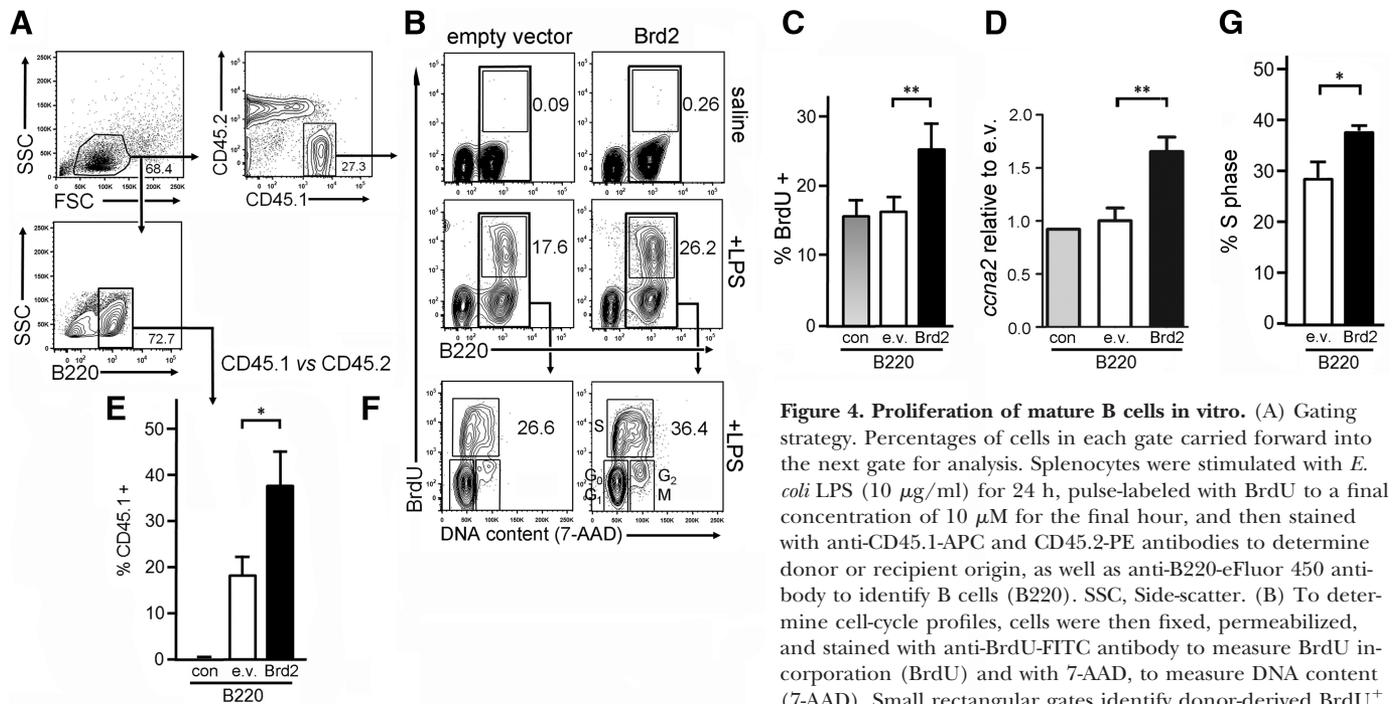
4A–C). Control studies showed that B cells derived from vector-transduced SPs responded the same as nonchimeric control C57BL/6J (CD45.2) mice that had not been irradiated or reconstituted (Fig. 4C). Cell-cycle analysis (Fig. 4B and F) supported the conclusion that Brd2 expression increased significantly the mitogenic response of B cells to LPS, expressed as the fraction of B220<sup>+</sup> cells that were in S-phase (Fig. 4G). Measurement of the ratio of CD45.1 and CD45.2 staining in the proliferation assay confirmed that donor SP-derived B cells accounted for the increased percentage of lymphocytes if the donor SPs had been transduced with Brd2-expressing lentivirus compared with empty-vector lentivirus (Fig. 4E).

Brd2 interacts directly with cyclin A promoter chromatin to promote *cyclin A* transactivation in fibroblasts [27, 35]. To test whether enhanced transactivation of *cyclin A* drives increased B cell proliferation, we measured cyclin A expression in cultured splenocytes, treated with B or T cell mitogens (LPS or  $\alpha$ CD3/ $\alpha$ CD28, respectively). The cyclin A mRNA levels were higher in LPS-stimulated B cells derived from Brd2-transduced donor SPs compared with control B cells derived from empty vector-transduced donor SPs and with nonchimeric control B cells (Fig. 4D). However, there was no difference in S-phase content (Supplemental Fig. 3B), BrdU<sup>+</sup> content of donor-derived cells (Supplemental Fig. 3C and D), or cyclin A expression (Supplemental Fig. 3E) among T cells derived from Brd2-transduced donor SPs, empty vector-transduced donor SPs, or nonchimeric control stimulated with  $\alpha$ CD3/CD28. In addition, no differences were observed in basal cyclin A mRNA expression if lymphocytes were not stimulated (Supplemental Fig. 3F). Taken together, these data indicate that the increased mitogenic response that we observed in B cells derived from Brd2-transduced donor SPs is driven through cyclin A up-regulation. Increased responsiveness does not occur through up-regulation of basal cyclin A expression but is revealed only upon B cell stimulation.

We used B cells from E $\mu$ -Brd2 Tg mice [9] to confirm that up-regulation of cyclin A was downstream of Brd2 overexpression. Importantly, cells were obtained from animals prior to development of B cell malignancy. In agreement with data from BM-reconstituted mice, Tg B cells were significantly more responsive to LPS than non-Tg B cells, as indicated by greater S-phase content (Supplemental Fig. 4A and B). Moreover, cyclin A protein levels were increased in stimulated Tg B cells compared with non-Tg B cell controls, as measured by intracellular staining (Supplemental Fig. 4C and D). These results confirm independently that constitutive Brd2 expression induces proliferation of B cells through up-regulation of cyclin A.

### Brd2 interacts with the cyclin A promoter in B cells

We have obtained a number of indirect clues that link cyclin A-directed proliferation with Brd2 function. To test directly whether Brd2 regulates cyclin A expression in B cells, we performed ChIP with B cells isolated from WT mice (Supplemental Fig. 5). We found that after a 24-h exposure to LPS (a time-point when up to one-quarter of primary B cells is about to enter S-phase of the cell cycle, a stage that is governed primarily by cyclin A), Brd2 precipitates chromatin of the cyclin



**Figure 4. Proliferation of mature B cells in vitro.** (A) Gating strategy. Percentages of cells in each gate carried forward into the next gate for analysis. Splenocytes were stimulated with *E. coli* LPS (10  $\mu$ g/ml) for 24 h, pulse-labeled with BrdU to a final concentration of 10  $\mu$ M for the final hour, and then stained with anti-CD45.1-APC and CD45.2-PE antibodies to determine donor or recipient origin, as well as anti-B220-eFluor 450 antibody to identify B cells (B220). SSC, Side-scatter. (B) To determine cell-cycle profiles, cells were then fixed, permeabilized, and stained with anti-BrdU-FITC antibody to measure BrdU incorporation (BrdU) and with 7-AAD, to measure DNA content (7-AAD). Small rectangular gates identify donor-derived BrdU<sup>+</sup> B cells; percentages of gated events are shown. One representative set of contour plots was selected from 10 replicates. (C) Quantitation of B cell-specific BrdU incorporation in B. Mice reconstituted with empty vector lentivirus (e.v.; open bar;  $n=10$ ) and Brd2 lentivirus (Brd2; solid bar;  $n=10$ ) were compared with nonchimeric, nonreconstituted control CD45.2 mice (con; gray bar;  $n=10$ ; \*\* $P<0.01$ ). (D) Cyclin A2 expression by RT-qPCR in B cells (B220) stimulated with 10  $\mu$ g/ml LPS for 24 h. Cyclin A2 mRNA (*ccna2*) was measured in cells from control ( $n=1$ ), empty-vector lentivirus-reconstituted ( $n=5$ ), and Brd2 lentivirus-reconstituted ( $n=5$ ) mice, calculated relative to GAPDH mRNA and normalized to averaged empty vector (bars as specified in C; \*\* $P<0.01$ ). (E) B cell (B220) chimerism in proliferation assay ( $n=10$ ; bars as specified in C; \* $P<0.05$ ). Nonchimeric CD45.2 control mice (con), by definition, did not harbor CD45.1<sup>+</sup> cells. (F) Cell-cycle kinetics of donor-derived (CD45.1<sup>+</sup>) B cells. Small rectangular gates identify donor-derived B cells in S-phase; percentages of gated events are shown; cell-cycle phases are specified (G<sub>0</sub>/G<sub>1</sub>, S, G<sub>2</sub>/M). (G) Quantitation of percentage of B cells (B220) in S-phase as shown in F ( $n=10$ ; bars as specified in C; \* $P<0.05$ ).

relative set of contour plots was selected from 10 replicates. (C) Quantitation of B cell-specific BrdU incorporation in B. Mice reconstituted with empty vector lentivirus (e.v.; open bar;  $n=10$ ) and Brd2 lentivirus (Brd2; solid bar;  $n=10$ ) were compared with nonchimeric, nonreconstituted control CD45.2 mice (con; gray bar;  $n=10$ ; \*\* $P<0.01$ ). (D) Cyclin A2 mRNA (*ccna2*) was measured in cells from control ( $n=1$ ), empty-vector lentivirus-reconstituted ( $n=5$ ), and Brd2 lentivirus-reconstituted ( $n=5$ ) mice, calculated relative to GAPDH mRNA and normalized to averaged empty vector (bars as specified in C; \*\* $P<0.01$ ). (E) B cell (B220) chimerism in proliferation assay ( $n=10$ ; bars as specified in C; \* $P<0.05$ ). Nonchimeric CD45.2 control mice (con), by definition, did not harbor CD45.1<sup>+</sup> cells. (F) Cell-cycle kinetics of donor-derived (CD45.1<sup>+</sup>) B cells. Small rectangular gates identify donor-derived B cells in S-phase; percentages of gated events are shown; cell-cycle phases are specified (G<sub>0</sub>/G<sub>1</sub>, S, G<sub>2</sub>/M). (G) Quantitation of percentage of B cells (B220) in S-phase as shown in F ( $n=10$ ; bars as specified in C; \* $P<0.05$ ).

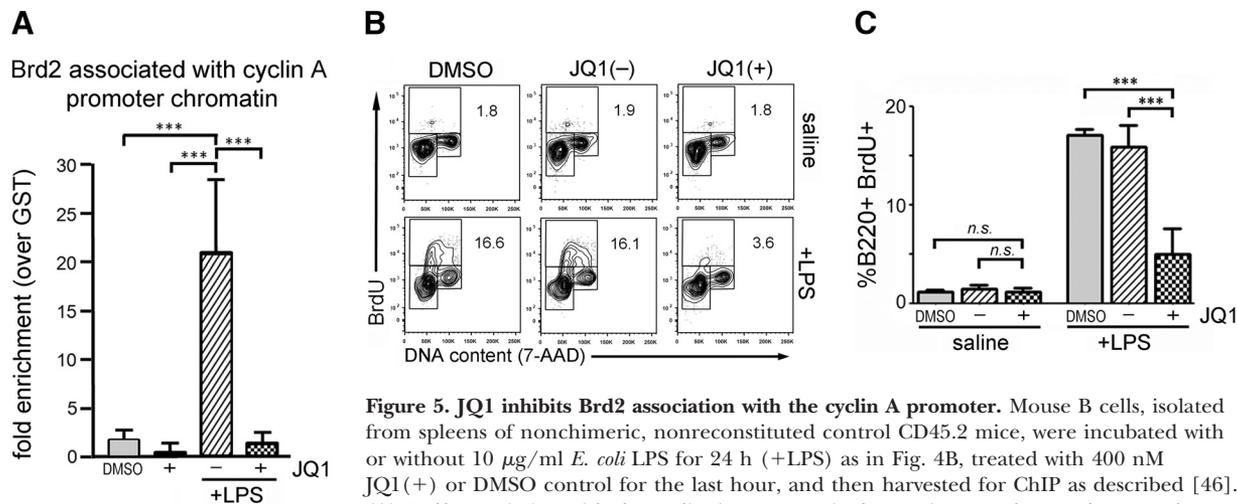
A promoter, whereas Brd2 is not associated with cyclin A promoter chromatin in nonstimulated cells (Fig. 5A).

To test if the interaction between Brd2 and the cyclin A promoter is druggable, we added JQ1, a small molecule inhibitor of BET proteins [28] to the LPS-stimulated B cell cultures, 1 h before harvest for ChIP. Brd2 did not associate with the cyclin A promoter chromatin in the presence of JQ1 (Fig. 5A). Moreover, when added simultaneously with LPS, JQ1 blocks B cell proliferation, as shown in a BrdU pulse-label experiment (Fig. 5B, right panels). Lack of effect of the inactive enantiomer of JQ1 (-) demonstrated the specificity of the JQ1(+) effect (Fig. 5B, middle panels). Finally, JQ1 is not cytotoxic to normal B cells, only cytostatic, as demonstrated by Annexin V/7-AAD measurement of B cell viability (Supplemental Fig. 6). We thus confirm that Brd2 associates with cyclin A promoter chromatin in proliferating, primary B cells and that disruption of this interaction by BET inhibitors has an antiproliferative effect on B cells.

## DISCUSSION

Abnormal transcription factor function has long been implicated in hematologic malignancy. In particular, specific oncoprotein fusions that arise from chromosomal translocations lead to dysfunction of differentiation programs and a reactiva-

tion of proliferation programs in progenitor cells of the blood, which commonly results in leukemia. However, compared with the research performed on sequence-specific DNA-binding transcription factors in cancer, far less consideration has been given to the chromatin environment in which these corrupted, oncogenic transcription factors work and to the critical role of chromatin status in the development of cancer. Translocations that deregulate factors that specifically act on chromatin or histone acetylation form a prominent, distinct class of oncogenic lesion. New attention has been focused recently on the role of bromodomain-containing transcriptional coregulators, because of recent success in the design of small molecule inhibitors [28, 29] that interfere with the binding between the bromodomain and the acetylated histone groups in nucleosomes of transcriptionally active chromatin. This approach proves that chromatin-binding surfaces, such as the bromodomain, are druggable targets [1]. However, model systems for misregulated BET protein expression are not widely available for the testing of these novel agents; apart from  $E\mu$ -Brd2, no BET protein-Tg mice have been characterized [36, 45]. We built on those reports with a stem-cell reconstitution model for Brd2-driven B cell expansion from progenitor cells and increased mitogen responsiveness of mature B cells in the periphery. We find that (1) Brd2 is essential for all of hemato-



**Figure 5. JQ1 inhibits Brd2 association with the cyclin A promoter.** Mouse B cells, isolated from spleens of nonchimeric, nonreconstituted control CD45.2 mice, were incubated with or without 10  $\mu\text{g/ml}$  *E. coli* LPS for 24 h (+LPS) as in Fig. 4B, treated with 400 nM JQ1(+) or DMSO control for the last hour, and then harvested for ChIP as described [46].

(A) Brd2 association with the cyclin A promoter is shown. (Mean values and SD are shown;  $n=4$ ;  $***P<0.001$ ). (B) To determine the proliferative response to mitogen, splenocytes from nonchimeric, nonreconstituted control CD45.2 mice were stimulated (+LPS) for 24 h, as in Fig. 4B; then stained with anti-B220-eFluor 450 antibody; fixed; permeabilized; stained for BrdU incorporation (BrdU) and DNA content (7-AAD) as in Fig. 4F; and compared with unstimulated control (saline). Cultures were exposed to 400 nM JQ1(+) (the active enantiomer; checked bars in C); 400 nM JQ1(-) (the inactive enantiomer; hatched bars in C); or DMSO vehicle (gray bars in C) at the same time as LPS. Plots from one representative experiment of six replicates are shown. (C) Quantitation of B ( $n=6$ ;  $***P<0.001$ ).

poiesis, yet overexpression preferentially promotes B cell expansion (T lymphopoiesis and mitogenesis are not enhanced by Brd2); (2) Brd2 promotes mature B cell proliferation in response to mitogen; and (3) the Brd2 effect in B cells is mediated through cyclin A. These results establish an acute model for chromatin-directed, cell-cycle stimulation that could become one of the “first hits” in B cell cancers.

Early in our characterization of this model, outlined in Fig. 2, we noted a lopsided ratio of chimeric B cells. Donor-derived cells in the periphery significantly exceeded the numbers of recipient-derived cells if the SPs had been transduced with Brd2-expressing lentivirus but not with control lentivirus. Conversely, we detected a significant, dramatic reduction in the number of donor-derived cells in the periphery if the SPs had been transduced with Brd2 shRNA-expressing lentivirus but not with control lentivirus. In the shRNA case, the lethally irradiated recipients survived, as they were also reconstituted with competitor CD45.2 BM cells. If reconstituted with only donor-derived SPs transduced with Brd2 shRNA, no recipients survived the critical period before adequate repopulation, which follows whole-body irradiation and retro-orbital injection of HSCs (data not shown). In separate experiments [46], we enumerated the major immune-cell subsets in spleen and BM of Brd2 hypomorphic (“brd2 lo”) mice [20] but noted no significant departures from populations enumerated in age- and sex-matched controls, likely because this genetic model shows compensation in the studied heterozygotes. Considering these data together, we conclude that Brd2 expression is important for the ability of SPs to reconstitute the immune system and that increased expression of Brd2 expands the B lineage.

HSCs, isolated from mouse BM by the SP method, are a heterogeneous cellular population that exhibits different lineage biases during long-term reconstitution [42], depending on their properties of Hoechst 33342 dye efflux [41]. Specifically,

SP cells with the greatest ability to efflux dye show the least Hoechst red and blue fluorescence and in flow cytometry bivariate plots, show lower fluorescence; Challen et al. [41] termed these “lower-SP”, whereas SP cells with greater fluorescence were termed “upper-SP”. The distribution of efflux capability defines a spectrum of functional ability to reconstitute lymphoid and myeloid lineages, with the lower-SP population significantly biased toward myeloid reconstitution and the upper-SP population significantly biased toward lymphoid reconstitution [41]. In view of the hypothesis that Brd2 expression promotes B cell expansion, we chose an unbiased approach that worked neither against myeloid expansion by restricting our SP gate to the upper SP cells nor against lymphoid expansion by restricting our SP gate to the lower SP cells. Therefore, we defined a SP gate of reduced stringency relative to the upper and lower SP gates reported previously [41] and included both populations as source cells for lentivirus transduction. The functional variable of interest here was Brd2 versus control rather than the SP subtype. As our SP cells were isolated into a pool by MoFlo cell sorting and then divided equally in separate cultures for Brd2 and control lentivirus transduction, the experimental design controlled for functional differences among subpopulations of SP cells and any lineage bias that might exist in the source population of cells. Thus, we interpret donor B cell expansion to be a result of Brd2 expression and not of our choice of SP gate. The fact that we found that Brd2 forced expression provides a proliferative advantage to mature B cells but not T cells or myeloid cells, whereas basal Brd2 expression is essential for all hematopoietic lineages, indicates that there are unstudied factors that synergize with Brd2 in B cells. These factors, once identified, may provide additional therapeutic targets for the treatment of B cell cancers.

Taken together, our results support the established model that Brd2 couples mitogenic signals to the transcription of target

genes that regulate proliferation [27, 35, 47]. Decreasing Brd2 levels or chromatin binding would be expected to promote immune cell-cycle arrest and differentiation at the expense of proliferation. The phenotype observed in mice reconstituted with Brd2 shRNA-transduced SPs introduces a striking parallel between our model and a model of cyclin A ablation in hematopoietic cells. With the use of an inducible Cre system (cyclin A1<sup>-/-</sup>A2<sup>f/f</sup>) in MxCre mice, Kalaszczynska et al. [48] show that ablation of cyclin A in HSCs promotes BM failure in mice, whereas in a BM transplantation assay with competitor BM from WT mice, cyclin A ablation leads to complete absence of cyclin A-null donor cells in reconstituted animals. Thus, we propose that cyclin A transcription provides a mechanistic link between Brd2 activity and proliferation.

We speculate that the ability of Brd2 to promote lineage expansion identifies a novel, useful therapeutic target for B cell deficiencies. Although many recent studies have focused on elucidating the underlying molecular mechanisms involved in HSC fate (i.e., quiescence, self-renewal, differentiation, or exhaustion), the underlying molecular mechanisms involved have yet to be clarified fully. Recent studies have focused on how chromatin modification directs stem-cell fate. Specifically, proteins of the PcG (and associated proteins, such as Bmi-1) and the trithorax group, which oppose each other, determine stem-cell fate [49]. Furthermore, core components of the switch/sucrose nonfermenting chromatin-remodeling complex play a critical role in embryonic stem cell pluripotency. Importantly, *female sterile (1) homeotic*, the *Drosophila* homolog of Brd2 [43], activates trithorax [50, 51], which in humans, is disrupted in 11q23 chromosomal translocations that are linked to MLL [52]. Thus, increased Brd2 activity would be likely to oppose PcG function [1], suggesting that Brd2 is a candidate protein to regulate HSC 'stemness' and proliferation during hematopoiesis. Such a therapeutic approach would need to be regulated carefully in view of the leukemogenic risk of elevated Brd2 expression [53].

Although we show that the small molecule BET protein inhibitor JQ1 (+) ablated cell cycle in normal peripheral B cells stimulated with LPS, JQ1 does not resolve roles of individual BET proteins, reviewed recently [1]. Like Brd2, Brd4 is also important for proliferation, but Brd2 lacks the long, unstructured carboxy-terminal interaction domain of Brd4 [1, 12], and only Brd4 interacts with P-TEFb [21]. The ability of JQ1 to inhibit B cell proliferation [54] is narrowly and incorrectly assumed to work only through Brd4; we report that Brd2 specifically mediates the inhibitory effects of JQ1 at promoters of inflammatory cytokine genes [46]. Targeting this pathway with therapeutic drugs may therefore confer multiple, diverse benefits in the treatment of hematologic malignancy, B cell-mediated disease, and inflammation.

## AUTHORSHIP

A.C.B. and W.P.B. performed the experiments. A.C.B. and G.V.D. wrote the paper in consultation with W.P.B. and B.S.N. G.V.D. designed the research and provided the funding for the study.

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## DISCLOSURES

The authors declare no conflict of interest.

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KEY WORDS:

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