Published in final edited form as:
J Immunol. 2014 December 1; 193(11): 5515–5524. doi:10.4049/jimmunol.1401798.

Deleted in Breast Cancer 1 (DBC1) is a Suppressor of B cell Activation by Negatively Regulating Alternative NF-κB Transcriptional Activity

Sinyi Kong[1], **Muthusamy Thiruppathi**[2], **Quan Qiu**[1], **Zhenghong Lin**[1], **Hongxin Dong**[1], **Eduardo N. Chini**[3], **Bellur S. Prabhakar**[2], and **Deyu Fang.**[1]

[1]Department of Pathology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave, Chicago IL 60611, USA

[2]Department of Microbiology and Immunology, University of Illinois College Of Medicine Chicago, 835 South Wolcot Ave, Chicago IL 60612, USA

[3]Laboratory of Signal Transduction, Department of Anesthesiology and Robert and Arlene Kogod Center on Aging, Mayo Clinic College of Medicine, Rochester, MN 55905, U.S.A

Abstract

CD40 and BAFF receptor (BAFFR) signaling plays important roles in B cell proliferation and immunoglobulin production. In this study we found that B cells from mice with deletion of *Dbc1* gene (*Dbc1*$^{-/-}$) show elevated proliferation, and IgG1 and IgA production upon in vitro CD40 and BAFF, but not BCR and LPS stimulation, indicating that DBC1 inhibits CD40/BAFF-mediated B cell activation in a cell-intrinsic manner. Microarray analysis and Chromatin Immunoprecipitation (ChIP) experiments reveal that DBC1 inhibits B cell function by selectively suppressing the transcriptional activity of alternative NF-κB members RelB and p52 upon CD40 stimulation. As a result, when immunized with Nitrophenylated-Keyhole Limpet Hemocyanin (NP-KLH), *Dbc1*$^{-/-}$ mice produce significantly increased levels of germinal center B cells, plasma cells, as well as antigen-specific immunoglobulin. Finally, loss of DBC1 in mice leads to higher susceptibility to Experimental Autoimmune Myasthenia Gravis (EAMG). Our study identifies DBC1 as a novel regulator of B cell activation by suppressing the alternative NF-κB pathway.

Introduction

B cell receptor (BCR)-mediated signaling upon recognition of specific antigen initiates humoral immune response. In addition, costimulatory signaling mediated by CD40 and B-cell activating factor (BAFF) receptor, plays important roles in B cell proliferation and immunoglobulin production (1–5). B cell activation is tightly regulated to limit unwanted responses to self-antigen (6, 7). While B cell activation and immunoglobulin production is

[#]Corresponding Author: Deyu Fang, Department of Pathology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave, Chicago IL 60611, USA, fangd@northwestern.edu. Tel, 312-503-3021; Fax, 312-503-3023.

Disclosure of Conflicts of Interest. The authors declare no competing financial interests.

Author contributions: SK performed the experiments and analyzed the data; CCSC contributed to development of reagents and animal models; SK, ENC & DF wrote the manuscript.

required for an optimal immune response against pathogens, dysregulated B cell activation can lead to increased immunoglobulin production and susceptibility to autoimmune disease (8). Myasthenia Gravis (MG) is primarily driven by autoantibodies against self-antigens such as nicotinic Acetylcholine Receptor (AChR), blocking receptor signaling and resulting in muscle weakness (9). Although the role of autoantibodies is well-defined in MG, the molecular pathways driving B cell activation and autoimmunity are less understood.

The Nuclear Factor kappa B (NF-κB) pathway is a central signaling pathway in the B cell immune response (10). NF-κB signaling can be divided into two branches: classical signaling that utilizes RelA, c-Rel and p50 dimers, and alternative signaling that utilizes RelB and p52 dimers. While the classical signaling pathway is required for the survival of most cell types, the alternative pathway is particularly essential for stages of B cell activation such as cell cycle progression, isotype switching and plasma cell survival (10–14). In addition, the classical and alternative NF-κB signaling pathways are differentially activated: In B cells, the classical signaling pathway is activated by B cell receptor (BCR), Toll-like receptors (TLRs) and inflammatory cytokines IL-1, IL-6 and TNF-α, whereas the alternative pathway is activated by CD40, BAFFR and LTβR(10). Although the NF-κB members have overlapping homology, the two branches of signaling maintain distinct transcriptional activities and cellular functions (10, 12). How the two signaling branches of NF-κB maintain distinct functions despite their structural similarities is still relatively unknown.

The *Dbc1* gene was initially identified as part of the 8p21 chromosome locus that is deleted in breast cancers (15). DBC1 has been shown to regulate gene transcription by interacting with nuclear receptors (16–18) and epigenetic modifiers (19–22). While DBC1 has been studied mostly in the context of cancer and metabolism, little is known about its role in the immune response. Sirt1, a target of DBC1, has been shown to play a regulatory role in the immune response, particularly in the function of macrophages and T cells through the suppression of classical NF-κB member RelA (23–25), prompting us to investigate the role of DBC1 in the immune response. Unexpectedly, we found that DBC1 regulates proliferation and immunoglobulin production of B cells, by suppressing the transcriptional activity of alternative NF-κB members RelB and p52. Consequently, loss of DBC1 in mice results in increased antigen-specific antibody production when immunized with the protein antigen NP-KLH without adjuvant, as well as increased susceptibility to immunoglobulin-mediated autoimmune disease Experimental Autoimmune Myasthenia Gravis (EAMG). Our findings identify DBC1 as a suppressor of B cell activation, through a novel mechanism by selectively suppressing the alternative NF-κB pathway.

Methods

Mouse and Cell Lines

HEK293T and NIH3T3 cells lines were maintained in DMEM (Gibco) supplemented with 10% Fetal Calf Serum (FCS) and 1% Penicillin/Streptomycin. EL4 cell line was maintained in RPMI supplemented with 10% FCS and 1% penicillin/streptomycin. *Dbc1*$^{-/-}$ mice were used as previously reported (26). DBC1/Sirt1 DKO mice were generated by crossbreeding with *Sirt1*$^{fl/fl}$ *CD19-Cre* mice. All mice used in this study were maintained and used at the

Northwestern University mouse facility under pathogen-free conditions according to institutional guidelines and animal study proposals approved by the Institutional Animal Care and Use Committee.

Plasmids, Antibodies and Reagents

PcDNA-Myc-DBC1 plasmid was purchased from Addgene (27). Antibodies used for immunoblotting and co-immunoprecipitation were from Bethyl laboratories (anti-p30/DBC1), Santa Cruz (anti-RelB(C-19) (D-4), RelA (F-6), C-Rel (B-6), p105/p50 (H-119), p100/p52 (C-5), and Myc (A-14), (9E10)), Sigma (anti-Flag (F7425) (F1804)) and Calbiochem (anti-Tubulin (DM1A)). Antibodies for Chromatin Immunoprecipitation were anti-RelB (C-19) and anti-RelA (C-20) (Santa Cruz). NP-KLH and NP-BSA was purchased from Bioresearch Technologies.

Primary B cell Isolation and Culture

Primary B cells were negatively isolated from 8–12 week old mice using Dynabeads® Mouse CD43 (Untouched B cells) (Life Technologies) per manufacturer's instructions. Purified B cells were maintained at 10^6/mL in RPMI (Dibco) supplemented with 10% Fetal Bovine Serum, 50μM β-mercaptoethanol, 100mM sodium pyruvate, 100mM HEPES buffer, and 1% penicillin/streptomycin. B cells were activated with goat $F(ab)_2$ anti-mouse IgM (10μg/ml; Jackson ImmunoResearch), anti-CD40 (1μg/ml; eBioscience), IL4 (10ng/ml), LPS (500ng/ml), BAFF (100ng/ml; Peprotech) as indicated.

For cell proliferation and immunoglobulin production assays, purified B cells were stained with Cell Trace Carboxyfluorescein diacetatesuccinimidyl ester (CFSE, 5μm; Life Technologies), and cultured at 10^6 cells/ml for 5 days with indicated stimuli. After 5 days, cells were subjected to flow cytometry and analysis.

Flow cytometry

Single-cell suspensions were Fc blocked with anti-CD16/32 antibody (eBioscience), stained with the appropriate fluorophore-conjugated antibodies then collected in an Accuri C6 Flow Cytometer or FACS Canto (BD Biosciences). Fluorescence labeled-antibodies used include fluorescein-isothiocyanate-conjugated anti-mouse IgA, phycoerythrin-conjugated anti-CD138, allophyocyanin-conjugated anti-IgG1 (BD Biosciences), Peridinin-chlorophyll Cy5.5-conjugated anti-B220 (Biolegend). Where appropriate, biotin-labeled anti-IgA and peanut agglutinin (PNA) (Vector labs) was used followed by allophyocyanin-conjugated streptavidin (Biolegend). For intracellular staining of immunoglobulin, cells were fixed and permeabilized using the CytoFix/Perm Kit (BD Biosciences) per manufacturer's instructions, then incubated with 1:400x dilution of isolated serum in 1% Bovine Serum Albumin, followed by staining with the appropriate fluorophore-conjugated antibodies.

Cell cycle analysis

2×10^6 B cells were incubated with 5-ethynhl-2′-deoxyuridine (Life Technologies) at 37C, 5% CO_2 for 2 hours, then fixed in 4% formaldehyde at RT for 15 minutes. Cells were then washed with PBS, permeabilized with 0.1% Triton X-100, and stained with Alexa488-

coupled azide for 45 minutes. Cells were washed then stained with 7-amino actinomycin D (7AAD) 5 minutes prior to flow cytometry analysis.

For Propidium Iodide (PI) staining, cultured primary B cells were fixed overnight at −20C in 70% ethanol, then washed with PBS twice prior to incubation with Propidium Iodide/ RNaseA solution (Biolegend) for 30 minutes RT. Cells were analyzed by flow cytometry.

TUNEL staining

Cells were fixed with 4% Formaldehyde for 15 minutes, washed, with PBS, then permeabilized with 0.5% Tween-20 for 15 minutes. Cells were then washed again before labeling with 50μL TdT and biotin-conjugated dUTP cocktail (Millipore) for 45 minutes at 37C. Cells were then washed and stained with FITC-conjugated avidin, and analyzed by flow cytometry for apoptosis.

Immunizations

For NP-KLH immunization studies, 8–12 week old WT and *Dbc1*$^{-/-}$ littermates were immunized subcutaneously (s.c) with 100μg NP-KLH (Biosearch-Technologies). In a separate experiment, 100μg NP-KLH was emulsified 1:1 in Complete Freund's Adjuvant before s.c. immunization. Blood serum was collected from the saphenous vein on days 0,7,14,21 and serum immunoglobulin measured by ELISA. Splenocytes were isolated on day 21 and processed for flow cytometry.

For EAMG induction, 8–12 week old WT and *Dbc1*$^{-/-}$ littermates were immunized s.c. with 100ug of AChR purified from the electric organ of *Torpedo californica* (tAChR). On day 35, mice were given a second dose of 100ug tAChR through s.c. administration. For clinical examination, mice were examined on a flat platform and exercised by gently dragging them across a cage top grid repeatedly (20–30 times). Mice were graded according to the following scale as previously described: 0= normal posture and mobility at baseline and after exercise; 1 = normal at rest but with muscle weakness shown by hunchback posture, restricted mobility, and difficulty in raising the head after exercise; 2= muscle weakness without exercise during observation period; 3= dehydrated and moribund with muscle weakness; and 4= dead(28). Sera were collected on days 14, 28 and 42 for ELISA, and splenocytes isolated on day 42 for flow cytometry analysis.

ELISA

96-well flat bottom plates were coated overnight at 4ºC with 1μg of NP-BSA, 0.1μg *Torpedo californica*-derived AChR (tAChR), or 0.1μg *Mus musculus*-derived (mAChR) per well to detect antibodies against NP-KLH, tAChR, and mAChR respectively. For detection of total immunoglobulin levels, 50 ng purified anti-mouse antibodies for the specific isotypes were used. Wells were then blocked with 1% BSA at RT for 2 hours, then incubated with mouse sera at 1:200 to 1:10000 dilutions overnight at 4ºC. Biotin-coated secondary antibodies against specific immunoglobulin were added and incubated for 1 hour at RT, followed by avidin-HRP, and TMB substrate (Thermo Scientific). Absorbance at 450nm was detected using FilterMax F5 microplate reader (Molecular Devices).

Bone marrow chimera

Bone marrow was isolated from 2 pairs of *Dbc1*$^{-/-}$ (45.2) and congenic B6.SJL (45.1) mice, mixed at a 1:1 ratio, and 5×10^6 cells were administered intravenously into each lethally irradiated B6.SJL recipient mice. After 6 weeks, mice were sacrificed and reconstituted splenocytes analyzed by flow cytometry. Recipient mice were fed with water supplemented with sulmethoxazole and trimetroprim during the entire duration.

RNA Isolation and Microarray analysis

5–10 $\times10^6$ primary B cells pooled from 3 pairs of mice were cultured with indicated stimuli for 4 hours at 37C, 5% CO_2. RNA was then isolated using the Qiagen RNAeasy kit per manufacturer's instructions. 1μg of isolated RNA was subjected to microarray analysis using the MouseRef8-v2.0 BeadChip Kit (Illumina) at the University of Chicago Genomics Core Facility. Heatmap was generated using Cluster (Eisen) and Treeview software. Functional annotation analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7. Response elements on genes were identified from *Genecards.org.* Microarray data has been submitted to GEO database and can be accessed at http://www.ncbi.nlm.nih.gov/geo (accession number GSE61029).

Real Time PCR

10^6 cells were lysed in Trizol (Invitrogen), and RNA isolated per manufacturer's instructions. 1μg of isolated RNA was then reversed transcribed using qScript cDNA synthesis kit (Quanta BioSciences). Real Time PCR was performed in duplicate wells using the iCycler Sequence and SsoFast SYBR Green Supermix (BioRad). Relative expression was normalized to expression of *Actb*. Primers used in this study are shown as in supplemental Table 1.

DNA-binding assay

Nuclear extracts from cultured primary B cells were isolated using the Nuclear Extract Kit (Active Motif) per manufacturer's instructions, and nutated with biotin-conjugated 21-mer DNA probe (5ng; Life Technologies), streptavidin-conjugated agarose (Sigma-Aldrich) in PBS containing protease inhibitor cocktail. Agarose-DNA-protein complex was then washed, boiled in 4x Laemlli's buffer, then subjected to SDS-PAGE and Western Blot.

Chromatin Immunoprecipitation (ChIP)

Primary B cells were stimulated with anti-CD40 and IL4 for 4 or 16 h, cross-linked with 10% formalin, and subjected to ChIP using the Chromatin Immunoprecipitation Assay Kit (Millipore) per manufacturer's instructions. In brief, 2×10^6 cells were lysed with SDS lysis buffer. Cell lysates were sonicated, and 3% of cell lysate was removed and used to determine the total amount of target DNA in input. Remaining cell lysates were diluted in ChIP dilution buffer. Immunoprecipitation was performed with each of the indicated antibodies (3 μg) at 4 °C overnight. Immune complexes were then mixed with salmon sperm DNA/protein agarose 50% slurry at 4 °C for 1 h. After immunoprecipitates were washed sequentially with low salt buffer, high salt buffer, LiCl wash buffer, and Tris EDTA, DNA-protein complexes were eluted with elution buffer, and cross-linking was reversed. Genomic

DNA was extracted using phenol/chloroform, and ethanol-precipitated DNA was resuspended in Tris EDTA. Primer sequences available upon request.

Statistical analysis

Student's t-test was used to calculate statistical significance for ELISA, cell cycle analysis, proliferation assay, flow cytometry analysis and western blot densitometry analysis. Fisher's exact test was used to calculate enrichment of genes in our functional annotation analysis. A value of $p \leq 0.05$ was considered significant.

Results

Dbc1$^{-/-}$ B cells have increased proliferation and immunoglobulin production

We used a mouse model bearing a targeted deletion of *Dbc1* gene to investigate the function of DBC1 in the immune system (26). Development of lymphoid and myeloid cells is grossly normal, as populations of T, B cells, macrophages and DCs are comparable to WT littermates (Supplemental Fig. 1A). Immature, transitional, follicular, marginal zone and B1 B cell subpopulations are also normal in numbers (Supplemental Fig. 1B–G).

To study the role of DBC1 in B cells, we cultured splenic B cells isolated from WT and *Dbc1*$^{-/-}$ (KO) mice with various stimuli. *Dbc1*$^{-/-}$ B cells have enhanced proliferation compared to WT when stimulated with alternative NF-κB agonists anti-CD40 F(ab)$_2$ fragment or BAFF (29), whereas proliferation was indistinguishable from WT upon anti-BCR and lipopolysaccharide (LPS) stimulation (Fig. 1A & B). We further examined whether the increased percentage of dividing cells in *Dbc1*$^{-/-}$ B cells was due to accelerated cell cycle progression or protection from apoptosis. The increased proliferation in *Dbc1*$^{-/-}$ B cells correlated with accelerated cell cycle progression, since the number of G2/M-phase cells were increased when cultured with anti-CD40 (Fig. 1C & D). In contrast, deletion of DBC1 does not affect apoptosis, since the number of apoptotic cells in *Dbc1*$^{-/-}$ B cells measured by TUNEL staining were comparable to WT (Supplemental Fig. 2A & B). Therefore, DBC1 negatively regulates B cell proliferation, likely by suppressing cell cycle progression initiated by CD40 stimulation.

In addition to B cell proliferation, CD40 and BAFFR signaling are known to promote isotype switching in B cells (30, 31). Indeed, although IgM levels were comparable between WT and *Dbc1*$^{-/-}$, IgG1 levels in *Dbc1*$^{-/-}$ B cells were increased when cultured in IgG1-driving condition, i.e. with CD40 agonist and IL-4 (Fig. 1E). Specifically, there was an increase in the number of IgG1-producing cells, but the Mean Fluorescence Intensity (MFI) of IgG1was comparable between WT and *Dbc1*$^{-/-}$ B cells, suggesting that the number of IgG1^{+} cells in increased, but IgG1 production per cell basis is unchanged (Supplemental Fig. 2C & D). In addition, *Dbc1*$^{-/-}$ B cells showed significantly increased IgA production when stimulated with LPS plus the alternative NF-κB agonist BAFF, but not with LPS alone (Fig. 1E). Similar to IgG1, the number of IgA-producing cells is increased, whereas IgA per cell basis is comparable between WT and *Dbc1*$^{-/-}$ B cells (Supplemental Fig. 2E & F). Interestingly, when stimulated with LPS and APRIL, which signals through the classical NF-κB pathway (32), or TGFβ plus IL-5, which utilizes SMAD and Runx signaling (33),

IgA production was indistinguishable between WT and *Dbc1*$^{-/-}$ B cells (Supplemental Fig. 2E & F), suggesting that DBC1 specifically regulates alternative NF-κB signaling. These results suggest that DBC1 suppresses B cell activation downstream of specific receptors such as CD40 and BAFF receptor.

DBC1 regulates B cell activation in a cell-intrinsic manner

Since DBC1 is systemically deleted in our mouse model, we performed a bone chimera experiment to study whether DBC1 inhibits B cell activation in a cell-intrinsic manner. Bone marrow cells derived from *Dbc1*$^{-/-}$ mice were mixed at a 1:1 ratio with those from congenic B6.SJL mice, transferred into lethally irradiated B6.SJL recipients and analyzed 6 weeks after transfer. *Dbc1*$^{-/-}$ (CD45.2) lymphocytes were significantly increased in proportion compared to WT (CD45.1) lymphocytes, which is predominantly due to an increase in KO B220^{+} cells (Fig. 2A & D). To confirm this, a significantly increased percentage of B220^{+}IgM^{+} B cells were detected in the CD45.2^{+} lymphocyte population compared to the CD45.1^{+} cells (Fig. 2B & E), suggesting that DBC1 has an intrinsic suppressive function on B cell proliferation. Moreover, consistent to our in vitro B cell activation results (Fig. 1), *Dbc1*$^{-/-}$ B cells from the recipient mice showed increased proliferation and IgG1 and IgA expression upon in vitro stimulation with α-CD40 and BAFF respectively when compared to that of WT B cells (Fig. 2C, F–H). These results reveal that DBC1 has an intrinsic suppressive function in B cell proliferation and IgG1 and IgA production.

DBC1 suppression of B cell activation is independent of Sirt1

One of the best-characterized functions of DBC1 is its suppression of Sirt1, a histone deacetylase that inhibits classical NF-κB activity by interacting with RelA (34–36). To test if DBC1 regulates B cell proliferation through Sirt1, we generated a B cell-specific double knock out of DBC1 and Sirt1 (DKO), by crossing *Dbc1*$^{-/-}$ mice with mice bearing loxp-flanked Sirt1 (*Sirt1*$^{fl/fl}$) and crossing to mice with CD19 promoter-driven Cre recombinase (*CD19-Cre*). However, when stimulated with anti-CD40, DKO B cells showed a similar increase in proliferation, cell cycle progression and IgG1 production to that observed in *Dbc1*$^{-/-}$ B cells (Supplemental Fig. 3). As a control, Sirt1-null B cells responded to CD40 stimulation at similar levels to WT B cells. Thus, Sirt1 appears to be dispensable for B cell response upon CD40 stimulation, and DBC1 suppression of B cell proliferation and immunoglobulin production is independent of Sirt1. Taking the bone marrow chimera and double knock out experiments together, we conclude that DBC1 regulates B cell proliferation through a B cell-intrinsic, Sirt1-independent mechanism.

Microarray analysis reveals that proliferative genes and NF-κB target genes are upregulated in *Dbc1*$^{-/-}$ B cells

To investigate the molecular mechanism underlying DBC1 regulation of B cell function, we used a microarray approach to examine the gene expression profile of *Dbc1*$^{-/-}$ B cells. Gene expression levels in activated *Dbc1*$^{-/-}$ B cells were normalized to levels in the naïve state, and the fold change was compared to WT B cells. We identified 154 and 121 genes that were differentially upregulated and downregulated respectively by >4 fold in *Dbc1*$^{-/-}$ B cells upon CD40 activation. Functional annotation analysis further revealed that the gene

categories most significantly enriched in *Dbc1*$^{-/-}$ B cells compared to WT were involved in proliferation, mitosis and cell division (Fig. 3A). In contrast, many genes involved in inflammation were downregulated (Fig. 3A). In support of our discovery that *Dbc1* gene deletion leads to increased B cell growth upon CD40 stimulation, the significant enrichment of proliferative genes was observed under CD40, but not under BCR-stimulated conditions (Fig. 3B & C). We attempted to identify specific signaling pathways affected by the deletion of *Dbc1* gene by analyzing Response Elements (RE) present in genes differentially expressed by > 4 fold in *Dbc1*$^{-/-}$ B cells. Among the genes that were upregulated and downregulated respectively, NF-κB REs were present in the largest percentage of genes, both in upregulated and downregulated gene groups (Fig. 3D), indicating that NF-κB signaling is indeed dysregulated in *Dbc1*$^{-/-}$ B cells. Thus, these microarray results affirm our observation that DBC1 suppresses the expression of cell cycle-related genes in B cells, in particular those stimulated by CD40 and NF-κB signaling.

DBC1 selectively suppresses alternative NF-κB pathway in B cells

Our findings so far implicate that the alternative NF-κB pathway is regulated by DBC1 in B cells. Interestingly, based on our microarray data, NF-κB targets were both upregulated and downregulated in *Dbc1*$^{-/-}$ B cells, leading us to speculate that DBC1 might differentially regulate different branches of NF-κB signaling. To test if DBC1 selectively inhibits specific NF-κB members, we incubated the cell lysates of WT and *Dbc1*$^{-/-}$ B cells with a biotinylated DNA probe containing a κB-consensus sequence, and avidin-conjugated agarose. DNA-bound NF-κB members in WT and *Dbc1*$^{-/-}$ B cell lysates were then pelleted and detected by Western Blot. Indeed, we found that the DNA-binding activity of alternative NF-κB members RelB and p52 was significantly increased in activated *Dbc1*$^{-/-}$ B cells, whereas the DNA-binding levels of classical NF-κB transcription factors RelA, c-Rel and p50 were comparable to WT (Fig. 4A & B), supporting our initial speculation that DBC1 selectively suppresses alternative NF-κB signaling. In contrast, protein levels of each NF-κB member in the nuclear extracts of WT and *Dbc1*$^{-/-}$ B cells were comparable (Fig. 4A, input panels), indicating that loss of DBC1 results in increased RelB: p52 DNA-binding activity, but not its nuclear translocation.

DBC1 suppresses RelB binding at target gene promoters

To confirm that DBC1 suppresses transcriptional activity of RelB at the promoters of NF-κB-target genes, we compared RelB promoter-binding activity between WT and *Dbc1*$^{-/-}$ B cells by ChIP. We chose Birc5, cyclinB1 (CCNB1) and CDC20, which are proliferation-inducing genes differentially upregulated in *Dbc1*$^{-/-}$ B cells (Fig. 3E), and have predicted NF-κB response elements, as our ChIP gene targets. We observed minimal promoter-binding of RelB in WT B cells during the early stage of CD40 stimulation (Fig. 5A). In contrast, *Dbc1*$^{-/-}$ B cells had increased RelB binding at the promoters of all 3 selected genes at early time points of CD40 activation, even in unstimulated B cells (Fig. 5C), indicating that the loss of DBC1 leads to increased RelB promoter binding activity in B cells. RelB binding at the promoter region of *Ciap2*, a known target of both classic and alternative NF-κB (37), was also increased, confirming that RelB DNA-binding activity is increased at target gene promoters (Fig. 5A). Interestingly, the promoter-binding activity of RelB in WT B cells increases to similar levels 16 hours upon CD40 stimulation, suggesting that DBC1 is

important in suppressing alternative NF-κB activity during the early phase of B cell activation. In contrast, while an increase in RelA binding activity was detected in *Dbc1*$^{-/-}$ B cells in two of the three selected promoters, RelA DNA-binding levels were close to background in unstimulated *Dbc1*$^{-/-}$ B cells (Fig. 5B), indicating that DBC1 preferentially suppresses RelB DNA-binding activity. Thus, DBC1 likely functions as a selective transcriptional suppressor of alternative NF-κB activity in B cells upon CD40 stimulation.

Dbc1$^{-/-}$ mice have aberrant immunoglobulin production

To investigate the role of DBC1 *in vivo*, we tested the antigen-specific immune response of WT and *Dbc1*$^{-/-}$ mice. Upon immunization with NP-KLH emulsified with the complete Freund's adjuvant (CFA) and boosted with NP-KLH/IFA on day 7, the levels of NP-specific antibodies in the sera of *Dbc1*$^{-/-}$ mice were similar to those of WT mice (Fig. 6A). However, when mice were immunized with NP-KLH without adjuvant, *Dbc1*$^{-/-}$ mice showed an increased antigen-specific response, since the levels of NP-specific immunoglobulin including the IgG1, IgG2a, IgG3 and IgA, but not IgM isotypes were significantly increased in *Dbc1*$^{-/-}$ mice (Fig. 6B). As expected, when immunized with NP-KLH without adjuvant, WT mice showed minimal increase in total serum immunoglobulin. The increased serum IgG and IgA levels in *Dbc1*$^{-/-}$ mice are supported by detection of increased NP-specific IgG1- and IgA- expressing B cells in the spleen by flow cytometry (Fig. 6C & D). In addition, an increase in B220^{LO}CD138^{+} plasma cells and B220^{+}PNA^{+} Germinal Center B cells was observed in *Dbc1*$^{-/-}$ mice (Fig. 6E & F), supporting the observation of increased isotype switching and immunoglobulin production. In summary, our experiments show that DBC1 has a suppressive function in B cells *in vivo*, as deletion of DBC1 in mice results in increased antigen-specific immunoglobulin production upon NP-KLH immunization. Interestingly, the expansion of antigen-specific B cells and increased immunoglobulin production is detected only upon immunization without adjuvant, suggesting that DBC1 acts as a checkpoint regulatory protein of B cell activation, to negatively regulate unwarranted B cell activation, such as during immunization without adjuvant.

Loss of DBC1 results in higher susceptibility to EAMG in mice

Our experiments so far indicate that loss of DBC1 leads to increased alternative NF-κB gene transcription, resulting in hyperactivation of B cells, and increased proliferation and immunoglobulin production. Interestingly, when immunized with antigen without adjuvant, loss of DBC1 in mice results in increased immunoglobulin production. The increase in immunoglobulin production in *Dbc1*$^{-/-}$mice upon NP-KLH immunization prompted us to ask whether *Dbc1*$^{-/-}$ mice are more susceptible to B cell-mediated autoimmune disease. EAMG is a model of human Myasthenia Gravis, in which mice immunized with *Torpedo* Aceytlcholine receptor (tAChR) supplemented with CFA develop cross-reactive autoantibodies against murine AChR (mAChR) resulting in muscle weakness, similar to human MG patients (38, 39). We tested if *Dbc1*$^{-/-}$ mice were more prone to EAMG by immunizing with tAChR without adjuvant. After 42 days, 80% of *Dbc1*$^{-/-}$ mice developed limb muscle weakness (Fig 7A). As a control, WT littermates immunized under the same conditions did not develop disease symptoms. In agreement with the clinical score, increased plasma cell population was detected in *Dbc1*$^{-/-}$ mice 42 days after immunization

(Fig 7 B–D). Similar to NP-KLH immunization, antigen-specific IgG1 and IgA against tAChR was increased in *Dbc1*$^{-/-}$ mice sera (Fig. 7E). Most interestingly, *Dbc1*$^{-/-}$ mice sera had significantly higher levels of cross-reactive IgG1 and IgG2a against mAChR (Fig. 7F), indicating that *Dbc1*$^{-/-}$ mice are indeed more prone to autoimmunity through production of cross-reactive autoantibodies.

Based on our discovery, we propose a working model of DBC1 suppression of unwarranted B cell activation (Fig. 7G & H). DBC1 suppresses alternative NF-κB transcriptional activity, which in turn negatively regulates B cell activation, proliferation and immunoglobulin production. When DBC1 is absent in B cells, suppression of RelB:p52 is lost, allowing for gene transcription, even in suboptimal stimulation conditions, such as presence of antigen without adjuvant. The loss of DBC1 suppression of RelB:p52 results in B cell proliferation and immunoglobulin production, leading to increased immunoglobulin levels in mouse sera upon immunization, and higher susceptibility to autoimmune diseases such as EAMG. Therefore through this study, we identify DBC1 as a novel suppressor of B cell activation by inhibiting alternative NF-κB signaling pathway.

Discussion

In this study we identify a novel function of DBC1 as a negative regulator of B cell activation by suppressing alternative NF-κB transcriptional activity upon CD40 and BAFF stimulation. This conclusion is supported by the following discoveries: first, loss of DBC1 results in increased B cell proliferation as well as IgG1 and IgA production, specifically in response to CD40 and BAFFR stimulation. Second, bone marrow chimera and *Dbc1*$^{-/-}$*Sirt1* $^{fl/fl}$ *CD19-Cre* double knock out studies indicate that DBC1 suppresses B cell proliferation and immunoglobulin production in a B cell-intrinsic, Sirt1-independent manner. Third, microarray analysis reveals that DBC1 suppresses the expression of genes in cell cycle progression, most of which are NF-κB targets. Fourth, DBC1- deficiency leads to the elevated RelB:p52-binding activity on the promoters of target genes during the early stage of B cell activation upon CD40 and BAFFR stimulation. Finally, loss of DBC1 results in significantly increased levels of antigen-specific IgG and IgA in mice when immunized with antigen in the absence of adjuvant, as well as increased susceptibility to EAMG.

Accumulated evidence suggests that CD40 and BAFFR signaling are critical in regulating B cell activation (7, 40). Activation of CD40 and BAFF leads to the activation of alternative NF-κB signaling, which in turn promotes cell cycle progression, germinal center formation, isotype switching and plasma cell survival (12, 41, 42). We propose that the loss of DBC1 regulation of RelB activity results increased CD40 signaling. In support of our hypothesis, promoter-binding activity of RelB is enhanced in *Dbc1*$^{-/-}$ B cells upon early stage of CD40 stimulation. Also, *Dbc1*$^{-/-}$ B cells exhibit increased proliferation, cell cycle progression and IgG1 production upon CD40 stimulation.

We observed that DBC1 has a suppressive role on B cell proliferation upon in vitro stimulation with CD40 or BAFF. However, while *Dbc1*$^{-/-}$ mice produce increased levels of immunoglobulin when immunized with NP-KLH alone, we did not observe a significant difference in the humoral response of WT and *Dbc1*$^{-/-}$ littermates immunized with NP-KLH

and adjuvant. One possibility is that DBC1 plays a subtle regulatory role in B cells, such that its function in B cells is only evident when there is no inflammatory signal. Immunization with NP-KLH and CFA triggers activation of all immune cell types in both WT and *Dbc1*$^{-/-}$, providing a strong inflammatory signal, which occludes the regulatory role of DBC1 in B cells. In contrast, when immunized without adjuvant, it becomes apparent that DBC1 plays an important regulatory role in B cell activation, since *Dbc1*$^{-/-}$ mice aberrantly produce increased immunoglobulin, despite the lack of co-stimulation and inflammatory response in other immune cell types induced by adjuvant.

The increased immunoglobulin production in *Dbc1*$^{-/-}$ mice when immunized without adjuvant is particularly interesting, in light of the fact that protein immunization without adjuvant produces a minimal immune response, and can produce a tolerizing effect (43, 44). One of the reasons for the tolerizing effect of immunization without adjuvant is the absence of CD40 signaling (45). Conversely, increased CD40 signaling has been shown to overcome the tolerizing effects of immunization without adjuvant, at least in CD8^{+} Cytotoxic T Lymphocytes (44). Based on our observations that *Dbc1*$^{-/-}$ B cells have increased RelB:p52 activity even upon early stage of CD40 stimulation, and have enhanced response to CD40 stimulation, we propose that DBC1 functions as a suppressor of B cell activation under insufficient CD40 stimulation. As a result, loss of DBC1 leads to B cell activation and immunoglobulin production in mice even when immunized without adjuvant. Interestingly, loss of DBC1 does not enhance the full activity of RelB at the later stage of CD40 stimulation *in vitro*. Similarly, loss of DBC1 does not enhance the B cell response when immunized with protein antigen supplemented with adjuvant, indicating that DBC1 functions predominantly as a checkpoint to suppress unwanted RelB activity during early stage or under weak stimulation. Therefore, DBC1 appears to serve as a crucial checkpoint to ensure that alternative NF-κB signaling in response to CD40 is only activated when adequate stimuli is present.

Our microarray results show that loss of DBC1 results in the upregulation of a subset of NF-κB target genes, and downregulation of another subset of target genes. One possibility is that DBC1 differentially regulates the two branches of NF-κB signaling, i.e. indirectly promoting classical signaling through Sirt1 inhibition, while suppressing alternative signaling pathway through the direct inhibition of RelB:p52. As a result, while the proliferation- and differentiation- promoting effects of NF-κB are enhanced in the *Dbc1*$^{-/-}$ B cells, inflammatory cytokines and innate response proteins are downregulated. Similarly, *Dbc1*$^{-/-}$ mice have reduced inflammatory cytokine production in the liver when fed a high fat diet largely in a Sirt1-dependent manner (26). However, previous lab data using Sirt1-null mice, as well as our experiments using *Dbc1*$^{-/-}$ *Sirt1*$^{fl/fl}$ *CD19-Cre* mice indicate that Sirt1 has minimal effect on B cell function. In addition, using ChIP assays we did not detect decreased RelA DNA-binding activity in *Dbc1*$^{-/-}$ B cells, which one would expect with increased Sirt1 activity. Another possible reason for the decrease in inflammatory cytokine production is that alternative NF-κB signaling has anti-inflammatory functions in B cells. Indeed, alternative NF-κB signaling has been shown to control inflammation, at least in macrophages (46–48). Thus, our findings suggest that the suppressive function of DBC1 on B cells is an outcome of its selective inhibition of RelB activity, as opposed to an indirect

outcome of increased Sirt1 activity. In addition, DBC1 suppresses the alternative NF-kB transcription factors RelB/p52 unlikely due to the altered expression and/or transcriptional activities of other NF-kB transcription factors, because neither the protein expression levels of RelA, cRel and p50, nor their promoter binding activity in DBC1-null B cells are changed comparing to that of WT controls (Fig. 4).

The non-canonical NF-κB pathway is implicated in the regulation of B cell development, particularly marginal zone and transitional B cell populations (49–51). However, we did not detect increased populations in our mouse model (Supplemental Fig. 1). One possibility is that loss of DBC1 simultaneously regulates other cell types, which might mask the effects of increased RelB activation in B cell development. Indeed, while we did not see an increase in B cell populations in mice with germline deletion of DBC1, bone marrow chimera experiments show that *Dbc1*$^{-/-}$ B cell population is increased in the recipients 6 weeks after the adoptive transfer (Fig. 2A, B, D & E), suggesting that while DBC1 regulates B cell proliferation in a cell-intrinsic manner, loss of DBC1 in other cells types in the germline deletion mice might antagonize the B cell hyperproliferative phenotype.

We observed that the antigen-specific IgG1 and IgA are increased in *Dbc1*$^{-/-}$ mice, suggesting that DBC1 regulates isotype switching in B cells. While isotype switching in B cells is partly dependent on external cues such as T cell and DC priming (52–54), neither the activation nor differentiation of T cells was altered by *Dbc1* gene deletion in T cells (data not shown). Together with our data that isolated *Dbcl*$^{-/-}$ B cells produce more IgG1 and IgA but not IgM upon *in vitro* CD40 stimulation, our discoveries imply that DBC1 is involved in isotype switching at least in a cell-intrinsic manner. CD40L and BAFF have been widely reported to induce isotype switching, particularly these two isotypes (31, 55). The alternative NFκB signaling pathway has also been shown to be important in IgG and IgA class switching (50, 56). In addition, it is also possible that the increased production of class-switched isotypes is a result of accelerated cell cycle progression in *Dbc1*$^{-/-}$ B cells, since it has been shown that class switching is linked to cell division, with IgG1 and IgA expressed at higher frequency, whereas IgE requires more cell divisions (30). Thus, the increased IgG and IgA production in *Dbc1*$^{-/-}$ mice is probably a result of both accelerated cell cycle progression as well as increased sensitivity to BAFF or CD40L-induced isotype switching.

In summary, our study reveals that DBC1 suppresses B cell proliferation and immunoglobulin production. DBC1 suppresses B cell activation by regulating alternative NF-κB transcriptional activity at target gene promoters. As a result, *Dbc1*$^{-/-}$ B cells are hyperactivated, leading to increased immunoglobulin levels in *Dbc1*$^{-/-}$ mice upon immunization under suboptimal conditions, and increased susceptibility to EAMG. Taken together, our data reveal a novel regulatory mechanism by DBC1 on the alternative NF-κB pathway, as well a novel checkpoint function of DBC1 in B cell activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health (NIH) RO1 grants (AI079056, AI108634 and AR066634) to D.F and (DK-084055/DK/NIDDK NIH HHS/United States) to E.N.C.

References

1. Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. Annu Rev Immunol. 2003; 21:231–264. [PubMed: 12427767]
2. Crowley JE, Scholz JL, Quinn WJ 3rd, Stadanlick JE, Treml JF, Treml LS, Hao Y, Goenka R, O'Neill PJ, Matthews AH, Parsons RF, Cancro MP. Homeostatic control of B lymphocyte subsets. Immunol Res. 2008; 42:75–83. [PubMed: 18668213]
3. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol Rev. 2009; 229:152–172. [PubMed: 19426221]
4. Hostager BS, Bishop GA. CD40-Mediated Activation of the NF-kappaB2 Pathway. Frontiers in immunology. 2013; 4:376. [PubMed: 24298274]
5. Bishop GA, Hostager BS. Signaling by CD40 and its mimics in B cell activation. Immunol Res. 2001; 24:97–109. [PubMed: 11594459]
6. Zou, YR.; Diamond, B. Chapter One – Fate Determination of Mature Autoreactive B Cells. In: Frederick, WA., editor. Advances in Immunology. Academic Press; 2013. p. 1-36.
7. Meffre E, Wardemann H. B-cell tolerance checkpoints in health and autoimmunity. Current Opinion in Immunology. 2008; 20:632–638. [PubMed: 18848883]
8. Mauri C. Regulation of immunity and autoimmunity by B cells. Curr Opin Immunol. 2010; 22:761–767. [PubMed: 21087847]
9. Conti-Fine BM, Milani M, Kaminski HJ. Myasthenia gravis: past, present, and future. The Journal of Clinical Investigation. 2006; 116:2843–2854. [PubMed: 17080188]
10. Bonizzi G, Karin M. The two NF-[kappa]B activation pathways and their role in innate and adaptive immunity. Trends in Immunology. 2004; 25:280–288. [PubMed: 15145317]
11. Weih DS, Yilmaz ZB, Weih F. Essential Role of RelB in Germinal Center and Marginal Zone Formation and Proper Expression of Homing Chemokines. The Journal of Immunology. 2001; 167:1909–1919. [PubMed: 11489970]
12. Liou HC, Sha WC, Scott ML, Baltimore D. Sequential induction of NF-kappa B/Rel family proteins during B-cell terminal differentiation. Mol Cell Biol. 1994; 14:5349–5359. [PubMed: 8035813]
13. Wuerzberger-Davis SM, Chen Y, Yang DT, Kearns JD, Bates PW, Lynch C, Ladell NC, Yu M, Podd A, Zeng H, Huang TT, Wen R, Hoffmann A, Wang D, Miyamoto S. Nuclear Export of the NF-[kappa]B Inhibitor I[kappa]B[alpha] Is Required for Proper B Cell and Secondary Lymphoid Tissue Formation. Immunity. 2011; 34:188–200. [PubMed: 21333553]
14. Jin J, Xiao Y, Chang JH, Yu J, Hu H, Starr R, Brittain GC, Chang M, Cheng X, Sun SC. The kinase TBK1 controls IgA class switching by negatively regulating noncanonical NF-[kappa]B signaling. Nat Immunol. 2012; 13:1101–1109. [PubMed: 23023393]
15. Hamaguchi M, Meth JL, von Klitzing C, Wei W, Esposito D, Rodgers L, Walsh T, Welcsh P, King MC, Wigler MH. DBC2, a candidate for a tumor suppressor gene involved in breast cancer. Proceedings of the National Academy of Sciences. 2002; 99:13647–13652.
16. Koyama S, Wada-Hiraike O, Nakagawa S, Tanikawa M, Hiraike H, Miyamoto Y, Sone K, Oda K, Fukuhara H, Nakagawa K, Kato S, Yano T, Taketani Y. Repression of estrogen receptor [beta] function by putative tumor suppressor DBC1. Biochemical and Biophysical Research Communications. 2010; 392:357–362. [PubMed: 20074560]
17. Fu J, Jiang J, Li J, Wang S, Shi G, Feng Q, White E, Qin J, Wong J. Deleted in Breast Cancer 1, a Novel Androgen Receptor (AR) Coactivator That Promotes AR DNA-binding Activity. Journal of Biological Chemistry. 2009; 284:6832–6840. [PubMed: 19126541]

18. Chini CC, Escande C, Nin V, Chini EN. DBC1 (Deleted in Breast Cancer 1) modulates the stability and function of the nuclear receptor Rev-erbalpha. Biochem J. 2013; 451:453–461. [PubMed: 23398316]
19. Kim JE, Chen J, Lou Z. DBC1 is a negative regulator of SIRT1. Nature. 2008; 451:583–586. [PubMed: 18235501]
20. Zhao W, Kruse JP, Tang Y, Jung SY, Qin J, Gu W. Negative regulation of the deacetylase SIRT1 by DBC1. Nature. 2008; 451:587–590. [PubMed: 18235502]
21. Chini CCS, Escande C, Nin V, Chini EN. HDAC3 Is Negatively Regulated by the Nuclear Protein DBC1. Journal of Biological Chemistry. 2010; 285:40830–40837. [PubMed: 21030595]
22. Li Z, Chen L, Kabra N, Wang C, Fang J, Chen J. Inhibition of SUV39H1 Methyltransferase Activity by DBC1. Journal of Biological Chemistry. 2009; 284:10361–10366. [PubMed: 19218236]
23. Zhang J, Lee SM, Shannon S, Gao B, Chen W, Chen A, Divekar R, McBurney MW, Braley-Mullen H, Zaghouani H, Fang D. The type III histone deacetylase Sirt1 is essential for maintenance of T cell tolerance in mice. J Clin Invest. 2009; 119:3048–3058. [PubMed: 19729833]
24. Zhang R, Chen HZ, Liu JJ, Jia YY, Zhang ZQ, Yang RF, Zhang Y, Xu J, Wei YS, Liu DP, Liang CC. SIRT1 suppresses activator protein-1 transcriptional activity and cyclooxygenase-2 expression in macrophages. J Biol Chem. 2010; 285:7097–7110. [PubMed: 20042607]
25. Sequeira J, Boily G, Bazinet S, Saliba S, He X, Jardine K, Kennedy C, Staines W, Rousseaux C, Mueller R, McBurney MW. sirt1-null mice develop an autoimmune-like condition. Experimental Cell Research. 2008; 314:3069–3074. [PubMed: 18687325]
26. Escande C, Chini CC, Nin V, Dykhouse KM, Novak CM, Levine J, van Deursen J, Gores GJ, Chen J, Lou Z, Chini EN. Deleted in breast cancer-1 regulates SIRT1 activity and contributes to high-fat diet-induced liver steatosis in mice. J Clin Invest. 2010; 120:545–558. [PubMed: 20071779]
27. Hiraike H, Wada-Hiraike O, Nakagawa S, Koyama S, Miyamoto Y, Sone K, Tanikawa M, Tsuruga T, Nagasaka K, Matsumoto Y, Oda K, Shoji K, Fukuhara H, Saji S, Nakagawa K, Kato S, Yano T, Taketani Y. Identification of DBC1 as a transcriptional repressor for BRCA1. Br J Cancer. 2010; 102:1061–1067. [PubMed: 20160719]
28. Sheng JR, Li L, Ganesh BB, Vasu C, Prabhakar BS, Meriggioli MN. Suppression of Experimental Autoimmune Myasthenia Gravis by Granulocyte-Macrophage Colony-Stimulating Factor Is Associated with an Expansion of FoxP3+ Regulatory T Cells. The Journal of Immunology. 2006; 177:5296–5306. [PubMed: 17015715]
29. Berberich I, Shu GL, Clark EA. Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B. J Immunol. 1994; 153:4357–4366. [PubMed: 7525701]
30. Hasbold J, Lyons AB, Kehry MR, Hodgkin PD. Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. European Journal of Immunology. 1998; 28:1040–1051. [PubMed: 9541600]
31. Castigli E, Wilson SA, Scott S, Dedeoglu F, Xu S, Lam KP, Bram RJ, Jabara H, Geha RS. TACI and BAFF-R mediate isotype switching in B cells. The Journal of Experimental Medicine. 2005; 201:35–39. [PubMed: 15630136]
32. He B, Santamaria R, Xu W, Cols M, Chen K, Puga I, Shan M, Xiong H, Bussel JB, Chiu A, Puel A, Reichenbach J, Marodi L, Doffinger R, Vasconcelos J, Issekutz A, Krause J, Davies G, Li X, Grimbacher B, Plebani A, Meffre E, Picard C, Cunningham-Rundles C, Casanova JL, Cerutti A. The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. Nat Immunol. 2010; 11:836–845. [PubMed: 20676093]
33. Cerutti A. The regulation of IgA class switching. Nat Rev Immunol. 2008; 8:421–434. [PubMed: 18483500]
34. Li X. Act1 modulates autoimmunity through its dual functions in CD40L/BAFF and IL-17 signaling. Cytokine. 2008; 41:105–113. [PubMed: 18061473]
35. Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW. Modulation of NF-[kappa]B-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. 2004; 23:2369–2380. [PubMed: 15152190]

36. Yang SR, Wright J, Bauter M, Seweryniak K, Kode A, Rahman I. Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-κB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging. American Journal of Physiology – Lung Cellular and Molecular Physiology. 2007; 292:L567–L576. [PubMed: 17041012]
37. Hong SY, Yoon WH, Park JH, Kang SG, Ahn JH, Lee TH. Involvement of Two NF-κB Binding Elements in Tumor Necrosis Factor α-, CD40-, and Epstein-Barr Virus Latent Membrane Protein 1-mediated Induction of the Cellular Inhibitor of Apoptosis Protein 2 Gene. Journal of Biological Chemistry. 2000; 275:18022–18028. [PubMed: 10751398]
38. Huijbers MG, Lipka AF, Plomp JJ, Niks EH, van der Maarel SM, Verschuuren JJ. Pathogenic immune mechanisms at the neuromuscular synapse: the role of specific antibody-binding epitopes in myasthenia gravis. Journal of Internal Medicine. 2014; 275:12–26. [PubMed: 24215230]
39. Fuchs S, Aricha R, Reuveni D, Souroujon MC. Experimental Autoimmune Myasthenia Gravis (EAMG): From immunochemical characterization to therapeutic approaches. Journal of Autoimmunity.
40. Thien M, Phan TG, Gardam S, Amesbury M, Basten A, Mackay F, Brink R. Excess BAFF Rescues Self-Reactive B Cells from Peripheral Deletion and Allows Them to Enter Forbidden Follicular and Marginal Zone Niches. Immunity. 2004; 20:785–798. [PubMed: 15189742]
41. Stewart R, Wei W, Challa A, Armitage RJ, Arrand JR, Rowe M, Young LS, Eliopoulos A, Gordon J. CD154 Tone Sets the Signaling Pathways and Transcriptome Generated in Model CD40-Pluricompetent L3055 Burkitt's Lymphoma Cells. The Journal of Immunology. 2007; 179:2705–2712. [PubMed: 17709483]
42. Alderson MR, Armitage RJ, Tough TW, Strockbine L, Fanslow WC, Spriggs MK. CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. J Exp Med. 1993; 178:669–674. [PubMed: 7688031]
43. Ahlstedt S, Björkstén B. Specific Antibody Responses in Rats and Mice after Daily Immunization without Adjuvant. International Archives of Allergy and Immunology. 1983; 71:293–299.
44. Diehl L, den Boer AT, Schoenberger SP, van der Voort EIH, Schumacher TNM, Melief CJM, Offringa R, Toes REM. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. Nat Med. 1999; 5:774–779. [PubMed: 10395322]
45. Suhlmann JE, Foy TM, Aruffo A, Crassi KM, Ledbetter JA, Green WR, Xu JC, Shultz LD, Roopeslan D, Flavell RA, Fast L, Noelle RJ, Durie FH. In the absence of a CD40 signal, B cells are tolerogenic. Immunity. 1995; 2:645–653. [PubMed: 7540943]
46. Lawrence T, Bebien M, Liu GY, Nizet V, Karin M. IKKα limits macrophage NF-κB activation and contributes to the resolution of inflammation. nature. 2005; 434:1138–1143. [PubMed: 15858576]
47. McMillan DH, Baglole CJ, Thatcher TH, Maggirwar S, Sime PJ, Phipps RP. Lung-Targeted Overexpression of the NF-κB Member RelB Inhibits Cigarette Smoke–Induced Inflammation. The American journal of pathology. 2011; 179:125–133. [PubMed: 21703398]
48. Weih F, Carrasco D, Durham SK, Barton DS, Rizzo CA, Ryseck RP, Lira SA, Bravo R. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-κB/Rel family. Cell. 1995; 80:331–340. [PubMed: 7834753]
49. Guo F, Tänzer S, Busslinger M, Weih F. Lack of nuclear factor-κB2/p100 causes a RelB-dependent block in early B lymphopoiesis. Blood. 2008; 112:551–559. [PubMed: 18505785]
50. Franzoso G, Carlson L, Poljak L, Shores EW, Epstein S, Leonardi A, Grinberg A, Tran T, Scharton-Kersten T, Anver M, Love P, Brown K, Siebenlist U. Mice Deficient in Nuclear Factor (NF)-κB/p52 Present with Defects in Humoral Responses, Germinal Center Reactions, and Splenic Microarchitecture. The Journal of Experimental Medicine. 1998; 187:147–159. [PubMed: 9432973]
51. Guo F, Weih D, Meier E, Weih F. Constitutive alternative NF-κB signaling promotes marginal zone B-cell development but disrupts the marginal sinus and induces HEV-like structures in the spleen. Blood. 2007; 110:2381–2389. [PubMed: 17620454]

52. Gerloni M, Lo D, Zanetti M. DNA immunization in relB-deficient mice discloses a role for dendritic cells in IgM → IgG1 switch in vivo. European Journal of Immunology. 1998; 28:516–524. [PubMed: 9521061]

53. Gascan H, Gauchat JF, Roncarolo MG, Yssel H, Spits H, de Vries JE. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4+ T cell clones. The Journal of Experimental Medicine. 1991; 173:747–750. [PubMed: 1997653]

54. Coffman RL, Seymour BW, Lebman DA, Hiraki DD, Christiansen JA, Shrader B, Cherwinski HM, Savelkoul HF, Finkelman FD, Bond MW, et al. The role of helper T cell products in mouse B cell differentiation and isotype regulation. Immunol Rev. 1988; 102:5–28. [PubMed: 2966762]

55. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, Cerutti A. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol. 2002; 3:822–829. [PubMed: 12154359]

56. Lin SC, Wortis HH, Stavnezer J. The Ability of CD40L, but Not Lipopolysaccharide, To Initiate Immunoglobulin Switching to Immunoglobulin G1 Is Explained by Differential Induction of NF-κB/Rel Proteins. Molecular and Cellular Biology. 1998; 18:5523–5532. [PubMed: 9710636]

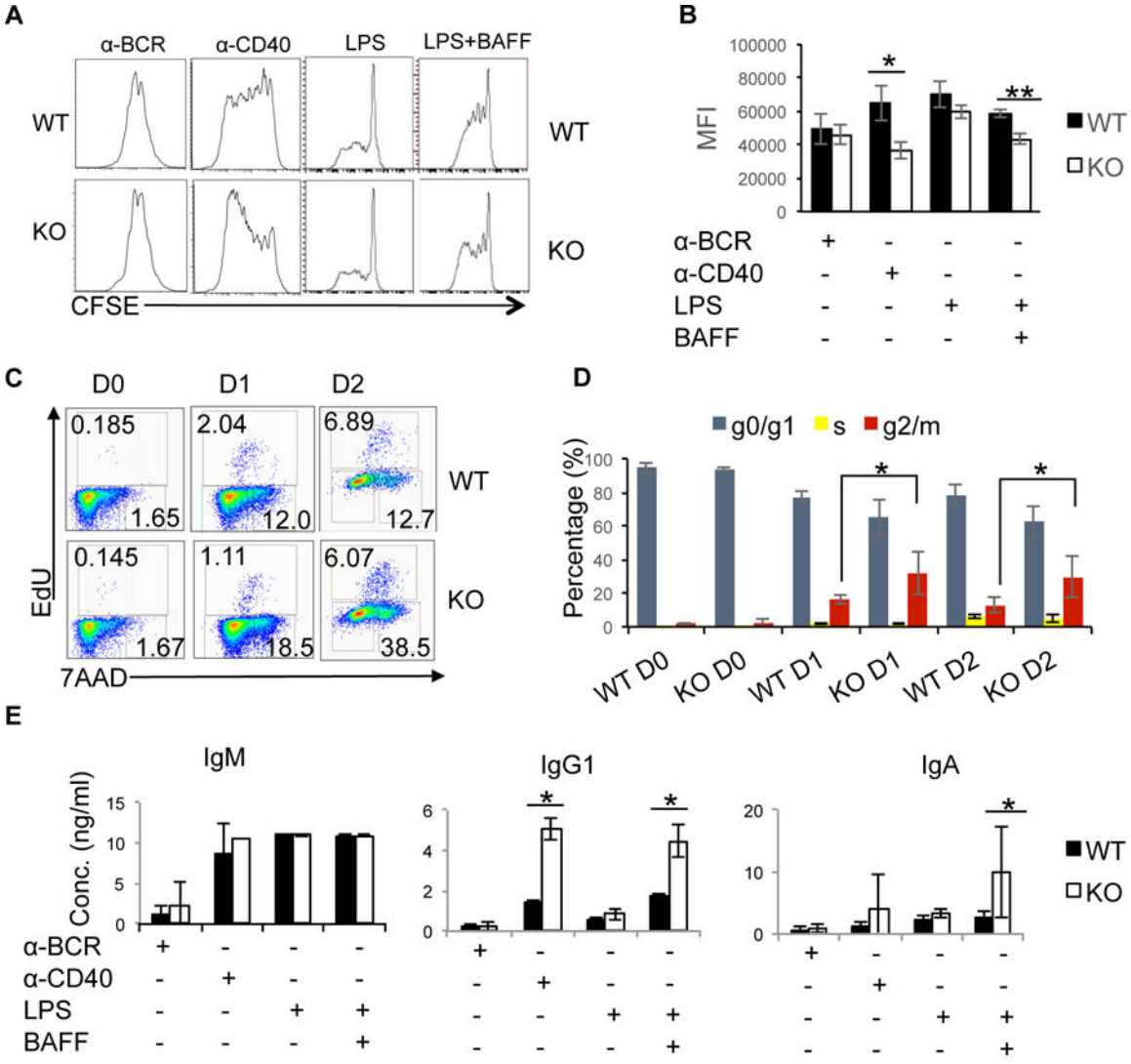

Figure 1. *Dbc1*$^{-/-}$ B cells have increased cell division and immunoglobulin production

(A) Splenic B cells were isolated from WT and *Dbc1*$^{-/-}$ mice, and cultured with: α-BCR, α-CD40 plus IL4, LPS, and LPS plus BAFF. Proliferation was measured after 5 days by CFSE dilution. **(B)** Proliferation measured by reduction in Mean Fluorescence Intensity (MFI) when cultured with indicated stimuli in (A). **(C)** Cell cycle progression of WT and KO B cells upon CD40 stimulation measured by 5′-ethynul-2′-deoxyuridine (EdU) and 7-AAD. Percentage of cells at S and G2/M- phase are shown at top left and bottom right. **(D)** Percentages of G0/G1, S, and G2/M- phase cells quantified from experiment in (C). **(E)** IgM, IgG1 and IgA levels in culture supernatant from (A) were measured by ELISA. Error bars represent SEM. A-D, N=7 independent experiments. E, N=5, *p<0.05, **p<0.01 ***p<0.001.

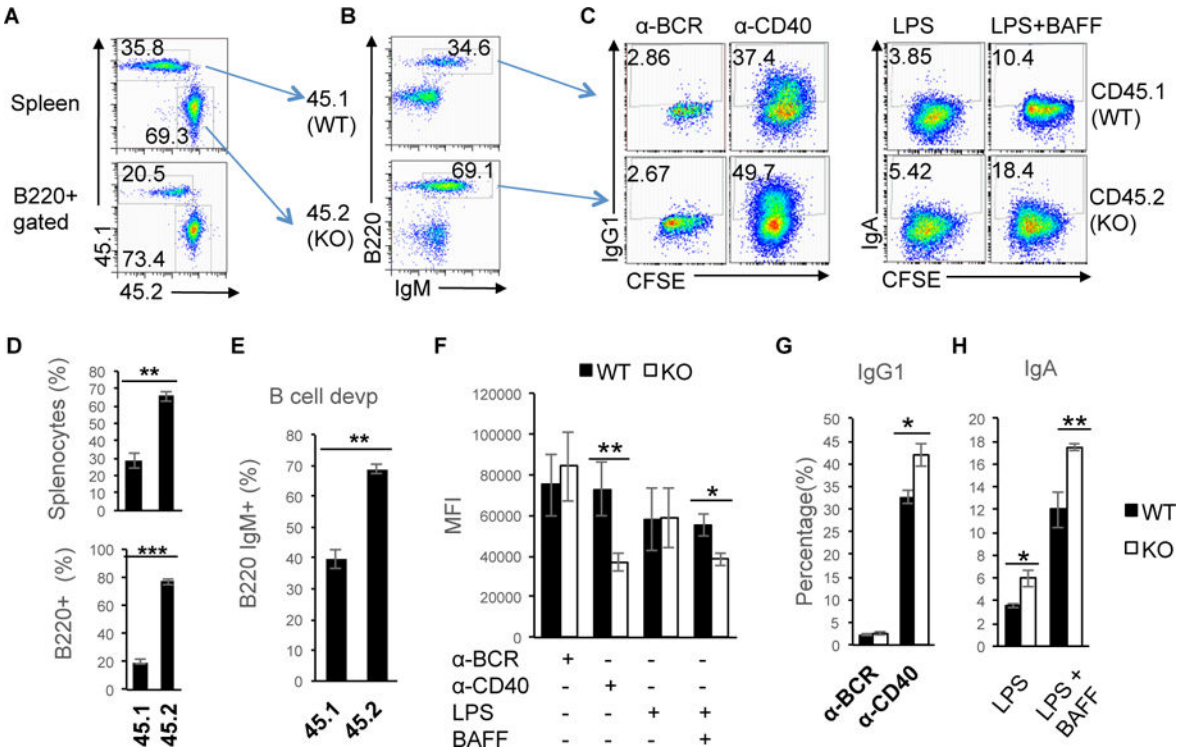

Figure 2. DBC1 regulates B cell proliferation and immunoglobulin production in a B cell-intrinsic manner

Bone marrow from congenic B6.SJL mice (CD45.1) and *Dbc1*$^{-/-}$ mice (CD45.2) were adoptively transferred into recipient B6.SJL mice. Reconstituted splenocytes were isolated 6–7 weeks after transfer, and analyzed for lymphocyte and B220^{+} cell percentages in **(A)**. **(B)** Percentages of B220^{+}IgM^{+} mature B cells from gated CD45.1 (WT) and CD45.2 (KO) populations. **(C)** Isolated CD45.1 (WT) and CD45.2 (KO) B cells were co-cultured with the indicated stimuli. Proliferation, IgG1 and IgA expression of WT and KO B cells were measured by CFSE and FACS. **(D)** Mean percentages of CD45.1 (WT)and CD45.2 (KO) cells based on total splenocytes (top) and B220+ gated cells (bottom). **(E)** Mean percentages of B220^{+}IgM^{+} cells based on gated CD45.1 (WT)and CD45.2 (KO) cells. **(F)** Proliferation as measured by reduction in MFI from (C). (**G)** Percentages of IgG1 – expressing cells from WT and KO populations as measured in (C). **(H)** Percentages of IgA-expressing cells from WT and KO populations as measured in (C). Error bars represent SEM. N=5. * P<0.05, **P<0.01, ***P<0.001.

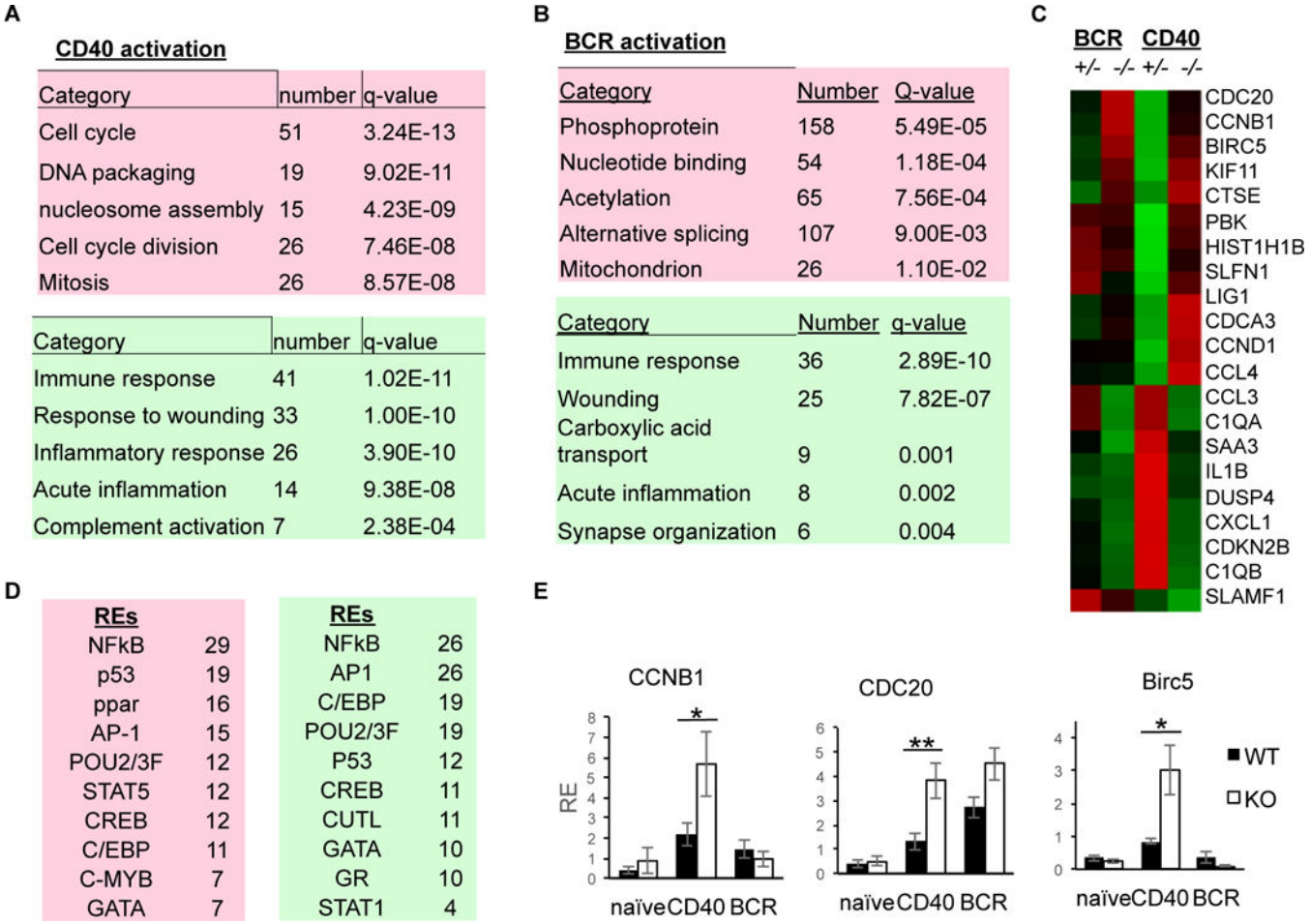

Figure 3. Microarray analysis of the gene expression profiles in *Dbc1*$^{-/-}$ B cells

Genes differentially upregulated (left) or downregulated (right) by > 4 fold in *Dbc1*$^{-/-}$ B cells upon CD40 stimulation were subjected to functional annotation analysis **(A)** Gene categories with highest enrichment from upregulated (top) or downregulated genes (bottom) are listed. **(B)** Gene categories that are enriched in upregulated (top) and downregulated (bottom) genes upon BCR stimulation. **(C)** Heat map of relevant differentially expressed genes upon BCR and CD40 stimulation. **(D)** Response Elements were identified for genes differentially regulated > 4 fold, and tallied (left: upregulated; right: downregulated). **(E)** Real-Time PCR (qPCR) was performed for 3 relevant proliferative genes CCNB1, CDC20 and Birc5. Error bars represent SEM. N=6 from 3 independent experiments. *P<0.05, **P<0.01.

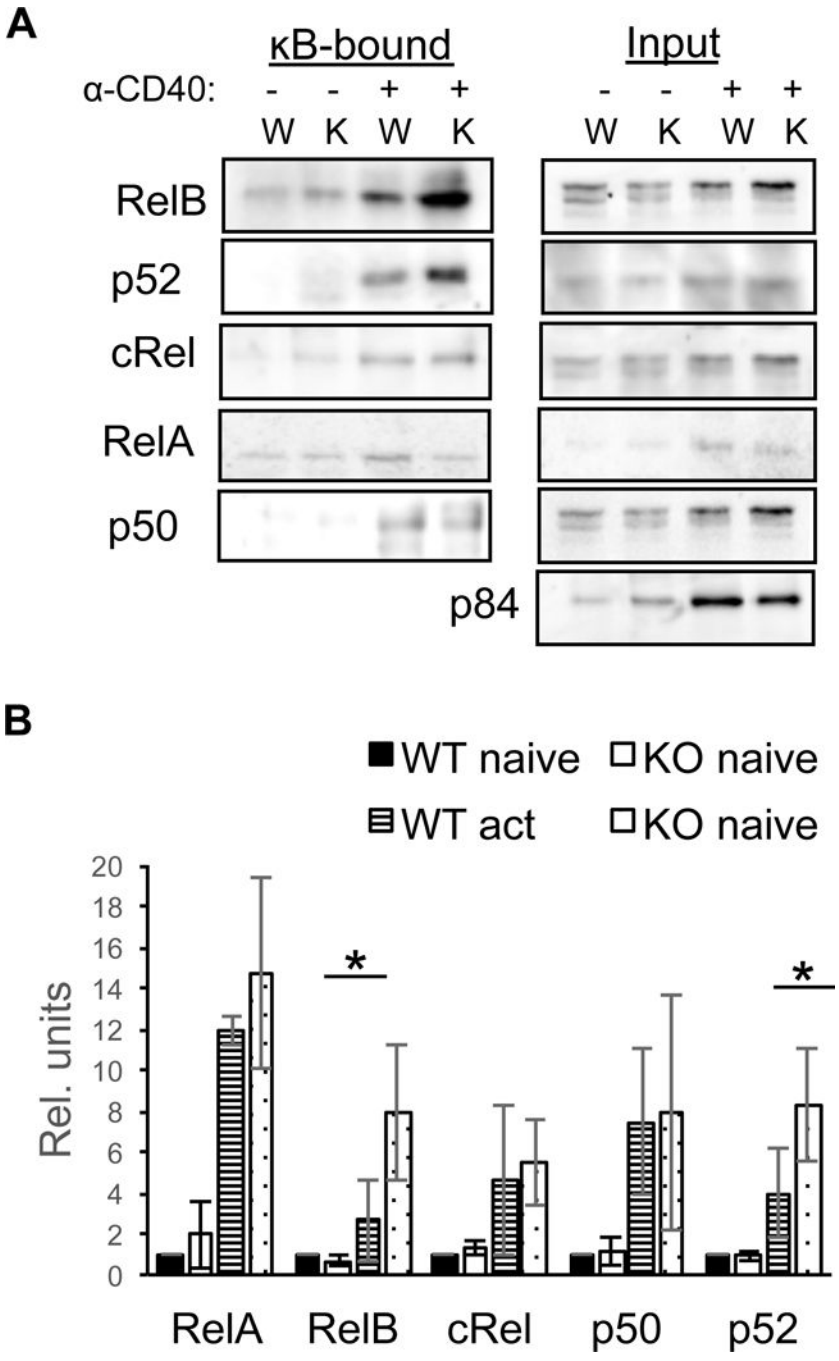

Figure 4. DBC1 selectively suppresses alternative NF-κB pathway in B cells

(A) Primary B cells were cultured with α-CD40 and IL-4 overnight. Cell lysates from naïve and activated WT and *Dbc1*$^{-/-}$ B cells were then incubated with biotinylated- DNA probe containing the κb-consensus sequence, and avidin-conjugated agarose beads. DNA-bound NF-κB members in naïve and CD40-activated WT and *Dbc1*$^{-/-}$ B cells were then pelleted, and detected by Western Blot. 10% of the nuclear extract not used in the binding assay was used as loading control. **(B)** Densitometry analysis of (A) for DNA-bound NF-κB members,

normalized to naïve WT. Error bars represent SEM. N=5 independent experiments. *=P<0.05.

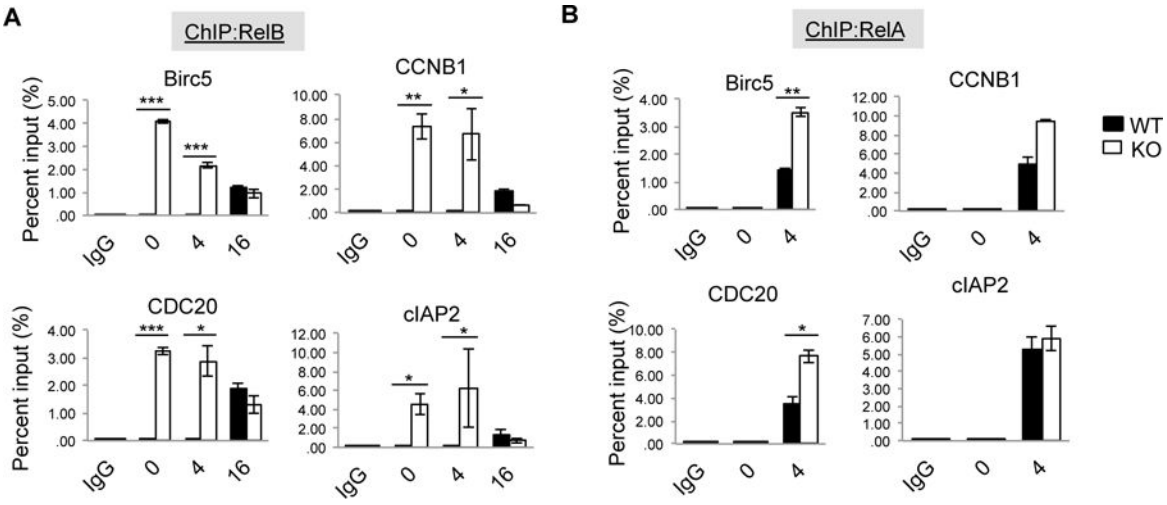

Figure 5. DBC1 suppresses RelB binding at target gene promoters

(A) WT and *Dbc1*$^{-/-}$ (KO) B cells were activated with α-CD40 for the indicated times, and RelB DNA-binding was assayed by ChIP. The promoter regions of Birc5, CCNB1, and CDC20 were quantified by qPCR. cIAP2 promoter region serves as positive control. Normal rabbit IgG was used as a negative control. **(B)** WT and KO B cells were activated as in (A), and ChIP was performed using α-RelA antibody. Error bars represent SEM. N=5 independent experiments. *=P<0.05.

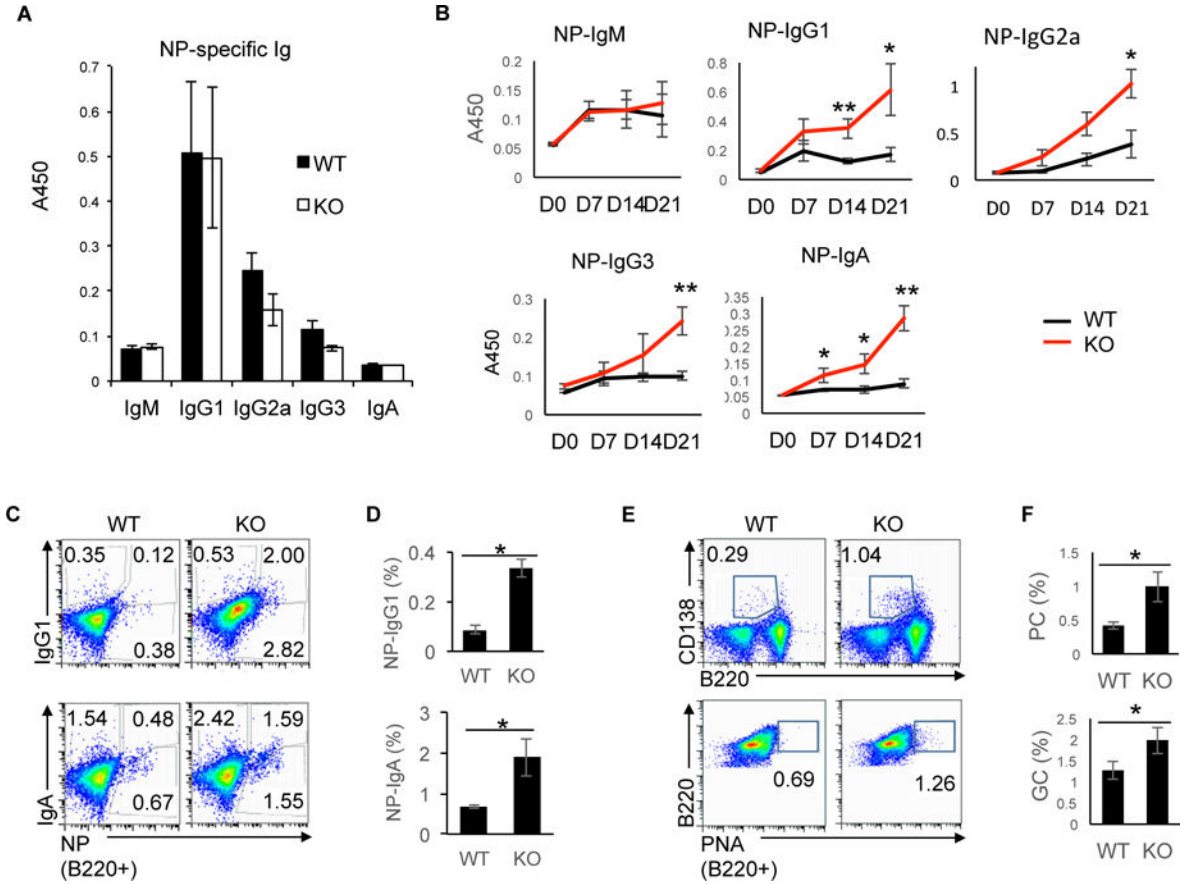

Figure 6. *Dbc1*$^{-/-}$ mice have aberrant immunoglobulin production

(A) WT and KO littermates were immunized with NP-KLH emulsified in Complete Freund's Adjuvant. NP-specific serum immunoglobulins were measured on day 14 by ELISA. **(B–F)** WT and *Dbc1*$^{-/-}$ littermates were immunized with NP-KLH in the absence of adjuvant. **(B)** NP-specific immunoglobulin of the indicated isotypes measured by ELISA. **(C)** Lymphocytes were gated on B220^{+} cells, and further analyzed for NP-binding IgG1^{+} (top) and IgA^{+} (bottom) cells by flow cytometry. **(D)** Average number of NP-binding IgG1^{+} and NP-binding IgA^{+} B cells as measured in (C). **(E)** Splenic plasma cells (CD138^{+}B220^{lo}) and germinal center B cells (B220^{+}PNA^{+}) in immunized WT and KO mice. **(F)** Mean percentages splenic plasma cells and germinal center B cells quantified from (E). Error bars represent SEM. N=6 mice from 2 independent experiments, * P<0.05, **P<0.01, ***P<0.005.

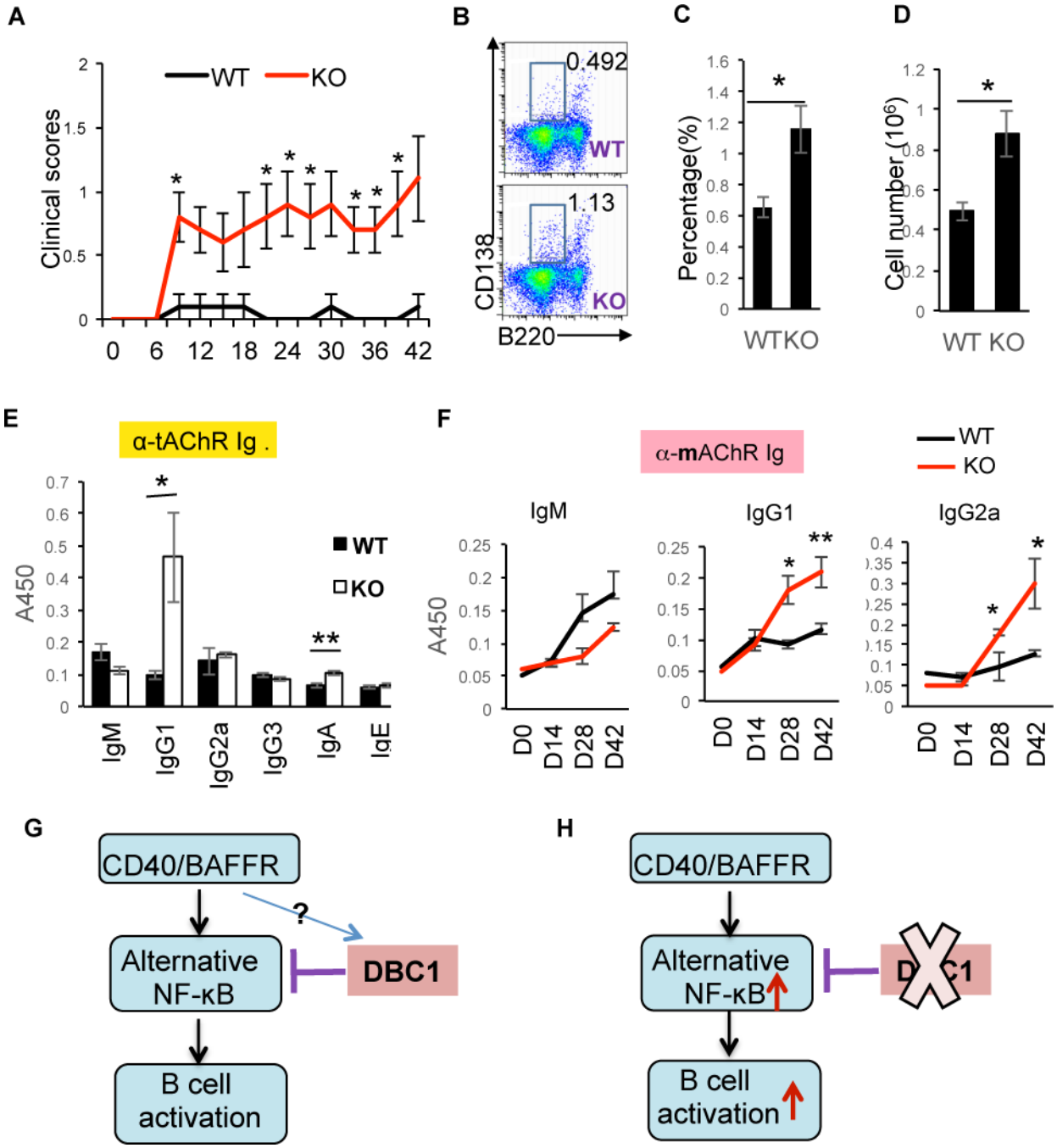

Figure 7. *Dbc1*$^{-/-}$ mice have increased clinical score and production of cross-reactive immunoglobulin in Experimental Autoimmune Myasthenia Gravis

WT and *Dbc1*$^{-/-}$ littermates were immunized with 100μg tAChR and muscle weakness was evaluated as described in the Methods section. (A) Mice were evaluated for muscle strength every 3 days as described in the Methods section, and mean clinical score of each group reported. (B) Splenocytes were isolated on day 42 and analyzed for plasma cells. (C) Percentage and (D) numbers of plasma cells from (B). (E) Antigen-specific response of WT and KO detected by measuring antibodies against tAChR. (F) Cross-reactive

immunoglobulin against murine AChR (mAChR) measured by ELISA. Error bars represent SEM. N=5, *=P<0.05, **=P<0.01. (G) DBC1 suppresses alternative NF-κB transcriptional activity, negatively regulating B cell activation and immunoglobulin production. (H) When DBC1 is absent in B cells, loss of alternative NF-κB suppression results in increased B cell activation, leading to increased B cell proliferation, and increased immunoglobulin levels in mice. Error bars represent SEM. N=6 from 2 independent experiments.