Histone Deacetylase Inhibitors Upregulate B Cell microRNAs That Silence AID and Blimp-1 Expression for Epigenetic Modulation of Antibody and Autoantibody Responses

Clayton A. White, Egest J. Pone, Tonika Lam, Connie Tat, Ken L. Hayama, Guideng Li, Hong Zan and Paolo Casali

*J Immunol* published online 12 November 2014
http://www.jimmunol.org/content/early/2014/11/11/jimmunol.1401702
Histone Deacetylase Inhibitors Upregulate B Cell microRNAs That Silence AID and Blimp-1 Expression for Epigenetic Modulation of Antibody and Autoantibody Responses

Clayton A. White,*‡,1,2 Egest J. Pone, ‡ Tonika Lam,,*‡ Connie Tat,*‡ Ken L. Hayama,‡ Guideng Li,*‡,3 Hong Zan,*‡,1 and Paolo Casali*‡

Class-switch DNA recombination (CSR) and somatic hypermutation (SHM), which require activation-induced cytidine deaminase (AID), and plasma cell differentiation, which requires B lymphocyte–induced maturation protein-1 (Blimp-1), are critical for the generation of class-switched and hypermutated (mature) Ab and autoantibody responses. We show that histone deacetylase inhibitors valproic acid and butyrate dampened AICDA/Aicda (AID) and PRDM1/Prdm1 (Blimp-1) mRNAs by upregulating miR-155, miR-181b, and miR-361 to silence AICDA/Aicda, and miR-23b, miR-30a, and miR-125b to silence PRDM1/Prdm1, in human and mouse B cells. This led to downregulation of AID, Blimp-1, and X-box binding protein 1, thereby inhibiting CSR, SHM, and plasma cell differentiation without altering B cell viability or proliferation. The selectivity of histone deacetylase inhibitor–mediated silencing of AICDA/Aicda and PRDM1/Prdm1 was emphasized by unchanged expression of HoxC4 and Irf4 (important inducers/modulators of AICDA/Aicda), Revl and Ung (central elements for CSR/SHM), and Bcl6, Bach2, or Pax5 (repressors of PRDM1/Prdm1 expression), as well as unchanged expression of miR-19a/b, miR-20a, and miR-25, which are not known to regulate AICDA/Aicda or PRDM1/Prdm1. Through these B cells–intrinsic epigenetic mechanisms, valproic acid blunted class-switched and hypermutated T-dependent and T-independent Ab responses in C57BL/6 mice. In addition, it decreased class-switched and hypermutated autoantibodies, ameliorated disease, and extended survival in lupus MRL/Fas(+/−) mice. Our findings outline epigenetic mechanisms that modulate expression of an enzyme (AID) and transcription factors (Blimp-1 and X-box binding protein 1) that are critical to B cell differentiation processes that underpin Ab and autoantibody responses. They also provide therapeutic proof-of-principle in autoantibody-mediated autoimmune disease.

Received for publication July 9, 2014. Accepted for publication October 19, 2014.

*Department of Microbiology and Immunology, University of Texas School of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229; and ‡Institute for Immunology, University of California, Irvine, CA 92697

1Current address: Allergan, Irvine, CA.

2Current address: California Institute of Technology, Pasadena, CA.

Address correspondence and reprint requests to Dr. Paolo Casali, Department of Microbiology and Immunology, University of Texas School of Medicine, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229. E-mail address: pcasali@uthscsa.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; AID, activation-induced cytidine deaminase; ANA, auto-antibody Ab; Blimp-1, B lymphocyte–induced maturation protein-1; Cd4, constant H chain; CD3, CD3 receptor; CSR, class switch DNA recombination; FBS-RPMI, RPMI 1640 medium with 10% FBS, 50 μg/mL gentamicin, and 1% antibiotic-antimycotic mixture; GC, germinal center; HDAC, histone deacetylase; HDAC inhibitor; IL, interleukin (human); KIR, K ligand; LPS, lipopolysaccharide; LTR, long terminal repeat; MHC, major histocompatibility complex; MRL, MRL-Fas(+/−) mice; miRNA, microRNA; mut, mutant; PNA, peanut agglutinin; qPCR, quantitative PCR; qRT-qPCR, quantitative RT-PCR; R2U, relative unit; SCF, stem cell factor; SHM, somatic hypermutation; TSA, trichostatin A; UTR, untranslated region; VPA, valproic acid; Xbp-1, X-box–binding protein 1.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00.
stimuli (e.g., IL-4, TGF-β, or IFN-γ), which induce selected intervening region (IgH)–S-Cγ4 germline transcription, thereby directing CSR to specific isotypes (1, 15). It is then downregulated in memory B cells and plasma cells to preserve the specificity, affinity, and isotype of the expressed BCR and Ab (1, 2). Terminal plasma cell differentiation is critically dependent on the transcriptional repressor Blimp-1. This extinguishes the proliferative mature B cell gene-expression program and drives the expression of X-box–binding protein 1 (Xbp-1), which induces secretory pathway genes for Ig secretion (4, 16, 17).

As we contended, an additional and critical level of regulation of AID expression would occur through epigenetic modifications and factors (2, 3, 18). As we also contended, epigenetic modifications and factors, including histone posttranslational modifications, DNA methylation, and microRNAs (miRNAs), interact with genetic programs to regulate B cell CSR, SHM, and plasma cell differentiation, thereby informing the Ab response (3). Accordingly, we suggested that, in addition to DNA methylation of the Aicda promoter (19) and histone acetylation of the Aicda locus (20), selected miRNAs provide a more important mechanism of modulation of AID expression (2, 3, 18). miRNAs likely play important roles in B cell development and peripheral differentiation, as well as T cell stage-specific differentiation and autoimmunity (18, 21–26).

Some miRNAs, including miR-155, miR-181b, and miR-361, would negatively regulate AID expression (2, 27–30), whereas miR-30a (31) and miR-125b (32, 33) would negatively regulate Blimp-1 expression. These miRNAs bind to evolutionarily conserved miRNA target sites in the 3′ untranslated region (UTR) of Aicda and Prdm1 miRNAs and cause degradation of the mRNA transcripts and/or inhibit their translation.

Histone deacetylase (HDAC) inhibitors (HDIs) were shown to alter gene expression by altering chromatin accessibility (34–37). In immune cells, these epigenetic modifiers exert modulatory effects, even at moderate concentrations. By using two well-characterized short-chain fatty acid (SCFA) HDIs, valproic acid (VPA) or sodium valproate (VPA) (38) and sodium butyrate (butyrate) (39), we tested the hypothesis that HDIs regulate intrinsic B cell functions that are critical in shaping effective Ab and autoantibody responses. VPA is a U.S. Food and Drug Administration–approved drug, which, as marketed under different brand names, is widely used as an anticonvulsant and a mood stabilizer. It selectively inhibits class I HDACs, particularly HDAC1 and HDAC2, and, less effectively, other members of class I and class IIa HDACs, of the four HDAC classes identified in mammals (38, 40). Butyrate is a major metabolite in the digestive tract, arising from bacterial fermentation of dietary fibers (41, 42), and it is widely available as a dietary supplement. Butyrate modulates gene expression by selectively inhibiting HDAC1, and, less effectively, other members of class I and class IIa HDACs (39).

We addressed the ability of VPA and butyrate to modulate AID and Blimp-1 expression, CSR, SHM, and plasma cell differentiation in human and mouse B cells in vivo and in vitro. In addition, we analyzed the role of HDIs as epigenetic modifiers of selected B cell miRNAs that silence Aicda and Prdm1 in Ab and autoantibody responses. Finally, we evaluated the impact of VPA on NP-CGG and NP-LPS class-switched and hypermutated Ab responses in normal mice, as well as on the autoantibody response in lupus-prone MRL/Fas+/− mice. Our findings outline important modulatory functions of epigenetic regulation of AID and Blimp-1 expression, unveil new mechanisms of modulating T-dependent and T-independent Ab responses, and provide a proof-of-principle therapeutic study in autoantibody-mediated autoimmunity.

**Materials and Methods**

**HDAC inhibitors**

For in vitro studies, VPA sodium salt (Sigma-Aldrich) was dissolved in drinking water at 0.8% w/v. This yields a stable VPA serum level (400–600 μM) in mice, comparable to the serum concentration in humans under long-term VPA treatment (300–900 μM) (43, 44). Drinking water containing VPA at the above concentration (HDI water) was received at an i.p. injection of 100 μg NP-CGG (Biosearch Technologies) in alum (Imject Alum; Pierce). Eight of the sixteen mice were on HDI water ad libitum, starting the day before and continuing throughout the duration of the experiment. The remaining eight mice were given untreated drinking water. Five mice drinking HDI water and five mice drinking untreated water were sacrificed at day 10; the remaining six mice were given a “booster” i.p. injection of 1 mg (500 μL) NP-LPS (Biosearch Technologies) in PBS. Four of these mice were on HDI water and four received untreated water throughout the duration of the experiment. All eight mice were given a “booster” injection of 25 μg NP-LPS (Biosearch Technologies) in PBS. Five of these mice were sacrificed at day 28. The Institutional Animal Care and Use Committees of the University of California, Irvine and the UT Health Science Center, San Antonio approved all animal protocols.

**Mouse B cells, CSR, and plasma cell differentiation**

Naive IgD+ B cells were isolated from 8- to 12-wk-old C57BL/6 mice, as described (9). B cells were resuspended in RPMI 1640 medium with 10% FBS, 50 mM 2-ME, and 1× antibiotic-antimycotic mixture (15240-062; Invitrogen) (RPMI) at 37°C in 48-well plates and stimulated with LPS (5 μg/ml) from Escherichia coli (055:B5; Sigma-Aldrich) for CSR to IgG1 (3 μg/ml) or CD154 (1 U/ml; obtained from melanoma fragments of baculovirus-infected SK2 insect cells (45)) + IL-4 (5 ng/ml; R&D Systems) for CSR to IgG1/IgE and plasma cell differentiation; or LPS (3 μg/ml) or CD154 (1 U/ml) + TGF-β (2 ng/ml; R&D Systems), IL-4 (5 μg/ml), IL-5 (3 ng/ml; R&D Systems), and anti-IgD dextran-Ab (Fina Biosolutions) for CSR to IgA. Nil, VPA [125–1000 μM], or CD154 (10 U/ml) + IL-21 (50 ng/ml; R&D Systems) or CD154 (10 U/ml), IL-4 (20 ng/ml; R&D Systems) and IL-21 (50 ng/ml) was added to cultures, and cells or supernatants were collected at various times.

**Human B cells, CSR, and plasma cell differentiation**

Human naive IgD+ B cells (>95% pure) were purified by negative selection, using the EasySep Human Naive B Cell Enrichment Kit (19254; STEMCELL Technologies), from healthy donor PBMCs, following the manufacturer’s instructions. Naive B cells were then cultured in FBS-RPMI and stimulated with CD154 (10 U/ml), IL-4 (20 ng/ml; R&D Systems) and IL-21 (50 ng/ml; R&D Systems) or CD154 (10 U/ml), IL-21 (50 ng/ml), and TGF-β (0.5 ng/ml) in the presence of nil, VPA, or butyrate for 120 h. B cells were then stained with 7-aminoactinomycin D (7-AAD), FITC–anti-IgM mAb (F5384; Sigma-Aldrich), PE–anti-CD19 mAb (HIB19; Biology), and alllophycocyanin–anti-IgG mAb (G18-145; BD Biosciences) or CD154 (1 U/ml), IL-4 (20 ng/ml; R&D Systems), IL-21 (50 ng/ml), IL-5 (3 ng/ml; R&D Systems), and anti-IgD dextran-Ab (Fina Biosolutions) for CSR to IgA. Nil, VPA [125–1000 μM]; doses that were similar to or below serum concentrations of VPA-treated mice (46), or butyrate (125–1000 μM) was added to cultures, and cells or supernatants were collected at various times.

**Flow cytometry**

For surface staining, B cells were reacted with PE–anti-B220 mAb (CD45R; RA3-6B2; eBioscience), Alexa Fluor 647–peanut agglutinin (PNA; Invitrogen), PE–anti-IgM mAb (AF6-78; BD Biosciences), FITC–anti-IgG1 mAb (A85-1; BD Biosciences), FITC–anti-IgG3 mAb (R40-82; BD Biosciences), FITC–anti-IgA mAb (C10-3; BD Biosciences), 7-AAD,
and biotin–anti-CD138 mAb (281–2; BD Biosciences), followed by FITC-streptavidin (11–4317-87; eBioscience) or PE-streptavidin (12–4317-87; eBioscience), FITC-anti-CD3 mAb (17A2; BioLegend), FITC-anti-CD4 (GK1.5; BioLegend), and/or allophycocyanin–anti-CD8 mAb (53-6.7; BD Biosciences). For intracellular staining, B cells were fixed in 150 μl formaldehyde (3.6%) for 10 min at 25°C. In the case of IgE intracellular staining, cells were trypsinized and then fixed (47). Cells were then permeabilized in cold methanol (90%) for 30 min on ice before staining with biotin–anti-CD19 and streptavidin, followed by PE-streptavidin, PE-Cy7–anti-B220 mAb (RA3-6B2; eBioscience), FITC–anti-IgM mAb (II/41; BD Biosciences), allophycocyanin–anti-IgG1 mAb (AS8-1; BD Biosciences), and/or PE–anti-IgE mAb (23G3; eBioscience). FACs was performed on single spleen cell suspensions. Cells were surface stained with PE–anti-CD19 (ID3; BD Biosciences), 7-AAD, and biotin–anti-CD138, followed by FITC-streptavidin. CD19+CD138+ plasma cells were then sorted using a Cytomation MoFlo cell sorter (Beckman Coulter) and frozen at −80°C until used for gene-expression analysis. Annexin V analysis for apoptotic cells was performed using the Annexin V/FTC Apoptosis Detection Kit II (55670; BD Biosciences), according to the manufacturer’s protocol. In all flow cytometry experiments, cells were appropriately gated on forward scatter and side scatter to exclude dead cells and debris. Cell analyses were performed using a FACScalibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (TreeStar). All experiments were performed in triplicate.

B cell proliferation, cell cycle, and cell division
To analyze C57BL/6 B cell proliferation in vivo, mice were injected i.p. with NP6-CGG injected i.p. 10 d later (twice within 16 h) with BrdU (1 mg) and sampled 4 h after the last i.v. injection. For in vivo Faslpr/lpr B cell proliferation, mice were provided with drinking water containing BrdU (0.8 mg/ml) for 10 d before sacrifice. Spleen cells were stained with PE–anti-B220 mAb. Incoroporated (intracellular) BrdU was stained with allophycocyanin–anti-BrdU mAb using the APC BrdU Flow Kit (BD Biosciences) and analyzed by flow cytometry, and the B cell cycle was analyzed with the same kit, according to the manufacturer’s instructions. Briefly, cells were stimulated and cultured as previously described, except that the cells were pulse-labeled with 10 μM BrdU during the last 30 min of the culture. The cells were then stained with PE–anti-B220 mAb before being fixed/permeabilized and stained for DNA content with 7-AAD and BrdU incorporation with allophycocyanin–anti-BrdU mAb. B cell division was analyzed by CFSE dilution using the CellTrace CFSE Cell Proliferation Kit (Invitrogen). Briefly, B cells were incubated for 3 min at 37°C in 1 μM CFSE in culture medium containing 100 μl of 1 x 10⁸ cells/ml and then washed in FBS-RPMI. Cells were cultured in the presence of LPS alone or LPS + IL-4 for 4 d before being stained with PE–anti-B220 mAb, 7-AAD, and allophycocyanin–anti-IgG1 mAb (5X6; BD Biosciences) or biotin–anti-IgG3 mAb (R40-82; BD Biosciences), followed by allophycocyanin-streptavidin (554067; BD Biosciences), and subsequently were analyzed by flow cytometry.

Secreted Ig and ELISPOT
Titers of IgG1, IgG3, IgA, and IgE in cell culture supernatants of in vitro–stimulated B cells or in vivo titers of circulating total and/or NP-binding IgM, IgG1, IgG2b, and IgG3 were measured using specific ELISAs, as we described (12, 14, 48). Titers of NP6-gor or NP,3-binding IgM, IgG1, and IgG3 also were determined, as previously described (12, 14, 48), and expressed as relative units (RU), defined as the dilution factor needed to reach 50% saturation binding, as calculated using GraphPad Prism software. Specific forward primer together with a reverse C region 3′-specific forward primer together with a reverse C region–specific primer (13) and Phusion high-fidelity DNA polymerase (New England BioLabs). PCR conditions for both the first- and second-round PCRs were 30 cycles of 98°C for 10 s, 60°C for 45 s, and 72°C for 1 min.

Quantitative RT-PCR of mRNAs and miRNAs
For quantification of mRNA, pri-miRNA, germline I H-CH, and post-recombination IgC–CH and mature Vδ1δ2δ3δ4 transcripts, RNA was extracted from 20–5 x 10⁶ cells using either TRIzol reagent (Invitrogen) or an RNeasy Plus Mini Kit (QiAGEN). Residual DNA was removed from the extracted RNA with gDNA elimination columns (QiAGEN). cDNA was synthesized from total RNA with the SuperScript III First-Strand Synthesis System (Invitrogen) using oligo–dT primer. Transcript expression was measured by quantitative RT-PCR (qRT-PCR) with the appropriate primers (Supplemental Table I) using a MyiQ Real-Time PCR Detection System to measure SYBR Green (QIAGEN). The fractionated proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad) overnight (30 V) at 4°C. After blocking and overnight incubation at 4°C with anti-AID (ZA001; Invitrogen), anti–Blimp-1 mAb (6D3; eBioscience), or anti-β-actin mAb (AC-15; Sigma-Aldrich), the membranes were incubated with HRP-conjugated secondary Abs. After washing with PBS–Tween 20 (0.05%), bound HRP-conjugated Abs were detected using Western Lightning Plus-ECL reagents (PerkinElmer Life and Analytical Sciences).

IgH locus SHM and deletions
To analyze Ig SHM induced in response to NP immunization, spleen B cells were isolated from C57BL/6 mice that were immunized with NP6-CGG and given untreated water or HDI water. Rearranged V186.2DJH-C DNA end was detected using the anti-IgM mAb (II/41; BD Biosciences), followed by biotin–anti-CD138 mAb, followed by PE-streptavidin (554067; BD Biosciences), and subsequently were analyzed by flow cytometry.

Immunoblotting
To analyze spontaneous SHM and deletion in MRL/Faslpr/lpr mice, CD19+PNAhi GC B cells were isolated from Peyer’s patches and cultured using a 40× objective lens with an Olympus Fluoview FV1000 Laser Scanning Confocal microscope. To analyze GC structure, 6-μm spleen sections were prepared by cryostat and loaded onto positively charged slides, fixed in cold acetone, and stained with FITC-PNA and PE–anti-B220 mAb for 1 h at 25°C in a moist chamber. Cover slips were mounted using ProLong Gold Antifade Reagent with DAPI before examination using an Olympus CKX41 fluorescence microscope.

Fluorescence and confocal fluorescence microscopy
To analyze IgG1-producing cells, B cells stimulated with LPS + IL-4 were immobilized on acid-washed cover slips coated with poly- β-lysine. Cells were fixed with formaldehyde (3.6%) for 10 min at 25°C, washed three times with TBS, permeabilized with Triton X-100 (0.5%) in TBS for 10 min at 25°C, washed three times with Triton X-100 (0.1%) in TBS, and then stained with FITC–anti-IgG1 mAb and biotin–anti-IgG3 mAb, followed by allophycocyanin-streptavidin or anti–Blimp-1 mAb (6D3; eBioscience), followed by Alexa Fluor 647–anti-IgG mAb (4418; Cell Signaling Technology) for 1 h at 25°C. After washing three times with Triton X-100 (0.1%) in TBS, cover slips were mounted with Pro-Long Gold Antifade Reagent with DAPI (Invitrogen). Fluorescent images were captured using a 40× objective lens with an Olympus Fluoview FV1000 Laser Scanning Confocal microscope. To analyze GC structure, 6-μm spleen sections were prepared by cryostat and loaded onto positively charged slides, fixed in cold acetone, and stained with FITC-PNA and PE–anti-B220 mAb for 1 h at 25°C in a moist chamber. Cover slips were mounted using Pro-Long Gold Antifade Reagent with DAPI before examination using an Olympus CKX41 fluorescence microscope.

Downloaded from http://www.jimmunol.org/ by guest on July 26, 2017

AEC Peroxidase Substrate Kit, following manufacturer’s protocol (SK-4200; Vector Laboratories). Plates were imaged and quantified using a CTL-ImmunoSpot Analyzer and software (Cellular Technology Limited).
94°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Data acquisition was performed during the 72°C extension step. Melting-curve analysis was performed from 72 to 95°C. For quantification of mature miRNA transcripts, RNA was extracted from 0.2–5 x 10⁶ cells using an miRNeasy Mini Kit and then reverse transcribed with a miScript II RT Kit (both from QIAGEN) using the miScript HiSpec buffer. A Bio-Rad MyiQ Real-Time PCR Detection System was used to measure SYBR Green (miScript SYBR Green PCR Kit; QIAGEN) incorporation, according to the manufacturer’s instructions. Mature miRNA forward primers (Supplemental Table I) were used at 250 nM in conjunction with the QIAGEN miScript Universal Primer and normalized to expression of small nuclear/nucleolar RNAs RNU6/RNU61/2, SORD61/SNORD61, SORD68/SNORD68, and SORD70/SNORD70. The ΔΔCt method was used for data analysis of qRT-PCR experiments.

Histone acetylation of miRNA host genes and Aicda promoter by chromatin immunoprecipitation and quantitative PCR

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (15, 48, 50). B cells (1 x 10⁷) were treated with formaldehyde (1% v/v) for 10 min at 25°C to cross-link chromatin, washed once in cold PBS with protease inhibitors (Roche), and resuspended in lysis buffer (20 mM Tris-HCl, 200 mM NaCl, 2 mM EDTA, 0.1% w/v SDS, and protease inhibitors [pH 8]). Chromatin was sonicated to yield DNA fragments (~200–1000 bp in length), precleared with protein A agarose beads (Pierce), H3 mAbs (H3K9ac/K14ac; 17-615; Millipore) at 4°C overnight. Immune complexes were precipitated with Protein A agarose beads, washed, and eluted (50 mM Tris-HCl, 0.5% SDS, 200 mM NaCl, 100 μg/ml proteinase K [pH 8]), followed by incubation at 65°C for 4 h. DNA was purified using a QIAquick PCR purification kit (QIAGEN). The miRNA host gene promoter region DNA was amplified from immunoprecipitated chromatin by quantitative PCR (qPCR) using appropriate primers (Supplemental Table I). Data were normalized to input chromatin DNA and depicted as relative abundance of each amplicon.

Luciferase 3’UTR reporter assays

The partial 3’UTR’s of Aicda miRNA (nt 691–1168 of NM_009645.2, National Center for Biotechnology Information) and Prdm1 miRNA (nt 2652–5101 of NM_007548.3, National Center for Biotechnology Information) were PCR amplified from spleen B cell cDNA and cloned into the pMIR-REPORT miRNA Expression Reporter Vector System (Invitrogen), which allows for analysis of 3’UTR-mediated regulation of firefly luciferase activity. The mutant (mut) Aicda 3’UTR containing point mutations (Supplemental Fig. 2E) was generated by PCR-based mutagenesis of the Aicda 3’UTR pMIR-REPORT vector using Phusion DNA polymerase (New England BioLabs.). Point mutations were introduced into the Prdm1 3’UTR, as described above, together with an additional deletion (nt 3680–5101) were deleted by PCR) to generate mutant Prdm1 3’UTR. The sequence of constructs was confirmed by two independent sequencing reactions. Reporter constructs were cotransfected with the pRL-TK vector (Promega), which drives constitutive expression of Renilla reniformis luciferase, into mouse CH12F3 B cells by electroporation (250V and 900V) with Renilla reniformis (Promega) which drives constitutive expression of firefly luciferase activity and normalized to Renilla luciferase activity, according to the manufacturer’s instructions, using the Dual-Luciferase Reporter Assay System (Promega).

Methylation analysis of Aicda promoter DNA

Genomic DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (QIAGEN), according to the manufacturer’s instructions. Bisulfite-treated DNA was amplified by PCR using GoTaq Hot Start Polymerase (Promega). The primers for bisulfite-sequencing PCR (Supplemental Table I) were designed using MethPrimer (http://www.urogene.methprimer/index1.html). PCR products were purified with a QIAquick PCR purification kit (QIAGEN) and sequenced before or after being cloned into the pCR-Blunt II-TOPO vector.

Lupus mice: autoantibodies, pathology, and disease

MRL/Fas/+ mice (The Jackson Laboratory) were housed in the University of California, Irvine and the UT Health Science Center, San Antonio vivaria and provided with autoclaved food and deionized water. MRL/Fas/+ mice were started on HDI water ad libitum at 6 or 17 wk of age or were on untreated water throughout their life and sacrificed when moribund. Anti-nuclear Ab (ANA) and anti-dsDNA Ab titers were determined in sera. For ANA assays, sera were serially diluted in PBS (from 1:40 to 1:160), incubated on ANA substrate slides (HEP-2 cell-coated slides; MBL-BION), and detected with a 1:1 mixture of FITC–anti-IgG1 and FITC–anti-IgG2a mAbs (R19-15; BD Biosciences). Images were acquired with a 40× objective on an Olympus CKX41 fluorescence microscope. Anti-dsDNA IgG and IgG2a Ab titers were measured in sera of MRL/Fas/+ mice by ELISA, as previously described (14). Titters were expressed in RU, defined as the dilution factor needed to reach 50% of binding. DAPI staining was performed using GraphPad Prism software (GraphPad). Skin lesions were scored on a scale of 0 to 3, with 0 = none, 1 = mild (snout and ears), 2 = moderate (<2 cm snout, ears, and intrascapular), and 3 = severe (>2 cm snout, ears, and intrascapular). To assess kidney pathology, kidneys from MRL/Fas/+ mice were either frozen in TissueTek O.C.T. compound (Sakura Finetek USA) on dry ice for immunofluorescence or fixed in paraformaldehyde (4%) and embedded in paraffin for H&E staining. For immunofluorescence, 4-μm sections were prepared by cryostat, loaded onto positively charged slides, fixed in cold acetone, and stained with a mixture of FITC-labeled rat mAb to mouse IgG1 or mouse IgG2a. Cover slips were mounted using ProLong Gold Antifade Reagent with DAPI before examination with an Olympus CKX41 fluorescence microscope. For H&E staining, kidneys were fixed overnight in paraformaldehyde (4%), serially passed into ethanol (30%) for 1 h, ethanol (50%) for 1 h, and ethanol (70%) overnight, and embedded in paraffin; 4-μm sections were cut for H&E staining.

Statistical analyses

All statistical analyses were performed using Excel (Microsoft) or GraphPad Prism software. Differences in Ig titers, CSR, and RNA transcript expression were analyzed with a Student paired (in vitro) or unpaired (in vivo) t test, assuming two-tailed distributions. Differences in the frequency and spectrum of somatic point mutations were analyzed with χ² tests. Differences in lifespan between mice that were administered untreated water or HDI water were compared by Kaplan–Meier curves and calculated using the Mantel–Cox log-rank test.

Results

CSR and SHM in Ab responses are inhibited by HDIs

To address the effect of HDIs on a T-dependent response, we injected C57BL/6 mice with NP₁₆-CGG, which preferentially induces NP-binding IgG1, 1 d after starting them on the HDI water; these mice drank HDI water at a comparable or higher rate than did mice drinking untreated water. Mice drinking HDI water showed a reduced IgG1 (including high-affinity NP₁₆-binding IgG1), but not IgM, response to NP, even after a second injection of NP₁₆-CGG (Fig. 1A, 1B); reduced class-switched NP-binding IgG1 titers occurred in the context of reduced total IgG1, IgG3, and IgG2b (IgG2b data not shown) but not IgM (Fig. 1C). They were associated with reduced proportions of GC IgG1⁺, but not IgM⁺, B cells and a reduced frequency of somatic point mutations (by >65%) in V₅₆₋₁₃-D₁₄-C₁₅ transcripts, with no significant alteration in spectrum of the residual mutations (Fig. 1D, 1E, Supplementary Fig. 1A), suggesting that this HDI reduced AID expression to impair the class-switched and hypermethylated Ab response. In these mice, HDI had no significant effect on T (CD3⁺) or B (B220⁺) cell number (Fig. 1F). A “direct” HDI effect on B cells was indicated by the reduced T-independent IgG3 response in HDI-treated mice injected with T-independent NP-LPS. These mice showed reduced titers of (high-affinity) NP₁₆-binding IgG3, as well as fewer total IgG3 B cells and NP₁₆-binding IgG3 Ab-forming cells (Fig. 1G, 1H). Thus, HDIs can dampen class-switched–specific T-dependent and T-independent Ab responses.

HDIs inhibit CSR without altering B cell viability or proliferation

To further define the impact of HDIs on CSR, we used appropriate stimuli to induce B cells to switch to IgG1, IgG3, IgA, or IgE in the presence of VPA, butyrate (0–1000 μM), or TSA (0–20 nM). These HDIs reduced, in a dose-dependent fashion, CSR to IgG1, IgG3, IgA, and IgE (Figs. 2A, 2B, 3A–C, Supplementary Fig. 1B).
without affecting B cell division (Fig. 2C, 2D), viability, or apoptosis in vivo or in vitro (Fig. 4A, Supplemental Fig. 1C, 1D).

Expression of the antiapoptotic genes Bcl2, Mcl1, and Bcl2l1 (Bcl2l1 encodes Bcl-xL), which enhance B cell and plasma cell survival, was unaltered or increased by HDIs in vivo and in vitro (Supplemental Fig. 1F, 1H). The reduction in IgG1+ and IgG3+ B cells reflected a lower proportion of class-switched cells/round of cell division (Fig. 2C, 2D, Supplemental Fig. 2A) and was associated with decreased titers of IgG1, IgG3, IgA, and IgE in culture fluids (Fig. 3D, 3E). HDI inhibition of CSR was further confirmed by decreased mature V_{H}DJ_{H}-C_{H}1, V_{H}DJ_{H}-C_{H}3, V_{H}DJ_{H}-C_{\alpha}, and V_{H}DJ_{H}-C_{e} transcripts and postrecombination I_{\mu}C_{H1}, I_{\mu}C_{H3}, I_{\mu}C_{\alpha}, and I_{\mu}C_{e} transcripts in the presence of normal levels of the respective germline I_{\mu}C_{H} transcripts, which are necessary for initiation of CSR (Supplemental Fig. 2B–D). Thus, HDIs significantly reduce CSR, without altering B cell viability or proliferation.

**FIGURE 1.** HDIs reduce CSR and SHM in Ab responses to NP-CGG and NP-LPS. Eight-week-old C57BL/6 mice were started on HDI water 1 d before injection with NP16-CGG or NP-LPS. (A) Titers of high-affinity NP3-binding IgM or IgG1 (RU) in serum 10 d after NP16-CGG injection. Each symbol represents an individual mouse (n = 5). (B) NP3-binding IgG1 titers (RU) in serum 28 d after initial NP16-CGG injection (7 d after booster NP16-CGG injection). Each symbol represents an individual mouse (n = 5). (C) Titers of total IgM, IgG1, or IgG3 in serum 10 d after NP16-CGG injection (n ≥ 5 mice). (D) Surface IgM and IgG1 expression in spleen B220+PNAhi GC B cells 10 d after NP16-CGG injection. Data are representative of three independent experiments. (E) Somatic point mutations in the V_{166.2} region of V_{166.2}DJ_{H}-C_{H}1 transcripts amplified from spleen B cells 10 d after NP16-CGG injection. Sequence data were pooled from three mice in each group. Pie charts depict the proportions of sequences that carry 1, 2, 3, and so on point mutations over the 294-bp V_{166.2} region of V_{166.2}DJ_{H}-C_{H}1 transcripts. In the center is the number of independent sequences analyzed; listed below the pie charts is the overall mutation frequency (changes/base). (F) Surface B220 and CD3 expression in spleen cells 10 d after NP16-CGG injection. Data are representative of three independent experiments. (G) NP3-binding IgG3 titers (RU) in serum 28 d after initial NP-LPS injection (7 d after booster NP-LPS injection). Each symbol represents an individual mouse (n = 4). (H) ELISPOT analysis of NP3-binding IgG3 Ab-forming cells (AFCs) in spleens 28 d after initial NP-LPS injection (7 d after booster NP-LPS injection). Data are from three independent experiments. Scale bars, 1 mm. Numbers indicate NP3-binding IgG3 AFCs/1 × 10^6 spleen cells (n = 3 mice). The p values were determined using an unpaired t test.
differentiation into plasma cells in vivo and in vitro (Fig. 4C–E). This was not associated with decreased plasma cell survival, as shown by normal plasma cell viability and apoptosis, as well as elevated Bcl2, Mcl1, and Bcl2l1 expression (Fig. 4D, Supplemental Fig. 1E, 1G, 1I) and normal transcripts of Il6 (data not shown), which enhances Blimp-1 expression (51). Thus, HDIs inhibit plasma cell differentiation without altering plasma cell survival.

The HDI-impaired Ab response was associated with reduced CSR and SHM, which are initiated by AID, and reduced plasma cell differentiation, which is orchestrated by Blimp-1. Expression of Aicda, Prdm1, and Xbp1 [Xbp1 is under control of Blimp-1 and its gene product promotes Ig secretion in plasma cells (16)] was significantly reduced by HDIs in vivo. Expression of Ung, which encodes for Ung that plays a role downstream of AID in CSR and SHM (1), and Bcl6, which encode Bcl-6, a master regulator of the GC reaction and a Prdm1 repressor (52, 53), were unchanged (Fig. 5A). Consistent with these in vivo findings, the expression of Aicda, Prdm1, and Xbp1 was silenced in a dose-dependent fashion by HDIs in stimulated B cells in vitro (Fig. 5B–D). This contrasted with the unchanged expression of Irf4 (encoding Irf4, a transcription factor that regulates CSR, SHM, and plasma cell differentiation) (54), Bcl6, Ung, HoxC4 (encoding the AID-inducing HoxC4 transcription factor) (12–14), Rev1 (encoding the CSR scaffold protein Rev1) (48), and Bach2 and Pax5 (encoding Bach2 and Pax5, both repressors of Prdm1) (55, 56) (Fig. 5C, data not shown). The downregulation of Aicda and Prdm1 transcripts greatly affected the expression of AID and Blimp-1 proteins (Fig. 5E). Thus, the inhibition of CSR, SHM, and plasma cell differentiation by HDIs reflects the HDI-mediated downregulation of AID and Blimp-1.

HDXIs upregulate miRNAs that target Aicda and Prdm1 transcripts in B cells

Enhancement of histone acetylation, a function of HDIs, which generally upregulate gene expression, is at odds with the decreased expression of AID/Aicda and Blimp-1/Prdm1 by HDIs in vivo and in vitro (Fig. 5). In fact, in B cells induced to undergo CSR, HDIs did not alter histone H3 acetylation in the Aicda promoter, nor did they alter DNA methylation [HDACs were suggested to interact with DNA methyltransferases (57)] (Fig. 6A–C), raising the possibility that HDIs upregulated the expression of gene(s), which, in turn, negatively regulated Aicda and Prdm1. HDIs can modulate expression of miRNAs (36), which silence target miRNAs by inducing their degradation and/or reducing their translation. In this context, Aicda can be silenced by miR-155, miR-181b, and miR-361 (2, 3, 18, 27–30); Prdm1 can be targeted by miR-23b (our prediction, using
mRNA 3
Prdm1
Aicda
mutation or deletion of the target sites of miR-155 and miR-181b of Aicda

tors. These were used to transfect mouse CH12F3 B cells that can

...nil or VPA. (E) IgG1, IgA, and IgE in culture fluids of B cells stimulated with LPS + IL-4 alone, LPS + TGF-β + IL-4 + IL-5 + anti-IgD dextran, or CD154 + IL-4, respectively, for 7 d in the presence of nil or increasing doses of VPA. (F) Proportions of IgG1+* plasmablasts (B220+CD138+), as measured by surface expression of B220, CD138, and IgG1 in B cells stimulated with LPS + IL-4 for 7 d in the presence of nil or VPA. (G) GC structure, as visualized by fluorescent microscopy (B220, red; PNA-binding lectin, green). Spleen sections prepared from mice that were on HDI water or untreated water and injected with NP16-CGG for 10 d (as in Fig. 1). GC B cells were identified by staining with PE-anti-B220 mAb and FITC-PNA. Data are representative of three independent experiments. Scale bars, 50 μm.

FIGURE 3. HDIs inhibit CSR, as well as IgG, IgA, and IgE production, in a dose-dependent fashion, but they do not affect GC formation. (A) Surface expression of B220 and IgA in B cells stimulated with CD154 or LPS + TGF-β + IL-4 + IL-5 + anti-IgD dextran in the presence of nil or increasing doses of VPA for 4 d. (B) Intracellular expression of IgE in B cells stimulated with CD154 + IL-4 in the presence of nil or increasing doses of VPA for 4 d. (C) Intracellular expression of IgM and IgG1 in B cells stimulated with LPS + IL-4 for 4 d in the presence of nil or VPA. (D) IgG1, IgG3, IgA, or IgE in culture fluids of B cells stimulated with LPS + IL-4, LPS alone, LPS + TGF-β + IL-4 + IL-5 + anti-IgD dextran, or CD154 + IL-4 + IL-5, respectively, for 7 d in the presence of nil or increasing doses of VPA. (E) IgG1, IgA, and IgE in culture fluids of B cells stimulated with CD154 + IL-4 or LPS + TGF-β + IL-4 + IL-5 + anti-IgD dextran for 7 d in the presence of nil or butyrate. Data are from three independent experiments (mean and SEM). (F) Proportions of IgG1+* plasmablasts (B220+CD138+), as measured by surface expression of B220, CD138, and IgG1 in B cells stimulated with LPS + IL-4 for 7 d in the presence of nil or VPA. (G) GC structure, as visualized by fluorescent microscopy (B220, red; PNA-binding lectin, green). Spleen sections prepared from mice that were on HDI water or untreated water and injected with NP16-CGG for 10 d (as in Fig. 1). GC B cells were identified by staining with PE-anti-B220 mAb and FITC-PNA. Data are representative of three independent experiments. Scale bars, 50 μm.

TargetScan.org, miRNA.org, and miRbase.org) and was suggested to be targeted by miR-30a and miR-125b (3, 18, 31–33) (Supplemental Fig. 2E). We found that miR-155, miR-181b, miR-361, miR-23b, miR-30a, and miR-125b were upregulated by VPA in B cells in vivo; in purified B cells induced to undergo CSR and plasma cell differentiation in vitro, irrelevant miRNAs miR-19a/b, miR-20a, miR-20b, and miR-25, which are not known to regulate Aicda, Prdm1, or Xbp1, were unchanged in B cells in vivo and in vitro (Fig. 7A, 7B). Primary miRNA transcripts pri–miR-155 and pri–miR-181b, which are processed by Drosha and Dicer to give rise to mature miRNAs, also were upregulated, suggesting that HDIs upregulate miRNA host gene transcription (Fig. 6D). Accordingly, miRNA upregulation in stimulated B cells was accompanied by increased overall histone H3 acetylation of the host genes encoding these miRNAs (Fig. 7C). Thus, HDIs upregulate miRNAs that target Aicda and Prdm1 mRNA 3’ UTRs and, therefore, can silence these miRNAs.

Selected B cell miRNAs upregulated by HDIs silence Aicda and Prdm1 transcripts

To prove that HDI inhibition of AID and Blimp-1 expression was mediated by upregulation of miRNAs that directly target Aicda and Prdm1 mRNA 3’UTRs, we cloned either wild-type or mutant 3’UTRs of Aicda and Prdm1 miRNAs (mut 3’UTRs were constructed by mutation or deletion of the target sites of miR-155 and miR-181b in Aicda mRNA 3’UTR or miR-23b, miR-30a, and miR-125b in Prdm1 mRNA 3’UTR) into pMIR-REPORT luciferase reporter vectors. These were used to transfect mouse CH12F3 B cells that can be induced to undergo CSR at a high rate. Like in primary B cells, VPA upregulated miR-155, miR-181b, miR-361, miR-23b, miR-30a, and miR-125b expression in CH12F3 B cells induced to undergo CSR (Fig. 7D). We then measured the ability of VPA to repress luciferase activity using reporter constructs containing wild-type or mutant 3’UTRs of Aicda and Prdm1 mRNAs that were transfected into CH12F3 B cells (Fig. 7E; Supplemental Fig. 2E). The luciferase reporter activity was reduced by VPA in B cells transfected with reporter constructs containing wild-type Aicda or Prdm1 3’UTRs but not those transfected with reporter constructs containing Aicda or Prdm1 mut 3’UTRs (Fig. 7F). In B cells transfected with reporter constructs containing wild-type Aicda or Prdm1 3’UTRs, the degree of inhibition of luciferase activity was significant, despite the relatively low dose of VPA used (250 μM) and the omission of some additional mRNA target sites in the 3’UTR of Aicda mRNA, thereby emphasizing the potency of the HDI-mediated upregulation of miRNAs on B cell gene expression. Thus, VPA silences AID and Blimp-1 expression in B cells through upregulation of selected miRNAs that directly target Aicda and Prdm1 mRNA 3’UTRs.

HDIs silence AICDA and PRDM1 in human B cells to inhibit CSR and plasma cell differentiation

Next, we determined whether HDIs also inhibit CSR and plasma cell differentiation in human B cells. We stimulated purified human IgD+ B cells with CD154 + IL-21 and IL-4 or TGF-β. Like in mouse B cells, VPA and butyrate effectively inhibited CSR to IgG, IgA, and IgE in human B lymphocytes, as well as plasma cell
differentiation in a dose-dependent fashion without altering B cell viability (Fig. 8A–D). VPA increased expression of miR-155, miR-181b, miR-361, miR-23b, miR-30a, and miR-125b, which [as reported or as predicted by us (2, 3, 18, 27–33)] target miRNA binding sites in the 3′UTR of human and mouse AICDA/Aicda or PRDM1/Prdm1, and decreased expression of AICDA, PRDM1, and XBP1 transcripts (Fig. 8E, 8F). Thus, HDIs (VPA and butyrate) modulate human B cell class-switching and plasma cell differentiation, as they do in mouse B cells.

HDIs dampen the autoantibody response, ameliorate disease, and increase survival in lupus mice

Lupus-prone MRL/Fas<sup>hpt/hpt</sup> mice spontaneously upregulate AID, Blimp-1, CSR, and SHM and generate great numbers of plasma cells, which produce large amounts of anti-dsDNA IgG and other autoantibodies, and develop age-dependent disease, which includes skin lesions and kidney pathology (2, 14, 49). In our female MRL/Fas<sup>hpt/hpt</sup> mice, ANAs and anti-dsDNA IgG autoantibodies appeared at 6 wk of age and reached high levels at 12 wk, in association with significant loads of point mutations and DNA deletions in the IgH locus. At 17 wk, all of these mice showed severe kidney immunopathology, and 75% of them displayed various skin lesions, which, in 55% of the cases, included the characteristic “butterfly” rash. To determine whether HDIs could inhibit the lupus class-switched and hypermutated autoantibody response, we assigned 80 3-wk-old female MRL/Fas<sup>hpt/hpt</sup> mice to three groups: a “non-treatment” group consisting of 50 mice that were given untreated water throughout their lives; an “early treatment” group of 15 mice that were given untreated water for the first 6 wk of life, at which time they were started on HDI water; and, a “late treatment” group of 15 mice that were given untreated water for the first 17 wk of life, at which time they were started on HDI water.

**FIGURE 4.** HDIs inhibit plasma cell differentiation. (A) Proportions of B220<sup>+</sup>PNA<sup>hi</sup> GC B cells, proliferating B cells (BrdU-stained B220<sup>+</sup> B cells), viable (7-AAD<sup>−</sup>) B220<sup>+</sup> B cells, and CD4<sup>+</sup> and CD8<sup>+</sup> T cells in spleen cells from mice that were on HDI water or untreated water and injected with NP<sub>16</sub>-CGG 10 d before analysis. (B) HDI does not alter B cell cycle. Mouse IgD<sup>+</sup> B cells were stimulated for 48 h with LPS or LPS + IL-4 in the presence of nil or VPA; during the last 30 min of culture, the cells were pulse-labeled with 10 μM of BrdU. The cells were then surface stained for B220 before intracellular staining with anti-BrdU mAb and 7-AAD. B220<sup>+</sup> cells are displayed, with gates indicating the percentage of cells in G0/G1, S, and G2/M phase. (C) Proportions of B220<sup>+</sup>CD138<sup>+</sup> (plasma) cells in spleen cells from C57BL/6 mice that were on HDI water or untreated water were analyzed 10 d after NP<sub>16</sub>-CGG injection. (D) Dose-dependent inhibition by VPA of plasma cell (B220<sup>+</sup>CD138<sup>+</sup>) differentiation (upper panels) in B cells stimulated for 4 d with LPS + IL-4, without alteration of plasma cell viability, as analyzed by 7-AAD staining (lower panels, proportions of 7-AAD<sup>−</sup> viable cells among B220<sup>+</sup>CD138<sup>+</sup> cells are indicated). (E) IgG1-producing plasma cells (IgG1<sup>+</sup>CD138<sup>+</sup> or IgG1<sup>+</sup>Blimp-1<sup>+</sup>) are reduced in cultures of IgD<sup>+</sup> B cells stimulated for 7 d with LPS + IL-4 in the presence of VPA (500 μM), as shown by confocal fluorescence microscopy. Cells were permeabilized and stained with DAPI (blue) to visualize nuclei and fluorescent mAbs to visualize IgG1 (green) and CD138 (red, upper set of panels) or Blimp-1 (red, lower set of panels). Arrows indicate IgG1-producing cells (yellow; CD138<sup>+</sup>/Blimp-1<sup>+</sup>IgG1<sup>+</sup>). Data are representative of three independent experiments. Scale bars, 10 μm.
At 12 wk of age, “early” HDI-treated MRL/Fas<sup>lpr/lpr</sup> mice displayed reduced levels of anti-dsDNA IgG, IgG1, IgG2a, and ANA (data not shown), but not IgM, autoantibodies; reduced overall IgG1/IgG2a but not IgM; significantly reduced (by >75%) somatic point mutations, with no significant alteration in spectrum; and a reduced load of DNA deletions in the IgH locus (Figs. 9A, 9B, 10A, 10B, Supplemental Fig. 3). They also exhibited reduced numbers of IgG2a<sup>+</sup> B cells, as well as reduced plasma cell differentiation, in the presence of normal B220<sup>+</sup> cell transcripts; and a reduced load of DNA deletions in the somatic point mutations, with no significant alteration in spectrum.

**Discussion**

As we have argued, epigenetic changes, such as histone post-translational modifications and DNA methylation, and epigenetic factors, such as miRNAs, can interact with genetic programs to regulate B cell functions, including CSR, SHM, and plasma cell differentiation, thereby informing Ab responses that are critical for the defense against microbial pathogens and tumor cells, as well as...
autoantibody responses that mediate autoimmunity and disease (3). We showed in this study that VPA and butyrate, two SCFA HDIs, inhibited CSR, SHM, and plasma cell differentiation by modulating intrinsic B cell mechanisms. They repressed AID and Blimp-1 expression in mouse and human B cells by upregulating selected miRNAs that silenced AICDA/Aicda and PRDM1/Prdm1 mRNAs, as demonstrated by multiple qRT-PCRs (this study) and further confirmed by mRNA-Seq and microRNA-Seq (H. Zan and P. Casali, unpublished observations). AID expression and CSR also were inhibited by TSA, a hydroxamic acid HDI. The doses at which VPA and butyrate inhibited B cell class switching, hypermutation, and plasma cell differentiation were within the range of those measured in humans for these HDIs (41, 43). By inhibiting AID and Blimp-1 expression, VPA dampened class-switched and hypermutated Abs in specific T-dependent and T-independent Ab responses in normal mice. This HDI also dampened class-switched and hypermutated autoantibody levels, reduced immunopathology, and extended survival in autoimmune MRL/Faslpr/lpr mice, a well-studied model of human lupus.

SCFA HDIs were suggested to display significant selectivity for different HDACs (58). For example, VPA targets class I HDACs, particularly HDAC1 and HDAC2, and, less effectively, class IIa HDACs; butyrate targets class I HDACs, mainly HDAC1, and, less effectively, other members of class I and class IIa HDACs (38, 39). HDAC activity is primarily associated with multiprotein complexes, the role and composition of which are often cell-type specific. HDAC-associated proteins specify the selectivity of HDIs, which display different affinities for different HDAC/cofactor complexes. HDIs with diverse chemical properties target different HDACs and HDAC/cofactor complexes, thereby regulating gene expression in a locus- and cell-type–specific fashion (58). Our findings indicate that, in B cells, HDIs modulate miRNAs selectively, possibly as a result of HDACs existing in unique contexts of HDAC/cofactor complexes, as occurring in these lymphocytes, particularly when in an activated state.

HDIs also may indirectly modulate Ab responses or mitigate autoimmunity by affecting elements other than B cells, such as innate immune cells (59) and T cells (regulatory T cells and Th1 and Th17 cells), or inhibiting proinflammatory cytokines (37, 46, 60, 61). However, as shown in this study, HDIs directly regulate B cell genes that are central to peripheral differentiation of these lymphocytes and maturation of Ab and autoantibody responses. Silencing AICDA/Aicda by HDIs was intrinsic to B lymphocytes and independent of other cellular elements, as shown by our in vitro experiments using purified human and mouse B cells, as well as our in vivo studies of the T-independent response to NP-LPS. In both in vivo and in vitro B cells, the HDI-mediated downregulation of AICDA/Aicda expression was associated with a concomitant increase in the respective targeting miR-155, miR-181b, and miR-361 (2), in a tight dose-dependent

FIGURE 6. HDIs do not alter DNA methylation and histone acetylation in the Aicda promoter. (A and B) CpG DNA methylation of the Aicda promoter was analyzed by bisulfite sequencing of genomic DNA from B cells stimulated for 4 d with LPS + IL-4 in the presence of increasing doses of VPA. (A) DNA sequencing of PCR products of bisulfite-treated genomic DNA. The sequence signal from dCs in CpG motifs is outlined. Because unmethylated dC nucleotides can be converted to dU (read as dT in DNA sequence), whereas methylated dC cannot, the ratio of the dC/dT (red) signal indicates the level of methylated dC at any given position. (B) Methylation pattern at each of the four dCs within CpG motifs from individually cloned sequences (each row is a unique sequence, and each dC is represented by a column of circles) is shown as an array of circles. ●, methylated dCs; ○, unmethylated dCs. (C) Abundance of acetylated histone H3 (H3K9ac/K14ac) in the Aicda promoter in B cells stimulated with LPS + IL-4 for 60 h in the presence of nil or VPA (1000 μM) was measured by ChIP and qPCR. (D) Primary (pri-) miRNA transcripts of miR-155 and miR-181b in B cells cultured for 60 h with LPS or LPS + IL-4 in the presence of nil or increasing doses of VPA were measured by qRT-PCR and normalized to Cd79b expression. Values for B cells cultured in medium containing VPA are depicted as relative to the values in B cells cultured in the absence of HDI, set as 1. Data are mean and SEM from three independent experiments. *p < 0.05, **p < 0.01 unpaired t test.
Our findings extend those suggesting a role for miR-155 in downregulating AID expression (28, 30, 62), in agreement with the demonstration that repression of this miRNA provides a mechanism of Bcl6-promoted positive regulation of AID and increased GC gene expression (30). As we showed, silencing of PRDM1/Prdm1 and XBP1/Xbp1 by HDIs also was intrinsic to B cells and independent of other cells. Like for AICDA, HDI-mediated downregulation of PRDM1/Prdm1 was associated with a concomitant increase in the respective B cell–targeting miRNAs (miR-23b, miR-30a, and miR-125b) (2), in vivo and in vitro, and in a tight dose-dependent fashion. HDI-induced downregulation of XBP1/Xbp1 could be secondary to decreased Blimp-1 expression and/or upregulation of selected miRNAs that we tentatively identified as silencers of XBP1/Xbp1 (H. Zan and P. Casali, unpublished observations). That HDIs downregulate Blimp-1 expression by upregulating miR-23b, miR-30a, and miR-125b that silence Prdm1 was further supported by our demonstration that HDIs slightly reduced or did not essentially alter the Prdm1 repressor genes Bach2, Bcl6, or Pax5.

The selectivity of HDI-mediated silencing of AICDA/Aicda and PRDM1/Prdm1 in B cells was further emphasized by the unchanged expression of HoxC4, Irf4, Rev1, and Ung, which play important roles in AICDA/Aicda regulation and/or CSR, as well as of miR-19a/b, miR-20a, and miR-25, which are not known to regulate AICDA/Aicda or PRDM1/Prdm1. We could not rule out the possibility that HDI regulated other B cell factors (e.g., NF-kB or Id2/3), which contributed to the reduction in AID or Blimp-1. The decrease in HDI-mediated repression of luciferase activity...
under the control of *Aicda* and *Prdm1* mRNA 3'UTRs bearing mutated miR-155, miR-181b, miR-23b, miR-30a, and miR-125b target sites demonstrated that miRNAs are indeed direct effectors of the HDI-mediated repression of such selected genes in B cells. The role of B cell miRNAs in mediating HDI suppression of AID and Blimp-1 expression, as well as in the dampening of Ab and autoantibody responses, could be further addressed using an integrated three-prong approach involving the generation of in vivo Argonaute–miRNA–*Aicda* or –*Prdm1* mRNA ternary complexes, knock-in mice lacking specific miRNA-targeting sites in *Aicda* or *Prdm1* 3'UTR, or mice with B cells specifically expressing “sponge” inhibitors of miR-155, miR-181b and miR-361, or miR-23b, miR-30a, and miR-125b. HDIs had no direct effect on the epigenetic status of the *Aicda* locus, because our acetylated histone ChIP and bisulfite sequencing experiments showed no alteration in histone acetylation or methylation of the *Aicda* promoter by VPA. Although it is possible that HDIs could also modify protein functions by increasing acetylation of nonchromatin proteins, our

FIGURE 8. HDIs inhibit CSR and plasma cell differentiation increases selected miRNAs and decreases target AICDA and PRDM1 mRNAs, as well as XBP1 mRNA, in human B cells. Human peripheral blood IgD+ B cells were stimulated with CD154, human IL-4, and human IL-21 (for CSR to IgG1 and IgE and plasma cell differentiation) or CD154, human IL-21, and TGF-β (for CSR to IgA) in the presence of nil, VPA, or butyrate for 60 h (for transcript or miRNA analysis) or 120 h (for flow cytometry or analysis of Ig titers in supernatants). (A) Proportions of IgG+ B cells, plasma cells (CD27⁺CD38⁺), or viable (7-AAD−) CD19⁺ cells. (B) Proportions of IgA+ B cells. (C) IgG and IgA titers in supernatants of B cells stimulated with CD154, human IL-4, and human IL-21 (upper panel) or CD154, human IL-21, and TGF-β (lower panel) and cultured in the presence of VPA or butyrate. Data are from three independent experiments (mean and SEM). *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t test. (D) Mature VDJH-Cα (in cells stimulated by CD154, human IL-21, and TGF-β) and VpJC5-Cα (in cells stimulated by CD154, human IL-21, and TGF-β) transcripts were analyzed by qRT-PCR and normalized to *HPRTI* transcripts. (E) AICDA, PRDM1, and XBP1 transcripts (in cells stimulated with CD154, human IL-4, and human IL-21) were analyzed by qRT-PCR and normalized to *HPRTI* transcripts. (F) miRNA expression was analyzed by qRT-PCR and normalized to expression of small nuclear/nucleolar RNAs RNU6-1/2, SNORD61, SNORD68, and SNORD70. Values for B cells cultured in the presence of HDIs are depicted as relative to the expression of each transcript or miRNA in B cells cultured in the absence of HDI, set as 1. Data are mean and SEM from three independent experiments. The p values were determined using an unpaired t test.
findings allow us to conclude that modulation of miRNAs leading to silencing of selected mRNAs is the mechanism by which HDIs mediate inhibition of the B cell differentiation processes that underpin the maturation of Ab responses.

Our findings in human and mouse B cells, in vitro and in vivo, greatly extend and provide a mechanistic underpinning for the limited data by Kienzler et al., suggesting that VPA reduces human naive B cell differentiation to (CD27hiCD38hi) plasmablasts and reduces IgG and IgA expression. However, at odds with our findings, they did not observe a decrease in CSR induction in human B cells in vitro by VPA. This could be explained by the different designs of their experiments compared with ours. Kienzler et al. (63) stimulated human B cells with CD154 + IL-21; we stimulated human naive B cells with CD154 + IL-21 + IL-4 or CD154 + IL-21 + TGF-β. IL-4 or TGF-β, both critical CSR-inducing stimuli, were missing in Kienzler et al.’s experiments, possibly resulting in relatively lower AICDA and PRDM1 expression. Analysis of the molecular events underpinning CSR was also missing, making it virtually impossible to provide a thorough explanation for the putative discrepancy between the data of Kienzler et al. and ours.

In vivo HDI inhibition of CSR and plasma cell differentiation “freezes” B cells at an IgM+ stage, as indicated by the higher proportion of IgM+ lymphocytes in normal and lupus-prone mice. Whether these IgM+ B cells remained “naive” B cells or underwent some degree of memory B cell differentiation is unclear. The much-reduced IgH locus mutational load in HDI-treated normal and lupus-prone mice supports the contention that those IgM+ B cells expressed primarily unmutated IgM natural Abs. These still would have been available for the response to microbial pathogens and might have played a protective role in systemic autoimmunity (64). In addition, in both normal and autoimmune mice, HDI treatment allowed for some residual AID expression, which resulted in a significant reduction in, but not ablation of, secondary Ab isotypes. Even at low titers, these can mediate a protective antimicrobial immunity, as suggested by the apparently normal risk for infections in Aicda−/− mice with reduced AID levels (65). Thus, in normal and
DNA deletions in intronic JH4-iE or untreated water (from 6 to 12 wk of age, at which time the mice were sacrificed, as in Fig. 9 and Supplemental Fig. 3). Experiments.

Relative to the expression of each transcript in the cells from mice that were on untreated water, set as 1. Data are mean and SEM from three independent experiments.

The number of independent sequences analyzed. Spleen cells from MRL/Faslpr/lpr mice that were on HDI water or untreated water were gated to analyze 7-AAD viable cells or annexin V+ apoptotic cells among CD19+CD138+ plasma cells. Expression of Bcl2, Mcl1, and Bcl2l1 transcripts in CD19+CD138+ plasma cells were measured by qRT-PCR and normalized to Gapdh expression. Values for the cells from mice that were on HDI water are depicted as relative to the expression of each transcript in the cells from mice that were on untreated water, set as 1. Data are mean and SEM from three independent experiments.

autoimmune mice, HDIs dampen the Ab or autoantibody response by efficiently inhibiting CSR, SHM, and plasma cell differentiation, while leaving an intact or even increased IgM pool, as well as residual IgG and IgA levels that may be sufficient for immune protection.

At higher doses than those used in our study, HDIs inhibit proliferation and induce apoptosis in cancer cells (66). They do so partially through induction of DNA damage, which healthy cells can repair but cancer cells cannot (67). In our in vitro and in vivo experiments, even the highest HDI doses did not reduce B cell and plasma cell viability or increase B cell or plasma cell apoptosis. Consistent with a recent finding that butyrate did not affect survival or proliferation of T cells (42), this SCFA HDI did not affect viability or proliferation of B lymphocytes at the concentrations used in this study. Similarly, VPA did not affect B cell viability, proliferation, cell cycle, or apoptosis, nor did it affect viability or survival of plasma cells. The reduction in the proportion of class-switched B cells/round of cell division (which is required for CSR) in the context of an unchanged overall cell number at each division further indicated that HDIs inhibited CSR without interfering with cell viability. This is consistent with the normal levels of lymphocytes in patients treated with VPA (46), in whom lymphopenia would have been expected to occur if this HDI impaired B and/or T cell viability.

Epigenetic dysregulation can compound genetic susceptibility to mediate autoantibody responses and autoimmunity (3). Epigenetic changes associated with autoimmune responses have been investigated in T cells but only marginally in B cells (68). We (14) and other investigators (69) showed that highly upregulated AID and Blimp-1 expression is an important feature of lupus patients and lupus-prone mice, including MRL/Faslpr/lpr mice. In these mice, dysregulation of AID and Blimp-1 causes aberrant rates of CSR and SHM, leading to increased loads of somatic point mutations and deletions/insertions in the IgH locus, as well as heightened Ig secretion rates that result in abundant production of pathogenic autoantibodies. Accordingly, increased AID and Blimp-1 expression in lupus patients is associated with high levels of mutated IgG autoantibodies, which heighten disease activity (14, 69). Conversely, AID deficiency in MRL/Faslpr/lpr Aicda−/− mice protected against disease (64, 70), and decreased AID expression in MRL/Faslpr/lpr HoxC4−/− and MRL/Faslpr/lpr Aicda+/− mice, which display 30–60% of the AID level of MRL/Faslpr/lpr mice, reduced autoantibody titers and delayed disease (14, 71). A role in promoting GC formation and generation of class-switched autoantibodies was suggested for miR-155 (62, 72, 73), and reduced autoantibody production and autoimmunity were reported in miR-155−/− deficient B6/Faslpr/lpr mice (74). However, this likely resulted from dysregulation of a variety of genes in multiple immune cells, including derepressed expression of SHIP-1 in B cells, which led to mitigation of B cell activation, proliferation, and autoantibody production. In our hands, HDIs did not
yield obvious alterations in GC formation or B cell SHIP-1 transcripts (data not shown).

PRDM1 has been identified as one of the risk loci for lupus in human genetic-association studies (75, 76), and increased Blimp-1 expression was shown to parallel a surge in circulating plasma cells during disease flares (77). Blimp-1 is required for generation of short-lived and long-lived plasma cells. Unlike short-lived plasma cells, long-lived autoreactive, malignant, or allergen-specific (IgE+) plasma cells are refractory to immunosuppression and irradiation (78). In our MRL/Fas<sup>lpr/lpr</sup> mice, HDI-mediated upregulation of miR-30a and miR-125b, which are highly expressed in GC B cells and downregulated in plasma cells (32, 79), along with HDI-mediated upregulation of miR-23b, which is reduced in lupus (80), silenced Blimp-1 expression, thereby impairing plasma cell differentiation and compounding the negative effect of decreased CSR and SHM on the production of high-affinity class-switched autoantibodies. Because Blimp-1 is required for formation and maintenance of plasma cells (4), HDI-mediated downregulation of Blimp-1 can lead to significantly long-lasting lower levels of autoantibodies by decreasing autoantibody-producing plasma cells. Accordingly, our preliminary data (data not shown) suggest that the beneficial effect of HDIs is long lasting after withdrawal of the drug from treated autoimmune mice.

Butyrate is one of the SCFAs produced by gut commensal bacteria through dietary fiber fermentation (41) and is the most potent HDI among the SCFAs. It modulates the function of intestinal macrophages (81) and acts on naive T cells to promote epigenetic changes that regulate the expression of genes responsible for differentiation into regulatory T cells and IL-10–producing T cells (42, 82). Our demonstration that butyrate (at a dose as low as 250 μM)
modulates AID expression and CSR to IgG, IgA, and IgE, as well as plasma cell differentiation through direct activity on B cells, indicates that this HDI can play an important role in modulating Ab responses of gut lymphoid organs (in which butyrate occurs at 1–20 mM). This would be particularly true of the cecal patch, a major intestinal lymphoid organ in the proximal colon, as well as in Peyer’s patches, which are highly represented in the ileum, the portion of intestine that is immediately afferent to the proximal colon. Both the cecal patch and Peyer’s patches contain vast numbers of B cells committed to the production of IgA, and to a lesser extent, IgE (83, 84). Butyrate may also play an important role in limiting AID expression in the inflamed colonic mucosa, in which AID is induced by proinflammatory cytokines (85). By suppressing AID, butyrate could suppress inflammation-mediated neoplastic transformation, leading to colorectal cancer (86), a process in which AID-mediated oncogenic mutagenesis plays a significant role.

Metabolites from intestinal microbiota are key determinants of host–microbe mutualism and, consequently, the health or disease of the intestinal tract, as well as other organs and tissues (87–89). It was suggested that SCFAs produced by gut commensal bacteria can distribute systemically and shape the immunological environment in the lung, thereby influencing the severity of allergic inflammation. Mice fed a high-fiber diet had increased circulating levels of SCFAs and were protected against allergic inflammation (mediated by IgE) of the lung, whereas a low-fiber diet decreased levels of SCFAs and increased allergic airway disease (87). A diverse microbial population, which would produce an appropriate amount of SCFA HDIs, particularly, butyrate, is required to maintain a baseline immune-regulatory state, including IgG, IgA, and IgE levels. Elevated serum IgE and CSR to IgE in B cells at mucosal sites in the absence of microbial colonization in germ-free mice and in mice with low-diversity gut microbiota further emphasize the important role for gut commensal bacteria–produced butyrate in modulating IgE levels (90, 91). Altered composition and decreased bacterial diversity of gut microbiota would lead to changes in absolute and relative numbers of B cells committed to the production of IgA, IgG, and IgE specificities and levels, which contribute to altered immunity and increased susceptibility to immune-mediated diseases.

Despite involving no human patient, our study provides a strong rationale and a mechanistic basis for the use of HDIs as epigenetic modulators of Ab responses, as well as therapeutics for systemic autoimmunity and, possibly, IgE-mediated allergic responses. In our experiments, HDI was administered in drinking water, rather than parenterally as in previous studies (46), resulting in steady and well-tolerated therapeutic levels of HDIs. These were comparable to those in patients taking HDIs per os (43) and without the concentration spikes associated with HDI injections (46). HDI administration in drinking water likely contributed to the effectiveness of the modulation of T-dependent and T-independent Ab responses in healthy mice, as well as to the damping of the systemic autoantibody response, reduction in immunopathology, and extended survival in lupus mice. Overall, our studies suggest a new and important therapeutic indication for VPA and butyrate, and, likely, other HDIs, such as TSA and suberoylanilide hydroxamic acid (approved by the U.S. Food and Drug Administration for the treatment of cutaneous T cell lymphoma). They also provide novel and significant mechanistic insights into epigenetic mechanisms of immunoregulation, as mediated by direct modulation of B cell–intrinsic functions, thereby offering new clues for further therapeutic approaches, as specifically targeted to B cells.

Acknowledgments

This paper is dedicated to the memory of our wonderful friend, Mr. Vic Braden. We thank Christie Mortales, Julia Taylor, Isabella Stenmark, Sonia Aghera, and Eun Je Kim for assistance in performing some experiments and Dr. Irene Pederson for the pMIR-REPORT luciferase vector.

Disclosures

The authors have no financial conflicts of interest.

References


