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*J Immunol* 2014; 193:5933-5950; Prepublished online 12 November 2014;
doi: 10.4049/jimmunol.1401702
http://www.jimmunol.org/content/193/12/5933

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/11/11/jimmunol.1401702.DCSupplemental

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

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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), Rev1 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 cell–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/Fasgtmgtm 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 the B cell differentiation processes that underpin Ab and autoantibody responses. They also provide therapeutic proof-of-principle in autoantibody-mediated autoimmunity. The Journal of Immunology, 2014, 193: 5933–5950.

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Received for publication July 9, 2014. Accepted for publication October 19, 2014. This work was supported by National Institutes of Health Grants AI079705, AI105813, and AI060573 (to P.C.), the Arthritis National Research Foundation (to H.Z.), and the Arthritis National Research Foundation (to H.Z.). 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, anti-nuclear Ab; Blimp-1, B lymphocyte–induced maturation protein-1; C4h, constant H chain; CSR, class switch DNA recombination; FBS-RPMI, RPMI 1640 medium with 10% FBS, 50 mM 2-ME, and 1× antibiotic-antimycotic mixture; GC, germinal center; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; IFN, interleukin of C4h gene; miRNA, microRNA; mut, mutant; PNA, peanut agglutinin; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; Rx, relative unit; SCFA, short-chain fatty acid; SHM, somatic hypermutation; TSA, trichostatin A; UTR, untranslated region; VPA, valproic acid; Xbp-1, X-box–binding protein 1.

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stimuli (e.g., IL-4, TGF-β, or IFN-γ), which induce selected intervening region (IgH)-S-C_H 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).

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 (sodium 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 US 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, class II 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 used as an anticonvulsant and a mood stabilizer. It selectively inhibits class I HDACs, particularly HDAC1 and HDAC2, and, less effectively, class II 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 used as a dietary supplement. Butyrate modulates gene expression by selectively inhibiting HDAC1, and, less effectively, other members of class I and class II 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/Fasʻ/ʻ mice. Our findings outline important modalities of epigenetic regulation of AID and Blimp-1 expression, unveil new approaches to modulation of T-dependent and T-independent Ab responses, and provide a proof-of-principle therapeutics study in autoantibody-mediated autoimmunity.

**Materials and Methods**

**HDAC inhibitors**

For in vivo 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 always well accepted by C57BL/6 and MRL/Fasʻ/ʻ mice. VPA and butyrate (obtained as sodium butyrate from Sigma-Aldrich) were directly diluted in culture media for in vitro experiments, at molarities ranging from 125 to 1000 μM. trichostatin A (TSA; Cayman Chemical) was dissolved in DMSO and diluted in culture media at 10 or 20 nM (~3 or 6 ng/ml) for in vitro experiments.

**Modulation of T-dependent and T-independent Ab responses by HDIs**

C57BL/6 mice (The Jackson Laboratory) were housed in a pathogen-free vivarium and provided with autoclaved food and deionized water. To study the T-dependent Ab response, 16 8-wk-old C57BL/6 mice received an i.p. injection of 100 μg NP-CGG (Biosearch Technologies) in alum at day 21 and were sacrificed at day 28. To study the T-independent Ab response, eight 8-wk-old C57BL/6 mice received an i.p. injection of 25 μg 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. 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 100 μg NP-CGG (Biosearch Technologies) in alum at day 21 and were sacrificed at day 28. To study the T-independent Ab response, eight 8-wk-old C57BL/6 mice received an i.p. injection of 25 μg 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 at day 21 and 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) (FBS-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 membrane fragments of baculovirus-infected Sf21 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 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 <0.5 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.

**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 PBS-RPMI and stimulated with CD154 (10 U/ml), IL-4 (20 ng/ml; R&D Systems) and IL-21 (50 ng/ml) by guest on July 26, 2017 http://www.jimmunol.org/ Downloaded from

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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-B220 mAb, followed by PE-streptavidin, PE-Cy7-anti-B220 mAb (RA3-6B2; eBioscience), FITC-anti-IgM mAb (II/41; BD Biosciences), allophycocyanin-anti-IgG1 mAb (AS5-1; 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 (1D3; BD Biosciences), 7-AAD, and biotin-anti-CD138, followed by FITC-streptavidin. CD19+CD138+ B cells (consisting of a large portion of germinal center [GC] B cells, referred to as “total” B cells) and 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/FITC Apoptosis Detection Kit II (556750; 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 previously injected i.p. with NP36-CGG were injected i.p. 10 d later (twice within 16 h) with BrdU (1 mg) and stained 4 h after the last injection. For in vitro proliferation, FoxP3+ 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. Incorporated (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, B 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 culture time. 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 ml CFSE stock solution (1 μM) and then incubated for 60 min at 37°C. After washing three times with FBS-RPMI and biotin–anti-CD138 mAb (281-2; BD Biosciences), followed by FITC–anti-IgG1 mAb (A85-1; BD Biosciences), and second-round PCRs were 30 cycles of 98°C for 10 s, 60°C for 45 s, and 72°C for 1 min; 72°C for 5 min. The intronic JH4-iE DNA region sequences downstream of JH4, which yielded DNA SHM and deletions

To analyze IgG SHM induced in response to NP immunization, spleen B cells were isolated from C57BL/6 mice that were immunized with NP43-CGG and given untreated water or HDI water. Rearranged V186.2DJH4-C region encoding the anti-NP IgH chain was amplified using a V186.2 leader-specific forward primer together with a reverse C-specific primer (13) and Phusion high-fidelity DNA polymerase (New England BioLabs). PCR conditions were 98°C for 10 s, 60°C for 45 s, and 72°C for 1 min for 30 cycles. To analyze spontaneous SHM and deletion in MRL/Fas−/− mice, CD19+PNAhi GC B cells were isolated from Peyer’s patches and cultured with HDI water (100 μM) for 60 h. Cells were harvested and lysed in Lysis buffer. Cell extracts containing equal amounts of protein (20 μg) were fractionated through SDS-PAGE (10%). 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-IgA (Z2001; Invitrogen), anti–IgM (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 (Pierce/Elmer Life and Analytical Sciences).

IgG locus SHM and deletions

To analyze IgG SHM induced in response to NP immunization, spleen B cells were isolated from C57BL/6 mice that were immunized with NP43-CGG and given untreated water or HDI water. Rearranged V186.2DJH4-C region encoding the anti-NP IgH chain was amplified using a V186.2 leader-specific forward primer together with a reverse C-specific primer (13) and Phusion high-fidelity DNA polymerase (New England BioLabs). PCR conditions were 98°C for 10 s, 60°C for 45 s, and 72°C for 1 min for 30 cycles. To analyze spontaneous SHM and deletion in MRL/Fas−/− mice, CD19+PNAhi GC B cells were isolated from Peyer’s patches and cultured with HDI water (100 μM) for 60 h. Cells were harvested and lysed in Laemmli buffer. Cell extracts containing equal amounts of protein (20 μg) were fractionated through SDS-PAGE (10%). 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-IgA (Z2001; Invitrogen), anti–IgM (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 (Pierce/Elmer Life and Analytical Sciences).

Quantitative RT-PCR of mRNAs and miRNAs

For quantification of mRNA, pri-miRNA, germline IGH, and post-recombination IGH, and mature VDJH4-C transcripts, RNA was extracted from 0.2–5 × 106 cells using either RNizol reagent (Invitrogen) or an RNeasy Plus Mini Kit (QiAGEN). Residual DNA was removed from the extracted RNA with gDNA elimation 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 real-time PCR (qRT-PCR) with the MyiQ Real-Time PCR Detection System to measure SYBR Green (IQ SYBR Green Supermix; both from Bio-Rad) incorporation with the following protocol: 95°C for 15 s, 40 cycles of
HDIs UPREGULATE miRNAs TO SILENCE AID AND BLIMP-1

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 × 10^6 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/nuclear RNAs Rnu6/RNU61/2, Snord61/SNORD61, Snord68/SNORD68, and Snord70/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 × 10^7) were treated with formaldehyde (1% w/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), and incubated with anti–acetyl histone H3 mAb (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, 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′UTRs of Aicda mRNA (nt 691–1168 of NM_009645.2, National Center for Biotechnology Information) and Prdm1 mRNA (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 a additional deletion (nt 3680–5101) were deleted by PCR to generate mut 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 900 Ω) with a Gene Pulser II (Bio-Rad). Transfected CH12F3 B cells were then cultured in FBS-RPMI for 1.5 h to allow for reporter gene expression. The ability of VPA to repress reporter activity was determined by 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.mthemailer/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+/p 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+/p 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 (R9-19; 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+/p mice by ELISA, as previously described (14). Titers were expressed in RU, defined as the dilution factor needed to reach 50% of binding. 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+/p mice were either frozen in tissue-Tek 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. 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). For immunofluorescence, 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 NP3-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 V_{H3},D_{H3},C_{y1} transcripts, with no significant alteration in spectrum of the residual mutations (Fig. 1D, 1E, Supplemental Fig. 1A), suggesting that this HDI reduced AID expression to impair the class-switched and hypermutated 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) NP3-binding IgG3, as well as fewer total IgG3* B cells and NP3-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, IgG3, or IgG3 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, IgG3, and IgG3 (Figs. 2A, 2B, 3A–C, Supplemental 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\text{DJH}-C^g1}, V_{H\text{DJH}-C^g3}, V_{H\text{DJH}-C^a}, and V_{H\text{DJH}-C^e} transcripts and postrecombination I_{m-C^g1}, I_{m-C^g3}, I_{m-C^a}, and I_{m-C^e} transcripts in the presence of normal levels of the respective germline I_{H-CH} transcripts, which are necessary for initiation of CSR (Supplemental Fig. 2B–D). Thus, HDIs significantly reduce CSR, without altering B cell viability or proliferation.

**HDIs inhibit plasma cell differentiation but not survival**

HDMI-mediated impairment of Ab responses was not due to alteration of GC development, because GC structure, GC B cell proportion, and B cell viability and proliferation were unaltered in NP-CGG-injected mice treated with VPA (Figs. 3G, 4A), nor did it stem from alteration of the cell cycle, as shown by the normal proportion of B cells in G0/G1, S, or G2/M upon stimulation in vitro (Fig. 4B). Rather, it reflected an HDI-mediated inhibition of B cell
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.

**Aicda, Prdm1, and Xbp-1 are silenced by HDIs**

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 AIID-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.

**HDIs 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 mRNAs by inducing their degradation and/or reducing their translation. In this context, Aicda can be silenced by miR-155, miR-181b, and miR-261 (2, 3, 18, 27–30); Prdm1 can be targeted by miR-23b (our prediction, using...
mRNA 3
Prdm1
in 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 be targeted by miR-30a and miR-125b (3, 18, 31–33) (Supplemental TargetScan.org, miRNA.org, and miRbase.org) and was suggested to 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 mut 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.

**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, respectively, for 7 d in the presence of nil or increasing doses of VPA. (E) IgG1, IgA, and IgG1 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. *p < 0.05, **p < 0.01, ***p < 0.001 unpaired t test.
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 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+/−Prdm−/− 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+/−Prdm−/− 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+/−Prdm−/− 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.
At 12 wk of age, “early” HDI-treated MRL/Faslpr/lpr 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 IgG3; significant reduction (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 B cells, as well as reduced plasma cell differentiation, in the presence of normal B220+ cell populations; and a reduced load of DNA deletions in the IgH locus (Figs. 9A, 9B). Early treated MRL/Faslpr/lpr mice downregulated Aicda, Prdm1, and Bcl6 transcripts in purified B cells treated with nil (0 μM of VPA) or VPA (250 or 1000 μM) and stimulated for 60 h with LPS + CD154 + IL-4, or LPS or CD154 + TGF-β + IL-4 + IL-5 + anti-IgD dextran were measured by qRT-PCR normalized to Cd79b expression. Values for the B cells from HDI (VPA)-treated mice are depicted as relative to the expression of each transcript in the B cells from untreated (nil) mice, set as 1. Data are mean and SEM from at least three pairs of mice. The p values were determined using an unpaired t test.

**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 manner.
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, Ifr4, 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 (CD22+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 VμDJH-Cα (in cells stimulated by CD154, human IL-21, and TGF-β) and VμDJH-Cγ1 and VμDJH-Cε (in cells stimulated with CD154, human IL-4, and human IL-21) 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, and decrease target Aicda and Prdm1 mRNAs, as well as Xbp1 mRNA, in MRL/Fas<sup>+/+</sup> mice. Female MRL/Fas<sup>+/+</sup> mice were started on HDI water or untreated water at the age of 6 wk and analyzed at the age of 12 wk. (A) Titers of circulating anti-dsDNA IgM, IgG, IgG1, and IgG2a and total IgM, IgG1, and IgG2a (n = 5 mice/group). (B) Frequency of somatic point mutations was reduced by 75% in MRL/Fas<sup>+/+</sup> mice given HDI water. A 700-bp intronic J<sub>H</sub>4-iE<sub>M</sub> DNA in CD19<sup>+</sup>Peyer’s patch GC B cells was amplified and sequenced (sequence data were pooled from three mice in each group). Pie charts depict the proportions of sequences that carried 1, 2, 3, and so forth point mutations (center of pie shows the number of independent sequences analyzed). Listed below the pie charts are overall mutation frequencies (changes/base). (C) Proportions of IgG2a<sup>+</sup> B cells and proliferation of spleen B220<sup>+</sup> cells (analyzed by administration of BrdU in drinking water at 10 wk of age). (D) CD19<sup>+</sup>CD138<sup>+</sup> plasma cells in spleens. Data are representative of three independent experiments. (E) Surface CD19 and CD3 expression in spleen cells. Data are from one of three independent experiments yielding comparable results. (F) Aicda and Ung transcripts (in spleen CD19<sup>+</sup>CD138<sup>+</sup> B cells, normalized to Cd79b transcript) and Prdm1 and Xbp1 transcripts (in spleen CD19<sup>+</sup>CD138<sup>+</sup> plasma cells, normalized to Gapdh transcript) were measured by qRT-PCR. Values from mice that were on HDI water are depicted as relative to the expression of each transcript in mice that were on untreated water, set as 1. Data are mean and SEM from four independent experiments. (G) miRNA expression in CD19<sup>+</sup>CD138<sup>+</sup> spleen B cells was measured by qRT-PCR and normalized to expression of small nuclear/nucleolar RNAs RNU6-1/2, SNORD61, SNORD68, and SNORD70. miRNA expression in the B cells from mice that were on HDI water are depicted as relative to the expression of each miRNA in the B cells from mice that were on untreated water, set as 1. Data are mean and SEM from three independent experiments. The p values were determined using an unpaired t test.
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/Fas1pr/lpr 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)

![Figure 11](http://www.jimmunol.org/)

**FIGURE 11.** HDIs reduce autoantibodies, immunopathology, and disease symptoms and extend the lifespan of MRL/Fas1pr/lpr mice. (A–E) Female MRL/Fas1pr/lpr mice were started on HDI water or untreated water at the age of 6 wk and analyzed at the age of 17 wk. (A) Rostral and dorsal images show characteristic lupus of skin lesion, including a “butterfly” rash. (B) Kidney pathology. Representative photomicrographs of kidney sections stained with H&E (left panels) or FITC-labeled rat mAb to mouse IgG1 and IgG2a (right panels). Scale bars, 100 μm. Data are from one of three independent experiments yielding similar results. (C) ANAs visualized by indirect immunofluorescence of HEp-2 cells that were incubated with sera (1:80 dilution) from MRL/Fas1pr/lpr mice using FITC-labeled rat mAb to mouse IgG1 and IgG2a. Scale bars, 20 μm. Data are from one of three independent experiments yielding similar results. (D) Skin lesion score in MRL/Fas1pr/lpr mice (n = 15; 11 of 15 untreated mice, but only 4 of 15 HDI-treated mice, developed skin lesions). (E) Titters of circulating anti-dsDNA IgG1 and IgG2a. (F–I) Fifty female MRL/Fas1pr/lpr mice were on untreated water throughout their lives; 15 male MRL/Fas1pr/lpr mice were on untreated water for the first 6 wk of life, after which they were started on HDI water; and 15 female MRL/Fas1pr/lpr mice were on untreated water for the first 17 wk of life, at which age they were showing active disease (skin lesions, proteinuria, anti-dsDNA autoantibodies) and were started on HDI water. (F) Dorsal images showing severe skin lesions in four female MRL/Fas1pr/lpr mice before (upper panels, 17 wk of age) and after (lower panels, 21 wk of age) 4 wk of HDI administration. (G) Skin lesion scores in 15 female MRL/Fas1pr/lpr mice before (17 wk of age) and after (21 wk of age) 4 wk of HDI administration. (H) Anti-dsDNA IgM, IgG, IgG1, and IgG2a autoantibody titers in female MRL/Fas1pr/lpr mice before (17 wk of age) and after (21 wk of age) 4 wk of HDI administration. (I) Survival of 50 female MRL/Fas1pr/lpr mice given untreated water throughout their lives (black line), 15 female MRL/Fas1pr/lpr mice started on HDI water at 6 wk of age (blue line), and 15 female MRL/Fas1pr/lpr mice started on HDI water at 17 wk of age when they already showed active disease (green line). The p values were determined using an unpaired t test.
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 IgG, IgA, 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 dampening 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.


Supplemental Figure 1
Supplemental Figure 1. HDI inhibit CSR and Aicda expression but do not alter the spectrum of somatic point-mutations as well as viability and apoptosis of B cells and plasma cells in vivo and in vitro. (A) Spectrum of somatic mutations in the V\textsubscript{186.2} region of V\textsubscript{186.2}DJ\textsubscript{H}-C\gamma\textsubscript{1} transcripts from mice that were on HDI-water or untreated water, as in Figure 1E. Values are expressed as the actual numbers of different nucleotide substitutions (top panels) or as the percentage of total point-mutations (bottom panels). (B) Dose-dependent inhibition of CSR and Aicda expression by HDI TSA in B cells. Mouse naïve B cells were stimulated with LPS plus IL-4 in the presence of 0, 10 or 20 nM (0, 3 or 6 ng/ml) TSA. IgG1\textsuperscript{+} B cells were analyzed 4 days after the stimulation. Aicda transcripts were measured by qRT-PCR and normalized to Cd79b expression, 60 hours after the stimulation. Values in B cells cultured in the presence of TSA are depicted as relative to Aicda transcript level in B cells cultured in the absence of TSA, set as 1. (C-F) Mouse naïve B cells were stimulated for 4 days with LPS plus IL-4 in the presence of nil or increased doses of HDI (VPA) to assess viability and apoptosis. Proportions of (C) viable (7-AAD\textsuperscript{-}) or (D) apoptotic (Annexin V\textsuperscript{+}) B220\textsuperscript{+} B cells are indicated. (E) Proportions of Annexin V\textsuperscript{+} B220\textsuperscript{lo}CD138\textsuperscript{+} plasma cells are indicated. (F) Expression of anti-apoptotic genes Bcl2, Mcl1 and Bcl2l1 measured by qRT-PCR and normalized to Gapdh expression. Values in cells treated with VPA are depicted as relative to the expression of each transcript in cells treated with nil, set as 1. Data are presented as mean and SEM from three independent experiments. (G-I) C57BL/6 mice on HDI-water or untreated water were injected with NP\textsubscript{16}-CGG 10 days before analysis. Proportions of (G) 7-AAD\textsuperscript{-} viable cells or Annexin V\textsuperscript{+} apoptotic cells among spleen B220\textsuperscript{lo}CD138\textsuperscript{+} plasma cells are indicated. Expression of Bcl2, Mcl1 and Bcl2l1 transcripts in (H) CD19\textsuperscript{+}CD138\textsuperscript{-} B cells and (I) CD19\textsuperscript{lo}CD138\textsuperscript{+} plasma cells were measured by qRT-PCR and normalized to Gapdh expression. Values in cells treated with HDI or cells isolated from mice that were on HDI-water are depicted as relative to the expression of each transcript in cells cultured in the absence of HDI, or cells isolated from mice that were on untreated water, respectively, set as 1. Data are presented as mean ± SEM from three independent experiments.
**Aicda mRNA 3'UTR**

\[5'...UGAAAGCAACCUCCUGGAAUGUC...GACCGCC\]
\[\text{miR-181b}\]
\[\text{miR-155}\]
\[3'...UGGGUGGCUGUCGUU\]
\[\text{miR-125b}\]
\[\text{miR-30a}\]
\[\text{miR-155}\]

**mut Aicda mRNA 3'UTR**

\[5'...UGAAAGCAACCUCCUGAAGUUUC...AGACGCCG-AGAGCAAGUAG...\]
\[\text{miR-181b}\]
\[\text{miR-155}\]
\[3'...UGGGUGGCUGUCGUU\]

**Prdm1 mRNA 3'UTR**

\[5'...AAUGUUACCAAAAUCUGGCG...GCAAGAUAUAAAAGAGUUAC...\]
\[\text{miR-125b}\]
\[\text{miR-30a}\]
\[\text{miR-23b}\]
\[\text{miR-30a}\]
\[\text{miR-30a}\]

**mut Prdm1 mRNA 3'UTR**

\[5'...AAUGUUACCAAAAUCUGGCG...GCAAGAUAUAAAAGAGUUAC...\]
\[\text{miR-125b}\]
\[\text{miR-30a}\]
\[\text{miR-23b}\]
Supplemental Figure 2. (A-D) HDI-mediated inhibition of CSR as indicated by reduced mature V_HDJ_H-C_H and post-recombination I_H-C_H transcripts in HDI-treated B cells. (A) Purified spleen B cells were labelled with CFSE and stimulated with LPS for 4 days in the presence of nil or HDI (VPA, 500 µM). The percentage of B220^IgG3^ B cells among total B220^ B cells that had completed the same number of divisions when treated with nil or HDI is depicted in the scatter plot. Data are representative of three independent experiments. (B) Germline Iγ3-Cγ3 transcripts in B cells stimulated for 60 hours with LPS in the presence of nil or increasing doses of VPA, measured by qRT-PCR and normalized to Cd79b transcripts (all other germline I_H-C_H transcripts were also normal, not shown). (C and D) B cells were stimulated with LPS plus IL-4 (for IgG1 and IgE), CD154 plus IL-4 (for IgE), LPS (for IgG3), or LPS or CD154 plus TGF-β, IL-4, IL-5 and anti-IgD dextran (for IgA) for 60 hours in the presence of nil or increasing doses of VPA. (C) Mature V_HDJ_H-Cγ1, V_HDJ_H-Cγ3, V_HDJ_H-Cα and V_HDJ_H-Cε transcripts and (D) post-recombination I_H-Cγ1, I_H-Cγ3, I_H-Cα and I_H-Cε transcripts, which are both hallmarks of completed CSR were analyzed by qRT-PCR and normalized to Cd79b transcripts. Data are from three independent experiments (mean and SEM). Values in B cells cultured in medium containing HDI are depicted as relative to the expression of each transcript in B cells cultured in the absence of HDI, set as 1. Data are presented as mean and SEM from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t-test. (E) Alignment of unmaturated and mut Aicda and Prdm1 mRNA 3’UTR sequences used in luciferase reporter assays and the miRNAs that target them. Unmaturated and mut 3’UTRs of the Aicda and Prdm1 mRNA 3’UTRs that were cloned into luciferase vectors for experiments in Figure 7E and 7F are diagramed. Base pairing of miRNAs that target the Aicda and Prdm1 mRNA 3’UTRs is represented by vertical lines. Grey boxes indicate the seed sequence of miRNAs. Mutations to predicted and known miRNA target sites were designed to disrupt miRNA binding to mRNA 3’UTRs and are shown in red. The latter three miRNA target sites in mut Prdm1 mRNA 3’UTR were deleted (rather than mutated).
Supplemental Figure 3. Spectrum and distribution of point-mutations in the intronic J_{H}4-iE_{H} region of MRL/Fas^{lpr/lpr} mice is not altered by HDI. A 700 bp sequences (outlined by pink line) of intronic J_{H}4-iE_{H} DNA in CD19^{+}PNA^{hi} GC B cells from Peyer’s patches of 12-week-old lupus-prone MRL/Fas^{lpr/lpr} mice that were on HDI-water or untreated water (starting at 6 weeks of age, as in Figure 9) were analyzed for nature and distribution of mutations. Red letters above the black germline sequence depict point-mutations in sequences from MRL/Fas^{lpr/lpr} mice that were on untreated water, blue letters below the germline sequence show point-mutations in sequences from mice that were on HDI-water.
<table>
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<tr>
<th>Transcripts Type</th>
<th>Gene/Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Mouse genes</td>
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<td>5'-AGAAAGTCACGCCTGGAGACC-3'</td>
<td>5'-CTCTCTCACCACGTAGCA-3'</td>
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<td>5'-TTGAGGAGAGGACACCTTG-3'</td>
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<td>Il-6</td>
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<td>5'-CCAGGATGCTATGGAATCAGAA-3'</td>
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<td>Prdm1</td>
<td>5'-GCTGCTGGCTCGCTGGTTTG-3'</td>
<td>5'-GGAGAGGAGGGCGCTTCCCCA-3'</td>
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<td>Xbp1</td>
<td>5'-AAGCCCCAGTAGGCGAGCTC-3'</td>
<td>5'-ACGGCCACCACGCTTACC-3'</td>
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<td>5'-CACCCACTACGGACCCGCAC-3'</td>
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<td>Pax5</td>
<td>5'-CCAACAAACCGCAAGAGG-3'</td>
<td>5'-CTGTGGAACAGCTTCCCC-3'</td>
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<td>Rev1</td>
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<td>Bcl2</td>
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<td>Mcl1</td>
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<td>5'-ATGGAGTTAGTGGGAGCAGC-3'</td>
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<td>Iμ-Cγ3</td>
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<td>Mature VδDJβ-Cε transcripts</td>
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<td>VδDJβ-Cα</td>
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<td>VδDJβ-Cε</td>
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<td>miR-181b-5p</td>
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<td>miR-361-5p</td>
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<td>miR-30a-5p</td>
<td>5'-TGTAACATCTCGACTGG-3'</td>
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miR-125b-5p  5'-TCCCTGAGACCCCTAACTTGTA-3'
miR-19a/b-3p  5'-TGTAAGAACTCTATGCAAAACTG-3'
miR-20a-5p  5'-AAAGTGCTTATAGTGCAAGTCT-3'
miR-25-3p  5'-CATCCTTGTCTCGGTTC-3'
Rnu6/RNU6-1/2  5'-GCTTCGGCAGCACATACTAAT-3'
Snord61/SNORD61  5'-CCACTGATCTTCCGACATGA-3'
Snord68/SNORD68  5'-GCTGTACTGACTTGATGA-3'
Snord70/SNORD70  5'-TTTTGGAACTGAATCTAAGTGATTT-3'

Human genes

V_{H} DJ_{H} FR3-C_{Y1}  5'-GACACGCGCYGTRTATTACTGTGCG-3'  5'-AGTAGTCTTTGACCAGGCAC-3'
V_{H} DJ_{H} FR3-C_{ε}  5'-GACACGCGCYGTRTATTACTGTGCG-3'  5'-CGGAGGTTGGCATGGGAAG-3'
V_{H} DJ_{H} FR3-C_{α}  5'-GACACGCGCYGTRTATTACTGTGCG-3'  5'-GTGGGAAGTTTCTGGCGGT-3'
AICDA  5'-CATCTCGGACTGGGACCTAGA-3'  5'-GGTTCCCTCGCAGAAAGTCG-3'
PRDM1  5'-AGCCCTGGGAAATACGGCAG-3'  5'-CGTTGTACGAGGGGATGAAAG-3'
XBP1  5'-GCAAGGCCCCAGTGTCACC-3'  5'-TGCCAGGCTCTGGGGAAGG-3'
HPRT1  5'-TGCTCGAGATGTGTAAGG-3'  5'-TCCCCCTTGATGCTCATT-3'

Somatic mutations

V_{H86.2-C_{γ1}}  5'-CATGCTCTTCTTGGCAGCAACAGC-3'  5'-GTGCAACCGCTGGGACAGGATCC-3'
JX13F-JX4R  5'-AGCCTGACATCTGAGGAC-3'  5'-TCTGATCGGCCATCTTGACTC-3'
JX15F-JX5R  5'-CATCTGAGGACTCTGCNGTCTAT-3'  5'-CCTCACTCCATTCCTCGGTTAAA-3'

miRNA host gene (HG) promoter ChIP

miR-155 HG  5'-AAGGTCATGAGTTCAAGGCCAGC-3'  5'-TGTCACACCGCTGGGACAGGATCC-3'
miR-181b HG  5'-GAGGTTGAATTTCCAGGAATGAG-3'  5'-ACTGCAAGGACCGCTGGGACAGGATCC-3'
miR-361 HG  5'-ACATGCGCTTTGCTTGAG-3'  5'-GGAGGTCAGTTATGGAGG-3'
miR-23b HG  5'-AGCTGTACCTGCCACCC-3'  5'-AAGCCCACTGATGGACAGC-3'
miR-30a HG  5'-TGCAAAAGACTAGTGGTGCTC-3'  5'-GTGAAAGGTGATGTGGTGG-3'
miR-125b HG-1  5'-TCTCTCCCGAGTCAATCTGCT-3'  5'-ACAGCCCTGTATATGGACACAC-3'
miR-125b HG-2  5'-CGTGTGCAGCTCCTCCAGT-3'  5'-ATCGAAGGACCGACAC-3'
Bisulfite PCR

Aicda promoter  5'-TGATTTTTGTTATTTTGGAATT-3'  5'-TACTCTTATAAAACTCTC-3'