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Overexpression of Activation-Induced Cytidine Deaminase in B Cells Is Associated with Production of Highly Pathogenic Autoantibodies¹

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Defective receptor editing or defective B cell checkpoints have been associated with increased frequency of multireactive autoantibodies in autoimmune disease. However, Ig somatic hypermutation and/or class switch recombination may be mechanisms enabling the development of pathogenic multireactive autoantibodies. In this study, we report that, in the BXD2 mouse model of autoimmune disease, elevated expression of activation-induced cytidine deaminase (AID) in recirculating follicular CD86⁺ subsets of B cells and increased germinal center B cell activity are associated with the production of pathogenic multireactive autoantibodies. CD4 T cells from BXD2 mice that expressed increased levels of CD28 and an increased proliferative response to anti-CD3 and anti-CD28 stimulation are required for this process. Inhibition of the CD28-CD86 interaction in BXD2 mice with AdCTLA4-Ig resulted in normalization of AID in the B cells and suppression of IgG autoantibodies. This treatment also prevented the development of germinal center autoantibody-producing B cells, suggesting that an optimal microenvironment enabling AID function is important for the formation of pathogenic autoantibodies. Taken together, our data indicate that AID expression in B cells is a promising therapeutic target for the treatment of autoimmune diseases and that suppression of this gene may be a molecular target of CTLA4-Ig therapy. *The Journal of Immunology*, 2007, 178: 5357–5365.

During establishment of self-tolerance, the self-reactive naive B cells are eliminated at two check points (1). Defects in these checkpoints have been shown to be associated with development of multireactive autoantibodies (autoantibodies) in the mature naive compartment of B cells in patients with systemic lupus erythematosus (SLE)³ (2) or rheumatoid arthritis (RA) (3). The self-reactive naive B cells do not produce pathogenic autoantibodies. In contrast, it is the Ig-secreting plasma cells and memory B cells that develop from the naive precursors after they are activated by Ag and have undergone further somatic hypermutation (SHM) and/or class switch recombination (CSR) that produce the pathogenic autoantibodies (4).

The BXD2 strain is one of the first set of BXD recombinant inbred strains of mice that were generated originally by Dr. B. A. Taylor (The Jackson Laboratory, Bar Harbor, ME) by inbreeding the intercross progeny of a cross between C57BL/6J and DBA/2J strains for >20 generations (5, 6). During the course of a survey to discover genetic loci that influence T cell senescence using a set of 20 of the BXD recombinant inbred strains, we noted an age-related development and progression of severe spontaneous arthritis in this strain (7). Other autoimmune hallmarks of BXD2 mice include the development of glomerulonephritis and high titers of immune complexes (7, 8). The autoantibody repertoire in a BXD2 mouse undergoes constant alteration throughout its life time. One-month-old BXD2 mice predominantly produce autoantibodies that are not multireactive and have a limited number of target autoantigens (autoantigens) including Ro/SSA (8). By 8 mo of age, there was a broad spectrum of IgG autoantibodies to multiple autoantigens. The onset of kidney and joint diseases correlates with an increase in the sera titers of circulating immune complexes and an increase in the autoantibodies targeting nucleosome proteins, metabolic enzymes, structural proteins, and heat shock proteins (HSPs) (8). We have previously produced 96 hybridoma clones from a BXD2 mouse with both arthritis and renal disease and observed that the pathogenicity of the individual autoantibody could act independently of CD4 T cells and was correlated with their ability to recognize multiple autoantigens (8).

In both humans and mice, Igs undergo SHM and CSR (4) in the germinal center (GC) through a process that is dependent on the activity of activation-induced cytidine deaminase (AID) in the B cells and is independent of RAG1 or RAG2 activity (9, 10). In normal mice, increased expression of AID mRNA can be detected after immunization and its expression is primarily in the GCs (9). Ectopic expression of AID in fibroblasts is sufficient to induce

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³ Abbreviations used in this paper: SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; SHM, somatic hypermutation; CSR, class switch recombination; HSP, heat shock protein; GC, germinal center; AID, activation-induced cytidine deamination; FWR, framework region; SA, streptavidin; PNA, peanut lectin agglutinin; MZ, marginal zone; FO, follicular; FM, follicular mature; SMLR, syngeneic mixed lymphocyte response.

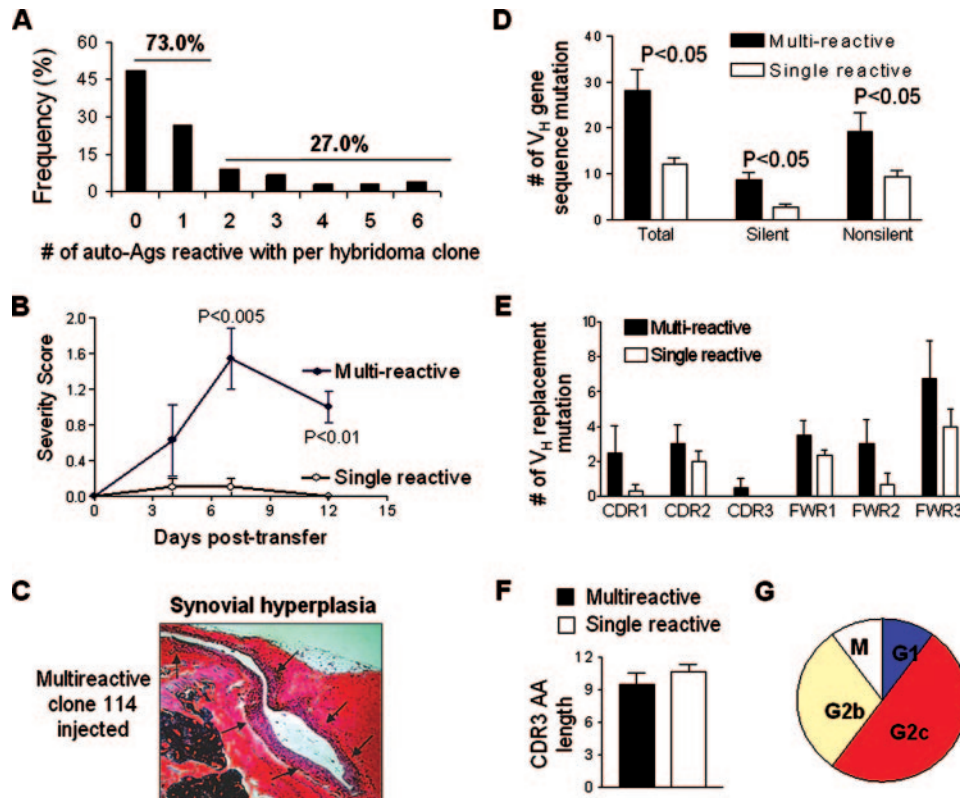


FIGURE 1. Increased multireactive autoantibodies with increased SHM and CSR in BXD2 mice. Hybridomas were generated from a 10-mo-old BXD2 mouse. **A**, Supernatants from all 213 clones of hybridomas were used as the primary Abs to detect the production of multireactive autoantibodies. Negative controls include sera from B6 mice and hybridoma culture supernatant obtained from the GK1.5 (anti-CD4) hybridoma. The ELISA OD value >2-fold of that obtained from the negative controls was considered as positive. The frequency of hybridomas that produce the IgM plus IgG isotype of autoantibodies that were reactive to different number of autoantigens was determined. **B**, Cloned hybridomas (six multireactive and three single reactive) were injected individually to Balb/c nude mice ($n = 3/\text{clone}$). Arthritis severity score in recipient mice was determined at the indicated time point following the injection. **C**, Gross anatomy and H&E staining (magnification, $\times 80$) of knee joint arthritis in a representative Balb/c nude mouse that was injected with hybridoma clone 114 at day 12. **D**, The frequency of mutations was calculated by the number of nonsilent (replacement) and silent nucleotide exchanges per base pair in the whole V_H sequence in multireactive ($n = 6$) vs single-reactive ($n = 3$) autoantibodies (mean \pm SEM). **E**, The average of nucleotide exchanges per base pair resulting in amino acid replacement mutation in each indicated FWR and CDR, comparing with the germline gene for multireactive vs single reactive Abs (mean \pm SEM). **F**, The average length of the CDR3 sequence in multireactive ($n = 6$) vs single-reactive ($n = 3$) autoantibodies. **G**, The frequency of different isotypes from the 10 most multireactive hybridomas (each reacts to more than or equal to five autoantigens) is shown as a pie chart.

SHM and CSR (11, 12), suggesting that AID is an important B cell-specific factor that is required for SHM and CSR.

In the present study, we analyzed the expression of AID in the B cells of BXD2 mice and found that the production of pathogenic IgG autoantibodies in these mice is associated with overexpression of AID and increased cycling GC B cells that exhibit high expression of CD86. This overexpression of AID could be induced in B cells by the CD28 costimulatory signal provided by CD4⁺ T cells of BXD2 mice. Administration of a single dose of AdCTLA4-Ig before the onset of disease led to both acute and chronic suppression of AID expression in B cells. This treatment also prevented the development of autoantibodies in BXD2 mice.

Materials and Methods

Mice

Female C57BL/6 and female BXD2 recombinant inbred strain of mice were obtained from The Jackson Laboratory. All mice were housed in the University of Alabama at Birmingham (UAB) mouse facility in specific pathogen-free conditions in a room equipped with an air-filtering system. The cages, bedding, water, and food were sterilized. All animal procedures were approved by UAB Institutional Animal Care and Use Committee.

Generation of hybridomas

Spleen cell fusion and hybridoma selection were performed using a standard protocol (13) by mixing BALB/c (H2^d) P3X63-Ag8.653 myeloma cells (American Type Culture Collection (ATCC)) with the spleen cells from a 10-mo-old female autoimmune BXD2 mouse that had developed both lupus and arthritis. Fused cells were suspended in hypoxanthine-aminopterin-thymidine medium and plated in 24-well plates. Supernatants from the 24-well plates were collected and tested for Ag binding by Western blot analysis. Wells containing Abs that recognized autoantigens were cloned by limiting dilution at 1, 0.5, and 0.25 cells/well. A total of 213 clones from positive wells were subjected to an additional round of limiting dilution subcloning and retested for reactivity to multiple autoantigens using an ELISA test.

Nine different hybridoma clones (six multireactive and three single reactive) were separately injected i.p. (10^7 cells/mouse) into Balb/c nude mice, which were analyzed at different time points for development of arthritis using the methods we previously described (7, 8).

Cloning and sequencing of V region genes

RNA was isolated from 10^7 hybridoma cells using the TRIzol reagent (Invitrogen Life Technologies). Following first-strand cDNA synthesis (Fermentas), the V_H genes were amplified by PCR with a primer set synthesized according to sequences provided (14). For the V_H genes, a set of 10 forward primers were used. The sequences of these primer pairs are: $V_H\text{fr}1\text{a}$: 5'-GTAAGACGTCCAGCTTCAGGAGTCAGGACC-3'; $V_H\text{fr}1\text{b}$: 5'-GTAA

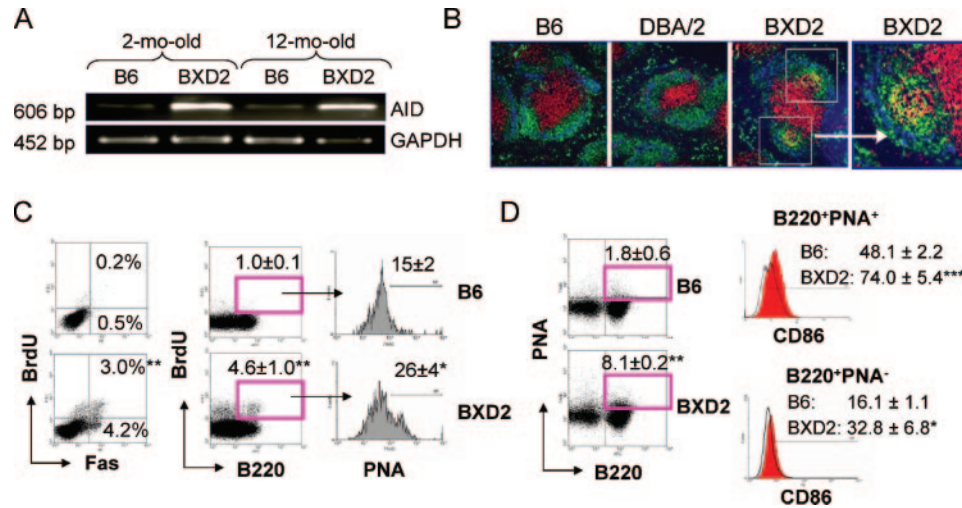


FIGURE 2. Increased cycling CD86⁺ GC B cells and AID expression in BXD2 mice. *A*, The expression of AID in purified unfractionated B cells from the spleen of 2- and 12-mo-old B6 and BXD2 mice was determined using RT-PCT. *B*, Spleens were obtained from 7-mo-old B6, DBA/2, or BXD2 mice. Immunohistochemistry analysis using a MOMA-1 (Rat, IgG2a, κ) developed with goat anti-rat IgG-AMCA (blue), a FITC-conjugated (green) anti-IgM, and a PE-conjugated (red) anti-CD4 plus anti-CD8. *C*, Single-cell suspensions prepared from the spleens of BrdU injected B6 and BXD2 mice (3 mo old) were stained with allophycocyanin-anti-B220, FITC-BrdU, PE-anti-Fas, or PE-anti-CD86, and biotin-anti-PNA followed by a secondary PE-Cy5 SA staining. The percentage of B cells that is Fas⁺BrdU⁺ or BudU⁺ for both strains is indicated (**, $p < 0.01$, $n = 5$). The percentage of PNA⁺ cells within the BrdU⁺B220⁺ cells (gated area) is further shown by a histogram for each strain (*, $p < 0.05$, $n = 5$). *D*, The expression of CD86 on either B220⁺PNA⁺ or B220⁺PNA⁻ cells from B6 (open area) and BXD2 (filled area) mice is shown (*, $p < 0.05$ or ***, $p < 0.005$, $n = 5$).

GACGTCCAGTGAAGGAGTCAGGACC-3'; V_Hfr1c: 5'-ATATGACGTC ATGCTGGTGGAGTCTGGGGGA-3'; V_Hfr1d: 5'-ATATGACGTC AAGC TGGTGGAATCTGGAGGA-3'; V_Hfr1e: 5'-ATATGACGTC AAGCTGGT GGAGTCTGGAGG-3'; V_Hfr1f: 5'-AATTGACGTC CAGCTGCAGCAGCC TGGG-3'; V_Hfr1g: 5'-AATTGACGTC CAGCTGCAGCAGTCTGGA-3'; V_Hfr1h: 5'-AATTGACGTC CAGCTGCAGCAGTCTGTG-3'; V_Hfr1i: 5'-AA TTGACGTC CAGCTGTGGAGACTGGAGG-3'; and V_Hfr1j: 5'-AATTGA CGTCCAGTGCAGCAGCCTGG-3'. The sequence of the reverse primer for the γ gene is 5'-ATTAAGTCGACCTTGGTGTCTGCTGGCCGGGTG-3'. Amplification was performed for 25 cycles (denaturation at 94°C for 1 min; annealing at 55°C for 1 min; primer extension at 72°C for 1 min; final incubation at 72°C for 10 min) in 100- μ l reaction volume containing 5 U of *Taq* polymerase (Promega) and 1.5 mM MgCl₂. Amplified products were excised from 1% agarose gels and purified with a QIAquick Gel Extraction kit (Qiagen). Purified products were ligated into pGEM-T Easy vector (Promega) and the mixture used to transform *Escherichia coli* XL-1 blue (Stratagene). Plasmids were isolated from positive bacterial colonies using a QIAprep Spin Miniprep kit (Qiagen) and those containing V_H genes were identified by *Eco*RI digestion. The sequence of each V_H gene was compared with the corresponding germline gene using the IMGT immunogenetics database (<http://imgt.cines.fr>) (15, 16). The frequency of mutation was calculated by the frequency of amino acid exchanges and the ratio of replacement vs silent nucleotide exchanges per base pair in each CDR and framework region (FWR).

RT-PCR analysis of AID

B cells were isolated from the spleens of B6 or BXD2 mice using a positive selection column (Miltenyi Biotec). RNA was isolated from 2 to 10 $\times 10^6$ purified splenic B cells using the TRIzol reagent. The isolated RNA was converted to cDNA using the First Strand cDNA Synthesis kit (Fermentas). The expression of AID was determined by RT-PCR according to the method of Jabara et al. (17). PCR conditions to detect AID were: 4 min denaturation at 95°C, amplification of cDNA for 36 cycles, each cycle programmed for denaturation at 94°C for 45 s, annealing at 46°C for 1 min and elongation at 72°C for 1.5 min, followed by a final extension phase of 10 min at 72°C. Primers were: AID (forward, 5'-GGA GAC CGA TAT GGA CAG CCT TCT G-3'; reverse, 5'-TCA AAA TCC CAA CAT ACG AAA TGC-3') as used by Jabara et al. (17). GAPDH (forward, 5'-ACC ACA GTC CAT GCC ATC AC-3'; reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3') was used as loading control. PCR conditions for GAPDH were 4 min denaturation at 95°C, amplification of cDNA for 30 cycles, each cycle programmed for denaturation at 95°C for 2 min, annealing at 60°C for 2 min and elongation at 72°C for 2 min, followed by a final extension phase of 3 min at 72°C. Sizes of the detected PCR products were 606 and 452 bp for AID and GAPDH, respectively.

Immunofluorescence and immunohistochemical analysis of tissue sections

For analysis of histology of the spleen, frozen sections were processed and stained as described by Martin et al. (18). Briefly, frozen spleen sections were stained with MOMA-1 (rat, IgG2a, κ), detected with goat anti-rat IgG-7-amino-4-methylcoumarin-3-acetic acid (AMCA; Jackson ImmunoResearch Laboratories), blocked with normal rat serum (Peel-Freeze), and washed and stained with a FITC-conjugated goat anti-mouse-IgM, and a mixture of biotin-anti-CD4 and biotin-anti-CD8, detected with PE-conjugated streptavidin (SA; Vector Laboratories).

Proliferation in vivo

Mice were injected i.p. with 1 mg of BrdU in a volume of 0.2 ml (BrdU Flow kit; BD Biosciences-BD Pharmingen) or 0.2 ml of buffered saline (PBS) as control and were sacrificed 1 h later. After cell preparation and counting, 2.0 $\times 10^6$ cells were stained for B220, CD80, CD86, CD95, peanut lectin agglutinin (PNA), and BrdU (anti-BrdU-FITC) according to the BrdU Flow kit manual (BD Biosciences-BD Pharmingen).

FACS sorting and flow cytometry analysis

FITC-conjugated anti-CD21 (clone 7G6) and anti-BrdU, PE-conjugated anti-CD23 (clone B3B4) and anti-CD95 (clone Jo2) were purchased from BD Pharmingen. PE-conjugated anti-CD19 (clone 6D5), anti-CD86 (clone GL-1), anti-CD28 (clone 37.51), and anti-CD69 (H1.2F3), allophycocyanin-conjugated anti-B220 (RA3-6B2) and anti-CD4 (GK1.5), and SA-Cy5 were purchased from Biolegend. Three- and four-color surface labeling was performed as we described previously (19). After cell surface staining, cells were either washed with PBS for FACS sorting of different subpopulations of B cells, or washed twice with FACS buffer and fixed in 1% paraformaldehyde/FACS solution for cell surface marker analysis. Cells (50,000–100,000/sample) were analyzed by flow cytometry on a FACScan (BD Biosciences). The analysis was performed using WinMDI Software (Trotter, The Scripps Research Institute, La Jolla, CA). Forward angle light scatter was used to exclude dead and aggregated cells. The results are presented as fluorescence histograms with the relative number of cells on a linear scale plotted vs the relative fluorescence intensity on a log scale.

Isolation and stimulation of B cells and CD4⁺ T cells

B cells or CD4⁺ T cells were isolated from the spleens of B6 or BXD2 mice using a B cell isolation kit (Miltenyi Biotec) or anti-CD4 Microbeads (Miltenyi Biotec) according to the MiniMacs protocol. Mixed syngeneic lymphocytes or CD4⁺ T cells were stimulated with anti-CD3 (clone

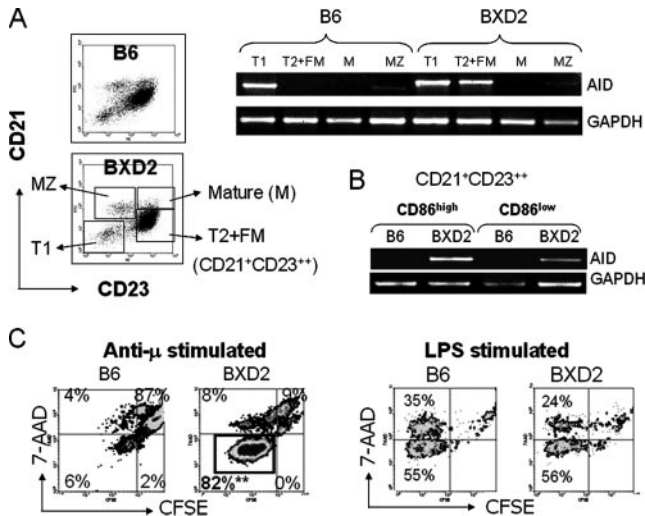


FIGURE 3. Increased AID expression in T2+FM B cells and defective anti-mu induced B cell apoptosis in BXD2 mice. **A**, B cells were purified from the spleen of a total of five 7-mo-old B6 and BXD2 mice. The purified B cells were stained with FITC-anti-CD21 and PE-anti-CD23. Different subpopulations of B cells were separated by FACS sorting based on the differential expression of CD21 and CD23 (T1, transition 1, T2+FM, transition 2, and follicular mature; and M, mature) (59). RNA was then isolated from each subpopulation of B cells for determination of the expression of AID using RT-PCR. **B**, CD86^{high} or CD86^{low} cells were sorted from recirculating CD21⁺CD23⁺⁺ purified B cells from 7-mo-old (five mice per group). The expression of AID and GAPDH was determined using RT-PCR. **C**, Decreased anti-mu-, but not LPS-, induced B cell apoptosis in BXD2 mice. Single-cell suspensions prepared from 2-mo-old B6 and BXD2 mice were labeled with CFSE and stimulated with either anti-mu (10 μ g/ml) or LPS (10 μ g/ml). After 5 days in culture at 37°C, proliferation (CFSE^{low}) and apoptosis (7-AAD^{high}) of B cells (gated within the PE-CD19⁺ cell population) were determined using FACS analysis (mean \pm SEM, $n = 5$ /group, **, $p < 0.01$).

145-2C11, 1 μ g/ml) plus anti-CD28 (clone 37.51, 10 μ g/ml) in the same culture medium with or without mouse CTLA4-Fc (1 μ g/ml; Axxora) for 48 h and were pulsed with [³H]thymidine (1 μ Ci/well; Amersham Biosciences) for the last 12 h. At the end of stimulation, the culture supernatants were collected, and cells were washed and counted.

For CD4 and B cell coculture experiments, 2×10^6 anti-CD3/anti-CD28 stimulated CD4⁺ T cells from either B6 or BXD2 mice were incubated with 5×10^6 B cells. B cells were isolated again by positive selection 60 h later. This procedure routinely yielded B cell preparations which were >95% CD19. RNA was isolated for determination of AID using RT-PCR as described above.

To analyze apoptosis of cycling B cells, single-cell suspensions prepared from B6 or BXD2 mice were labeled with 5 μ M CFSE (Molecular Probes) in PBS plus 5% FBS for 5 min at 37°C in dark, and washed three times with 5 ml of RPMI 1640–10% FBS. These labeled cells were then stimulated with either LPS (10 μ g/ml; Sigma/Aldrich) or F(ab')₂ of goat-anti-mouse IgM (μ -chain; Jackson ImmunoResearch Laboratories) (10 μ g/ml). After 5 days in culture at 37°C, proliferation (CFSE^{low}) and apoptosis (7-AAD^{high}) of B cells (PE-CD19⁺ cell population) were determined using FACS analysis.

Construction of rAd vectors, viral delivery, and analysis of expression

Adenovirus-expressing mouse CTLA4-mouse IgG3 (AdCTLA4-Ig) is a gift from Drs. P. Robbins and L. Lu (University of Pittsburgh Medical Center, Pittsburgh, PA) (20). AdCTLA4-Ig or control AdLacZ (10^9 PFU/mouse) was delivered through tail vein injection to BXD2 mice at different ages at one single dose (three to eight mice per group). Sera levels of CTLA4-Ig were analyzed by ELISA using an anti-mouse CTLA4 Ab (Biolegend) as the capture Ab and an HRP-conjugated goat-anti-mouse IgG3 as the detection Ab (Southern Biotechnology Associates).

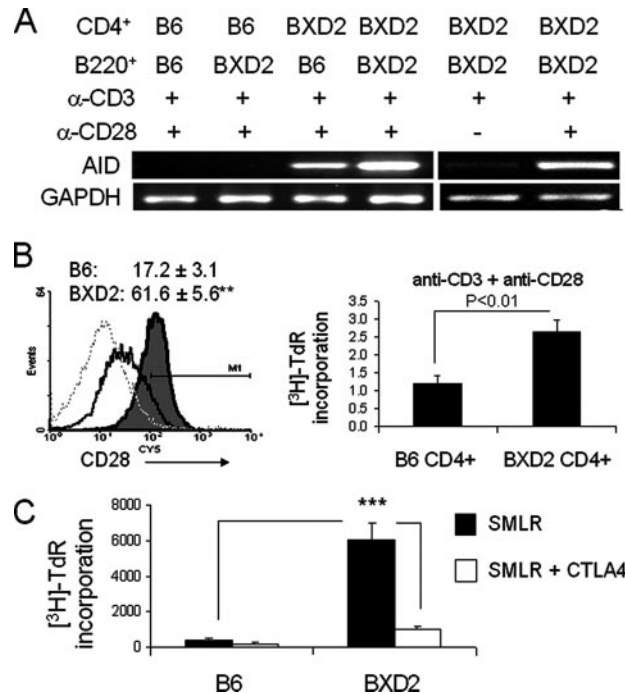


FIGURE 4. Increased costimulatory signals from CD4⁺ T cells of BXD2 mice. **A**, CD4⁺ T cells and B cells were purified from a total of five 7-mo-old B6 and five age-matched BXD2 mice. Cells were cultured under the indicated CD4⁺ T cells (2×10^6 cells) and B cells (5×10^6 cells) mixture condition. B cells were enriched after a 60-h coculture. RNA was isolated from B cells for determination of AID using RT-PCR. **B**, Single-cell suspensions prepared from the spleen of 2-mo-old B6 (open area) or BXD2 (shaded area) mice were stained for allophycocyanin-anti-CD4 and biotin-CD28 plus a secondary PE-Cy5 conjugated SA staining. Numbers (mean \pm SEM) indicate the percentage of cells in the gated area (**, $p < 0.01$; $n = 4$). CD4⁺ T cells purified from 2-mo-old mice were stimulated with anti-CD3 + anti-CD28. The proliferative response at day 2 after stimulation was determined using a [³H]thymidine method. **C**, Syngeneic mixed lymphocytes from the spleen (10^6) of indicated mice were cultured in the presence and absence of the CTLA4-Ig (1 μ g/ml). Proliferation was determined at the 48 h time point by a 12-h pulse of 1 μ Ci [³H]TdR. ***, $p < 0.005$ ($n = 4$).

ELISA quantification of autoantibodies

The reactivity of BXD2 sera or different hybridomas to different autoantigens was quantified using an ELISA. Each ELISA well was coated with 5 μ g/ml of the tested autoantigen (all HSP proteins were purchased from Stressgen-Assay Designs). All other protein autoantigens were purchased from Sigma-Aldrich. ELISAs were developed with either an HRP-labeled goat anti-mouse IgG or a goat anti-mouse IgM Ab (Southern Biotechnology Associates) and tetramethylbenzidine substrate (Sigma-Aldrich). OD₄₅₀ was measured on an Emax Microplate reader.

ELISPOT quantitation of autoantibody-producing B cells

To determine the frequency of autoantibody-producing B cells in the spleen, ELISPOT assay was performed using a protocol that we previously described with modification (21). Polyvinylidene difluoride-backed plates (96-well) (Millipore) were coated overnight with 5 μ g/ml of the indicated autoantigen at 4°C, washed, and then blocked with complete medium. B cells isolated from spleens of AdLacZ or AdCTLA4-Ig treated mice were washed and adjusted to a final volume of 200 μ l containing 2×10^5 cells/well. After 4 h of incubation, cells were washed three times with PBS/0.05% Tween 20, before overnight incubation at 4°C with 1 μ g/ml biotinylated goat anti-mouse IgM mAb (Southern Biotechnology Associates) or biotinylated goat anti-mouse IgG2b (Southern Biotechnology Associates) plus biotinylated goat anti-mouse IgG2c mAb (Southern Biotechnology Associates) in PBS/10% FCS. Plates were washed three times with PBS/0.05% Tween 20, before 40 ng of HRP-conjugated goat anti-biotin Ab (Vector Laboratories) was added, in 100 μ l of PBS/10% FCS, to each well.

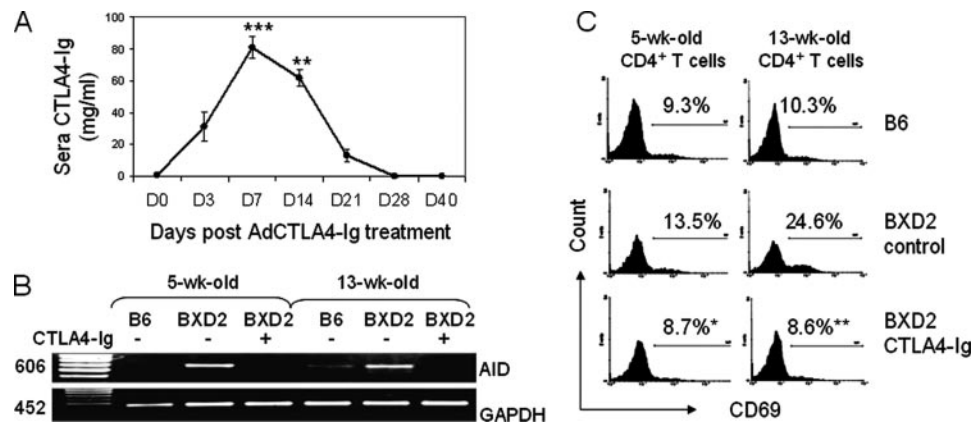


FIGURE 5. CTLA4-Ig suppressed AID in B cells and activation of CD4 T cells in BXD2 mice. *A*, AdCTLA4-Ig (1×10^9 PFU/mouse) was injected to 4-wk-old BXD2 mice and sera levels of CTLA4-Ig at the indicated time points were determined using a sandwich ELISA method. The CTLA4-Ig levels in each sample were quantitated by comparison of the absorbance value of the sample with that of the control CTLA4-Ig protein (mean \pm SEM, $n = 5$ /group, ** and *** indicates $p < 0.01$ and $p < 0.005$, respectively, compared with results obtained from the day 0 time point). *B*, The expression of AID and control GAPDH in purified B cells of 5- and 13 wk-old control B6 mice, AdLacZ-treated, or AdCTLA4-Ig-treated BXD2 mice at 4 and 12 wk of age was determined by RT-PCR. *C*, The expression of CD69 on CD4⁺ T cells in the spleen of indicated groups of mice was determined by flow cytometry. Numbers indicate the percentage of CD69⁺ cells in gated CD4⁺ T cells (* and ** indicate $p < 0.05$ and $p < 0.01$, respectively, comparing results from AdCTLA4-Ig-treated BXD2 vs those from AdLacZ-treated BXD2 mice, $n = 5$ /group).

Plates were incubated at room temperature for 1 h and washed with PBS/0.05% Tween 20 and then with PBS alone before spots were developed using 3-amino-9-ethylcarbazole. Plates were read on a CTL automatic ELISPOT reader and analyzed using Immunospot 3.1 software (CTL). All results were repeated in triplicate.

Statistical analysis

A two-tailed *t* test was used for statistical analysis of the expression of cell surface makers and the proliferative response of T and B cells. Significant differences in treatment effects of AdCTLA4-Ig vs AdLacZ at any one time point were determined by the Fisher exact test. Values of $p < 0.05$ were considered significant.

Results

Increased SHM and CSR of autoantibodies in BXD2 mice

We have previously shown that BXD2 mice developed significantly higher titers of autoantibodies compared with that of age-, H2-, and *IgH* locus-matched B6 and BXD19 strains of mice (8). One distinctive feature of the pathogenic autoantibodies produced by autoimmune BXD2 mice is that they can induce renal disease and/or arthritis and are multireactive. To characterize the frequency of multireactive autoantibodies, we have generated 213 subclones of hybridomas. The reactivity of the mAb produced from each hybridoma clone to a panel of six autoantigens that are commonly recognized by autoantibodies of BXD2 mice was determined. These autoantigens include DNA, histone, nitrotyrosine-modified enolase, BiP (HSPa5), HSP72, and Ro52 (8). Approximately 27% of cloned hybridomas (58 of 213 clones) isolated from BXD2 mice produced multireactive autoantibodies (Fig. 1*A*). There was a significant increase in the severity of arthritis induced in Balb/c nude mice after injection of cloned multireactive autoantibody hybridomas, compared with injection of single-reactive autoantibody hybridomas (Fig. 1, *B* and *C*).

Because the pathogenicity of BXD2 autoantibodies appears to be associated with their multireactivity, we determined whether multireactive autoantibodies exhibit increased SHM in the V_H genes compared with Ab genes from single reactive autoantibodies from the same mouse. There was a 2- to 3-fold increase in the number of total mutations in multireactive compared with single-reactive clones with a 2- to 3-fold increase in both silent and non-silent (replacement) mutations (Fig. 1*D*). In all but one of the mul-

tireactive clones analyzed, the number of replacement mutations were higher than the number of silent mutations. The frequency of replacement mutations was higher in the CDR1 of the multireactive clones compared with single-reactive clones (Fig. 1*E*). However, the length of CDR3 was equivalent between multireactive and single-reactive clones (Fig. 1*F*). Unlike most natural multireactive autoantibodies that are of the IgM isotype, the majority of multireactive autoantibodies from BXD2 mice have undergone CSR to the IgG isotype (Fig. 1*G*). These data suggest that abnormal SHM and CSR may contribute to the development of pathogenic multireactive autoantibodies in BXD2 mice.

Increased expression of AID and PNA⁺ GC B cells of BXD2 mice

Based on the premise that abnormal SHM and CSR occur, leading to the development of pathogenic autoantibodies in BXD2 mice, we speculated that up-regulated AID in B cells may be an important pathogenic mechanism. Higher levels of AID mRNA were observed in purified B cells from the spleens of 2- and 12-mo-old BXD2 mice compared with B cells from control C57BL/6 (B6) mice (Fig. 2*A*). Histological analysis of the spleen of BXD2 mice revealed spontaneous formation of multiple GC-like structures exhibiting close contact of T (red), B (green), and metallophilic macrophages (blue) which were not observed in the spleen of normal parental B6 and DBA/2 mice (Fig. 2*B*).

As GC B cells that exhibit high proliferation may accumulate higher numbers of mutations, we used FACS analysis to determine whether there were increased proliferating GC B cells in BXD2 mice. There was an increase in the percentage of Fas⁺ GC B cells and BrdU⁺-proliferating PNA⁺ GC B cells in the spleen of BXD2 mice compared with B6 mice (Fig. 2*C*). There was also an increase in the expression of CD86 on both B220⁺PNA⁺ B cells and B220⁺ PNA⁻ B cells of BXD2 mice compared with their counterparts from B6 mice (Fig. 2*D*), whereas CD80 expression was not increased on B cells of BXD2 mice (data not shown).

Increased AID in CD21⁺CD23⁺⁺CD86⁺ B cells in BXD2 mice

Ig maturation can occur in recirculating CD21⁺CD23⁺⁺ B cells located in follicles, and nonrecirculating CD21⁺CD23^{+/-} B cells enriched mainly in the marginal zone (MZ) of the spleen (22,

23). Follicular (FO) B cells contain both the transitional stage 2 (T2) and FO mature (FM) B cells that are involved in T-dependent Ab responses leading to generation of memory and plasma cells (23, 24). Analysis of the expression of AID in fractionated spleen B cells revealed that there was higher expression of AID in the (CD21⁺CD23⁺⁺) FO B cells from BXD2 mice than in the same population of cells obtained from control B6 mice (Fig. 3A), suggesting that CD4 T cells are important for the up-regulation of AID. The expression of AID was, however, equivalent in the BXD2 mice and the B6 mice in the earliest T1 compartment of the B cells (CD21⁻CD23⁻) and the MZ B cells (10) and was not detectable in the mature B cells from either strain (Fig. 3A). In BXD2 mice, AID expression in CD86^{high} B cells isolated from the recirculating CD21⁺CD23⁺⁺ FO B cell pool expressed higher levels of AID than the CD86^{low} B cells isolated from the same pool (Fig. 3B); however, the expression of AID was low in both CD86⁺CD21⁺CD23⁺⁺ and CD86⁻CD21⁺CD23⁺⁺ B cells from normal B6 mice (Fig. 3B). Thus, high AID expression is found in CD86^{high} B cells at a T-dependent stage of development in BXD2 mice.

To determine whether there was a defect in apoptosis after Ab cross-linking of B cells from BXD2 mice, B cells from B6 and BXD2 mice were treated with anti- μ or LPS *in vitro*. There was a decrease in apoptosis of BXD2 B cells compared with B6 B cells after treatment with anti- μ , but not after stimulation with LPS (Fig. 3C). This apoptosis defect of B cells in response to anti- μ may enable survival of B cells that have undergone extensive SHM and CSR (25).

CD4 T cells promote increased AID in GC B cells of BXD2 mice

To demonstrate that activated CD4 T cells from BXD2 mice exhibit an increased ability to stimulate the expression of AID in B cells, we have used a four-way coculture experiment. B cells were purified by positive selection after 60-h coculture with the T cells. Anti-CD3 plus anti-CD28 stimulation of CD4⁺ T cells from BXD2 mice induced expression of AID in B cells from either B6 or BXD2 mice (Fig. 4A, left panel). In contrast, coculture of stimulated CD4⁺ T cells from B6 mice did not induce detectable levels of AID in B cells from either B6 or BXD2 mice (Fig. 4A, right panel). In the absence of CD28 signaling, T cells from BXD2 mice were not able to stimulate AID expression in B cells from BXD2 mice (Fig. 4A, right panel). Compared with the CD4⁺ T cells from age-matched B6 mice, the CD4⁺ T cells from 2-mo-old BXD2 mice expressed significantly higher levels of CD28 (Fig. 4B, left panel). On stimulation *in vitro* with anti-CD3 and anti-CD28, the CD4⁺ T cells from BXD2 mice exhibited a significantly higher proliferative response (Fig. 4B, right panel). The syngeneic mixed lymphocyte response (SMLR) of lymphocytes obtained from 2-mo-old BXD2 mice is significantly higher than that of lymphocytes obtained from control mice and is abrogated by addition of CTLA4-Ig to the culture medium (Fig. 4C). Taken together, these results suggest that the high expression of AID in GC B cells is associated with external signals provided by anti-CD3 plus anti-CD28-stimulated CD4⁺ T cells.

CTLA4-Ig blocks AID expression *in vivo*

To test whether the CD28-CD80/CD86 interactions enhance AID expression in B cells *in vivo*, groups of BXD2 mice at 4 and 12 wk of age were administered a single dose of an adenovirus-expressing CTLA4-Ig (AdCTLA4-Ig, 1×10^9 PFU per mouse) or control AdLacZ. Administration of AdCTLA4-Ig resulted in high levels of circulating CTLA4-Ig at day 7 following the injection (Fig. 5A). The levels of AID in B cells of AdCTLA4-Ig-treated BXD2 mice

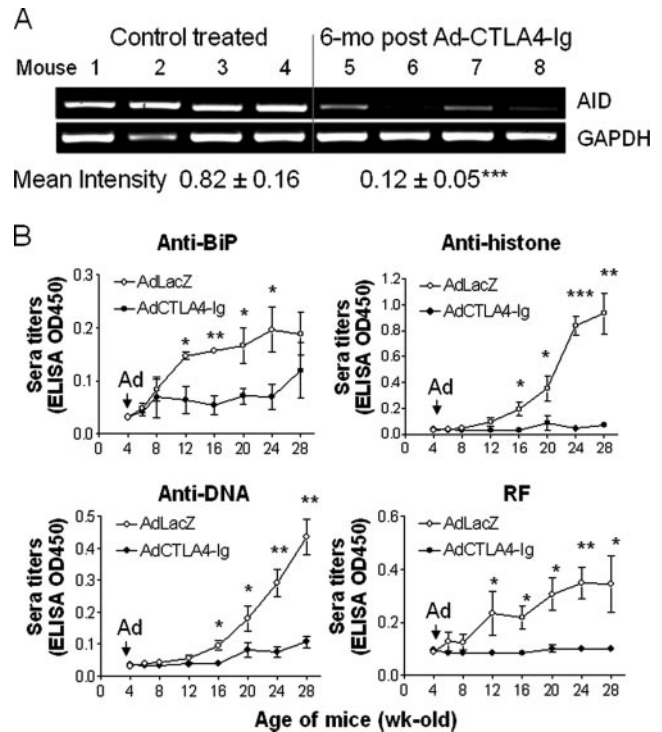


FIGURE 6. CTLA4-Ig treatment resulted in a long-term inhibition of AID and production of autoantibodies in BXD2 mice. AdLacZ or AdCTLA4-Ig (1×10^9 PFU/mouse) was injected into 4-wk-old BXD2 mice (four mice per group). *A*, The expression of AID and control GAPDH in purified B cells of 28-wk-old AdLacZ-treated or AdCTLA4-Ig-treated BXD2 mice was determined by RT-PCR. Each band represents the result from each individual mouse at the time of sacrifice (bands 1–4: 6-mo post-AdLacZ treated; and bands 5–8: 6-mo post-AdCTLA4-Ig treated). The intensity of each band was quantitated using the Image J 1.37v software (<http://rsb.info.nih.gov/ij/>). The intensity of AID relative to the intensity of GAPDH from each group was calculated and shown ($***, p < 0.005$, mean \pm SEM, $n = 4$). *B*, The titers of IgG isotype autoantibodies against the indicated autoantigen were determined using an ELISA method. Data (mean \pm SEM) are results obtained from the average of four mice in each group. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.005$, respectively, comparing results from AdCTLA4-Ig-treated mice vs those from age-matched mock-treated BXD2 mice at the same time point.

were rapidly reduced to undetectable levels 1 wk after treatment for both age groups of mice (Fig. 5B). There was an increase in the CD69⁺CD4 T cells in 13-wk-old BXD2 mice which was reduced by administration of AdCTLA4-Ig to levels equivalent to those observed in age-matched B6 mice (Fig. 5C). These studies indicate that interaction between CD28-CD80/CD86 is required for high expression of AID in the BXD2 B cells.

Long-term inhibition of AID after early administration of AdCTLA4-Ig

The above results show that expression of AID was significantly reduced at an early time point after administration of AdCTLA4-Ig. Surprisingly, this decreased expression of AID was long-lasting and there was a 4-fold reduction of AID in BXD2 mice 6 months after a single administration of AdCTLA4-Ig, although there was some return of AID expression compared with the almost complete suppression at an early time point after CTLA4-Ig (Fig. 6A). There was a significantly decreased production of IgG autoantibody with all four of the autoantigens tested, i.e., BiP (HSPa5 or GRP78), DNA, histone, and rheumatoid factor, as compared with the untreated controls, up to 28 wk of age (Fig. 6B). These results suggest

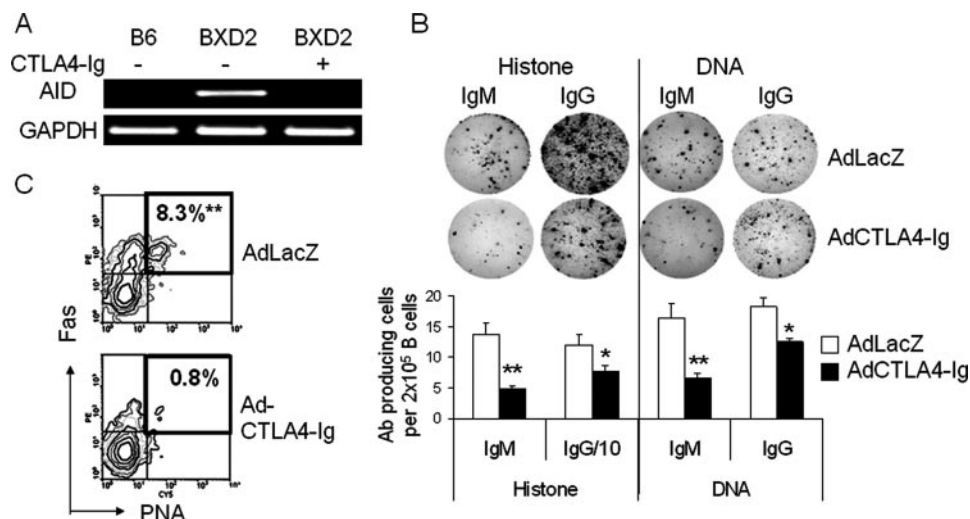


FIGURE 7. CTLA4-Ig suppressed IgM and IgG autoantibody-producing B cells and GC formation in BXD2 mice. AdLacZ or AdCTLA4-Ig (1×10^9 PFU/mouse each) was injected into 8-mo-old BXD2 mice ($n = 3$ /group). *A*, The expression of AID and control GAPDH in purified B cells 7 days after the treatment was determined by RT-PCR. *B*, At day 7 after the injection, the frequency of IgM or IgG anti-histone or anti-DNA autoantibody-producing B cells in purified B cells of mice was determined using an ELISPOT method. Results are shown as the number of autoantibody-producing B cells per 2×10^5 B cells added into each well. For IgG anti-histone-producing B cells, this number was divided by 10 for each mouse for easy comparison purpose (*, $p < 0.05$; **, $p < 0.01$). *C*, Single-cell suspensions were prepared from the spleen of AdLacZ or AdCTLA4-Ig injection (1×10^9 PFU/mouse each) injected 8-mo-old BXD2 mice at day 7 after the injection ($n = 3$ /group). Cells were stained with allophycocyanin-anti-B220, PE-anti-Fas, and biotin-anti-PNA followed by a secondary PE-Cy5 SA staining. The percentage of B cells (B220⁺) that is Fas⁺PNA⁺ was indicated (**, $p < 0.01$).

that early administration of Ad-CTLA4-Ig inhibited the microenvironment and molecular mechanisms that are required for the development of pathogenic autoantibodies.

AdCTLA4-Ig inhibited CSR and the development of autoantibodies

To determine whether down-regulation of AID by AdCTLA4-Ig could block the development of autoantibody-forming B cells, and CSR by these B cells, 8-mo-old BXD2 mice were treated with either AdLacZ or AdCTLA4-Ig (1×10^9 PFU/mouse). The expression of AID, as shown above, was dramatically suppressed in AdCTLA4-Ig-treated BXD2 mice 1 wk after treatment (Fig. 7*A*). There was a significant decrease in the number of B cells producing IgM anti-histone and anti-DNA autoantibodies in AdCTLA4-Ig-treated 8-mo-old BXD2 mice, compared with AdLacZ-treated, age-matched BXD2 control mice (Fig. 7*B*). There was also a significant decrease in the number of B cells producing IgG anti-histone and IgG anti-DNA autoantibodies in AdCTLA4-Ig-treated BXD2 mice, compared with AdLacZ-treated mice (Fig. 7*B*). AdCTLA4-Ig treatment dramatically decreased the percentage of PNA⁺Fas⁺B220⁺ GC B cells (Fig. 7*C*). Our results suggest that AdCTLA4-Ig inhibits the function of AID and the formation of GC in BXD2 mice that give rise to autoantibody-producing B cells.

Discussion

This is the first report describing increased AID in GC B cells associated with the development of pathogenic autoantibodies. Previous work has pinpointed the role of AID as being important in SHM and CSR (26–29). Overexpression of AID has been shown to result in mutations of certain target genes, including oncogenes *c-myc* and *pim1*, as well as other surface molecules, such as CD4 and CD5, and AID is up-regulated in GC and follicular B cell lymphoma (30–32). Ubiquitous overexpression of AID in a transgenic mouse results in T cell lymphoma (33). However, expression of AID in certain human lymphoma B cell lines was associated with hypermutation of Ig sequences (34, 35). The patho-

genic role of AID has not been widely studied in human autoimmune disease because the *AID* gene is mainly expressed by GC B cells and is down-modulated in circulating plasma and memory B cells (36). This is consistent with the present finding that AID expression was detected in developmental stages of B cells but not in mature B cells.

In the present study, we show that pathogenic multireactive autoantibodies have undergone a higher rate of SHM compared with non- or low pathogenic single-reactive autoantibodies. In both multireactive and single-reactive autoantibodies, there was an ~2:1 ratio of replacement to silent mutations. This is consistent with the 2:1 ratio of SHM in the V_H gene of the multireactive antiglomerular components autoantibodies isolated from lupus-prone MRL-*lpr* mice (37). However, the number of total replacement (R) and silent mutations in the V_H sequence of multireactive autoantibodies obtained in the present study is far more than that observed for the dual-reactive anti-Sm and anti-DNA autoantibodies isolated from MRL-*lpr/lpr* strain of mice (ranging from 3 to 19 per Ab with 0 to 11 nonsilent mutations and 0 to 3 silent mutations from 8 hybridoma clones analyzed). These Abs were predicted to be developed due to Ag-selected SHM (38). There was a high rate of mutations in the CDR1 region of the V_H gene of multireactive mAbs compared with single-reactive mAbs. This is consistent with previous results by Wilson et al. (39) that this may be the result of insertions and deletions. Although alteration in CDR3 length has been reported as a feature of anti-DNA autoantibodies (40–42), there was no significant difference in the CDR3 length in single-reactive, compared with multireactive, autoantibodies in BXD2 mice, even though most multireactive autoantibodies recognized dsDNA.

Our results support a recent paradigm shift that multireactive or cross-reactive autoantibodies are more pathogenic than single-reactive autoantibodies. For example, many investigators have demonstrated that lupus-inducing or nephritogenic autoantibodies tend to be multireactive or cross-reactive (42–44). The present result directly demonstrates that in a single mouse, there is increased SHM in the V_H germline sequence of multireactive pathogenic

autoantibodies compared with the V_H germline sequence of single-reactive nonpathogenic autoantibodies. Our results suggest that increased SHM is an important process leading to the development of multireactive autoantibodies in autoimmune disease. Increased SHM has also been observed in nonmalignant B cells isolated from GCs in the spleen (45) and multireactive autoantibodies have been found from spleen and PBMCs of human SLE and arthritis patients (2, 3). We propose that the development of multireactive autoantibodies in BXD2 mice is mediated by increased AID. Without AID, increased SHM and CSR leading to the development of IgG multireactive autoantibodies in BXD2 mice will not occur (46).

Honjo and colleagues (47) have previously generated conditional AID-transgenic mice that constitutively express AID only in B cells. The results show that the Tg AID, despite its abundance, is much less efficient for CSR and SHM than the endogenous AID. Also, the B cell development appears to be normal in AID-transgenic mice compared with wild-type control. These results indicate that up-regulation of AID, by itself, neither induces GC formation, nor promotes B cell survival (47). The results by Honjo and colleagues (47) are not contradictory to the present findings because our results suggest that factors produced from endogenously activated CD4 T cells are required to enable the excessive AID to induce abnormal SHM and CSR. We have found that both T1 and FO (T2 plus FM) B cells of BXD2 mice exhibit greatly increased expression of AID transcripts. This is in sharp contrast to the B cells from B6 mice in which, in the absence of Ag stimulation, transcripts of AID could only be detected in the relatively small subpopulation of T1 B cells. Defective down-regulation in AID may lead to increased SHM, CSR, and survival (48) of autoreactive B cells in BXD2 mice. GC B cells from BXD2 mice are more activated in vivo compared with B6 mice, shown by increased BrdU labeling and expression of CD86. However, because activated CD4 T cells from BXD2 mice are able to stimulate the expression of AID in B cells of both B6 and BXD2 mice, we conclude that AID is an important, but not the initiating, factor leading to the generation of pathogenic autoantibodies. Our results further indicate that CD28-CD86 engagement not only induces AID but also provides optimal signals for SHM and CSR within the autoreactive B cells. In the BXD2 GC and follicular B cell environment, enhanced autoantigen stimulation of B cells may induce a significantly increased survival of B cells that, in combination with the increased expression of AID, may provide a suitable microenvironment for the production of pathogenic autoantibodies as a result of abnormal SHM and CSR.

The use of CTLA4-Ig in the treatment of RA patients has shown encouraging initial results (49). Patient response to treatment, however, is variable, and the key immune mechanisms associated with the inhibition of autoimmune disease by CTLA4-Ig treatment remain to be identified. Activated CD4⁺ Th cells are the central regulators of the cytokines and factors for autoreactive B cell development. CD28 is a key CD4 T cell costimulatory molecule and blockade of CD28-CD80/CD86 signaling by CTLA4-Ig has been shown to down-regulate T cell activation (50). The ability of CTLA4-Ig to negatively regulate T cell function has led to its application in immune modulation. Long-term administration of CTLA4-Ig to NZB/W F₁ mice has been shown to prevent disease onset for a period of months (51). However, when AdCTLA4-Ig was given to 18- to 20-wk-old NZB/W F₁ mice (52), the therapeutic effect of AdCTLA4-Ig reported in NZB/W F₁ mice was not as effective as the results reported herein in BXD2 mice. This may be due to decreased efficacy when treatment is initiated after onset of disease compared with before onset of disease. We also observed that administration of AdCTLA4-Ig to 13-mo-old BXD2 mice resulted in an acute suppression of AID in B cells. This

treatment, however, did not eliminate pre-existing IgG autoantibodies in aged BXD2 mice (data not shown). These results suggest that treatment of young BXD2 mice before or during the early stage of disease development with CTLA4-Ig may provide a long-term therapeutic effect and this will correlate with down-regulation of AID and suppression of GC formation.

Based on the results observed here, we propose that an effective therapeutic strategy would be to inhibit AID expression in GC B cells. Our results further suggest that a subgroup of early stage SLE or RA patients that produce high levels of IgM multireactive autoantibodies (2, 3), exhibit increased expression of CD86 and AID in B cells, and express high levels of CD28 on CD4⁺ T cells (53, 54) would be candidates for such therapy. Blockade of AID expression can be achieved by inhibition of CD4⁺CD28⁺ T cells or their products such as CD40L and cytokines that can induce AID mRNA expression in B cells (55). Another novel therapeutic strategy would be to directly block AID activity in GC B cells by competitive inhibitors of cytidine deaminase such as tetrahydrouridine (9), or by cytidine analogs such as zebularine (56). Both drugs have been pursued for cancer therapy (56–58) but may be effective for treatment of autoimmune disease through their inhibitory effects on AID.

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Disclosures

The authors have no financial conflict of interest.

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