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EBV-Encoded Latent Membrane Protein 1 Cooperates with BAFF/BLyS and APRIL to Induce T Cell-Independent Ig Heavy Chain Class Switching[1]

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Abstract

By substituting the H chain C region of IgM with that of IgG, IgA, or IgE, class switching enables Abs to acquire new effector functions that are crucial for the neutralization of invading pathogens. Class switching occurs through class switch DNA recombination (CSR) and usually requires engagement of CD40 on B cells by CD40 ligand on Ag-activated $CD4^+$ T cells. CSR must be tightly regulated because abnormal IgG and IgA production favors the onset of autoimmunity, whereas increased switching to IgE leads to atopy. These inflammatory disorders can be triggered or exacerbated by EBV infection. In this study, we show that EBV induces CD40-independent CSR from C_μ to multiple downstream C_γ, C_α, and C_ϵ genes through latent membrane protein 1 (LMP1), a CD40-like viral protein that signals in a ligand-independent fashion. LMP1-induced CSR is associated with transcriptional activation of germline C_γ, C_α, and C_ϵ genes and triggers the up-regulation of activation-induced cytidine deaminase, a crucial component of the CSR machinery. In addition, LMP1 induces B cells to express B cell-activating factor of the TNF family and a proliferation-inducing ligand, two molecules that mediate B cell survival and T cell-independent Ab production. B cell-activating factor of the TNF family and a proliferation-inducing ligand cooperate with LMP1 to induce Ig class switching because their neutralization by appropriate soluble decoy receptors attenuates CSR in LMP1-expressing B cells. By showing that LMP1 triggers T cell-independent CSR, our findings suggest that EBV could play an important role in the pathogenesis of disorders with aberrant IgG, IgA, and/or IgE production.

Immunoglobulin H chain class switching diversifies the Ab effector functions by substituting the H chain C region (C_H) of IgM with that of IgG, IgA, or IgE (1). B cells undergo IgH chain class switching through class switch DNA recombination (CSR),[3] an intriguing process that involves the recombination of the switch μ (S_μ) region 5′ of the C_μ

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gene with an analogous S_γ , S_α, or S_ϵ region 5′ of C_γ , C_α, and C_ϵ, respectively (2). Most Ags, including complex viral and bacterial proteins, induce CSR by up-regulating CD40 ligand (CD40L) on $CD4^+$ T cells (3). Engagement of CD40 on IgD^+ naive B cells by CD40L triggers NF-κB-dependent transcriptional activation of I_H gene promoters that are located 5′ of each S region and encompass a noncoding I_H exon (2). The resulting germline I_H-C_H transcription increases the accessibility of the targeted S region to the CSR machinery. This enzymatic complex includes activation-induced cytidine deaminase (AID), a B cell-specific and CD40-inducible enzyme that induces CSR through an as-yet-elusive mechanism (2, 4).

T cell-dependent (TD) Ags trigger CD40-dependent CSR in B cells located within the germinal center (GC) of secondary lymphoid follicles (5, 6). These GC B cells subsequently differentiate to long-lived IgD^- memory B cells or Ab-secreting plasma cells (7, 8). T cell-independent (TI) Ags, such as viral glycoproteins and bacterial polysaccharides, elicit CD40-independent CSR and Ab production in extrafollicular marginal zone and intestinal B cells (9–11). This process requires B cell-activating factor of the TNF family (BAFF; also known as B lymphocyte stimulator) and a proliferation-inducing ligand (APRIL) (12–16), two CD40L-related molecules produced by myeloid cells (17–19). BAFF binds to three receptors specifically expressed on B cells, including transmembrane activator and calcium modulator and cyclophylin ligand interactor (TACI), B cell maturation Ag (BCMA), and BAFF-R (also known as BR3) (20–22). In addition to favoring Ab production, BAFF-R delivers survival signals that are crucial for the conservation of the peripheral B cell repertoire (23,24). Unlike BAFF, APRIL binds to TACI and BCMA, but not BAFF-R (25, 26). Similarly to CD40 (3), TACI, BCMA, and BAFF-R signal by recruiting TNFR-associated factors (TRAFs) to their cytoplasmic tails (24). By activating IκB kinase, TRAFs induce phosphorylation-dependent degradation of IκB, a cytoplasmic inhibitor of NF-κB (27, 28). The subsequent nuclear translocation of NF-κB transcriptionally activates genes involved in B cell proliferation, differentiation, and survival (29).

Dysregulated switching to IgG and IgA is central to the pathogenesis of autoimmune disorders such as systemic lupus erythematosus (SLE) (30), whereas aberrant switching to IgE underlies the pathogenesis of atopic disorders such as allergic asthma and atopic dermatitis (31). Both autoimmunity and atopy can be triggered or exacerbated by viral infections, including EBV infection (32–34). EBV is a B lymphotropic herpes virus that infects >90% of the human population during the first years of life (35). EBV infection is usually asymptomatic, because most EBV-containing B cells are eliminated by $CD8^+$ CTLs (36). However, a few latently infected B cells persist for the lifetime (36). In some predisposed subjects, latent EBV infection would favor production of IgG and IgA autoantibodies (37, 38). Abnormal switching to IgG, IgA, and IgE can be also observed in adolescents with infectious mononucleosis, a self-limiting lymphoproliferative disorder

[3]Abbreviations used in this paper: CSR, class switch DNA recombination; S, IgH switch region; CD40L, CD40 ligand; AID, activation-induced cytidine deaminase; TD, T cell dependent; TI, T cell independent; GC, germinal center; BAFF, B cell-activating factor of the TNF family; APRIL, a proliferation-inducing ligand; TACI, transmembrane activator and calcium modulator and cyclophylin ligand interactor; BCMA, B cell maturation Ag; TRAF, TNFR-associated factor; SLE, systemic lupus erythematosus; LMP, latent membrane protein; EBNA, EBV-encoded nuclear Ag; mBAFF, mouse BAFF; SC, switch circle; LUC, luciferase reporter plasmid; wt, wild type; CT, circle transcript; PB, peripheral blood; LCL, lymphoblastoid B cell line; BL, Burkitt's lymphoma; tet, tetracycline; CTAR, C-terminal activation region; BCR, B cell Ag receptor.

secondary to acute EBV infection (34), as well as in immunocompromised subjects with EBV-associated B cell lymphoproliferative disorders (39–41). It is unclear how EBV dysregulates the Ab response.

In the initial phase of the infection, EBV drives tonsillar IgD^+ naive B cells to undergo extrafollicular activation and proliferation through three latent membrane proteins (LMP1, −2A, and −2B) and six EBV-encoded nuclear Ags (EBNA1 to −6) (42, 43). This growth program, also known as latency III, allows the expansion of the viral episome in the B cell compartment until a strong antiviral T cell response is established (44). Later on, EBV induces infected IgD^+ blasts to switch to a default program, also known as latency II, which entails only EBNA-1, LMP1, and LMP2A, and allows infected B cells to differentiate to class-switched IgD^- memory B cells (42, 43, 45). By further down-regulating LMP1 and LMP2A, memory B cells acquire a latency program, also known as latency I, which includes only EBNA1 and allows the persistence of EBV in a transcriptionally quiescent state (43, 46). Periodic reactivation of LMP1 and LMP2A in the tonsillar microenvironment would generate growth and survival signals that enable latently infected memory B cells to persist for the lifetime (36). The mechanisms by which EBV-infected IgD^+ blasts differentiate to class-switched IgD^- memory B cells remain elusive.

Among EBV-encoded proteins, LMP1 is essential to induce B cell activation, proliferation, survival (35,47), as well as in vitro B cell transformation (48). The LMP1 cytoplasmic tail has extensive functional homology with CD40 and, like CD40, induces IκBα degradation and NF-κB nuclear translocation by recruiting TRAFs and IκB kinase (49–51). Unlike CD40, which delivers transient signals upon engagement by CD40L (3), LMP1 constitutively signals in a ligand-independent fashion (52). This observation prompted us to hypothesize that EBV might dysregulate IgG, IgA, and IgE production by delivering CD40-like signals to B cells.

In this study, we show that B cell infection by EBV actively induces CSR from C_μ to multiple C_γ, C_α, and C_ϵ genes through LMP1. This viral protein further dysregulates CSR by triggering aberrant BAFF and APRIL expression in B cells. Our findings suggest that neutralization of BAFF and APRIL by soluble TACI and BCMA decoy receptors may attenuate dysregulated IgG, IgA, and IgE production in certain patients with latent or active EBV infection.

Materials and Methods

Cells and reagents

IARC, BL16, Bjab, and HL-60 cell lines (from American Type Culture Collection (Manassas, VA) and R. Dalla-Favera (Columbia University, New York, NY)) were cultured in RPMI 1640 medium (Invitrogen, Carslbad, CA). IgD^+ B cells and monocytes were obtained from PBMCs as described (53). IgD^+ B cells were incubated with EBV (B95-8 strain) for 2 h at 37°C After virus removal, B cells were incubated for 3 wk at a density of 10^6 cells/ml. All cultures were conducted in RPMI 1640 medium supplemented with 10% FCS, antibiotics, and glutamine. Ramos subclones expressing EBV proteins (from R. Harris and M. Neuberger (Medical Research Council Laboratory of Molecular Biology,

Cambridge, U.K.)) were cultured in medium supplemented with 1 μg/ml puromycine (Sigma-Aldrich, St. Louis, MO). tet-LMP1 Bjab cells (from N. Lam and B. Sugden (University of Wisconsin-Madison, Madison, WI)) were cultured with medium supplemented with 1 μg/ml puromycine and 200 μg/ml geneticin (Invitrogen), and with or without 1 ng/ml doxycycline (Sigma-Aldrich). Control MOPC-21 (Sigma-Aldrich), TACI-Ig), BCMA-Ig (Alexis Biochemicals, San Diego, CA), and CD40-Ig (Ancell, Bayport, MN) were used at 30 μg/ml.

Flow cytometry

CD3, CD14, CD19, CD23 (BD PharMingen, San Diego, CA), IgM, IgG, and IgA (Southern Biotechnologies Associates, Birmingham, AL) were detected with PE- or FITC-conjugated Abs. Mouse BAFF (mBAFF) was labeled with a mouse Ab to BAFF (Alexis Biochemicals) and a PE-conjugated anti-mouse Ab (BD PharMingen). BAFF-Rs were labeled with a CD8-BAFF fusion protein (Ancell) and a PE-conjugated Ab to CD8 (BD PharMingen). Cells were acquired using a FACSCalibur analyzer (BD Immunocytometry Systems, San Jose, CA).

Genomic PCRs and RT-PCRs

DNA and RNA extractions were preceded by removal of dead B cells through Ficoll. Genomic DNA was extracted from 10×10^6 viable B cells by using the QIAmp DNA mini kit (Qiagen, Valencia, CA). Switch circles (SCs) were amplified from 500 ng of genomic DNA (13). Total RNA was extracted from 5×10^6 viable B cells by using the RNeasy total RNA kit (Qiagen). cDNA was reverse transcribed from 3 μg of total RNA (13). PCRs were made semiquantitative by varying the number of cycles and performing dilutional analysis so that there was a linear relationship between the amount of cDNA used and the intensity of the PCR product. Germline I_H-C_H transcripts, mature VDJ-C_H transcripts, AID, BAFF, APRIL, and β-actin were amplified as described (13). TACI, BCMA, BAFF-R, and LMP1 were amplified by using the following primer pairs: TACI, forward, 5′-AAGAAGAGGGGGGATCCCTGC-3′, and reverse, 5′-TTATGCACCTGGGCCCCC-3′; BCMA, forward, 5′-CTAAGGAA GATAAACTCTGAACCA-3′, and reverse, 5′-TTACCTAGCAGAAATT GATTTCTC-3′; BAFF-R, forward, 5′-GTGAGCTGGAGGCGGCGACAG-3′, and reverse, 5′-CTATTGTGCTCAGGGCCGGC-3′; and LMP1, forward, 5′-CTTCAGAAGAGACCTTCTCT-3′, and reverse, 5′-ACAATGCCTGT CCGTGCAAA-3′. The conditions were as follows: denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C.

Southern blots

PCR products were fractionated onto agarose gels, transferred overnight to nylon membranes, and hybridized with radiolabeled probes as described (13). SCs were hybridized with a probe recognizing the recombined S_μ region; circle transcripts (CTs) and mature VDJ-C_μ transcripts were hybridized with a probe encompassing nt 1–250 of the first C_μ exon; and mature VDJ-C_γ transcripts were hybridized with a 5′-CAGGGGGAA GACCGATGG-3′ oligoprobe recognizing a consensus C_γ sequence.

Vectors

−449/+265 and −291/+131 genomic DNA fragments encompassing the $I_{\gamma}3$ and I_{ϵ} promoters were inserted into a promoterless pGL3-Basic vector (Promega, Madison, WI) containing a luciferase (LUC) reporter gene (54, 55). Wild-type (wt) LMP1 and mutant LMP1, including DEL 187–351 LMP1, 187-STOP LMP1, and 231-STOP LMP1, were cloned into a pcDNA3 expression vector (Invitrogen) (56). The $\kappa B_{(2X)}$-LUC reporter vector is driven by an NF-κB-responsive minimal promoter with two NF-κB-binding κB sites. $\kappa B_{(2X)}$-LUC and the IκBα-pcDNA3 expression vector were provided by E. Cesarman (Weill Medical College of Cornell University, New York, NY). The −839/+232 genomic DNA segment encompassing the BAFF promoter was PCR amplified from placental genomic DNA by using sense 5′-CACAGGTCCACCAAGTCAACAA CAGA-3′ and antisense 5′-ATCACTACTTGAACTTTGAAGGTTGG-3′ primers with 5′ overhangs containing *Kpn*I (sense) and *Xho*I (antisense) restriction sites. The resulting DNA segment was sequenced and then cloned into the pGL3-Basic reporter vector. The MatInspector software (Genomatix Software, Munchen, Germany) was used to identify putative NF-κB-binding κB motifs.

Primer extension gene analysis

A Primer Extension System-Avian Myeloblastosis Virus Reverse Transcriptase (Promega) was used to identify the *BAFF* gene transcription initiation site. Briefly, total RNA from HL-60 or BL16 cells was reversed transcribed with an avian myeloblastosis virus reverse transcriptase and an end-labeled 5′-CAGTAGGTTTGCTGGCATTTACCCTC-3′ antisense primer recognizing a sequence immediately upstream of the first BAFF exon. The resulting cDNA was analyzed on a denaturing polyacrylamide gel to identify the number of bases between the labeled oligonucleotide and the 5′ end of the targeted RNA.

Luciferase reporter assays

Cells (20×10^6/ml) were transfected with 40 μl of plasmid DNA-Tris-EDTA solution containing 20 μg of pGL3-Basic vector and 200 ng of pRL-TK control vector (Promega). pGL3 or $\kappa B_{(2X)}$-LUC reporter vectors were cotransfected with 2 μg/ml pcDNA3.1 expression vectors containing wt or mutated LMP1. Electroporation was performed at 625 V/cm (HL-60 and BL16) or 525 V/cm (Bjab) and 950 μF using a Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA). Transfected cells (1×10^6/ml) were cultured for 48 h. The luciferase activity was measured with the Dual-Luciferase Assay System (Promega).

Immunoblots

Total proteins were fractionated onto a 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking, membranes were probed with Abs to BAFF (Upstate Biotechnology, Lake Placid, NY), APRIL, TACI, BCMA, actin (Santa Cruz Biotechnology, Santa Cruz, CA), and LMP1 (DAKO, Carpinteria, CA). Proteins were detected with an ECL detection system (Amersham, Little Chalfont, U.K.).

EMSAs

Cells (5×10^6) were lysed to extract nuclear proteins as reported (54). A double-stranded oligonucleotide probe overlapping the κB3 site (residues −236 to −216, GTGTCTGGACTCCCCCTCGCC) of the $I_\gamma 3$ promoter (54) was end labeled with [γ-^{32}P]ATP by T4 kinase and used at ∼30,000 cpm in each EMSA reaction. Reaction mixtures (20 μl) contained 1 ng of DNA probe, 4 μg of nuclear proteins, 2 μg of poly(dI-dC) (Sigma-Aldrich), and 2 μl of binding buffer (12.5 mM HEPES (pH 7.9), 5 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.05% Nonidet P-40). Reaction samples were incubated at room temperature for 15 min and then electrophoresed through a 6% nondenaturing polyacrylamide gel in 0.25 × Tris-borate EDTA buffer at 150 V.

Results

B cells up-regulate AID and undergo CSR from C_μ to C_γ C_α, and C_ϵ upon infection by EBV

CSR from C_μ to a downstream $C_\gamma 3$, $C_\gamma 1$, $C_\alpha 1$, $C_\gamma 2$, $C_\gamma 4$, $C_\alpha 2$, or C_ϵ gene is preceded by the transcription of that gene in the form of a noncoding germline I_H-C_H transcript that includes the I_H exon 5′ of the targeted S region and C_H (1). CSR also requires the up-regulation of the B cell-specific enzyme AID (2). By inducing looping-out deletion of the IgH DNA between S_μ and the targeted downstream S region, CSR generates a single-copy extrachromosomal reciprocal DNA recombination product, also known as SC (57). Because CSR does not target a consensus sequence within the S region (58), actively class-switching B cells generate multiple SCs with different sizes. After excision from the *IgH* locus, SCs transcribe short-lived chimeric I-C_μ CTs that include the promoter upstream of the targeted I_H exon, the I_H exon, and C_μ (58). These CTs often undergo posttranscriptional remodeling, thereby generating more than one band after PCR amplification (58). Together with AID and germline I_H-C_H transcripts, SCs and CTs constitute specific markers of ongoing CSR and, in healthy individuals, are usually detected only in IgD^- GC B cells (59). Ongoing CSR was analyzed in noninfected (EBV^-) normal peripheral blood (PB) IgD^+ naive B cells, which display S regions in an unrearranged configuration (13, 59) as well as in monoclonal IARC549 and IARC100 lymphoblastoid cell lines (LCLs) obtained by transforming normal polyclonal PB B cells with EBV in vitro. CSR was also studied in monoclonal BL16 cells, a neoplastic Burkitt's lymphoma (BL) B cell line that, like IARC549 and IARC100, expresses surface IgD on most of its elements and harbors a type-III EBV gene latency program.

Noninfected IgD^+ B cells from healthy subjects lacked total S_γ-S_μ and S_α-S_μ SCs (Fig. 1*A*), expressed no or low germline $I_\gamma 1$-$C_\gamma 1$, $I_\gamma 3$-$C_\gamma 3$, and $I_\alpha 1$-$C_\alpha 1$ transcripts, and lacked $I_\gamma 1/2$-C_μ, $I_\gamma 3$-C_μ and $I_\alpha 1/2$-C_μ CTs as well as AID transcripts (*B*). In contrast, lymphoblastoid and EBV^+ BL16 B cells contained total S_γ-S_μ and S_α-S_μ SCs, expressed large amounts of germline $I_\gamma 1$-$C_\gamma 1$, $I_\gamma 3$-$C_\gamma 3$, and $I_\alpha 1$-$C_\alpha 1$ transcripts, and contained $I_\gamma 1/2$-C_μ, $I_\gamma 3$-C_μ, and $I_\alpha 1/2$-C_μ CTs, and AID transcripts. In both lymphoblastoid and EBV^+ BL16 cells, 5–15% of the clonal elements expressed surface IgG or IgA, but not surface IgD and IgM (not shown), further suggesting ongoing CSR. Additional experiments were performed to verify whether infection of normal IgD^+ B cells by EBV induces CSR. Compared with noninfected IgD^+ B cells (Fig. 1*A*), EBV-infected IgD^+ B cells up-regulated AID transcripts and contained extrachromosomal $S_\gamma 1/2$-S_μ, S_γ 3-S_μ, $S_\gamma 4$-S_μ, $S_\alpha 1/$ 2-S_μ and S_ϵ-S_μ SCs (*C*), which reflect

ongoing CSR from C_μ to $C_\gamma 1/C_\gamma 2$, $C_\gamma 3$, $C_\gamma 4$, $C_\alpha 1/C_\alpha 2$, and C_ϵ respectively. These findings indicate that B cells undergo CD40-independent CSR from C_μ to multiple downstream C_γ, C_α, and C_ϵ genes upon infection by EBV.

EBV up-regulates germline I_H-C_H transcription, AID expression, and CSR through LMP1

To evaluate the mechanism by which EBV induces CSR, we took advantage of IgM^+ subclones established from the EBV^- BL cell line Ramos and stably expressing LMP1, LMP2A, EBNA1, EBNA2, or EBNA-LP expression vectors. Compared with control subclones transfected with empty expression vectors, B cell sub-clones expressing LMP1 contained $I_\gamma 1/2$-C_μ and $I_\gamma 3$-C_μ CTs as well as mature V_HDJ_H-$C_\gamma 1$ and V_HDJ_H-$C_\gamma 3$ transcripts (Fig. 2), the end product of CSR from C_μ to $C_\gamma 1$ and $C_\gamma 3$. B cell subclones expressing LMP2A contained lower amounts of $I_\gamma 3$-C_μ CTs and V_HDJ_H-$C_\gamma 3$ transcripts, but lacked $I_\gamma 1/2$-C_μ and V_HDJ_H-$C_\gamma 1$ transcripts. In contrast, B cell subclones expressing EBNA1, EBNA2, or EBNA-LP lacked $I_\gamma 1/2$-C_μ and $I_\gamma 3$-C_μ CTs as well as mature V_HDJ_H-$C_\gamma 1$ and V_HDJ_H-$C_\gamma 3$ transcripts. Finally, all B cell sub-clones expressed β-actin transcripts as well as mature V_HDJ_H-C_μ transcripts. These findings suggest that LMP1 and, to a lesser extent, LMP2A trigger CD40-independent CSR.

The CSR-inducing activity of LMP1 was further evaluated in an EBV^- BL cell line, Bjab, stably expressing a tetracycline (tet)-inducible LMP1 expression vector. Like Ramos B cells, Bjab B cells express IgM but not IgG, IgA, or IgE on the surface. Bjab-tet-LMP1 B cells up-regulated LMP1 (shown below) and activated luciferase reporter vectors containing the $I_\gamma 3$ gene promoter ($I_\gamma 3$-LUC), the I_ϵ gene promoter (I_ϵ-LUC), or $\kappa B_{(2X)}$-LUC upon incubation with doxycycline for 2 days (Fig. 3*A*). Overexpression of the NF-κB inhibitor IκBα inhibited doxycycline-induced activation of $I_\gamma 3$-LUC, I_ϵ-LUC, and $\kappa B_{(2X)}$-LUC. In addition to activating the $I_\gamma 3$ and I_ϵ promoters, doxycycline up-regulated the expression of germline $I_\gamma 1$-$C_\gamma 1$,$I_\gamma 3$-$C_\gamma 3$, $I_\alpha 1$-$C_\alpha 1$, and I_ϵ-C_ϵ transcripts (Fig. 3*B*). Furthermore, Bjab-tet-LMP1 B cells exposed to doxycycline for 4 days up-regulated AID transcripts and induced $I_\gamma 1$/ 2-C_μ $I_\gamma 3$-C_μ $I_\alpha 1/2$-C_μ and I_ϵ-C_μ CTs (Fig. 3*C*), which reflect ongoing CSR from C_μ to $C_\gamma 1/C_\gamma 2$, $C_\gamma 3$, $C_\alpha 1/C_\alpha 2$, and C_ϵ, respectively. The induction of CSR by doxycycline was associated with up-regulation of surface IgG and IgA and down-regulation of surface IgM (Fig. 3*D*). These findings indicate that B cells undergo NF-κB-dependent germline I_H-C_H transcription and CSR upon activation by LMP1.

LMP1 induces BAFF and APRIL expression in B cells

We have recently found that dendritic cells up-regulate BAFF and APRIL, two inducers of TI CSR, upon engagement of CD40 by CD40L (13). Given its ability to mimic CD40 signaling, LMP1 might up-regulate BAFF and APRIL in B cells as CD40 does in dendritic cells. Compared with control subclones, Ramos B cell subclones expressing LMP1 or, to a lesser extent, LMP2A contained more BAFF and APRIL transcripts and proteins (Fig. 4*A*). In contrast, Ramos B cell subclones expressing EBNA1, EBNA2, or EBNA-LP contained BAFF and APRIL transcripts and proteins in amounts comparable with those detected in control subclones. Additional experiments evaluated the expression of BAFF and APRIL in Bjab-tet-LMP1 B cells. When exposed to tet, Bjab-tet-LMP1 B cells up-regulated LMP1 as well as BAFF and APRIL transcripts and proteins (Fig. 4*B*). tet also up-regulated surface

CD23, a canonical LMP1-inducible B cell-activation protein, BAFF, as well as the total BAFF-binding activity (Fig. 4*C*), which reflects the surface density of TACI, BCMA, and BAFF-R receptors. In contrast, tet did not up-regulate CD3, a T cell-restricted component of the TCR complex, or CD19, a component of the B cell receptor complex expressed by all mature B cells. These findings indicate that LMP1 up-regulates BAFF and APRIL in B cells.

LMP1 activates the BAFF gene promoter through NF-κB

NF-κB is crucial for the activation of B cells by LMP1 (35, 51). We took advantage of Bjab-tet-LMP1 B cells to verify whether LMP1 up-regulates BAFF through NF-κB. DNA sequence analysis showed that the *BAFF* gene promoter contains at least six NF-κB-binding κB sites (Fig. 5*A*). Bjab-tet-LMP1 B cells activated a luciferase reporter vector carrying the *BAFF* gene promoter (BAFF-LUC) as well as $\kappa B_{(2X)}$-LUC upon incubation with doxycycline for 2 days. Overexpression of IκBα inhibited the induction of BAFF-LUC and $\kappa B_{(2X)}$-LUC by doxycycline (Fig. 5*B*), suggesting that NF-κB is critical to up-regulate BAFF. Because LMP1 recruits TRAFs and activates NF-κB through C-terminal activation region (CTAR)-1 and CTAR-2 (49, 56), we verified whether disruption of one or both CTARs affects the up-regulation of BAFF by LMP1. wt Bjab B cells activated BAFF-LUC as well as $\kappa B_{(2X)}$-LUC upon transfection with wt LMP1, which contains both CTAR-1 and CTAR-2. In contrast, 187-STOP LMP1, which lacks both CTAR-1 and CTAR-2, failed to activate BAFF-LUC as well as control $\kappa B_{(2X)}$-LUC. Furthermore, both BAFF-LUC and $\kappa B_{(2X)}$-LUC were activated by DEL 187–351 LMP1, which lacks only CTAR-1, or 231-STOP LMP1, which lacks only CTAR-2. These findings suggest that the up-regulation of BAFF by LMP1 requires the integrity of at least one CTAR domain. Finally, transfection of Bjab B cells with graded amounts of IκBα progressively inhibited the activation of BAFF-LUC and $\kappa B_{(2X)}$-LUC by wt LMP1, further indicating that NF-κB is crucial for the up-regulation of BAFF by LMP1.

B cells express BAFF and APRIL upon infection by EBV

Additional experiments were performed to verify whether purified B cells up-regulate BAFF and APRIL upon EBV infection. Purified noninfected IgD^+ B cells lacked BAFF and APRIL transcripts (Fig. 6*A*) as well as BAFF and APRIL proteins (*B*). Similar normal B cells expressed CD19, but most of them lacked the EBV (LMP1)-inducible Ag CD23 as well as mBAFF and the myeloid Ag CD14 (Fig. 6*C*). In contrast, purified EBV-infected IgD^+ B cells contained BAFF and APRIL transcripts and proteins and coexpressed CD19, CD23, and mBAFF on the surface. Similar EBV-infected normal B cells lacked CD14, indicating a lack of contaminating monocytes and macrophages. The expression of BAFF and APRIL was also measured in B cell lines harboring a type-III EBV latency gene program. IARC504 lymphoblastoid B cells and neoplastic EBV^+ BL16 B cells expressed BAFF and APRIL transcripts and proteins in amounts comparable with those expressed in myeloid cells, including HL60 AML cells. Moreover, lymphoblastoid and malignant BL16 B cells, which contain LMP1 (Fig. 6, *A* and *B*), expressed more BAFF and APRIL transcripts than Akata and Mutu I (not shown), two EBV^+ BL B cell lines that, unlike LCLs and BL16, express a type-I EBV latency gene program and therefore lack LMP1. Finally, all B cell types under study but not HL60 AML cells expressed TACI, BCMA, and BAFF-R

transcripts (Fig. 6*A*) and proteins (*B*). These findings indicate that B cells express TACI, BCMA, and BAFF-R, and aberrantly up-regulate BAFF and APRIL upon infection by EBV.

BAFF and APRIL up-regulate AID and enhance CSR in EBV-infected B cells

The above findings prompted us to hypothesize that engagement of TACI, BCMA, and/or BAFF-R by autocrine BAFF and APRIL enhances LMP1-induced CSR in EBV-infected B cells. To verify this, we took advantage of soluble TACI-Ig and BCMA-Ig decoy receptors, which prevent the binding of BAFF and APRIL to cell-bound TACI, BCMA, and BAFF-R. Lymphoblastoid IARC549 B cells down-regulated total S_γ-S_μ and S_α-S_μ SCs (Fig. 7*A*), $I_\gamma 1/2$-C_μ, $I_\gamma 3$-C_μ, and $I_\alpha 1/2$-C_μ CTs, as well as AID transcripts upon exposure to soluble TACI-Ig and BCMA-Ig decoy receptors for 2 days (*B*). This down-regulation was specific, as similar lymphoblastoid B cells did not attenuate CSR upon exposure to a control Ig or CD40-Ig, which blocks CD40L-CD40 interaction. These findings suggest that EBV triggers CD40-independent CSR not only through LMP1 but also through endogenous (LMP1-induced) BAFF and APRIL.

BAFF and APRIL enhance NF-κB activation and germline I_H-C_H transcription in EBV-infected B cells

Additional experiments were set up to assess whether BAFF and APRIL released by EBV-infected LMP1-expressing B cells activate NF-κB. Exposure of lymphoblastoid IARC549 B cells to BCMA-Ig but not control Ig or CD40-Ig down-regulated the binding of nuclear NF-κB to an oligonucleotide encompassing a DNA sequence crucial for the activation of the human $I_\gamma 3$ promoter by CD40 (Fig. 7*C*). As expected, this effect was associated with down-regulated expression of germline $I_\gamma 3$-$C_\gamma 3$ transcripts (Fig. 7*D*). In contrast, the expression of germline $I_\gamma 3$-$C_\gamma 3$ transcripts was not affected by control Ig or CD40-Ig. These results indicate that engagement of TACI, BCMA, and/or BAFF-R by autocrine BAFF and APRIL activates NF-κB and enhances the expression of germline I_H-C_H transcripts in EBV-infected LMP1-expressing B cells.

Discussion

We have shown that EBV-encoded LMP1 induces CD40-independent CSR from C_μ to multiple $C_\gamma C_\alpha$, and $C_\in$ genes in B cells. This induction is associated with NF-κB-dependent activation of downstream C_H gene promoters and up-regulation of germline I_H-C_H transcripts and AID transcripts. LMP1 up-regulates also BAFF and APRIL through an NF-κB-dependent mechanism that requires at least one CTAR domain. By engaging TACI, BCMA, and BAFF-R on B cells, BAFF and APRIL activate NF-κB and further enhance CSR. These findings suggest that EBV could play an important role in the pathogenesis of disorders associated with aberrant IgG, IgA, and/or IgE production.

EBV is thought to initially infect naive IgD^+ B cells in the mantle zone of lymphoid follicles located beneath the tonsillar epithelium (44). By expressing a full set of EBNA and LMP proteins, EBV induces IgD^+ B cells to become blasts, which proliferate outside the GC (43). Subsequent down-regulation of most EBV proteins but EBNA1, LMP1, and LMP2A would enable infected IgD^+ blasts to enter the GC and differentiate to class-switched IgD^- memory

B cells (43, 45). After further down-regulation of LMP1 and, to a lesser extent, LMP2A, latently infected memory B cells would leave the tonsil and enter the circulation (43,46). It remains unclear whether IgD^+ blasts undergo IgH class switching upon stimulation by viral proteins or as a result of CD40-dependent progression through the GC in response to a TD Ag. By showing that EBV induces CD40-independent CSR, our data suggest that at least some infected IgD^+ B cells rapidly undergo TI class switching to IgG, IgA, or IgE outside the GC.

Viruses induce CD40-independent IgH class switching through mechanisms that remain largely unknown (60–62). Our data indicate that EBV transcriptionally activates downstream germline C_H gene promoters, including $C_\gamma 3$ and C_ϵ, through LMP1. This CD40-like viral protein would transactivate $C_\gamma 3$ and C_ϵ genes through an NF-κB-dependent mechanism. Consistent with this, overexpression of the NF-κB inhibitor IκBα or (not shown) disruption of both NF-κB-activating CTAR domains within the LMP1 cytoplasmic tail impairs the induction of germline I_H-C_H transcription by LMP1. In LMP1-expressing B cells, germline I_H-C_H transcription is associated with up-regulation of AID transcripts and induction of CSR from C_μ to multiple downstream C_γ , C_α, and C_ϵ genes. These findings provide a mechanistic explanation for previous studies showing that enforced LMP1 expression restores TD IgG, IgA, and IgE production in CD40-deficient mice (63), which otherwise show severely impaired TD IgH class switching (64). Whereas CD40 transmits transient ligand-dependent signals (3), LMP1 continuously signals in a ligand-independent fashion (52). This implies that LMP1-induced CSR is subject to less regulatory constraints than CD40-induced CSR. By showing that EBV-infected LMP1-expressing IgD^+ B cells constitutively express high levels of I_H-C_H transcripts and AID and continuously undergo CSR, our data extend recent findings indicating that artificial S_μ and $S_\gamma 3$ DNA substrates undergo spontaneous recombination in lymphoblastoid B cells (65). By triggering unrestrained CSR, LMP1 may play a key role in the pathogenesis of dysregulated IgG, IgA, and IgE production occurring in certain EBV-infected individuals, including immunocompromised HIV-infected subjects and transplant recipients (39–41).

In normal B cells, switching from IgM to IgG, IgA, or IgE requires two signals, one delivered by CD40L and the other delivered by a cytokine (1, 2). Among cytokines, IL-4 induces switching to IgG and IgE (66–69), IL-10 to IgG and IgA (13, 66, 70, 71), and TGF-β to IgA (71–73). In EBV-infected LMP1-expressing B cells, CSR to C_γ and C_α occurs in the absence of exogenous cytokines. This does not imply that cytokines do not play any role in EBV-induced CSR, because EBV induces B cells to produce large amounts of autocrine IL-10 through LMP1 (74) as well as small nonpolyadenylated viral RNAs, also referred to as EBER1 and EBER2 (75). Consistent with this, neutralization of IL-10 by a specific blocking Ab partially inhibits switching to IgG and IgA in LMP1-expressing B cells (not shown). In addition to up-regulating endogenous IL-10, EBV produces an IL-10-like protein through a viral gene known as *BCRF1* (76). Furthermore, EBV-infected B cells produce IL-13 (77), which, like IL-4, activates switching from IgM to IgG4 and IgE (78, 79). This might explain our finding that EBV-infected LMP1-expressing B cells actively switch to $C_\gamma 4$ and C_ϵ in the absence of external IL-4.

Unlike CD40, which mediates CSR in GC B cells (3, 5), LMP1 elicits IgG and IgA production outside the GC of secondary lymphoid follicles (63). This extrafollicular pattern is also associated with B cell responses to TI Ags with repetitive structure, including envelope glycoproteins from viruses and capsular polysaccharides from bacteria (10, 60, 80). By showing that LMP1 up-regulates BAFF and APRIL, two mediators of TI Ab production (13–16), our data suggest that EBV exploits an otherwise physiological TI pathway to maximize CSR in infected IgD^+ B cells. Engagement of TACI, BCMA, and BAFF-R by LMP1-induced BAFF and APRIL would enhance CSR from C_μ to a targeted downstream C_H gene by activating the germline transcription of that gene through NF-κB. Consistent with this, neutralization of autocrine BAFF and APRIL by soluble TACI-Ig and BCMA-Ig decoy receptors attenuates NF-κB activation and down-regulates the expression of downstream germline I_H-C_H transcripts in LMP1-expressing B cells. In similar cells, TACI-Ig and BCMA-Ig decoy receptors down-regulate AID and impair CSR. Thus, autocrine BAFF and APRIL would cooperate with LMP1 to trigger NF-κB-dependent germline I_H-C_H transcription and CSR.

In addition to transactivating downstream C_H genes, NF-κB would play an important role in the LMP1-mediated up-regulation of BAFF. Consistent with this, overexpression of the NF-κB inhibitor IκBα interferes with the transcriptional activation of the *BAFF* gene promoter in LMP1-activated B cells. Furthermore, disruption of the two NF-κB-activating CTARs within the LMP1 cytoplasmic tail severely impairs the activation of the *BAFF* gene promoter by LMP1. Although playing a key role, NF-κB may not be the only transcription factor involved in LMP1-mediated up-regulation of BAFF. Consistent with this, the *BAFF* gene promoter includes several putative STAT-binding γ-IFN-activated sequences (not shown), which could be activated by STAT proteins induced by LMP1 (81). STAT proteins could be also induced by IL-10 (82), an LMP1-inducible cytokine that up-regulates BAFF in myeloid cells (74, 83). Thus, LMP1-induced NF-κB and STAT transcription factors might synergistically activate the *BAFF* gene promoter upon binding to cooperative κB and γ-IFN-activated sequence sites.

LMP1 is not the only CSR-inducing viral protein, because LMP2A triggers CSR from C_μ to $C_\gamma 3$ and up-regulates BAFF and APRIL, although to a lesser extent than LMP1. Unlike LMP1, LMP2A activates B cells by mimicking signaling through the B cell Ag receptor (BCR) (36). Consistent with this, the cytoplasmic tail of LMP2A encompasses immunoreceptor tyrosine-based activation motifs similar to those found in the Igα and Igβ signal-transducing subunits of the BCR complex (36). In addition to modulating B cell proliferation and survival (84), signals emanating from BCR modulate IgH class switching. For instance, BCR engagement by certain TI Ags induces CD40-independent switching to IgG3 both in vivo and in vitro (80, 85). In addition, BCR engagement cooperates with BAFF and APRIL to induce CD40-independent IgG production (13,19,25). Thus, it is conceivable that LMP2A triggers TI CSR to Cγ3 through a BCR-like pathway. This pathway would cooperate with LMP1 as well as endogenous BAFF and APRIL to optimize IgH class switching in EBV-infected B cells.

Our findings raise the possibility that IgH class switching confers a specific functional advantage to EBV. When engaged by Ag, surface IgM and IgD (i.e., BCR) deliver

proliferation and survival signals through the Igα-Igβ heterodimer (84). These signals are negatively regulated by CD22, an inhibitory coreceptor that contains typical immunoreceptor tyrosine-based inhibitory motifs (86). Recent studies indicate that B cells become resistant to CD22-mediated inhibitory signals upon switching from IgM and IgD to IgG (87). In this fashion, IgG^+ (IgD^-) memory B cells become more sensitive to BCR-driven proliferation and survival signals upon exposure to Ag. It is tempting to speculate that EBV triggers TI CSR in extrafollicular IgD^+ B cells to rapidly generate a pool of IgD^- B cells expressing protective BCRs, such as IgG. Due to their lower sensitivity to CD22-mediated inhibitory signals, these de novo class-switched B cells would facilitate the initial expansion of the viral episome.

In addition to inducing TI CSR, LMP1 and LMP2A deliver signals that are essential for the survival of infected B cells (36). Our findings imply that these signals might be greatly amplified by autocrine BAFF, a powerful inducer of B cell survival (24). BAFF exerts most of its prosurvival activity through BAFF-R (21, 23), which is expressed in large amounts by both EBV-infected and noninfected B cells. By engaging BAFF-R on bystander self-reactive B cells, BAFF expressed on and released by latently infected tonsillar B cells might facilitate the onset of autoimmune disorders, including SLE (33, 34, 37). Consistent with this, SLE patients display increased levels of circulating soluble BAFF (24), and mice overexpressing BAFF develop an SLE-like syndrome with kidney deposition of IgG and IgA autoantibodies (22). Finally, BAFF and APRIL might also be implicated in the pathogenesis of autoimmune and IgE-mediated atopic disorders arising in certain HIV-infected subjects and transplant recipients with EBV-associated B cell lymphoproliferative disorders (35, 40, 41, 88). In these immune-compromised individuals, neutralization of BAFF and APRIL by soluble decoy receptors or blocking Abs might attenuate production of self-reactive IgG and IgA, dysregulated switching to IgE, and aberrant B cell accumulation.

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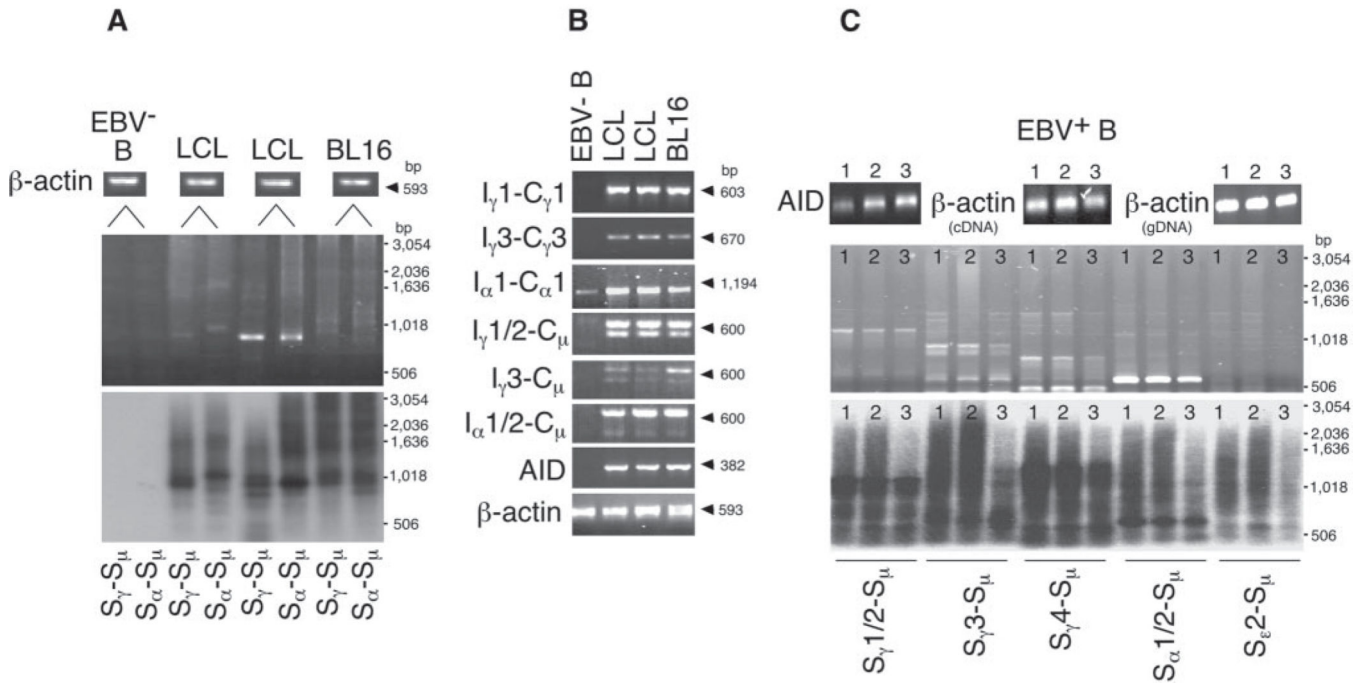

FIGURE 1.

B cells undergo CSR from C_μ to C_γ,C_α, and C_ϵ upon infection by EBV. *A*, Total extrachromosomal S_γ-S_μ and S_α-S_μ SCs and genomic β-actin DNA in normal IgD^+ B cells, IARC549 and IARC100 LCLs, and EBV^+ BL16 cells. *B*, Germline $I_\gamma1$-$C_\gamma1$, $I_\gamma3$-$C_\gamma3$, and $I_\alpha1$-$C_\alpha1$ transcripts, $I_\gamma1/2$-C_μ, $I_\gamma3$-C_μ, and $I_\alpha1/2$-C_μ CTs, AID transcripts, and β-actin transcripts in normal IgD^+ B cells, IARC549 and IARC100 LCLs, and EBV^+ BL16 cells. *C*, AID transcripts, β-actin transcripts, genomic β-actin DNA, and extrachromosomal $S_\gamma1/2$-S_μ, $S_\gamma3$-S_μ, $S_\gamma4$-S_μ, $S_\alpha1/2$-S_μ, and S_ϵ-S_μ SCs in EBV-infected IgD^+ B cells from three healthy subjects. Data depicted in *A*-*C* represent one of three similar experiments. IgD^+ B cells were from three healthy donors.

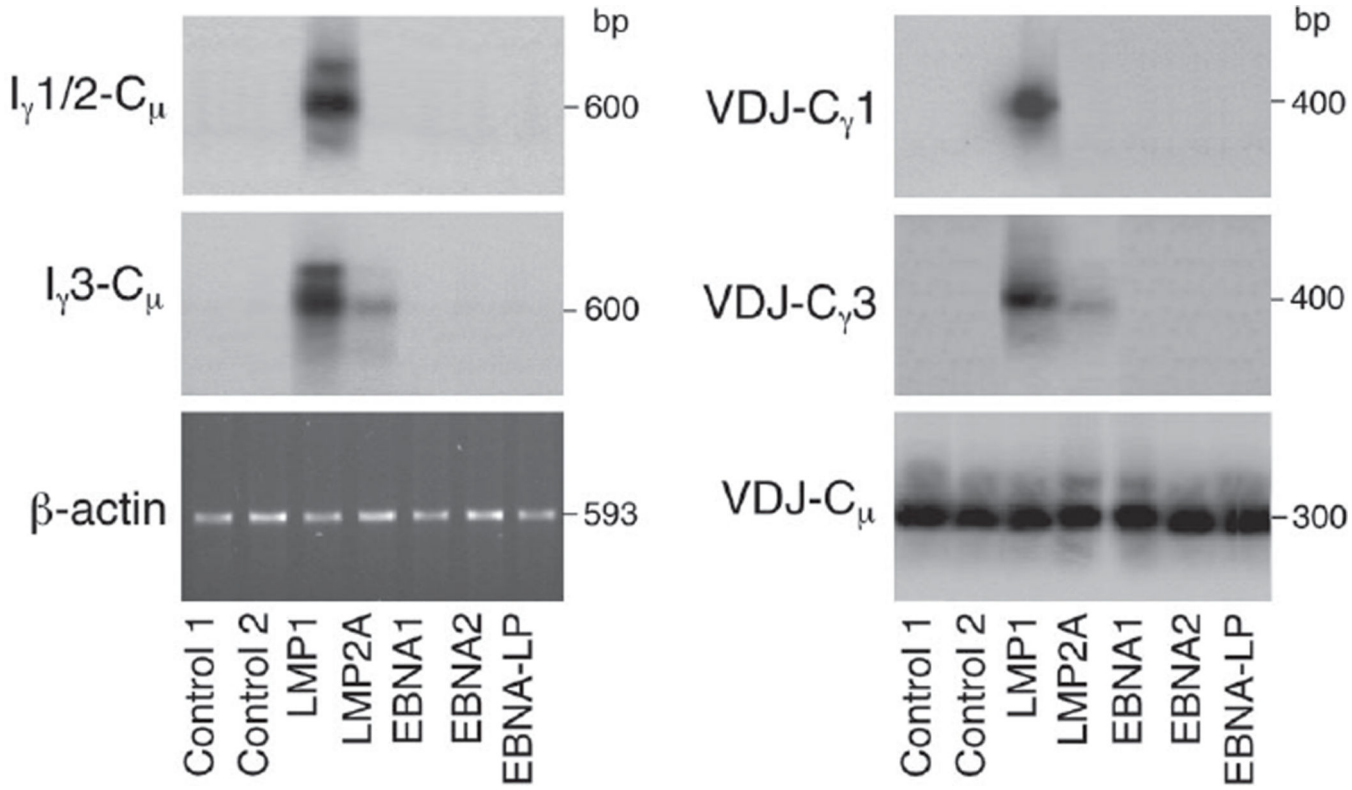

FIGURE 2.

EBV-encoded LMP1 and LMP2A proteins induce CSR in B cells. $I_\gamma 1/2\text{-}C_\mu$ and $I_\gamma 3\text{-}C_\mu$ CTs, and VDJ-$C_\gamma 1$, VDJ-$C_\gamma 3$, and VDJ-C_μ transcripts in Ramos B cell subclones stably transfected with pRH132 (control 1), pRH132-pSG5 (control 2), pRH132-LMP1, pRH132-LMP2A, pRH132-EBNA1, pRH132-EBNA2, or pRH132-pSG5-EBNA-LP expression vectors. Data represent one of three similar experiments.

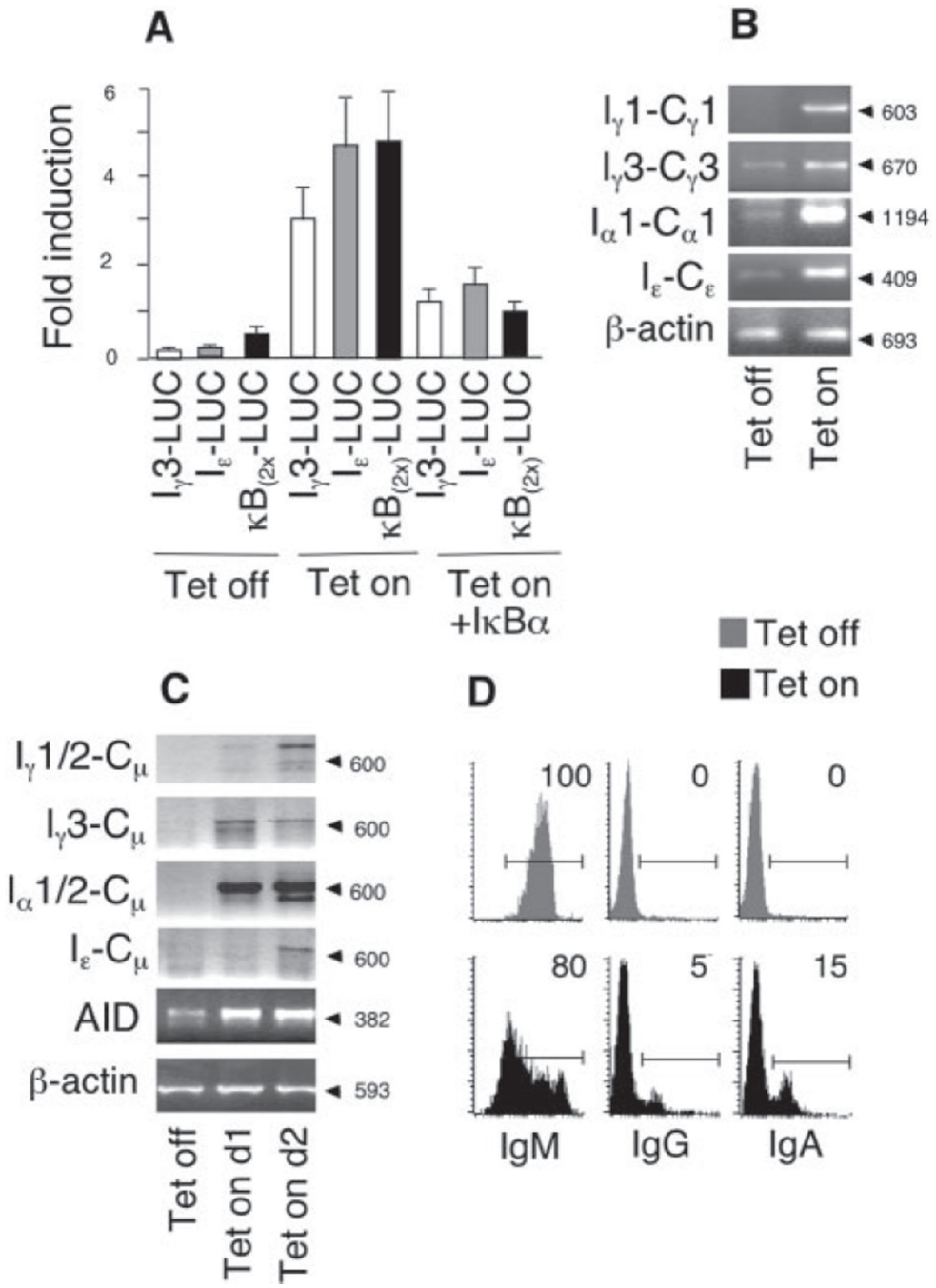

FIGURE 3.

LMP1 induces NF-κB-dependent germline I_H-C_H transcription and CSR in B cells. *A*, Bjab-tet-LMP1 B cells transfected with I_γ3-LUC, I_ϵ-LUC, or $\kappa B_{(2X)}$-LUC were incubated without (tet off) or with (tet on) doxycycline. Luciferase activity was measured after 2 days. *B*, Germline I_γ1-C_γ1, I_γ3-C_γ3, I_α1-C_α1, and I_ϵ-C_ϵ transcripts in Bjab-tet-LMP1 B cells incubated without (tet off) or with (tet on) doxycycline for 2 days. *C*, I_γ1/2-C_μ, I_γ3-C_μ, I_α1/2-C_μ, and I_ϵ-C_μ CTs, AID transcripts, and β-actin transcripts in Bjab-tet-LMP1 B cells incubated without (tet off) or with (tet on) doxycycline for 4 days. *D*, Surface IgM, IgG, and

IgA on Bjab-tet-LMP1 B cells incubated without (tet off; shaded profiles) or with (tet on; solid profiles) doxycycline for 4 days. Numbers indicate percentage of positive cells. Data depicted in *A–D* represent one of three similar experiments, and bars indicate SD.

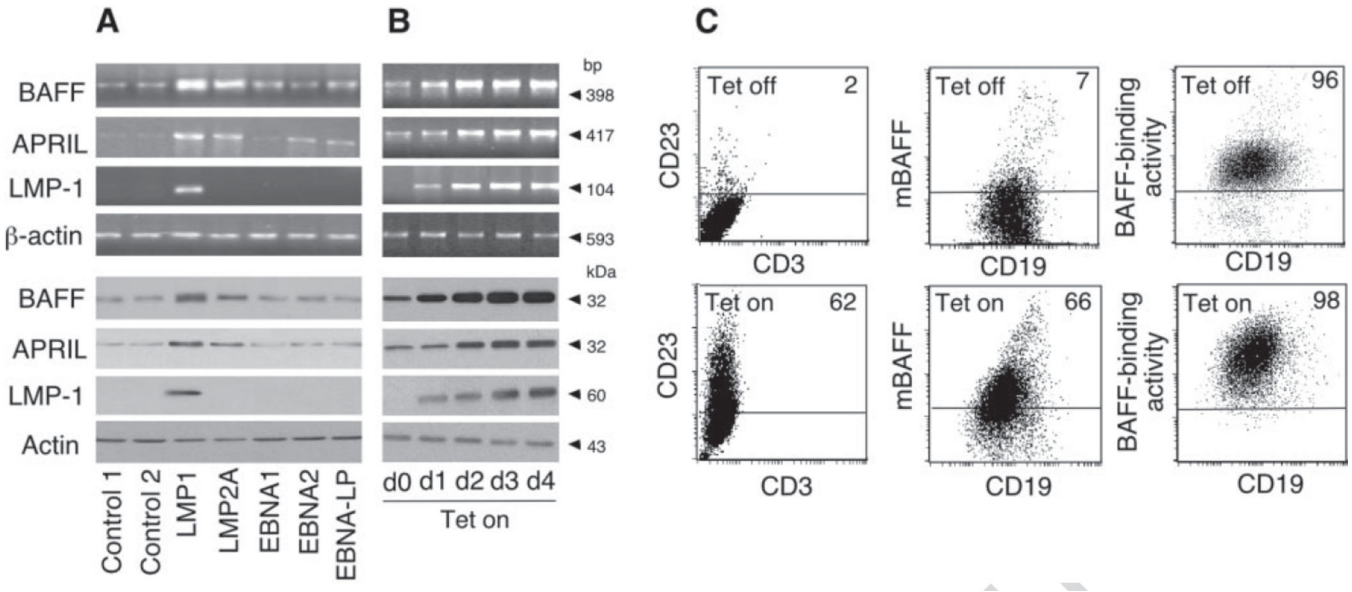

FIGURE 4.

LMP1 up-regulates BAFF and APRIL. *A*, BAFF, APRIL, LMP1, and actin transcripts and proteins in EBV$^-$ Ramos B cell subclones stably transfected with pRH132 (control 1), pRH132-pSG5 (control 2), pRH132-LMP1, pRH132-LMP2A, pRH132-EBNA1, pRH132-EBNA2, or pRH132-pSG5-EBNA-LP expression vectors. *B*, BAFF, APRIL, LMP1, and actin transcripts and proteins in Bjab-tet-LMP1 B cells incubated without (d 0) or with (tet on) doxycycline. *C*, CD3, CD23, CD19, mBAFF, and BAFF-binding activity on Bjab-tet-LMP1 B cells incubated without (tet off) or with (tet on) doxycycline for 2 days. Numbers indicate percentage of positive cells. Data depicted in *A*—*C* represent one of three similar experiments.

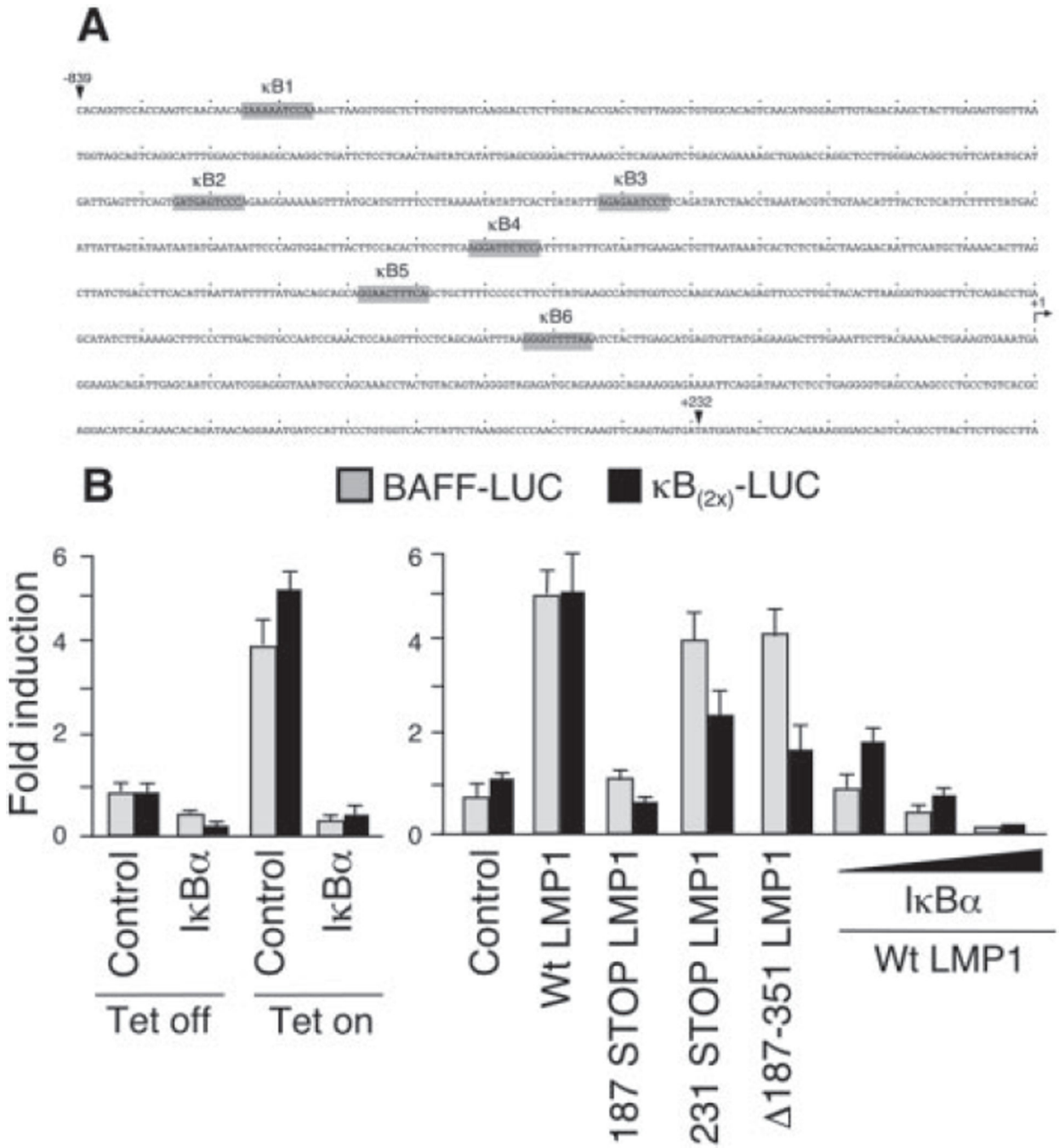

FIGURE 5.

LMP1 elicits NF-κB-dependent up-regulation of BAFF and APRIL in B cells. *A*, DNA sequence of the BAFF promoter (GenBank accession no. AY129225). +232 indicates the 3′ end of the promoter, a turned arrow indicates the major initiation site (+1), and boxes depict putative κB motifs. *B*, *Left*, Bjab-tet-LMP1 B cells transfected with BAFF-LUC (▨) or $\kappa B_{(2}X)$-LUC (■) in the presence or absence of IκBα-pcDNA3.1 were incubated without (tet off) or with (tet on) doxycycline. *Right*, wt Bjab B cells were cotransfected with BAFF-LUC (▨) or $\kappa B_{(2)}$-LUC (■) and wt LMP1, 187-STOP LMP1, 231-STOP LMP1, ΔEL 187–351

LMP1, or 20, 10, and 2 μg of IκBα-pcDNA3.1. The luciferase activity was measured after 2 days. Data represent one of three similar experiments, and bars indicate SD.

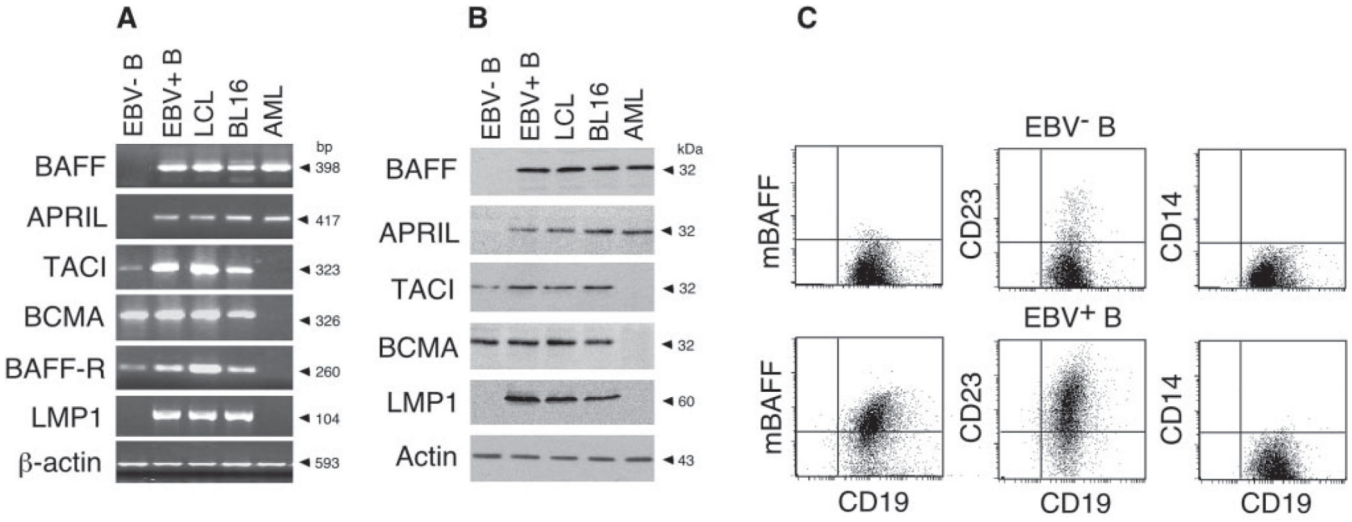

FIGURE 6.

Normal B cells express BAFF and APRIL upon EBV infection. *A*, BAFF, APRIL, TACI, BCMA, BAFF-R, LMP1, and β-actin transcripts in noninfected or EBV-infected IgD^+ B cells from the PB of a healthy subject, IARC549 lymphoblastoid B cells, neoplastic EBV^+ BL16 cells, and neoplastic HL60 AML cells. *B*, BAFF, APRIL, TACI, BCMA, LMP1, and actin proteins in noninfected or EBV-infected IgD^+ B cells from the PB of a healthy subject, IARC549 lymphoblastoid B cells, neoplastic EBV^+ BL16 cells, and neoplastic HL60 AML cells. *C*, mBAFF, CD19, CD23, and CD14 in purified noninfected and EBV-infected IgD^+ B cells from the PB of a healthy subject. Data depicted in *A*-*C* represent one of three similar experiments. IgD^+ B cells were from three independent healthy donors.

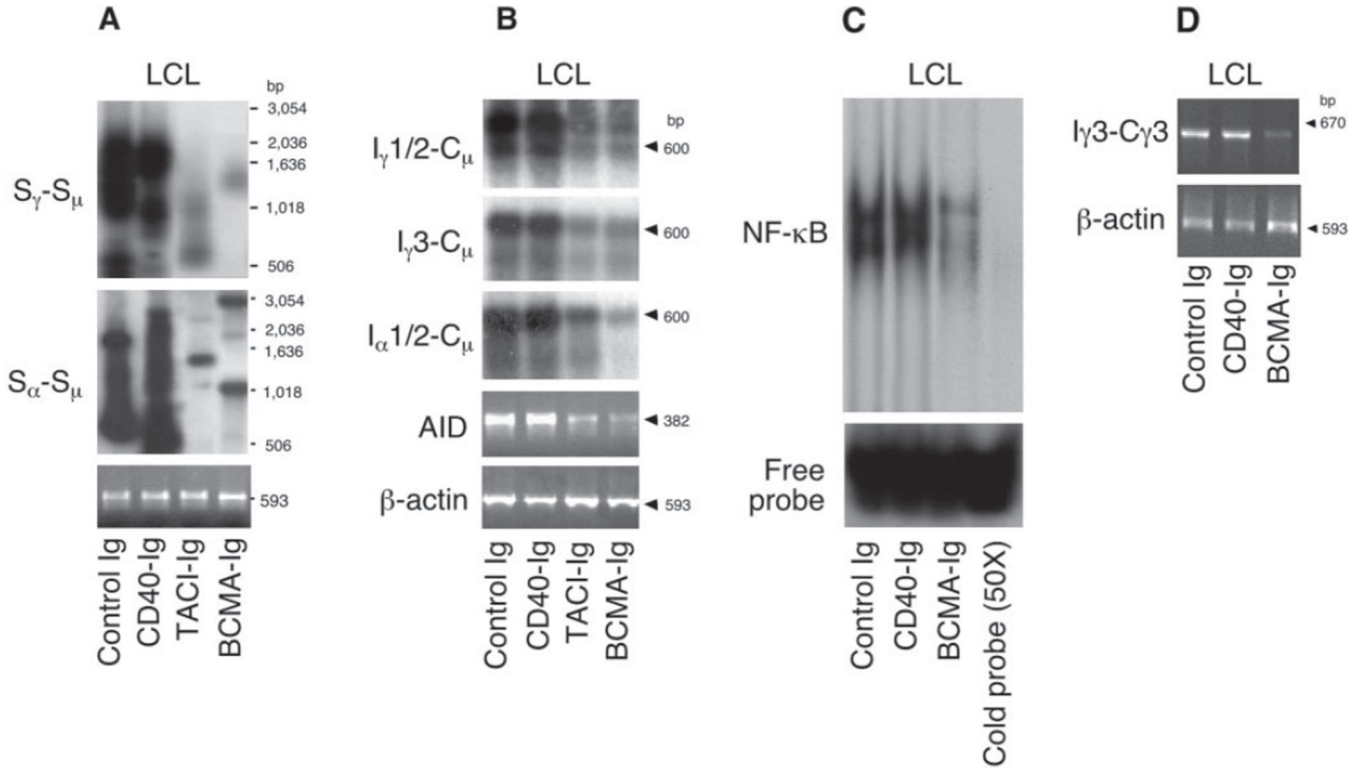

FIGURE 7.

Neutralization of BAFF and APRIL attenuates CSR in EBV$^+$LMP1$^+$ B cells. *A*, Total S_γ-S_μ and S_α-S_μ SCs and genomic β-actin DNA in IARC549 lymphoblastoid B cells incubated for 3 days with 30 μg/ml control MOPC-21 Ig, CD40-Ig, TACI-Ig, or BCMA-Ig. *B*, $I_\gamma1/2$-C_μ, $I_\gamma3$-C_μ, and $I_\alpha1/2$-C_μ CTs, AID transcripts, and β-actin transcripts in IARC549 lymphoblastoid B cells cultured as above. *C*, Binding of nuclear NF-κB to a radiolabeled DNA sequence encompassing the CD40-responsive element from the *$I_\gamma3$* gene promoter in IARC549 lymphoblastoid B cells incubated for 2 days with control Ig, CD40-Ig, or BCMA-Ig. The specificity of shifted bands and their identity to NF-κB were established by preincubating nuclear proteins with cold probe. *D*, Germline $I_\gamma3$-$C_\gamma3$ transcripts and β-actin transcripts in IARC549 lymphoblastoid B cells cultured as above. Data depicted in *A*—*D* represent one of three similar experiments.