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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

Bing He,* Nancy Raab-Traub,† Paolo Casali,‡ and Andrea Cerutti2*†

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 Cμ to multiple downstream Cγ, Cα, and Cε 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 Cγ, Cα, and Cε 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. The Journal of Immunology, 2003, 171: 5215–5224.

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2 Address correspondence and reprint requests to Dr. Andrea Cerutti, Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021. E-mail address: acerutti@med.cornell.edu
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 cyclophillin 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.
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 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 epismes 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 IkBα degradation and NF-κB nuclear translocation by recruiting TRAFs and IkB 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 Cμ to multiple Cκ, Cα, and Cε 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, Carlsbad, 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 glucose. 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), tetr-1MP1 Bjab cells (from N. Lam and B. Sugden (University of Wisconsin-Madison, Madison, WI)) were cultured with medium supplemented with 1 µg/ml puromycin and 200 µg/ml genetin (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 a mouse Ab to BAF (Alexis Biochemicals) and a PE-conjugated anti-mouse Ab (BD PharMingen). BAF-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 Ficol. 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 IgC transcripts, mature VDJ-C 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’-AAGAAGGGGGGATCCCTGC-3’, and reverse, 5’-TTATGACCTGCGCCC3’; BCMA, forward, 5’-CTAAAGGAA GATAAACCTGAGGCA-3’, and reverse, 5’-TTACCTCTGAGCAATT GATTTTC-3’; BAFF-R, forward, 5’-GGTGAGCTGGAGGCGGCGACAG-3’, and reverse, 5’-ATTCATGGTCCTAGGCGCGC-3’; and LMP1, forward, 5’-CTTACAGAGAACCTTCCTC-3’, and reverse, 5’-AATAATGCGT CCCTGACAA-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 Sc region; circle transcripts (CTs) and mature VDJ-Sc transcripts were hybridized with a probe encompassing nt 1–250 of the first Sc exon; and mature CTs, transcripts were hybridized with a probe encompassing nt 35–265 and −291/+131 genomic DNA fragments encompassing the I3 and I1 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 187–233, were cloned into the pGL3-Basic reporter vector. The MatInspector software (Genomatix Software, Munchen, Germany) was used to identify putative NF-κB sites.

Vectors

The LMP1 ORF was inserted into a pLNCX vector (Promega, Madison, Wl) containing a selectable neo gene. The LMP1 was inserted into the pGL3-Basic vector (Promega, Madison, Wl) containing a luciferase (LUC) reporter gene (54, 55). Wild-type (wt) LMP1 and mutant LMP1, including DEL 187–233, were cloned into the pGL3-Basic reporter vector. The MatInspector software (Genomatix Software, Munchen, Germany) was used to identify putative NF-κB sites.

Flow cytometry

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 Bjab cells was reversed transcribed with an avian myeloblastosis virus reverse transcriptase and an end-labeled 5’-CAGTAGGTTCGCGATTTAACACTG-3’ antisense primer recognizing a sequence immediately upstream of the first BAFF exon. The resulting cDNA was analyzed on a denaturing polyacrylamide gel.
B cells up-regulate AID and undergo CSR from Cγ to Cα upon infection by EBV

CSR from Cμ to a downstream Cγ3, Cα1, Cα2, and Cα3 gene is preceded by the transcription of that gene in the form of a noncoding germline Iγ3-Cα1 transcript that includes the Iγ4 exon 5' of the targeted S region and Cα5 (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 Sγ 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 Iγ4-Cα3 CTs that include the promoter upstream of the targeted Iγ4 exon, the Iγ4 exon, and Cα5 (58). These CTs often undergo posttranscriptional remodeling, thereby generating more than one band after PCR amplification (58). Together with AID and germline Iγ4-Cα3 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 unarranged 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 Sγ-Sα and Sγ-Sα SCs (Fig. 1A), expressed no or low germline Iγ1-Cα1, Iγ3-Cα3, and Iγ1-Cα1 transcripts, and lacked Iγ1/Iγ2-SCs, Iγ3/SCs, and Iγ1/Iγ2-SCs as well as AID transcripts (B). In contrast, lymphoblastoid and EBV+ BL16 B cells contained total Sγ-Sα and Sγ-Sα SCs, expressed large amounts of germline Iγ1-Cα1, Iγ3-Cα3, and Iγ1-Iγ2-SCs, and contained Iγ1-Iγ2-SCs, Iγ3/SCs, and Iγ1-Iγ2-SCs, 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. 1A), EBV-infected IgD+ B cells up-regulated AID transcripts and contained extrachromosomal S1/2/3-S, S3/3-S, S4/-S, S2/-S, S2-S, Scs (C), which reflect ongoing CSR from Cμ to Cμ/C2, C3, C4, Cμ/Cμ, and Cμ, respectively. These findings indicate that B cells undergo CD40-independent CSR from Cμ to multiple downstream Cμ, Cν, and Cε genes upon infection by EBV.

**EBV up-regulates germline Iμ-Cμ 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 subclones expressing LMP1 contained I1/2-Cμ and I3-Cμ CTs as well as mature VμDJμ1-Cμ and VμDJμ1-Cμ3 transcripts (Fig. 2), the end product of CSR from Cμ to Cμ/C2 and Cμ/C3. B cell subclones expressing LMP2A contained lower amounts of I3-Cμ CTs and VμDJμ1-Cμ3 transcripts, but lacked I1/2-Cμ and VμDJμ1-Cμ1 transcripts. In contrast, B cell subclones expressing EBNA1, EBNA2, or EBNA-LP lacked I1/2-Cμ and I3-Cμ CTs as well as mature VμDJμ1-Cμ1 and VμDJμ1-Cμ3 transcripts. Finally, all B cell subclones expressed β-actin transcripts as well as mature VμDJμ1-Cμ3 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. Compared with untreated B cells, Bjab B cells express IgM but not IgG, IgA, or IgE on the surface. Bjab-tet-LMP1 B cells up-regulated LMP1 mRNA (shown below) and activated luciferase reporter vectors containing the Iγ2 gene promoter (Iγ2-LUC), the λ5 gene promoter (λ5-LUC), or κBκκκ-LUC upon incubation with doxycycline for 2 days (Fig. 3A). Overexpression of the NF-κB inhibitor IκBα inhibited doxycycline-induced activation of Iγ2-LUC, λ5-LUC, and κBκκκ-LUC. In addition to activating the Iγ2 and λ5 promoters, doxycycline up-regulated the expression of germline I1-Cμ1, I3-Cμ3, I1-Cμ1, and Iε-Cμ transcripts (Fig. 3B). Furthermore, Bjab-tet-LMP1 B cells exposed to doxycycline for 4 days up-regulated AID transcripts and induced I1/2-Cμ, Iγ3-Cμ, Iε1/2-Cμ, and Iε-Cμ CTs (Fig. 3C), which reflect ongoing CSR from Cμ to Cμ/C2, C3, C4, Cμ/Cμ, and Cμ, respectively. The induction of CSR by doxycycline was associated with up-regulation of surface IgG and IgA and down-regulation of surface IgM (Fig. 3D). These findings indicate that B cells undergo NF-κB-dependent germline Iμ-Cμ 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. 4A). 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. 4B). Tet also up-regulated surface...
CD23, a canonical LMP1-inducible B cell-activation protein, BAFF, as well as the total BAFF-binding activity (Fig. 4C), 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 sites (Fig. 5A). Bjab-tet-LMP1 B cells activated a luciferase reporter vector carrying the BAFF gene promoter (BAFF-LUC) as well as NF-κB (2X) -LUC upon incubation with doxycycline for 2 days. Overexpression of IκBα inhibited the induction of BAFF-LUC and NF-κB (2X) -LUC by doxycycline (Fig. 5B), 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 NF-κB (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 NF-κB (2X) -LUC. Furthermore, both BAFF-LUC and NF-κB (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 NF-κB (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. 6A) 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

**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-ERNA-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 κB (❄) -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 κB (❄) -LUC (❄) and wt LMP1, 187-STOP LMP1, 231-STOP LMP1, DEL 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.
Ag CD14 (Fig. 6C). 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 than the normal B cells.

**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 Sγ-Sμ and Sμ-Sμ 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, lγ1/2-Cμ, lγ3-Cμ, and lγ1/2-Cμ 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 Iγ3 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 lγ3-Cγ3 transcripts and β-actin transcripts in IARC549 lymphoblastoid B cells cultured as above. Data depicted in A–D represent one of three similar experiments.
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 NL60 AML cells
expressed TACI, BCMA, and BAFF-R transcripts (Fig. 6A) 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 recep-
tors, which prevent the binding of BAFF and APRIL to cell-bound
TACI, BCMA, and BAFF-R. Lymphoblastoid IARC549 B cells
down-regulated total Sγ, Sμ, and Sμ, Sδ, SCs (Fig. 7A). Iγ, Iγ/II, III,
III, and Iγ/II, III C Ts, 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-xb activation and germline
Iγ/Cμ 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 activ-
ate NF-xb. Exposure of lymphoblastoid IARC549 B cells to
BCMA-Ig but not control Ig or CD40-Ig down-regulated the bind-
ing of nuclear NF-xb to an oligonucleotide encompassing a DNA
sequence crucial for the activation of the human Iγ promoter by
CD40 (Fig. 7C). As expected, this effect was associated with
down-regulated expression of germline Iγ/Cμ, Iγ/II/Cμ, III/Cμ,
and Iγ/II, III C Ts, 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.

**Discussion**

We have shown that EBV-encoded LMP1 induces CD40-indepen-
dent CSR from Cμ to multiple Cγ, Cδ, and Cε genes in B cells.
This induction is associated with NF-xb-dependent activation of
downstream Cμ gene promoters and up-regulation of germline
Iγ/Cμ transcripts and AID transcripts. LMP1 up-regulates also
BAFF and APRIL through an NF-xb-dependent mechanism that
requires at least one CTAR domain. By engaging TACI, BCMA,
and BAFF-R on B cells, BAFF and APRIL activate NF-xb 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 pro-
teins, EBV induces IgD+ B cells to become blasts, which prolif-
erate outside the GC (43). Subsequent down-regulation of most
EBV proteins but EBNA1, LMP1, and LMP2A would enable in-
fected IgD+ blasts to enter the GC and differentiate to class-
switched IgD+ memory B cells (43, 45). After further down-reg-
ulation 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 un-
dergo 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 in-
dicate that EBV transcriptionally activates downstream germline
Cμ gene promoters, including Cγ, Cδ, and Cε, through LMP1. This
dCμ-like viral protein would transactivate Cγ, Cδ, and Cε genes
through an NF-xb-dependent mechanism. Consistent with this,
overexpression of the NF-xb inhibitor IκBα or (not shown) dis-
ruption of both NF-xb-activating CTAR domains within the
LMP1 cytoplasmic tail impairs the induction of germline Iγ/Cμ
transcription by LMP1. In LMP1-expressing B cells, germline
Iγ/Cμ transcription is associated with up-regulation of AID tran-
scripts and induction of CSR from Cμ to multiple downstream Cγ,
Cδ, and Cε genes. These findings provide a mechanistic explana-
tion 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-depend-
ent signals (3), LMP1 continuously signals in a ligand-independ-
ent fashion (52). This implies that LMP1-induced CSR is subject
to less regulatory constraints than CD40-induced CSR. By show-
ing that EBV-infected LMP1-expressing IgD+ B cells constitu-
tively express high levels of Iγ/Cμ transcripts and AID and con-
 tinuously undergo CSR, our data extend recent findings indicating
that artificial Sμ and Sδ DNA substrates undergo spontaneous
recombination in lymphoblastoid B cells (65). By triggering un-
restrained 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 de-

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-ex-
pressing B cells, CSR to Cε occurs in the absence of ex-
genous 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 pro-
tein through a viral gene known as BCRF1 (76). Furthermore,
EBV-infected B cells produce IL-13 (77), which, like IL-4, acti-
vates switching from IgM to IgG4 and IgE (78, 79). This might
explain our finding that EBV-infected LMP1-expressing B cells
actively switch to Cε, Cδ, and Cμ 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 lymph-
oid follicles (63). This extracellular pattern is also associated with
B cell responses to TI Ags with repetitive structure, including
envvelope 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 $C_m$ to a targeted downstream $C_\mu$ gene by activating the germline transcription of that gene through NF-$\kappa$B. Consistent with this, neutralization of autocrine BAFF and APRIL by soluble TACI-Ig and BCMA-Ig decoy receptors attenuates NF-$\kappa$B activation and down-regulates the expression of downstream germline $Ig_m - C_{\mu}$ 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-$\kappa$B-dependent germline $Ig_m - C_{\mu}$ transcription and CSR.

In addition to transactivating downstream $C_\mu$ genes, NF-$\kappa$B would play an important role in the LMP1-mediated up-regulation of BAFF. Consistent with this, overexpression of the NF-$\kappa$B inhibitor IxB$\alpha$ interferes with the transcriptional activation of the BAFF gene promoter in LMP1-activated B cells. Furthermore, disruption of the two NF-$\kappa$B-activating CTARs within the LMP1 cytoplasmic tail severely impairs the activation of the BAFF gene promoter by LMP1. Although playing a key role, NF-$\kappa$B 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 $\gamma$-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-$\kappa$B and STAT transcription factors might synergistically activate the BAFF gene promoter upon binding to cooperative $\kappa$B and $\gamma$-IFN-activated sequence sites.

LMP1 is not the only CSR-inducing viral protein, because LMP2A triggers CSR from $C_m$ to $C_\lambda^3$ 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 receptor (BCR) (36). Consistent with this, the cytoplasmic tail of LMP2A encompasses immunoreceptor tyrosine-based activation motifs similar to those found in the Igox and Ig$\beta$ signal-transducing sub-units 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 T1 CSR to $C_\gamma^3$ 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 IgD-Ig$\beta$ 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 T1 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 epimorphose.

In addition to inducing T1 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 inductor of B cell survival (20). 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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References


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