Evasion of affinity-based selection in germinal centers by Epstein–Barr virus LMP2A

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Epstein–Barr virus (EBV) infects germinal center (GC) B cells and establishes persistent infection in memory B cells. EBV-infected B cells can cause B-cell malignancies in humans with T- or natural killer-cell deficiency. We now find that EBV-encoded latent membrane protein 2A (LMP2A) mimics B-cell antigen receptor (BCR) signaling in murine GC B cells, causing altered humoral immune responses and autoimmune diseases. Investigation of the impact of LMP2A on B-cell differentiation in mice that conditionally express LMP2A in GC B cells or all B-lineage cells found LMP2A expression enhanced not only BCR signals but also plasma cell differentiation in vitro and in vivo. Conditional LMP2A expression in GC B cells resulted in preferential selection of low-affinity antibody-producing B cells despite apparently normal GC formation. GC B-cell–specific LMP2A expression led to systemic lupus erythematosus-like autoimmune phenotypes in an age-dependent manner. Epigenetic profiling of LMP2A B cells found increased H3K27ac and H3K4me1 signals at the zinc finger and bric-a-brac, tramtrack domain-containing protein 20 locus. We conclude that LMP2A reduces the stringency of GC B-cell selection and may contribute to persistent EBV infection and pathogenesis by providing GC B cells with excessive prosurvival effects.

LMP2A | germinal center | plasma cell differentiation | B cells | autoimmune diseases

Epstein–Barr virus (EBV) is a B-lymphotropic human herpesvirus associated with a variety of hematopoietic cancers, including endemic Burkitt lymphoma, Hodgkin lymphoma, lymphoproliferative disorders in immune-compromised people, nasopharyngeal carcinoma, and some gastric cancers (1, 2). Nevertheless, EBV establishes lifelong latent infection by adolescence in more than 95% of adults worldwide. EBV efficiently infects resting human B cells in vitro, leading to their activation and proliferation. EBV-infected B cells grow in vitro as immortalized lymphoblastoid cell lines (LCL). LCLs express EBV-encoded proteins including the EBV nuclear antigens (EBNAs) EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNALP and the latent membrane proteins LMP1, LMP2A, and LMP2B. Of these EBV gene products, EBNA2, EBNALP, EBNA3A, EBNA3C, and LMP1 are essential for B-cell transformation (3), whereas EBNA1 is necessary for EBV episome persistence (4).

LMP2A consists of an N-terminal cytoplasmic domain, 12 membrane-spanning domains, and a short C-terminal cytoplasmic domain. The N-terminal cytoplasmic domain has an immune receptor tyrosine-based activation motif (ITAM), which can recruit the protein tyrosine kinases Syk and Src (5). In LCLs or mouse cells that ectopically express LMP2A, LMP2A suppresses B-cell antigen receptor (BCR) signaling (6–8). In mice lacking BCR expression, transgenic LMP2A can partially rescue B-cell differentiation (9–11), indicating that LMP2A can mimic tonic BCR signals necessary for B-cell survival in vivo (12). Likewise, LMP2A is essential for growth transformation of germinal center (GC)-derived BCR^- B cells (13). Depending on its B-cell expression level in murine models, constitutive LMP2A expression alters B-cell development or augments the differentiation and activation of antigen-driven B cells (9, 10, 14–16).

In the peripheral blood of persistently infected people, EBV resides exclusively in IgD^- memory B cells (17). However, in tonsils, EBV is found not only in memory B cells but also in GC and IgD^+ resting B cells in which EBNA1, LMP1, and LMP2A are expressed (17, 18). Thereby, EBV may use antigen-driven B-cell activation and differentiation in mucosal secondary lymphoid organs, particularly GC reactions, to establish persistent infection in the memory B-cell pool. Together with LMP1, which constitutively activates CD40 signaling (19, 20), LMP2A may provide a survival advantage for EBV-infected B cells by modifying GC B-cell selection. However, B-lineage–specific expression of LMP1 inhibits GC formation (20) or causes B-cell ablation through immune surveillance (21). LMP2A does not affect affinity maturation of B cells in GCs when expressed in B cells of a transgenic mouse line (22). Constitutive expression in transgenic mice used in these previous studies may be inappropriate for studying the influence of infection-induced virus proteins on B-cell differentiation, particularly in GCs. To date, the impact of LMP2A on differentiation and selection of GC B cells is still controversial.

Significance

Epstein–Barr virus (EBV) is a human herpesvirus that establishes persistent infection of the B-cell compartment. EBV is associated with autoimmune diseases, including systemic lupus erythematosus (SLE). However, the molecular mechanisms by which EBV contributes to autoimmunity remain unclear. We used previously undescribed mouse models to study the role of EBV-encoded latent membrane protein 2A (LMP2A), which mimics B-cell receptor signaling. Interestingly, LMP2A not only enhanced B-cell survival but also upregulated the transcription factor zinc finger and bric-a-brac, tramtrack domain-containing protein 20 and promoted plasma cell differentiation. When expressed late in B-cell development, LMP2A also caused prominent features of SLE, including autoantibody production with kidney immune complex deposition. Our findings suggest that LMP2A has important roles in B-cell activation and differentiation and in the development of EBV-associated autoimmune diseases.

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Data deposition: The sequences reported in this paper have been deposited in the Gene Expression Omnibus database (accession nos. GSE70521 and GSE71408).

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To explore the possibility that LMP2A contributes to persistent EBV infection by modifying GC B-cell selection, we generated an experimental mouse system in which LMP2A is conditionally expressed in GC B cells, similar to LMP2A expression in EBV-infected human B cells. Using this system, we analyzed the effects of LMP2A expression on B-cell entry into and differentiation within GCs.

Results

LMP2A Expression in B Cells Results in B-Cell Hyperactivation and Impaired Humoral Responses. To characterize the effects of LMP2A expression on humoral immune responses, we generated mice in which LMP2A was conditionally expressed in B cells. We produced two knockin mice, $LMP2A^{STOP}$ mice and control GFP^{STOP} mice, into whose ROSA26 locus one of two cassettes was inserted (Fig. S1*A*). $LMP2A^{STOP}$ mice received a cassette containing floxed neomycin phosphotransferase cDNA (NEO) with a stop codon, N-terminal HA-tagged LMP2A, an internal ribosome entry site sequence (IRES), and monomeric enhanced GFP (mEGFP). GFP^{STOP} mice received a cassette containing floxed NEO with a stop codon, an IRES, and mEGFP (Fig. S1*A*). Subsequently, $LMP2A^{STOP}$ mice and GFP^{STOP} mice were crossed with *CD19-Cre* mice to generate mice that express both LMP2A and GFP ($LMP2A^{CD19}$) or only GFP (GFP^{CD19}) in all B-lineages. Approximately 80% of B-lineage cells expressed GFP in $LMP2A^{CD19}$ mice and GFP^{CD19} mice. HA-tagged LMP2A expression in $LMP2A^{CD19}$ B cells was confirmed also (Fig. S1 *B* and *C*). Analysis of proliferative activity revealed that B cells from $LMP2A^{CD19}$ mice were more responsive to BCR stimulation than B cells in GFP^{CD19} mice (Fig. 1*A*), although LMP2A expression did not affect B-cell differentiation (Fig. S1 *D*–*F*). Consistent with B-cell responses in $LMP2A^{CD19}$ mice, tyrosine phosphorylation of proteins, including Syk, was significantly higher in anti-IgM–stimulated $LMP2A^{CD19}$ B cells despite normal NF-κB activation (Fig. 1*B* and Fig. S2*A*), suggesting that LMP2A may affect only some BCR signals.

To assess the overall effect of LMP2A expression on humoral immune responses, $LMP2A^{CD19}$ mice and GFP^{CD19} mice were immunized with (4-hydroxy-3-nitrophenyl) acetyl chicken gamma globulin (NP-CGG). In the initial phase of humoral immune responses, some antigen-committed B cells proliferate and differentiate into low-affinity antibody-secreting plasmablasts in the extrafollicular area, whereas others migrate to the follicle and start to form GCs. An enzyme-linked immunospot (ELISPOT) analysis demonstrated significant increases in number of antibody-secreting cells (ASCs), particularly IgM^+ cells in spleens of $LMP2A^{CD19}$ mice 1 wk after immunization (Fig. S2*B*), indicating that LMP2A promotes extrafollicular B-cell differentiation into plasma cells. Immunohistochemical analysis of spleen sections revealed defective GC formation in $LMP2A^{CD19}$ mice (Fig. S2*C*) and fewer $GL7^+$ and peanut agglutinin (PNA)-binding GC B cells in $LMP2A^{CD19}$ mice than in GFP^{CD19} mice 2 wk after immunization. (Fig. 1*C*). Antigen-specific IgG1 antibody responses also were severely impaired in $LMP2A^{CD19}$ mice, although IgM responses in these mice were either unaffected or augmented (Fig. 1*D* and Fig. S2*D*). These data indicate that constitutive LMP2A expression enhances the differentiation of antigen-committed B cells into ASCs but suppresses either GC B-cell expansion or entry into GCs.

LMP2A Expression in GC B Cells Results in Decreased Antigen-Specific B Cells Without Decreased GC Formation. To determine more precisely how LMP2A expression affects GC B-cell differentiation, $LMP2A^{STOP}$ mice and GFP^{STOP} mice were crossed with *Aicda-Cre* mice (23). The *Aicda* locus, which encodes the enzyme activation-induced cytidine deaminase (AID), is activated selectively in GC B cells (24). The resulting strains express LMP2A and GFP ($LMP2A^{AID}$ mice) or only GFP (GFP^{AID} mice) upon

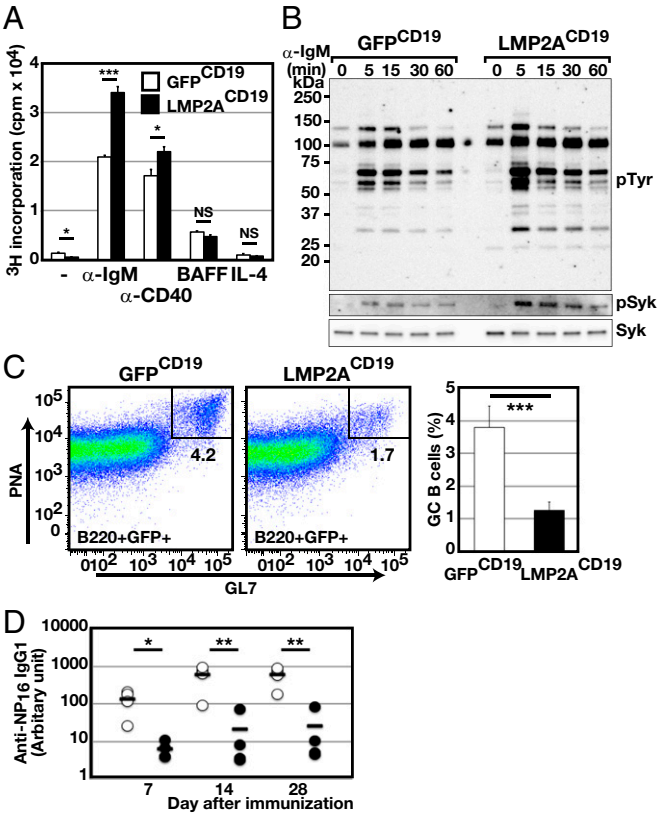

Fig. 1. LMP2A expression in B-lineage cells accelerates B-cell activation but causes impaired humoral responses. (*A*) Purified B cells from spleens of $LMP2A^{CD19}$ or GFP^{CD19} mice were cultured with the indicated stimuli. Cultures were pulsed with ^{3}H-thymidine, and ^{3}H-thymidine incorporation was analyzed. Data are representative of three independent experiments. (*B*) Purified B cells from spleens of $LMP2A^{CD19}$ or GFP^{CD19} mice were stimulated with α-IgM F(ab′)$_2$ for the indicated periods, and the expression of phosphorylated tyrosine (pTyr), phosphorylated Syk (pSyk), and total Syk were analyzed by Western blot. Data are representative of three independent experiments. (*C* and *D*) $LMP2A^{CD19}$ and GFP^{CD19} mice were immunized i.p. with NP-CGG in alum. (*C*, *Left*) Flow cytometric analysis of splenic GC B cells ($CD19^+GL7^{hi}PNA^{hi}$) from $LMP2A^{CD19}$ or GFP^{CD19} mice 14 d after immunization ($n = 4$). The numbers indicate the frequencies of cells in the gates. (*Right*) Columns (open: GFP^{CD19}; closed: $LMP2A^{CD19}$) indicate mean frequencies. (*D*) The serum titers of NP-specific IgG1 were analyzed by ELISA (open circles: GFP^{CD19}; filled circles: $LMP2A^{CD19}$; $n = 4$). IgG1 with affinity for NP was captured using NP_{16}-BSA. The bar in each time point indicates the average. Error bars show the means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

activation of the *Aicda* locus. In these mice, most B cells were GFP^-, although some splenic B cells were GFP^+ (Fig. S3*A*). These GFP^+ B cells acquired LMP2A and GFP coexpression as a consequence of differentiation in the bone marrow, where a fraction of immature B cells express AID (25). Small numbers of GFP^+ cells were observed in immature bone marrow B cells (Fig. S3*B*). There was little or no difference in the surface phenotype of GFP^+ and GFP^- splenic B cells of unimmunized $LMP2A^{AID}$ mice (Fig. S3 *C*–*E*), suggesting that inducible LMP2A expression did not affect B-cell development in $LMP2A^{AID}$ mice. When immunized with NP-CGG, $LMP2A^{AID}$ mice, unlike $LMP2A^{CD19}$ mice, formed GCs morphologically indistinguishable from those of GFP^{AID} mice (Figs. S2*C* and S4*A*). Flow cytometry analysis revealed that GC B cell frequencies were comparable in $LMP2A^{AID}$ mice and GFP^{AID} mice (Fig. S4*B*). As expected, in both groups of mice more than 85% of GC B cells expressed GFP (Fig. S4*C*). The only difference detected between these two groups of mice was a slightly but significantly lower frequency of light-zone GC B cells in $LMP2A^{AID}$ mice than in GFP^{AID} mice (Fig. S4*D*). Following positive selection by follicular

dendritic cell (FDC)-presented antigen, light-zone GC B cells either reenter the dark zone for further cell division or leave the GC to differentiate into plasma cells or memory B cells (26). To determine the effects of LMP2A expression on post-GC B-cell function, we characterized memory B cells and plasma cells following immunization. The frequency of GFP^+, $B220^-$, and $CD138^+$ splenic plasma cells was significantly higher in $LMP2A^{AID}$ mice than in GFP^{AID} mice (Fig. 2*A*), although there was no difference in GFP^+, $B220^+$, IgD^-, $GL7^{lo}$, and $CD38^+$ splenic memory B cells between the two groups (Fig. S4*E*).

Frequencies of Antigen-Specific B Cells and Serum Levels of Antigen-Specific Antibodies in $LMP2A^{AID}$ and GFP^{AID} Mice. Despite morphologically normal GC formation and elevated plasma cells, the frequency of NP-binding B cells was significantly reduced in GCs of $LMP2A^{AID}$ mice (Fig. 2*B*). Serum levels of NP-specific IgG1, particularly, high-affinity IgG1, also were significantly lower in $LMP2A^{AID}$ mice than in GFP^{AID} mice, although the serum level of NP-specific IgM in $LMP2A^{AID}$ mice was comparable to that in GFP^{AID} mice (Fig. S4*F*). ELISPOT assays revealed that frequencies of NP-specific IgG1-secreting cells were significantly reduced in $LMP2A^{AID}$ mice 16 d after immunization, although the numbers of total IgM-secreting cells were elevated (Fig. 2*C*). These results indicate that *Aicda-Cre*–dependent expression of LMP2A strongly inhibits antigen-specific antibody responses but not GC formation, while enhancing plasma cell differentiation.

B Cells of $LMP2A^{AID}$ and WT Mice Have Comparable Ability to Expand in GCs. To compare the ability of LMP2A-expressing and normal B cells to expand and differentiate in GCs under more competitive conditions, equal numbers of bone marrow cells from $LMP2A^{AID}$ mice, GFP^{AID} mice ($CD45.2^+$), and WT mice ($CD45.1^+$) were cotransferred into irradiated recombination activating gene 2 (*Rag2*)-knockout mice to generate $LMP2A^{AID}$/WT or GFP^{AID}/WT chimeric mice (Fig. S5*A*). The resulting bone marrow chimeric mice were immunized with NP-CGG and then were analyzed for GC formation 2 wk after immunization. Almost equal numbers of $LMP2A^{AID}$-derived and WT-derived GC B cells were detected in immunized $LMP2A^{AID}$/WT chimeras (Fig. S5 *B* and *C*). The frequencies of $LMP2A^{AID}$-derived light-zone GC B cells were slightly but significantly reduced in $LMP2A^{AID}$/WT chimeras, as in $LMP2A^{AID}$ mice (Figs. S4*D* and S5 *B* and *C*). The frequencies of $LMP2A^{AID}$-derived cells were significantly lower than those of WT cells among NP-binding B cells in $LMP2A^{AID}$/WT chimeras (Fig. S5 *B* and *C*). These findings suggest that $LMP2A^{AID}$-derived B cells and WT B cells do not differ in their ability to expand in GCs. However, LMP2A-expressing B cells were less reactive to antigens.

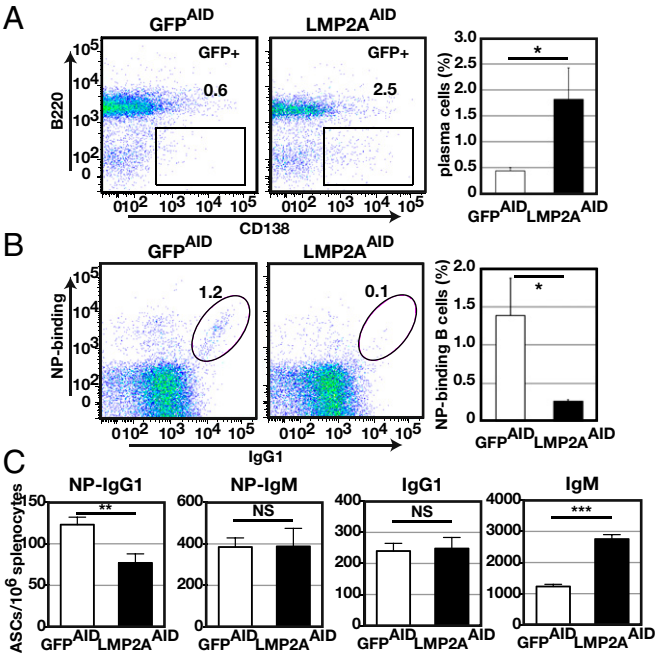

Fig. 2. LMP2A expression in GC B cells reduces the generation of antigen-specific B cells. $LMP2A^{AID}$ and GFP^{AID} mice were immunized i.p. with NP-CGG in alum. (*A* and *B*, *Left*) Flow cytometric analysis of plasma cells ($GFP^+B220^-CD138^+$) (*A*) or NP-binding$^+$ B cells ($CD4^-CD8^-CD11b^-CD3\varepsilon^-Gr\text{-}1^-IgM^-IgD^-IgG1^+GFP^+CD19^+$NP-binding$^+$) (*B*) in the spleen 14 d after immunization ($n = 4$). The numbers indicate frequencies of cells in the gates. (*A* and *B*, *Right*) Columns (open: GFP^{AID}, closed: $LMP2A^{AID}$) indicate mean frequencies. (*C*) The number of NP-specific $IgG1^+$ (NP-IgG1) and IgM^+ (NP-IgM) ASCs or total ASCs in the spleen was analyzed by ELISPOT assay ($n = 5$). Sixteen days after immunization, splenocytes were prepared from either $LMP2A^{AID}$ or GFP^{AID} mice and used for the assay. Error bars show the means ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not statistically significant.

LMP2A Expression in GC B Cells Suppresses the Selection of High-Affinity B Cells Expressing the $V_H186.2$ Gene Segment. To investigate LMP2A effects on the selection of high-affinity antibody-producing B cells in GCs, single NP-binding B cells from spleens of immunized $LMP2A^{AID}$ mice or GFP^{AID} mice were sorted by FACS. The Ig heavy-chain $V_H186.2$ genes, which are preferentially used by high-affinity NP-specific B cells in C57BL/6 mice, were amplified by RT-PCR, and then PCR direct sequencing was performed (27–30). As shown in Table S1, the use of the $V_H186.2$ gene segment in NP-binding B cells was more than 2.5-fold lower in $LMP2A^{AID}$ mice than in GFP^{AID} mice. However, the replacement mutation/silent mutation (R/S) ratio of complementarity-determining regions (CDR) 1 and 2, the frequency of *DFL16.1* use, the frequency of YYGS sequence insertion in CDR3, and the frequency of W-to-L mutation in CDR1 in $V_H186.2^+$ B cells was not affected by the expression of LMP2A in GC B cells. Thus, it appears that LMP2A expression severely impairs selection of high-affinity B cells expressing the $V_H186.2$ gene but not somatic hypermutation in GCs.

LMP2A Expression Enhances Differentiation of B Cells into ASCs. The increased numbers of ASCs, particularly IgM-secreting cells, suggested that LMP2A may affect the differentiation of B cells into plasma cells or affect Ig isotype class-switching. To test this possibility, B cells isolated from $LMP2A^{CD19}$ mice were cultured in the presence of IL-4, IL-5, and soluble CD40 ligand (sCD40L). Both IgM and IgG production were more rapidly inducible in $LMP2A^{CD19}$ B cells than in GFP^{CD19} B cells (Fig. 3*A*). Furthermore, significantly higher levels of interferon regulatory factor 4 (*Irf4*) and B lymphocyte-induced maturation protein 1 (*Blimp1*) mRNAs, which encode key transcription factors involved in plasma cell differentiation, were induced in $LMP2A^{CD19}$ B cells before Ig secretion. Nonetheless, *Irf4* and *Blimp1* mRNAs levels were lower in $LMP2A^{CD19}$ B cells than in control B cells before stimulation (Fig. 3*B*). CD138 staining, in combination with a dye-dilution assay, also revealed a higher frequency of $CD138^+$ cells in $LMP2A^{AID}$ B cells after several cell divisions (Fig. 3*C*). These in vitro findings suggest that LMP2A accelerates the differentiation of B cells into antibody-forming cells, which may explain the increased numbers of plasma cells and ASCs in spleens of immunized $LMP2A^{AID}$ mice.

LMP2A Expression in GC B Cells Leads to Autoantibody Production and Development of Glomerulonephritis. It has been suggested that impaired affinity maturation in GCs may lead to autoantibody production (31). EBV titers have been reported to correlate with disease activity in patients with systemic lupus erythematosus (32–36), suggesting a possible involvement of EBV infection in autoantibody production. Indeed, we detected anti-dsDNA antibodies only in sera of $LMP2A^{AID}$ mice (Fig. 4*A*). Anti-cardiolipin

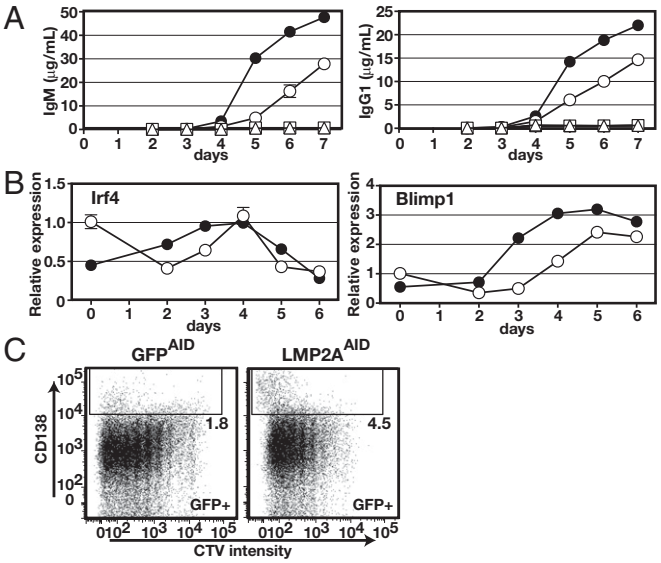

Fig. 3. LMP2A expression enhances B-cell differentiation into ASCs. (*A*) Purified splenic B cells from LMP2A^{CD19} or GFP^{CD19} mice were cultured for 7 d with the indicated stimuli (triangle: unstimulated; square: IL-4 and IL-5; circle: IL-4, IL-5, and sCD40L), and the cultured supernatants were analyzed by ELISA (open symbols: GFP^{CD19}; filled symbols: LMP2A^{CD19}; $n = 3$). Error bars show the means ± SEM. Data are representative of three independent experiments. (*B*) Expression of the indicated genes was analyzed by RT-qPCR. Purified splenic B cells from either LMP2A^{CD19} or GFP^{CD19} mice were cultured with anti-CD40 antibody and IL-4 for the indicated times (open circles: GFP^{CD19}, filled circles: LMP2A^{CD19}; $n = 3$). Error bars show the means ± SEM. (*C*) Purified splenic B cells from either LMP2A^{AID} or GFP^{AID} mice were labeled with CellTrace Violet (CTV) and cultured with anti-CD40 antibody and IL-4 for 4 d. Then CD138 expression was analyzed by flow cytometry. The numbers indicate the frequencies of GFP$^+$CD138$^+$ cells in the gates. Data are representative of three independent experiments.

antibodies also were detected in sera of LMP2A^{AID} mice and, to a lesser extent, in sera of LMP2A^{CD19} mice (Fig. 4*B*). Staining of kidney sections from either 6-mo-old unimmunized LMP2A^{AID} mice or GFP^{AID} mice with H&E or anti-IgG and IgM revealed subendothelial deposition of antibodies and development of glomerulonephritis-like lesions in LMP2A^{AID} mice (Fig. 4*C*).

LMP2A Expression Induced Epigenetic Changes in B Cells That Correlate with Plasma Cell Differentiation. To investigate the effects of LMP2A expression on the B-cell epigenetic landscape, histone 3 lysine 4 monomethylation (H3K4me1) enhancer marks, histone 3 lysine 4 trimethylation (H3K4me3) promoter marks, and histone 3 lysine 27 acetylation (H3K27ac) active promoter or enhancer marks were analyzed. ChIP sequencing (ChIP-seq) analysis was done on biological replicate samples of splenic B220$^+$ B cells from LMP2A^{CD19} mice and GFP^{CD19} mice. ChIP-seq reads were first mapped to the mouse genome mm9. Model-based analysis for ChIP-seq (MACS) identified 37,150 significant H3K27ac peaks in GFP^{CD19} B cells and 37,497 significant H3K27ac peaks in LMP2A^{CD19} B cells. Altogether, 58,342 significant H3K4me1 peaks in GFP^{CD19} B cells and 55,293 significant H3K4me1 peaks in LMP2A^{CD19} B cells were identified, along with 27,881 significant H3K4me3 peaks in GFP^{CD19} B cells and 26,481 significant H3K4me3 peaks in LMP2A^{CD19} B cells.

Unexpectedly, LMP2A expression in B cells resulted in increased H3K4me1 and H3K27ac signals by ≥1.5-fold at only a small number of gene loci, including zinc finger and bric-a-brac, tramtrack (BTB) domain containing protein 20 (*Zbtb20*) and tripartite motif-containing 12A (*Trim12a*) (Fig. 5*A* and Table S2). Consistent with this result, quantitative PCR (qPCR) showed significantly increased Zbtb20 and Trim12a transcript levels in LMP2A^{CD19} B cells ($P = 0.01$ and $P < 0.0001$, respectively) (Fig. 5*B*). Zbtb20 encodes a zinc finger protein that has been shown to be important for B-cell differentiation into ASCs and for plasma cell longevity (37, 38). Therefore, the induction of Zbtb20 expression by LMP2A in B cells may at contribute, at least in part, to the observed LMP2A effects on B-cell differentiation into antibody-secreting plasma cells.

Pathways Affected by LMP2A-Induced Epigenetic Changes. In contrast to the limited number of sites that were positively affected by LMP2A, we observed a generally repressive role of LMP2A on global histone modifications. Most sites that differed between LMP2A^{CD19} B cells and GFP^{CD19} B cells were repressed in LMP2A^{CD19} B cells, with ≥1.5-fold decreased H3K27ac ChIP-seq signal at more than 300 sites. The top 300 loci that differed in H3K27ac, H3k4me1, or H3K4me3 signals between LMP2A^{CD19} B cells and GFP^{CD19} B cells were analyzed further for pathway enrichment. H3K27ac, H3K4me1, and H3K4me3 sites affected by LMP2A expression were linked to their nearest genes. IntPath analysis (39) of genes with decreased H3K27ac signal found enrichment for the TNFα/NF-κB pathway. Genes with decreased H3K4me1 signal were enriched for B-cell receptor signaling pathway and apoptosis (Table S3).

Discussion

In this study, we analyzed the effects of conditional expression of LMP2A on B-cell functions and humoral immune responses. LMP2A constitutively activates Syk in vitro and transduces BCR-like signals in vitro and in vivo (5, 10, 11). Indeed, in B cells derived from LMP2A^{CD19} mice, LMP2A enhanced Syk tyrosine phosphorylation and B-cell proliferation in response to BCR stimulation, further supporting the existence of crosstalk between LMP2A and BCR signals. Moreover, B cells from both LMP2A^{CD19} and LMP2A^{AID} mice displayed significantly accelerated in vitro differentiation into ASCs when stimulated with anti-CD40 antibody or sCD40L in combination with IL-4. These results are consistent with the reports that LMP2A expression facilitates plasma cell differentiation in vitro and in vivo in response to CD40 stimulation or Toll-like receptor agonists (40, 41). These findings strongly suggest that LMP2A affects BCR signals as well as CD40 signals.

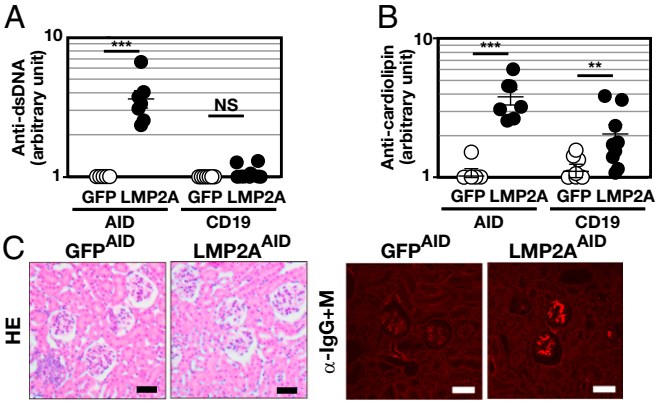

Fig. 4. LMP2A^{AID} mice develop a lupus-like autoimmune phenotype. (*A* and *B*) The serum titers of anti-dsDNA (*A*) or anti-cardiolipin (*B*) antibody in 8- to 20-wk-old mice were measured by ELISA. (*Left*) open circles: GFP^{AID}; filled circles: LMP2A^{AID}; (*Right*) open circles: GFP^{CD19}, filled circles: LMP2A^{CD19}. $n = 6$–8. Error bars show the means ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$; NS; not statistically significant. (*C*) Paraffin-embedded kidney sections from 6-mo-old LMP2A^{AID} or GFP^{AID} mice were stained with H&E (*Left*) or anti-IgG/IgM (*Right*). (Scale bars, 50 μm.) Data are representative of three independent experiments.

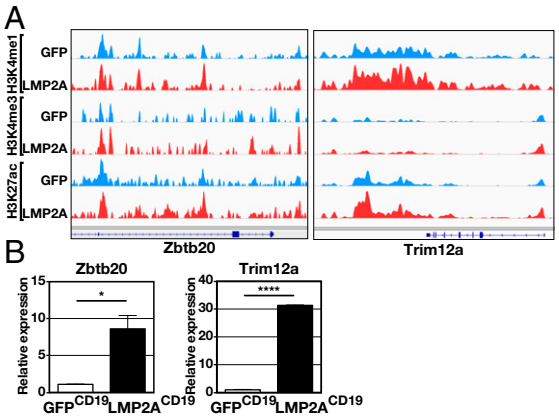

Fig. 5. LMP2A-mediated epigenetic and transcription effects on Zbtb20 and Trim12a. (*A*) H3K4me1, H3K4me3, and H3K27ac ChIP-seq signals at the Zbtb20 (*Left*) and Trim12a (*Right*) enhancers from LMP2A^{CD19} and GFP ^{CD19} B cells are shown. (*B*) Expression of *Zbtb20* and *Trim12a* genes in LMP2A^{CD19} and GFP^{CD19} B cells was evaluated by RT-qPCR. Error bars show the means ± SEM. $*P < 0.05$; $****P < 0.0001$.

The expression of LMP2A resulted in the impairment of antigen-specific antibody responses without affecting GC formation in immunized LMP2A^{AID} mice. Defective humoral immune responses appeared to be a consequence of the preferential selection of low-affinity B cells but not high-affinity B cells, as underscored by the reduced use of the $V_H186.2$ gene segment by NP-binding GC B cells of LMP2A^{AID} mice. Bone marrow chimera experiments revealed that LMP2A^{AID}-derived B cells had the ability to expand and survive in GCs and that this ability was comparable to that of normal B cells, even though they expressed mostly low-affinity BCR. This selection and survival advantage of LMP2A^{AID}-derived low-affinity B cells could be attributed to LMP2A-mediated enhancement of BCR signals. It also is noteworthy that increased numbers of plasma cells and IgM-secreting cells, detected by flow cytometry and ELISPOT, respectively, were observed in LMP2A^{AID} mice 2 wk after immunization, whereas the frequencies of antigen-specific cells in total IgM- and IgG1-secreting cells were decreased compared with those of immunized control mice. This finding is consistent with LMP2A-mediated enhancement of in vitro plasma cell differentiation and suggests that LMP2A may accelerate the differentiation of GC B cells into plasma cells, even before affinities of antibodies mature sufficiently, leading to the reduction in high-affinity antibody-producing B cells in LMP2A^{AID} mice.

Numbers of ASCs were significantly higher in LMP2A^{CD19} mice than in GFP^{CD19} mice 1 wk after immunization, and LMP2A expression suppressed subsequent GC formation. These observations suggest that LMP2A expression from an early stage of B-cell development may skew the fate of B-cell development. In this context, LMP2A may shift early B-cell–lineage cells from being antigen-committed in the initial phase of the immune response (42) toward extrafollicular plasma cell differentiation. Interestingly, in the acute phase of infectious mononucleosis (IM), 70–80% of circulating EBV-infected B cells differentiate toward plasmacytoid cells (43). Also of note, in patients with IM, EBV-infected B cells are preferentially located in the extrafollicular area of lymphoid tissues, and some exhibit a plasmacytoid phenotype and express the replication-associated protein BZLF (44). Our results suggest that LMP2A may have important roles in establishing these B-cell phenotypes.

Although LMP2A expression had limited effect on the epigenetic landscape, we observed robust up-regulation of *Irf4* and *Zbtb20* expression in LMP2A-expressing B cells. Zbtb20 was up-regulated at the mRNA level by approximately eightfold. Interestingly, IRF4 binds to the Zbtb20 promoter and up-regulates Zbtb20 expression (37, 38). Thus, LMP2A- and IRF4-mediated up-regulation of Zbtb20 may be an important mechanism by which EBV promotes plasma cell survival. Recent studies revealed that Zbtb20, which is a Bcl6 homolog and a member of the broad complex BTB zinc finger family, is a transcriptional repressor that has critical roles in plasma cell differentiation and survival (37, 45). Zbtb20 is physiologically expressed in B1 and GC B cells, with highest expression in long-lived bone marrow ASCs (37). Zbtb20 enhances primary B-cell terminal differentiation into ASCs and enhances plasma cell survival in a B-cell–intrinsic manner (45). Interestingly, MCL1 levels are reduced in Zbtb20-deficient plasma cells relative to WT controls, suggesting a key Zbtb20 anti-apoptotic role. Taken together, our findings suggest that LMP2A may be an important viral factor that enhances plasmacytoid differentiation of EBV-infected B cells, which can trigger the viral replication program.

LMP2A robustly induced Trim12a expression in B cells. TRIM12a is a member of the tripartite motif family, which contains RING, B-box, and coiled coil domains. Although Trim12a function remains largely uncharacterized, it is notable that Trim12 was previously implicated as a candidate gene in defective T-cell–negative selection in the nonobese diabetic (NOD) mouse strain (46). TRIM12 expression was reduced by ∼300-fold in NOD thymocytes relative to controls. A major objective of future research will be to characterize further how TRIM12a may underlie LMP2A effects on B-cell development.

Despite these effects on Zbtb20 and Trim12a, most LMP2A effects on the B-cell epigenetic landscape suggested that LMP2a represses many B-cell loci. Consistent with this observation, LMP2A coexpression reversed LMP1-induced B-cell hyperactivation phenotypes in a murine model (22). Pathways suppressed by LMP2A expression included apoptosis, Toll-like receptor signaling, B-cell receptor signaling, and the TNFα/NF-κB pathway (Table S3). Does LMP2A activate signaling molecules downstream of BCR signals? We also performed microarray analysis to determine the change in gene expression by LMP2A in B cells. We found marked similarities in the gene-expression profiles of LMP2A- and BCR-activated B cells (Fig. S6) (47). However, quantitative analysis of these gene expressions will be required to confirm these possibilities.

LMP2A^{AID} mice produced autoantibodies against dsDNA and cardiolipin and had detectable immune complex deposition in the kidney; LMP2A^{CD19} mice also produced anti-cardiolipin antibodies. EBV titers correlate with systemic lupus erythematosus disease activity in some patients (32–36). Both LMP2A^{CD19} and LMP2A^{AID} mice exhibited hyperimmunoglobulinemia (Fig. S7), which often is associated with autoantibody production in murine autoimmunity models (31, 48). Likewise, autoantibody production often is observed in IM patients (49, 50). We speculate that LMP2A may have an important role in the generation of self-reactive B cells by enabling EBV-infected B cells to bypass GC selection. Interestingly, EBV-infected memory B cells obtained from IM patients express lower levels of self-reactive antibodies than EBV-uninfected memory cells (51). Therefore, additional mechanisms may shape the repertoire of EBV-infected memory B cells, and LMP2A-mediated changes in GC B-cell selection may be particularly important for the generation of low-affinity antibody-producing plasma cells.

In summary, conditional LMP2A expression in mice reduced the stringency of B-cell selection in GCs and resulted in preferential generation of low-affinity antibody-producing cells, possibly by augmenting BCR signals. These data strongly support the hypothesis that LMP2A contributes to persistent EBV infection in memory B cells by providing a survival advantage to EBV-infected GC B cells. In addition, LMP2A accelerated plasma cell differentiation in vitro and in vivo through enhanced Zbtb20 expression.

These findings provide insights into the mechanism of persistent EBV infection in lymphoid tissues.

Materials and Methods

To generate LMP2A^{STOP} mice, the targeting vector used to insert the HA-tagged LMP2A gene cassette into the ROSA26 locus was introduced into BALB/c ES cells. The mutant ES cells were microinjected into C57BL/6 mice, and heterozygous offspring were intercrossed into C57BL/6 mice. Mice were maintained in pathogen-free animal facilities at the Research Institute for Microbial Diseases, Osaka University, and were immunized i.p. with 100 μg NP-CGG (Biosearch Technologies) in alum following the guidelines of Osaka University for animal studies. This study was approved by the Osaka University Institutional Animal Care and Use Committee. Western blotting, ELISA, and ELISPOT assays were performed following standard or published protocols. ChIP-seq assay and data analysis were performed as previously described (52). For more details, see *SI Materials and Methods*.

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