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EBV Latent Membrane Protein 2A Induces Autoreactive B Cell Activation and TLR Hypersensitivity¹

Hongsheng Wang,² Matilda W. Nicholas, Kara L. Conway, Pradip Sen, Ramiro Diz, Roland M. Tisch, and Stephen H. Clarke³

EBV is associated with systemic lupus erythematosus (SLE), but how it might contribute to the etiology is not clear. Since EBV-encoded latent membrane protein 2A (LMP2A) interferes with normal B cell differentiation and function, we sought to determine its effect on B cell tolerance. Mice transgenic for both *LMP2A* and the Ig transgene 2-12H specific for the ribonucleoprotein Smith (Sm), a target of the immune system in SLE, develop a spontaneous anti-Sm response. LMP2A allows anti-Sm B cells to overcome the regulatory checkpoint at the early preplasma cell stage by a self-Ag-dependent mechanism. LMP2A induces a heightened sensitivity to TLR ligand stimulation, resulting in increased proliferation or Ab-secreting cell differentiation or both. Thus, we propose a model whereby LMP2A induces hypersensitivity to TLR stimulation, leading to activation of anti-Sm B cells through the BCR/TLR pathway. These data further implicate TLRs in the etiology of SLE and suggest a mechanistic link between EBV infection and SLE. *The Journal of Immunology*, 2006, 177: 2793–2802.

Systemic lupus erythematosus (SLE)⁴ is a chronic autoimmune disorder characterized by the production of autoantibodies and multiorgan inflammatory damage. Both genetic and environmental factors contribute to the etiology of SLE, but a clear understanding of how these factors contribute to disease is still lacking. Among the environmental risk factors, EBV is the most closely associated with SLE. SLE patients have higher titers of anti-EBV Abs than control populations (1–4), and EBV infection is more common among juvenile and adult SLE patients than among control populations (5, 6). Some cases of SLE appear to directly result from acute EBV infection (4, 7) or reactivation of EBV (8). In addition, SLE patients have a 40-fold higher EBV viral load in peripheral blood leukocytes than control populations due to poor cytotoxic T cell (CTL) responses (9) and a higher frequency of infected B cells (10).

Experimental studies support a role for EBV in the induction of SLE. EBV induces infectious mononucleosis causing B cell activation and autoantibody production (11). Some Abs elicited by EBV cross-react with nuclear Ags, suggesting that antigenic mimicry is involved in the appearance of certain autoantibodies (12–14). This possibility is strengthened by the observation that EBV-encoded protein, EBNA-1, contains linear epitopes similar to those

of common self-antigenic targets in SLE, the B/B' and D peptides of ribonucleoprotein Smith (Sm) (15) and the Ro protein (16). Moreover, animal immunizations with plasmids for in vivo expression of EBNA-1 peptides cross-reactive with Sm and Ro elicit anti-Sm and anti-Ro responses that are characterized by epitope spreading. The spreading involves other regions of the autoantigen, as well as additional nuclear Ags, including dsDNA, and is associated with the development of clinical symptoms of SLE (16–18). Thus, EBV Ags could induce anti-nuclear Abs in SLE, providing a basis for EBV as an etiologic factor.

EBV exists as a lifelong latent B cell infection in >90% of the world's population. The latent membrane protein (LMP) 2A plays a critical role in regulating viral latency by mimicking a constitutively activated BCR (19). The cytoplasmic domain of LMP2A contains ITAMs, the same signaling domains found in BCR subunits Ig α and Ig β . This allows LMP2A to recruit the protein tyrosine kinases Lyn and Syk, sequestering them from the BCR and thereby inhibiting BCR signal transduction (20, 21). It also activates the PI3K/Akt pathway, which enhances survival by increasing Bcl-x_L expression (19). A *LMP2A* transgene interferes with B cell development by permitting B cells that lack a BCR to leave the bone marrow and migrate to the spleen (22), indicating that it provides survival signals independent of the BCR.

The effects of LMP2A on B cell development and activation raise the possibility that it affects the regulation of autoreactive B cells. Recently, LMP2A expression has been shown to affect anergy induction (23). In this report, we sought to determine whether LMP2A affects the regulation of B cells specific for an Ag characteristically targeted in SLE. We have investigated the regulation of anti-Sm B cells in mice transgenic (Tg) for an anti-Sm H chain, 2-12H (24, 25). 2-12H Tg mice develop a high frequency of anti-Sm B cells, some of which are negatively regulated by developmental arrest and anergy (24). Other anti-Sm B cells are activated in 2-12H mice and begin plasma cell (PC) differentiation, but arrest at an early pre-PC stage (26). Differentiative arrest occurs before the up-regulation of the transcription factor B lymphocyte maturation protein 1 (Blimp-1), which is required for PC differentiation (27). However, these anti-Sm pre-PCs differentiate to Ab-secreting PCs on the autoimmune MRL/*lpr* background (26).

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⁴ Abbreviations used in this paper: SLE, systemic lupus erythematosus; ASC, Ab-secreting cell; Blimp-1, B lymphocyte-induced maturation protein-1; FO, follicular; HPRT, hypoxanthine phosphoribosyltransferase; LMP, latent membrane protein; MZ, marginal zone; ODN, oligonucleotide; PC, plasma cell; Sm, Smith; Tg, transgenic; XBP-1, X-box binding protein 1.

Thus, in 2-12H mice, anti-Sm B cell regulation occurs at multiple checkpoints and both before and after activation.

Since LMP2A affects BCR signaling and B cell differentiation, we examined whether it interferes with tolerance to SLE-specific Ags leading to autoantibody production. Using mice with both the anti-Sm 2-12H transgene and the EBV-derived *LMP2A* transgene, we demonstrate that EBV contributes to autoantibody production by allowing anti-Sm B cells to bypass the pre-PC tolerance checkpoint. Moreover, *LMP2A* increases the reactivity and sensitivity of B cells to TLR activation. We propose that *LMP2A* facilitates autoreactive B cell activation by the BCR/TLR pathway through enhancement reactivity to TLR ligands.

Materials and Methods

Mice

Anti-Sm 2-12H Tg mice and *LMP2A* TgE mice have been described previously (22, 24). Both 2-12H and *LMP2A* Tg mice are on a mixed background of C57BL/6 and CB17. 2-12H/*LMP2A* double Tg mice were generated by crossing 2-12H with *LMP2A* Tg mice. Offspring carrying transgenes were identified by tail genomic DNA as described previously (22, 24). All animal protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Flow cytometry

Cells were prepared and stained as reported previously (28). Fluorochrome-conjugated or biotinylated mAbs specific for mouse B220, IgM, IgM^a, IgM^b, CD43, CD5, CD19, CD23, CD21/CD35, CD138, and MHC class II (I-A^b) were obtained from BD Pharmingen. For staining of TLRs, splenic B cells were fixed in 1.5% paraformaldehyde and permeabilized with methanol according to standard protocols. The cells were stained with CD19-FITC (eBioscience) and IgM-allophycocyanin (BD Pharmingen) plus one of the following: anti-TLR4-biotin (eBioscience), anti-TLR9-FITC (Imgenex), or anti-RP105-biotin (eBioscience). PerCP-labeled streptavidin was used as a secondary reagent for biotinylated Abs. Cells were analyzed using a FACSCalibur (BD Biosciences). Data were analyzed by WinMDI software (The Scripps Institute). In some experiments, anti- μ -stimulated cells were fixed with 1% formaldehyde and methanol, followed by staining with PerCP-labeled anti-B220 and Alexa 647-labeled anti-phospho-ERK(p44/42) Ab (Cell Signaling Technology) according to a previous protocol (29). The level of phospho-ERK was detected by flow cytometry on gated B220⁺ B cells. Cells pretreated with 20 μ M of a phospho-ERK inhibitor, PD98059 (Calbiochem), for 20 min at 37°C were used as a control.

For cell sorting experiments, splenic cells were stained with Abs as described in each experiment and sorted on a MoFlo high-speed sorter (DakoCytomation). Sorted populations were >90% pure as determined by reanalysis.

ELISA and ELISPOT

Quantitation of anti-Sm Abs and total IgM in mouse serum was done by ELISA as previously described (24). Quantitation of anti-Sm secreting Ab-secreting cells (ASCs) was performed using ex vivo ELISPOT assays described elsewhere (30).

Real-time PCR

Total RNA from sorted CD138⁻, CD138^{int}, and CD138^{high} B cells was isolated using a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. A DNA digestion step is included before elution of RNA. Approximately 200 ng of total RNA in 20 μ l was reverse-transcribed with SuperScript II reverse transcriptase and random hexamer primers (Invitrogen Life Technologies). The PCR was performed in an ABI Prism 7000 Sequence Detection System with the SYBR Green PCR Master Mix reagents (Applied Biosystems). The cDNAs were serially diluted (50/10/5/1) to verify the efficiency of the PCR to amplify *Blimp-1*, X-box binding protein 1 (*XBP-1*), *PAX-5*, and the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT*) transcripts. The PCR products were revealed by gel electrophoresis to verify the specificity of the PCR. Calculations were performed with the ABI Prism 7000 SDS software and using *HPRT* as an internal control. Quantification was performed relative to the expression levels of non-Tg CD138⁻ cells. PCR primer sequences were as follows: *Blimp-1* forward, 5'-TGTTGGATCTTCTCTGGAAAA-3'; *Blimp-1* reverse, 5'-GTGTAAAGTAGACTGCCTTGA-3'; *XBP-1* forward, 5'-GGTGCAGGCCAGTTGTC-3'; *XBP-1* reverse, 5'-TCCA

GAATGCCAAAAGGAT-3'; *PAX-5* forward, 5'-CAGCACTACTCTGACATCTT-3'; *PAX-5* reverse, 5'-GTTGGCTTTCATGTCATCC-3'; *HPRT* forward, 5'-GTTCTTTGCTGACCTGCTGGAT-3'; and *HPRT* reverse, 5'-GTCCCCCGTTGACTGATCAT-3'.

EMSA and Western blotting

Splenic B cells were purified using a Mouse B cell Recovery Column kit (Cedarlane Laboratories) according to manufacturer's instruction with a slight modification. The purity of B cells is monitored by flow cytometry and was always \geq 90%. Nuclear and cytoplasmic extracts were prepared as described previously (31). EMSA was performed as described using ³²P-labeled dsDNA probes containing NF- κ B binding sites (32). Bands were visualized using a PhosphorImager (Molecular Dynamics). For κ B Western blotting, 40 μ g of cytoplasmic extract was resolved by SDS-PAGE using a 10% gel. For phosphorylation Western blotting, 3×10^6 of purified B cells were stimulated with 20 μ g/ml goat F(ab')₂ anti-mouse μ Abs for 3 and 12 min at 37°C. After lysis, the cell lysate was resolved by 10% gels. Proteins were transferred to nitrocellulose membrane and detected with Abs against κ B α , κ B β , κ B ϵ , ERK, phospho-ERK (Santa Cruz Biotechnology), phosphotyrosine (Ab-2; Oncogene Research Products), and β -actin (Sigma-Aldrich), followed by an HRP-labeled secondary Abs. The bands were visualized using ECL development reagents (Amersham Biosciences).

Proliferation assay

Column-purified B cells were labeled with CFSE (Molecular Probes) at a concentration of 1 μ M for 10 min at room temperature. The cells were then cultured in 10% FBS/RPMI 1640 at 1×10^6 /ml in 24-well plates in the presence and absence of LPS (InvivoGen), imiquimod-R837 (InvivoGen), and CpG oligonucleotides (ODNs) (InvivoGen) at concentrations as indicated for 3 days. The cells were then stained with annexin V-Cy5 or 7-aminonactinomycin D (BD Pharmingen) and analyzed by a FACSCalibur.

Ab secretion assay

A total of 2×10^5 of purified B cells was cultured in 96-well plates in 200 μ l of RPMI 1640 medium for 3 or 5 days. CpG ODNs, imiquimod, or LPS was added at different concentrations as indicated. The supernatant was analyzed by ELISA for anti-Sm and total IgM.

Statistical analysis

The Student *t* test was used to assess the significance of the differences between groups. A value of *p* < 0.05 was considered significant.

Results

LMP2A induced a spontaneous anti-Sm response

To determine whether *LMP2A* disrupts anti-Sm B cell regulation, we generated mice carrying both the *LMP2A* and 2-12H transgenes. As shown in Fig. 1A, 2-12H/*LMP2A* mice produced significantly higher levels of serum anti-Sm than 2-12H and non-Tg controls, indicating that *LMP2A*-expressing anti-Sm B cells are activated in otherwise nonautoimmune mice. Control 2-12H mice had higher levels of serum anti-Sm than non-Tg littermates. This result differs from those of earlier studies, which found that Tg mice had levels similar to non-Tg mice (24, 33), and could be due to a subclinical infection or to background gene differences resulting from continued backcrossing to C57BL/6 mice. Total IgM levels in 2-12H/*LMP2A* mice did not differ from those of 2-12H Tg or non-Tg controls (Fig. 1A), suggesting that anti-Sm production was selective. Consistent with increased serum anti-Sm, there were significantly higher numbers of anti-Sm ASCs in the bone marrow and spleens of 2-12H/*LMP2A* mice compared with control mice.

LMP2A induced an expansion of the splenic B cell population and a contraction of the peritoneal B cell population

In keeping with previous studies (22), we found that the population of immature IgM⁺ B cells was markedly reduced in the bone marrow of *LMP2A* Tg mice (data not shown) and that they were replaced by an aberrant population of μ H chain-negative B lineage

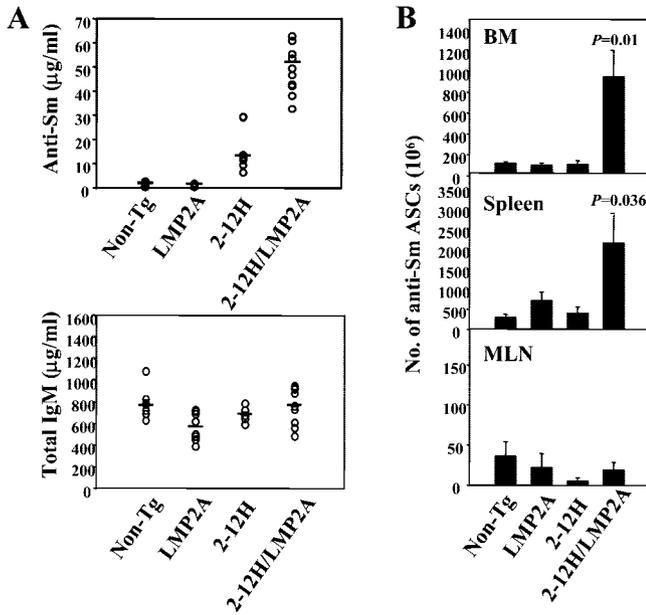


FIGURE 1. Activation of anti-Sm B cells in 2-12H/LMP2A mice. *A*, Serum anti-Sm IgM and total IgM levels were measured by ELISA. Each symbol represents an individual mouse (age 2–12 mo). The amount of anti-Sm IgM was significantly higher in 2-12H/LMP2A mice than in 2-12H mice ($p < 0.0001$). The total IgM level was significantly lower in LMP2A mice than other groups ($p < 0.05$). *B*, The number of Sm-specific ASCs in bone marrow (BM), spleen, and mesenteric lymph node (MLN) in mice of the indicated strains. ASCs were detected by a Sm-specific ELISPOT assay. Four to five mice from each group (means \pm SEM) have been examined.

cells that survive by virtue of a constitutive, LMP2A-directed survival signal. In contrast, IgM⁺ B cell differentiation was not reduced in the marrow of 2-12H/LMP2A Tg mice since all B cells express the 2-12H transgene (data not shown).

Further studies showed that the splenic B cell population of 2-12H/LMP2A mice was ~3-fold larger than that of 2-12H mice (Table I) and that, consistent with previous reports (22, 34), there were significantly fewer splenic B cells in LMP2A Tg mice compared with their non-Tg littermates. Between 50 and 80% of the CD19⁺ cells in the spleens of LMP2A Tg mice were IgM⁻, whereas almost all CD19⁺ B cells in spleens of 2-12H/LMP2A mice were IgM⁺ due to the expression of the 2-12H transgene (Fig. 2A). The frequency of splenic anti-Sm B cells in 2-12H and 2-12H/LMP2A mice was similar, while the number of anti-Sm B cells was higher in 2-12H/LMP2A mice, commensurate with the increased number of B cells (Fig. 2A and Table I).

To determine whether B cell differentiation in 2-12H mice was altered by LMP2A, we examined B cells for CD23 and CD21 expression to discriminate transitional, follicular (FO), and marginal zone (MZ) B cells (Fig. 2A and Table I). We also examined expression of CD5 and CD43 because splenic B cells of LMP2A mice were reported to have a CD5⁺ B-1 phenotype (34). Thus, it was unexpected that most LMP2A splenic B cells showed an ambiguous phenotype. Most were CD23⁺CD21^{int}, similar to FO B cells, and some were CD23^{low/-}CD21^{high}, similar to MZ B cells (Fig. 2B). LMP2A splenic B cells had a modestly elevated level of CD5 expression compared with B cells from non-Tg mice, but CD43 expression was high mimicking the CD43 level on B-1 cells. The phenotype of splenic B cells from 2-12H/LMP2A Tg mice was similar to that seen in LMP2A mice, although CD5 expression was not increased and CD43 expression was somewhat lower (Fig. 2A and data not shown). This CD23^{low}CD43⁺CD5^{+/-} phenotype was

Table I. Splenic B cell subsets

	B220 ⁺ Sm ⁺ ($\times 10^6$)					B220 ⁺ Sm ⁺ ($\times 10^6$)						
	Total	FO ^a	MZ	Tr	CD138 ⁺	CD138 ^{high}	Total	FO	MZ	Tr	CD138 ⁺	CD138 ^{high}
Non-Tg	51.3 ^b (6.7)	44.0 (6.1)	2.5 (0.5)	3.5 (0.7)	7.7 (1.3)	0.2 (0.04)	3.8 (0.5)	2.6 (0.3)	0.4 (0.09)	0.7 (0.13)	0.7 (0.03)	0.04 (0.01)
LMP2A	10.3 ^{c*} (1.7)	5.3* (1.3)	2.0 (0.6)	1.7* (0.6)	1.5* (0.3)	0.5 (0.2)	0.5* (0.2)	0.1* (0.03)	0.2* (0.1)	0.1* (0.03)	0.1* (0.1)	0.1 (0.03)
2-12H	36.3 (7.8)	24.1 (5.7)	2.4 (0.2)	3.1 (0.9)	4.3 (0.9)	0.3 (0.1)	7.1 (1.5)	3.6 (0.9)	1.3 (0.1)	1.2 (0.4)	0.7 (0.2)	0.1 (0.01)
2-12H/LMP2A	101.7* (12.7)	56.1* (7.5)	9.2* (1.5)	22.2* (6.4)	27.6* (12.5)	2.9* (0.5)	28.2* (6.1)	9.4* (1.1)	4.3* (0.6)	7.4* (1.1)	5.7* (2.3)	0.9* (0.3)

^a FO, MZ, and transitional (Tr) B cells were gated as depicted in Fig. 2A. CD138⁺ and CD138^{high} B cells were gated as shown in Fig. 3A.

^b Numbers are means \pm SEM (in parentheses) of four to six mice aged from 2 to 8 mo.

^c Statistical comparisons were made between LMP2A and non-Tg mice and between 2-12H/LMP2A and 2-12H mice. * $p < 0.05$.

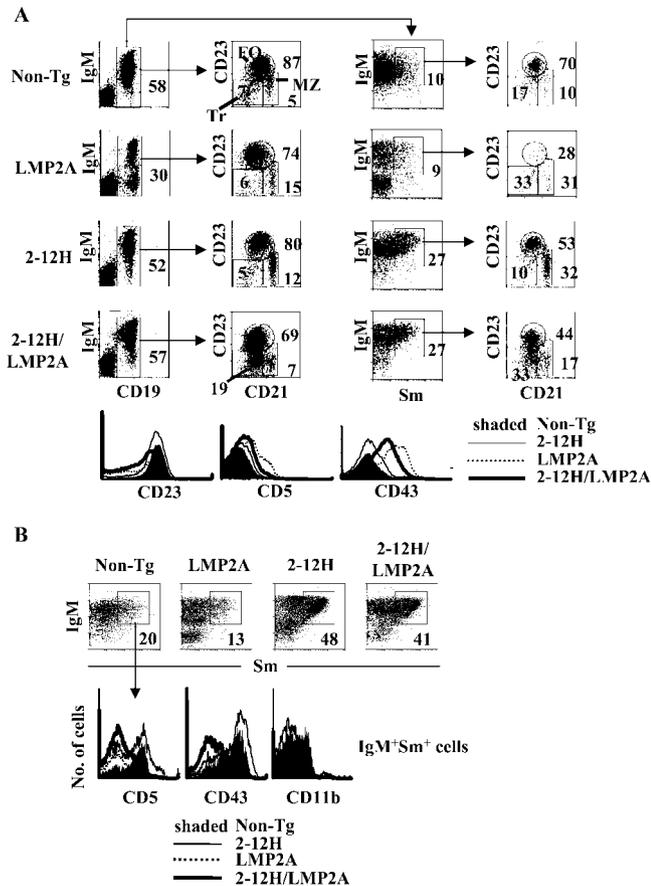


FIGURE 2. LMP2A significantly affects B cell phenotype analysis. *A*, Splenic cells from each group were stained with indicated Abs to identify B cell subsets. The histograms in the first column are gated on lymphocytes, and those of the remaining columns are gated on B cell subsets as illustrated. The single parameter overlays are gated on CD19⁺IgM⁺ cells. *B*, Analysis of peritoneal B cells. The histograms of the *top row* are gated on lymphocytes, and the single parameter histograms are gated on the anti-Sm B cells as shown. Single parameter overlays are gated on CD19⁺IgM⁺ anti-Sm B cells. These analyses of bone marrow, splenic, and peritoneal B cells are representative of three or more experiments. MZ, marginal zone B cells; Tr, transitional B cells; and FO, follicular B cells.

previously observed with B cells that had received a partial signal for B-1 cell differentiation (35), suggesting that they might be arrested at an intermediate step in B-1 differentiation.

The number of peritoneal B cells was significantly reduced in LMP2A and 2-12H/LMP2A mice, although this effect of LMP2A expression was partially ameliorated by the 2-12H transgene (Fig. 2*B* and Table II). We also found that LMP2A had significant effects on anti-Sm B cells. The majority of 2-12H and 2-12H/LMP2A anti-Sm B cells had the CD5⁺CD43⁺CD11b⁺ phenotype

Table II. Peritoneal anti-Sm B cells

Mice	CD19 ⁺ (×10 ⁵)	B-1a (CD19 ⁺ CD5 ⁺) (×10 ⁵)	Sm ⁺ B-1a (×10 ⁵)
Non-Tg	15.3 ± 4.4 ^a	8.7 ± 3.2	2.7 ± 1.0
LMP2A	2.6 ± 1.1 ^{b**}	1.1 ± 0.4 ^{**}	0.2 ± 0.04 ^{**}
2-12H	10.6 ± 3.6	5.0 ± 2.2	3.2 ± 0.6
2-12H/LMP2A	6.2 ± 1.0 [*]	2.2 ± 0.4 [*]	1.6 ± 0.5 [*]

^a Numbers are means ± SEM of four to five mice aged from 2 to 4 mo.

^b Statistical comparisons were made between LMP2A and non-Tg mice and between 2-12H/LMP2A and 2-12H mice. *, $p \leq 0.05$; **, $p < 0.01$.

of B-1 cells (33) (Fig. 2*B*), but those in 2-12H/LMP2A mice had lower levels of CD5 and CD43 (Fig. 2*B*).

Anti-Sm B cell regulation at the pre-PC stage was bypassed

We recently identified a checkpoint in B cell regulation at an early pre-PC stage (IgM⁺CD138^{int}), beyond which anti-Sm B cells do not mature in nonautoimmune mice (26). However, anti-Sm B cells on the autoimmune MRL/lpr background can bypass this checkpoint to become CD138^{high} pre-PCs and fully mature PCs (26). To assess the effect of LMP2A on regulation at the IgM⁺CD138^{int} checkpoint, we examined pre-PC differentiation in 2-12H/LMP2A mice. As shown in Fig. 3*A* and Table I, 2-12H/LMP2A mice had ~6-fold more CD138^{int} and ~10-fold more

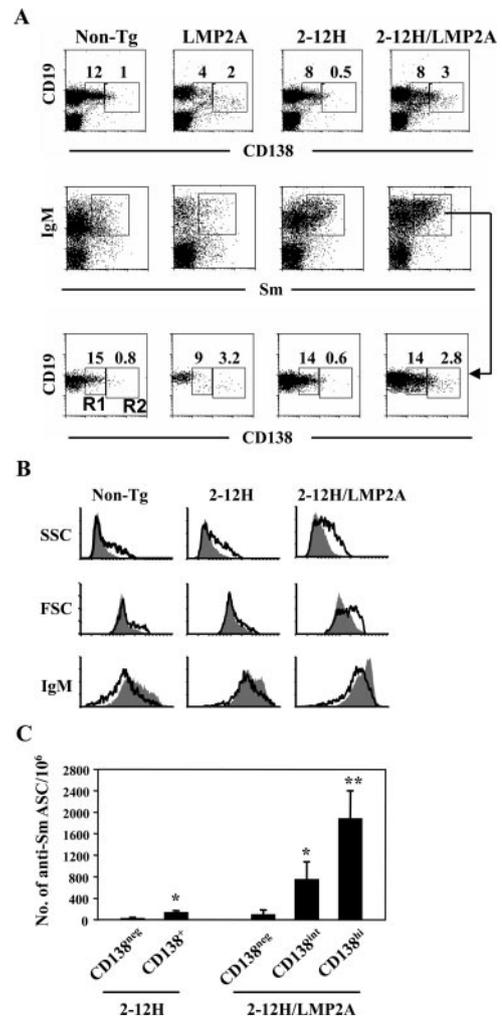


FIGURE 3. Analysis of CD138-expressing B cells indicates that LMP2A allows differentiation beyond the pre-PC checkpoint. *A*, CD138^{int} and CD138^{high} B cells in LMP2A and nonexpressing mice. Splenic cells were stained with Sm and Abs to CD19, IgM, and CD138. Percentages of CD138^{int} and CD138^{high} B cells are indicated. The dot plots of the *top* and *middle rows* are gated on lymphocytes, and those on the *bottom row* are gated on anti-Sm B cells, as indicated. The data are representative of four to six mice analyzed. *B*, Forward (FSC) and side (SSC) scatter and surface IgM are compared for CD138⁻ (shaded) and CD138⁺ B cells (line). *C*, ELISPOT analysis of sorted CD138⁻, CD138^{int}, and CD138^{high} B cells to determine anti-Sm ASC frequency. The CD138⁺ B cells were sorted using the gates depicted in *A*. The CD138⁻ B cells used were all remaining B cells. Shown for each group ($n = 4$) is the average number (± SEM) of Sm-specific ASCs per million B cells. *, $p < 0.05$ and **, $p < 0.001$ compared with CD138⁻ B cells, respectively.

CD138^{high} pre-PCs than 2-12H mice. Similar increases were evident in anti-Sm CD138^{int} and CD138^{high} pre-PCs. As noted above, *LMP2A* mice have significantly fewer B cells than the double Tg mice and therefore had fewer CD138^{int} B cells than non-Tg mice (Fig. 3A and Table I). Despite reduced splenic B cells, *LMP2A* mice had a larger number of CD138^{high} pre-PCs than non-Tg control mice (Table I). The anti-Sm CD138^{int} and CD138^{high} B cells were IgM^{low} and were larger and more granular than CD138⁻ B cells (Fig. 3B), all features shared with anti-Sm pre-PCs in 2-12H *MRL/lpr* mice (26). Thus, expression of *LMP2A* enhanced the differentiation of anti-Sm B cells to the CD138^{high} pre-PC stage.

An anti-Sm ELISPOT assay was used to determine whether anti-Sm B cells and pre-PCs were ASCs. Sorted CD138⁻ B cells, CD138^{int}, and CD138^{high} pre-PCs were compared for the frequency of anti-Sm ASCs. As shown in Fig. 3C, the frequency of anti-Sm ASCs among CD138⁻ B cells of both 2-12H and 2-12H/*LMP2A* mice was low. In contrast, there was a progressive increase in anti-Sm ASCs among CD138^{int} and CD138^{high} pre-PCs of 2-12H/*LMP2A*, but only a small increase among CD138^{int} pre-PCs of 2-12H mice. Thus, anti-Sm pre-PCs of 2-12H/*LMP2A* mice differentiated to ASCs.

In keeping with their differentiation to ASCs, CD138⁺ B cells from 2-12H/*LMP2A* mice exhibited changes in gene expression characteristic of normal PC differentiation. As determined by real-time PCR, Blimp-1 mRNA levels increased progressively as anti-Sm B cells differentiated from CD138⁻ to CD138^{int} and CD138^{high} pre-PCs (Fig. 4). Blimp-1 activates *XBP-1*, which controls a variety of PC-specific functions, and represses *PAX-5*, which drives the expression of a series of B cell-specific genes. *XBP-1* mRNA levels mirrored the progressive increase in Blimp-1 expression, whereas *PAX-5* transcripts progressively decreased during PC differentiation (Fig. 4). *LMP2A* mice showed the same pattern of Blimp-1 and *PAX5* expression, whereas CD138⁻ and CD138^{int} B cells of 2-12H and non-Tg mice were unchanged in their expression of these genes (Fig. 4).

LMP2A had no apparent effect on 2-12H BCR stimulation

LMP2A is reported to severely impair the ability of B cells to signal through the BCR (20, 36). The observation that anti-Sm B cells from 2-12H/*LMP2A* mice can differentiate to ASCs suggests that BCR signaling in these animals is relatively intact. To evaluate this possibility, we examined BCR signaling in 2-12H/*LMP2A* and control mice. In comparison to B cells from non-Tg mice, few B cells from 2-12H Tg mice proliferated in response to stimulation with anti-IgM (68 vs 19%, respectively). The failure of the majority of 2-12H B cells to proliferate in response to BCR ligation is consistent with anergy as a major mechanism of anti-Sm B cell regulation (24, 25). 2-12H/*LMP2A* B cells had a higher background proliferative rate than B cells from 2-12H mice (4 vs 10%; $p < 0.01$) and mice from the other two strains (5–6%; $p < 0.01$) (Fig. 5A). However, while BCR ligation induced an ~5-fold increase in proliferation of 2-12H B cells ($p < 0.05$), it did not induce significantly higher proliferation of 2-12H/*LMP2A* B cells. *LMP2A* B cells did not proliferate in response to BCR ligation by anti-IgM, which is consistent with the observations that many were BCR negative and that *LMP2A* interferes with BCR signaling (20, 36). Thus, for B cells of 2-12H Tg mice, *LMP2A* induced a higher background proliferation but inhibited anti-BCR-induced proliferation.

The poor proliferative response of 2-12H/*LMP2A* B cells to BCR cross-linking would appear to contradict the selective increase in serum anti-Sm Ab and number of anti-Sm CD138^{high} ASCs in 2-12H/*LMP2A* mice, both of which are indicative of B cell activation and differentiation in these animals. To compare BCR signaling by 2-12H/*LMP2A* and 2-12H B cells, we examined

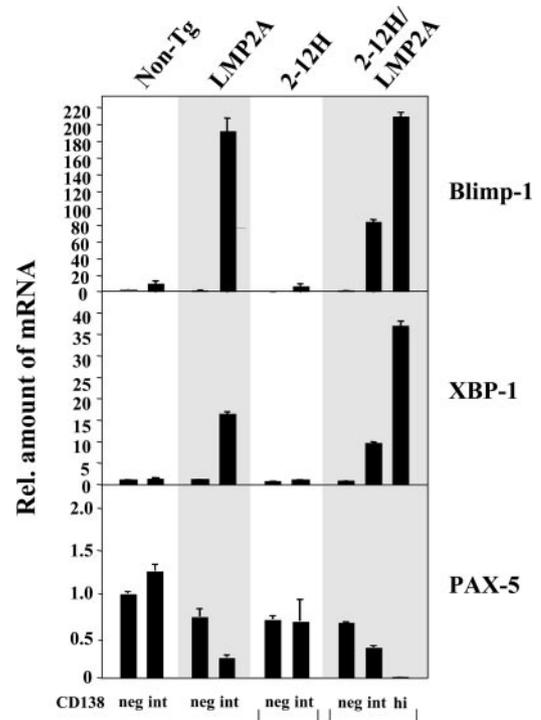


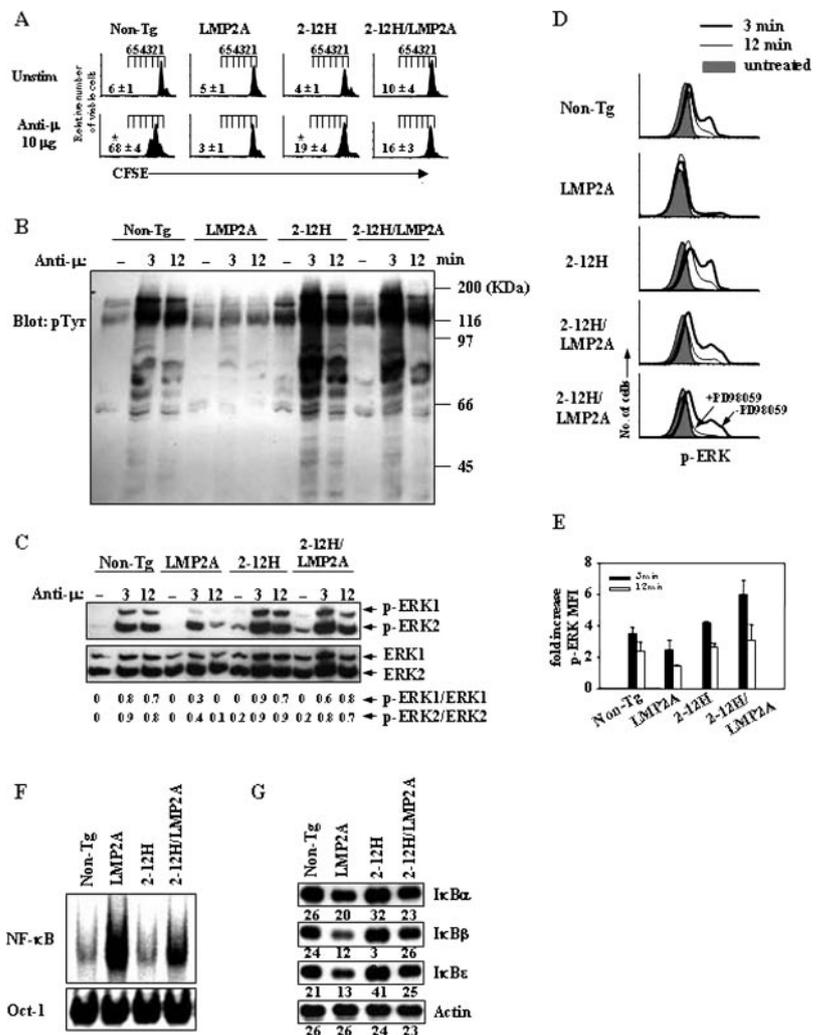
FIGURE 4. Real-time PCR comparison of Blimp-1, XBP-1, and PAX-5 expression in sorted B cell subpopulations indicates differentiation beyond the pre-PC tolerance checkpoint. Splenic B220⁺CD138⁻, CD138^{int}, and CD138^{high} B cell populations were sorted from multiple mice of each group based on gates depicted in Fig. 3A. CD138⁺ cells from non-Tg and *LMP2A* Tg mice were sorted to include both CD138^{int} and CD138^{high} subsets. Total RNA was extracted and reverse transcribed to cDNA, followed by real-time PCR to amplify Blimp-1, XBP-1, and PAX-5 as well as HPRT. Data are relative amount of mRNA to non-PC cells that were set to 1.0. Data are means \pm SEM of triplicate assays. One of two independent experiments with similar results is shown.

both BCR ligation-induced intracellular protein tyrosine phosphorylation and ERK phosphorylation. Splenic B cells from 2-12H and 2-12H/*LMP2A* mice responded to anti-IgM stimulation with similar overall protein tyrosine phosphorylation and ERK-specific phosphorylation (Fig. 5, B and C), and this response was similar to that of non-Tg B cells. There was little evidence of protein tyrosine phosphorylation in BCR-activated *LMP2A* B cells (Fig. 5B), and the level of ERK phosphorylation was weak (Fig. 5, C–E), particularly ERK1 phosphorylation (Fig. 5C). This difference is likely due to low percentage of BCR-expressing B cells in these mice (Fig. 2A). Interestingly, B cells from 2-12H and 2-12H/*LMP2A* mice had higher basal intracellular protein tyrosine phosphorylation and ERK phosphorylation than B cells from non-Tg mice (Fig. 5, B and C), suggestive of chronic BCR engagement (37). Thus, although BCR ligation-induced signaling in 2-12H/*LMP2A* B cells is equal to or greater than that seen in non-Tg B cells, proliferation is not significantly induced.

LMP2A-expressing B cells exhibit constitutive NF- κ B activation

Longnecker and colleagues (23) showed that NF- κ B is constitutively activated in B cells expressing *LMP2A*. Since NF- κ B is involved in B cell activation and differentiation, we examined resting 2-12H/*LMP2A* B cells for evidence of NF- κ B activation and/or I κ B degradation. Fractionated cytoplasmic and nuclear proteins were examined for the levels of the NF- κ B cytoplasmic inhibitors I κ B α , β , and ϵ and the amount of nuclear NF- κ B DNA binding

FIGURE 5. 2-12H/LMP2A B cells do not proliferate in response to BCR signals but are nevertheless BCR signaling competent. *A*, Column-purified splenic B cells were CFSE labeled and stimulated with 10 $\mu\text{g/ml}$ F(ab')₂ anti-mouse μ . After 3 days, cultured cells were analyzed by flow cytometry to determine CFSE content of CD19⁺ viable B cells. Data are means \pm SEM of two to four mice in each group. *, $p < 0.05$ compared with nonstimulated controls. *B*, Protein tyrosine phosphorylation. Column-purified B cells from mice of each line were stimulated with F(ab')₂ anti- μ for 3 and 12 min. Cytoplasmic proteins were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine (pTyr) Abs. The molecular weights of protein bands are indicated on the right. Data are representative of two independent experiments. *C*, ERK phosphorylation was determined by Western blot analysis. The membranes from *B* were sequentially stripped and reprobbed with anti-phospho-ERK (*top panel*) and anti-ERK (*lower panel*) Abs. The ratios of the intensities of phospho-ERK over total ERK are given at the *bottom*. *D*, ERK phosphorylation was also assessed by flow cytometry. Purified B cells from each strain were stimulated as in *B*, fixed with formaldehyde, and treated with methanol. Cells were then stained with B220-PerCP and phospho-ERK-Alexa 647 Abs and analyzed by flow cytometry. Shown is the pERK staining on gated B220⁺ B cells. *Bottom panel*, The ERK phosphorylation for 2-12H/LMP2A B cells 3 min after anti-IgM stimulation in the presence or absence of the inhibitor of ERK phosphorylation PD98059 as a control for the specificity of the anti-pERK Ab in this assay. *E*, The fold change over unstimulated cells (means \pm SD of two independent experiments) in the mean fluorescence intensity (MFI) of the cells in *D* is presented. *F*, NF- κ B DNA binding activity assay. Nuclear extracts from purified B cells were analyzed by EMSA. *Lower panel*, Oct-1 DNA binding acting as a control for protein loading. *G*, I κ B expression. Cytoplasmic protein extracts from *E* were analyzed by immunoblotting using Abs recognizing I κ B α , β , ϵ , and actin. The numbers at the *bottom* are densities of the band. Data are representative of two independent experiments.



activity. As expected, B cells from 2-12H/LMP2A mice had significantly more nuclear NF- κ B activity and less cytoplasmic I κ B inhibitors than B cells from 2-12H mice (Fig. 5, *F* and *G*). A similar difference was seen in comparisons of B cells from LMP2A and non-Tg mice. Thus, B cells from 2-12H/LMP2A mice exhibited constitutive activation of NF- κ B.

LMP2A-expressing B cells were hypersensitive to TLR activation

TLR signaling has been suggested to be important to the activation of autoreactive B cells in autoimmunity (38, 39). Since NF- κ B is activated by TLR signaling, we hypothesized that LMP2A affects TLR activation. Thus, we examined the responsiveness of B cells from 2-12H/LMP2A and control mice to stimulation through TLR4/RP105 by LPS, TLR9 by hypomethylated CpG ODNs, and TLR7 by imiquimod. As shown in Fig. 6*A*, B cells from both 2-12H and 2-12H/LMP2A mice proliferated equally well at high concentrations (1 $\mu\text{g/ml}$) of CpG ODNs, whereas B cells from 2-12H/LMP2A mice exhibited increased proliferation compared with 2-12H B cells in response to a low CpG ODN concentration (0.1 $\mu\text{g/ml}$) ($p < 0.01$). B cells from LMP2A mice showed a similar hypersensitivity to CpG ODN stimulation when compared with B cells from non-Tg mice (Fig. 6*A*). In contrast, we did not observe a statistically significant difference in proliferation between 2-12H/LMP2A and 2-12H in response to LPS or imiquimod (Fig. 6*A*). Thus, LMP2A enhanced the sensitivity of B cells to activation by TLR9.

In contrast to proliferation, LMP2A enhanced Ab secretion by all three TLR ligands. TLR stimulation of B cells from 2-12H/LMP2A mice with CpG ODNs, imiquimod, and LPS secreted 2- to 3-fold more IgM than B cells from non-Tg mice and \sim 7-fold more IgM than B cells from 2-12H Tg mice (Fig. 6*B*). For CpG ODNs and LPS, increased secretion was seen at concentrations suboptimal for the activation of non-Tg B cells. Much of the Ab produced in response to these stimuli was anti-Sm in cultures of B cells from 2-12H/LMP2A and 2-12H Tg mice but not in cultures of B cells from non-Tg. Thus, LMP2A induces a heightened sensitivity to CpG ODN- and LPS-induced ASC differentiation by autoreactive B cells.

LMP2A affected TLR expression

To determine whether LMP2A alters TLR expression, we detected the expression of LPS receptors, TLR4 and RP105, and the CpG-ODN receptor, TLR9, by flow cytometry. As shown in Fig. 7, B cells from 2-12H/LMP2A and LMP2A Tg mice expressed slightly higher levels of TLR4 than B cells from normal and 2-12H mice but considerably higher levels of RP105, a homolog of TLR4. TLR9 expression was also slightly increased on B cells expressing LMP2A (Fig. 7). IgM⁺ B cells from LMP2A Tg mice have increased expression of each of these receptors compared with IgM⁻ B cells from the same mice, supporting a functional role for the BCR in regulation of TLR expression in LMP2A-expressing B

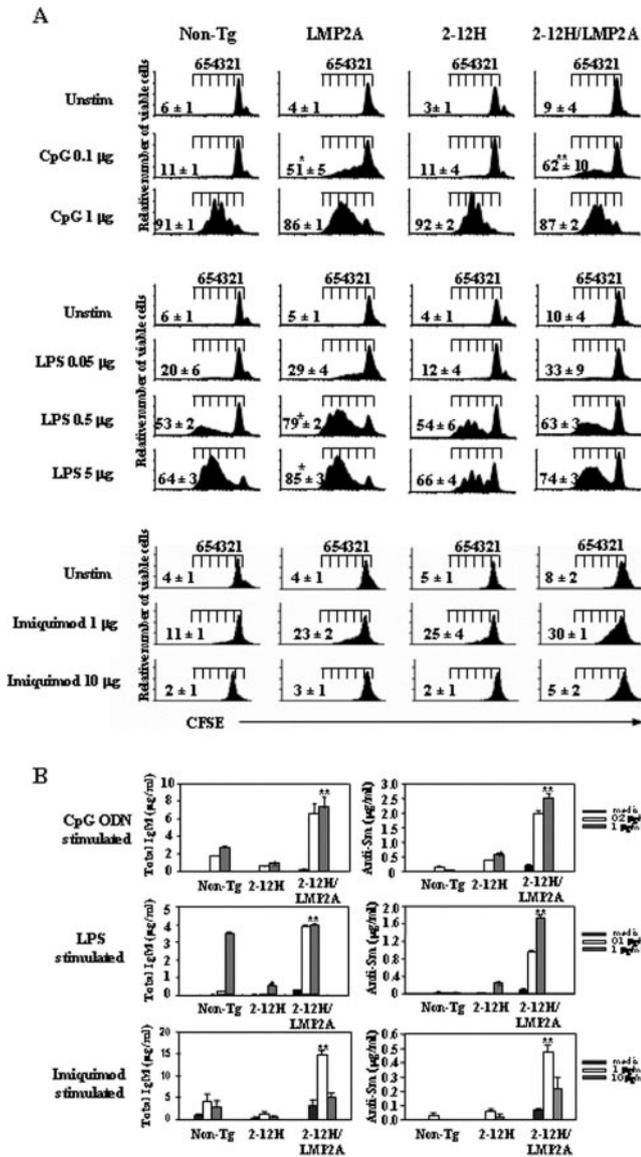


FIGURE 6. LMP2A induces B cell hyperresponsiveness to CpG ODN, LPS, and imiquimod. *A*, CpG ODN induced proliferation. Column-purified B cells were labeled with CFSE, stimulated with CpG ODN, LPS, or imiquimod at the indicated concentrations for 3 days, and analyzed by flow cytometry to determine the CFSE content of CD19⁺ B cells. The number of cell divisions is indicated on the top of each panel. The numbers (means ± SEM of three to five mice) indicated in each histogram are percentages of cells that undergo more than two divisions. All cells are gated on live cells (annexin V negative). Values of $p < 0.01$ compared with non-Tg (*) or 2-12H Tg (**) mice. *B*, Ab secretion by CpG ODN, imiquimod, or LPS-stimulated cells. Purified B cells stimulated with different concentrations of CpG ODN for 5 days (top panel) or imiquimod and LPS for 3 days (lower panel). The total IgM (left panel) and anti-Sm IgM (right panel) in the supernatant were measured by ELISA. Data are means ± SEM of triplicate assays. Data are representative of three independent experiments. *, $p < 0.001$ compared with non-Tg mice. **, $p < 0.0001$ compared with 2-12H and non-Tg mice.

cells. Thus, LMP2A induces a BCR-dependent increase in TLR expression on B cells.

Discussion

This study demonstrates that the EBV gene, *LMP2A*, induces a spontaneous anti-Sm response in nonautoimmune mice and induces a loss of tolerance at the CD138^{int} pre-PC checkpoint.

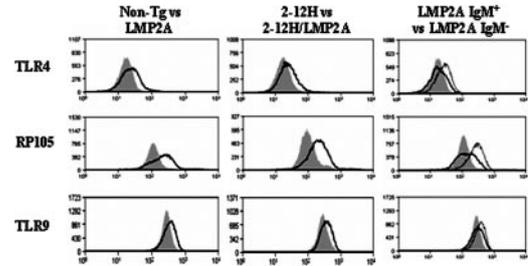


FIGURE 7. LMP2A induces higher TLR expression levels. One parameter histograms are shown for TLR4, RP105, and TLR9 expression. The first column is a comparison of B cells from non-Tg (shaded) and *LMP2A* (line) mice. The second column is a comparison of expression by B cells from 2-12H (shaded) and 2-12H/*LMP2A* (line). Non-Tg and 2-12H B cells do not differ in expression of these receptors. The third column is a comparison of non-Tg CD19⁺ B cells (shaded) with IgM⁺ (thin line) and IgM⁻ (thick line) B cells of *LMP2A* mice. The data are representative of three mice of each strain.

Sorted anti-Sm CD138^{int} pre-PCs of 2-12H/*LMP2A* mice become ASCs and differentiate into CD138^{high} PCs, resulting in an increased frequency relative to B cells of 2-12H mice (Fig. 3A and Table I). Differentiation to the PC stage could be inferred from the high frequency of anti-Sm ASCs in the bone marrow (Fig. 1B), the residence of long-lived PCs in normal mice (40). While the CD138^{int} B cells of 2-12H mice exhibit a gene expression profile of a B cell (PAX-5⁺, Blimp-1⁻, XBP-1⁻), the same cells of 2-12H/*LMP2A* mice exhibit a profile consistent with differentiation to a PC (PAX-5^{-low}, Blimp-1⁺, XBP-1⁺) (Fig. 4). B cells from *LMP2A* mice showed a similar increase in differentiation to the CD138^{high} pre-PC stage and changes in gene expression. Thus, the block in expression of PC-specific genes that occurs in B cells from nonautoimmune mice was lost in B cells from 2-12H/*LMP2A* mice. These changes in differentiation and gene expression mirrored the changes observed in B cells from autoimmune 2-12H MRL/*lpr* mice (26). Whether *LMP2A* disrupts other regulatory checkpoints in this model, such as anergy and developmental arrest, has yet to be systematically addressed. Longnecker and colleagues (23) reported that expression of *LMP2A* prevented induction of anergy to soluble hen egg lysozyme, suggesting that multiple checkpoints are affected.

An important question raised by these findings is whether tolerance is lost by an Ag-dependent or -independent mechanism. A role for Ag is possible because 2-12H/*LMP2A* B cells can signal in response to BCR ligation similar to those from 2-12H Tg mice (Fig. 5B). Since all B cells in 2-12H/*LMP2A* mice expressed the *LMP2A* transgene, the presence of naive splenic B cells suggests the need for additional signals. Moreover, there was a disproportionate increase in serum anti-Sm; 2-12H/*LMP2A* mice had a ~5-fold higher level of serum anti-Sm Abs than 2-12H mice, even though the levels of total serum IgM were similar. Thus, while these data suggest Ag dependence, a definitive answer will require a model system in which Ag can be removed.

The hypersensitivity of B cells from 2-12H/*LMP2A* mice to TLR-induced differentiation to ASCs suggests a possible mechanism for the anti-Sm response in these mice. Marshak-Rothstein and colleagues (38) identified a BCR/TLR9-dependent activation pathway for autoreactive B cells, in which activation of B cells producing rheumatoid factor or anti-DNA occurs when both the BCR and TLR9 are engaged (38, 39). Whether TLR signaling is required for anti-Sm B cell activation, and if so, which TLR is involved, is not known, although a recent study demonstrates that TLR9 and TLR3 are not required for an anti-Sm response by

MRL/*lpr* mice (41). We suggest that the sensitivity to TLR-induced activation caused by LMP2A expression increases the likelihood that anti-Sm B cells and possibly other autoreactive B cells such as anti-DNA and rheumatoid factor B cells will be activated through a BCR/TLR pathway. Since activation by this pathway requires a BCR signal, it would be consistent with the evidence for Ag dependence. Experiments to test this hypothesis in mice that lack functional TLRs are underway. A role for LMP2A in anti-Sm B cell activation is not exclusive of the antigenic mimicry hypothesis (16–18) and in fact could facilitate autoreactive B cell activation by EBV Ags.

The activation and increased sensitivity of 2-12H/LMP2A B cells to TLR stimulation is unlikely to be explained by changes in receptor levels alone. TLR-induced proliferation and secretion do not necessarily correlate (e.g., LPS; Fig. 6) and neither do changes in sensitivity and receptor level (Fig. 7). Thus, we suggest that a more likely explanation for the increased reactivity by LMP2A-expressing B cells is that LMP2A induces a change in TLR signaling. LMP2A expression causes constitutive activation of the Ras/PI3K/Akt pathway (19, 42, 43) and of NF- κ B (23). Since TLRs, including TLR4, TLR7, and TLR9, also trigger the PI3K/Akt pathway leading to increased NF- κ B activation (44, 45), activation of this pathway by LMP2A may reduce the TLR signaling threshold. The increase in Ras activity, which activates the Raf/MEK/ERK pathway in addition to the PI3K/Akt pathway, may be responsible for the up-regulation of RP105 and TLR4 expression. This is based on the understanding that PMA, a protein kinase C activator that triggers the MEK/ERK cascade, dramatically increases the expression of RP105 but increases TLR4 and TLR9 expression only modestly (46).

Whether these findings reflect the action of LMP2A in EBV-infected human B cells remains to be tested. There are important differences between this model system and EBV-infected human B cells that may affect the action of LMP2A in human B cells; whereas in humans EBV infects a small number of mature B cells (9, 47), LMP2A is expressed in all LMP2A Tg B cells throughout differentiation because LMP2A expression is under the control of an E μ enhancer and promoter (22). Hypersensitivity to TLR ligands by autoreactive B cells may require that LMP2A be expressed before anergy induction. Since this is unlikely to occur in humans, EBV-infected B cells may not be hypersensitive to TLR ligand stimulation. Alternatively, TLR hypersensitivity may be independent of the functional status of the B cell, and thus, LMP2A may have a role in human autoimmune disease. In this case, based on the data presented here, multiple TLRs may be affected, resulting in responses to multiple autoantigens characteristic of SLE. It should be noted that LPS is unlikely to play a role because human B cells respond poorly to LPS, as TLR4 expression is low (48). We have focused on an SLE-specific autoantigen, but since this mechanism could act on B cells of specific for autoantigens targeted in other autoimmune diseases, EBV could have a role in the etiology of multiple autoimmune diseases such as Sjögren's syndrome (49), rheumatoid arthritis (50), multiple sclerosis (51), and autoimmune hepatitis (52).

Increased sensitivity to TLR ligands may facilitate EBV latency. EBV infects naive B cells in the oral cavity and induces memory B cell differentiation (reviewed in Ref. 53), due in part to the ability of LMP1 and LMP2A to mimic CD40 and BCR signals, respectively (19, 54). Once established as memory B cells, EBV can exist as a latent infection for as long as the lifetime of the individual. LMP2A's ability to increase sensitivity to TLR stimulation, as suggested here, may affect this process in two ways. First, it could facilitate the activation of naive B cells because TLR stimulation is required for efficient activation of naive human B

cells (55). Second, increased TLR sensitivity may provide a survival advantage to EBV-infected memory B cells because periodic TLR stimulation may be required for long-term survival of memory B cells (56).

Since EBV infection is present in >90% of the population worldwide, additional genetic and environmental factors are obviously required for chronic autoantibody production and development of SLE (57, 58). One relevant factor that could account for a role in SLE is the frequency of EBV-infected B cells. SLE patients have a higher EBV load (9) and increased frequency of infected B cells compared with healthy controls (10). We have previously shown (59) that the frequency of anti-Sm B cells affects the penetrance of the anti-Sm response in murine SLE. Thus, a high EBV infection frequency in humans could increase the chances for autoreactive B cell activation through a hypersensitive BCR/TLR activation pathway, particularly if, as indicated, genetic predisposition leads to an increase in autoreactive B cells in the periphery (60).

A question raised by the LMP2A-driven activation of autoreactive B cells is whether EBV-infected B cells are in sufficient number to account for autoantibody production in disease, even taking into account the higher frequency of EBV-infected B cells in SLE (10). This possibility cannot be ruled out given extrafollicular (61) and germinal center expansion of autoreactive B cells. EBV-infected PCs have been thought to undergo lysis due to the activation of the EBV lytic genes and virus production, casting doubt on the possibility of EBV-infected B cells generating serum autoantibody. However, recent evidence suggests that as many as 80% of infected PCs do not activate the lytic genes and are not replicating virus (62). Thus, a response by EBV-infected B cells may be able to generate autoantibody production.

Alternatively, the activation of autoreactive EBV-infected B cells could also lead to the activation of uninfected B cells through epitope spreading (63, 64), a mechanism that does not require PC differentiation. By this mechanism, the development of a response to one Ag (e.g., Sm B/B') could lead to the activation of B cells specific for other epitopes on the same Ag, epitopes on associated proteins, and even DNA (64). Thus, an initial response by EBV-infected B cells could lead to an autoimmune response composed of both EBV-infected and uninfected B cells.

In summary, we report that LMP2A induces an anti-Sm B cell response, increases B cell sensitivity to TLR activation, and allows ASC differentiation by otherwise regulated B cells. We suggest that alterations in TLR ligand reactivity and sensitivity increase the likelihood autoreactive B cell activation through a BCR/TLR activation pathway. This may have implications for multiple autoimmune diseases in which an association with EBV is indicated. The targeting of therapeutic strategies to the pathways activated by LMP2A may prove useful for the treatment of these diseases, as well as EBV-associated B cell tumors and lymphoproliferative disorders.

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Disclosures

The authors have no financial conflict of interest.

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