Epstein-Barr Virus LMP2A Enhances B-Cell Responses In Vivo and In Vitro

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Epstein-Barr virus (EBV) establishes latent infections in a significant percentage of the population. Latent membrane protein 2A (LMP2A) is an EBV protein expressed during latency that inhibits B-cell receptor signaling in lymphoblastoid cell lines. In the present study, we have utilized a transgenic mouse system in which LMP2A is expressed in B cells that are specific for hen egg lysozyme (E/HEL-Tg). To determine if LMP2A allows B cells to respond to antigen, E/HEL-Tg mice were immunized with hen egg lysozyme. E/HEL-Tg mice produced antibody in response to antigen, indicating that LMP2A allows B cells to respond to antigen. In addition, E/HEL-Tg mice produced more antibody and an increased percentage of plasma cells after immunization compared to HEL-Tg littermates, suggesting that LMP2A increased the antibody response in vivo. Finally, in vitro studies determined that LMP2A acts directly on the B cell to increase antibody production by augmenting the expansion and survival of the activated B cells, as well as increasing the percentage of plasma cells generated. Taken together, these data suggest that LMP2A enhances, not diminishes, B-cell-specific antibody responses in vivo and in vitro in the E/HEL-Tg system.

Epstein-Barr virus (EBV) is a lymphotropic gammaherpesvirus that is harbored by a significant percentage of the population. EBV infects B cells and initially induces their proliferation and expansion. The infected B cells transition from this expansion phase in which numerous viral gene products are expressed to a latent phase in which very few or no viral proteins are expressed (12, 25, 29). EBV is normally maintained without symptoms, but latent EBV infection is associated with a number of malignancies of B-cell origin, such as Hodgkin's lymphoma, Burkitt's lymphoma, and lymphoproliferative diseases in immunocompromised individuals (16, 25, 29). Therefore, understanding the life cycle and proteins utilized by EBV to create and maintain latent infection in B cells may lead to both treatment and prevention of EBV-associated malignancies.

EBV encodes latent membrane protein 2A (LMP2A), which has been identified in latently infected B cells (1, 2, 6, 12, 24, 25, 30). However, much of our knowledge of LMP2A function results from experiments using lymphoblastoid cell lines (LCLs) (17–20). From these studies, it was shown that LMP2A acts as a B-cell receptor (BCR) mimic by phosphorylating proteins involved in normal BCR signal transduction. However, by activating these proteins, LMP2A sequesters these proteins from the BCR in LCLs and inhibits their activation by the BCR (7–9). BCR cross-linking of LCLs that express LMP2A fails to phosphorylate Lyn and Syk; fails to activate phosphatidylinositol 3-kinase (PI3K), phospholipase C gamma, and flux calcium; and fails to reactivate lytic EBV replication (17–20). LMP2A has a 118-amino-terminal tail with tyrosines critical for LMP2A function (8, 9). Tyrosines 74 and

85 form an immunoreceptor tyrosine activation motif (ITAM) that binds Syk, and tyrosine 112 binds to Lyn. All three of these tyrosines are required for LMP2A to block BCR signal transduction (8, 9). From these studies using LCLs, it has been proposed that LMP2A blocks the lytic reactivation of the virus and maintains EBV in the latent state by inhibiting BCR signal transduction.

In a transgenic mouse model that expresses LMP2A in B cells (TgE), LMP2A globally alters the transcription factors required for normal B-cell development to generate B cells that lack a BCR (4, 22). In this system, BCR-negative B cells are protected from apoptosis by the LMP2A-mediated activation of the PI3K/Ras pathway (23). More recently, we crossed these LMP2A transgenic mice (TgE) with a strain of mice that expresses a rearranged BCR specific for hen egg lysozyme (HEL-Tg) to generate mice that produce LMP2A-positive B cells with a BCR specific for a known antigen (E/HEL-Tg) (28). In these mice, LMP2A is not able to protect B cells from BCR-induced apoptosis in response to autoantigen, suggesting that LMP2A allows BCR signaling to occur. Furthermore, in response to a weaker autoantigen, LMP2A bypassed tolerance induction of B cells by providing additional signals that changed a tolerogenic BCR-induced signal into a functional BCR signal (28). These data suggest that the effect of LMP2A on BCR-derived signals may be positive or negative, depending on the context in which the signals are received.

In the current study, we sought to extend these findings using the E/HEL-Tg mouse model. We evaluated the splenic B-cell population and found that E/HEL-Tg mice had a dramatic basal increase in the numbers of B cells and B-cell follicles. We immunized E/HEL-Tg mice to evaluate the effect of LMP2A on the antigen-dependent antibody response. Not only did E/HEL-Tg mice produce antibody after immunization, but they also demonstrated increases in serum immunoglobulin M (IgM) levels in comparison to those of HEL-Tg mice. Furthermore, E/HEL-Tg mice contained an increased

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percentage of antibody-secreting plasma cells after immunization, indicating that LMP2A enhanced the B-cell response to antigen *in vivo*. Finally, the increase in antibody production in E/HEL-Tg B cells is intrinsic to the B cells, since B cells activated *in vitro* with antigen and an antibody that cross-links CD40 demonstrated enhanced HEL-specific IgM production. *In vitro* studies indicate that multiple mechanisms are responsible for the increased antibody response, including increased expansion and survival of LMP2A-positive B cells, as well as increased generation of plasma cells after activation. Taken together, these data indicate that LMP2A enhances a BCR-dependent function (e.g., the antibody response), in contrast to previous observations in EBV-transformed LCLs grown *in vitro*.

MATERIALS AND METHODS

Mice. Hen egg lysozyme immunoglobulin-transgenic (HEL-Tg [MD4]) mice are available from the Induced Mutant Repository of the Jackson Laboratory. HEL-Tg mice express anti-HEL immunoglobulin of the IgM^a and IgD^a allotype (11). Latent membrane protein 2A-transgenic (TgE-Tg) animals were previously described (4). Transgenic mice were bred together in order to generate the desired double-transgenic animals (E/HEL-Tg). The E_μLMP2A transgene was always donated from an LMP2A male. Littermate controls that do not express either HEL-Tg or LMP2A-Tg are considered wild-type controls for these experiments.

Multiplex PCR analysis. Genomic tail DNA from transgenic animals was subjected to multiplex PCR using primers specific for each transgene. Genomic tail DNA was prepared as previously described (4). Primers and the protocol for the PCR were performed as previously described (28).

Immunization. Six- to 8-week-old HEL-Tg and E/HEL-Tg mice were immunized with 100 μg of HEL (Sigma, St. Louis, MO) emulsified in complete Freund's adjuvant (Sigma). Blood was isolated by retro-orbital bleeding 1 day before immunization and at day 10 postimmunization. Sera were analyzed for IgM by radioimmunoassay as described previously (13). Levels were confirmed to be HEL specific by enzyme-linked immunosorbent assay (ELISA) as described below.

ELISPOT analysis. Serial dilution of total spleen cells from HEL-Tg and E/HEL-Tg B cells were cultured on Whatman UniFilter 350 plates coated with hen egg lysozyme for 4 h at 37°C with 5% CO₂. Plates were washed and incubated with biotin-labeled rat anti-mouse IgM antibody (Pharmingen, San Diego, CA) overnight at 4°C. The plates were subsequently incubated with an alkaline phosphatase-conjugated anti-biotin antibody (Vector Labs, Burlingame, CA) for 2 h at room temperature and developed with NBT/BCIP (nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate) as described previously (31). Spots were counted using an enzyme-linked immunospot (ELISPOT) plate reader and software (Cellular Technologies, Cleveland, OH).

Immunohistochemistry. Spleens were snap-frozen in an ethanol bath containing dry ice and stored at -80°C. Spleens were embedded, and frozen sections were cut at 4 μm and mounted on charged slides. Slides were fixed in 75% acetone-25% ethanol and then washed. Endogenous peroxidase was blocked by incubation with 0.3% H₂O₂-sodium azide. Endogenous biotin was blocked with avidin, and endogenous avidin was blocked with biotin (DakoCytomation, Glostrup, Denmark). The slides were additionally blocked with protein block (DakoCytomation) to block nonspecific antibody staining. Primary antibody (rat anti-mouse B220 [clone RA3-6B2]; Pharmingen, San Diego, CA) was added for 30 min, and the slides were washed. Slides were then incubated with biotinylated rabbit anti-rat antibody (DakoCytomation) before incubation with streptavidin-horseradish peroxidase. Diaminobenzidine substrate was added to the slides before they were rinsed with deionized water. Slides were counterstained in filtered Mayer's hematoxylin before they were finally dehydrated through graded ethanol, cleared in xylene, and applied to coverslips with permanent mounting medium.

Flow cytometry staining. Spleens were dissociated between frosted slides in cold staining buffer (10 mg/ml bovine serum albumin, 1× phosphate-buffered saline [PBS], 10 mM HEPES) to prepare single-cell suspensions. Red blood cells were lysed in a 155 mM ammonium chloride solution (Sigma). Approximately 2 × 10⁶ cells were incubated in staining buffer with previously optimized concentrations of the indicated antibodies on ice for 30 min. Cells were washed and analyzed by flow cytometry using a Becton-Dickinson FACScan and Cellquest analysis software. All antibodies were purchased from Pharmingen.

***In vitro* B-cell antibody production.** CD19⁺ B cells were purified as described previously to approximately 90 to 95% purity (21, 23). CD19⁺ purified B cells were cultured at a concentration of 1 × 10⁵ cells/ml in complete RPMI (consisting of RPMI [Life Technologies], 10% heat-inactivated fetal calf serum, L-glutamine, 5 × 10⁻⁴ M mercaptoethanol, and penicillin and streptomycin) in the presence of hen egg lysozyme (10 μg/ml) (Sigma) and 1 μg/ml rat anti-mouse CD40 monoclonal antibody (Pharmingen) for the indicated length of time (day 3, 5, or 7) at 37°C and 5% CO₂. Supernatants were collected and analyzed by ELISA. Briefly, flat-bottom 96-well plates were coated with 2 μg/ml hen egg lysozyme diluted in PBS overnight at 4°C. After multiple washings, the plates were blocked with PBS-5% bovine serum albumin (Sigma) for 1 h at 37°C. The plates were incubated with the supernatants for 2 h at 37°C before the plates were washed with PBS-0.05% Tween 20. The wells were then incubated with horseradish peroxidase-conjugated rat anti-mouse IgM (Pharmingen) for 1 h before development with TMB One solution (Promega, Madison, WI). The colorimetric reaction was stopped with 1 N HCl, and the plate was read within 5 min using a UVmax kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA) at a wavelength of 450 nm.

RESULTS

E/HEL-Tg mice have enlarged spleens with increased numbers of B cells. We previously generated a transgenic mouse model in which B cells express both LMP2A and a BCR specific for hen egg lysozyme (E/HEL-Tg) (28). Bone marrow-derived B cells from these mice indicated that LMP2A allowed BCR signaling to occur in response to autoantigen. We wanted to extend these findings to determine if E/HEL-Tg mice produce antibody after immunization with hen egg lysozyme as a foreign antigen.

Before immunization, we initially analyzed the spleens from E/HEL-Tg mice and compared them to those of their HEL-Tg littermates that do not express LMP2A. The spleens of E/HEL-Tg mice demonstrated splenomegaly with an increase in spleen size (Fig. 1a) and in total cell number (Fig. 1b) in comparison to HEL-Tg mice. The increase in spleen size in E/HEL-Tg mice compared to their littermate controls (HEL-Tg mice) is the opposite to what is observed with the LMP2A TgE mice compared to nontransgenic littermate controls (Fig. 1a and b) (4, 13). Further analysis demonstrated that there was a significant increase in the number of B lymphocytes in the spleens of E/HEL-Tg mice compared to the spleens from control HEL-Tg mice (Fig. 1c). However, this was not the case when the numbers of splenic B cells were compared between TgE mice and nontransgenic littermate controls, in which the numbers of LMP2A⁺ B cells had decreased (Fig. 1c) (4, 12).

To further confirm the increased number of B cells in the spleens of E/HEL-Tg mice, we performed immunohistochemical analysis of spleen sections from HEL-Tg and E/HEL-Tg mice. The spleens from E/HEL-Tg mice showed a significant increase in staining for B220⁺ B cells as compared to HEL-Tg mice (Fig. 2b versus a). The spleens from nonimmunized E/HEL-Tg mice also demonstrate that despite increased B-cell numbers, there were no gross alterations in B-cell organization, as the B cells were localized to B-cell follicles (Fig. 2b).

We sought to determine if the B cells in follicles from E/HEL-Tg mice would reorganize into germinal centers after immunization. Immunohistological analysis showed that E/HEL-Tg mice have larger and more defined polarized follicles on day 10 after immunization, which is consistent with germinal center formation (Fig. 2d versus c). Additional flow cytometric analysis indicated that there was an increased number of germinal center B cells (GL-7⁺ CD19⁺) in the spleens of

FIG. 1. Increased number of total cells and B cells in E/HEL-Tg Mice. (a and b) Spleens were aseptically removed and dissociated as described in Materials and Methods. Total cell number was determined by trypan blue exclusion. (c) Total spleen cell suspensions were stained for CD19 and the IgM^a transgene and analyzed by flow cytometry. The number of B cells was calculated by multiplying the percentage of CD19⁺ IgM⁺ B cells by the total number of spleen cells for each mouse. For panels b and c, each symbol indicates the result from an independent mouse. WT, wild type.

E/HEL-Tg mice after immunization (Fig. 2e). However, the actual percentages of cells that become GL-7⁺ CD19⁺ were equivalent between HEL-Tg and E/HEL-Tg mice after immunization (HEL-Tg, 29.7% $\pm$ 1.2%, versus E/HEL-Tg, 28.3% $\pm$ 5%), suggesting that LMP2A does not increase the likelihood of B cells becoming germinal center B cells. Taken together, these findings demonstrate that LMP2A allows for normal reorganization of B-cell follicles after immunization, without increasing the percentage of germinal center B cells.

E/HEL-Tg mice produce an enhanced antibody response to antigen. Due to the ability of E/HEL-Tg mice to respond to antigen by reorganizing B-cell follicles and forming germinal centers, we next investigated if E/HEL-Tg B cells would generate an antibody response after immunization with hen egg lysozyme. As shown in Fig. 3a, E/HEL-Tg showed a significant increase in IgM after immunization with antigen, demonstrating that LMP2A does not inhibit the ability of E/HEL-Tg B cells to respond to antigen. Also, the level of IgM produced after immunization in E/HEL-Tg was significantly higher than that in HEL-Tg mice. One explanation for the increase in serum IgM in E/HEL-Tg mice after immunization is that the E/HEL-Tg mice contain an increased number of antibody-secreting plasma cells. Therefore, we plated equal numbers of input cells in an ELISPOT analysis and determined that E/HEL-Tg mice contain significantly more antibody-secreting

FIG. 2. Architecture of spleens from HEL-Tg and E/HEL-Tg mice. (a to d) Spleens were isolated either before or 10 days after immunization with 100 μ g/ml hen egg lysozyme. (a to d) Spleens were snap-frozen before sectioning and stained with anti-B220 before visualization with horseradish peroxidase-dependent oxidation of diaminobenzidine to produce a brown substrate. (c and d) Polarized follicles in germinal centers from immunized E/HEL-Tg mice contain many more B cells than germinal centers from immunized HEL-Tg mice (see red arrows in panel d versus c) (e) Spleens from immunized mice were made into single-cell suspensions before being stained with phycoerythrin-labeled anti-CD19 antibody and fluorescein isothiocyanate-labeled anti-GL7 antibody and analyzed by flow cytometric analysis. The graph shows the average number of CD19⁺ GL7⁺ B cells from three to four independent mice.

cells after immunization than HEL-Tg mice (average HEL-Tg result of 40 spots/12,500 cells plated versus average E/HEL-Tg result of 560 spots/12,500 cells plated), which likely explains the increase in serum IgM in E/HEL-Tg mice (Fig. 3b). However, the increase in antibody-secreting plasma cells in E/HEL-Tg spleen cells could simply be due to the presence of more B cells in the input cells used for the ELISPOT assay. If LMP2A directly increases the percentage of cells that become antibody-secreting plasma cells, then we would expect to see an increase in the percentage of CD138⁺ CD19^{lo} cells, which is a marker for murine plasma cells. The data demonstrate both increased percentages and numbers of CD138⁺ CD19^{lo} cells in the spleens from immunized E/HEL-Tg B cells (Fig. 3c). Therefore, the increase in antibody production in E/HEL-Tg mice is likely due not only to an increased number of B cells, but also to an increased percentage of activated B cells that become antibody-secreting plasma cells. Taken together, these findings suggest that LMP2A expression in B cells does not

FIG. 3. Antibody response in E/HEL-Tg mice in vivo. (a) Sera were collected before immunization and 10 days after immunization and analyzed for IgM levels as described in Materials and Methods. PI refers to preimmune sera, and D10 refers to sera collected 10 days after immunization. (b) Spleens from immunized mice were dissociated into single-cell suspensions, and equal numbers of cells were plated for ELISPOT analysis. The spots were developed and counted using an ELISPOT counter as described in Materials and Methods. The data represent the average number of antibody-secreting cells (ASC) from three HEL-Tg mice and three E/HEL-Tg mice after immunization. (c) Spleens from immunized mice were made into single-cell suspensions before staining with phycoerythrin-labeled anti-CD19 antibody and fluorescein isothiocyanate-labeled anti-CD138 antibody and analyzed by flow cytometric analysis. The graph is the average number of CD19⁺ CD138⁺ B cells from three to four independent mice.

inhibit B cells from responding to antigen in vivo and in fact increases the percentage and number of cells that become antibody-secreting plasma cells.

E/HEL-Tg B cells produce more antigen-specific antibody in vitro. If the increase in antibody production in vivo was simply due to unequal numbers of B cells, then equal numbers of E/HEL-Tg and HEL-Tg B cells activated in vitro should produce equal levels of antibody after activation. To address this prediction, equal numbers of purified B cells were activated with antigen and an antibody that cross-linked CD40 in vitro for 7 days. As shown in Fig. 4a, E/HEL-Tg B cells produced significantly more cumulative HEL-specific IgM than HEL-Tg B cells over the course of the in vitro assay. These findings suggest that B cells that express LMP2A produce more antibody than non-LMP2A-expressing B cells. One possible mechanism for the increase in antibody production in vitro could be due to an LMP2A-mediated increase in the expansion and survival of activated B cells. To test this possibility, the total numbers and percentages of viable HEL-Tg and E/HEL-Tg B cells activated with antigen and anti-CD40 antibody were cal-

FIG. 4. Antibody response of E/HEL-Tg B cells in vitro. Equal numbers of HEL-Tg and E/HEL-Tg B cells were activated with 10 μ g/ml hen egg lysozyme and 1 μ g/ml anti-CD40 antibody for 3, 5, or 7 days at 37°C with 5% CO₂. (a) Supernatants from individual wells for each time point were harvested and analyzed for the production of cumulative HEL-specific IgM by ELISA as described in Materials and Methods. The data are an average of the optical density (OD) readings of multiple wells for HEL-specific IgM $\pm$ standard error. (b and c) The total cell number and percent viability were determined by trypan blue exclusion at the indicated time points. (d) HEL-Tg and E/HEL-Tg B cells were activated for either 3 or 5 days, and viable cell numbers were determined. Equal numbers of viable cells were plated for ELISPOT analysis for 4 h as described in Materials and Methods. The percentage of plasma cells was determined by the following equation: (no. of spots counted/no. of cells plated) $\times$ 100. The data are an average of multiple wells $\pm$ standard error. The percentage of plasma cells was not determined on day 7 by ELISPOT analysis due to the lack of an adequate number of viable cells for the assay. For panels a to d, the data are representative of two to three experiments with similar results.

culated by trypan blue exclusion. As shown in Fig. 4b, E/HEL-Tg B cells expand to a greater extent than HEL-Tg B cells by day 3, but the total numbers of cells become equivalent by day 5, suggesting that LMP2A provides an initial proliferative advantage. However, the E/HEL-Tg B cells show a survival advantage over the course of the experiment (Fig. 4c), indicating that the cultures containing E/HEL-Tg B cells have more viable cells present to produce antibody throughout the course of the experiment. Additionally, the increase in antibody production by E/HEL-Tg B cells could be explained by an LMP2A-mediated increase in the percentage of activated B cells that become plasma cells. Therefore, we determined the number of antibody-secreting plasma cells by ELISPOT analysis on days 3 and 5 after activation. The percentage of antibody-secreting cells was not determined on day 7 due to inadequate numbers of viable cells for the assay. As shown in Fig. 4d, when equal numbers of viable B cells were compared on

days 3 and 5, the E/HEL-Tg B cells contained a significantly higher percentage of antibody-secreting cells than HEL-Tg B cells. Taken together, these data indicate that, in the E/HEL-Tg system, LMP2A enhances the B-cell antibody response by increasing the percentage of B cells that differentiate into plasma cells and by increasing the expansion and survival of the activated B cells.

DISCUSSION

Previous studies using LCLs suggest that the role of LMP2A in latently-infected B cells was to block BCR signal transduction to inhibit viral lytic reactivation. More recent studies indicate that LMP2A may allow BCR signaling (28) and even act as a BCR mimic to induce lytic reactivation (27). Therefore, additional models are needed to dissect the effect of LMP2A on B cells. As new systems become available to test LMP2A function, many new exciting functions are being discovered.

In this study, we analyzed the peripheral B-cell population that was generated in E/HEL-Tg mice. We first observed that E/HEL-Tg mice demonstrated an increase in spleen size and the number of B cells, which was confirmed by immunohistochemistry. Additionally, the immunohistochemistry indicated no gross changes in splenic architecture, which is not surprising based on previous immunohistochemistry of TgE spleens performed by our laboratory (data not shown) and another LMP2A transgenic mouse produced by Rajewsky and colleagues that demonstrated normal B-cell localization in the spleen (5). It is currently not known if the increase in the B cells is due to an increase in the number of B cells produced and/or an increase in the survival of LMP2A-positive B cells. Bone marrow data obtained with our E/HEL-Tg mice demonstrate that there are no differences in the numbers and/or percentages of B cells in the bone marrow of E/HEL-Tg mice compared to HEL-Tg mice (data not shown), making it unlikely that E/HEL-Tg mice are producing more B cells. However, our previous data obtained with TgE mice (21) demonstrated that LMP2A could provide a survival signal in the absence of the BCR. We would propose that in the E/HEL-Tg mouse model system, LMP2A cooperates with the tonic survival given by the HEL-specific transgenic BCR to provide enhanced survival of peripheral B cells.

Previous data using LCLs would suggest that LMP2A would block the ability of E/HEL-Tg B cells to respond to antigen. We tested this prediction by exposing E/HEL-Tg B cells to antigen both *in vivo* and *in vitro*. The data indicate that LMP2A allows B cells to respond to antigen after immunization *in vivo* by the following criteria: after immunization, E/HEL-Tg mice demonstrate (i) an increase in the percentage and number of plasma cells and (ii) an increase in the levels of serum IgM. The *in vitro* data further confirm that LMP2A does not inhibit B cells from responding to antigen, since E/HEL-Tg B cells after antigen exposure also demonstrate (i) an increase in the percentage and number of plasma cells, (ii) an increase in the levels of HEL-specific IgM, and (iii) expansion. Therefore, in contrast to the predictions from LCLs, LMP2A-expressing B cells respond to antigen both *in vivo* and *in vitro*.

We further compared the antibody response from E/HEL-Tg mice to that from HEL-Tg mice to test if LMP2A affected the

level of antibody produced. The *in vivo* studies indicated that LMP2A enhanced the antibody response, since E/HEL-Tg mice contained an increased percentage of plasma cells and higher levels of serum IgM compared to HEL-Tg mice. However, it is difficult to completely rule out the contribution of increased B-cell numbers in E/HEL-Tg mice to the enhanced IgM serum response compared to HEL-Tg mice. Therefore, we expanded our experiments to include *in vitro* studies that addressed the antibody responses between E/HEL-Tg and HEL-Tg B cells using equal numbers of cells for the assays. *In vitro*, E/HEL-Tg B cells produced higher levels of antibody at all time points tested when equal numbers of cells were initially plated. We determined that the increase in antibody response was due to multiple mechanisms. B cells from E/HEL-Tg mice demonstrated an increase in expansion and survival at all time points tested when compared to HEL-Tg B cells, suggesting that there were more E/HEL-Tg B cells that could respond to antigen and become antibody-secreting cells throughout the culture period. Additionally, the data indicate that an increased percentage of B cells become antibody-secreting cells over the course of the experiment. Taken together, the data indicate that LMP2A enhances the B-cell response, which may be important for EBV pathogenesis, such as the generation of autoantibodies.

It is interesting to note that E/HEL-Tg mice contain an increased percentage of antibody-secreting plasma cells *in vivo* and *in vitro* after exposure to antigen. One interpretation of this finding is that LMP2A promotes plasma cell development after stimulation through CD40 and the BCR. This possibility is supported by previous data that demonstrate LMP2A positively regulates transcription factors that promote plasma cell differentiation (21). The possibility that LMP2A promotes plasma cell differentiation is interesting, in that Thorley-Lawson and colleagues have provided evidence that lytic reactivation requires differentiation into plasma cells (15). When combined with recent evidence that LMP2A may act as a BCR mimic to promote lytic reactivation (27), the data together suggest that LMP2A may be important for viral reactivation by promoting plasma cell differentiation when stimulated through the BCR and CD40.

However, the data also indicate that LMP2A enhanced the expansion of B cells after stimulation through CD40 and the BCR, suggesting an alternative mechanism to maintain latency, namely, by increasing the number of latently infected B cells. Previous studies have addressed whether LMP2A promotes the expansion of LCLs *in vivo* (26). Findings from this study indicate that LMP2A⁻ LCLs showed no defect in expansion in SCID mice, demonstrating that LMP2A is not important for the expansion of EBV-transformed B cells in this model. Even though LMP2A does not affect LCL expansion, our data indicate that LMP2A may influence the expansion of nontransformed B cells when stimulated through the BCR and CD40. Therefore, our data suggest that LMP2A will augment the expansion of resting memory cells that receive a stimulus to proliferate, which effectively would increase the number of latently infected B cells.

Previous data using LCLs also would predict that LMP2A would protect B cells stimulated through the B-cell receptor from apoptosis (10). The *in vitro* data in Fig. 4 further suggest that LMP2A can protect B cells from BCR-induced death,

since more E/HEL-Tg B cells were viable at all time points tested throughout the assay. These findings further support a role for LMP2A to protect latently infected memory B cells from antigen-induced death *in vivo*.

The observation that E/HEL-Tg B cells produce antibody when stimulated only through the CD40 molecule (Fig. 4a) suggests that LMP2A is acting as a BCR mimic in this system to allow for antibody produced in the absence of any BCR-induced signal to the B cells. Previous studies using LCLs show that LMP2A does not activate the phosphorylation of tyrosines to a level induced by cross-linking IgM on the surface of these cells. However, E/HEL-Tg B cells produced as much antibody when they were stimulated through CD40 alone compared to HEL-Tg B cells stimulated through CD40 and the BCR. It is possible that the low level of signaling induced by LMP2A may be able to synergize with the CD40 signal to allow for antibody production in the absence of BCR signals.

It is also interesting to ponder whether LMP2A could potentially synergize with CD40 signals to promote class switch recombination (CSR). Unfortunately, we are unable to test this possibility with our E/HEL-Tg system due to the transgenic B-cell receptor. CSR requires increased levels or activity of activation-induced cytidine deaminase (14). Therefore, we would hypothesize that LMP2A would not enhance the effect of CSR because LMP2A increases the level of transcription factors that negatively regulate activation-induced cytidine deaminase expression (21). Therefore, LMP2A may synergize with some, but not all, outcomes of CD40 signaling.

An increase in the antibody response in LMP2A-positive cells is not predicted from studies using LCLs. However there are inherent differences between LCLs and E/HEL-Tg B cells that could explain the different outcomes of LMP2A on B-cell activation. First, the LCLs were only stimulated through the BCR and did not receive stimulation through CD40. It is possible that LMP2A may inhibit some aspects of BCR signal transduction but allows for the integration of low levels of BCR signals with secondary signals for B-cell activation. Second, the level of LMP2A is lower in our transgenic model than is found in LCLs (3). Therefore, the current model affords the ability to test the effect of LMP2A on B cells when LMP2A expression is not high. This scenario is possible *in vivo*, since the level of LMP2A likely varies during the viral life cycle based on changes in the levels of transcription factors that regulate LMP2A expression. A third reason for the different outcomes between LCL and E/HEL-Tg B cells is that LMP2A is expressed in the absence of other viral proteins in the E/HEL-Tg system. Interestingly, it recently was demonstrated that there is a small percentage of latently infected human B cells that exclusively express LMP2A (12). Therefore our mouse model allows for the testing of IgM⁺ LMP2A⁺ B cells that are found *in vivo* in limiting numbers. Finally, LMP2A is expressed in a primary B cell in our system that is very different from LCLs, which are immortalized, proliferating cells. Therefore, subtle changes by LMP2A in B-cell function may be lost due to the innate characteristics of LCLs.

The findings from the present study using E/HEL-Tg mice demonstrate that B cells that express LMP2A expand, survive, and differentiate to plasma cells to a greater degree than B cells that do not express LMP2A after exposure to BCR cross-linking with antigen. Taken together, both the E/HEL-Tg

mouse model and LCL models provide unique insights into the function of LMP2A under different conditions that may be identified *in vivo*.

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