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Latent Membrane Protein 2A, a Viral B Cell Receptor Homologue, Induces CD5+/B-1 Cell Development

Akiko Ikeda, Mark Merchant, Lori Lev, Richard Longnecker, and Masato Ikeda

The latent membrane protein 2A (LMP2A) is an EBV-encoded protein that has been implicated in regulating viral latency and pathogenesis within infected cells (1). Upon primary infection, the virus enters a latent life cycle characterized by the expression of nine latency-associated viral proteins, including LMP2A (2, 3). LMP2A is one of the most consistently detected mRNAs found in humans infected with EBV, suggesting that LMP2A plays an important role in regulating viral persistence and in the development of EBV-associated diseases (4). Our studies have demonstrated that LMP2A functionally mimics a constitutively active B cell Ag receptor (BCR) and modulates BCR-mediated signal transduction during B cell development (5) and activation (6).

Consistent with a role in functionally mimicking BCR signaling, LMP2A has been shown in EBV-immortalized human lymphoblastoid cell lines to interact with cellular proteins and localize to subcellular compartments in a fashion similar to that of an activated BCR. The amino-terminal tail of LMP2A contains several tyrosine residues important for these functions of LMP2A. Two of these tyrosines encode a conserved immunoreceptor tyrosine activation motif (ITAM), which has been shown to be critical for the ability of LMP2A to interact with the protein tyrosine kinases Lyn and Syk. In addition to mimicking a constitutive BCR signal, LMP2A is also capable of blocking BCR-induced signaling by recruiting Lyn and Syk (7, 8). The recruitment of Lyn and Syk by LMP2A results in the constitutive phosphorylation of these kinases independent of ligand binding. Further evidence that LMP2A is co-opting the BCR signaling pathway was found when LMP2A was shown to constitutively localize to lipid rafts. Therein, LMP2A is thought to form self-aggregates that inhibit the translocation of the BCR into lipid rafts, thus blocking subsequent activation of the BCR (9). Together these data show that LMP2A imparts multiple functions, which allow it to both mimic the BCR signal required for B cell survival and also eliminate the ability of the BCR to properly activate infected B cells.

Transgenic mice expressing LMP2A downstream of the μIg heavy-chain enhancer and promoter (Eμ) have highlighted the ability of LMP2A to impart developmental and survival signals to developing and mature B cells (5). In EμLMP2A transgenic line E (TgE), which expresses high levels of LMP2A, there is a developmental alteration characterized by a bypass of BCR expression resulting in BCR-negative (BCR−) B cells in peripheral lymphoid organs (10). The lack of BCR expression in TgE mice results from the absence of Ig heavy chain rearrangement (10). Normally, B cells lacking a cognate BCR rapidly undergo apoptosis; however, in TgE mice, BCR− cells persist. In EμLMP2A transgenic line 6 (Tg6), which expresses lower levels of LMP2A, there are no dramatic differences in B cell development compared with wild-type animals other than a slight reduction in the number of BCR+ B cells in the spleen of LMP2A transgenic mice, indicating that there is likely an expression threshold that must be overcome to observe a developmental phenotype (10). Furthermore, LMP2A requires the association and activation of Syk to alter B cell development because LMP2A transgenic mice bearing mutations in the ITAM of LMP2A show a B cell phenotype indistinguishable from that of wild-type mice (11). These results indicate that LMP2A positively mediates developmental signals in B cells and that Syk plays a key role in mediating LMP2A signaling. However, the unusual BCR− peripheral B cell subset in the EμLMP2A− mice has not been fully characterized with respect to other B cell subsets.

Two distinct mature B cell populations, B-1 and B-2, are present in the mouse and human periphery. B-1 cells are found mainly in the peritoneal and pleuroperticardial cavities, whereas B-2 cells are...
mainly found in spleen, lymph nodes, and peripheral blood. B-1 cells are subdivided into CD5⁺ (B-1a) or CD5⁻ (B-1b) cells and are distinguishable from B-2 cells by cell surface marker expression (IgM<sup>bigh</sup>, IgD<sup>low</sup>, B220<sup>low</sup>, CD43<sup>+</sup>, and CD23<sup>low</sup>) (12, 13). B-1 cells are more likely to produce autoantibodies and may be involved in T cell-independent responses to common environmental Ags. Another unique property of B-1 cells is the capacity for self-replenishment. B-2 cells are responsible for T cell-dependent responses to exogenous Ags and for generating memory B cells. Developmentally, immature B cells emigrate from the bone marrow to the splenic periaortiolar sheath, forming transitional type 1 B cells (14). Only a small percentage of type 1 cells develop into transitional type 2 cells that reside in the spleen primary follicle. Type 2 B cells then differentiate into follicular B-2 cells localized in the primary lymphoid follicle or marginal zone B cells in the perifollicular marginal zones (14). Despite the wealth of information in regard to the development of B-2 cells, the source of B-1 cells has been controversial. Initially, it was thought that B-1 cells develop as a separate lineage that is distinct from conventional B-2 cells. Thus, B-1 cells develop in early life as a distinct population (the lineage hypothesis) (15). However, recent studies suggest that B-1 cells result from a pathway potentially available to all developing B cells. In this model, a different strength and context of BCR signaling are required for the development of each B cell subset (the differentiation hypothesis) (15).

In this study, the peripheral and bone marrow B cell subsets in LMP2A transgenic mice were analyzed to clarify how LMP2A alters B cell development. This analysis indicates that LMP2A expression results in the exclusive generation of B cells that have a phenotype indicative of the CD5⁺ B-1 subset or B-1a cell type. The development of these cells is independent of a functional BCR, indicating that LMP2A signals replace those normally derived from the BCR, resulting in a developmental switch to the B-1 cell lineage. These data support the differentiation model of CD5⁺ B-1 cell development because signals derived from LMP2A during early B cell development are capable of switching B cells normally committed to B-2 cell development to become B-1 lineage cells. Furthermore, these data suggest that signals derived from LMP2A during latent infection may help EBV establish a unique B cell reservoir, a step that could be important during early pathogenesis of the virus.

**Materials and Methods**

**Mice**

Wild-type C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Construction of EqaLMP2A TgE, Tg6, and ITAM mutant transgenic mice (TgΔITAM) have been previously described (5, 11). For analysis of flow cytometry and cell proliferation, 4- to 8-wk-old mice were used. All animals were housed at the Northwestern University Center for Experimental Animal Resources in accordance with university animal welfare guidelines.

**Isolation of primary lymphoid cells and flow cytometry**

Bone marrow cells were flushed from femur and tibia using IMDM (Life Technologies, Rockville, MD). Spleens and lymph nodes were dissociated between frosted slides in RPMI 1640 medium (BioWhittaker, Walkersville, MD) to prepare single cell suspensions. Peritoneal cells were obtained by peritoneal lavage with HBSS. Peripheral blood was drawn from ophthalmic veins. RBCs were lysed in RBC lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM NaEDTA (pH 7.2)). Cells were suspended in staining buffer (1% PBS, 10 mM HEPES, 1% FBS, and 0.1% sodium azide). For surface staining, 1 × 10⁶ cells in 100 µl were incubated with previously optimized concentrations of FITC-ε, PE-, PerCP-, APC-, and biotin-labeled Abs at 4°C for 30 min. For a second step when required, cells were resuspended in 100 µl of appropriate secondary reagent and incubated at 4°C for 30 min. Cells were washed with 1× PBS and analyzed by using FACSCalibur (BD Biosciences, Mountain View, CA) and CellQuest Pro analysis software (BD Biosciences). The Abs and secondary reagents used were anti-CD5, anti-B220, anti-CD43, anti-CD23, anti-c-Kit, anti-CD24, anti-BP-1 Ag, anti-IgM, and streptavidin-PerCP (BD Biosciences). All data represent cells that fall within the lymphocyte gate determined by forward and side scatter. A total of 5,000–10,000 events in the lymphocyte gate were analyzed.

**Cell culture**

A total of 1 × 10⁶ bone marrow cells were cultured in 3 ml of IMDM, 30% FBS, 100 µM 2-ME, 2 mM L-glutamine, and 10 ng/ml recombinant mouse IL-7 (R&D Systems, Minneapolis, MN). After 4-day culture, medium was changed to 3 ml of fresh medium supplemented with or without IL-7. Cells were cultured for an additional 2 days and were used for flow cytometric analysis.

**Proliferation assay**

Splenic and peritoneal B cells were purified by positive selection with anti-CD19 (Miltenyi Biotec, Auburn, CA). Purified B cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Cells (0.5 × 10⁶/well) in triplicate were stimulated with various concentrations of LPS or PMA in a 96-well tissue culture plate for 24 h. After stimulation, cells were labeled with [³H]thymidine (1 µCi/well) for 12 h. A microplate cell harvester (FilterMate 196; Packard Instrument, Meriden, CT) was used to collect cells, and a microplate scintillation counter (TopCount NXT; Packard Instrument) was used to assess proliferation.

**Serum IgM levels**

Total IgM levels in mouse serum were quantified by radial immunodiffusion at Prairie Diagnostic Services (Saskatoon, SK, Canada).

**Results**

**LMP2A⁺ BCR⁻ B cells display the surface markers of CD5⁺ B-1 subset**

Recent studies with LMP2A transgenic mice have shown that, in addition to the loss of IgM expression, CD19 expression is high in splenic B cells from TgE mice (16). High CD19 expression is also found on B-1 cells within the peritoneal cavity (17). This commonality suggests that TgE B cells may share other features of B-1 lineage cells. To test this idea, TgE splenic B cells were analyzed by flow cytometry for expression of the lymphocyte differentiation markers CD5 and B220. TgE is a representative LMP2A transgenic line expressing high levels of LMP2A (10). In wild-type mice, B-1 cells (CD5⁺ and B220⁺) are readily detected in the peritoneal cavity and represent only a small proportion of splenic B cells (Fig. 1A, WT). Approximately 10% of the peritoneal B cells from wild-type mice are CD5⁺ (Fig. 1B and Table I). This is somewhat lower than what is observed for other strains such as BALB/c (data not shown), in which the number can be as high as 60% (18). In contrast, splenic and peritoneal B cells from TgE mice are both predominantly CD5⁺ and B220⁺ (Fig. 1A, TgE-LMP2A), indicating that most B cells in TgE mice display a B-1α phenotype. In addition, as would be expected from the previously described TgE-LMP2A phenotype (5), the majority of these cells are surface Ig negative (data not shown).

To further characterize the origin and trafficking of the splenic CD5⁺ population cells in TgE mice, bone marrow B cells from wild-type and TgE mice were examined (Fig. 1A, WT and TgE-LMP2A). Interestingly, bone marrow B cells from TgE mice also exhibited a significant increase in CD5⁺ B cells (Fig. 1A). CD5⁺ and B220⁺ B cells were also the predominant population of B cells in both the inguinal lymph node and in the peripheral blood in TgE-LMP2A mice, whereas the majority of B cells in wild-type mice were conventional B-2 cells (CD5⁻ and B220⁺; Fig. 1B, WT and TgE-LMP2A). Increases in absolute numbers of CD5⁺ B cells were also observed in each organ of TgE mice (Table I). These results indicate that LMP2A signaling allows for the development of a unique population of CD5⁺ B cells most closely related to the B-1a lineage.
High levels of LMP2A expression are required for LMP2A-mediated differentiation of CD5<sup>+</sup> B-1 cells

Previous studies have indicated that high levels of LMP2A expression are required for the generation and survival of BCR<sup>−</sup> B cells in the periphery of LMP2A transgenic lines, whereas lower levels of LMP2A expression result in transgenic lines with no dramatic developmental alteration, except for a reduction in the normal number of BCR<sup>−</sup> peripheral B cells (10). To determine whether high levels of LMP2A expression are required for the generation of cells bearing a B-1 lineage phenotype, B cells from the spleen, peritoneal cavity, and bone marrow from the Tg6 line, a representative low-expressing LMP2A transgenic line, were analyzed. B cells harvested from the spleen and bone marrow from Tg6 transgenic mice do not show an increase in CD5<sup>+</sup> cells when compared with TgE or wild-type mice (Fig. 1A). B cell development is normal in TgE mice (11). B cell development is normal in TgE mice, high-LMP2A-expressing transgenic (TgE-LMP2A), and ITAM-mutant-LMP2A transgenic (ΔITAM-LMP2A) mice were stained with CD5 and B220. Lymphocyte populations determined by forward and side scatter were plotted with CD5 and B220. The percentages of CD5<sup>+</sup> or CD5<sup>−</sup> B cells to total lymphocytes are indicated. Representative experiments are shown from eight TgE-LMP2A, four Tg6-LMP2A, or three ΔITAM-LMP2A.

Syk is required for LMP2A-mediated differentiation of CD5<sup>+</sup> B-1 cells

We have shown that the Syk protein tyrosine kinase interaction with LMP2A is required for the LMP2A-mediated bypass of B cell developmental checkpoints (11). To investigate whether Syk is also essential for LMP2A-mediated B-1a development, B cells were analyzed for LMP2A TgΔITAM. The LMP2A transgene in these mice contains a mutation in the LMP2A ITAM that prevents the association of LMP2A with Syk. This transgenic line, as well as others, expresses levels of LMP2A comparable with those of the TgE line (11). B cell development is normal in TgΔITAM spleen and bone marrow B cell populations. To determine whether the interaction of Syk with LMP2A is important for the generation of CD5<sup>+</sup> B-1 lineage B cells in TgE mice, flow cytometry was performed on spleen, bone marrow, and peritoneal cavity cells collected from wild-type and TgΔITAM mice. In TgΔITAM mice, there was a complete absence of the unique CD5<sup>+</sup> and B220<sup>+</sup> B cell populations present in the spleen and bone marrow samples of TgE mice (Fig. 1A, compare TgΔITAM-LMP2A with TgE-LMP2A). Absolute numbers of total and CD5<sup>+</sup> B cells in each organ of TgΔITAM mice did not show any difference when compared with wild type (data not shown). These results indicate that the interaction of Syk with LMP2A is essential for LMP2A-mediated B-1a development.

TgE B cells are CD5<sup>+</sup>, CD43<sup>+</sup>, and CD23<sup>−</sup>, which is characteristic of B-1a cells

To further verify the B-1a lineage phenotype of B cells from TgE mice, B220<sup>−</sup> gated B cells from the spleen, peritoneal cavity, and

<table>
<thead>
<tr>
<th>Bone marrow</th>
<th>Total Cells</th>
<th>B220&lt;sup&gt;+&lt;/sup&gt; CD5&lt;sup&gt;+&lt;/sup&gt;</th>
<th>B220&lt;sup&gt;+&lt;/sup&gt; CD5&lt;sup&gt;−&lt;/sup&gt;</th>
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<tr>
<td>TgE-LMP2A (n = 6)</td>
<td>53.3 ± 9.9</td>
<td>16.3 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.2 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Spleen</td>
<td>Wild type (n = 13)</td>
<td>83.9 ± 1.85</td>
<td>4.6 ± 0.4</td>
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<tr>
<td>TgE-LMP2A (n = 12)</td>
<td>60.5 ± 16.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.8 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peritoneal cavity</td>
<td>Wild type (n = 5)</td>
<td>3.6 ± 1.1</td>
<td>0.37 ± 0.02</td>
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<tr>
<td>TgE-LMP2A (n = 2)</td>
<td>2.9 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.81 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> The absolute number (×10<sup>6</sup>) of each cell population was determined by flow cytometry. Values are given as the mean ± SD. Student’s t tests were calculated as transgenic vs wild-type mice.

<sup>b</sup> p < 0.001.

<sup>c</sup> p < 0.005.

<sup>d</sup> p < 0.05.
bone marrow were analyzed for CD43, CD23, and CD5 expression. The majority of splenic B cells from wild-type mice display a B220+, CD5−, CD43+, and CD23+ surface marker expression profile (Fig. 2A, WT). In contrast, TgE splenic B cells display a surface marker expression profile characteristic of B-1a cells, with the majority of the B cells staining B220+, CD5−, CD43+, and CD23− (Fig. 2A, TgE-LMP2A). This population was also negative for Mac-1 (data not shown), confirming that the B cells from the TgE mice had a splenic B-1 phenotype, because Mac-1 is negative in the splenic B-1 cells but positive in the peritoneal cavity B-1 cells (19). In contrast with conventional splenic B-1 cells, which are usually IgMhigh and IgDlow, TgE B cells lack expression of IgM or IgD due to the previously characterized block in IgH rearrangement (5).

Because TgE splenic B cells appeared to resemble B-1-like lineage cells, we were interested in analyzing the cell surface marker expression profile of peritoneal B cells. As expected, a large fraction of the B cells from the peritoneal cavity from wild-type mice were B220+, CD5+, CD43+, and CD23−, confirming that these cells display the expected B-1 cell surface profile (Fig. 2B, WT). Likewise, B cells from the peritoneal cavity from TgE mice were almost entirely B220+, CD5+, CD43+, and CD23−, confirming their B-1 lineage phenotype (Fig. 2B, TgE-LMP2A). Similar to the spleen, there was a dramatic increase in the number of these cells when compared with wild-type mice (compare WT and TgE-LMP2A in Fig. 2, A and B). Interestingly, the peritoneal B cells from TgE mice were positive for Mac-1, which is comparable with B-1 cells found in the peritoneum in wild-type mice (data not shown).

Because TgE B cells appear to display a B-1-like expression profile in the spleen and the peritoneum, we were interested in analyzing the bone marrow to determine whether the apparent skewing toward the B-1 lineage occurs there, as suggested by previous data (Fig. 1). In the TgE mice, there was an appearance of B cells in the bone marrow that had a B-1-like phenotype, as indicated by B220+, CD5+, CD43+, and CD23− cell populations (Fig. 2C, TgE-LMP2A). This population of cells was almost entirely absent in the bone marrow of wild-type mice when compared with the TgE mice (Fig. 2C, WT and TgE-LMP2A). In summary, the expression of lymphocyte differentiation markers indicates that the majority of B cells from the spleen, peritoneal cavity, bone marrow, inguinal lymph node, and peripheral blood from TgE mice have a B-1a phenotype.

LMP2A+ B-1a cells are committed in bone marrow at the large pre-B stage

CD5 expression in TgE bone marrow B cells suggests that LMP2A-mediated B-1 development starts within bone marrow. To determine the exact stage at which the commitment to B-1 cells is made, CD5 expression was examined in pro-, pre-, and immature B cells from TgE bone marrow. Typically, CD43 and IgM are used to developmental stage bone marrow B cells, but because TgE bone marrow B cells do not express Ig and B-1 cells are positive for CD43, we instead chose to use c-Kit, CD24, and BP-1, which distinguish different B cell developmental stages. As previously described, different B cell developmental subsets can be distinguished by the following expression profile: early pro-B (c-Kit+, CD24−), late pro-B (c-Kit+, CD24low, pre-B (c-Kit−, CD24high, BP-1+), and immature B (c-Kit−, CD24high, BP-1−) (12). The percentage of both early and late pro-B cells from B220+ gated cells was the same in wild-type and TgE bone marrow, indicating that TgE mice display normal pro-B cell development (Fig. 3A, WT and TgE-LMP2A). As expected, CD5 expression in these pro-B populations was negative in TgE and wild-type mice (Fig. 3A, WT and TgE-LMP2A). These data indicate that LMP2A-mediated B-1 lineage commitment does not occur at or before the pro-B stage.

Next, pre-B and immature B cell stages were examined by staining TgE and wild-type bone marrow cells for CD24 and BP-1 (Fig. 3B). Expression of CD5 was observed in TgE pre-B cells (CD24high and BP-1+), as well as in TgE immature B cells (CD24high and BP-1−), indicating that CD5 expression begins during the pre-B cell stage. Interestingly, the BP-1+ population of cells dramatically decreased in TgE mice. The nature of this dramatic decrease in pre-B cells is not known, but may be due to more rapid transition through this B cell developmental stage. It is also possible that LMP2A dramatically alters normal B cell developmental signals due to LMP2A expression and/or lack of a cognate BCR.

To further examine LMP2A-mediated B-1 lineage development, large pre-B cells were selectively cultured in medium containing IL-7. IL-7 is a cytokine required for the survival of the early B cell lineage, and culture conditions have been established and extensively used to selectively expand primary large pre-B cells (20, 21). Upon removal of IL-7 from culture, large pre-B cells can progress into the small pre-B stage, with a small portion of these

**FIGURE 2.** B-1 immunophenotypes of TgE-LMP2A B cells. Cells isolated from spleen (A), peritoneal cavity (B), and bone marrow (C) from wild-type (WT) and high-LMP2A-expressing transgenic (TgE-LMP2A) mice were stained simultaneously with B220, CD5, and either CD43 or CD23. B cells (B220+) were gated from total lymphoid population and plotted with CD5 and CD43 (upper panels) or CD5 and CD23 (lower panels). The percentages of B-1 cells (CD5+ CD43+ or CD5− CD23−) and B-2 cells (CD5− CD23−) to total B cells are indicated. One of four comparable experiments is shown.
cells proceeding further into the immature B cell stage (22). This developmental progression is characterized by a decrease in the BP-1^{high} expressing population with a subsequent decrease in cell size (Fig. 4, WT). Bone marrow cells cultured from TgE mice contained large pre-B cells that already expressed CD5 (Fig. 4, compare 9% and 10% for WT with 43% and 34% for TgE-LMP2A). Interestingly, TgE bone marrow cells developed normally through the pre-B stage, in that cultured cells from TgE bone marrow maintained high BP-1 expression and showed a decrease in cell size upon removal of IL-7 from the culture medium (Fig. 4, TgE-LMP2A). Thus, in TgE bone marrow, pre-B development was normal and both large and small pre-B cells were positive for BP-1, which is similar to wild type. This clearly indicates that the decrease in the BP-1^{high} population (Fig. 4B) is not due to LMP2A down-regulating BP-1 expression, but rather is due to a reduction in the pre-B population.

LMP2A^{+} B-1 cells proliferate in response to LPS but not to PMA

It is known that B-1 and B-2 cells respond differentially to mitogens (15), providing an important means to evaluate the B-1 phenotype of the B cells in the TgE mice. Peritoneal B-1 cells proliferate in response to LPS and to phorbol esters (PMA), whereas B-2 cells proliferate only in response to LPS and not to PMA (23). Recently, studies have revealed that the response of B-1 cells to mitogens differs with respect to whether they were derived from the spleen or the peritoneal cavity (24). Splenic B-1 cells appear to function much like B-2 cells in that they respond to LPS but not to PMA, unlike peritoneal B-1 cells. Therefore, to functionally characterize TgE B-1-like cells, thymidine incorporation assays were performed on purified B cells from spleens isolated from TgE and wild-type mice using LPS or PMA as mitogens. TgE splenic B-1-like cells responded to LPS significantly more than did wild-type B-2 cells (Fig. 5). In contrast, PMA caused proliferation of both wild-type and TgE peritoneal B cells (Fig. 5). However, neither wild-type splenic B cells nor TgE splenic B-1-like cells responded to PMA (Fig. 5). This is similar to the increase in proliferation that was observed in Ig-expressing B-1 cells (24). To our surprise, TgE...
peritoneal B-1 cells did not respond to LPS alone, which contrasts with typical splenic B-1 cells. The mechanism of LMP2A-mediated B-1 development in the peritoneal cavity remains to be elucidated. In summary, the phenotype of TgE splenic B-1-like cells is consistent with that of Ig-expressing splenic B-1 cells.

**Serum IgM levels increase in LMP2A mice**

Although the majority of splenic B cells in TgE mice are B220<sup>−</sup> IgM<sup>+</sup>, we consistently observe a very small percentage of B220<sup>−</sup> IgM<sup>−</sup> B cells in TgE spleens. Typically, this number is between 10% and 30% of the B220<sup>−</sup> IgM<sup>−</sup> B cells (Fig. 6A, TgE-LMP2A; compare 7% B220<sup>−</sup> IgM<sup>+</sup> with 23% B220<sup>−</sup> IgM<sup>−</sup>). To further characterize the nature of these cells, CD5 expression was analyzed on B220<sup>−</sup> IgM<sup>+</sup> and B220<sup>−</sup> IgM<sup>−</sup> B cells from the spleen. Compatible with our earlier results, CD5 expression was greatly increased in TgE B220<sup>−</sup> IgM<sup>−</sup> B cells (Fig. 6A, TgE-LMP2A). Interestingly, there was also enhanced CD5 expression in TgE B220<sup>−</sup> IgM<sup>−</sup> B cells, indicating that the IgM<sup>−</sup> B cells in TgE spleens also display a B-1a-like phenotype (Fig. 6A, TgE-LMP2A). Because TgE animals were capable of making surface IgM, albeit at low levels, we wanted to analyze serum IgM levels in TgE mice because B-1 cells are known to be the main source for natural IgM production. Previous studies have shown high levels of serum IgM in Src homology 2-containing protein tyrosine phosphatase-1<sup>−/−</sup> (25) and CD22<sup>−/−</sup> (26, 27) mice, in which there is a considerable increase in the number of B-1 cells in the periphery. Similar to these knockout mice, TgE mice also have high levels of serum IgM (Fig. 6B), providing additional functional data supporting the observation that TgE B cells display a B-1a phenotype. It is interesting to note that the small number of B cells that do manage to recombine their Ig genes and express IgM still have a B-1 phenotype, indicating that the LMP2A signal is dominant to the BCR signal.

**Discussion**

We have demonstrated that LMP2A TgE mice exhibit an unusual distribution and development of B-1a cells. In wild-type adult mice, B-1 cells are found in the peritoneal cavity, represent only a small proportion of splenic B cells, and are absent from lymph nodes and peripheral blood. In TgE mice, however, B-1-like cells are the principal B cell population in bone marrow, spleen, and periphery. Most interesting is the existence of a large B-1-like subset in the TgE bone marrow, indicating that LMP2A redirects B cell development to favor B-1a differentiation. This developmental alteration in TgE mice is dependent on the association of LMP2A with Syk, indicating that normal BCR signaling pathways initiated by Syk are used for this phenotype. In addition, this phenotype is only observed in transgenic mice with high expression levels of LMP2A, indicating that strength of signal may be important for the LMP2A-mediated B-1a-like lineage commitment. These studies clearly indicate that progenitor B cells in the bone marrow are capable of differentiating into B-1 cells in the bone marrow microenvironment if stimulated with the appropriate signals such as the signals derived from LMP2A.

B-1 cells in normal mice are frequently autoreactive and include cells with specificities for IgG (rheumatoid factor), ssDNA, and the common membrane phospholipid phosphatidyl choline (PtdC), specificities all of which are rare among B-2 cells (12, 13). The B-1 subset also harbors cells specific for bacterial carbohydrate Ags and phosphoryl choline. Two main hypotheses have been proposed to explain the generation of B-1 cells: the lineage model and the differentiation model. The lineage model is based on cell transfer experiments of progenitor cells. Fetal liver can reconstitute both the B-1 and B-2 compartments of irradiated mice, whereas...
adult bone marrow is generally limited to the generation of B-2 cells (28, 29). Progenitors for B-1 cells are selectively lost when neonatal B cells are depleted by treatment with anti-IgM, whereas B-2 recovery begins as soon as the treatment Ab disappears (30). Thus, the lineage model suggests that B-1 cells arise from progenitor cells found in the fetal liver and are maintained long-term by self-replenishment in the peritoneal cavity. The first indication that B-1 cells might arise from differentiation of a common B cell progenitor cell arose from experiments in which continuous BCR cross-linking in the absence of T cell help caused splenic B-2 cells to develop into B-1-like CD5+ cells as a consequence of thymus-independent type 2 Ag-like activation (31). Recent Ig-transgenic studies support such a differentiation model and have indicated the importance of particular BCR specificity in generating B-1 or B-2 cells. In B-1-specific Ig transgenic mice (VH12 (anti-PtC), VH11 (anti-PtC), and VH3609 (anti-Thy-1)), transgene-expressing B cells are predominantly the B-1 subset in spleen and peritoneal cavity (32–34). In contrast, B cells are limited to the B-2 subset in B-2-specific Ig transgenic mice (3-83 (anti-H-2k/H-2d) and HyHEL10 (anti-hen egg lysozyme)) (33, 35). Importantly, VH3609 mice reveal that the generation of B-1 cells is dependent upon Ag association with the BCR. Introduction of the VH3609 transgene results in an increase in CD5+ B cells reactive with T cell glycoprotein Thy-1 (34). However, when VH3609 mice are crossed onto a Thy-1 background, anti-Thy-1 Ab-producing B cells and transgene-expressing B-1 cells are absent in the periphery (36). Thus, engagement of the BCR by Ag, in this case Thy-1, is necessary for B-1 development.

Inducible disruption of the BCR complex in mature B cells results in the absence of all mature B cell subsets, revealing that BCR signaling is required for both B-1 and B-2 B cell differentiation (37). In mice lacking key intracellular signaling intermediates (i.e., Bruton’s tyrosine kinase (Btk), B cell linker protein (BLNK), Vav, phospholipase C γ2, phosphatidylinositol 3-kinase p85α, protein kinase C β, and CD19), B-1 cells are dramatically reduced in the peritoneal cavity (12, 13). Conversely, the loss of inhibitory regulators, such as Src homology 2-containing protein tyrosine phosphatase-1, CD22, and CD72, is associated with increased numbers of peritoneal B-1 cells (12, 13). Interestingly, changes of B-1 cell levels are commonly correlated in splenic and peritoneal B cells in these knockout/transgenic mice. These studies lead to a model in which a stronger BCR signal is required for the generation of peritoneal B-1 cells. Indeed, the observations that mice bearing B-1 cells engineered to respond to specific Ags develop B-2 cells when there are defects in normal BCR-mediated signal transduction or when there is a lack of the Ig-specific Ag indicate that the quality of the signal through the BCR drastically affects B cell development. In VH12/stid mice, deficiency of Btk blocks the increase of B-1 cells that is observed in the wild-type background VH12 mice (38). VH12/VH4/stid mice develop B-2 cells despite the expression of B-1-specific VH12/Vk4 Ig (38). In addition, selective B-2 B cell generation is found in VH3609/Thy-1−/− mice that lack expression of specific Thy-1 Ag for VH3609 Ig (36). These studies clearly indicate that signals with appropriate strength and timing are required for B-1 lineage commitment. Taken together, these data suggest that an intermediate BCR signal favors B-2 differentiation, whereas a stronger signal favors B-1 cell differentiation.

Because there is a lack of animal models for EBV infection and latency, transgenic models have been indispensable to clarify the role of specific viral proteins in viral infection and subsequent latency. To investigate the involvement of LMP2A in B-1 cell development, three representative lines of LMP2A transgenic mice were used. Previous studies have shown that LMP2A transgenic mice display two distinct phenotypes. LMP2A transgenic mice expressing higher levels of LMP2A in the bone marrow, such as TgE, show a dramatic phenotype characterized by the development of B cells that lack expression of IgM (10). LMP2A transgenic mice expressing lower levels of LMP2A in the bone marrow, such as Tg6, exhibit fairly normal B cell differentiation (10). In this study, we have shown that high expression of LMP2A, as observed in TgE B cells, correlates with the selective differentiation toward a B-1-like lineage, whereas low levels of LMP2A expression, as observed in Tg6 B cells, do not show an increase in skewing toward B-1 differentiation.

The LMP2A signal in many ways mimics a normal BCR signal. Our previous studies have shown that LMP2A self-aggregates in lipid rafts in the plasma membrane and constitutively associates with Src family protein tyrosine kinases and the Syk tyrosine kinase, using specific phosphotyrosine motifs found in the LMP2A amino terminus (7, 8). In addition, LMP2A, like the BCR, requires Btk and BLNK, indicating similar cellular requirements for both BCR and LMP2A signaling (16, 39). Indeed, the results obtained in the ΔITAM-LMP2A mice reveal that Syk and its downstream pathway are also essential for the LMP2A-mediated B-1 phenotype. However, unlike the signals thought to stem from a developing BCR, those stemming from LMP2A are thought to be constitutive, resulting in B cells that are chronically stimulated. Commitment of cells into the B-1 lineage in the bone marrow has been suggested from several lines of study, and our current study provides further evidence that this can occur. In VH12 mice, bone marrow, a large portion of VH12 Ig+ B cells, are CD5− and defined as circulating mature cells (32). This CD5− population may contain some portion of pre-B or immature stage of B-1 cells. In another example, VH14/VH9 bone marrow reconstitutes splenic B-1 population in irradiated mice (33). In addition, CD5− cells emerge from in vitro culture of VH14/VH9 bone marrow cells in the presence of IL-7 (33). These studies suggest that bone marrow cells expressing B-1-specific Ig have a potential to progress into the B-1 subset. Our data further support the finding that bone marrow B cells can express CD5 and commit into the B-1 lineage at the pre-B stage as the result of LMP2A expression.

Commonly, it has been thought that B-1 lineage commitment occurs at a transitional stage in the periphery (40, 41). Therefore, the B-1 commitment at the pre-B stage shown in our study is earlier than generally thought. The LMP2A transgene is cloned downstream of Eμ. In μ-chain expression, μ0 transcripts are detected at the early pro-B stage before productive rearrangement (42). The rearranged μ-chain is complexed with surrogate light chain as the pre-BCR at the clonal proliferating pre-B stage. It is likely that expression of LMP2A protein also starts at the early pro-B stage, like other transgenes regulated by Eμ. Therefore, it is likely that LMP2A can substitute for the function of pre-BCR at the pre-B stage.

Can the B-1 lineage commitment observed in TgE mice be generalized into B-1 differentiation mediated through normal developmental signals? During normal B cell development, it is thought that the pre-BCR signals as soon as it is expressed on the cell surface because pre-BCR-expressing cells are rarely isolated from the bone marrow (43). Thus, it is commonly believed that an assembled functional pre-BCR is all that is required for pre-BCR signaling and that specific Ags are not necessary. If the μ-chain fails to assemble with surrogate light chain, pro-B cells are not positively selected to progress into pre-B cells (44). Despite this, the majority of B cells in the bone marrow of TgE mice display a B-1 phenotype, whereas most B-1-specific Ig transgenic mice demonstrate no B-1 commitment within the bone marrow. This difference can be explained in several ways. First, it is possible that
LMP2A mediates a stronger signal than does the BCR. Second, LMP2A competes with the BCR and blocks normal BCR signaling (45). Our in vivo studies indicate that LMP2A provides a constitutive BCR-like signal (6, 46). Thus, in TgE mice, a strong LMP2A signal could favor differentiation into B-1-like lineage cells in the bone marrow. This scenario seems likely because of the similarity of cellular factors that are required downstream of LMP2A and the BCR for proper signal transduction, such as Syk (11), BLNK (39), and Btk (16). However, we cannot formally exclude the possibility that the LMP2A signal induces B-1-like development in a manner that is independent of signals that mimic the BCR. It is also possible that LMP2A simply blocks the B-2 development by acting as a dominant-negative competitor of the BCR signaling. In the latter case, it may be unnecessary for LMP2A to mediate a stronger signal for the B-1 development.

EBV is a ubiquitous human oncogenic herpesvirus associated with a number of proliferative disorders, including Burkitt’s lymphoma, Hodgkin’s disease, immunoblastic lymphoma, oral hairy leukoplakia, and nasopharyngeal carcinoma (2). Besides these known associations, there has been an interest in determining whether EBV may directly or indirectly be involved in other human proliferative disorders. Chronic lymphocytic leukemia (CLL) may be one such example. The surface expression of CLL cells is characterized by expression of CD5 (47) and the production of polyreactive autoantibodies (48). Furthermore, CLL B cells are believed to be the transformed counterparts of normal B-1 cells. Transformation into high-grade large cell non-Hodgkin’s lymphoma (NHL), designated Richter’s syndrome, occurs in ∼3–10% of CLL patients (49, 50). In most cases, the high-grade component is considered to represent a blast transformation of the CLL cells (51). However, in rare cases, the high-grade component includes characteristics of Hodgkin’s disease (52). Although EBV has not been detected in CLL cells, 16% of NHL (53) and 92% of Hodgkin and Reed-Sternberg (H-RS)-like cells (54) are positive for EBV, suggesting that EBV may be involved in the high-grade transformation of CLL. However, it is not known whether the H-RS cells arise from transformation of the underlying CLL cells or from a different pathological process. Nevertheless, in a few of these cases, NHL or H-RS-like cells carry similar somatic mutations when compared with CLL, demonstrating a clonal relationship of CLL with NHL (55, 56) or H-RS-like cells (57–60). Because our data reveal that LMP2A of EBV can skew B cells toward the development of a B-1-like lineage, it may be informative to examine whether EBV is involved in the etiology of CLL and its related diseases.

Although EBV can virtually infect all B cells isolated from human donors and found in all of the B cell subsets, including CD5+ populations in the tonsils where infectious virus is produced, EBV is tightly restricted to long-lived memory B cells (IgD+CD27+CD5−) in the peripheral blood of individuals harboring EBV latent infections (61, 62). However, how EBV gains access to the latent compartment is not yet fully delineated. An intriguing possibility that arises from our results is that bone marrow cells may provide a reservoir for EBV-infected cells in humans. Although human bone marrow B cells may lack expression of CD21, an important coreceptor for EBV infection, bone marrow B cells can be infected as previously shown (63). In addition, EBV can enter B cells in an apparently CD21-independent manner. Recent studies have shown that deletion of gp350/220, the viral ligand for CD21, from the viral genome results in virus that can still infect human B cells (64). Finally, bone marrow transplantation can result in a life-threatening EBV-associated lymphoproliferative disease (65–67). Thus, the presence of EBV in the bone marrow may be associated with the generation of CLL-related malignancies via skewing toward the B-1 lineage during development.

In conclusion, our current studies using LMP2A transgenic mice show that B-1 lineage commitment can occur in bone marrow B cell progenitors. These findings imply that the quantity and quality of BCR-like signals are what drive the B-1, B-2 lineage commitment decision. Furthermore, our results may shed light on a novel association between EBV and some human cancers displaying a B-1 lineage phenotype.

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References


