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Influence of EBV on the Peripheral Blood Memory B Cell Compartment

Tatyana A. Souza,* B. David Stollar,† John L. Sullivan,‡ Katherine Luzuriaga,‡ and David A. Thorley-Lawson2*

Peripheral blood memory B cells latently infected with EBV bear somatic mutations and are typically isotype switched consistent with being classical Ag-selected memory B cells. In this work, we performed a comparative analysis of the expressed Ig genes between large sets of EBV-infected and uninfected peripheral blood B cells, isolated from the same infectious mononucleosis patients, to determine whether differences exist that could reveal the influence of EBV on the production and maintenance of these cells. We observed that EBV+ cells on average accumulated more somatic hypermutations than EBV− cells. In addition, they had more replacement mutations and a higher replacement-silent ratio of mutations in their CDRs. We also found that EBV occupies a skewed niche within the memory compartment, due to its exclusion from the CD27+IgD+IgM+ subset, but this skewing does not affect the overall structure of the compartment. These results indicate that EBV impacts the mutation and selection process of infected cells but that once they enter memory they cannot be distinguished from uninfected cells by host homeostasis mechanisms. The Journal of Immunology, 2007, 179: 3153–3160.

The virus EBV is a γ herpesvirus that maintains lifelong infection in 95% of the adult world population (reviewed in Refs. 1 and 2). It is notorious for its ability to latently infect B lymphocytes in culture and drive their growth and transformation through the expression of nine latent proteins (3). Primary infection with EBV can cause a self-limiting lymphoproliferative disease, infectious mononucleosis (IM),3 in roughly one-third of newly infected adolescents. In some rare cases, EBV infection can lead to death, as observed in patients with X-linked lymphoproliferative disease. EBV is also associated with numerous human B cell neoplasias, as might be expected given its ability to drive cellular growth (1, 2). Nevertheless, in the vast majority of cases, persistent EBV infection does not cause a threat to the host. Therefore, a delicate balance exists between the virus and the host which, if perturbed, can have catastrophic results.

For many years, after the description of growth transformation by EBV, it was assumed that this was the state in which the virus persisted in vivo. However, this idea was overturned by the discovery that, in vivo, EBV persists in resting CD20+CD27+ memory B cells in which the virus is quiescent (4–6). The absence of viral latent protein expression in these cells, which therefore are nonpathogenic, explains why EBV can persist benignly for life in virtually every adult human. This discovery in turn created an apparent contradiction: EBV always expresses latent proteins and drives B cell proliferation in newly infected cells in culture, yet it is able to persist quiescently in resting memory B cells in vivo. A resolution was suggested by studies of viral latent protein expression in vivo (reviewed in Refs. 5 and 7). It was found that the growth-promoting EBV latent proteins are expressed only in infected naive B cells in tonsils from healthy donors, suggesting that these are the initial targets of the virus. Furthermore, in germinal center (GC) B cells, EBV expresses only the latent membrane proteins 1 and 2a (LMP1 and LMP2a) and the viral episome-tethering protein, EBV nuclear Ag 1. LMP1 and LMP2a have been shown, either in vitro or in transgenic mice, to potentially be capable of providing the necessary signals, in the absence of Ag, to allow a latently infected B cell to enter a follicle (8), initiate somatic hypermutation (SHM; Ref. 8) and class switching (CSR; Ref. 9), characteristics of the GC reaction, receive appropriate survival/rescue signals (10, 11) and leave as a memory cell (12). This led to the model that EBV infects naive B cells in the tonsils and activates them so that they can differentiate through a GC, using LMP1 and LMP2a signaling, to become resting memory B cells (5). Thus, growth transformation is a transient state that the virus induces to gain access through the GC to the final site for latent persistent infection, the resting memory B cell. This mechanism closely mimics the normal differentiation of Ag-activated naive B cells into the memory compartment.

In the model as originally proposed, it was assumed that, given that LMP1 and LMP2a theoretically have the capacity to initiate and complete the GC reaction, they would do so. This meant that the cells would not undergo the normal process of Ag selection and could express aberrant or nonfunctional Isgs. Such a conclusion was reinforced by studies in transgenic mice confirming that not only could LMP2a rescue autoreactive B cells (13) but it could even allow the survival of B cells that completely lacked surface Ig expression (10). However, when the expressed Ig genes of latently...
infected memory B cells from peripheral blood (PB) were analyzed, they showed all the hallmarks of SHM expected for cells that had undergone classical Ag selection (14). This created a new question: why would the virus encode for latent proteins that can usurp the GC process if ultimately it persists in memory cells that have undergone a normal Ag-driven selection process? A simplistic explanation might be that EBV infects memory cells directly as proposed by Kurth et al. (15), but this idea does not explain why EBV possesses the proteins LMP1 and LMP2a that are expressed in latently infected cells with a GC phenotype in vivo and have the ability to provide GC-specific signals. We predicted that if EBV-infected cells truly transit the GC and the viral latent proteins promote this Ag-driven process, then the EBV+ memory B cells should bear some viral induced hallmarks. Additionally, because the EBV+ cells resemble typical memory B cells, it is unclear how these cells impact the structure of the whole memory B cell compartment, especially in IM patients whose frequency of infected cells in PB can be as high as 1 in 2 memory cells. Subtle effects of viral protein signaling on EBV+ memory B cells and the subsequent impact of these cells on the memory compartment might not have been detected in the limited data set we analyzed previously (14) but could be revealed through a detailed comparative analysis of a much larger data set generated from single EBV+ and EBV− memory B cells isolated from the same donors. In the current study, we have performed such an analysis.

Materials and Methods

Primary B cells

Primary B cells were obtained from IM patients as described previously (16). PB mononuclear cells (2 × 10⁷ cells/ml) were stained with anti-human CD20 PE (BD Pharmingen) or anti-human CD19 Cy (DAKO), anti-human IgD PE (BD Pharmingen), and anti-human CD27 FITC (BD Pharmingen). Single-memory (CD20+/CD27− or CD19+/CD27− IgD−) marginal zone (CD19+/CD27− IgD+), or naive (CD19+/CD27− IgD+) B cells were sorted with a Cytomation MoFlo FACS into 10 µl of 1 × first-strand buffer (In Vitrogen Life Technologies) in 96-well plates, immediately frozen on dry ice, and stored at −80°C. All studies were approved by the Human Studies Committee at the University of Massachusetts and Tufts University Medical Schools.

Limiting dilution analysis

Limiting dilution analysis was used to determine the frequency of EBV-infected cells in isolated PB populations as described (16).

cDNA synthesis

Single-cell cDNA synthesis was performed according to the protocol of Wang and Stollar (17), with the exception of adding 5 pmol of EBV-encoded mRNA 1 (EBER1) RNA-specific primer (AGGACCTACGCT GCCTCTAGA) and 5 pmol of Ig Cα RNA-specific primer (GAGGCT CAGCGGGAAGAC) to the primer mixture already containing specific primers to the Cμ, Cγ, Cκ, Cα Ig constant chain regions. Eight wells containing all buffers from the time of sorting, minus the single cells, served as negative controls.

Single-cell PCR analysis of amplified Ig V region genes

EBER1 PCR, performed as described (16), was used to detect infected PB cells. Ig gene RT-PCR was performed according to the protocol of Wang and Stollar (17) with modifications (14). Ig V region PCR products were excised from agarose gels and DNA extracted using the QIAquick gel extraction kit (Qiagen) and the DNA was sequenced by Tufts University Core Facility with corresponding constant region primers. Sequences determined in this study can be found in the GenBank database under accession numbers DQ205136–DQ205184 and DQ206025–DQ206654. Sequences were aligned using the VBase database (http://vhbase.mrc-cpe.cam.ac.uk/) and the IMGT database (the international ImMunoGeneTics information system; http://imgt.cines.fr ref) which contain all the known human Ig genes. We analyzed 294 bps (framework regions 1 through 3) from each Ig Vγ region sequence for frequency and characteristics of the mutations. Mutations in the primer-binding regions were disregarded. Only base substitutions were counted. The probability that an excess or scarcity of replacement (R) mutations in CDRs or framework regions (FWR) was due to chance alone was calculated using the multinomial distribution model of Lossos (18).

Results

EBV occupies a skewed niche in the memory B cell compartment but does not affect its overall structure

To determine how EBV-infected cells impact the structure of the PB memory B cell compartment, we analyzed and compared large sets of EBV+ and EBV− memory B cells from three IM patients with large fractions of their memory B cell pool infected with EBV (20–50%). Single PB memory B cells (CD20+/CD27+; Ref. 19) were sorted by flow cytometry, and cDNA was prepared from each cell. The presence or absence of EBV was confirmed by real-time PCR analysis for EBER1(16) and the expressed VH regions were then amplified from the cDNA of single EBER1+ and EBER1− cells (14). Using Ig gene RT-PCR for the three prevalent isotypes, Igα, IgG and IgM, we examined the isotype distribution between the EBV+ and EBV− memory B cell subcompartments. Typically, the PB memory B cell compartment, as defined by CD27 expression, consists of 50–60% IgM+ B cells and 40–50% IgG+ plus IgA+ B cells (19, 20). However, the isotype usage by EBV+ memory B cells revealed that they were highly skewed against the IgM isotype with only 13% of the cells expressing IgM (Fig. 1, IM1–3 combined; first column). More interesting, however, was the finding that the EBV+ population demonstrated a complementary skewing in favor of IgM+ cells (Fig. 1, IM1–3 combined, center column) such that when the CD27+ compartment was reconstituted, by recombining the EBV+ and EBV− subcompartments, the distribution of the isotypes was normal with 54% of the cells bearing IgM and 46% of the cells bearing IgG or IgA isotypes (Fig. 1, IM1–3 combined, right column). Similarly, each IM patient analyzed exhibited these same trends but with more variability, as expected, for each individual (Fig. 1, IM1, IM2, and IM3). These results lead to the conclusion that EBV+ memory cells reside in a selective niche of the memory B cell compartment but the overall structure of the compartment, in terms of isotype usage, is not affected by the skewing within the EBV+ subcompartment. These results provide experimental support for the prediction (6) that these cells are perceived as normal by the mechanisms that regulate memory B cell homeostasis.

EBV is preferentially excluded from CD27+ IgD+ IgM+ B cells during acute infection

The CD27+ PB memory B cell compartment consists of IgG and IgA isotype-switched GC-derived memory B cells as well as unswitched IgM-only B cells, which some have proposed to also originate in the GC (21). In addition, the CD27+ PB B cell compartment also consists of a population of cells which have been suggested to represent the circulating counterparts of splenic marginal zone cells (19, 20). These express IgD and IgM, occupy up to 50% of the CD27+ memory B cell compartment, and have been suggested to originate independently of GCs (21). The exclusion of EBV from these cells has been demonstrated in healthy carriers of the virus (4) and likely also occurs in acute infection given that EBV is preferentially enriched in IgD− cells and CD27− cells (16). However, the double-sorted population has never been directly tested during acute infection, where extremely high levels of latently infected memory cells are present (20–50% compared with 0.001–0.0001% in healthy carriers). Therefore, we checked whether exclusion of EBV from the CD27+ IgD+ B cells during acute infection could account for the low frequency of EBV+ memory cells bearing the IgM isotype, due to the observation that the majority of CD27+ IgD+ PB cells coexpress IgM+ (>90%; Ref. 19) and comprise the majority of the IgM+ memory B cell
EBV* cells are preferentially excluded from B cells of the IgM isotype, whereas EBV− cells are skewed toward B cells of the IgM isotype in the CD27+ memory B cell pool. Ig gene RT-PCR was performed for the three prevalent C\textsubscript{H} chains (\(\mu\), \(\alpha\), and \(\gamma\)) on cell lysates from single EBV+ (left column) and EBV− (middle column) CD27+ memory B cells isolated from three IM patients. The percent of single cells expressing each isotype was calculated for each population. Right columns, enumeration of the isotype use of the whole CD27+ compartment calculated by combining the results for the EBV+ and EBV− subsets taking into account the relative proportions of each based on the measured frequencies of EBV-infected cells within the compartment. Data on the far right represent the average from three IM patients. The \(\chi^2\) test between the EBV+ and the whole IM compartment had a significant \(p\) value of <0.001.

**FIGURE 2.** EBV+ cells are excluded from the CD27+IgD− B cells. A, PB cells from IM patients were stained for CD19, IgD, and CD27 expression. CD19 B cells were gated, and either whole CD27+ or CD27− IgD+ or CD27− IgD− B cell subpopulations were sorted. B, Limiting dilution analysis using EBER1 was performed on each population for each patient, when possible. The frequency of EBV-infected cells is shown per 1000 cells of each B cell population.

**Table I.** Frequency of EBV+ cells per 1000 CD19+ B cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD27+IgD+IgM+ Fraction</th>
<th>CD27+IgD− Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM1</td>
<td>316</td>
<td>385</td>
</tr>
<tr>
<td>IM2</td>
<td>357</td>
<td>370</td>
</tr>
<tr>
<td>IM3</td>
<td>455</td>
<td>333</td>
</tr>
</tbody>
</table>

\(p = 0.8\) by paired \(t\) test.
ory B cells, while the EBV which was correct. Therefore, we analyzed the SHM frequency in and the IgD/H11001 pattern of the SHMs in the expressed Ig genes of a large data set of the CD27/CD19/H11001 compartment in the IM patients. Fig. 1 are consistent with EBV delineating the IgM-only subset of the CD27+ B cell compartment in the IM patients.

EBV+ memory B cells have more somatic mutations than their EBV− counterparts

To determine whether the presence of EBV impacted the EBV+ memory B cell pool, we analyzed and compared the level and pattern of the SHMs in the expressed Ig genes of a large data set generated from single EBV+ and EBV− PB memory B cells from three IM patients. By comparing matched populations from the same donors, we could ensure that any differences we observed were due to the presence of the virus. However, we have shown above that the EBV+ memory pool includes only the IgD− memory B cells, while the EBV− memory pool contains both the IgD− and the IgD+ memory B cells, and a discrepancy exists in the literature over the amount of SHMs found in the Vh genes of circulating CD27+ IgD− IgM+ B cells. Weill’s group (20) has reported that the CD27+IgD+IgM+ putative marginal zone B cells carry fewer mutations than the GC-derived memory cells, whereas Rajewsky’s group (19) reported no difference. Before comparing the infected and uninfected populations, it was important to resolve which was correct. Therefore, we analyzed the SHM frequency in the Vh regions of single CD27+ IgD+ IgM+ B cells and compared them with those of the CD27+ IgD− memory B cells bearing the IgM, IgG, or IgA isotypes. The analysis revealed that the CD27+ IgD+ IgM+ B cells did, indeed, accumulate one-half to one-third of the SHMs found in the IgD− memory B cells (Fig. 3). CD27+ IgD− cells of the IgM isotype accrued, on average, 4.8 mutations per 100 bp in the IgVh regions compared with the 2.2 mutations per 100 bp in the CD27+ IgD+ IgM+ B cells (p < 0.001, Student’s t test, two-tailed). CD27+ B cells of the IgG or IgA isotypes, on average, acquired 6 mutations per 100 bp in their Vh genes. This level of SHMs was significantly higher than that of either the IgM only (p = 0.002, Student’s t test, two-tailed) or the IgD+IgM+ (p < 0.001, Student’s t test, two-tailed) memory B cell populations confirming the observations of Weill et al.

Therefore, to avoid any bias in our analysis, due to the CD27+ IgD+ IgM+ carrying less SHMs and being solely in the EBV− subcompartment, we examined the effects of viral protein signaling by comparing only the IgD+ EBV+ and -EBV− memory B cell populations. EBER1− memory B cells were identified as before, and the expressed Vh regions were amplified (14). The resultant products were sequenced, and mutations were identified by comparison with published germline sequences. Single cells of the IgM isotype were compared only when isolated from the CD19+CD27− IgD− fraction, whereas single cells of the IgG and IgA isotypes were analyzed from the CD20+CD27+ fraction. The Vh genes isolated from EBV+ cells were all mutated and contained no aberrant mutations but had, in fact, acquired significantly (~15%) more SHMs than the uninfected cells, containing an average of 6 mutations per 100 bp, whereas EBV− cells had an average of 5.2 mutations per 100 bp (p = 0.02, one-tailed t test; Table II). Similarly, when compared by isotype, the EBV+ cells acquired significantly more SHMs than the EBV− cells (Table III; p = 0.04, one-tailed, paired-sample t test). Thus, memory B cells carrying latent EBV accumulate more SHMs in their Vh genes than their uninfected counterparts.

To confirm the findings above, we also compared the SHM frequency between the whole CD27+ EBV+ and -EBV− subpopulations without excluding the IgD+ cells. If the IgD+ IgM+ putative marginal zone memory cells were present only in the EBV+ subcompartment, then the SHM frequency of the whole EBV− CD27+ population should be even further reduced than that of the EBV+ population. This was indeed the case with the overall frequency of Vh gene SHMs in the EBV+ CD27+ B cell population.

<table>
<thead>
<tr>
<th>No. of Sequences Analyzeda</th>
<th>Mutation Frequency/100 bp (mean; median)</th>
<th>R Mutations in CDRsa</th>
<th>R/S CDR</th>
<th>R/S FWR</th>
<th>% Selected by Multinomialb</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM1 EBV+</td>
<td>184</td>
<td>5.9; 5.8</td>
<td>5.4</td>
<td>3.3</td>
<td>1.9</td>
</tr>
<tr>
<td>IM1 EBV−</td>
<td>54</td>
<td>5.2; 4.6</td>
<td>5.1</td>
<td>3.7</td>
<td>2.3</td>
</tr>
<tr>
<td>IM2 EBV+</td>
<td>37</td>
<td>6.7; 6.1</td>
<td>5.4</td>
<td>3.6</td>
<td>1.6</td>
</tr>
<tr>
<td>IM2 EBV−</td>
<td>16</td>
<td>4.4; 4.6</td>
<td>5.0</td>
<td>3.2</td>
<td>1.8</td>
</tr>
<tr>
<td>IM3 EBV+</td>
<td>46</td>
<td>5.9; 5.4</td>
<td>5.9</td>
<td>3.8</td>
<td>1.8</td>
</tr>
<tr>
<td>IM3 EBV−</td>
<td>46</td>
<td>5.5; 4.8</td>
<td>4.1</td>
<td>2.2</td>
<td>1.7</td>
</tr>
<tr>
<td>EBV+</td>
<td>267</td>
<td>6.0 (5.9)c</td>
<td>5.5</td>
<td>3.6</td>
<td>1.8</td>
</tr>
<tr>
<td>EBV−</td>
<td>116</td>
<td>5.2 (4.2)c</td>
<td>4.9</td>
<td>3.0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

aData were compiled from each IM patient and for three IM patients combined. Single cells without SHMs were not included in the analysis. Except where indicated, the IgD+ cells were not included.

bMean number of replacement (R) mutations in CDR1 and CDR2 regions. p = 0.04 by one-tailed t test for EBV+ vs EBV−.

cPercent of cells having significantly less R mutations in FWRs (p < 0.05) and significantly more R mutations in CDRs (p < 0.05) than chance alone, calculated using the multinomial distribution (18). p < 0.001 by binomial distribution analysis for EBV+ vs EBV−.

dThe values in parentheses include the IgD+ cells, p < 0.001 for EBV+ vs EBV−; p = 0.02 for EBV+ vs EBV− when excluding the IgD+ cells (one-tailed t test).
In patients with acute infection, up to one-half of their memory B cells may be carrying the virus (16). We therefore investigated whether this was achieved through the displacement of extant memory cells or by transiently expanding the memory compartment, which could accommodate the large number of infected memory cells. To this end, we measured the number of memory B cells per milliliter of blood as IM resolves. As seen in Table V, the number of GC-derived memory B cells per milliliter of blood was 1.5- to 4.5-fold elevated at the time of presentation but had already fallen and leveled off 1 wk later. The EBV-infected memory cells behaved similarly, demonstrating a very rapid fall in absolute numbers during the first week. However, as we have shown previously, the infected cells differ in that they continue to decline, relative to the uninfected cells, for up to 1 yr, although the decrease in the memory B cell compartment increases during acute infection.

Table IV.  Frequency of EBV\(^+\) and EBV CD27\(^+\)IgD\(^−\) B cells fitting the criteria for Ag selection based on \(V_H\) gene usage

<table>
<thead>
<tr>
<th>(V_H) gene (a)</th>
<th>EBV(^+)</th>
<th>EBV(^−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{H1})</td>
<td>Ratio (b)</td>
<td>%</td>
</tr>
<tr>
<td>VH1–18</td>
<td>7/15</td>
<td>54</td>
</tr>
<tr>
<td>VH1–46</td>
<td>7/10</td>
<td>70</td>
</tr>
<tr>
<td>VH2–5</td>
<td>3/8</td>
<td>38</td>
</tr>
<tr>
<td>VH3–11</td>
<td>6/13</td>
<td>46</td>
</tr>
<tr>
<td>VH3–15</td>
<td>1/6</td>
<td>17</td>
</tr>
<tr>
<td>VH3–23</td>
<td>10/27</td>
<td>37</td>
</tr>
<tr>
<td>VH3–30</td>
<td>3/10</td>
<td>30</td>
</tr>
<tr>
<td>VH3–33</td>
<td>5/11</td>
<td>45</td>
</tr>
<tr>
<td>VH3–48</td>
<td>4/8</td>
<td>50</td>
</tr>
<tr>
<td>VH3–7</td>
<td>4/10</td>
<td>40</td>
</tr>
<tr>
<td>VH4–34</td>
<td>0/6</td>
<td>0</td>
</tr>
<tr>
<td>VH4–59</td>
<td>1/14</td>
<td>7</td>
</tr>
<tr>
<td>VH5–51</td>
<td>6/28</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>57/164</td>
<td>35(%)</td>
</tr>
</tbody>
</table>

\(a\) EBV\(^+\) and EBV\(^−\) \(V_H\) sequences from three IM patients were analyzed based on \(V_H\) gene usage. \(V_H\) gene groups that had four or more cells in each population were analyzed. \(V_H\) gene nomenclature is according to V-Base.

\(b\) Shown are the number of cells having significantly less R mutations in FWRs \((p < 0.05)\) and significantly more R mutations in CDRs \((p < 0.05)\) than chance alone, calculated using the binomial distribution (18), divided by the total number of cells that carried the given \(V_H\) gene.

\(c\) \(p = 0.02\) by one-tailed, paired \(t\) test. Binomial distribution analysis of totals 57 of 164 (35\%) vs 20 of 84 (24\%); \(p < 0.001\).

The memory B cell compartment increases during acute infection.

We have previously shown that the EBV\(^+\) memory B cells have patterns of mutations consistent with having undergone Ag selection (14). Through the accumulation of a large data set, comparing EBV\(^+\) and EBV\(^−\) cells, we have now shown that EBV\(^+\) cells accumulate more mutations. To further assess the impact of EBV, we have used this data set to see whether the patterns of mutation accumulation in the CDRs can increase the specificity of the Ig receptor (S) mutations in the FWRs or in CDRs. Because replacement mutations in the CDRs can increase the specificity of the Ig receptor for its Ag, they proposed that an R:S ratio higher than ~3 in the CDRs is indicative of positive Ag selection. Conversely, preservation of the Ig protein fold is necessary in the FWRs; therefore, the R:S ratio is usually ~1.5. Applying these criteria, we found (Table II) that EBV\(^+\) memory B cells had significantly more (~12\%) R mutations in their CDRs than did their EBV\(^−\) counterparts \((p = 0.043\), one-tailed \(t\) test). EBV\(^+\) memory B cells also had, on average, a higher R:S ratio in the CDRs than the uninfected cells (3.6 vs 3.0), whereas the R:S ratios in the FWRs were essentially identical (1.9 vs 1.8), suggesting that a proper Ig fold was necessary for the EBV\(^+\) cells to survive.

Lossos et al. (18) later proposed a more stringent test of SHM patterns for evidence of Ag selection, using the multinomial distribution model and taking into consideration the intrinsic capacity of CDR codons to produce R mutations (23). When this analysis was applied to the EBV\(^−\) GC-derived memory B cell population, which should have undergone Ag selection (Table II), it was observed that 20\% of the cells met the criteria for Ag selection. By comparison, significantly more, 33\%, of the EBV\(^+\) population met the criteria (binomial distribution analysis \(p < 0.001\)). We also compared the proportion of cells in the EBV\(^+\) and EBV\(^−\) populations meeting the criteria when subdivided based on \(V_H\) gene usage. As shown in Table IV, again significantly more EBV\(^+\) cells meet the criteria for Ag selection than their EBV\(^−\) counterparts do (one-tailed paired \(t\) test, \(p = 0.02\)). Therefore, by the criteria of Schlomchik et al. and Lossos et al., the EBV\(^+\) cells have accumulated more mutations of the type and pattern expected of an Ag-selected cell.

Table III.  Mean number of mutations per 100 bp in EBV\(^+\) or EBV CD27\(^−\) IgD\(^−\) B cells of each isotype

<table>
<thead>
<tr>
<th>Isotype (a)</th>
<th>EBV(^+)</th>
<th>EBV(^−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>5.1 (5.4)(c)</td>
<td>4.4 (3.9)</td>
</tr>
<tr>
<td>IgG</td>
<td>6.0 (5.4)</td>
<td>5.8 (5.1)</td>
</tr>
<tr>
<td>IgA</td>
<td>6.2 (5.8)</td>
<td>5.5 (5.1)</td>
</tr>
</tbody>
</table>

\(a\) Data were compiled from three IM patients. Single IgG\(^+\) and IgA\(^+\) cells were identified by H chain-specific PCR on single CD20\(^−\)CD27\(^−\) sorted by flow cytometry. Single IgM-only cells were identified by H chain-specific PCR on single CD19\(^+\)IgD\(^−\)CD27\(^−\) cells.

\(b\) One-tailed paired sample \(t\) test was performed to compare the mean numbers of mutations for each isotype between the EBV\(^+\) and EBV\(^−\) populations; \(p = 0.04\).

\(c\) Numbers in parentheses, median.

(4.2 mutations per 100 bps) being 33\% lower than in the EBV\(^+\) population (5.9 mutations per 100 bps) \((p < 0.001\), Student’s \(t\) test, one-tailed). This confirms that IgD\(^+\)IgM\(^−\) putative marginal zone memory cells are largely restricted to the EBV\(^−\) subpopulation and that the EBV\(^+\) subpopulation is mainly composed of isotype-switched and IgM-only memory B cells, which acquire more SHMs.

EBV\(^+\) memory B cells display patterns of SHM similar to those of their EBV\(^−\) counterparts but with more R mutations and higher R:S in their CDRs.

In patients with acute infection, up to one-half of their memory B cells may be carrying the virus (16). We therefore investigated whether this was achieved through the displacement of extant memory cells or by transiently expanding the memory compartment, which could accommodate the large number of infected memory cells. To this end, we measured the number of memory B cells per milliliter of blood as IM resolves. As seen in Table V, the number of GC-derived memory B cells per milliliter of blood was 1.5- to 4.5-fold elevated at the time of presentation but had already fallen and leveled off 1 wk later. The EBV-infected memory cells behaved similarly, demonstrating a very rapid fall in absolute numbers during the first week. However, as we have shown previously, the infected cells differ in that they continue to decline, relative to the uninfected cells, for up to 1 yr, although the decrease in...
absolute numbers is extremely small compared with the first week (16). We conclude that the memory B cell compartment increases during acute infection and can thus accommodate the newly generated EBV\(^+\) memory B cells. These observations are also consistent with the conclusion that the EBV\(^+\) cells are subject to the same homeostasis that regulates the size of the entire memory compartment.

**Discussion**

The goal of this study was to produce and analyze a large data set of expressed Ig sequences from matched EBV\(^+\) and EBV\(^-\) memory cells that could then be used to identify the impact of EBV on the production of latently infected B cells and to analyze the effect these cells have on the whole-memory B cell compartment. Cells were isolated from patients experiencing acute infection, during which EBV can occupy up to 50% of the memory B cell compartment, and its impact can thus be more easily assessed. On the basis of the isotype use, we conclude that EBV-carrying memory cells occupy a skewed niche within the whole CD27\(^+\) B cell compartment. However, this did not affect the overall structure of the compartment, given that the EBV\(^-\) subcompartment compensates for the skewing created by the EBV\(^+\) cells. As a consequence, these studies provide the first direct evidence that EBV\(^+\) memory cells are not perceived as different by the host and are maintained by homeostasis mechanisms as a part of the entire memory B cell pool. This was originally predicted previously based on the observation that all viral protein expression is turned off in these cells and that they divide at the same rate as uninfected memory cells (6). Further support for this idea comes from the kinetic studies showing that the EBV-infected memory B cell numbers contract rapidly with the whole compartment, during the first week. This result must be interpreted with caution, however, because we do not know to what extent levels of cells in the periphery represent what is happening in the tissue or what the relative contributions of immune surveillance and viral reactivation may be to the loss of the latently infected cells.

We have confirmed our previous finding that, even under conditions of acute infection, EBV preferentially resides in GC-derived (IgD\(^-\)CD27\(^+\)) over the putative marginal zone (IgM\(^+\) IgD\(^+\)CD27\(^+\)) memory cells. It was possible that EBV infects the IgM\(^+\)IgD\(^+\)CD27\(^+\) B cells and down-regulates surface IgD expression as has been observed in vitro (24). However, IgM down-regulation has not been observed in vitro; therefore, if down-regulation of IgD occurred in vivo, we should see normal numbers of IgM-bearing cells in the EBV\(^+\) compartment, but we do not. EBV-infected cells were highly skewed against both IgM and IgD.

It has been suggested that IgM\(^+\)IgD\(^-\)CD27\(^+\) cells are the circulating counterparts of splenic marginal zone B cells and that they arise independently of the GC (20). This proposal, taken together with our observation that EBV is preferentially excluded from these cells, is consistent with our model that EBV-infected cells must traverse the GC to establish long-term persistence in memory B cells. Interestingly, transient persistence of EBV has been observed in the putative circulating marginal zone cells from a patient lacking GC-derived memory cells (25). This suggests that EBV can enter, but not persist in, marginal zone cells explaining the low but possibly significant levels of virus we have detected in this population during acute infection.

Although EBV-infected cells do not appreciably impact the structure of the memory compartment, analysis of our large data set shows a clear impact of EBV on the infected cells themselves. Thus, EBV\(^+\) memory cells have significantly more SHMs and specifically more R mutations with a higher R/S ratio in the CDRs, and a higher fraction of them meet the criteria of the multinomial test. Although the statistical significance of these differences is not very robust, nevertheless these differences are real, significant, and observed within each individual. This is most likely due to the fact that the difference itself is small in value; hence, a large N is needed from the three patients to improve the statistical power. The meaning of these differences is open to interpretation. Traditional statistical analyses would suggest that the EBV\(^+\) cells have undergone Ag selection (18, 22). In this case, the differences might imply that the EBV\(^+\) cells have undergone more stringent selection or more rounds of proliferation and selection. This in turn implies that the EBV latent membrane proteins augment cognate Ag signaling rather than drive the whole process. It is difficult to conceive how this augmentation could result in more stringent selection; however, it could lead to more rounds of expansion and survival as suggested by an LMP2a/hen egg lysosome transgenic mouse model (25). Because LMP2a alone cannot drive proliferation, Ag recognition would be necessary to initiate the GC reaction; then LMP2a could cooperate with Ag to allow the survival of latently infected B cells in the GC even if they generated a suboptimal BCR signal (10, 13, 26). Alternatively, LMP1 is known to provide a constitutive T\(_{\text{h}1}\) signal, and it is known that extended Th signaling preferentially drives GC B cells to become memory rather than plasma cells (27). Plasma cell differentiation is known to signal the reactivation of the virus (28), which would prevent the establishment of latent persistence. Therefore, the role of LMP1 could be to ensure that latently infected GC cells are directed away from plasma cell differentiation and driven into memory. Thus, the combination of Ag, LMP2a, and LMP1 signaling could provide EBV with access to Ag-selected GC cells while also giving both an advantage in the GC and a predisposition to become memory cells.

The statistical analysis of SHMs to predict Ag selection has been challenged recently (29). Shapiro et al. have shown that codon positions 1 and 2 of the CDRs and position 3 of the FWRs have high rates of mutations due to the actions of the mutation machinery, which would yield high numbers of R mutations in the CDRs and of S mutations in the FWRs (30). This led Bose and Sinha (29) to argue that a high number of R mutations in the CDRs does not necessarily result from positive Ag selection but could reflect the codon composition and the intrinsic bias of the mutation machinery. In addition, too many R mutations in the CDRs could actually be disadvantageous and lead to loss of Ag binding (31). Therefore, it could be argued that the EBV\(^+\) cells have been allowed to acquire more R mutations in their CDRs because they have not undergone stringent Ag selection and mutations have simply accumulated in regions of high mutability. In this case, the latent membrane proteins could be driving the whole GC process consistently with their known properties (8–12). Although this could result in the production of memory B cells with no surface Ig, such cells would be deleted as soon as LMP2a expression is turned off when the cells enter the periphery (6). This is different from the results with LMP2a-transgenic mice where constitutive expression allows such cells to artifically survive in the periphery.

Our studies therefore provide a cautionary note with respect to interpreting experiments performed with transgenic mice expressing single EBV latent proteins, especially from constitutive promoters. In humans, the pathogenic potential of EBV latent proteins is tightly controlled such that to all intents and purposes the latently infected memory B cells during acute infection or in healthy carriers appear to the host as normal. By comparison, constitutive expression of LMP1 in transgenic mice blocks germinal center formation and leads to lymphoma (32), whereas constitutive LMP2a expression allows for the survival and expansion of surface Ig- and autoantibody B cells (10, 13, 26). Because none of these phenomena is observed in human carriers of the virus, these
results most likely constitute artifacts, with respect to the mechanism of EBV persistence, created by constitutive expression of an isolated latent gene rather than the regulated expression in the context of the whole virus that occurs in vivo. Such studies may be more relevant to the relatively rare occasion when EBV infection leads to pathogenesis and cancer due to constitutive and/or inappropriate expression of its latent proteins.

The ability of EBV to efficiently drive latently infected cells into memory explains why, during the acute phase of the disease, EBV + cells enter the memory compartment in such large numbers that up to 50% may be latently infected (16). We have shown that the size of the peripheral memory B cell compartment increases during acute infection and is thus able to accommodate the large numbers of newly generated EBV + memory cells without displacing the pre-existing memory repertoire. This keeps the host safe from reinfections that could arise had the repertoire been displaced by the EBV + cells. Whether this acute expansion of the memory compartment is simply part of the immune response to a virus infection or whether EBV actively stimulates the expansion is not known.

Kurth et al. (15, 33) have proposed an alternative to the GC model whereby EBV enters the PB memory compartment by direct infection and expansion of memory B cells. This was based on their observations that expanding populations in the GCs of tonsils from IM patients were driven by EBV, not by a GC reaction. Their model has several weaknesses. Most notable was that the cells were identified as GC solely on the basis of location. The expression of GC-specific markers and the restricted form of latency associated with infected GC cells were either not tested or not detected, and controls were not performed to exclude the possibility that rare cells expressing these properties had been missed. Their model also does not explain why EBV would have preference for persisting in memory over naive B cells, because it can infect both, or why EBV encodes latent proteins, LMP1 and LMP2a, which provide GC functions including the ability of LMP2a to drive B cells to enter mucosal lymphoid tissue and initiate GCs in the absence of cognate Ag (8). It is also difficult to reconcile their model with our current data that EBV occupies a skewed niche within the memory compartment and that EBV-infected cells accumulate more SHMs. The expanding memory B cells infected with EBV they detected in the tonsils of IM patients did not accumulate any further mutations (33). To accommodate these findings, it would be necessary to propose that EBV specifically targets a skewed subset of memory B cells that had already acquired more somatic mutations.

The key to the correct interpretation of the Kurth et al. studies is that by the time the patients present with clinical symptoms, the number of infected B cells in the PB is already decaying exponentially, virus shedding has plateaued (16, 34, 35) and cells replicating the virus are extremely rare (36, 37), suggesting that the majority of the infection and expansion into the memory compartment is already over. In the tonsils of IM patients, the disruption of the tonsil architecture, visible in the analyzed tonsil sections (33), either allows the direct infection of bystander cells such as GC B cells or the invasion of GCs by extracellular EBV-transformed lesions. These infected cells cannot differentiate out of the virally driven cell cycle and thus continue to expand until the CTL response deletes them. Thus, when patients present with symptoms, it is evident why these proliferating clones are observed as the dominant population of infected cells in the tonsils. As predicted by this scenario, in tonsils analyzed from patients 2 wk after symptom presentation, the number of EBV expanding clones in IM tonsils had significantly dropped (15). This was probably due to the CTL response, and at this time one clone that resembled a GC founder undergoing intraclonal diversification was observed.

In conclusion, we have shown that EBV + cells in the PB inhabit a skewed subpopulation within the normal memory compartment and that viral latent proteins impact the SHM process. The notion that EBV uses the GC reaction to convert newly infected lymphoblasts into memory cells was originally proposed to account for the fact that EBV was a promiscuous activating and transforming virus in vitro yet persisted in resting memory cells in vivo. The current studies raise questions about whether Ag plays a role in the production of these cells and, if so, the identity of the Ag and how it might interact with viral latent protein signaling.

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