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# EBV and Systemic Lupus Erythematosus: A New Perspective<sup>1</sup>

Andrew J. Gross,<sup>2\*</sup> Donna Hochberg,<sup>†</sup> William M. Rand,<sup>‡</sup> and David A. Thorley-Lawson<sup>3†</sup>

**We have proposed that EBV uses mature B cell biology to access memory B cells as a site of persistent infection. A central feature of this model is that EBV adapts its gene expression profile to the state of the B cell it resides in and that the level of infection is stable over time. This led us to question whether changes in the behavior or regulation of mature B cells would alter the state of EBV persistence. To investigate this, we studied the impact of systemic lupus erythematosus (SLE), a disease characterized by immune dysfunction, on EBV infection. We show that patients with SLE have abnormally high frequencies of EBV-infected cells in their blood, and this is associated with the occurrence of SLE disease flares. Although patients with SLE have frequencies of infected cells comparable to those seen in immunosuppressed patients, in SLE the effect was independent of immunosuppressive therapy. Aberrant expression of viral lytic (BZLF1) and latency (latency membrane proteins 1 and 2a) genes was also detected in the blood of SLE patients. We conclude that the abnormal regulation of EBV infection in SLE patients reflects the sensitivity of the virus to perturbation of the immune system. *The Journal of Immunology*, 2005, 174: 6599–6607.**

**T**he immune system is intimately involved in the regulation of EBV. As is the case for most viral infections, cell-mediated immunity by CD4 and CD8 cells is essential for controlling the proliferation of cells newly infected with EBV and in limiting viral replication (1–3). However, EBV regulation must be maintained by other mechanisms as well, because T cell immunosurveillance cannot regulate the latently infected memory B cells, which do not express T cell targets (1, 4).

We have proposed that a more subtle level of EBV regulation comes from its interaction with the B cell in which it resides (5, 6). This is evident from our previous studies in which we have shown that defined patterns of EBV gene expression are associated with specific populations of mature B cells. In the tonsils of healthy individuals, naive B cells, which are thought to be newly infected, express all nine known latency proteins (Epstein-Barr nuclear Ag 1 (EBNA1),<sup>4</sup> EBNA2, EBNA3a, EBNA3b, EBNA3c, and LP and latent membrane protein (LMP)1, LMP2a, and LMP2b) (7, 8); infected germinal center and memory B cells express a limited set of three latency proteins (LMP1, LMP2a, and EBNA1(Q-K)) (8, 9); and infected memory B cells in the blood cease expression of all viral proteins, with the exception that they express EBNA1

when they divide (4). Viral replication occurs in tonsillar plasma cells, resulting in expression of the lytic genes including BZLF1 (10). These findings have led us to propose a comprehensive model of how EBV uses mature B cell biology to establish and maintain persistent infection (5, 6). The implication of this line of thinking is that the biology of the virus is intimately interwoven with and responsive to mature B cell biology. Because the virus is sensitive to the state of the B cell it infects, changes in the behavior or regulation of mature B cells may be reflected in changes in the state of EBV persistence.

To pursue whether EBV regulation can be affected by B cell dysfunction, we investigated EBV infection in patients with systemic lupus erythematosus (SLE). SLE is considered a prototypical systemic autoimmune disease (SAID) because fundamental defects in immune function result in damage to multiple organ systems. Central to the immunological dysfunction in SLE are abnormalities in T and B lymphocytes, which result in the production of autoantibodies to a variety of nuclear Ags, ultimately leading to organ damage from immune complex deposition (for general review, see Ref. 11). A variety of defects in B cell function have been described in SLE patients, which could potentially impact EBV infection, including the presence of unusual B cell subsets, expression of activation markers, and perturbation of intracellular signaling (12–17). In addition, defects in CTL function have also been described in SLE, which could affect EBV persistence as well (18). Therefore, if any disease is likely to have an effect on EBV, it is SLE.

Previously, it has been reported that EBV infection is deregulated in patients with SLE, because viral loads were found to be elevated in the blood (19, 20) and saliva (20, 21). In those studies, viral load was defined as the total genome copy number and did not distinguish between genomes in virions and genomes in latently infected cells. This is a critical distinction because latently infected cells express very low numbers of viral genomes per cell (two to five copies), whereas a single cell replicating the virus and producing virions contains thousands of genomes. A large increase in the viral load in the blood could therefore be due to a large increase in the frequency of latently infected cells or a very small increase in the fraction of infected cells replicating the virus. The former would reflect an increase in the absolute numbers of latently infected cells, whereas the latter would reflect a change in

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<sup>4</sup> Abbreviations used in this paper: EBNA, Epstein-Barr nuclear Ag; SLE, systemic lupus erythematosus; SAID, systemic autoimmune disease; LMP, latent membrane protein; SLEDAI, SLE disease activity index; ANCOVA, analysis of the covariance; IS, immunosuppression; ln, natural log.

the regulation of viral replication. Mechanistically, these two outcomes may have very different causes. Because the previous reports used techniques that were unable to distinguish the two possibilities, the origin of the increased viral load in SLE (more latently infected cells or more viral replication) is not known. Furthermore, these reports did not examine critical measures of viral persistence, such as viral gene expression and the phenotype of infected cells, which define more precisely the state of viral regulation.

We decided to revisit the issue of EBV perturbation in SLE, because a more thorough and complete survey of changes in viral persistence in SLE could characterize the nature of the perturbation and thereby provide new insight into the mechanisms involved in regulating the virus. In this study, we confirm definitively that EBV infection is perturbed in the setting of SLE. We now show that the increased viral load is due to an increase in the numbers of latently infected cells, particularly in patients with active disease. Furthermore, for the first time, we demonstrate that patients with SLE have aberrant expression of viral latent and lytic genes in the blood. We conclude that immune dysfunction in SLE alters the regulatory mechanisms of EBV persistence, and we discuss how B cell deregulation may impact viral persistence.

## Materials and Methods

### Patient populations

Patient studies were approved by the institutional review board of Tufts-New England Medical Center (T-NEMC). Thirty-five patients attending the adult rheumatology clinic and the nephrology clinic at T-NEMC who met the American College of Rheumatology 1997 revised criteria for the diagnosis of SLE were recruited for this study. At the time of blood collection, patients' medical records were reviewed, and current medications, pertinent laboratory data, SLE disease history, as well as evidence of disease activity as documented by the treating physicians were recorded. From these data, a SLE disease activity index (SLEDAI) was calculated (22) (median SLEDAI, 2; range, 0–13). A patient was considered to have a disease flare if the SLEDAI was  $>4$  (23). The time interval since patients' last SLE disease flare (SLEDAI  $>4$ ) was also determined (months since last flare: median, 28; range, 0–122). (For patient demographics, see Table I.)

Forty-four healthy volunteers were recruited from Tufts University School of Medicine. Seventeen patients attending the rheumatology clinic who met the American College of Rheumatology criteria for other SAIDs were recruited as another control group. These patients generally had recent onset of these conditions ( $<3$  mo) and were generally untreated, except for three who were treated with prednisone (5–7.5 mg/day) and three who were treated with sulfasalazine. One patient with Crohn's disease, diagnosed by colonic biopsy, not treated with any immunosuppressive medications, was recruited for this study from the gastroenterology clinic. (For healthy and SAID patient demographics, see Table I.) (Unlike SAID patients, most SLE patients had longstanding disease (mean disease duration,  $11 \pm 8$  years; 30 patients diagnosed with SLE  $\geq 2$  years before study), but there was no correlation between SLE disease duration and frequency of EBV-infected cells in the blood ( $p = 0.3$ ; data not shown).)

Table I. Patient demographics for the three study groups

|                                | SLE        | Healthy    | SAID       |
|--------------------------------|------------|------------|------------|
| No. of patients                | 35         | 44         | 17         |
| Age, mean (range) <sup>a</sup> | 40 (21–73) | 29 (21–61) | 48 (24–80) |
| % Female <sup>b</sup>          | 91         | 43         | 64         |
| Race <sup>c</sup>              |            |            |            |
| % Caucasian                    | 49         | 68         | 73         |
| % African-American             | 14         | 0          | 20         |
| % Asian                        | 20         | 7          | 0          |
| % Other                        | 17         | 25         | 7          |

<sup>a</sup>  $p < 0.001$  for SLE and for SAID compared to healthy.

<sup>b</sup>  $p < 0.001$  for SLE compared to healthy;  $p = 0.13$  for SAID compared to healthy.

<sup>c</sup> Age and race unavailable for two patients in SAID group.

The patient and control populations were not matched. This was because we have not previously observed any relationship between age, sex, or race, and frequency of EBV-infected cells (our unpublished data). Data from the current study maintains that sex and race have no significant influence on the frequency of infected cells among healthy individuals ( $p = 0.4$  and  $p = 0.14$ , respectively; data not shown). The current data do indicate that there is a slight but statistically significant rise in frequency of infected cells with age observed in our measurement on healthy individuals ( $R^2 = 0.16$ ;  $p = 0.01$ ). Whether this trend is biologically significant or an epiphenomenon of this particular data set is unclear.

### Primary cells and cell lines

Sixty milliliters of patients' blood was collected by routine venipuncture into a syringe containing 1000 USP units of sodium heparin. Blood was layered onto Ficoll-Hypaque Plus (Pharmacia) and centrifuged at 2000 rpm for 30 min at 20°C, and the buffy coats containing the PBMCs were removed. PBMCs were then kept on ice, except where indicated. PBMCs were washed twice with  $1 \times$  PBS/1.0% BSA (PBSA) and then resuspended at a desired concentration in the same buffer.

EBV-negative tonsil cells were used as negative controls as well as "filler" cells for RNA preparation. Tonsils were obtained from patients undergoing routine tonsillectomies for obstructed breathing disorders at the Massachusetts General Hospital. The tonsils were minced thoroughly, and connective tissue was removed by filtering through silkscreen. Cells were resuspended in PBSA and purified as described above. Tonsils were repeatedly confirmed to be EBV negative by DNA PCR (W-repeat sequence) and RT-PCR (EBER1 transcripts) (described below).

The lymphoblastoid cell lines ER, IP, and AT, generated by our laboratory, were used as positive controls for EBNA2, LMP1, LMP2, and EBER expression at the RNA level, as well as for W-repeat DNA PCR. The EBV-positive Burkitt lymphoma line AKATA (gift from E. Keiff, Harvard Medical School, Boston, MA) was used as positive controls for EBNA1(Q-K) expression. The EBV-positive marmoset cell line B95.8 (gift from E. Keiff) was used as a positive control for expression of the lytic cycle gene *BZLF1*. The EBV-negative B cell line, BJAB (gift from E. Keiff), was used as a negative control for these studies. All cell lines were cultured at 37°C with 5% CO<sub>2</sub> in RPMI 1640 supplemented with 10% FCS, 2 mM sodium pyruvate, 2 mM glutamine, and 100 IU of penicillin/streptomycin.

### Cell separations

B cells were purified by negative selection using the Stem Sep System (StemCell Technologies) as described by the manufacturer. Briefly, PBMCs were resuspended to a concentration of  $5 \times 10^7$  cell/ml and stained with 100  $\mu$ l/ml B cell enrichment Ab mixture (StemCell Technologies) for 15 min at room temperature. The Ab mixture contains Abs directed against all types of peripheral blood cells except B cells. Cells were then stained with 60  $\mu$ l/ml magnetic colloid labeled Ab (StemCell Technologies) for 15 min at room temperature. The sample was then passed over a column, in the presence of a magnet, which allowed the population of interest to be collected as the flow-through fraction. Flow cytometric analysis was used to assay the purity of all isolated populations. Both fractions were stained with anti-CD20 FITC (DakoCytomation), anti-IgD PE (Southern Biotechnology Associates), and/or anti-CD27 PE (BD Pharmingen), and analyzed using a FASCalibur (BD Biosciences). Data were analyzed with WinList 4.0 software (Verity Software House). FACS analysis allowed for the determination of both the recovery and purity of the isolated populations. B cell population purity following negative selection was 65–99% and typically was  $>90\%$ .

Populations of naive and memory B cells were purified by FACS separation. PBMCs, prepared as described above, were resuspended to a concentration of  $2 \times 10^7$  cells/ml and stained for 30 min at 4°C with Abs to the desired cell surface markers, indicated above. After staining, cells were washed twice with PBSA and resuspended to a concentration of  $1 \times 10^8$  cells/ml. Cell sorting was performed on a MoFlo FACS sorter (DakoCytomation). FACS reanalysis allowed for the determination of the isolated population purity.

### Limiting dilution DNA PCR

Limiting dilution analysis was used to determine the frequency of EBV-infected cells for each patient. The details of this assay using DNA PCR have been published previously (24). It can detect the presence of a single EBV genome in a background of as many as  $1 \times 10^6$  EBV-negative cells. Dilutions of isolated B cell populations were distributed into the wells of V-bottom microtiter plate (Nunc) in replicates of eight. The cells were pelleted, the cell pellets were lysed, and the extract was subjected to DNA PCR specific to the W repeat region of the EBV genome exactly as described previously. PCR conditions were as follows: 5  $\mu$ l of sample DNA,

0.2 mM dNTPs, 0.20 pM each primer, 2.0 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl, and 1 U of *Taq* (Applied Biosystems) in a total volume of 50  $\mu$ l. The reaction was performed in a Geneamp 9600 thermocycler (PerkinElmer) for 35 cycles of 95°C for 15 s and 65°C for 1 min followed by 1 cycle of 72°C for 5 min. Specific products were resolved by electrophoresis, Southern blotted, and detected using radiolabeled purified specific PCR product from IB4 cells. The frequency of infected cells was determined from the distribution of wells with positive PCR signals (exemplified in Fig. 5, B and C), by applying Poisson statistics (for details of the quantification, sensitivity, and confidence limits of the analysis, see Ref. 25).

The absolute number of infected cells per milliliter of blood was calculated by multiplying the frequency of EBV-infected B cells by the fraction of CD20<sup>+</sup> B cells in PBMCs (determined by FACS analysis) and number of PBMCs per milliliter of blood (from the number of PBMCs obtained following Ficoll extraction of blood).

#### RT-PCR

If patients had sufficient numbers of B cells isolated from the blood, EBV gene expression was determined in addition to the frequency of infected cells. RT-PCR was used to determine the expression of EBV genes indicative of viral replication and the three latency programs. The details of this assay have been published previously (7). Serial dilutions of isolated cell populations were prepared and aliquoted into Eppendorf tubes. EBV-negative tonsillar cells were added, when needed, to each tube to bring the total number of cells to  $5 \times 10^6$ . The 10<sup>6</sup> EBV<sup>+</sup> lymphoblastoid cell line cells were used as a positive control. RNA was isolated by the TRIzol method (Invitrogen Life Technologies), and cDNA was prepared as described previously (8). PCR for the EBV genes *EBNA2*, *EBNA1(Q-K)*, *LMP1*, *LMP2*, *EBERs*, and *BZLF1* were performed by taking one-tenth of the cDNA (20  $\mu$ l) for each gene. This allowed for simultaneous PCR for all of the genes on the same sample. Primers used were as follows: *EBNA1(Q-K)*, TGC CCCCTCGTCAGACATGATT and AGCGTGCCTACGGAT (26); *EBNA2*, CATAGAAGAAGAAGAGGATAGAGA and ATAGGGGATTC GAGGAATTACTGA (27); *LMP1*, TTGGTGTACTCCTACTGAT GATCACC and AGTAGATCCAGATACCTAAGACAAGT (27); *LMP2*, ATGACTCATCTCAACACATA and CATGTTAGGCAAATTGCAA (28); *EBERs*, AAAACATGCGGACCACCAGC and AGGACCTACGCT GCCCTAGA (28); and *BZLF1*, TTCCACAGCCTGCACCAGTG and GGCAGCAGCCACCTCACGGT (28). Reactions were performed as described previously (8). The reaction for *BZLF1* was performed in a final volume of 50  $\mu$ l containing 50 mM KCl, 20 mM Tris (pH 8.4), 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, and 20 pM each primer under the following conditions: 40 cycles of 95°C for 15 s, annealing temperature of 59°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min.

Specific products were resolved by electrophoresis, Southern blotted, and detected using radiolabeled-purified specific PCR product (amplified from B95.8 for *BZLF1*, AKATA for *EBNA-1(Q-K)*, and IB4, ER, and IP for *EBNA-2*, *LMP1*, and *LMP-2a*). In every case, it was possible to detect the mRNA from a single infected cell in the presence of  $5 \times 10^6$  uninfected tonsil B cells, except for *BZLF1* from B95.8 because only 5–10% of these cells express lytic gene transcripts when unstimulated.

#### CMV DNA detection

The 10<sup>7</sup> PBMCs were digested with 1 mg/ml proteinase K (Sigma-Aldrich) at 55°C for at least 6 h. DNA was isolated by the phenol/chloroform/isoamyl alcohol method. One microgram of DNA was used for quantitative PCR. The primers used were GACTAGTGTGATGCTGGCCAAG and GCTACAATAGCCTCTTCTCATCTG, and the TaqMan probe used was 56-FAM/AGCCTGAGGTTATCAGTGAATGAAGCGCC/36-TAMNph (29). PCR conditions were as follows: 24  $\mu$ l of sample DNA, 0.1 mM dNTPs, 200 pM each primer, 100 pM probe, 1 $\times$  TaqMan buffer supplemented with 5.0 mM MgCl<sub>2</sub>, and 1 U of Amplitaq G (Applied Biosystems) in a total volume of 50  $\mu$ l. The reaction was performed in a TaqMan 5700 (Applied Biosystems) for 50 cycles of 95°C for 15 s, and 60°C for 1 min, followed by 1 cycle of 72°C for 5 min. PBMCs from immunosuppressed transplant patients with confirmed CMV viremia were provided by A. Rabson (T-NEMC).

#### Statistical analysis

Comparisons between patient groups of data with normal distributions (e.g., natural log (ln) frequencies of infected cells) were performed with independent *t* tests or one-way ANOVA followed by Student Newman-Keuls post hoc testing where appropriate. Analysis of the covariance (ANCOVA) was used to compare ln frequencies of infected cells between patient groups while controlling for age. Comparisons between patient groups of data with skewed distributions (e.g., frequency of infected cells)

was performed with the Kruskal-Wallis test. The effect of patient group and patient age on the frequency of EBV-infected cells was determined by ANCOVA. Pearson correlation was used to examine the relationship between two continuous variables. ANOVA and the  $\chi^2$  test were used to test for differences in age and sex of the patient groups. Statistical analysis was performed using SPSS software (version 12.0; SPSS).

## Results

### *The frequency of EBV-infected B cells in the blood of SLE patients is high*

In healthy individuals, tight regulation of EBV infection is evident in the blood, where the frequency of infected cells remains low but relatively stable over long periods of time. Therefore, we investigated whether the immune dysfunction present in SLE would affect the frequency of virus-infected cells.

We quantified precisely the frequency of virus-infected cells using a limiting dilution EBV DNA PCR assay that we have described previously (30). Because this assay detects intact infected cells, it provides a measure of the number of virus-infected cells that is independent of the number of viral genomes per cell (usually two to five copies). A limiting dilution assay prevents bias by the inclusion of even a single lytically infected cell that may have thousands of genome copies and would therefore generate a very large increase in the viral load without affecting the frequency of infected cells.

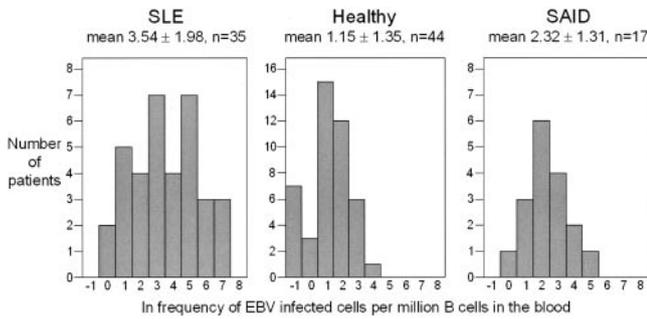
Using this technique, we found elevated frequencies of virally infected cells in the peripheral blood of 35 patients meeting the American College of Rheumatology criteria for the diagnosis of SLE, as compared with 44 healthy individuals (Table II). Similar to our previous findings in healthy individuals, the frequency distribution in patients with SLE is skewed; therefore, it is not possible to calculate a meaningful mean value or SD for comparison between populations. However, the data show a normal distribution when the ln frequencies are derived, and analysis of the transformed data demonstrates a significant difference between these groups (mean ln frequency/10<sup>6</sup> B cells for SLE patients =  $3.54 \pm 1.98$ ; for healthy controls =  $1.15 \pm 1.35$ ;  $p < 0.001$ ) (Fig. 1). Taking the antilogs, these values translate into an average 10-fold increase, which is comparable to the average 12-fold increase that we have reported previously in immunosuppressed organ transplant patients (31).

Patients with SLE were significantly older than healthy controls (Table I) ( $p < 0.001$ ). Although there is no precedence for an age-related change in the frequency of infected cells, we did detect a small but statistically significant rise in the ln frequency of EBV-infected cells with advancing age in the healthy control group ( $R^2 = 0.16$ ;  $p = 0.008$ ) but not the patient groups ( $p = 0.7$ ; data not shown). It is unclear whether this is a real trend or an epiphenomenon of this one data set. However, to exclude the possibility that the level of infected cells increases because of this uncertain age effect, ANCOVA was performed. This confirmed that patient group had a strong effect on the frequency of infected cells ( $F =$

Table II. Summary of frequencies of EBV-infected cells per million B cells in the blood of three patient groups: patients with SLE, healthy individuals, and patients with other SAIDs<sup>a</sup>

|         | <i>n</i> | Median | Range    |
|---------|----------|--------|----------|
| SLE     | 35       | 26.0   | 1.1–1775 |
| Healthy | 44       | 3.3    | 0.3–30   |
| SAID    | 17       | 9.4    | 1.5–99   |

<sup>a</sup> Because the distribution of the data in each of these groups is skewed, the medians and ranges are provided instead of the means and SDs.  $p < 0.001$  for all group comparisons.



**FIGURE 1.** Frequencies of EBV-infected cells in patients with SLE, healthy individuals, and patients with other SAIDs. The histograms show the distribution in the frequencies of infected cells per million B cells for each group. The frequencies are plotted as ln values because the data distributions within each group are skewed (see *Results*). The frequencies of infected cells in the SLE group is significantly higher than healthy individuals or patients with other SAIDs ( $p < 0.001$ ).

16.8;  $p < 0.001$ ), whereas age had no significant effect ( $F = 0.6$ ;  $p = 0.4$ ).

There are two ways that the frequency of infected cells could become elevated: either the absolute number of infected cells in the circulation increases or the number of uninfected cells decreases. SLE patients are often lymphopenic (low numbers of lymphocytes in the blood), and similar to the findings of others (13, 32), the number of CD20<sup>+</sup> B cells per milliliter of blood was slightly lower in our lupus patient population compared with healthy individuals (SLE,  $8.8 \times 10^4$  B cells/ml blood  $\pm 8.4 \times 10^4$ , healthy,  $14.1 \times 10^4 \pm 7.5 \times 10^4$ ;  $p = 0.01$ ).

To see whether the reduced numbers of B cells in the blood of SLE patients could account for the observed increase in the frequency of infected cells, the absolute number of infected cells per milliliter of blood was calculated by normalizing the frequency of infected B cells to the number of B cells per milliliter of blood (SLE patients: median, 1.1; infected cells per milliliter of blood, range, 0.1–93; healthy patients: median, 0.4; range, 0.1–5; based on data from 28 healthy patients). Like the data for the frequency of infected cells, the distribution of the data for EBV-infected cells per milliliter of blood is skewed but is normalized by deriving the ln of the number of infected cells for each patient. Analysis of the transformed data demonstrates that patients with SLE have significantly more infected cells per milliliter of blood than healthy individuals (SLE, mean ln infected cells per milliliter of blood,  $0.51 \pm 1.92$ ; healthy,  $-1.12 \pm 1.53$ ;  $p < 0.001$ ) (Fig. 2). Therefore, the high frequency of infected cells in SLE patients was not

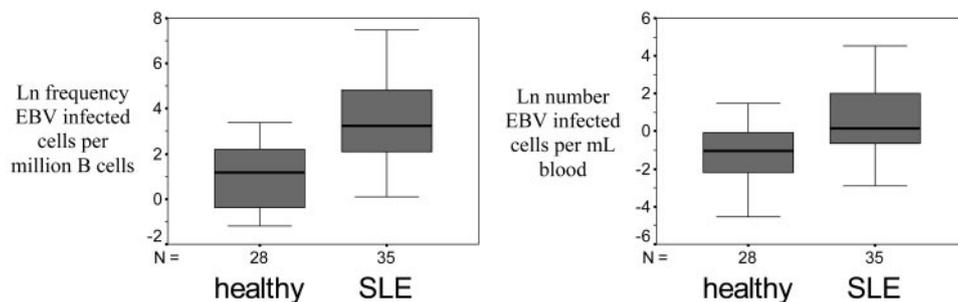
a consequence of B lymphopenia, but reflects an expansion in the absolute number of EBV-infected cells in the blood of these patients.

*Treatment of SLE patients with immunosuppressive agents does not significantly affect the frequency of EBV-infected cells in the blood*

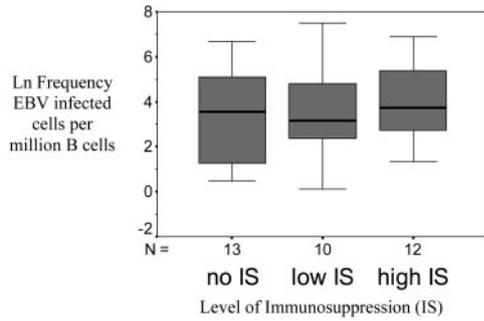
Our laboratory and others (31, 33, 34) have shown previously that patients treated with immunosuppressive agents following organ transplant have high frequencies of EBV-infected cells in their blood. SLE patients are also treated with immunosuppressive drugs, albeit with fewer agents and at lower doses than patients who have received organ transplants. To investigate whether the high frequencies of infected cells observed in patients with SLE were a consequence of iatrogenic immunosuppression (IS), patients were stratified into three groups based on levels of IS at the time of phlebotomy: treatment with no agents other than hydroxychloroquine (no IS); treatment with prednisone, 2.5–7.5 mg/day (low IS); and treatment with prednisone,  $\geq 10$  mg/day, and/or another immunosuppressive agent such as methotrexate, azathioprine, cyclosporin, mycophenolate mofetil, or cyclophosphamide (high IS). We found no difference in the frequencies of EBV-infected B cells among these three groups (mean ln frequency/ $10^6$  B cells: no IS,  $3.27 \pm 2.15$ ,  $n = 13$ ; low IS, mean,  $3.35 \pm 2.11$ ,  $n = 10$ ; and high IS, mean,  $4.00 \pm 1.75$ ,  $n = 12$ ;  $p = 0.6$ ) (Fig. 3). Taking the antilog values, this represents, at best, a 2-fold increase (which is not statistically significant) between the no IS and the high IS groups, showing that the effect of SLE on the frequency of EBV-infected cells is independent of treatment with immunosuppressive medications.

*Patients with SLE have higher frequencies of EBV-infected cells than patients with other SAIDs*

To determine whether the effect of SLE on the frequency of EBV-infected cells in the blood is specific to SLE, we investigated EBV infection in 17 patients with other SAIDs. This included patients with rheumatoid arthritis ( $n = 11$ ), spondyloarthropathy ( $n = 2$ ), systemic sclerosis ( $n = 2$ ), Sjogren's syndrome ( $n = 1$ ), and Crohn's disease ( $n = 1$ ). Many of these patients had been diagnosed recently with their diseases, had evidence of active inflammation, and were treated with no or very low doses of immunosuppressive medications. Although the frequencies of infected cells in these patients were higher than those of healthy individuals ( $p < 0.05$ ), they were significantly lower than the frequency of EBV-infected cells in the blood of patients with SLE (SAID, mean ln frequency/ $10^6$  B cells =  $2.32 \pm 1.31$ ;  $p < 0.05$ ) (Table II and



**FIGURE 2.** Summary of the frequency of EBV-infected cells and the absolute number of EBV-infected cells per milliliter of blood in patients with SLE and healthy individuals. Box plots show the distribution in the frequency of infected cells per million B cells (*left*) and the absolute number of infected cells per milliliter of blood (*right*) for each population. The line inside each box represents the median. The ends of each box define the 25th to 75th percentiles. Lines outside the boxes define the 5th and 95th percentiles. Number of patients (N) in each group is shown. The ln values are used because the data distributions are skewed. Patients with SLE had significantly higher numbers of infected cells per milliliter of blood ( $p < 0.001$ ), as well as higher frequencies of infected cells ( $p < 0.001$ ) compared with healthy individuals.



**FIGURE 3.** Distribution of the Ln frequencies of EBV-infected B cells per million B cells in SLE patients categorized by IS level: no IS, low IS, or high IS (see *Results* for definitions). There is no significant difference between groups ( $p = 0.6$ ). Data are shown as box plots.

Fig. 1). These results suggest that EBV infection may be perturbed in a broad range of autoimmune disease but that the most profound effect occurs with SLE.

*SLE disease activity affects the frequency of virally infected cells*

SLE is a disease of flares and remissions. Although the cause of this is unknown, it is thought that disease flares represent times of greatest immune dysfunction (35). To investigate whether changes in the level of immune dysfunction could affect the regulation of EBV infection, the frequency of virally infected cells was studied in patients with active and inactive disease. Disease activity was quantified using the SLEDAI (22). A flare was defined as a score of  $>4$  (23). Fifteen patients with recently active disease (flare within 3 mo of study) had significantly higher frequencies of infected cells than 16 patients with inactive disease (no flare within 12 mo of study) (mean Ln frequency/ $10^6$  B cells: recent flare =  $4.44 \pm 2.19$ ; no recent flare =  $2.60 \pm 1.48$ ;  $p = 0.01$ ) (Fig. 4). (Patients with active disease were no more likely to be treated with immunosuppressive medications than patients with inactive disease ( $p = 0.9$ ), because many patients with disease flares were studied before the initiation of therapy.)

To further investigate whether disease activity affects EBV infection, we measured the frequency of EBV-infected cells over time in individual patients. Unlike healthy individuals in whom the frequency of infected cells is relatively stable for years (25), we saw large fluctuations in the frequency of infected cells in the blood of seven patients over the course of 9–17 mo, and these were usually associated with changes in disease activity as indicated by the SLEDAI score (Table III). Interestingly, one patient (no. 7) had a rise in their frequency of infected cells, which was associated

**Table III.** Change in the frequency of EBV-infected cells in the blood of seven SLE patients at two time points (visits 1 and 2)<sup>a</sup>

| Patient | Visit 1   |                | Visit 2   |        | Interval (mo) |
|---------|-----------|----------------|-----------|--------|---------------|
|         | Frequency | SLEDAI         | Frequency | SLEDAI |               |
| 1       | 1240      | 12             | 250       | 4      | 14            |
| 2       | 760       | 4              | 100       | 2      | 11            |
| 3       | 129       | 2              | 144       | 2      | 15            |
| 4       | 30        | 4              | 10        | 2      | 14            |
| 5       | 392       | 0 <sup>b</sup> | 37        | 0      | 17            |
| 6       | 410       | 7              | 500       | 2      | 11            |
| 7       | 120       | 0              | 470       | 5      | 9             |

<sup>a</sup> The SLEDAI score at each visit is indicated as well as the time interval between visits. Frequency is the number EBV-infected cells per million B cells.

<sup>b</sup> Patient 5 had a disease flare 1 mo before clinic visit no. 1.

with a disease flare rather than a change in their immunosuppressive medications. These findings show that the effect of SLE on the regulation of EBV persistence is related to the presence of SLE disease activity and illustrates the sensitivity of the virus to changes in immune function.

*There is no relationship between frequency of EBV-infected cells and specific clinical or serological features of SLE*

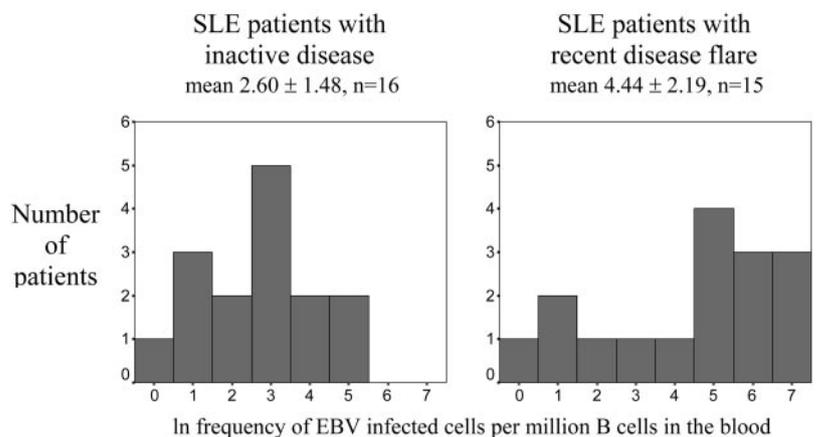
Because disease activity affects the frequency of infected cells, we wondered whether there was an association between serological markers of SLE disease activity and the frequency of infected cells. However, we found no correlation between the frequency of EBV-infected cells and levels of anti-dsDNA Ab (in patients who produce this Ab), complement levels, or erythrocyte sedimentation rate.

Patients with SLE can be stratified into subsets based on various clinical and immunological manifestations of the disease. We investigated whether the high frequencies of EBV-infected cells observed in many SLE patients were associated with particular disease subsets. No relationship was seen between the frequency of EBV-infected cells and the presence of nephritis ( $n = 14$  of 35 patients with data available;  $p = 0.4$ ), the presence of antiphospholipid Ab syndrome ( $n = 7$  of 32;  $p = 0.9$ ), or the production of certain autoantibodies documented at any point in patients' disease history, including the following: anti-dsDNA Abs ( $n = 27$  of 35;  $p = 0.8$ ), anti-Smith Ab ( $n = 6$  of 26;  $p = 0.8$ ), and anti-ribonucleoprotein Ab ( $n = 8$  of 18;  $p = 0.6$ ) (data not shown).

*Patients with SLE have aberrant latent and lytic gene expression in the blood*

Another indicator of EBV regulation in healthy carriers is the absence of viral gene expression in the blood (4). If EBV is affected

**FIGURE 4.** Distribution of the Ln frequencies of EBV-infected cells per million B cells in SLE patients with recently active disease (flare in  $\leq 3$  mo) and inactive disease (no flare in  $\geq 12$  mo). The Ln frequencies of infected cells in the recent flare group are significantly higher than those in the inactive disease group ( $p = 0.01$ ).



by SLE immune dysfunction, it could result in inappropriate expression of viral genes in the blood. We investigated EBV gene expression in B cells from the peripheral blood of SLE patients using RT-PCR (Table IV). The immediate early lytic gene, *BZLF1*, which is involved in viral reactivation, was detected in 6 of 17 patients (35%). *EBNA2* is the first latency gene activated following infection of a B cell *in vitro*; it was not detected in the blood of 23 SLE patients tested, indicating that newly infected cells are not present in the blood. *LMP1* and *LMP2a* are viral genes expressed in all stages of EBV latency in tonsillar B cells and are of interest because they deliver surrogate B cell survival and differentiation signals (CD40 and BCR, respectively) (5, 36–38). *LMP1* was detectable in 5 of 17 SLE patients' blood (29%), and *LMP2a* was detected in 4 of 22 patients (18%). Although transcripts of *LMP2a* are found occasionally in the blood of healthy individuals, *LMP1* is never detected (24, 27, 28, 39, 40). *EBNA1* expression from the Q promoter (*EBNA1(Q-K)*) was also tested because this transcript is expressed along with *LMP1* and *LMP2a* in a discrete transcription program, termed the default program, which is used in germinal center and memory B cells in the tonsils of healthy individuals as well as several tumors. Transcripts of *EBNA1(Q-K)* were detected in only 1 of 17 SLE patients, which is similar to what is observed in the blood of healthy people where *EBNA1* is rarely detected. From these data, it can be concluded that EBV gene expression is aberrantly regulated in the blood of some patients with SLE. It is important to note that EBV regulation is not completely disrupted, because expression of the EBV growth program gene, *EBNA2*, was not detected.

To exclude the possibility that *BZLF1*, *LMP1*, and *LMP2a* expression was detected in the blood of lupus patients because of the relatively large quantities of EBV-infected cells that could be tested in these patients, limiting dilution analysis of gene expression was performed. This allows for the calculation of the frequency of infected cells expressing viral genes. Two SLE patients had sufficient numbers of samples with *BZLF1* expression at various B cell dilutions to perform this analysis. Two and 0.5% of the infected cells in the blood of these patients expressed this lytic gene. Although only a small fraction of infected cells express *BZLF1*, such levels of expression are very abnormal. In comparison, we have never detected *BZLF1* expression in the blood of healthy patients. We have detected *BZLF1* expression in the blood of patients with infectious mononucleosis, but the frequency of infected cells expressing this lytic gene was considerably less (0.02 to <0.001%;  $n = 5$ ) (data published in Ref. 41) than those in the two lupus patients. These findings confirm that *BZLF1*, and probably *LMP1*, are abnormally regulated in some patients with SLE.

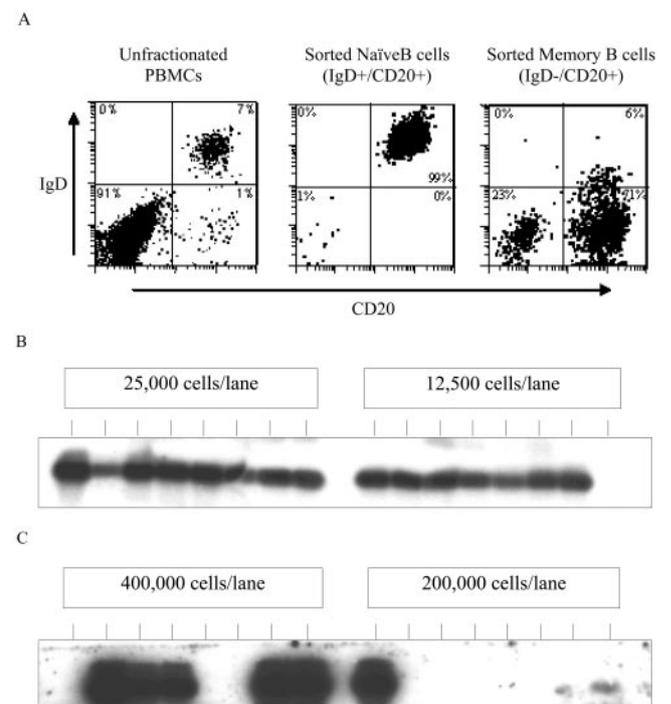
#### The infected cells in the blood of patients with SLE are memory B cells

A hallmark of EBV infection in healthy individuals is the restriction of the virus to the  $IgD^-$  population of memory B cells in the

blood (24, 34, 42). The appearance of EBV in peripheral blood naive B cell would represent a profound deregulation of persistent infection. Therefore, we investigated whether EBV remained restricted to  $IgD^-$  B cells in the blood of SLE patients. B cells (positive for the pan-B cell marker CD20) were fractionated by FACS into  $IgD^+$  (which is expressed primarily on naive B cells) and  $IgD^-$  (memory cells) populations (Fig. 5A). The frequency of virus-infected cells was then assayed in these two populations (Fig. 5, B and C, and Table V). Essentially, all of the virally infected cells were in the  $IgD^-$  memory B cell population. These findings indicate that the perturbations of the immune system in SLE are not so profound as to disrupt the mechanism responsible for restricting EBV to memory B cells in the blood.

#### CMV DNA is not present in the blood of patients with SLE

To investigate whether the immune dysfunction in SLE specifically affects EBV or whether it can affect other herpesviruses, we also studied CMV. CMV infects 70% of humans, but generally the infection is tightly controlled, and virus is not detectable in the blood. CMV can cause clinical disease in immunosuppressed patients, at which times viremia is present in the blood. We performed quantitative real-time PCR for CMV on 1.0 and 0.1  $\mu g$  of



**FIGURE 5.** EBV-infected cells are restricted to  $IgD^-$  memory B cells in the blood of patients with SLE. PBMCs were sorted into  $IgD^-$  (memory) and  $IgD^+$  (naive) B cell populations by FACS. EBV DNA PCR was performed on serial dilutions of  $IgD^-$  and  $IgD^+$  B cell populations and analyzed by Southern blot. By measuring the fraction of samples negative at each cell dilution and applying Poisson statistics, the frequency of EBV-infected cells in each population of cells can be determined (shown in Table V). A, FACS reanalysis of  $IgD^+$  (naive) and  $IgD^-$  (memory) B cell subsets separated from unfractionated PBMCs of a representative SLE patient by flow cytometry sorting using the pan-B cell marker CD20 (FITC) and  $IgD$  (PE). B and C, Following FACS sorting, memory (B) and naive (C) B cells were serially diluted, and replicates of each dilution were subject to DNA PCR for the W repeat of viral DNA. PCR products were detected by Southern blot with a radiolabeled probe specific for the W repeat. The number of cells tested per sample and the position of each lane containing a sample is indicated. Three positive and eight negative controls were performed for each B cell type tested (data not shown).

Table IV. EBV gene expression in the blood of SLE patients<sup>a</sup>

| EBV Gene          | No. Patients Positive/Tested | % Patients Positive |
|-------------------|------------------------------|---------------------|
| <i>BZLF1</i>      | 6/17                         | 35                  |
| <i>LMP1</i>       | 5/17                         | 29                  |
| <i>LMP2a</i>      | 4/22                         | 18                  |
| <i>EBNA1(Q-K)</i> | 1/17                         | 6                   |
| <i>EBNA2</i>      | 0/23                         | 0                   |

<sup>a</sup> In the blood of healthy individuals, *BZLF1*, *LMP1*, *EBNA1*, and *EBNA2* expression has not been detected, and *LMP2a* transcripts are rarely detected (Refs. 27, 28, and 40, and our unpublished observations).

DNA from PBMCs of five SLE patients who were seropositive for CMV infection and five immunosuppressed transplant patients with known CMV viremia. SLE patients with a broad range of frequencies of EBV-infected cells in the blood (range, 2–977) were used to determine whether there was any correlation between deregulation of EBV infection and CMV infection. Although CMV DNA could be detected in the PBMCs of the five immunosuppressed transplant patients, CMV DNA could not be detected in PBMCs of any SLE patient (Table VI). Because CMV does not appear to be deregulated in patients with SLE, it can be concluded that immune abnormalities in SLE specifically deregulate EBV, further demonstrating the sensitivity of EBV to the immune dysfunction present in this autoimmune disease.

## Discussion

The goal of our study was to test the prediction that diseases affecting the immune system will alter the state of EBV persistence. In agreement with this prediction, we have demonstrated a marked perturbation of EBV infection in patients with SLE, as indicated by high frequencies of infected B cells, and aberrant expression of viral genes. The logical explanation for this finding is that host factors related to SLE disrupt EBV. Because EBV dwells in and is regulated by the immune system, it is sensitive to immune dysfunction, as seen in immunosuppressed patients, e.g., organ transplant recipients. Thus, any host factor that perturbs EBV must, directly or indirectly, act on or through the immune system. We can conclude therefore that immune dysfunction associated with SLE is disrupting EBV persistence.

The alternative explanation is that the perturbed state of EBV infection causes SLE. However, this begs the question, what perturbs EBV in the first place, leading to some or all of the symptomatology of SLE? It must be either the virus or the host. It is unlikely that there is anything different about the virus, because there is no evidence for disease-specific strains, and viral perturbation has not been described in healthy individuals. Therefore, the logical conclusion is that it is a function of SLE that disrupts EBV and therefore that the altered behavior of EBV reflects the sensitivity of the virus to immune dysfunction in SLE. This concept can be generalized to state that any disease that affects the immune system may change the status of EBV persistence.

It has been suggested that EBV infection has a causative role in SLE (43). Our experiments do not directly address this possibility. The changes in EBV behavior we observe can most simply be attributed to defects in immune function in SLE patients, without the need to invoke or deny a causative role. Whether these changes in EBV infection are an intermediate and essential step in the pathogenesis of SLE or merely an epiphenomenon of disrupted immune function remains to be answered (Fig. 6). What can be concluded is that deregulated EBV infection manifested as increased frequencies of infected cells, increased viral loads, and viral gene expression, cannot be interpreted per se as supporting the possibility that EBV plays a role in SLE.

To evaluate how host immune factors can affect the virus, viral biology needs to be considered. Persistent infection by EBV is characterized by a relatively stable frequency of virus-infected cells in the peripheral blood (25). To maintain this level, EBV, like all pathogens that establish chronic persistent infections, must evade immunosurveillance mechanisms. EBV achieves this by persisting in resting memory B cells where all viral protein expression is shut down (27, 28, 31, 40), thereby eliminating all potential targets for immune surveillance. The corollary, implicit in the lack of viral protein expression, is that the virus can have no influence on the behavior of the latently infected memory cells. Based on these considerations, we have hypothesized that the

Table V. Frequency of EBV-infected naive and memory B cells in the blood of SLE patients

| Patient | B Cell Population         | Frequency/10 <sup>6</sup> Cells |
|---------|---------------------------|---------------------------------|
| 1       | IgD <sup>+</sup> (naive)  | 5                               |
|         | IgD <sup>-</sup> (memory) | 590                             |
| 2       | IgD <sup>+</sup> (naive)  | 2                               |
|         | IgD <sup>-</sup> (memory) | 2400                            |
| 3       | IgD <sup>+</sup> (naive)  | 4                               |
|         | IgD <sup>-</sup> (memory) | 4500                            |
| 4       | IgD <sup>+</sup> (naive)  | <3                              |
|         | IgD <sup>-</sup> (memory) | 450                             |

steady-state level of infected memory B cells in the blood is regulated by two factors. The first is the rate at which newly infected cells are produced. We assume this accrual is inversely related to the levels of immunosurveillance because cells producing infectious virus and newly infected B cells are both vigorously surveilled by CTL (1, 44). The second form of regulation is related to the homeostasis of memory B cells. Newly latently infected cells cease expression of viral proteins when they enter the memory compartment, so EBV should be responsive to the signaling mechanisms that maintain normal memory B cell homeostasis. We propose that the increased frequency of infected memory cells in the blood of SLE patients is due to a defect in the regulation of memory B cell homeostasis rather than in impaired immunosurveillance.

It is generally accepted that immunosurveillance plays an important role in regulating the levels of EBV-infected cells. This is based on studies of immunosuppressed organ transplant recipients who have high numbers of EBV-infected cells in the blood (31, 33, 45–47) and are at risk for EBV lymphoproliferative diseases (48). In this study, we have shown that patients with SLE also have elevated frequencies of infected cells in the blood, similar to those seen in immunosuppressed organ transplant recipients (31). Unlike organ transplant recipients, however, there is little evidence to suggest that this is caused by diminished immune surveillance in SLE. Kang et al. (19) have reported no change in the frequency of EBV-specific CD8 T cells in SLE patients and noted an increase in the frequency of EBV-specific CD4 T cells. Our studies indicate that CMV infection is well controlled in patients with SLE. This is consistent with the clinical observation that symptomatic CMV infection is rarely observed in SLE except in patients treated with immunosuppressive agents (49). Earlier studies of CTL function in SLE have reported debilitated cytotoxic killing, but these claims are not consistent with the findings of Kang et al. (19), possibly because those earlier studies did not measure Ag-specific, MHC-restricted responses (18).

There is also considerable clinical evidence to suggest that immune surveillance functions are relatively intact in SLE patients. Significant defects would be expected to have broad effects on a variety of infections, including herpesviruses. Although common in transplant recipients, reactivated herpesvirus infections are not usually seen in SLE. An increased incidence of reactivation by varicella zoster virus is observed, but like healthy individuals, these events are typically benign and self-limited (50). Other opportunistic infections that frequently cause clinical disease in the setting of IS, such as fungal infections, are rare in SLE patients (49).

Most directly pertinent to our assumption that immune surveillance of EBV is relatively normal in SLE is the lack of EBV-associated lymphoproliferative diseases in these patients. These diseases are usually manifested as B cell lymphomas that occur in patients who are highly immunosuppressed, either by medicines or by infection with HIV. They are thought to arise when EBV-driven cellular proliferation occurs in the absence of effective immunosurveillance (48). Strikingly, such tumors have not been reported in SLE patients, except in

Table VI. Results from real-time PCR amplification for CMV DNA<sup>a</sup>

| SLE Patients     |                    | Immunosuppressed Transplant Recipients |                    |
|------------------|--------------------|--|--------------------|
| 1 $\mu$ g of DNA | 0.1 $\mu$ g of DNA | 1 $\mu$ g of DNA                       | 0.1 $\mu$ g of DNA |
| >50              | >50                | 36.0                                   | 42.4               |
| >50              | >50                | 38.5                                   | 40.3               |
| >50              | >50                | 35.3                                   | 38.9               |
| >50              | >50                | 33.2                                   | 37.7               |
| >50              | >50                | 38.2                                   | 46.4               |

<sup>a</sup> The results indicate the number of PCR cycles necessary to detect a positive signal.

those treated with strong IS regimens. Taken together, these observations imply that immunosurveillance of EBV is effective in SLE, that the high frequencies of infected cells seen in SLE are not due to diminished immunosurveillance, and that high frequencies of EBV-infected cells are not per se a risk factor for EBV lymphomagenesis. For these reasons, we conclude that deregulated homeostasis, not IS, is responsible for the elevated frequency of EBV-infected cells found in the blood of SLE patients.

We have shown previously that EBV gene transcription patterns are dictated by the differentiation state of the B cell (4, 8). Therefore, we suspected that EBV gene expression might also be sensitive to the disruptions of normal B cell function seen in SLE patients. These include the presence of unusual B cell subsets, expression of activation markers, and perturbation of intracellular signaling (12–17). We found two unusual features of EBV gene expression in SLE: detection of the latent gene *LMP1* and the lytic gene *BZLF1*. This aberrant viral gene expression is likely a function of the B cell abnormalities present in the blood of these patients. *BZLF1* is the gene that initiates viral replication, and cells expressing it are usually absent from the blood (4). The presence of cells expressing *BZLF1* in the blood of SLE patients may be a consequence of abnormal B cell activation or B cell differentiation. Significant numbers of plasma cell precursors (plasmablasts) have been detected in the blood of patients with SLE but not in healthy individuals (3, 13, 32). The replication of EBV is believed to occur in response to the differentiation of the infected cell into a plas-

ma-blast (10). Therefore, if EBV were present in one of the circulating plasmablasts, it would be expected to begin replicating.

In a similar vein, expression of *LMP1* and *LMP2a* may be from activated memory B cells. *LMP1* and *LMP2* each provide pro-survival signals to B cells (36–38), and the expression of either of these genes could be a natural response of the virus to prevent activation induced cell death of stimulated cells. Alternatively, high B cell turnover from germinal centers in SLE or abnormal homing mechanisms may push tonsil memory B cells, expressing *LMP1* and *LMP2a*, into the blood before gene expression is down-regulated.

Although we do not favor it, the alternate explanation for our results is that, despite the considerations discussed above, there is a defect in immunosurveillance in SLE patients. It was striking that the frequency of infected cells is the same in SLE patients irrespective of treatment with immunosuppressive agents and that the frequency of infected cells are elevated similarly to what is seen in immunosuppressed organ transplant patients (31). This suggests that if there is a defect in immunosurveillance in SLE, its effect on EBV is equivalent to high levels of IS. In addition, a defect in immunosurveillance would need to be specific for EBV because other infections are not nearly so profoundly deregulated. There is precedence for unique sensitivity of EBV to immune dysfunction, as seen in X-linked lymphoproliferative disease. Males with this disease have mutations in the gene *SH2D1A*, predisposing them to the development of EBV lymphoproliferative disease (51–53). Therefore, our results might imply that of all the infections to which the human race is exposed, EBV is under the most precarious control.

Our studies to date have been limited to the status of EBV in the blood of SLE patients. It would be especially interesting to examine the behavior of EBV in the lymphoid tissues of these patients where much of the viral life cycle occurs. Unfortunately, such material is not usually available.

In conclusion, we have demonstrated perturbation of EBV infection in patients with SLE. Future studies will need to address whether specific B cell abnormalities in SLE are associated with the changes in EBV infection observed in these patients. Regardless of the outcome of such studies, it is evident that the immune dysfunction of SLE will serve as an excellent tool for investigating the mechanisms of EBV regulation, and EBV may perhaps also be useful in investigating aspects of the immune dysfunction in SLE.

## Acknowledgments

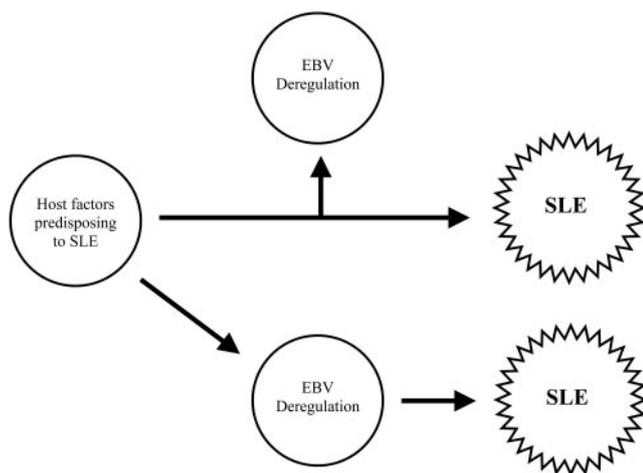
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## Disclosures

The authors have no financial conflict of interest.

## References

1. Khanna, R., and S. R. Burrows. 2000. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. *Annu. Rev. Microbiol.* 54: 19–48.
2. Catalina, M. D., J. L. Sullivan, R. M. Brody, and K. Luzuriaga. 2002. Phenotypic and functional heterogeneity of EBV epitope-specific CD8<sup>+</sup> T cells. *J. Immunol.* 168: 4184–4191.
3. Amyes, E., C. Hatton, D. Montamat-Sicotte, N. Gudgeon, A. B. Rickinson, A. J. McMichael, and M. F. Callan. 2003. Characterization of the CD4<sup>+</sup> T cell response to Epstein-Barr virus during primary and persistent infection. *J. Exp. Med.* 198: 903–911.
4. Hochberg, D., J. M. Middeldorp, M. Catalina, J. L. Sullivan, K. Luzuriaga, and D. A. Thorley-Lawson. 2004. Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo. *Proc. Natl. Acad. Sci. USA* 101: 239–244.
5. Thorley-Lawson, D. 2001. Epstein-Barr virus: exploiting the immune system. *Nat. Rev. Immunol.* 1: 75–82.
6. Thorley-Lawson, D. A., and A. J. Gross. 2004. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. *N. Engl. J. Med.* 350: 1328–1337.



**FIGURE 6.** Models of EBV deregulation and its involvement in the pathogenesis of SLE. Abnormalities in immune function associated with SLE affect EBV, perturbing the infection. Deregulation of viral persistence may be a side effect of the immune dysfunction that causes SLE. Alternatively, perturbed EBV infection may be a necessary factor in the development of symptomatic SLE.

7. Joseph, A. M., G. J. Babcock, and D. A. Thorley-Lawson. 2000. Cells expressing the Epstein-Barr virus growth program are present in and restricted to the naive B-cell subset of healthy tonsils. *J. Virol.* 74: 9964–9971.
8. Babcock, G. J., D. Hochberg, and A. D. Thorley-Lawson. 2000. The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. *Immunity* 13: 497–506.
9. Babcock, G. J., and D. A. Thorley-Lawson. 2000. Tonsillar memory B cells, latently infected with Epstein-Barr virus, express the restricted pattern of latent genes previously found only in Epstein-Barr virus-associated tumors. *Proc. Natl. Acad. Sci. USA* 97: 12250–12255.
10. Laichalk, L. L., and D. A. Thorley-Lawson. 2005. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *J. Virol.* 79: 1296–1307.
11. Hahn, B. 1997. An overview of the pathogenesis of systemic lupus erythematosus. In *Dubois' Lupus Erythematosus*. D. Wallace and B. Hahn, eds. Williams & Wilkins, Baltimore, p. 69–75.
12. Liossis, S. N., B. Kovacs, G. Dennis, G. M. Kammer, and G. C. Tsokos. 1996. B cells from patients with systemic lupus erythematosus display abnormal antigen receptor-mediated early signal transduction events. *J. Clin. Invest.* 98: 2549–2557.
13. Arce, E., D. G. Jackson, M. A. Gill, L. B. Bennett, J. Banchemareau, and V. Pascual. 2001. Increased frequency of pre-germinal center B cells and plasma cell precursors in the blood of children with systemic lupus erythematosus. *J. Immunol.* 167: 2361–2369.
14. Grammer, A. C., R. Slota, R. Fischer, H. Gur, H. Girschick, C. Yarburo, G. G. Illei, and P. E. Lipsky. 2003. Abnormal germinal center reactions in systemic lupus erythematosus demonstrated by blockade of CD154-CD40 interactions. *J. Clin. Invest.* 112: 1506–1520.
15. Folzenlogen, D., M. F. Hofer, D. Y. Leung, J. H. Freed, and M. K. Newell. 1997. Analysis of CD80 and CD86 expression on peripheral blood B lymphocytes reveals increased expression of CD86 in lupus patients. *Clin. Immunol. Immunopathol.* 83: 199–204.
16. Huck, S., R. Le Corre, P. Youinou, and M. Zouali. 2001. Expression of B cell receptor-associated signaling molecules in human lupus. *Autoimmunity* 33: 213–224.
17. Liossis, S. N., E. E. Solomou, M. A. Dimopoulos, P. Panayiotidis, M. M. Mavrikakis, and P. P. Sfikakis. 2001. B-cell kinase lyn deficiency in patients with systemic lupus erythematosus. *J. Invest. Med.* 49: 157–165.
18. Stohl, W., A. S. Hamilton, D. M. Deapen, T. M. Mack, and D. A. Horwitz. 1999. Impaired cytotoxic T lymphocyte activity in systemic lupus erythematosus following in vitro polyclonal T cell stimulation: a contributory role for non-T cells. *Lupus* 8: 293–299.
19. Kang, I., T. Quan, H. Nolasco, S. H. Park, M. S. Hong, J. Crouch, E. G. Pamer, J. G. Howe, and J. Craft. 2004. Defective control of latent Epstein-Barr virus infection in systemic lupus erythematosus. *J. Immunol.* 172: 1287–1294.
20. Moon, U. Y., S. J. Park, S. T. Oh, W. U. Kim, S. H. Park, S. H. Lee, C. S. Cho, H. Y. Kim, W. K. Lee, and S. K. Lee. 2004. Patients with systemic lupus erythematosus have abnormally elevated Epstein-Barr virus load in blood. *Arthritis Res. Ther.* 6: R295–R302.
21. Strauch, B., L. L. Andrews, N. Siegel, and G. Miller. 1974. Oropharyngeal excretion of Epstein-Barr virus by renal transplant recipients and other patients treated with immunosuppressive drugs. *Lancet* 1: 234–237.
22. Bombardier, C., D. D. Gladman, M. B. Urowitz, D. Caron, and C. H. Chang. 1992. Derivation of the SLEDAI: a disease activity index for lupus patients: the Committee on Prognosis Studies in SLE. *Arthritis Rheum.* 35: 630–640.
23. Petri, M., J. Buyon, and M. Kim. 1999. Classification and definition of major flares in SLE clinical trials. *Lupus* 8: 685–691.
24. Babcock, G. J., L. L. Decker, M. Volk, and D. A. Thorley-Lawson. 1998. EBV persistence in memory B cells in vivo. *Immunity* 9: 395–404.
25. Khan, G., E. M. Miyashita, B. Yang, G. J. Babcock, and D. A. Thorley-Lawson. 1996. Is EBV persistence in vivo a model for B cell homeostasis? *Immunity* 5: 173–179.
26. Schaefer, B. C., J. L. Strominger, and S. H. Speck. 1996. A simple reverse transcriptase PCR assay to distinguish EBNA1 gene transcripts associated with type I and II latency from those arising during induction of the viral lytic cycle. *J. Virol.* 70: 8204–8208.
27. Chen, F., J. Z. Zou, L. di Renzo, G. Winberg, L. F. Hu, E. Klein, G. Klein, and I. Ernberg. 1995. A subpopulation of normal B cells latently infected with Epstein-Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1. *J. Virol.* 69: 3752–3758.
28. Tierney, R. J., N. Steven, L. S. Young, and A. B. Rickinson. 1994. Epstein-Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state. *J. Virol.* 68: 7374–7385.
29. Tanaka, N., H. Kimura, K. Iida, Y. Saito, I. Tsuge, A. Yoshimi, T. Matsuyama, and T. Morishima. 2000. Quantitative analysis of cytomegalovirus load using a real-time PCR assay. *J. Med. Virol.* 60: 455–462.
30. Miyashita, E. M., B. Yang, K. M. Lam, D. H. Crawford, and D. A. Thorley-Lawson. 1995. A novel form of Epstein-Barr virus latency in normal B cells in vivo. *Cell* 80: 593–601.
31. Babcock, G. J., L. L. Decker, R. B. Freeman, and D. A. Thorley-Lawson. 1999. Epstein-Barr virus-infected resting memory B cells, not proliferating lymphoblasts, accumulate in the peripheral blood of immunosuppressed patients. *J. Exp. Med.* 190: 567–576.
32. Odendahl, M., A. Jacobi, A. Hansen, E. Feist, F. Hiepe, G. R. Burmester, P. E. Lipsky, A. Radbruch, and T. Dörner. 2000. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *J. Immunol.* 165: 5970–5979.
33. Qu, L., M. Green, S. Webber, J. Reyes, D. Ellis, and D. Rowe. 2000. Epstein-Barr virus gene expression in the peripheral blood of transplant recipients with persistent circulating virus loads. *J. Infect. Dis.* 182: 1013–1021.
34. Rose, C., M. Green, S. Webber, D. Ellis, J. Reyes, and D. Rowe. 2001. Pediatric solid-organ transplant recipients carry chronic loads of Epstein-Barr virus exclusively in the immunoglobulin D-negative B-cell compartment. *J. Clin. Microbiol.* 39: 1407–1415.
35. Bermas, B. L., M. Petri, D. Goldman, B. Mittleman, M. W. Miller, N. I. Stocks, C. S. Via, and G. M. Shearer. 1994. T helper cell dysfunction in systemic lupus erythematosus (SLE): relation to disease activity. *J. Clin. Immunol.* 14: 169–177.
36. Kilger, E., A. Kieser, M. Baumann, and W. Hammerschmidt. 1998. Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. *EMBO J.* 17: 1700–1709.
37. Caldwell, R. G., J. B. Wilson, S. J. Anderson, and R. Longnecker. 1998. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity* 9: 405–411.
38. Zimmer-Strobl, U., B. Kempkes, G. Marschall, R. Zeidler, C. Van Kooten, J. Banchemareau, G. W. Bornkamm, and W. Hammerschmidt. 1996. Epstein-Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival. *EMBO J.* 15: 7070–7078.
39. Miyashita, E. M., B. Yang, G. J. Babcock, and D. A. Thorley-Lawson. 1997. Identification of the site of Epstein-Barr virus persistence in vivo as a resting B cell. *J. Virol.* 71: 4882–4891.
40. Qu, L., and D. T. Rowe. 1992. Epstein-Barr virus latent gene expression in uncultured peripheral blood lymphocytes. *J. Virol.* 66: 3715–3724.
41. Hochberg, D., T. Souza, M. Catalina, J. L. Sullivan, K. Luzuriaga, and D. A. Thorley-Lawson. 2004. Acute infection with Epstein-Barr virus targets and overwhelms the peripheral memory B-cell compartment with resting, latently infected cells. *J. Virol.* 78: 5194–5204.
42. Joseph, A. M., G. J. Babcock, and D. A. Thorley-Lawson. 2000. EBV persistence involves strict selection of latently infected B cells. *J. Immunol.* 165: 2975–2981.
43. James, J. A., K. M. Kaufman, A. D. Farris, E. Taylor-Albert, T. J. Lehman, and J. B. Harley. 1997. An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. *J. Clin. Invest.* 100: 3019–3026.
44. Khanna, R., D. J. Moss, and S. R. Burrows. 1999. Vaccine strategies against Epstein-Barr virus-associated diseases: lessons from studies on cytotoxic T-cell-mediated immune regulation. *Immunol. Rev.* 170: 49–64.
45. Crawford, D. H., J. M. Edwards, P. Sweny, A. V. Hoffbrand, and G. Janossy. 1981. Studies on long-term T-cell-mediated immunity to Epstein-Barr virus in immunosuppressed renal allograft recipients. *Int. J. Cancer* 28: 705–709.
46. Crawford, D. H., P. Sweny, J. M. Edwards, G. Janossy, and A. V. Hoffbrand. 1981. Long-term T-cell-mediated immunity to Epstein-Barr virus in renal-allograft recipients receiving cyclosporin A. *Lancet* 1: 10–12.
47. Yao, Q. Y., A. B. Rickinson, J. S. Gaston, and M. A. Epstein. 1985. In vitro analysis of the Epstein-Barr virus: host balance in long-term renal allograft recipients. *Int. J. Cancer* 35: 43–49.
48. Hopwood, P., and D. H. Crawford. 2000. The role of EBV in post-transplant malignancies: a review. *J. Clin. Pathol.* 53: 248–254.
49. Kang, I., and S. H. Park. 2003. Infectious complications in SLE after immunosuppressive therapies. *Curr. Opin. Rheumatol.* 15: 528–534.
50. Kahl, L. E. 1994. Herpes zoster infections in systemic lupus erythematosus: risk factors and outcome. *J. Rheumatol.* 21: 84–86.
51. Seemayer, T. A., T. G. Gross, R. M. Egeler, S. J. Pirruccello, J. R. Davis, C. M. Kelly, M. Okano, A. Lanyi, and J. Sumegi. 1995. X-linked lymphoproliferative disease: twenty-five years after the discovery. *Pediatr. Res.* 38: 471–478.
52. Sullivan, J. L., and B. A. Woda. 1989. X-linked lymphoproliferative syndrome. *Immunodef. Rev.* 1: 325–347.
53. Purtilo, D. T., C. K. Cassel, J. P. Yang, and R. Harper. 1975. X-linked recessive progressive combined variable immunodeficiency (Duncan's disease). *Lancet* 1: 935–940.