Defective Control of Latent Epstein-Barr Virus Infection in Systemic Lupus Erythematosus

Insoo Kang, Timothy Quan, Helena Nolasco, Sung-Hwan Park, Myung Sun Hong, Jill Crouch, Eric G. Pamer, John Greg Howe and Joe Craft

*J Immunol* 2004; 172:1287-1294; doi: 10.4049/jimmunol.172.2.1287
http://www.jimmunol.org/content/172/2/1287

**References**
This article cites 57 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/172/2/1287.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Defective Control of Latent Epstein-Barr Virus Infection in Systemic Lupus Erythematosus

Insoo Kang, Timothy Quan, Helena Nolasco, Sung-Hwan Park, Myung Sun Hong, Jill Crouch, Eric G. Pamer, John Greg Howe, and Joe Craft

EBV infection is more common in patients with systemic lupus erythematosus (SLE) than in control subjects, suggesting that this virus plays an etiologic role in disease and/or that patients with lupus have impaired EBV-specific immune responses. In the current report we assessed immune responsiveness to EBV in patients with SLE and healthy controls, determining virus-specific T cell responses and EBV viral loads using whole blood recall assays, HLA-A2 tetramers, and real-time quantitative PCR. Patients with SLE had an ~40-fold increase in EBV viral loads compared with controls, a finding not explained by disease activity or immunosuppressive medications. The frequency of EBV-specific CD69⁺ CD4⁺ T cells producing IFN-γ was higher in patients with SLE than in controls. By contrast, the frequency of EBV-specific CD69⁺ CD8⁺ T cells producing IFN-γ in patients with SLE appeared lower than that in healthy controls, although this difference was not statistically significant. These findings suggest a role for CD4⁺ T cells in controlling, and a possible defect in CD8⁺ T cells in regulating, increased viral loads in lupus. These ideas were supported by correlations between viral loads and EBV-specific T cell responses in lupus patients. EBV viral loads were inversely correlated with the frequency of EBV-specific CD69⁺ CD4⁺ T cells producing IFN-γ and were positively correlated with the frequencies of CD69⁺ CD8⁺ T cells producing IFN-γ and with EBV-specific, HLA-A2 tetramer-positive CD8⁺ T cells. These results demonstrate that patients with SLE have defective control of latent EBV infection that probably stems from altered T cell responses against EBV.
CD8+ T cells (21). Likewise, CD4+ T cell-mediated cytotoxicity is important for control of EBV infection (22–25), with activated CD4+ T cells expressing Th1-type cytokines able to kill EBV-transformed, autologous lymphoblastoid cells (LCL) (24).

Several new methods have been developed to quantify Ag-specific T lymphocytes directly from clinical samples, bypassing the need for long term in vitro tissue culture. One of these methods is intracellular staining of Ag-stimulated lymphocytes, followed by multiparameter flow cytometric analysis (26). This method is highly effective and accurate in quantifying Ag-specific T cells at the single-cell level while simultaneously demonstrating cytokine synthesis even in whole blood. This assay has been used to quantify and characterize HIV-specific CD4+ T cells and CMV- and VZV-specific CD4+ and CD8+ T cells in prospective studies, discerning a difference of <0.1% of virus-specific T cells among patients (20, 27, 28).

In the present study we investigated the control of latent EBV infection in patients with SLE, measuring EBV viral loads in PBMCs and EBV-specific CD4+ and CD8+ T cell responses using quantitative real-time PCR and multiparameter flow cytometry, respectively. We demonstrate that patients with lupus, compared with healthy controls, have an ~40-fold increase in EBV viral loads in PBMCs, a finding that was independent of B cell numbers, medications, disease activity, and the presence of lupus nephritis. At the same time such patients maintained robust EBV-specific CD4+ T cell responses compared with controls, albeit in the setting of a tendency toward decreased EBV-specific CD8+ T cell responses. These findings suggest that patients with SLE have defective control of latent EBV infection stemming from decreased CD8+ T cell responses against EBV that is compensated by increased EBV-specific CD4+ T cell responses.

Materials and Methods

Patients and determination of disease activity

Patients with SLE (n = 42) who met the 1982 revised American College of Rheumatology criteria for SLE and healthy controls (n = 32) were enrolled in the study. The mean age was similar between patients with SLE and healthy controls (mean ± SD, 38.6 ± 9.1 and 38.8 ± 14.3 years; p = 0.354, by Mann-Whitney U test). The female to male ratio was similar between patient and control groups (38 females and four males in the SLE and 28 females and eight males in the control group; p = 0.074, by χ2 test). Not all patients and controls were included in every analysis; rather, subsets of both were chosen. As in the entire group, the age and gender of lupus patients and controls were not different in subsets. The Yale University Human Investigation Committee approved the study, and informed consent was obtained from each patient and control before enrollment. Demographic and clinical data, including medications, were recorded upon enrollment. Subjects were screened for anti-EBV viral capsid Ag, anti-EBNA IgG Abs, and anti-CMV IgG Abs using an indirect immunofluorescence assay kit (Gibco Laboratories, Salt Lake City, UT) and an ELISA kit (Wampole Laboratories, Cranbury, NJ), respectively, according to the protocols of the manufacturers. Disease activity in patients with SLE was assessed using the SLE disease activity index (SLEDAI), a validated clinical activity test that scores a weighted index of nine organ systems, including central nervous, vascular, renal, musculoskeletal, serosal, dental, immunologic, constitutional, and hematological systems (29). We assigned those patients with an SLEDAI score of 4 or lower to the group with milder disease activity and those with an SLEDAI score of 5 or higher to the group with more severe disease. We divided patients with SLE into two groups according to use, or not, of potentially immunosuppressive therapies (cyclophosphamide, azathioprine, mycophenolate mofetil, and methotrexate). We arbitrarily assigned patients taking 20 mg/day prednisone or higher or its equivalent to the former group (potentially immunosuppressive therapy) and patients taking smaller doses or not taking prednisone to the latter group.

Defective control of latent EBV infection in SLE

These assays were performed as previously described (26). Briefly, 1 ml of heparinized blood was aliquoted into 15-ml conical polypropylene tubes (BD Biosciences Labware, Franklin Lakes, NJ) containing 200 U recombinant anti-CD28 (BD PharMingen, San Diego, CA) and anti-CD49d (BD Biosciences Immunocytometry Systems, San Jose, CA) mAbs at a concentration of 1 μg/ml. Abs were added at optimal stimulatory concentrations (EBV Abs from U.S. Biological (Swampscott, MA) and CMV Abs from MicrobiX (Toronto, Canada)), with lysates of uninfected cells used as a negative control. As positive controls, we used PMA (final concentration, 25 ng/ml/ionomycin (final concentration, 1 μg/ml) and/or staphylococcal enterotoxin B (final concentration, 10 μg/ml). Culture tubes were incubated in a humified, 37°C, 5% CO2 incubator for a total of 6 h, with the last 4 h of incubation in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, MO), a secretion inhibitor to enhance the accumulation of cytokines in the cytoplasm. Blood samples were then lysed and fixed with FACS Lysing Solution (BD Biosciences Labware). Cells were subsequently resuspended in FACS Permeabilization Solution (BD Biosciences Labware) and stained for a typical four-color analysis of CD4+ and CD8+ T cell cytokine responses with a staining Ab mixture of anti-CD4-PE, anti-CD8-allophycocyanin, anti-CD69-PE, and anti-IFN-γ or anti-TNF-α-FTTC (BD PharMingen). Specific cytokine expression occurring within the CD69+ (activated) cell subset was analyzed to enhance the identification of Ag-specific T cells.

In our assays we stimulated whole blood for 6 h with viral Ags based on the results of previous studies (26, 30); such short term stimulation also avoids potential activation-induced cell death (26, 30). Pilot experiments measuring virus-specific T cell responses at various time points from 5–18 h showed no significant differences in results; thus, we picked the 6 h point for our analyses (data not shown).

Measurement of EBV viral loads in PBMCs of patients with SLE and in healthy controls

PBMCs were purified by standard Ficol-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation. As described previously (31), PBMCs were treated with 60 μg/ml proteinase K (Roche, Indianapolis, IN), and DNA was extracted from aliquots of 105 PBMCs. DNA was amplified using primers 1100 and 1181 (Operon, Alameda, CA) for the EBV BMLF1 gene in a PerkinElmer 9600 thermal cycler (Wellesley, MA). The 50 μl of PCR components (Roche) included 10× PCR buffer, 1.5 mmol/liter MgCl2; 0.025 mmol/liter each of dATP, dCTP, dGTP, and dTTP; and 1.0 U Tag DNA polymerase. The EBV-containing Raji cell line was used to develop a standard curve. Thermal cycler parameters were 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR product was hybridized to a BMLF1 probe labeled with Tris rutherium II chelate electrochemiluminescent label (Baron Biotech, Milford, CT). The hybridized PCR product was quantified using a PerkinElmer QPCR 5000. All samples were amplified using β-globin primers to test for DNA integrity.

In some samples the percentage and absolute number of B cells in peripheral blood were measured along with the determination of EBV viral loads. Blood was collected in EDTA-treated tubes. Cells in whole blood were stained for 15 min with anti-CD19 Abs (BD PharMingen). RBCs were lysed using RBC lysing buffer (BD Biosciences Labware) and washed. Cells were then fixed with 2% formaldehyde. Absolute B cell numbers were calculated based on the complete blood count and percentage of B cells.

Measurement of BMLF1-specific CD8+ T cells using HLA-A2 tetramers

Soluble HLA-A2 tetramers were produced as previously described (31, 32). Recombinant MHC class I H chains and β2-microglobulin protein were produced in Escherichia coli transformed with the relevant expression plasmid. Expression of the H chain is limited to the extracellular domain, whereas the C terminus of this domain is modiﬁed by the addition of a substrate sequence for the biotinylating enzyme BirA (Avidity, Boulder, CO). HLA-A2 H chains and human β2-microglobulin were dissolved in 8 mol/liter urea and refolded in the presence of the HLA-A2-restricted BMLF1 lytic peptide (GLCTLVAML) from EBV at 60 μg/ml with protease inhibitors (31). Soluble monomeric complexes were purified by gel filtration over a Superdex 200HR column (Amersham Pharmacia Biotech). Purified monomeric complexes were biotinylated at room temperature for

Analysis of EBV- and CMV-specific CD4+ and CD8+ T cells by multiparameter flow cytometry after short term in vitro Ag stimulation

Several new methods have been developed to quantify Ag-specific T lymphocytes directly from clinical samples, bypassing the need for long term in vitro tissue culture. One of these methods is intracellular staining of Ag-stimulated lymphocytes, followed by multiparameter flow cytometric analysis (26). This method is highly effective and accurate in quantifying Ag-specific T cells at the single-cell level while simultaneously demonstrating cytokine synthesis even in whole blood. This assay has been used to quantify and characterize HIV-specific CD4+ T cells and CMV- and VZV-specific CD4+ and CD8+ T cells in prospective studies, discerning a difference of <0.1% of virus-specific T cells among patients (20, 27, 28).

In the present study we investigated the control of latent EBV infection in patients with SLE, measuring EBV viral loads in PBMCs and EBV-specific CD4+ and CD8+ T cell responses using quantitative real-time PCR and multiparameter flow cytometry, respectively. We demonstrate that patients with lupus, compared with healthy controls, have an ~40-fold increase in EBV viral loads in PBMCs, a finding that was independent of B cell numbers, medications, disease activity, and the presence of lupus nephritis. At the same time such patients maintained robust EBV-specific CD4+ T cell responses compared with controls, albeit in the setting of a tendency toward decreased EBV-specific CD8+ T cell responses. These findings suggest that patients with SLE have defective control of latent EBV infection stemming from decreased CD8+ T cell responses against EBV that is compensated by increased EBV-specific CD4+ T cell responses.
4 h in the presence of 9 μg of BirA enzyme, 200 μmol/L biotin, and 10 mmol/L ATP. Excessive biotin was removed by gel filtration, and monomeric complexes were tetramerized by PE-conjugated streptavidin (Molecular Probes, Sunnyvale, CA) at a 4:1 molar ratio. For tetramer staining, PBMCs were incubated on ice for 1 h in staining buffer (PBS with 0.5% BSA and 0.02% sodium azide) containing PE-conjugated tetramer loaded with the BMLF1 peptide and with anti-CD3-allophycocyanin and anti-CD8-Cy Abs. Stained cells were fixed with 2% formaldehyde and analyzed by flow cytometry.

Statistical analysis
The Mann-Whitney U test was used to compare EBV viral load and the frequency of EBV- and CMV-specific CD4+ and CD8+ T cells between patients with SLE and healthy controls. Spearman correlation analysis was performed to determine bivariate correlations. All statistical analyses were performed using SPSS software (version 10.1; SPSS, Chicago, IL).

Results
Assessment of EBV- and CMV-specific T cell responses in patients with SLE and healthy controls
We first analyzed EBV-specific T cell responses in 32 patients with SLE and in 19 healthy controls, all with anti-EBV viral capsid Ag and anti-EBNA Abs, using whole blood in vitro Ag stimulation, followed by flow cytometric analysis (Fig. 1, A and B, representative examples). The percentages of CD69/CD4+ and CD69/CD8+ T cells that secreted cytokines in samples stimulated with uninfected cell lysates were subtracted from the percentages of T cells that produced cytokines in samples stimulated with viral Ags as previously described (20, 27, 28). In our hands, T cell responses to uninfected cell lysates were quite low; the mean frequencies of CD69/CD4+ T cells producing IFN-γ and TNF-α ± SEM were 0.06 ± 0.005 and 0.09 ± 0.009%, respectively, and the mean frequencies of CD69/CD8+ T cells producing IFN-γ and TNF-α were 0.09 ± 0.013 and 0.09 ± 0.016%, respectively.

Patients with SLE had a higher frequency of EBV-specific CD69/CD4+ T cells producing IFN-γ compared with healthy controls (p = 0.018, by Mann-Whitney U test) and had a trend toward a higher frequency of EBV-specific CD69/CD4+ T cells producing TNF-α (Fig. 1, A and B, representative examples, and Table I). By contrast, the frequencies of EBV-specific CD69/CD8+ T cells producing IFN-γ and TNF-α tended to be lower in patients with SLE than in healthy controls, although these differences did not reach significance (Fig. 1, A and B, representative examples, and Table I).

We next conducted a stratification analysis for EBV-specific T cell responses in patients with SLE based on disease activity, the presence or absence of lupus nephritis, and the use of potentially immunosuppressive therapies (see Materials and Methods). The frequencies of EBV-specific CD69/CD4+ and CD69/CD8+ T cells producing cytokines were not statistically different between patients with mild or more severe activity of disease (n = 18 and n = 14, respectively), patients with and without nephritis (n = 9 and n = 23, respectively), and patients receiving or not receiving potentially strong immunosuppressive therapies (n = 15 and n = 17, respectively).

We then determined the frequency of CMV-specific CD69/CD4+ and CD69/CD8+ T cells producing cytokines in subsets of the same patients (n = 17) and healthy controls (n = 9), all of whom had Abs to CMV. Both groups had comparable frequencies of CMV-specific CD69/CD4+ T cells that secreted IFN-γ and TNF-α. The frequency of CMV-specific CD69/CD8+ T cells producing IFN-γ and TNF-α tended to be lower in patients with SLE than in healthy controls; however, this difference was not statistically significant (Table II).

These data suggest that patients with SLE have a more robust EBV-specific CD4+ T cell memory response than healthy controls, and a trend toward weaker EBV-specific, and CMV-specific, CD8+ memory responses, findings that are not explained by disease severity or immunosuppressive medications.

Measurement of EBV viral loads in PBMCs
EBV viral loads in PBMCs were next measured in a group of 22 consecutive patients with SLE and 21 healthy controls and compared with disease activity and medications in the former group. Patients with SLE had a higher number of copies of EBV DNA in PBMCs than did healthy controls (mean ± SEM, 192.78 ± 95.10 vs 4.65 ± 3.78 copies/10^6 PBMCs, respectively; p = 0.001, by Mann-Whitney U test; Fig. 2A). Based on SLEDAI, the patients with SLE were divided into two groups: mild disease with a SLEDAI score of 4 or less, and more severe disease with a score of 5 or greater (n = 14 and n = 8, respectively). Differences in EBV viral loads were not evident between patients with mild vs more severe disease (means ± SEM, 156.18 ± 147.0 and 81.59 ± 40.13 EBV DNA copies/10^6 PBMC, respectively; p = 0.600, by Mann-Whitney U test; Fig. 2B). Those with and without nephritis (n = 6 and n = 16, respectively) also had similar levels of EBV viral loads (mean ± SEM, 166.48 ± 85.51 and 202.51 ± 128.3 EBV DNA copies/10^6 PBMCs, respectively; p = 0.910, by Mann-Whitney U test; Fig. 2C). EBV viral loads were also compared between patients receiving immunosuppressive therapies (n = 9) and those not receiving these therapies (n = 13). EBV viral loads were similar in these groups (mean ± SEM, 166.48 ± 85.51 and 202.51 ± 128.3 EBV DNA copies/10^6 PBMCs, respectively; p = 0.910, by Mann-Whitney U test; Fig. 2D).

To rule out the possibility that the increased EBV viral loads in PBMCs of patients with SLE were secondary to an increased number of B cells, we compared EBV viral loads and the absolute number and percentage of B cells in a randomly selected subset of patients with SLE (n = 9) and of healthy controls (n = 6). In this
experiment the patients with SLE had higher EBV viral loads compared with healthy controls (mean ± SEM, 120.18 ± 62.6 vs 0.33 ± 0.33 EBV DNA copies/10^3 PBMCs; p = 0.005, by Mann-Whitney U test); however, the absolute number and percentage of B cells in peripheral blood were not different between patients with SLE and healthy controls (mean ± SEM, 162 ± 31.2 × 10^3 and 240 × 10^3 ± 29.0 absolute B cell count/μl, respectively; p = 0.113, by Mann-Whitney U test; mean B cell percentage of lymphocytes ± SEM, 10.8 ± 1.16 and 13.67 ± 1.05%, respectively; p = 0.145, by Mann-Whitney U test).

These data indicate that patients with SLE have higher viral loads in PBMCs than healthy controls, a finding not explained by disease severity or medications or by numbers of peripheral B cells that potentially harbor latent virus.

**Correlation of EBV viral loads and EBV-specific T cells in patients with SLE**

Given our observation that lupus patients had more robust CD4^+ T cell memory responses against EBV, and perhaps less robust CD8^+ responses, we next attempted to correlate viral loads in PBMCs of patients with SLE with Ag-specific cellular responses, determining the latter with flow cytometry after short term in vitro stimulation with EBV lysates. There was an inverse correlation between EBV viral loads in PBMCs and the frequency of EBV-specific CD69^+ CD4^+ T cells producing IFN-γ (r = −0.618; p = 0.014 by Spearman correlation; Fig. 3A). A similar trend was noticed between EBV viral loads and the frequency of EBV-specific CD69^+ CD4^+ T cells producing TNF-α, although this trend did not reach statistical significance (r = −0.290; p = 0.294, by Spearman correlation; Fig. 3B).

By contrast, patients with higher EBV viral loads in PBMCs had a higher frequency of EBV-specific CD69^+ CD8^+ T cells producing IFN-γ, with a positive correlation between these two parameters (r = 0.575; p = 0.031, by Spearman correlation; Fig. 3C). Likewise, there was a trend toward a correlation of EBV viral loads and the frequency of EBV-specific CD69^+ CD8^+ T cells producing TNF-α, although this did not reach statistical significance (r = 0.474; p = 0.087, by Spearman correlation; Fig. 3D).

We also measured BMLF1-specific CD8^+ T cells in patients with SLE who were HLA-A2 positive using HLA-A2 tetramers (Fig. 4, representative example); the frequency of BMLF1-specific CD8^+ T cells measured by tetramers in our hands was comparable to those reported in other studies (0.4–3.0%) (32, 33). We found a positive correlation between viral loads in PBMCs and BMLF1 peptide-specific CD8^+ T cells in patients with SLE (r = 0.900; p = 0.037, by Spearman correlation; Fig. 5).

We also asked whether there were correlations between EBV viral loads and the frequencies of EBV-specific CD69^+ CD4^+ and CD69^+ CD8^+ T cells producing cytokines in healthy controls (n = 12). Correlations were not noted in these analyses, probably a consequence of the undetectable viral loads in the majority of control individuals. Similarly, there was no correlation between EBV viral loads and the frequency of the frequency of BMLF1-specific CD8^+ T cells measured by tetramers (n = 5).

**Discussion**

We have measured EBV viral loads in PBMCs and EBV-specific T cell responses in patients with SLE and healthy controls using quantitative real-time PCR, short term recall assays, and HLA-A2 tetramers. Patients with SLE, compared with healthy controls, had an ~40-fold increase in EBV viral loads in PBMCs, a finding independent of B cell numbers, medications, disease activity, and the presence of lupus nephritis. In addition, compared with controls, patients with SLE had increased EBV-specific CD4^+ T cell responses and perhaps decreased EBV-specific CD8^+ T cell responses. These results demonstrate that patients with SLE have defective control of latent EBV infection that probably stems from altered T cell responses against this virus.

The elevation of EBV viral loads in patients with SLE compared with healthy controls was striking. As we reflected upon this finding, it was perhaps not surprising, given that Miller and colleagues (34) reported some 30 years ago that patients with SLE taking immunosuppressive drugs for renal transplantation secreted more EBV in their saliva compared with patients with chronic uremia and healthy individuals. Although these earlier findings could be explained by disease severity and/or immunosuppression, the explanation could as well be alterations in T cell immune responses that are present in SLE, an explanation in concert with the findings of Tsokos and colleagues (9) that lupus patients have decreased cytokoticity against EBV-infected B cells. Alternatively, the increased viral loads in PBMCs of patients with SLE could be secondary to an increased number of peripheral B cells, a reservoir for

<table>
<thead>
<tr>
<th>EBV-Specific T Cells (Mean % ± SEM)</th>
<th>CD69^+ CD4^+</th>
<th>CD69^+ CD4^+</th>
<th>CD69^+ CD8^+</th>
<th>CD69^+ CD8^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>1.20 ± 0.50</td>
<td>0.37 ± 0.19</td>
<td>0.22 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>MNF-α</td>
<td>0.46 ± 0.13</td>
<td>0.77 ± 0.33</td>
<td>0.44 ± 0.15</td>
<td></td>
</tr>
</tbody>
</table>

Table I. Frequency of EBV-specific T cells in patients with SLE and healthy controls

<table>
<thead>
<tr>
<th>CMV-Specific T Cells (Mean % ± SEM)</th>
<th>CD69^+ CD4^+</th>
<th>CD69^+ CD4^+</th>
<th>CD69^+ CD8^+</th>
<th>CD69^+ CD8^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>0.89 ± 0.25</td>
<td>1.26 ± 0.55</td>
<td>0.58 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>MNF-α</td>
<td>0.61 ± 0.26</td>
<td>0.434</td>
<td>1.06 ± 0.50</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Frequency of CMV-specific T cells in patients with SLE and healthy controls

* Determined by Mann-Whitney U test.
latent EBV; however, this possibility seems unlikely because we did not find differences in the absolute numbers and percentages of B cells between patients and controls. Moreover, patients with SLE frequently have leukopenia, including lymphopenia, rather than leukocytosis (35), a trend noted in our lupus cohort.

It was of interest that EBV viral loads did not correlate with immunosuppressive medications in SLE, especially as the adverse effect of immunosuppression in controlling EBV viral loads is well recognized (31, 36, 37). The discrepancy between a correlation of immunosuppression and viral loads in transplant recipients and in patients with SLE may stem from the fact that immunosuppressive therapies used in the latter are not as strong as those used in patients with organ transplantation (22, 38). Moreover, as we suspect and as we will discuss, it may also reflect intrinsically abnormal viral specific T cell responses in SLE that is present regardless of the level of immunosuppression. We also take note of a recent study of patients with rheumatoid arthritis that reported increased EBV viral loads in PBMCs compared with healthy controls and patients with other inflammatory diseases, although SLE patients were not included in this work (39). This suggests that our observation of increased EBV viral loads in PBMCs of patients with SLE may not be a phenomenon specific for SLE, at least in the rheumatic diseases.

**FIGURE 3.** Correlations of EBV viral loads with EBV-specific T cell responses in patients with SLE (n = 15). EBV viral loads were correlated with the frequency of EBV-specific CD69+ CD4+ T cells producing IFN-γ and TNF-α (A and B) and with the frequency of CD69+ CD8+ T cells producing IFN-γ and TNF-α (C and D). Values on the x-axis are the frequency (percentage) of EBV-specific T cells. Values on the y-axis are natural log-transformed EBV DNA copies per 10^6 PBMCs. The p values were determined by the Spearman correlation.

**FIGURE 4.** BMLF1-specific T cells in HLA-A2-positive and -negative lupus patients. PBMCs from HLA-A2-positive (A) and HLA-A2-negative (B) lupus patients with anti-EBV Abs were stained with Abs to CD3 and CD8 and HLA-A2 tetramers loaded with HLA-A2-restricted BMLF1 peptide. Cells were gated on the CD3+ CD8+ population. The percentage of CD8+ and BMLF1 tetramer-positive T cells is indicated to the right of the rectangular region of each plot.

**FIGURE 5.** Correlation of EBV viral loads with the frequency of BMLF1 lytic peptide-specific CD8+ T cells in patients with SLE (n = 5). Values on the x-axis are the frequency (percentage) of BMLF1 lytic peptide-specific CD8+ T cells measured by HLA-A2 tetramers loaded with the HLA-A2-restricted BMLF1 lytic peptide. Values on the y-axis are natural log-transformed EBV DNA copies per 10^6 PBMCs. The p values were determined by the Spearman correlation.
Cellular immunity is crucial in maintaining EBV latency. As inferred above, EBV-related lymphoproliferative disease has been observed after organ transplantation and in association with AIDS (13, 14), and the infusion of EBV-specific CD4+ and CD8+ T cells prevent and lead to resolution of EBV-associated lymphoma in patients following bone marrow transplantation (22). The development of EBV-associated lymphoproliferative disease closely correlates with the decline of EBV-specific T cell responses and the rise of EBV viral loads (40). Notably, however, EBV viral loads in our patients with SLE, at ~200 DNA copies/10^6 PBMCs, were not as high as those in patients with EBV-associated lymphoproliferative disease, where they may reach 10,000 DNA copies/10^6 PBMCs (40, 41) (J. G. Howe, unpublished observations). This finding is in concert with the fact that patients with SLE do not appear to have an increased incidence of EBV-associated disease (42, 43).

To specifically address EBV-specific T cell immunity in patients with SLE, we analyzed Ag-specific CD4+ and CD8+ T cells using short term recall assays of whole blood for determination of viral specific T cell responses and HLA-A2 tetramers to enumerate BMLF1-specific CD8+ T cells. These methods appear physiologic in evaluating memory T cell responses, avoiding the addition of exogenous materials and long term tissue culture (20, 27, 28). For example, EBV-transformed LCL in long term culture can secrete various cytokines, including IL-10, thereby potentially affecting cytotoxicity results (44, 45). Conversely, the T cells responsible for cytotoxicity are the ones that produce cytokines, in particular Th1 cytokines such as IFN-γ or TNF-α (the ones measured using the short term stimulation assay in our study (33)), with the frequency of these cells correlated with EBV viral loads (24, 41, 46, 47). Thus, we believe that measurement of activated T cells producing Th1 cytokines is a valid marker for T cell-mediated control of latent EBV infection; however, we do note that the frequency of cytokine-producing CD8+ T cells measured in this study may be an underestimate, because we stimulated cells by adding soluble Ags that are processed by the exogenous MHC class I pathway (48–51).

Using these assays, we demonstrated that patients with SLE, compared with healthy controls, had an increased frequency of EBV-specific memory CD69+ CD4+ T cells producing IFN-γ compared with healthy controls. A similar trend, without reaching a level of statistical significance, was noticed in the frequency of EBV-specific CD69+ CD4+ T cells producing TNF-α. Although it is possible that increased EBV-specific CD4+ T cell responses are secondary to the generalized T cell hyper-responsiveness seen in patients with SLE (52–54), this seems unlikely, because we found that the frequency of CMV-specific CD69+ CD4+ T cells producing IFN-γ and TNF-α was similar between patients with SLE and healthy controls.

Several studies have shown a critical role for CD4+ T cells, in particular, Th1 cells, in controlling EBV infection (22–25, 55). CD4+ T cells from fetal cord blood also inhibit lymphoblastoid transformation of EBV-infected B cells (24). Thus, the increased frequency of EBV-specific CD69+ CD4+ T cells producing IFN-γ compared with controls probably reflects an appropriate response to increased EBV viral loads in PBMCs of patients with SLE. The inverse correlation between viral loads and the frequency of EBV-specific CD69+ CD4+ T cells producing IFN-γ supports the latter idea, suggesting that patients with SLE who mount stronger CD4+ T cell immune responses against EBV can better control viral replication. This idea finds support in the observation that CD4+ T cells producing IFN-γ suppress EBV-induced B cell transformation (46).

The increased frequency of EBV-specific CD4+ T cell responses in SLE also could be a compensatory mechanism for defect control of EBV with CD8+ T cells, as suggested by earlier work (9). Although we were not able to specifically identify such a CD8+ T cell defect, we did note some decrease in the frequency of EBV-specific CD69+ CD8+ T cells producing IFN-γ and TNF-α in patients with SLE. These findings suggest a possible CD8+ T cell defect in controlling latent EBV infection in SLE, which leads to increased EBV viral loads in PBMCs. It is not clear whether any defect in the EBV-specific CD8+ T cell responses in SLE is an EBV-restricted or a global phenomenon. The latter is certainly possible, as we also noted a tendency toward decreased CMV-specific memory CD8+ T cell responses in SLE (Table II). Moreover, recent work has demonstrated that patients with SLE have decreased production of IFN-γ and IL-2 and an increased production of IL-4 and IL-10 by CD8+ T cells (56) and decreased cytotoxic T cell function following anti-CD3 triggering (57, 58).

Although patients with SLE may have defective EBV-specific CD8+ T cell responses compared with healthy controls, the numbers of Ag-specific CD8+ T cells did rise in response to increased EBV viral loads. EBV viral loads correlated positively with the frequency of EBV-specific CD69+ CD8+ T cells producing IFN-γ (with a similar trend in CD8+ T cells producing TNF-α) and with the frequency of BMLF1 lytic peptide-specific CD8+ T cells, as measured by HLA-A2 tetramers. A positive correlation between EBV viral loads and EBV-specific CD8+ T cell responses is consistent with findings from studies of patients with HIV infection and organ transplantation (31, 40, 41). In HIV-infected patients with long term nonprogression, EBV viral loads in PBMCs were positively associated with the number of functional EBV-specific CD8+ T cells measured by ELISPOT (41). EBV viral loads in PBMCs also correlate with the frequency of EBV-specific T cells following allogenic peripheral blood stem cell transplantation (31). Thus, the rise of CD8+ T cells in response to increased EBV viral loads in SLE does not appear to be sufficient to suppress EBV viral loads to the level of healthy controls, as EBV viral loads in PBMCs are still high, and the frequency of EBV-specific CD69+ CD8+ T cells tends to be low in SLE. Thus, the CD8+ T cell response, although appropriate, is seemingly inadequate.

The results of our study demonstrate that patients with SLE have increased EBV viral loads in PBMCs that probably stem from inadequate CD8+ T cell responses against EBV. However, patients with SLE can mount appropriate EBV-specific CD4+ T cell responses that account for the lack of development of EBV-associated lymphoproliferative diseases. Although our work does not directly address the intriguing idea that EBV plays a pathogenic role in SLE, it does suggest that patients with SLE have a defective control of latent EBV infection with increased EBV viral loads.

Acknowledgments
We thank Dr. George Miller and Sarah Nikiforow for their helpful discussions, and the clinical faculty (Drs. Gordon Hutchinson, Barbara Roach, Robert McLean, and Mark Schwartz) and fellows (Drs. Rodica Van Solingen, Lisa Suter, Abha Gupta, Jeff Chung, and Sou-Pan Wu) of Yale Rheumatology for help with patient recruitment. We also thank our patients and healthy controls for their kind donations of blood.

References


17. von Herrath, M. G., M. Yokoyama, J. Dockey, M. B. Oldstone, and J. L. Whitton. 1996. CD4-deficient mice have reduced levels of memory cytotoxic T lymphocytes after immunization and show diminished resistance to subsequent virus challenge. J. Virol. 70:1072.


