Distribution and Phenotype of Epstein-Barr Virus-Harboring Cells in Hodgkin’s Disease

By Hermann Herbst, Erik Steinbrecher, Gerald Niedobitek, Lawrence S. Young, Louise Brooks, Nikolaus Müller-Lantzsch, and Harald Stein

The Epstein-Barr virus (EBV) had been associated with Hodgkin’s disease (HD) by various lines of serological and epidemiological evidence prior to the demonstration of EBV antigens and DNA in isolated cases of HD in 1985 and 1987, respectively. Since then, many investigators found EBV genomes in up to 25% of DNA extracts from HD tissue biopsies analyzed by direct filter hybridization methods. Because of the limited sensitivity of this approach, these studies were extended by the application of the polymerase chain reaction (PCR) technique, which showed DNA sequences in considerably larger proportions of cases, ranging between 40% and 80% depending on the sensitivity limits applied. Although most investigators found approximately 55% to 60% of the HD cases to contain substantial amounts of EBV DNA, the significance of these findings remained controversial. Several investigators favored tumor cells, rather than cells of the reactive admixture, as the most probable origin of the EBV DNA, and this view was substantiated by the visualization of EBV DNA by in situ hybridization with [35S]-labeled single-stranded RNA probes and nonisotopically labeled EBER probe showed a phenotype of mature B lymphocytes and a polyclonal composition for a large proportion of the EBER+ small cells. However, in contrast to noninfected cells, CD20 expression was not detectable in many of these cells, which may indicate downregulation of certain differentiation antigens in latently EBV-infected small lymphoid cells.

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by formalin fixation and paraffin embedding. In six cases, snap-frozen aliquots of the tissues, stored at -80°C, were available. Development of the disease was unrelated to human immunodeficiency virus (HIV) infection, and none of these cases was incorporated into previous studies. Histological typing followed the Rye classification. Ten lymph nodes, removed during mastectomy or prostatectomy and showing a regular histology with only moderate sinus histiocytosis, were used as controls.

**Immunohistology.** The production and specificity profiles of the monoclonal antibodies specific for LMP (clones CS1, -2, -3, and -4) and of a rabbit antiserum directed against LMP, were described elsewhere. Monoclonal antibodies against CD20 (L26), CD30 (Ber-H2), and CD68 (KP-1), as well as CD3-specific rabbit antibodies, were obtained from Dakopatts (Glostrup, Denmark). The anti-T-cell receptor β-chain antibody, βF1, was from T-Cell Sciences (Cambridge, MA), and the CD45RO-specific antibody A633 was a kind gift of Dr G.G. Aversa (Stanford, CA). Sections (4 µm) of either formalin-fixed/paraffin-embedded or snap-frozen tissue blocks were stained by the immunoalkaline phosphatase method. Affinity-purified mouse anti-rabbit immunoglobulin, rabbit anti-mouse immunoglobulin antibodies, and APAAP complex (diluted 1:20) were obtained from Dakopatts. Incubation with the CD3-specific antibodies was performed overnight. Formalin-fixed sections required a proteolytic treatment with 1 mg/mL Streptomyces griseus protease (Sigma, Deisenhofen, Germany) for 10 minutes before incubation with the monoclonal antibodies βF1 and Ber-H2.

**Probes.** EBER1- and EBER2-specific fragments were derived from the plasmids pJJl and pJJ2, kindly provided by Dr J. Arrand (Manchester, UK), and subcloned into the BamHI/EcoRI and EcoRI/HindIII sites, respectively, of the transcription vector pBluescriptKS (Promega-Biotech, Madison, WI). For the preparation of IgLC RNA probes, the 550-bp SrfI fragment containing the human IgLCx gene constant segment, and the 600-bp BglII/BamHI fragment containing the IgLCx gene C2 constant segment, respectively, were subcloned into pGEM1 (Promega Biotech). Phages with the IgLCx and IgLCx genomic fragments were kindly provided by Dr P. Leder, (Cambridge, MA). After linearization with the appropriate restriction enzymes (GIBCO-BRL, Karlsruhe, Germany), [3²P]-labeled antisense or sense (control) run-off transcripts were generated using either SP6, T3, or T7 RNA polymerases and [3²P]-uridine-5'-triphosphate (1,250 Ci/mmol; New England Nuclear, Dreieich, Germany) or, alternatively, digoxigenin-11-uridine-5'-triphosphate (Boehringer Mannheim, Mannheim, Germany). A mixture of EBER1- and EBER2-specific RNA probes was applied to increase the sensitivity. The hybridization procedure with either [³²P]-labeled, digoxigenin-labeled, or combinations of [³²P]- and digoxigenin-labeled probes on frozen and paraffin-sections, washing steps including RNase digestion of nonspecifically bound probe, and autoradiography were performed as described. Immobilized digoxigenin was detected using a monoclonal digoxigenin-specific antibody (Boehringer Mannheim; dilution 1:20) and the APAAP procedure. All sections were processed in parallel using the same batches of reagents and probes. Abrogation of specific signals in hybridizations preceded by micrococcal nuclease digestion verified that the targets of the hybridizations were RNA molecules. The specificity of the EBER probes was further tested on six HD-derived cell lines, of which only the line L591, known to harbor EBV, expressed EBER1 and EBER2.

Sequential immunohistochernistry and in situ hybridization. Cryosectionst sections fixed in 4% paraformaldehyde were incubated in 2 mg/mL predigested pronase/1X phosphate-buffered saline (PBS) at 37°C for 5 minutes. Slides were rinsed twice with 0.1 mol/L glycine/1X Tris-buffered saline (TBS). Antibodies were used in freshly prepared RPMI 1640 medium (GIBCO-BRL), pH 7.5, containing 10 mg/mL bovine serum albumin, 1.0 mg/mL yeast tRNA, and 5,000 U/mL heparin ammonium salt (Sigma) to inhibit RNase activity. Incubation was 30 minutes at room temperature each with the monoclonal antibody, rabbit anti-mouse immunoglobulin antibody, and APAAP complex (Dakopatts), the latter two in 1:20 dilution, using RPMI dilution buffer as described above. The last two incubation steps were repeated once for 10 minutes each. Washing after each incubation step was done in 1x TBS. Immobilized antibodies were visualized as described above and slides were immediately subjected to the in situ hybridization procedure. Paraffin sections were similarly treated following dewaxing in xylene and predigestion in 0.5 mg/mL protease VIII (Sigma) in PBS for 8 minutes at room temperature; these further procedures did not differ from those for cryostat sections. Autoradiographic exposure was 4 to 6 days. To estimate the RNA loss during immunostaining procedures, adjacent tissue sections were subjected to in situ hybridization not preceded by immunostaining. The autoradiographic exposure times for slides subjected to the double-labeling procedure were adjusted accordingly.

**RESULTS**

Histological diagnoses and phenotype characteristics of 46 HD cases are summarized in Table 1. Twenty-three cases showed EBER expression in neoplastic cells, while the other 23 cases did not display an EBER-specific signal in any of the H-RS cells even after prolonged autoradiographic exposure. The intensity of the EBER-specific in situ hybridization signal over H-RS cells varied considerably from cell to cell; short exposure times (6 to 12 hours of autoradiography) were particularly useful to assess the atypical nuclear morphology of EBER+ H-RS cells, whereas extended exposure times (> 4 days) resulted in exhaustion of the capacity of the autoradiographic emulsion over most EBER+ cells and were useful to establish the total number of such cells. The number of EBER+ cells was matched by the number of CD30+ cells in adjacent tissue sections. Thus in these HD cases, virtually all H-RS cells, and not only a proportion, expressed EBER transcripts. Hybridization with sense (control) probes resulted in background signals over all cells. Eighteen of 23 cases with EBER+ H-RS cells displayed LMP expression in 10% to 80% of the neoplastic cells, as detected in paraffin sections. In comparison with the monoclonal antibodies, CS1-4, the LMP-specific rabbit antiserum stained smaller percentages of neoplastic cells. Unlike LMP expression, EBER transcripts were not restricted to H-RS cells. In most cases, small lymphoid cells with unsuspicious nuclear morphology were found in small

| Table 1. Phenotype of Hodgkin and Reed-Sternberg Cells in 46 HD Cases |
|-----------------------------|-------|--------|--------|--------|--------|
| Diagnosis*                  | EBER  | LMP    | CD30   | CD20   | CD3   |
| HDlnp                      | 2/21  | 2/2    | 2/2    | 2/2    | 0/2   |
| HDns                       | 10/24 | 8/24   | 24/24  | 2/24   | 3/24  |
| HDmc                       | 10/18 | 7/18   | 18/18  | 1/18   | 2/18  |
| HDld                       | 1/2   | 1/2    | 2/2    | 0/2    | 0/2   |
| Total                      | 23/46 | 18/46  | 46/46  | 5/46   | 5/46  |

*Histotypes of HD: lp, lymphocyte predominance, nodular subtype; ns, nodular sclerosis; mc, mixed cellularity; iid, lymphocyte depletion.†Number of cases with labeled H-RS cells per number of cases studied.
Table 2. Numbers of EBER-Expressing Cells in 46 HD Cases With EBER+ or EBER- H-RS Cells

<table>
<thead>
<tr>
<th>No. of EBER+ cells/0.5-cm² Section Area</th>
<th>0</th>
<th>1-20</th>
<th>21-50</th>
<th>51-100</th>
<th>101-150</th>
<th>151-200</th>
<th>201-250</th>
<th>&gt;250</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD cases with EBER+ H-RS cells</td>
<td></td>
<td></td>
<td>2</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HD cases with EBER- H-RS cells</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>5</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>All HD cases</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>5</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
</tbody>
</table>

Elevated numbers of EBER+ cells (> 100) cells were found in 26 of 46 cases: in all those HD cases carrying EBER+ H-RS cells (23 cases), as well as in three cases with EBER- tumor cells.

numbers (Table 2), regardless of whether the neoplastic cells were EBV-infected (Fig 1). In two cases with EBER+ H-RS cells, no small EBER+ cells could be identified. In two additional biopsies, no EBER+ cells were present at all, even in serial sections; three cases, two of which did not show EBV infection of the tumor cells, displayed high numbers of EBER-expressing small lymphoid cells, often accumulated in incoherent clusters. Two of the 10 control tissues with intact nodal architecture displayed only few interfollicular EBER+ cells (up to 10 per 0.5-cm² section area); the other tissues did not show EBER-specific signals. Hybridizations with IgLC gene probes were performed with all tissues that did not contain EBER+ cells. These cases displayed intense labeling of plasma cells within 48 hours of exposure, thus excluding the possibility of a failure to detect EBER+ cells because of RNA degradation.

Simultaneous isotopic/nonisotopic in situ hybridization double-labeling was used to characterize EBV-infected cells in each six of HDmc and HDns cases with LMP+ tumor cells and larger numbers of EBER+ small cells. With respect to EBER-specific labeling, the results were identical to those previously obtained with [35S]-labeled EBER-specific probes. In all cases, the tumor cells did not display any IgLC transcripts, whereas approximately each 30% of the small EBER+ cells showed IgLCx or IgLCα gene RNA, respectively (Fig 2).

To further characterize EBER-expressing cells, nine HD cases, particularly cases with either an EBER- or EBER+/CD20-/CD45RO-/TcRβ- H-RS cell phenotype, were subjected to the sequential immunohistology/isotopic in situ hybridization double-labeling procedure on frozen and paraffin sections using antibodies against CD20, CD30, CD45RO, CD68, LMP, and the TcRβ-chain. In cases with EBER+ H-RS cells, CD30-specific staining virtually matched with EBER expression (Fig 3, Table 3). LMP staining was always associated with EBER-specific autoradiographic signals, and CD68 reactivity was not coexpressed with EBER in any of the cases. Approximately 6 of 10 EBER+ reactive cells were detected by the CD20 antibody L26 in...
Fig 2. Detection of EBER (red substrate) and IgL<sub>Cx</sub> gene transcripts (silver grains) by simultaneous in situ hybridization showing EBER<sup>+</sup>/IgL<sub>Cx</sub><sup>-</sup> H-RS cells, an EBER<sup>+</sup>/IgL<sub>Cx</sub><sup>-</sup> small B cell (arrow) with colocalization of both signals, and several EBER<sup>-</sup>/IgL<sub>Cx</sub><sup>+</sup> small cells. Paraffin section, in situ hybridization with [<sup>35</sup>S]-labeled RNA antisense IgL<sub>Cx</sub> probe and digoxigenin-labeled antisense EBER probes, APAAP procedure, autoradiographic exposure 5 days, original magnification x540.

Fig 3. Detection of EBER1 and EBER2 transcripts in tissue from mixed cellularity type HD, in situ hybridization preceded by immunostaining with the antibody Ber-H2 (CD30). EBER transcripts are almost exclusively associated with CD30<sup>-</sup> atypical cells. The autoradiographic signal is heterogeneous, ranging from densely labeled cells to cells with no or only few grains due to the short exposure time of 24 hours. (Cryostat section; original magnification x410.)

Fig 4. Detection of EBER1 and EBER2 transcripts in tissue from mixed cellularity type HD, in situ hybridization preceded by immunostaining with the antibody L26 (CD20). EBER transcripts are associated with CD20<sup>-</sup> atypical cells and few CD20<sup>-</sup> small cells (arrows). Cryostat sections, in situ hybridization, [<sup>35</sup>S]-labeled RNA antisense probes, autoradiographic exposure 24 hours. (Original magnification x290.)

Fig 5. In situ hybridization with EBER-specific antisense probes preceded by immunostaining with the antibody L26 (CD20). EBER-specific autoradiographic signal and immunostaining colocalized in a small lymphoid cell. H-RS cells are EBER<sup>+</sup> (arrow). HD, nodular sclerosing type, paraffin section, autoradiographic exposure 48 hours. (Original magnification x250.)

Fig 6. In situ hybridization with EBER-specific antisense probe preceded by immunostaining with the antibody L26 (CD20) showing a cluster of EBER<sup>+</sup> reactive cells with weak or absent immunohistological signal. HD, mixed cellularity type, paraffin section, autoradiographic exposure 48 hours. (Original magnification x120.)

Fig 7. Detection of EBER1 and EBER2 transcripts in a mixed cellularity type HD case, in situ hybridization preceded by immunostaining with the antibody A6 (CD45RO). EBER transcripts in H-RS and reactive cells are absent from CD45RO<sup>+</sup> cells. Paraffin section, in situ hybridization with [<sup>35</sup>S]-labeled RNA antisense probes, autoradiographic exposure 48 hours. (Original magnification x280.)
whereas neighboring EBER-/CD20+ cells were still strongly reactive, second time point by CD30-specific staining only, because the nuclear activity of EBER+ small cells. Unambiguous reactivity of mere technical problem resulting in reduced immunoreactivity of EBER+/CD20+ small cells was considerably lower than of EBER+ cells with the TcRp-specific antibody PF1 or cryostat sections (Fig 4). In paraffin sections, the proportion of EBER+/CD20+ small cells was considerably lower and the staining was reduced to weak signal intensity, whereas neighboring EBER-/CD20+ cells were still strongly labeled (Figs 5 and 6). Furthermore, an HD case with CD20+/EBER+ H-RS cells showed an intense CD20 staining of the EBER-labeled tumor cells, further excluding a mere technical problem resulting in reduced immunoreactivity of EBER+ small cells. Unambiguous reactivity of EBER+ small cells with the TcRβ-specific antibody BF1 or the antibody A6 (CD45RO) was not found in any of the tissues (Fig 7). EBER+ reactive cells were thus either of B-cell phenotype (CD20+/IgLC+) or could not be assigned to a particular lineage.


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### DISCUSSION

Detection of the highly abundant EBER transcripts represents a considerable technical advancement in the study of EBV infection, because it permits visualization of practically all latently EBV-infected cells in tissue sections. In contrast to DNA-DNA in situ hybridization, use of single-stranded antisense EBER-specific RNA probes and removal of nonspecifically bound probe by ribonuclease digestion result in low background signal and facilitate discrimination between EBER+ and EBV− cells. It is thus possible to address some of the questions that remained open in previous studies, eg, as to the presence of EBV in mainly neoplastic or reactive cells in HD tissue biopsies, and their number, distribution, and phenotype.

In situ hybridization for the detection of EBER transcripts showed the presence of EBV in H-RS cells of 23 of 46 HD cases that were not included in any of our previous reports. Except for the two cases of HD1p, which may be not representative, HDnc was the histological type of the disease most frequently associated with the virus, followed by HDs. These results are well in agreement with a recently published study describing EBER+ H-RS cells in 11 of 23 cases of typical HD. LMP was detectable by immunohistochemistry in variable proportions of EBER+ neoplastic cells of 18 cases. This figure supports the view that infection of malignant CD30+ cells. Some cases did not display any EBER+ cells in only very small numbers, or even their absence, in most cases is likely to explain why EBER+ reactive cells were not described in a previous report on EBER expression in HD. With respect to the normal lymph node tissues, similar results were obtained in a study focusing on nonmalignant lymphoid lesions. The observation of EBER+ cells in only a low proportion of normal lymph nodes is in apparent contradiction to the fact that worldwide more than 90% of the population is seropositive for EBV-specific antibodies. Quantitative evaluation of EBV-infected peripheral blood cells, on the other hand, showed a very low frequency of EBV+ cells (1 to 5 infected cells per 10⁸ mononuclear leukocytes) in healthy donors, which is in good agreement with the histomorphological findings. Furthermore, antibody titers are unlikely to be determined only by EBV infection of lymphoid cells, because epithelial cells also represent targets for this herpes virus.

### Table 3. Summary of Double-Labeling Results in a Case of Nodular Sclerosing Type HD With EBER+ H-RS Cells

<table>
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<tr>
<th>Combination</th>
<th>Autoradiographic Exposure Time</th>
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<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>EBER+/CD30− cells</td>
<td>90</td>
</tr>
<tr>
<td>EBER−/CD30− cells</td>
<td>2</td>
</tr>
<tr>
<td>EBER+/CD30+ cells</td>
<td>8</td>
</tr>
<tr>
<td>EBER+ and/or CD30+ cells</td>
<td>100</td>
</tr>
</tbody>
</table>

Per 100 EBER+ and/or CD30+ cells; H-RS cells were identified by morphology and immunophenotype at the first time point, and at the second time point by CD30-specific staining only, because the nuclear morphology was concealed by the autoradiographic signal.

EBV was not restricted to neoplastic cells in biopsies from HD patients. Variable, usually small numbers of EBER+ reactive lymphoid cells were found in HD cases with EBER+ or EBER− H-RS cells. Absolute numbers could be determined precisely in cases without EBV infection of H-RS cells. In addition to the cytomorphic aspect, sequential double-labeling using markers not expressed by the neoplastic cell population proved helpful to estimate the frequency of EBER+ reactive cells in tissues with EBER+ H-RS cells. Some cases did not display any EBER+ cells even in serial sections. This absence of EBER expression did not represent a problem of tissue preservation, because IgLC gene transcripts could be detected in plasma cells and B cells. The presence of EBER+ reactive cells in only very small numbers, or even their absence, in most cases is likely to explain why EBER+ reactive cells were not described in a previous report on EBER expression in HD. With respect to the normal lymph node tissues, similar results were obtained in a study focusing on nonmalignant lymphoid lesions. The observation of EBER+ cells in only a low proportion of normal lymph nodes is in apparent contradiction to the fact that worldwide more than 90% of the population is seropositive for EBV-specific antibodies. Quantitative evaluation of EBV-infected peripheral blood cells, on the other hand, showed a very low frequency of EBV+ cells (1 to 5 infected cells per 10⁸ mononuclear leukocytes) in healthy donors, which is in good agreement with the histomorphological findings. Furthermore, antibody titers are unlikely to be determined only by EBV infection of lymphoid cells, because epithelial cells also represent targets for this herpes virus.
Counting of EBER+ cells per tissue section area showed elevated numbers (>100) of such cells in all cases with EBER+ H-RS cells and, additionally, in three cases with EBER− neoplastic cells, ie, in 26 of 46 HD cases (56%; Table 2). This proportion is in good agreement with the results of several semiquantitative PCR studies11,12,14,18 showing increased numbers (104 internal repetitive fragment target sequences11,12) of EBV genomes in 55% to 60% of the cases. The results presented here support the previous interpretation of PCR data that an increased EBV genome content of HD tissues is most likely related to the presence of EBV in tumor cells, and in only rare instances due to proliferations of EBV-infected reactive cells.11

Simultaneous double-labeling experiments helped to characterize the majority of EBER+ reactive cells as IgLC+ mature B lymphocytes. The presence of both types of IgLC, κ and λ, indicates an oligoclonal or polyclonal composition of these cells; however, it does not provide information about the size of individual clones. H-RS cells did not display IgLC transcripts, which supports previous observations,47 suggesting their origin from immature B cells, T cells, or other cells.6 Immunophenotyping of reactive EBER+ cells was problematic and, particularly in paraffin sections, virtually impossible for the vast majority of these cells. In frozen sections, most of the small EBER+ cells could be stained with the antibody L26 (CD20). However, in paraffin sections, the number of these cells was reduced. CD20+/EBER− reactive cells and CD20+/EBER+ tumor cells, on the other hand, could be detected easily with our immunohistological procedure. These observations, in conjunction with the IgLC+ phenotype of most EBER+ small cells and the established immunophenotype of resting and activated, noninfected B lymphocytes as constantly CD20+ cells,48 led us to conclude that expression of the CD20 antigen may be downregulated in latently EBV-infected small lymphoid cells in vivo. The CD20 antigen directly regulates transmembrane ion flux in B lymphocytes and is a key structure for antigen-independent B-cell activation.49 Downregulation of this activation-associated antigen may thus have specific advantages for EBV-harboring cells. To test this hypothesis, it will be necessary to quantitate the density of CD20 and other antigens on suspended cells, and nonisotopic EBER-specific labeling may facilitate the identification of EBV-infected cells in such experiments. LMP expression was not observed in any of the reactive cells, which is in keeping with published data.50,51 and may facilitate evasion of these cells from immune recognition. This interpretation is also supported by the previously established absence of the EBNA2 latent gene product13,21,52 and EBV lytic cycle antigens (early antigen,53 viral capsid antigen,50 and gp250/350 membrane antigen13) from any cells in HD tissues.

In general, the majority of EBER+ cells were of (CD20+) /IgLC+/LMP− phenotype, whereas EBER+/TcRβ+ or EBER+/CD45RO+ cells, ie, mature T cells, were not detectable. This raises the possibility that immature lymphoid cells, ie, cells with incomplete or missing antigen receptor gene rearrangements, were among the EBER+/IgLC− subpopulation.51 However, our estimate of the proportions of EBER+ B and T cells is largely based on cases with elevated numbers of EBER+ reactive cells, which may not be representative for HD cases with small numbers of EBER+ reactive cells. The presence of CD43+ EBV-infected cells in addition to occasional B cells was recently observed in some cases of HD, and interpreted as evidence for a T-cell nature of these CD43+ cells.27 However, CD43 antigen is not lineage-specific; moreover, it is found on many EBV+ lymphoblastoid B-cell lines.52 Thus, whereas EBER+/TcRβ+/CD45RO+ small cells arising in certain pediatric immunodeficiency syndromes can be identified with our methodology (H. Herbst, unpublished observation), it must be concluded that such EBER+ mature T cells, if present at all, appear to be extremely rare in lymph nodes from normal adults and HD patients.

The frequency of EBER+ cells in the reactive admixture was slightly higher when compared with nonhyperplastic control lymph nodes, but their phenotypic properties were similar. The two groups of HD, with or without EBV-harboring H-RS cells, respectively, did not differ from each other with regard to the occurrence, frequency, and phenotype of reactive EBER+ cells. Thus, in the majority of HD patients, the postulated specific isolated immune defect that permits proliferation of tumor cells expressing a viral neo-antigen, LMP, is obviously not accompanied by an unrestricted proliferation of reactive EBER+ lymphoid cells. This is in sharp contrast to the pattern seen during infectious mononucleosis,53 as well as in lymph nodes of immunosuppressed transplant recipients or HIV-infected individuals, who generally display larger numbers of EBER+ cells.46 It is thus likely that there are distinct immunological differences between patients with global immunodeficiencies, which are at risk to develop EBV-induced oligoclonal or monoclonal non-Hodgkin’s lymphoma,54,55 on the one side, and HD patients on the other.

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