Inhibition of Epstein–Barr virus-induced growth proliferation by a nuclear antigen EBNA2-TAT peptide

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Epstein–Barr virus (EBV) causes infectious mononucleosis and is associated with cancers in immunocompromised populations. Antiviral drugs targeted against lytic viral replication have limited efficacy in these disease settings. EBV infection of peripheral blood mononuclear cells induces growth proliferation and the EBV latency Epstein–Barr virus-encoded nuclear antigen (EBNA)2 transcriptional transactivator (TAT) is essential for this response. EBNA2 targets the cellular DNA-binding protein CBFI to mimic activated Notch signaling. A 10-aa peptide from the CBFI interaction domain of EBNA2 was synthesized as a fusion with the protein transduction domain of HIV-1 TAT. The EBNA2-TAT peptide blocked EBNA2-CBFI interaction in an in vitro GST affinity assay and labeling with fluorescein confirmed that the EBNA2-TAT peptide efficiently entered cultured B cells. Neither EBNA2-TAT, nor a mutant peptide with a 2-aa substitution that was unable to block the EBNA2-CBFI interaction, significantly affected the growth of non-EBNA2-expressing EBV(−) B cells or Burkitt’s lymphoma Akata cells. However, treatment of an EBV-immortalized lymphoblastoid cell line with the EBNA2-TAT peptide stopped cell growth and reduced cell viability. RT-PCR analyses of gene expression in the peptide-treated lymphoblastoid cell line cultures revealed that EBNA2-TAT treatment down-regulated the EBNA2-responsive viral LMP1 and LMP2 genes and cellular CD23, intercellular adhesion molecule 1, BATF, and Cdk1 genes while up-regulating expression of the cyclin-dependent kinase inhibitor p21. EBV-induced outgrowth of B cells from cultured peripheral blood mononuclear cells was also blocked in a dose-responsive manner by the EBNA2-TAT peptide. This study suggests that cell-permeable EBNA2 peptides may have potential as novel anti-EBV therapeutics.

EBNA2 is essential for EBV-immortalization of B cells and contributes to the process as a transcriptional activator and a mediator of cell survival. In both of these roles, EBNA2 mimics aspects of activated Notch signaling (12–16). EBNA2 is one of the first viral genes expressed after infection and regulates the expression of the other EBNA and LMP genes as well as reprogramming cell gene expression. Like activated Notch, EBNA2 targets responsive promoters predominantly through interaction with the CSL family [CBF1, Su(H), Lag-1] DNA-binding protein CBF1 (17–20) with additional interactions with proteins such as Spi/Pu.1 (21, 22), AUF1 (23), SKIP (24), DP103 (25), the SWI/SNF complex (26), and histone acetyl transferases (27) also influencing promoter responsiveness. The critical importance of the EBNA2–CBFI interaction is highlighted by the observation that EBV carrying a mutated EBNA2 unable to bind CBF1 is incapable of immortalizing B cells (28). The antiapoptotic function shared by EBNA2 and activated Notch is mediated through binding to the immediate-early response factor Nur77 and prevention of Nur77-induced cell death (29, 30).

The dependence on CBF1 as a partner for EBNA2 promoter targeting raises the possibility that pharmacological disruption of their interaction might represent a way to impact on the B cell growth proliferative response induced by latent EBV infection. Comparative protein sequence analysis revealed several short amino acid sequences within the EBNA2–CBFI interaction domain that are highly conserved across the different EBV strains and EBV-related lymphocryptoviruses (31–33). In earlier work, peptides representing these conserved sequences were tested as competitors for EBNA2 binding by using an in vitro EMSA assay. A 10-aa peptide was identified that was an effective competitor for EBNA2 binding to CBF1. A version of this peptide in which the two tryptophan residues were changed to serine and arginine did not affect the interaction (34). However, assessing the potential of this reagent to disrupt EBNA2 function in EBV-infected cells requires a means of delivering the peptide across the cell plasma membrane and into the nucleus.

Recently, short basic regions termed protein transduction domains (PTDs) have been identified and shown to be able to transport linked cargo such as peptides, proteins, or nucleic acid into living cells (35–37). Three proteins that contain PTDs are HIV transcriptional transactivator (TAT), Drosophila antennapedia, and herpes simplex virus VP22 (38–41). A synthetic oligoarginine peptide also acts as an effective PTD (42–44). We generated an EBNA2-TAT PTD peptide and tested this reagent.
for its ability to enter B cells and to affect B cell outgrowth induced by EBV infection. The peptide had minimal toxicity even at high concentration and antagonized EBNA2 activity in both established EBV⁺ LCL cell lines and in primary B cell outgrowth assays. The results suggest that, with further development, PTBD-based peptides may have therapeutic potential as anti-EBV reagents in situations of immunocompromise or severe acute disease where the EBV growth proliferative program places the individual at risk for subsequent development of EBV-associated malignancies.

Materials and Methods

Peptide Synthesis and Detection. Peptides were synthesized by standard fluorenylmethoxycarbonyl methodology, were purified by RP-HPLC, and were analyzed by MS. Concentrations were based on peptide mass. An N-terminal aminohexanoic acid linker to fluorescein was incorporated to reduce intramolecular interactions.

Intracellular localization of an N-terminal fluorescein-conjugated version of the EBNA2-TAT peptide (F-EBNA2-TAT) was performed by pulsing the cells with 100 μM peptide in PBS, pH 7.4, plus 2% FCS for 20 min at room temperature. The cells were then washed with PBS, were resuspended in 50 μl of PBS, were spotted onto slides, were fixed in 4% paraformaldehyde, were mounted with 4’,6-diamidino-2-phenylindole (Vector Laboratories), and were imaged by using a fluorescence microscope.

For cellular uptake and stability, the cells were pulsed with 50 μM peptide in RPMI medium 1640 with 10% FCS, were washed with PBS, were lysed in Tris-tricine loading buffer [1 M Tris–HCl (pH 6.8)/0.4% (vol/vol) glycerol/14% (wt/wt) SDS/0.3 M DTT/0.06% (vol/vol) Coomassie blue stain], were normalized by a BCA protein assay (Pierce), and were run on a 10% Tris-tricine gel. Gel peptide levels were imaged and quantitated with FluorChem (Alpha Innotech, San Leandro, CA).

GST Affinity Assay. In vitro transcription/translation of CBF1 was carried out by using a TNT T7 quick-coupled transcription/translation system (Promega) and the plasmid pSG5-CBF1 (JH261). GST-tagged EBNA2 (252–425) (PDL115) was prepared by standard procedures. Purified GST-EBNA2 (252–425) was incubated with 2 μl of 35S-labeled CBF1 in the absence or presence of competitor EBNA2-TAT peptides at 0.1, 1, 10 or 100 μg for 1 h at room temperature. The beads were washed three times in NETN buffer [100 mM NaCl/1 mM EDTA/0.2% Nonidet P-40/20 mM Tris-HCl (pH 8.0)/0.2 mM PMSF], and was added to 30 μl of sample buffer. Samples were boiled and electrophoresed through SDS/12% PAGE gels, which were dried and exposed to x-ray film. Images were quantitated with FluorChem (Alpha Innotech).

Growth and Survival Assays. DG75, Akata, and an Akata virus-immortalized LCL were fed daily with fresh medium or medium containing peptide. Cell viability was determined by using Trypan blue exclusion, and metabolic activity was measured by using the CellTiter-Glo (Promega) assay.

Proliferation Assays. The EBV virus was obtained by treating Akata or Akata Bx1 cells [gift of L. Hutt-Fletcher (Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, LA) (45)] with 50 μg/ml anti-IgG (Cappel)]. Virus supernatant was concentrated by using Centricon Plus-80 filters (Millipore). PBMCs (Johns Hopkins Oncology Center Cell Procurement Bank) were infected with concentrated virus for 3 h and were cultured in RPMI medium 1640 plus 10% FCS for 7 days. Medium, plus or minus peptide, was replaced daily. Absorbance was performed in 1- or 10-well replicates. Colony outgrowth and EBV-GFP(+) cells were monitored by using fluorescence microscopy. For fluorescence-activated cell sorter analysis, PBMCs were incubated with anti-CD19 phycoerythrin-conjugated antibody (Becton Dickinson Pharmingen).

RNA Extraction and RT-PCR. RNA was extracted from untreated and peptide-treated LCLs by using RNeasy (Qiagen, Valencia, CA). RNA was treated with Dnase (Invitrogen) according to manufacturer’s instructions. cDNA synthesis was carried out with AMV RT (Promega). PCR conditions were 2 min at 95°C, 30 sec at 55°C, 30 sec at 72°C, 30 sec at 95°C for 35 cycles, then for 10 min at 72°C. The following PCR primers were used: LMP1 5’-GTGATCTTGAGAAGCCAGGAG-3’ and 5’-CGTGGGCCGAGAGCCCCACTAC-3’; LMP2A 5’-GACATCTACCTAGAGTCGAAG-3’ and 5’-CTGACAGAGATGAAGAGTCCGAG-3’; B-cell translocation gene 5’-GGTGAATGATGCTCTGG-3’ and 5’-AGGAACCTTCTTACCTACT-3’; BATF 5’-GACAAGAGACGCCAGAGGTTG-3’ and 5’-TGAAGGCGGATGCTTCTGGT-3’; p21 5’-GTCCGTCAGAACCCTATGGCCG-3’ and 5’-TGACAGGTCCATACCTGTTCT-3’; PDL115, 5’-TGGCCTTGGTGGTTACGAGG-3’ and 5’-ATGGAAAGCGATGATG-3’; and TATA box-binding protein 5’-CACGAACCACTGACTT-3’ and 5’-TTTTCTTGATGGCCAGTCTG-3’. Southern blots were performed to confirm the specificity of the RT-PCR products by using the following oligonucleotide probes: LMP1 5’-GTCTCCTCGGTCCTTCTTGAG-3’ and 5’-GTGGCTCTGCTCTCCTTGAG-3’; LMP2A 5’-GGTACAAACGGTACTAACTG-3’ and 5’-CTCCGGAGAATCACAACGAG-3’; and Cdk1 5’-CTACATAATTGATGAC-3’.

Results

Synthesis of EBNA2-TAT Peptides. Previously (34), we had identified a peptide sequence from conserved region 6 of EBNA2, which blocked interaction in vitro between viral EBNA2 and the cellular protein CBF1 that targets EBNA2 to responsive promoters. We wished to determine whether this peptide would be capable of interfering with EBNA2 function in EBV-infected cells. However, such an analysis requires that the peptide be delivered across the cell membrane and into living B cells. We synthesized the EBNA2 peptide as a fusion with TAT amino acid 47–57 (35) (SPGPWWPPV-YGRKKRRQRRR). Two control peptides were also synthesized; F-EBNA2-TAT, in which a fluorescein moiety was conjugated through an aminohexanoic acid linker and an EBNA2-TAT fusion in which the two tryptophans in the EBNA2 peptide were changed to serine and arginine to generate a mutant peptide [EBNA2(mt)-TAT] (SPGPWWPPV-YGRKKRRQRRR). HPLC analysis revealed a single peptide species in each case and MS analysis confirmed the identity of the peptides (data not shown).

To verify that the addition of a TAT fusion partner did not alter the ability of the EBNA2 peptide to interfere with EBNA2 binding to CBF1, a GST affinity assay was performed and the EBNA2-TAT peptide and EBNA2(mt)-TAT peptides were tested as competitors. The CBF1-binding domain of EBNA2, was expressed as a GST fusion protein, GST-EBNA2 (252–425), and interaction between GST-EBNA2 (252–425) and in vitro-translated 35S-labeled CBF1 was demonstrated (Fig. 1A). Added EBNA2-TAT peptide was able to compete with 35S-labeled CBF1 for binding to GST-EBNA2 (252–425) with an IC50 of <10 μM, whereas the control EBNA2(mt)-TAT peptide was an ineffective competitor.
EBNA2-TAT Peptide Affects the Growth and Survival of EBNA2-Expressing LCLs. The effect of different concentrations of EBNA2-TAT and EBNA2(mt)-TAT peptides on the growth of EBV(−) and EBV(+) B cell lines was next examined. Over a 4-day period, treatment of EBV(−) DG75 cells with either peptide at 1-, 25-, or 50-μM concentrations had little effect on cell proliferation (Fig. 2 A and B). Akata cells, which are EBV(+) but are EBNA2(−), were also not significantly affected by treatment with either peptide (Fig. 2 C and D). A newly established EBV− LCL cell line was also insensitive to treatment with mutant peptide at the same three concentrations (Fig. 2F). However, treatment with wild-type EBNA2-TAT peptide at 25- or 50-μM concentrations dramatically reduced proliferation of the LCL cells (Fig. 2E). The metabolic activity of LCLs treated with 25 μM EBNA2-TAT or EBNA2(mt)-TAT peptides was examined in a CellTiter-Glo assay in which intracellular ATP levels are measured by using a luciferase readout. Cells treated with EBNA2-TAT peptide had significantly reduced metabolic activity at both 24 and 48 h after treatment (Fig. 2G). In contrast, cells treated with mutant peptide showed metabolic activity comparable to controls in this assay (Fig. 2G). EBV + LCLs have up-regulated surface adhesion molecules and form macroscopic clumps in culture. Examination of the peptide treated versus the untreated LCL cultures revealed that the EBNA2(mt)-TAT peptide did not affect this growth phenotype, whereas in the EBNA2-TAT-peptide-treated culture, the clumps were completely dispersed (Fig. 2H).

In culture, EBV(+) LCLs express the type III latency genes, which include the membrane proteins LMP1 and LMP2A (1). LMP1 is particularly important for cellular proliferative responses and is also responsible for the up-regulation of cell adhesion antigens (46). Because LMP1 and LMP2A expression in B cells is driven by EBNA2, we examined the effect of the EBNA2-TAT peptides on their expression by using RT-PCR, followed by Southern blotting of the PCR products. LCLs were treated with 50-μM concentrations of either wild-type or mutant peptide for 24 and 48 h. Treatment with EBNA2-TAT peptide significantly reduced the level of both transcripts at 48 h, whereas treatment with mutant peptide did not alter transcript abundance (Fig. 3A). EBNA2 also modulates cellular gene expression and cell genes known to be EBNA2-responsive include CD23 and Cdk1. These genes were also expressed at reduced levels in EBNA2-TAT peptide-treated LCLs (Fig. 3A). RNA expression in peptide-treated cells was also examined by real-time RT-PCR (Fig. 3B). LMP1, CD23, and Cdk1 were down-regulated in this analysis, as was BATF and ICAM-1. In contrast, expression of p21 was increased in peptide-treated cells at both 24 and 48 h.

EBNA2-TAT Peptide Blocks Outgrowth of Colonies from EBV-Infected PBMCs. The EBNA2-TAT peptide interfered with the proliferation of already immortalized B cells that depended on the type III latency program for continued growth. To evaluate the ability of the EBNA2-TAT peptide to prevent EBV-induced initiation of B cell proliferation, a PBMC proliferation assay was established. Human PBMCs were incubated in the presence or absence of EBV virus obtained from induced Akata BX-1 cells. This virus contains a GFP marker inserted in the BXLF1 ORF (45). After 7 days of incubation, the wells were examined for outgrowth of clumps of self-adherent proliferating cells. In wells that were left uninfected, most of the cells were dying and no macroscopic colonies were visible. In contrast, wells that were infected with EBV virus contained large numbers of macroscopic colonies that were GFP(+), demonstrating EBV infection (data not shown). The effect of the EBNA2-TAT and EBNA2(mt)-TAT peptides on B cell outgrowth was assessed by using 1-, 10-, and 50-μM concentrations of peptide. The peptide was added immediately after virus infection and replenished daily. The EBNA2(mt)-TAT peptide had no discernable effect on B cell colony outgrowth at any of the three doses, and in each case, five of five wells contained macroscopic B cell colonies (Fig. 4A). On the other hand, the EBNA2-TAT peptide had a dose-responsive effect on colony formation. At the lowest concentration (1 μM), four of five wells contained visible colonies; at 10 μM, only one of five wells was positive and no colonies were detected in the wells receiving 50 μM EBNA2-TAT peptide (Fig. 4A). The IC50 of the EBNA2-TAT peptide was ∼10 μM, which is consistent with the concentration required to block the interaction of EBNA2 and CBF1 in a GST affinity assay (Fig. 1A).

To verify that the assay was measuring B cell outgrowth, untreated PBMCs were taken at day 0 and at 7 days after EBV infection, and were analyzed by fluorescence-activated cell sorter for expression of the pan B cell marker CD19 (Fig. 4B).
by EBNA2-TAT peptide at 25- and 50-M concentrations for 1 week after which the cultures were switched to peptide free medium for another 3 weeks. No colony growth of a regimen of short-term exposure followed by culture in the absence of EBNA2-TAT peptide was therefore examined. Such a mechanism of physical interference should require the continuous presence of the peptide. The effect on B cell outgrowth of a regimen of short-term exposure followed by culture in the absence of EBNA2-TAT peptide was therefore examined. Ten of 10 wells treated initially with 100-M EBNA2-TAT peptide contained macroscopic colonies at the 4-week postinfection time point. This result indicates that continuous exposure to peptide is needed to interfere with EBNA2 function and provides additional support for the proposed mechanism of action of the EBNA2-TAT peptide.

Discussion

PTDs have the ability to mediate cell entry in a concentration-dependent manner that is independent of receptors or transporters and consequently operates in a wide variety of cell types. The mechanism of cell entry is not fully understood but may involve avid binding of the positively charged residues to cell-surface polyanions such as heparin sulfate and internalization through an endocytosis related process (35, 47). PTDs have been used to deliver fusion proteins and peptides that have shown biological activity (48–58). The EBNA2-TAT peptide severely impaired the growth of cultured EBV(+)-LCLs. The LCL cultures used in our experiments were newly immortalized and the W latency promoter was still active (data not shown). The W latency promoter is not CBF1- or EBNA2-responsive and hence the W latency promoter-driven EBNA2 expression would not be affected by the EBNA2-TAT peptide. The use of newly immortalized LCLs in these experiments allowed us to evaluate the impact of interfering with EBNA2 activation of CBF1-responsive promoters in a setting in which normal EBNA2 expression was retained. This result differs from previous analyses of EBNA2 function that have studied the effects of loss of EBNA2 expression. LCLs are dependent on the transcriptional activation function of EBNA2 for continued growth. LCLs immortalized with a virus expressing an estrogen-regulated EBNA2 stop growing when estrogen is removed from the culture medium (59). These cells can be rescued by transduction with a vector expressing...
the wells showing B cell outgrowth is indicated. B cell outgrowth was not
oligonucleotide probes specific
stained
the relative fold difference between LCLs treated with 50
EBNA2-TAT; mt, 50 mM EBNA2(mt)-TAT. (Fig. 4.) Changes in gene expression in
cells. Expression of the CD19 B cell marker at days 0 and 7 after EBV infection
wild-type EBNA2, but cannot be rescued by an EBNA2 that is
mutated in the conserved region 6 motif and is unable to interact
with CBF1 (13). The estrogen-regulated LCLs can also be
rescued by transduction of activated intracellular domain of
Notch (NotchIC) in circumstances in which LMP1 is either also
provided or is selectively up-regulated (13, 14, 60). This observation highlights both the high degree of overlap in EBNA2- and
NotchIC-regulated cell genes and the importance for B cell
growth of EBNA2 activation of the viral LMP1 gene (which is
poorly responsive to NotchIC). RT-PCR analyses revealed that
LMP1 was down regulated by the EBNA2-TAT peptide and this
is likely to be a significant component of the EBNA2-TAT
peptide’s negative effect on LCL growth. Although EBNA2 and
NotchIC both alter cellular gene expression through interactions
with CBF1, the EBNA2-TAT peptide is designed to be specific
for the EBNA2–CBF1 interaction. EBNA2 and NotchIC bind to
adjacent but distinct regions of CBF1, and mutagenesis studies
have identified amino acids that affect only NotchIC or EBNA2
interaction (61, 62).

The EBNA2-regulated viral LMP2A gene also showed re-
duced expression in the presence of the EBNA2-TAT peptide. LMP2A inhibits lytic viral reactivation and provides cell survival
signals through activation of Akt (63, 64). Cell genes tested that
were known to be either EBNA2-regulated or responsive to the
combination of EBNA2 and LMP1 were also down-regulated in
the presence of the EBNA2-TAT peptide. ICAM-1 (65) medi-
ates cell–cell contacts and contributes to B cell growth as clumps
in culture, soluble CD23 (46) acts as an autocrine growth factor,
and BATF (66), an AP-1 family member, may have a role in
repression of the EBV lytic cycle. Cdk1/Cdc2 (59) interacts with
A and B cyclins to regulate the mitotic phase of the cell cycle (67)
and was the most significantly affected of the cellular genes
evaluated here. The Cdk1 promoter is regulated by nuclear
factor Y, and nuclear factor Y has recently been found to be
up-regulated by EBNA2 in conditionally EBNA2-expressing
cells (68). With the exception of Cdk1, the real-time RT-PCR
analyses showed a relatively small down-regulation of the tested
genes by the EBNA2-TAT peptide. This observation suggests that
cessation of EBV-driven LCL growth can be achieved
through the accumulated effects of down-regulating multiple
genes without necessarily completely ablating expression of
individual EBNA2-regulated genes. The increased expression of
p21 observed in the peptide treated cells is consistent with
induction of growth arrest.

The EBNA2-TAT peptide also prevented the proliferation of
primary B cells infected in vitro with EBV. The effect was specific
in that the EBNA2(mt)-TAT peptide did not have this property
and continuous exposure to peptide was required to block
outgrowth of proliferating B cell colonies. Improved bioavail-
ability of the peptide could be addressed in the future by using
peptidomimetic approaches or modifications such as cyclization. Peptidomimetics contain nonnatural building blocks such as
D-amino acids or β-amino acids (69–71). The ability of the
EBNA2-TAT peptide to affect the growth of EBV-infected
LCLs as well as to prevent expansion of newly infected B cells
suggests that the peptide may have therapeutic potential. Early-
onset posttransplant lymphoproliferative disease is strongly
EBV-associated and has a high mortality. The disease is a

Table 1. Continuous EBNA2-TAT is required to block EBV-induced
B cell proliferation

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>1 week</th>
<th>3 weeks</th>
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<tr>
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<td>10 of 10</td>
<td>10 of 10</td>
<td>9 of 9</td>
</tr>
<tr>
<td>Uninfected</td>
<td>0 of 10</td>
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<tr>
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<td>Infected, 100 μM EBNA2-TAT*</td>
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*Treated for 1 week.

Fig. 3. EBNA2-TAT down-regulates expression of known EBNA2-responsive
viral and cellular genes. (A) Southern blots of RT-PCR products amplified using
primers for the EBNA2-regulated EBV LMP1 and LMP2A genes and cell CD23
and Cdk1 genes. The amplified products were hybridized with 32P-labeled
oligonucleotide probes specific for the individual genes. Ethidium bromide-
stained β-actin cDNA served as a loading control. U, untreated; wt, 50 mM
EBNA2-TAT; mt, 50 mM EBNA2(mt)-TAT. (B) Changes in gene expression in
peptide-treated LCLs as measured by real-time RT-PCR. Results are shown as
the relative fold difference between LCLs treated with 50 μM EBNA2-TAT or
EBNA2 (mt)-TAT, with TATA box-binding protein as the internal standard.
Cells were treated for 24 h (open bar) or 48 h (filled bar). The data are representative of three experiments.

Fig. 4. EBNA2-TAT prevents EBV-induced B cell proliferation. (A) Phase
contrast photomicrographs showing PBMC 7 days after EBV infection in the
presence of 1-, 10-, or 50-μM concentrations of EBNA2-TAT or EBNA2(mt)-TAT
peptide. The infections were performed in five-well replicates. The fraction of
the wells showing B cell outgrowth is indicated. B cell outgrowth was not
affected by EBNA2(mt)-TAT but was reduced at 1- and 10-μM EBNA2-TAT
concentrations and was abolished by 50 μM EBNA2-TAT peptide. (B) Fluores-
cence-activated cell sorter profile showing that proliferating colonies are B
cells. Expression of the CD19 B cell marker at days 0 and 7 after EBV infection
is shown. Bar, CD19+ cells.
particular problem in children who are more likely to be EBV-seronegative at the time of transplant (6). There is some heterogeneity in viral gene expression in the tumor cells but EBNA2-driven expansion is a significant component of the disease. Current treatment centers on reduction in immunosuppression, which carries an associated risk of graft rejection. Other treatments such as adoptive immunotherapy and clearance of B cells by using anti-B cell antibodies show promise but there remains a need for additional treatment options (72). Infectious mononucleosis normally resolves with only symptom-specific treatment. However, infectious mononucleosis can have an extended recovery time in severe cases and new intervention strategies targeting the expansion of latently infected cells may also be relevant to primary EBV-associated disease.

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