EBV Biology in the Pathogenesis of PTLD

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Biology of EBV

Infection

EBV is a double-stranded DNA herpesvirus first identified by Epstein, Achong, and Barr in tissue obtained from a patient with Burkitt lymphoma [1]. Today we know that over 90% of the world's population is infected with EBV. Typically the virus is transmitted through the saliva, and infection is asymptomatic, although infectious mononucleosis can result in adolescents and young adults. In the setting of clinical transplantation, EBV can also be transmitted via an organ from a seropositive donor to a seronegative recipient. The 172 kilobase pair EBV genome is packaged in a nucleocapsid surrounded by a viral tegument and enclosed within a lipid bilayer envelope containing glycoprotein spikes. The major viral envelope glycoprotein is gp350/220, which participates in viral infection by interacting with the CD21 molecule (complement receptor 2) on B cell membranes, thereby mediating the initial attachment of the virion to the cell. The interaction of gp350/220 and CD21 also induces capping of CD21 on the membrane and triggers endocytosis of the virus. Viral entry into the cell requires fusion of the viral envelope with the B cell membrane, a process mediated by interaction between the viral envelope glycoprotein, gp42, and major histocompatibility complex (MHC) class II proteins (HLA-DR, –DQ, or –DP) expressed on the cell membrane. gH, and gL are other viral envelope glycoproteins required for the fusion event [2]. While B cells are the predominant cellular host for EBV, epithelial cells are also susceptible to infection. Whereas gH and gL have been shown to be essential for epithelial cell infection by EBV, gp350/

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gp220 and gp42, which are necessary for infection of B cells by EBV, are not required for infection of epithelial cells. Recently, the Ephrin receptor A2 (EphA2) was shown to be important for EBV infection of epithelial cells and was found to associate with gH/gL and gB [3]. EphA2 appears to play a role in facilitating the internationalization and fusion of membrane-associated EBV. Interestingly, EphA2 is also a receptor for Kaposi sarcoma-associated herpesvirus (KSHV) that shares a similar cell tropism as EBV.

Other cell types that EBV has been reported to infect include T cells, NK cells, and possibly monocytes, although the mechanisms of viral entry into these cell types are not well understood. Nevertheless, rare cases of EBV-associated T cell or NK cell post-transplant lymphomas have been reported [4].

The Viral Life Cycle

EBV is generally acquired through close contact with oral secretions from a carrier. The virus initially infects epithelial cells in the oropharynx and undergoes productive replication whereby infectious viral particles are produced. These viral particles can be the source of transmission of the virus to another host and can also go on to infect B cells in the vicinity [5]. Ultimately, EBV persists for the lifetime of the host in a subset of circulating memory B cells. As with other herpesviruses, EBV persistence is linked to viral latency, although infected memory B cells that differentiate to plasma cells activate the lytic program to release new viral particles. Thus the EBV life cycle takes two forms: the latent phase in which the virus remains dormant within B lymphocytes and the lytic phase, in which the virus is actively replicated and infectious virions are released that can go on to infect bystander cells or be shed to infect naive individuals. Using this life cycle strategy, EBV is highly successful at achieving widespread infection of the human population while perpetuating viral survival and minimizing the pathologic consequences for the host. An important component of this strategy is that EBV has achieved a seemingly harmonious state with the host immune system though this is likely a delicately balanced co-existence [6]. Indeed, disruption of the viral-host equilibrium predisposes individuals to the development of EBV-associated B cell lymphomas as in immunosuppressed transplant recipients with PTLD or immunocompromised people co-infected with HIV.

How EBV gains access to the memory B cell compartment has been studied extensively [7]. One prominent model is that the virus initially infects a naïve B cell and then exploits the normal B cell differentiation process [8, 9] (Fig. 3.1). During the early stages of infection of B cells, the linear EBV genome circularizes and is subsequently maintained as an extrachromosomal episome. The first of several latent cycle gene programs is triggered within 12–16 hours of infection, and nine key latent cycle genes are expressed that lead to cellular activation and autonomous proliferation of the infected cell. This program of viral gene expression has been termed latency type III (or the growth program) and is characterized by expression of Epstein-Barr nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C, LP, and latent

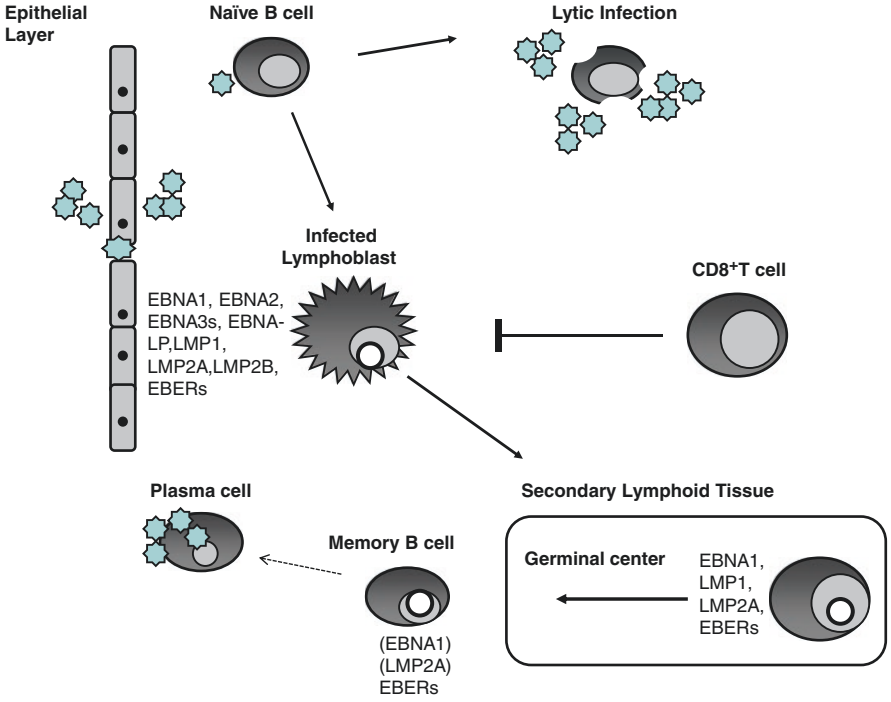

Fig. 3.1 EBV exploits the normal B cell differentiation process to gain access to memory B cells. EBV typically is transferred through oral secretions and then passes through the epithelial layer of the oropharynx. Viral particles infect naïve B cells and can establish a lytic infection where infectious viral particles are produced to subsequently infect other bystander cells or are shed to infect naïve individuals. Infection of naïve B cells can lead to establishment of a latent infection in which B cells take on a lymphoblastic phenotypic, associated with expression of the full complement of latent cycle genes. These infected lymphoblasts can proliferate autonomously but are under stringent control by EBV-specific $CD8^+$ T cells. Infected lymphoblasts can migrate to secondary lymphoid tissue where the viral gene expression shifts to a more restricted group of latent cycle genes that facilitate survival and transit through the germinal center reactions. Once infected cells exit the germinal center and emerge in the memory B cell pool, minimal viral genes are expressed to enhance viral persistence. The differentiation of infected memory B cells to plasma cells can elicit reactivation of the virus and production of infectious particles to perpetuate the viral life cycle

membrane protein (LMP1, LMP2A, LMP2B) in addition to polyadenylated viral RNAs (EBERS 1 and 2) and a group of transcripts from the BamH1A region of the genome whose function is unknown. The resulting infected B cells resemble antigen-activated B lymphoblasts. In immunocompetent individuals the outgrowth of the EBV-activated lymphoblasts is controlled by a robust, anti-viral cytotoxic T lymphocyte (CTL) response. However, disruption of host immunity can lead to development of EBV^+ B cell lymphomas, as seen in PTLD, that are also characterized by the latency III program of viral gene expression. Finally, the same viral gene expression program is found in B cells infected with EBV in vitro, resulting in the generation of immortalized lymphoblastoid cell lines (LCL).

In the normal course of EBV infection, the activated lymphoblasts can migrate to the B cell follicles of secondary lymphoid tissue where the growth program is silenced and replaced by latency type II (default program) characterized by expression of EBNA1, LMP1, and LMP2A. In conventional T cell-dependent immune responses, the follicles are sites where activated B cell blasts that have encountered antigen undergo isotype switching and somatic mutation of immunoglobulin genes to differentiate into antibody forming cells or memory cells bearing high-affinity B cell receptors (BCR). This process of differentiation depends upon encounter with antigen-presenting follicular dendritic cells and T helper cells. Cells expressing BCR that do not bind antigen die via apoptosis. In type II latency, the expression of EBNA1, LMP1, and LMP2A provides key signals that ensure survival of the infected cell and drive it through the B cell differentiation process associated with the GC reaction without requirement for interaction with antigen, follicular dendritic cells or T helper cells. Infected memory cells that emerge from GC then switch to type I, or latency program, where either no viral genes are expressed or only EBNA1 is expressed during cell division to ensure maintenance of the EBV episome. At the same time, the viral life cycle can be perpetuated when memory B cells harboring the virus recirculate to the lymphoid tissues in the epithelium and differentiate to plasma cells, eliciting reactivation of the virus.

The virtual absence of viral gene expression when EBV is harbored in resting memory B cells in the periphery promotes viral persistence and escape from host anti-viral immune mechanisms. This scenario suggests that EBV has co-evolved with the host immune system and utilizes its ability to induce autonomous proliferation of infected B cells only transiently as a means to exploit the process of B cell development to transit safely through to the memory B cell compartment [10]. Thus, the development of EBV-associated B cell lymphomas, including PTLD, may be an inadvertent and unintended consequence of this process in the context of impaired immunity or when additional mutations arise.

Latent Cycle Genes of EBV

How do latent cycle proteins shepherd EBV through the process of B cell differentiation? In this section the key properties of the 11 gene products expressed during type III latency and in PTLD-associated B cell lymphomas are summarized (Table 3.1).

(i) EBNA1: a DNA-binding protein that attaches to the origin of plasmid replication (OriP) of EBV and is required for episomal replication of the viral genome. Further, EBNA tethers the viral genome to mitotic chromosomes and is sufficient to ensure passage of the viral genome to daughter cells during cell division. EBNA1 also plays a role in transcription activation of latent viral genes and host cell genes.
(ii) EBNA2: a transcriptional activator that regulates the function of several viral genes including LMP1 and LMP2A as well as numerous cellular genes.

Table 3.1 Latent cycle genes expressed in EBV-associated PTLD B cell lymphomas and in type III latency

Gene	Function/activity
LMP1	Major oncogene of EBV; activates several signal transduction pathways to provide growth and survival signals to B lymphoblasts; constitutively active mimic of CD40
LMP2A	Acts as constitutively active mimic of BCR to provide key survival signals; inhibits BCR signaling by sequestering signaling proteins normally utilized by BCR
LMP2B	Participates in regulation of LMP2A function
EBNA1	Required for maintenance of viral genome as an episome; binds to mitotic chromosomes
EBNA2	Major viral transactivator of EBV; involved in expression of numerous cellular and viral genes
EBNA3A, B, and C	EBNA3A and EBNA3C are required for B cell transformation in vitro; like EBNA3A and EBNA3C, EBNA3B is involved in transcriptional regulation
EBNA-LP	Enhances the function of EBNA2
EBERs	Highly abundant, small nonpolyadenylated RNAs that can modulate apoptosis pathways, induce IL-10 production, and alter the host immune response

EBNA2 does not directly interact with DNA regulatory sequences but instead mimics Notch signaling by interacting with the DNA-binding protein, RBP-Jκ, to prevent B cell differentiation. EBNA2 is required for transformation of human B cells in vitro.

(iii) EBNA3A, 3B, 3C: encoded by genes that lie in tandem within the EBV genome. All three proteins interact with cellular DNA-binding protein RBP-Jκ and modulate transactivation by EBNA2. EBNA3A inhibits differentiation of B cells to plasma cells. EBNA3A and EBNA3C are required for immortalization of B cells in vitro, but EBNA3B is dispensable. EBNA3A and EBNA3C promote tumorigenesis, while EBNA3B can suppress tumorigenesis. EBNA3C, through interaction with cyclin proteins, can disrupt cell cycle checkpoints. EBNA3s are the primary target of the host $CD8^+$ T cell immune response.

(iv) EBNA-LP: the initial latent cycle gene expressed following infection of B cells. Important in transformation of B cells in vitro and enhances the ability of EBNA2 to transactivate cellular and viral genes.

(v) LMP1: the major oncogene of EBV since it is sufficient to transform rodent fibroblasts in vitro and is required for generation of LCL from human B cells. LMP1 is an integral membrane protein with a short intracellular N-terminal tail, six membrane-spanning domains, and a long cytoplasmic C-terminal tail. Within the intracellular tail are three carboxy-terminal activating regions (CTAR) that interact with cellular adaptor proteins to activate multiple cellular signaling pathways. In this way LMP1 mimics a constitutively active member of the tumor necrosis factor receptor (TNFR) superfamily and activates NF-κB; the mitogen-activated protein (MAP) kinases p38, Erk, and JNK; and PI3K/Akt through the use of the cellular adaptor proteins TRAF

and TRADD. LMP1 signaling induces expression of cell adhesion molecules, anti-apoptotic proteins including bcl-2, cFLIP, A-20, and the production of the B cell lymphoma autocrine growth factor IL-10 [11, 12]. Thus, LMP1 provides critical growth and survival signals to infected B cells. Indeed, the ability of LMP1 to inhibit apoptosis through death receptors [13] suggests it may play an important role in survival of B cells through the GC in the absence of encounter with antigen by providing signals normally delivered through T cell help. Mice expressing a transgene for LMP1 under the control of the immunoglobulin promoter develop lymphomas at three times the frequency as LMP1-negative, control mice [14], and expression of LMP1 in B cells of mice that are depleted of T cells leads to lymphomagenesis [15].

(vi) LMP2A: expressed in the membrane of infected B cells and contains immunoreceptor tyrosine-based activation motifs (ITAM) similar to the BCR. Like LMP1, LMP2A is aggregated in the membrane and constitutively signals. LMP2A sequesters key tyrosine kinases, including Syk, from the BCR and thus inhibits BCR-mediated cell activation, thereby inhibiting entry into the lytic phase of infection. However, LMP2A supplies the tonic signals normally provided by the BCR for cell survival and can drive cellular proliferation and production of the growth factor IL-10.

(vii) LMP2B: the second isoform of LMP2 and is controlled by a separate promoter from LMP2A. Neither LMP2A nor LMP2B is essential for B cell transformation in vitro. LMP2B has been one of the most enigmatic of the EBV latent cycle proteins. Recent studies suggest the LMP2B can physically associate with LMP2A [16] and negatively regulates the ability of LMP2A to inhibit switching from the latent to the lytic cycle [17].

(viii) EBER: EBERs 1 and 2 are small polyadenylated, non-coding RNAs expressed in each of the three forms of latency. Commonly used as targets for in situ hybridization to establish the presence of EBV in clinical specimens. Abundant in EBV-transformed cells, contribute to oncogenesis, and can modulate the immune response. They have been reported to inhibit apoptosis [18], induce IL-10 production in Burkitt lymphoma cells [19], promote cell cycle transition [20], and activate the PI3K/Akt signaling pathway [21].

EBV Genetics and Oncogenic Mechanisms

PTLD

PTLD represents a heterogeneous group of disorders, the majority of which are B cell proliferations associated with EBV. Within the B cell proliferations, various malignant subtypes have been defined and will be discussed in another chapter. PTLD tumors can arise during primary infection with EBV or as a result of viral reactivation. Further, EBV-*associated* PTLD lymphomas can be polyclonal or monoclonal, with polyclonal tumors arising more often in the early post-transplant period, while tumors that occur more than 1 year post-transplant tend to be of the

monoclonal variety but are more biologically heterogeneous. Thus, the factors contributing to the pathogenesis of PTLD are multiple and complex and include an immunosuppressed host, a virus with the ability to confer autonomous growth on infected cells and to invoke clever strategies of immune evasion, and the direct effects of immunosuppressive drugs on virally infected or transformed cells – all in the setting of alloreactivity. Despite these common factors, most transplant recipients do not develop PTLD. How then does PTLD arise?

Transplant recipients generally maintain higher EBV loads than healthy individuals, have increased numbers of latently infected memory B cells, and have increased frequency of viral reactivation [22]. The elevated viral loads and lytic replication could lead to more viral infection events in naïve B cells raising the number of cells that initially express the latency III growth program. If these cells cannot exit the cell cycle or fail to successfully progress through the differentiation program, then lymphomas could arise. Similarly, infection of bystander GC B cells or memory B cells could lead to aberrant expression of the growth program, without the ability to differentiate, and subsequent clonal expansion. Alternately, latently infected GC B cells or memory B cells could inappropriately turn on the growth program, perhaps due to accumulated mutations or as yet unidentified signals [10]. Coupled with the impaired T cell response, the autonomous growth properties of EBV^+ lymphoblasts that result in each of these scenarios could culminate in PTLD. In support of this, analysis of immunoglobulin gene sequences shows that PTLD tumors can originate from naïve B cells, GC cells, or memory cells. Extensive molecular and phenotypic studies of EBV^+ monoclonal PTLD indicate that the majority appears to be GC-experienced cells that reflect different stages of B cell differentiation [23]. The high rate of proliferation in these cells could lead to additional mutations that further drive oncogenesis, in some cases perhaps independent of EBV. Sporadic alterations in c-Myc [24], p53 [25] and other oncogenes have been described in PTLD lesions; however, it is unclear whether they contribute to enhanced tumor cell growth [26]. Monoclonal forms of PTLD tend to carry a higher frequency of mutations in tumor suppressor genes and altered proto-oncogene expression.

A variety of other factors could influence the development and progression of PTLD-associated B cell lymphomas including viral determinants that drive tumor growth and survival, viral mechanisms of immune evasion or subversion, and EBV genomic diversity and microRNA (miRNA). The following section will highlight some specific examples pertinent to each of these categories.

Viral Determinants That Drive Growth and Survival of PTLD-Associated B Cell Lymphomas

EBV has evolved to effectively co-opt several cellular signaling pathways within the host B cell to promote growth and survival of infected cells. The cellular cytokines IL-6 and IL-10 are both well-described autocrine growth factors in EBV^+ B cell lymphomas [12, 27]. In addition, elevated levels of IL-6 and IL-10 are found in the circulation of patients with PTLD [28, 29]. In the case of IL-10, it has been definitively shown that the EBV-encoded protein, LMP1, activates the cellular

mitogen-activated protein kinase p38 and the PI3K/Akt pathway to induce production of IL-10 [11]. The latent cycle protein, LMP2A, acts as a constitutively active mimic of the BCR to deliver tonic signals to EBV-infected B cells through activation of the Syk pathway. Furthermore, LMP2A can provide signals for survival and differentiation of B cells in the absence of BCR signaling through constitutive activation of the ERK/MAPK pathway [30, 31]. Other cell signaling pathways including NF-κB are constitutively active in EBV$^+$ B cell lymphomas in PTLD. Finally, the EBNA3A and EBNA3C proteins function to promote cell cycle progression in B cells. Together, these virally induced mechanisms likely support ongoing cell survival and proliferation.

Viral Mechanisms of Immune Evasion or Subversion and the Tumor Microenvironment

EBV-encoded proteins that can counter apoptotic signals are a common theme in viral subversion strategies. LMP1 can actively block apoptotic signals delivered through the Fas/Fas ligand and TRAIL death receptor pathways. This function of LMP1 could help ensure survival of infected cells through the process of B cell differentiation and could also prevent elimination of EBV$^+$ lymphoblasts by viral-specific CTL. LMP1 is also able to block apoptotic signals in EBV-infected B cells through upregulation of a variety of survival proteins including bcl-2, A20, mcl-1, and bfl-1. The EBV lytic cycle gene, BHRF1, encodes a viral homolog of bcl-2 that can inhibit apoptosis induced by multiple stimuli including anti-Fas antibodies and TNF-α. EBNA1 can block apoptosis induced by p53 expression which may be particularly relevant in Burkitt lymphoma where EBNA1 is the sole latent cycle protein expressed [32]. A second EBV nuclear antigen, EBNA2, interferes with apoptosis induced by some stimuli through the intrinsic pathway by sequestering Nur77 in the nucleus and preventing its translocation to the cytoplasm where it can induce cytochrome C release from the mitochondria [33].

Immunomodulatory cytokines or their receptors, either encoded by EBV or induced by EBV, are also an important tactic utilized by the virus to evade host immunity. The lytic cycle gene BCRF1 encodes viral IL-10 (vIL-10), a functional homolog of cellular IL-10. vIL-10 is expressed early following infection of B cells by EBV and, because of its immunosuppressive properties, may facilitate transformation by impairing T cell and macrophage responses. In particular, vIL-10 can inhibit production of IFN-γ by T cells and production of IL-12 by monocytes. As discussed earlier, LMP1 induces cellular IL-10, which acts as an autocrine growth factor for EBV$^+$ B cell lymphomas. Cellular IL-10 can also have potent inhibitory effects on host T cells and monocytes during viral latency as in PTLD-associated lymphomas. The lytic cycle EBV gene, BARF1, encodes a functional, soluble receptor for colony-stimulating factor 1 (CSF-1) that can interfere with the ability of CSF-1 to augment monocyte/macrophage proliferation and produce IL-12. Finally, EBV infection of B cells induces expression of a cellular protein, EB13, that is a functional homolog of the IL-12 p40 subunit. Thus, it has been suggested that EB13 can antagonize IL-12 activity [34]. EB13 can also pair with p28, an IL-12p35-related protein, to form the cytokine IL-27. IL-27 is a

complex cytokine with diverse pro- and anti-inflammatory properties, but strong evidence exists to indicate that IL-27 can inhibit a variety of effector functions by T cells [35].

Other immunomodulatory pathways that could alter the tumor microenvironment have been described. LMP1 has been shown to upregulate PD-L1 through an NF-κB-dependent pathway [36, 37]. The expression of PD-L1/2 on B cell lymphomas and identification of amplification of 9p24.1 using comparative genomic hybridization leading to overexpression of PD-L1/2 on EBV$^+$ PTLD clinical specimens [38] indicate that immunomodulation of PD-1$^+$ T cells in the tumor microenvironment is plausible [38].

Clearly, there are multiple avenues by which EBV can modulate host immunity that could impact on the development and progression of PTLD.

EBV microRNAs and Host Cell microRNAs

In 2004, EBV was the first virus shown to encode microRNA (miRNA), a family of small non-coding single-stranded RNA of ~22 nucleotides that are post-transcriptional regulators of gene expression predominantly via complementary base pairing with mRNA transcripts. Since that time, more than 40 EBV miRNAs have been identified and localized within the BART and BHRF1 clusters of the EBV genome. EBV miRNAs are expressed in the lytic and the latent cycle of infection and can modulate a number of processes relevant to oncogenesis and viral persistence. For example, EBV miRNA can regulate expression of EBV latent cycle genes, inhibit transition to the lytic phase, inhibit cellular apoptosis, inhibit tumor suppressor genes, and modulate the host innate and adaptive immune responses [39]. In addition to the expression of virally encoded miRNA, EBV infection can markedly alter the cellular miRNA profile and disrupt host cell homeostasis. The latent cycle gene LMP1, in particular, has been shown to alter the expression of numerous host cell miRNAs important in processes ranging from production of cytokines [40], activation of signal transduction pathways, to the immunogenicity of the infected cell. Finally, the viral and host cell miRNA can be transported as cargo in exosomes from the EBV-infected cell to other cells, thereby potentially modulating the tumor microenvironment.

EBV Genomic Diversity

There has been ongoing interest in understanding the extent of EBV genome diversity in health and disease with the possibility of identifying specific variants linked to pathogenesis. Targeted sequencing of the cytoplasmic domain of LMP1 identified gain-of-function mutations at amino acids 212 and 366 that were commonly present in EBV isolated from B cell lines derived from patients with EBV+ PTLD [41]. These mutations were shown to elicit sustained ERK MAPK activation and cFOS induction suggesting another potential pathway of signal transduction dysregulation that may contribute to oncogenesis. More broadly, early classification schemes based on genomic sequences of EBNA2, EBNA3s [42], and LMP1 [43] were established, but it has been difficult to demonstrate definitive links between viral subtypes and EBV-driven tumorigenesis.

The advent of next-generation sequencing approaches has rapidly expanded the number of whole genome sequences available for EBV including sequences from FFPE sections of PTLD lesions [44] as well as B cell lines established from PTLD patients [45, 46]. It is likely that additional PTLD-derived whole-genome EBV sequences will become available and, coupled with computational analysis, may reveal PTLD-associated variants of interest that can be exploited as biomarkers for increased risk of PTLD and potential targets of cellular immunotherapy.

Conclusion

EBV is a highly successful virus that has developed effective strategies to persist in memory B cells of healthy individuals with minimal clinical consequences. However, disruption of the delicate balance between EBV and anti-viral immunity, as in transplant recipients, can result in the development of EBV^+ B cell lymphomas. Host-viral interactions play an important role in the development of EBV-associated PTLD. Elucidating the underlying host-viral mechanisms in the pathogenesis of PTLD could identify new therapeutic opportunities for the treatment of EBV-associated PTLD.

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