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The Interplay Between Epstein Barr Virus and B Lymphocytes: Implications for Infection, Immunity, and Disease

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Abstract

Human B cells are the primary targets of Epstein Barr virus (EBV) infection. In most cases EBV infection is asymptomatic because of a highly effective host immune response but some individuals develop self-limiting infectious mononucleosis, while others develop EBV-associated lymphoid or epithelial malignancies. The viral and immune factors that determine the outcome of infection are not understood. The EBV life cycle includes a lytic phase, culminating in the production of new viral particles, and a latent phase, during which the virus remains largely silent for the lifetime of the host in memory B cells. Thus, in healthy individuals there is a tightly orchestrated interplay between EBV and the host that allows the virus to persist. To promote viral persistence EBV has evolved a variety of strategies to modulate the host immune response including inhibition of immune cell function, blunting of apoptotic pathways, and interfering with antigen processing and presentation pathways. In this article we focus on mechanisms by which dysregulation of the host B cell, and immune modulation, by the virus can contribute to development of EBV+ B cell lymphomas.

Keywords

B cells; Epstein Barr virus; Latent Membrane Protein 1; microRNA; signal transduction

Introduction

Epstein Barr virus (EBV), also known as human herpes virus 4, has infected more than 90% of adults worldwide. Typically, EBV infection is asymptomatic in healthy individuals and is controlled by a robust immune response with CD8+ T cells playing a major role. However, EBV infection can also cause infectious mononucleosis (IM), a self-limiting lymphoproliferative disorder in adolescents and young adults. EBV is also associated with several malignancies including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinomas (NPC) and B cell lymphomas in individuals who are immunosuppressed or immunocompromised including transplant recipients, the elderly, and AIDS patients. Occasionally, EBV has been identified in peripheral T cell lymphomas and nasal NK/T cell lymphomas. A major goal in the field is to understand the immune and viral factors that

determine whether EBV co-exists harmoniously with the immune system as it does in the vast majority of humans, or whether infection results in the development of one of a variety of EBV-associated lymphoid and epithelial malignancies.

The Viral Life Cycle

To fully understand the host-viral interactions following infection with EBV requires a closer look at the complex life cycle of this virus (Fig 1). EBV is transmitted from the carrier through the saliva and enters the host via the oropharynx region. B cells are the principal targets of EBV infection, primarily due to their expression of CD21, the major receptor for the virus. However, EBV can also infect epithelial cells through distinct processes including transfer from infected B cells (1). Epithelial cells are important sites of lytic infection, producing viral progeny that amplify cell to cell spreading and enable transmission to new hosts. Ultimately, the virus persists for the lifetime of the host in subsets of memory B cells, both IgD+CD27+ non-class switched and IgD-CD27+ class switched, but not in naïve B cells (2). In this setting the virus maintains a latent state as an episome and expresses no viral genes thereby allowing EBV to remain hidden from the immune system. Periodically, the virus may become reactivated through mechanisms that are unclear, but the host immune response typically is sufficient to maintain control. How EBV selectively persists in memory cells has been an area of much interest and various models have been proposed including direct infection of memory cells (3) or germinal center (GC)-dependent processes in which naïve B cells infected with EBV rely on various latent cycle proteins to traverse through GC reactions and emerge as memory cells harboring the virus (4). The latter model invokes coordinated expression of specific viral latency genes that are sequentially expressed such that Latency 0 and Latency 1, also termed Latency Program, are restricted to memory B cells and are characterized by the lack of expression of any viral genes, or expression of the weakly immunogenic Epstein Barr nuclear antigen-1 (EBNA-1), required for mitotic segregation of the episome during cell division. Latency II, also called the Default Program, (EBNA-1, LMP-1, LMP-2A, -2B) is expressed in infected germinal center centroblasts, while Latency III, or the Growth Program, (EBNA-1, -2, -3, -4, -5, -6, LMP1, LMP-2A, -2B) is expressed in lymphoblasts. Interestingly, these same viral latent cycle programs are also expressed in various EBV malignancies. Latency 1 is found in Burkitt's lymphoma, Latency II in Hodgkin's disease, NPC, T/NK cell lymphoma, and Latency III is characteristic of IM and the B cell lymphoblastoid lymphomas found in transplant recipients and AIDS patients.

The Immune Response to EBV

Clearly, the complexity of the viral life cycle has been a major factor in the inability, so far, to produce an effective vaccine for EBV. Nevertheless, we are accumulating extensive information on the T cell response to EBV in health and disease. CD8+ T cells with specificity for immunodominant epitopes of lytic and latent cycle proteins are readily detected in the circulation of EBV-infected, healthy individuals. Multiple lytic viral proteins, particularly immediate early, and early, cycle proteins are recognized by host CD8+ T cells (5). EBNA3A, -3B, -3C, and LMP2-A are the predominant latent proteins recognized by host CD8+ T cells, although EBNA-1 and LMP-1-specific T cells have also been identified.

Tetramer studies of peripheral blood mononuclear cells (PBMC) from healthy, seropositive individuals revealed that CD8⁺ T cells specific for individual immunodominant epitopes of lytic or latent cycle proteins can constitute as much as 2% of the CD8⁺ T cell subset indicating a significant proportion of the T cell repertoire is devoted to maintaining control of EBV (6). Further, analysis of PBMC from IM patients revealed that a massive expansion (upwards of 50%) of EBV-specific T cells occurs, with T cells reactive to lytic cycle proteins more abundant than T cells reactive to latent cycle proteins (7). MHC class II tetramers have also been utilized to visualize the CD4⁺ T cell response to EBV in IM and healthy blood donors. These studies indicate that both lytic and latent proteins are targeted by CD4⁺ T cells, and while relatively high frequencies of CD4⁺ T cells can be detected during IM (8), the overall magnitude of the response is diminished compared to CD8⁺ T cells.

Our laboratory has focused on understanding the pathogenesis of EBV-associated B cell lymphomas in immunosuppressed and immunocompromised populations. An important question to address in these individuals is whether the T cell response is intact and functional. Our group published early studies on the direct identification of EBV-specific CD8⁺ T cells in transplant recipients using first generation MHC/peptide tetramers (9). Specifically, we measured the frequency of CD8⁺ T cells that bind to tetramers loaded with immunodominant peptides of the EBV latent cycle antigen EBNA3A (FLR), the immediate-early lytic cycle antigen, BZLF1 (RAK) or the early lytic cycle antigen, BMLF1 (GLC). All six patients analyzed demonstrated CD8⁺ T cell binding to the BZLF1 peptide (range=0.9%–3.9%) and to the EBNA3A epitope (range=0.3–0.6%). Fifteen of eighteen patients analyzed had detectible CD8⁺ T cells specific for the lytic cycle BMLF1 protein (range=0.1–0.8%). Overall, 21 of 24 patients analyzed had EBV-specific CD8⁺ T cells that displayed an activated/memory phenotype despite immunosuppressive therapy. On the basis of available serology, at least 10 of these patients were seronegative at transplant but acquired the virus from the graft donor at the time of transplant. Furthermore, in three of the patients that received a graft from a seropositive donor but were seronegative at transplant, and for which sequential samples were available, EBV-specific CD8⁺ T cells were detected by 4 weeks, 6 weeks, and 12 weeks, respectively, post-transplant. These results indicate that transplant recipients can mount a primary T cell response to EBV despite the use of potent immunosuppressive medication. Moreover, the proportion of EBV-specific T cells and the dominance of lytic responses resembled features of the CD8⁺ T cell response in healthy individuals. More recently, we have utilized cytometry by time-of-flight (CyTOF) mass cytometry to investigate the phenotypic and functional properties of T cells in the circulation of EBV-infected transplant recipients (unpublished data). Our findings indicate that polyfunctional T cells specific for lytic and latent proteins can be detected but the overall magnitude of the response is attenuated compared to healthy donors. These findings are consistent with studies by Metes and colleagues (10) who have suggested that the inability to control EBV load levels in transplant recipients is associated with the presence of EBV-specific T cell populations that display features of cellular exhaustion.

While T cells are thought to constitute the principal effector component of the immune response to EBV, there is mounting evidence that NK cells also play a role. Elevated NK cell numbers are associated with lower viral loads in individuals with IM (11). Studies in

immunodeficient mice reconstituted with human cells indicate that NK cells are particularly important in controlling lytic EBV infection (12). Munz and colleagues suggest that a $\text{IFN-}\gamma^{\text{high}}$, $\text{CD56}^{\text{bright}}$ $\text{NKG2A}^+\text{CD94}^+\text{CD54}^+\text{CD62L}^-$ subset of NK cells found in tonsils of EBV carriers are critical in restricting transformation of B cells (13). NK cells also appear to have a role in controlling chronic viral infection. Individuals with XMEN, a primary immunodeficiency associated with defects in NK cell function, exhibit high levels of EBV and are at increased risk for EBV+ lymphoproliferative disorders and life-threatening IM. Control of chronic EBV infection in these patients has been linked to the NK cell receptor, NKG2D (14). Further, males with X-linked lymphoproliferative disease (XLP) have defects in the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP), that is crucial for NK cell cytotoxic function, and are unable to control EBV infections (15). While the XLP and XMEN immunodeficiencies can also affect the T cell response to EBV, other rare immunodeficiencies that are NK cell specific are also associated with the development of EBV+ malignancies and life-threatening IM (16, 17). It will be important in future studies to gain more insight into the molecular interactions that occur between NK cells and EBV-infected cells, especially given the panoply of NK cell receptors and the ability of viruses to modulate NK cell ligands.

EBV and Immune Evasion Strategies

Like other herpes viruses, EBV utilizes a multitude of strategies to evade detection and elimination by the host immune system (18). Immune evasion strategies of EBV have been recently reviewed (19, 20) and will not be extensively detailed here, but can be broadly classified into three categories, those that modulate: 1) immune cell function, 2) antigen presentation pathways or 3) apoptotic pathways. With regard to modulation of immune cell function, EBV encodes the lytic cycle protein BCRF1, also termed viral IL-10, that can suppress the production of $\text{IFN-}\gamma$, IL-2 and IL-6 by anti-viral CD4^+ T cells (21). BARF1, a soluble version of the colony stimulating factor (CSF-1) receptor, indirectly inhibits production of the anti-viral molecule $\text{IFN-}\alpha$ through neutralization of CSF-1 (22).

A variety of EBV proteins target the processing and presentation of viral antigens thereby promoting immune evasion. Along these lines, EBNA-1 contains a glycine-alanine repeat that inhibits processing and presentation by HLA class I (23, 24). The early lytic cycle protein BNLF2a prevents peptide loading of HLA class I molecules through interaction with the Transporter associated with Antigen Processing (TAP) (25), while BGLF5, a DNase/exonuclease with host shutoff function, blocks synthesis of new HLA class I and class II molecules (26) and BILF1 downregulates cell surface class I by targeting them for lysosomal degradation (27). Finally, EBV has evolved several tactics to prevent apoptosis of the infected cell in order to augment viral persistence. A functional bcl-2 homolog encoded by BHRF1 can inhibit apoptosis induced by a range of stimuli at least in part by binding to the pro-apoptotic protein Bim (28). Our group demonstrated that EBV-infected B cell lymphomas are resistant to induction of apoptosis through the death receptor mediated pathways, Fas/Fas ligand and TRAIL/DR (29). This process was dependent upon signaling by latent membrane protein 1, LMP1, a latent cycle protein that plays a central role in transformation, survival, and proliferation of EBV-infected B cells. LMP1 signaling in human B lymphoma cell lines induces expression of the cellular protein c-FLIP that

interferes with formation of the death inducing signaling complex (DISC), required to initiate activation of caspase 8 following ligation of death receptors. Induction of cFLIP by LMP1 is NF- κ B dependent and provides a mechanism for EBV to subvert signals through the extrinsic, death receptor-mediated pathway of apoptosis and host cellular effector pathways (30). LMP1 modulation of cell death pathways may be especially relevant to the germinal center model for EBV discussed above. In this scenario LMP1, a functional homologue of CD40, can provide survival signals, in concert with another latent cycle protein, LMP2a, which mimics B cell receptor (BCR) signaling, to infected B cells that are otherwise destined to die in the absence of encounter with antigen. Provision of survival signals, and perturbation of the apoptotic pathway, by LMP1 and LMP2a could be relevant to the oncogenesis of Hodgkin's disease that originate from germinal center B cells. LMP1 signaling can also upregulate expression of several cellular anti-apoptotic factors including A-20, bcl-2, Mcl-1 and IAP-2, primarily through induction of NF- κ B activity (31–34). These are just a few salient examples of the myriad maneuvers through targeting of immune cell function, antigen presentation, and cell death pathways, by which EBV modulates the host immune response. Understanding the manner in which EBV-encoded proteins cooperate to evade and subvert the immune response during lytic and latent infection is crucial to advancing new approaches to vaccine development and to understand the pathogenesis of EBV-associated malignancies.

A Closer Look at the EBV Oncogene LMP1

To gain more insight into how EBV coopts host cell function during viral latency and lymphomagenesis we have focused extensively on LMP1, the major oncogene of EBV. LMP1 is required for transformation of human B cells (35) and is sufficient to transform rodent fibroblasts *in vitro* (36). LMP1 is an integral membrane protein containing six transmembrane-spanning domains and a long C-terminal tail (Fig. 2A). The transmembrane domain acts to induce oligomerization of LMP1 complexes in the membrane to aggregate in lipid rafts. This clustering of LMP1 proteins brings individual C-terminal tails into proximity, creating suitable docking sites for cytoplasmic signaling adaptor proteins, thereby allowing LMP1 to signal in a constitutive fashion. We have examined in detail the signal transduction pathways elicited by LMP1 and the corresponding downstream functions in latently infected B cells. In particular we utilized EBV-induced IL-10 production as a model system to investigate how EBV alters human B cell function. In early studies we showed that EBV-infected B cell lymphomas produce human IL-10 that is required for cellular proliferation such that blockade of the autocrine human IL-10 pathway by neutralizing anti-IL-10 mAbs or soluble IL-10 receptor significantly inhibited the proliferation of EBV⁺ B cell lines from patients with post-transplant lymphoproliferative disorder (PTLD) (37). We also determined that elevated levels of human IL-10 and viral IL-10 are found in the circulation of transplant recipients with EBV viremia (38) and in the circulation of SCID mice injected with EBV⁺ B cell lines from patients with PTLD (39). It is likely that IL-10 produced by EBV infected B cells also acts to negatively regulate the immune response, akin to regulatory IL-10 producing B cells, that have been more recently described to play a role in peripheral tolerance in autoimmunity, cancer, and organ transplantation (40). In the case of EBV-infected B cells, we demonstrated that LMP1 signaling is sufficient to elicit

IL-10 production through p38 MAPK and PI3K-dependent pathways (41) and that activation of PI3K depends upon the Syk tyrosine kinase and the Src family kinase Fyn (42) (Fig 2B). Moreover, LMP1-induced PI3K activation drives B cell survival by preventing loss of XIAP induced by the mitochondrial protease HtrA2 (43). By understanding the signal transduction pathways elicited by LMP1 we have been able to pinpoint specific molecules within key signaling nodes as potential therapeutic targets. Along these lines, rapamycin a potent mTOR inhibitor that acts downstream of PI3K, partially blocks growth of EBV⁺ B cell lymphomas *in vivo* and *in vitro* (39, 44). We also demonstrated that p110 δ is the predominant isoform of PI3K expressed in EBV⁺ B cell lymphomas and that combined blockade of mTOR and the PI3K pathway significantly inhibits lymphoma proliferation (45). Our work on the basic aspects of EBV pathogenesis and LMP1 indicate that the PI3K/Akt/mTOR axis is a critical cellular signaling node that has been co-opted by EBV to promote B cell survival and proliferation.

The B Cell MicroRNA Landscape and EBV Infection

EBV was the first virus shown to encode microRNA (miRNA) (46), a family of small, approximately 22 nucleotide, non-coding RNA that post-transcriptionally regulate gene expression for control of cellular events. Subsequently it has been established that virally-encoded miRNA participate in viral-host cell interactions including immune evasion, prolonging survival of infected cells, regulation of viral genes and potentially in the pathogenesis of viral-associated disease (47). We investigated the possibility that EBV could not only influence host cell function through virally-encoded miRNA, but also by usurping the host cell microRNA machinery and that these changes could contribute to lymphomagenesis in EBV⁺ lymphomas (48). For example, miR-155 is upregulated in B cells upon activation via the BCR and its deletion suppresses both the GC response and the generation of GC B cells (49). Interestingly, overexpression of miR-155 is characteristic of many B cell lymphomas (50, 51) and in a mouse model constitutive expression of miR-155 in B cells lead to uncontrolled proliferation of pre-B cells and subsequent malignancy (52). Similarly, activation of the BCR in the presence of IL-4, results in the upregulation of miR-21 (53). miR-21 has been termed an oncomir because it can negatively regulate a variety of tumor suppressors including PTEN (54) and because transgenic mice overexpressing miR-21 develop pre-B cell lymphomas (55). Thus, miRNA are involved in normal B cell development and perturbation of miRNA is sufficient to initiate B cell lymphomagenesis.

We and others have found that EBV infection induces the expression of specific host B cell miRNAs, including miR-155 and miR-21, which potentially play a role in viral oncogenesis (56). Moreover, we demonstrated that activation of LMP1 is sufficient to significantly alter the expression of B cell miRNA that regulate IL-10 production and cell survival (Fig. 2B). Interestingly, the microRNA profile that is induced by LMP1 signaling varies depending on sequence heterogeneity within the cytoplasmic signaling tail of LMP1. We previously demonstrated that LMP1 molecules isolated from patients with EBV⁺ B cell lymphomas exhibit sequence diversity including two mutations at position 212 (G->S) and 366 (S->T) of the cytoplasmic tail of LMP1 that are shared by each of the tumor-derived LMP1 molecules but are distinct from the B95.8 form of LMP1 isolated from a patient with IM

(57). Further, these gain of function mutations in tumor-derived LMP1 mediate sustained ERK MAPK signaling, c-FOS activation and AP-1 activity. These studies suggest that further analysis of viral diversity and the impact on cellular function may provide new insights in to the underlying viral mechanisms that drive B cell lymphomagenesis and may be exploited to develop biomarkers for disease.

EBV Diversity: Implications for the Immune Response and Disease

The DNA genome of EBV is approximately 170 kilobase in size and encodes more than 85 genes. The diversity within the EBV genome has long been recognized but has primarily been studied in the context of EBV+ NPC tumors. In particular, a high proportion of LMP1 molecules isolated from cell lines derived from Chinese patients with NPC were shown to have a characteristic 30 base pair deletion, compared to the B95.8 strain of EBV, in the signaling tail of LMP1 (58) which results in the loss of 10 amino acids (aa343–352). Further, the deletion variant form of LMP1 exhibited increased tumorigenicity in fibroblast transformation assays (59) and increased activity in NF- κ B reporter assays compared to the B95.8 form of LMP1 (60). Consequently, it was proposed that the 30 base pair deletion may be important in development of NPC, however, subsequent studies showed a similarly high frequency of this deletion in LMP1 isolated from healthy, seropositive Chinese subjects (61).

More broadly, there are two defined types of EBV, type I and type 2, based on sequence differences identified in the EBNA-2 and EBNA-3A, -3B, and -3C genes and these differences are manifest in transforming and reactivation abilities in the subtypes. Type I EBV predominates throughout the world although type I and type 2 are equally represented in Africa (61). Several other classification schemes for EBV have been described and at least three systems are based on amino acid changes in the C-terminal region of LMP1. Raub-Traub and colleagues (62) proposed a system based on sequences from aa189–aa377 of LMP1 variants compared to the laboratory strain, B95.8 and named seven forms (Alaskan, China 1, China 2, China 3, Mediterranean+ (Med), Med-, and North Carolina) depending on the geographic region of origin of the specimen. Another classification scheme focused on amino acid sequences in short segments surrounding the 33 bp repeat and identified 25 variant forms (63). Finally, the Sandvej et al (64) classification scheme is based on LMP1 sequences in isolates from healthy Europeans and patients without EBV disease and yielded four variants: A, B, C, and D. The various criteria used for each of these classification schemes and the limited number of samples that were analyzed make it challenging to draw comparisons across studies and to establish whether specific variants or sequences of the EBV genome are linked to disease. Furthermore, it is important to also consider that 80–100% of healthy, seropositive individuals may be co-infected with multiple forms of EBV (61, 65) and that immunosuppressed or immunocompromised patients have increased susceptibility to concurrent infections (61). With respect to the immune response, there is evidence that genetic diversity can correlate with differences in T cell immunity such that LMP1 variants derived from NPC tumors elicit augmented T regulatory cell function and diminished cytokine production (66, 67).

The full impact of variation in EBV genotypes, with respect to the immune response and to disease, has not likely been appreciated yet, in part, because the analyses have focused on distinct parts of the viral genome. The whole genome sequence of the B95.8 form of EBV was originally reported in 1984 (68) and only recently, with the advent of high-throughput sequencing technology, have studies begun to investigate EBV diversity on a genome-wide scale. Analysis of a recent publicly available sequence repository of EBV+ samples revealed not only high genomic diversity both within and across samples, but also strong evidence for positive selection, as would be expected for a virus under strong selective pressure from the host immune response. Current technologies including high throughput sequencing platforms are likely to provide a more comprehensive, integrated assessment of EBV genotypes and may lead to an improved understanding of the relationship between EBV diversity, health, and disease.

In conclusion, EBV infection initiates a complex, ongoing interplay between the virus, the host B cell and the immune response. EBV has successfully employed a variety of strategies to promote viral persistence in healthy individuals, however, dysregulation of these pathways, or perturbation of host immunity, may contribute to the development of EBV-associated malignancies. Future studies to elucidate the mechanisms by which EBV coopts B cell function, the immune response to EBV, and the significance of viral diversity will be important in understanding the outcome of EBV disease.

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Figure 1. Model of the life cycle of EBV and interactions with the host immune response
 EBV is transmitted from the carrier through the saliva and enters the host via the oropharynx region where it can infect naïve tonsillar B cells via interaction of the viral envelope glycoprotein gp350 and CD21/C3d expressed on B cells. Lytic infection can produce new viral particles and epithelial cells can also become infected. Eventually, infection enters into a latent phase in the periphery and a specific set of viral genes, including LMP1, LMP2a, EBNA1 and EBERs, is expressed (latency III). Infected B cells progress through germinal center reactions in latency II where LMP1 and LMP2a can provide surrogate signals for CD40 and B cell receptor signaling, respectively. EBV+ B cells emerge from the germinal center and EBV persists in a subset of memory B cells without viral gene expression (latency 0) or with EBNA1 expression (latency 1) during cell division. Periodic reactivation of the virus can occur leading to production of new viral particles but host CD4+ and CD8+ T cells specific for lytic cell proteins are effective at controlling this process.

Figure 2. Schematic diagram of (A) LMP1 and (B) induction and regulation of IL-10 by LMP
 LMP1 contains six membrane-spanning domains and a cytoplasmic region containing both TRAF and TRADD binding sites. LMP1 contains two C terminal activating regions (CTAR) and both Box 1 and Box 2 domains. Mutations at AA212 and AA366 were identified in LMP1 molecules isolated from transplant recipients with EBV+ B cell lymphomas when compared to LMP1 isolated from the B95.8 strain of EBV. LMP1 is a constitutively active mimic of CD40 and can activate a variety of signaling pathways including PI3K/AKT via Fyn and Syk kinases. The viral protein LMP2a also activates PI3K/Akt via Lyn kinase. Activation of the PI3K/AKT as well as the p38 MAPK is critical to the constitutive production of the autocrine growth factor human IL-10. LMP1 signaling also modulates host cell miRNA machinery to augment IL-10 production.