Published in final edited form as:
Curr Opin Organ Transplant. 2008 December ; 13(6): 563–568. doi:10.1097/MOT.0b013e3283186ba9.

Epstein Barr Virus, Rapamycin, and Host Immune Responses

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Abstract

Purpose of review—To summarize recent advances that contribute to our understanding of the pathobiology of Epstein Barr virus (EBV)-associated post-transplant lymphoproliferative disease (PTLD), the host immune response to virally-infected B cells, and the molecular basis for the effects of mTOR inhibitors on EBV^+ B cell lymphomas.

Recent Findings—Cytogenetic and genomic analyses support the concept that the underlying biology of EBV-associated PTLD is complex. Transplant recipients can generate and maintain significant populations of EBV-specific $CD8^+$ memory T cells but the function of these cells may be impaired. EBV invokes multiple strategies to subvert and evade the host immune response. The PI3K/AKT/mTOR signal transduction pathway is a nexus for growth and survival signals in PTLD-associated EBV^+ B cell lymphomas.

Summary—Multiple factors influence the development of EBV-associated PTLD including the host immune response to EBV, virally induced effects on the infected cell and the host immune system, and the type and intensity of immunosuppression.

Keywords

PTLD; EBV; Rapamycin; B cell lymphoma

Introduction

Epstein Barr Virus (EBV) is a highly successful gammaherpes virus that has infected over 90% of the world's population. In the vast majority of cases infection is asymptomatic, but EBV is linked to a variety of malignancies of lymphoid and epithelial origin including B cell lymphomas in transplant recipients. EBV has potent transforming ability such that infection of B lymphocytes results in the generation of immortalized lymphoblastoid cell lines (LCL) *in vitro* and autonomously proliferating lymphoblasts *in vivo*. Immunocompetant hosts readily control the expansion of EBV^+ lymphoblasts through vigorous anti-viral T cell immunity. However, in the setting of transplantation these EBV-infected lymphoblasts can give rise to potentially fatal EBV^+ B cell lymphomas. The prevailing paradigm to explain the development of these tumors is that immunosuppressive drugs intended to prevent allograft rejection enable outgrowth of EBV-infected B cells because viral-specific T cells are inadvertently inhibited as well. While impaired host immunity is clearly a principal factor in development of post-transplant malignancies, emerging evidence indicates that a much more complex and dynamic interplay between pharmacologic, viral, and host immune components impacts upon the pathogenesis of post-transplant EBV-associated lymphomas. Rapamycin, an mTOR inhibitor, has drawn much interest recently because of its potential to suppress alloimmune responses

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while simultaneously providing anti-tumor activity. These dual effects are possible because mTOR, a serine/threonine kinase, is responsible for integrating nutrient, hormone, and growth signals with cellular functions including protein synthesis, cell cycle progression, and apoptosis. In this article we discuss recent discoveries that provide insight into the pathogenesis of post-transplant EBV^+ B cell lymphomas, the host immune response to EBV and viral strategies of immune evasion, and the rationale for using mTOR inhibitors as a therapeutic approach.

1. Basic Biology of EBV-associated B Cell Lymphomas in Post-Transplant Lymphoproliferative Disease (PTLD)

In addressing the basic biology of EBV-associated malignancies in transplant recipients it is important to emphasize the heterogeneity of tumor types that exist. PTLD encompasses a spectrum of morphologically distinct lymphoid abnormalities, which are predominantly of B cell origin and in most cases are EBV^+, the focus of this article. The World Health Organization (WHO) classification is increasingly used to describe PTLD subtypes [1]. Three basic categories have been defined by WHO; early lesions, polymorphic PTLD, and monomorphic PTLD. Early lesions include plasmacytic hyperplasia and infectious mononucleosis-like proliferations that are mostly polyclonal and usually regress upon withdrawal of immunosuppression. Polymorphic PTLD are the most common form of PTLD and can be either polyclonal or monoclonal, can originate from B cells at various points along the B cell maturation process, do not usually contain oncogene mutations and have variable response to withdrawal of immunosuppression. Monomorphic PTLD resemble non-Hodgkin's lymphomas and are composed of several subtypes, including some derived from T cells. Those of B cell origin are diffuse large B cell lymphoma, Burkitt's or Burkitt's-like lymphoma, and plasma cell myeloma. Monomorphic PTLD tend to occur later post-transplant and are usually monoclonal proliferations often with cytogenetic abnormalities and mutations in p53, Ras, or c-myc. Monomorphic PTLD is less likely to respond to reduction in immunosuppression.

2. Developments in Understanding the Pathogenesis of PTLD

A key issue in the field is to define the biologic relationship between the various PTLD subtypes. The goal in delineating the underlying molecular and cellular characteristics of the various forms of PTLD is to enable development of more effective therapeutic strategies and the ability to design more specific treatments for individual patients. An obvious approach towards achieving these aims is to utilize genomics and proteomics to analyze gene and protein expression patterns in tumor specimens that represent the various categories of PTLD. A few studies of this type have been recently published. Craig et al. [2] performed gene expression profiling on a small panel of EBV^+ and EBV^- monomorphic PTLD tumors. Over fifty genes showed elevated expression in EBV^+ PTLD and, not surprisingly, were associated with virally-induced immune responses. In contrast, over 200 genes were underexpressed in EBV^+ PTLD compared to EBV^- PTLD. These findings emphasize the importance and impact of EBV on the tumor biology of PTLD. Perhaps the most significant observation from this study is that EBV^+ PTLD could clearly be distinguished from EBV^- PTLD suggesting they are biologically distinct. Moreover, the lack of any viral-associated alterations in the EBV^- PTLD are consistent with the concept that these tumors are in fact, EBV-independent, and are not the result of another oncogenic virus.

Vakiani et al [3] used immunophenotyping, genetic and genomic expression analysis on a panel of PTLD tumors representing the monomorphic and polymorphic subtypes, as well as other B cell non-Hodgkin's lymphomas (B-NHL) from immunocompetant individuals. The authors found that non-GC (naïve or memory cell) PTLD tumors represent a distinct type of B-NHL but are mostly derived from antigen-experienced, memory-like cells. However, EBV^+ and

EBV$^-$ PTLD showed no significant difference in gene expression profiles in contrast to the data of Craig et al [2]. These discordant findings may be related to the relatively small sample size analyzed in each report and the heterogeneity of tumors. This is a common issue that hampers PTLD studies and calls for efforts to conduct larger, collaborative studies. Nevertheless, it is important to note that both studies [2,3] found that gene expression patterns of PTLD tumors were similar to gene expression patterns of EBV-infected LCL, indicating that LCL represent a good *in vitro* model to examine the effects of the virus on cell function.

Recent cytogenetic studies revealed that karyotypic abnormalities are more frequent in monomorphic PTLD compared to polymorphic PTLD [4], however, these chromosomal imbalances did not correlate with EBV infection, clinical outcome or PTLD phenotype. The chromosomal abnormalities identified in PTLD are not unique but are characterized by a reduced frequency and complexity compared to conventional B-NHL, thus underscoring the biological differences in cytogenetic progression of these malignancies.

Dysregulation of signal transduction pathways is a common characteristic of hematopoietic malignancies and potentially could be exploited to develop new therapeutics. Protein microarray analyses in monomorphic PTLD tumor specimens were compared to benign lymph node specimens and revealed dysregulation in the PI3K/AKT/mTOR, mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) pathways [5]. The overactivation of PI3K/AKT/mTOR signaling axis in PTLD tumors provides additional support for the use of the mTOR inhibitor Rapamycin (RAPA) as a therapeutic option in PTLD. This topic will be discussed below in Section 4.

3. EBV and Host Immunity

For the most part the host-pathogen interaction in PTLD pathogenesis has been thought of as unidirectional, with the dominant focus on the debilitated CD8$^+$ T cell response due to immunosuppression. In spite of this it is clear that significant populations of EBV-specific CD8$^+$ T cells can be generated in immunosuppressed graft recipients, and that significant populations of pre-existing anti-viral memory CD8$^+$ T cells are preserved in immunosuppressed graft recipients [6,7]. On the other hand, there has been relatively little attention paid, until recently, to the role of CD4$^+$ T cells in the response to EBV, the functionality of EBV-specific T cells in transplant recipients, and the tactics of EBV to promote immune evasion. Each of these factors is likely to impact significantly on the pathogenesis of PTLD and should be taken into account when designing new therapeutic approaches.

One of the difficulties in studying the basic immune response to EBV is the paucity of good animal models. However, a recent report demonstrated that NOD/SCID mice implanted with human fetal liver and thymic tissue, followed by sublethal irradiation and transplantation of autologous CD34$^+$ cells obtained from fetal liver systemically reconstituted human B cells, T cells, monocytes, macrophages and dendritic cells [8]. Moreover, when the humanized mice were infected with EBV a marked increase in memory T cells was detected and these cells could generate class I- and class II-restricted IFN-γ responses to EBV peptides presented by autologous LCL *in vitro*. This model could prove useful for characterizing the phenotype, specificity, and function of EBV-specific CD4$^+$ and CD8$^+$ T cells *in vivo* and for examining the effects of immunosuppressive drugs on this response.

Humanized SCID (huSCID) mice reconstituted with PBMC from healthy, seropositive donors can give rise to EBV$^+$ B cell lymphomas. The huSCID model has been useful in understanding the factors required for the development of EBV$^+$ B cell lymphomas, however the incidence of tumors in this model is highly variable. Early work showed that autologous T cells are required to generate B cell tumors in this model. New studies now show that plasmacytoid dendritic cells (pDC) appear to be important in EBV immunity because depletion of pDC in

humanized NOD-SCID mice increases mortality from disseminated EBV disease [9]. Conversely, supplementing pDC in humanized mice that were challenged with EBV delayed mortality from EBV^+ B cell lymphomas. In this model pDC produce IFN-α and promote activation of NK cells and $CD3^+$ T cells. These findings demonstrate that there are important cellular interactions that extend beyond host T cells and infected B cells, and that NK cells and DC can also influence the development of EBV^+ B cell lymphomas.

Recent reports examined the function of EBV-specific $CD4^+$ and $CD8^+$ T cells in transplant recipients [7,10]. Several important findings emerge from these studies and related prior studies. First, transplant recipients have easily detectible levels of circulating EBV-specific $CD8^+$ T cells, similar to levels observed in healthy, seropositive individuals. Second, the proportion of $CD8^+$ T cells specific for lytic cycle antigens exceeds the proportion specific for latent cycle antigens. Third, functional activity of EBV-specific cells, on the basis of IFN-γ production, is somewhat diminished in transplant recipients compared to healthy individuals. Fourth, the proportion of functional, EBV-specific $CD8^+$ T cells can increase when immunosuppression is reduced for treatment of PTLD and can coincide with regression of the tumor.

There are indications that the cellular response to EBV can lead to generation of T cells with regulatory activity. Characterization of the $CD4^+$ T cell response to purified LMP1 and LMP1-derived peptides in healthy, seropositive individuals reveals that the response is heavily skewed towards production of IL-10 rather than IFN-γ [11]. In transplant recipients, the use of type I DC to reactivate autologous T cells *in vitro* leads to the expansion of EBV-specific $CD8^+$ T cells that produce both IFN-γ and IL-10, upregulate FOXP3, and can suppress noncognate $CD4^+$ T cell proliferation [12]. It is interesting to note that T cell responses during chronic exposure to HBV, HCV and HIV display an “exhausted” phenotype. Specifically, these T cells fail to respond to encounter with antigen and are marked by the expression of the membrane protein, programmed death -1 (PD-1). In a murine model of chronic viral infection with LCMV Ahmed and colleagues have shown that antibody blockade of the PD-1/PD-1 ligand pathway can restore the ability of the exhausted T cells to proliferate, secrete cytokines, and kill virally-infected cells [13]. It will be of interest to examine the expression of PD-1 on EBV-specific T cells in transplant patients and determine whether their function is impaired.

One of the most intriguing properties of the gammaherpes viruses, including EBV, is the ability to actively evade or subvert host immune responses to promote viral persistence [14]. Several new examples of viral trickery have recently emerged. The discovery that human viruses express microRNAs (miRNA) was made in a Burkitt’s lymphoma cell line latently infected with EBV [15]. miRNA are small non-coding RNAs that regulate gene expression by inhibiting target gene translation or by inducing degradation of the target gene transcript. A recent report showed that the EBV miRNA, BHRF1–3, targets the IFN-inducible T cell attracting chemokine *CXCL-11/TAC* [16]. Suppression of *CXCL-11/TAC* expression by EBV could alter immune responses to tumors. Further, it has become clear that EBV proteins can modulate expression of cellular miRNA. For example, latent membrane protein 1 (LMP1) of EBV induces expression of the cellular miRNA miR-146a [17]. Thus, miRNA provides yet another tool in the arsenal of EBV to modulate cellular function and host immunity.

LMP1, a classic oncogene of EBV, is capable of modulating host immunity on a number of levels including protecting the host B cell from apoptosis. Snow et al. [18] showed LMP1 blocks propagation of the apoptotic signal delivered by cell surface death receptors through NF-κB-dependent induction of the cellular protein cFLIP. In this way, EBV can escape elimination via the Fas/Fas ligand or TRAIL/death receptor effector pathways often used by cytotoxic T cells and NK cells.

Most of the EBV evasion strategies that have been described to date are active during the latent phase of infection, as is seen in PTLD lymphomas. However, viral replication during the lytic phase is potentially susceptible to a strong cellular immune response since numerous highly immunogenic viral proteins are expressed. Indeed, there have been several findings recently describing mechanisms by which EBV dampens the immune response during the lytic phase. It is now clear that EBV, like other herpes viruses, has developed strategies to target multiple sites along the antigen-presentation pathway. The early lytic cycle EBV protein BNLF2a can interact with the TAP complex to prevent peptide- and ATP-binding functions, thereby interfering with $CD8^+$ T cell recognition through HLA class I proteins [19]. Similarly, the early lytic cycle EBV gene product BGLF5 impairs syntheses of HLA class I and class II molecules through a host shutoff mechanism [20,21]. The decrease in HLA proteins can compromise the ability of virus-specific $CD8^+$ T cells to recognize viral peptides presented by infected cells. These maneuvers by EBV can enhance production of infectious viral progeny during primary infection or reactivation and may contribute to elevations in viral load in transplant patients.

4. Molecular Basis for the Use of mTOR Inhibitors in EBV Disease

Clearly, a major dilemma in treating PTLD patients is that the potential benefit of restoring host immunity by withdrawing or reducing immunosuppression is countered by the possibility of precipitating graft rejection. Experimental findings that the immunosuppressive drug RAPA may also have anti-tumor properties suggest a potential opportunity to preserve the graft while treating PTLD. Most of the studies using RAPA, or other mTOR inhibitors, attribute the anti-tumor efficacy to effects on angiogenesis and inhibition of VEGF production [22–24]. However, there is emerging evidence that mTOR inhibitors can also have potent cell autonomous effects that may be particularly relevant in the setting of EBV-associated PTLD. Both RAPA and its derivative everolimus have been shown to inhibit proliferation of EBV^+ B LCL [25,26]. Further, RAPA significantly inhibits production of IL-10, an autocrine growth factor for EBV^+ B cell lymphomas. Our group has recently elucidated the underlying mechanism that accounts for IL-10 production in PTLD-associated B cell lymphomas [27]. Using an inducible signaling system we showed that the EBV-encoded protein LMP1 is sufficient to stimulate IL-10 production in EBV^- B cell lymphomas. The production of IL-10 as a result of LMP1 activation is complex and involves multiple cellular signaling pathways including the p38 MAPK and the PI3K/AKT/mTOR axis. Specifically, LMP1 activates p38 leading to phosphorylation of the transcription factor, CREB, likely involved in IL-10 gene expression. LMP1-signaling also triggers activation of PI3K that induces IL-10 though at least two pathways. First, PI3K inactivates the regulatory protein GSK3β, thereby allowing enhanced CREB function. Second, PI3K activates mTOR, leading to phosphorylation of the mTOR substrate p70-S6 kinase and likely enhancing IL-10 expression through translational mechanisms. The later pathway of IL-10 production is targeted by RAPA but each of these pathways could potentially be exploited as therapeutic targets for treatment of PTLD.

RAPA also inhibits constitutive activation of the Jak/STAT pathway [26] a pathway often dysregulated in hematopoietic malignancies. Finally, RAPA has direct effects on the expression of cell cycle proteins that could account for its ability to induce arrest of EBV^+ B cell lymphomas in the G1 phase of the cell cycle [28]. Together, these effects on EBV^+ B cell lymphomas by RAPA culminate in profound inhibition of tumor cell proliferation. Importantly, these studies were all conducted with concentrations of RAPA (5–10 ng/ml) that are pharmacologically achievable in patients, further emphasizing the relevance of these findings.

Another interesting twist with respect to RAPA is that the PI3K/AKT/mTOR axis appears to be intimately linked to the development of T regulatory cells (Treg) because inhibition of mTOR signaling by RAPA can confer FoxP3 expression in T cells [29,30]. Indeed, RAPA has been shown to be an effective agent for *in vitro* expansion of T cells with regulatory function.

On the one hand, induction of Treg could be advantageous to graft survival, but effects of Treg on immune-mediated elimination of EBV-infected B cells could be potentially deleterious.

5. Clinical Reports on Rapamycin and PTLD

As outlined above, mounting experimental evidence supports the rationale for utilizing mTOR inhibitors as a means to reduce the incidence of PTLD in patients at risk for EBV disease or as alternative to minimizing immunosuppression in patients diagnosed with PTLD. In addition, the direct examination of clinical PTLD tissue samples demonstrates constitutive activation of mTOR [31]. However, whether or not mTOR inhibitors affect the incidence of PTLD in transplant recipients has been controversial. Several studies report a lower overall incidence of malignancies (including skin, PTLD, and other neoplasms) in graft recipients receiving RAPA-based maintenance therapy compared to calcineurin inhibitor (CNI)-based therapies [32–34]. On the other hand, either no difference, or an increased incidence, of PTLD in transplant recipients on RAPA-based maintenance therapy has also been reported [35–37]. However, in one of the studies the patient number was noted to be small [37] and in another study over-immunosuppression with RAPA, CNI, basiliximab, and steroids in a high risk group likely contributed to the high incidence of PTLD [36]. These findings again raise the question of whether the overall intensity of immunosuppression rather than use of any particular agent may be an overriding factor impacting the onset of de novo malignancies. Thus, at this point it is difficult to draw definitive conclusions regarding the effect of RAPA on the incidence of PTLD despite the strong experimental evidence demonstrating inhibitory effects on EBV^+ B cell lines. With respect to conversion to RAPA following diagnosis of PTLD, several small series describe a beneficial effect with complete remission often observed [38–41]. However, it remains to be seen if tumor regression is related to the direct effects of RAPA or to withdrawal of the initial immunosuppression regimen. Interestingly, the RAPA derivative everolimus has shown efficacy in reducing cardiac allograft vasculopathy and this effect was associated with a decreased risk for CMV infection [42]. In addition, another RAPA derivative, temsirolimus, has shown significant anti-tumor activity in patients with relapsed mantle cell lymphoma [43]. Both of these agents, like RAPA, act to inhibit mTOR and it will be of interest to determine if they have similar effects, on a cellular and molecular level, on the transformation and growth of EBV-infected B cells.

6. Conclusion

The pathogenesis of EBV^+ B cell lymphomas in PTLD involves immune, viral and pharmacologic factors. In order to develop more effective therapeutic options for patients it will be necessary to better define the biologic relationship between the different forms of EBV^+ PTLD tumors, to characterize host-pathogen interactions, and to understand, on a molecular level, the effects of immunosuppressive drugs on EBV-infected B cells and on development of PTLD in transplant recipients.

Acknowledgements

Supported by NIH awards: RO1 AI41769 and RO1 CA105157

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