T-Cell Responses to EBV

Andrew D. Hislop and Graham S. Taylor

Abstract Epstein–Barr virus (EBV) is arguably one of the most successful pathogens of humans, persistently infecting over ninety percent of the world's population. Despite this high frequency of carriage, the virus causes apparently few adverse effects in the vast majority of infected individuals. Nevertheless, the potent growth transforming ability of EBV means the virus has the potential to cause malignancies in infected individuals. Indeed, EBV is thought to cause 1 % of human malignancies, equating to 200,000 malignancies each year. A clear factor as to why virus-induced disease is relatively infrequent in healthy infected individuals is the presence of a potent immune response to EBV, in particular, that mediated by T cells. Thus, patient groups with immunodeficiencies or whose cellular immune response is suppressed have much higher frequencies of EBV-induced disease and, in at least some cases, these diseases can be controlled by restoration of the T-cell compartment. In this chapter, we will primarily review the role the αβ subset of T cells in the control of EBV in healthy and diseased individuals.

Contents

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Abbreviations

BL	Burkitt lymphoma
CMV	Cytomegalovirus
DLBCL	Diffuse large B-cell lymphoma
E	Early
EBNA	Epstein–Barr nuclear antigen
EBV	Epstein–Barr virus
ENKTL	Extranodal NK/T-cell lymphoma
GCa	Gastric carcinoma
HL	Hodgkin lymphoma
HLA	Human leucocyte antigen
IE	Immediate early
IM	Infectious mononucleosis
iNKT	Invariant natural killer T cell
L	Late
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
MHC	Major histocompatability complex
NK	Natural killer
NPC	Nasopharyngeal carcinoma
PTLD	Post-transplant lymphoproliferative disease
SAP	Signaling lymphocytic activation molecule-associated protein
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
VCA	Viral capsid antigen
XIAP	X-linked inhibitor of apoptosis protein
XLP	X-linked lymphoproliferative disease

1 Introduction

Like all herpesviruses, the EBV replication cycle oscillates between phases of either lytic or latent viral gene expression in infected cells. During lytic replication, up to 80 genes are expressed in a temporally regulated manner resulting in the production of new viral particles, the death of the infected cell and transmission of the virus to other cells or spread to new hosts. Latent gene expression, in the case of EBV, can induce growth transformation of infected cells; these genes include the six Epstein–Barr nuclear antigens (EBNAs) and the two latent membrane proteins (LMPs). Here, there is no virus production and ultimately the virus can enter a quiescent state where there is minimal, if any, viral gene expression allowing amplification of a reservoir of infected cells capable of reactivating virus in the future. These two patterns of gene expression give the immune system two potential sets of antigens that can be targeted to control the virus. Furthermore, in the normal biology of infection both sets of genes are likely expressed in different cellular backgrounds and different anatomical locations. Thus, latent antigens are expressed in B lymphocytes within lymphoid tissue, while lytic antigens are expressed also in B lymphocytes but likely more frequently expressed in epithelial cells of the oropharynx (Hadinoto et al. 2009). These different cellular backgrounds and anatomical locations likely impact on the virus-specific T-cell response in terms of the function and subset of antigen-specific T cells which traffic to these sites.

2 T-Cell Response in Primary Infection

Primary infection with EBV mostly occurs in childhood and in the main results in an infection with few if any symptoms of acute virus infection. However, if infection is delayed until adolescence, some 25–75 % of infected individuals will develop the primary infection syndrome infectious mononucleosis (IM): an acute self-limiting febrile illness characterised by the development of lymphadenopathy, sore throat and associated with the massive expansion of activated CD8+ T lymphocytes (Balfour et al. 2013; Crawford et al. 2006). Most of what we know about primary infection and the host response comes from the study of IM patients as the symptoms provide a convenient marker to identify people undergoing primary infection.

2.1 T-Cell Response in Infectious Mononucleosis

People who develop infectious mononucleosis are thought to have been infected with the virus for up to six weeks before the symptoms became apparent. Why primary EBV infection can result in IM is presently unclear, but several proposals

have been put forward. These include the development of T-cell specificities induced by non-EBV epitopes that cross-react with EBV epitopes and contribute to the expanded CD8+ T-cell response in IM (Clute et al. 2005; Selin et al. 2011). However, ex vivo analysis of such cross-reactive T-cell responses show they are low in frequency (Cornberg et al. 2010) and so their contribution to IM requires clarification. Others have proposed that IM may develop in part due to the reduced frequency of regulatory T cells found in IM patients compared to healthy donors (Wingate et al. 2009). Two recent epidemiological studies have suggested that a genetic component may be responsible for the development of IM. Thus, same sex twins have a higher incidence of IM compared to first-degree relatives (Rostgaard et al. 2014), while the concordance for development of IM was twice as frequent in monozygotic twins compared to dizygotic twins (Hwang et al. 2012). What form these genetic differences may take are unclear; however, polymorphisms or genetic markers associated with IM in immune response loci have been identified including polymorphisms in the promoters of IL-10 and IL-1 (Helminen et al. 1999; Hatta et al. 2007), polymorphisms in the TGF-β gene (Hatta et al. 2007) and presence of certain microsatellite markers in the HLA locus (McAulay et al. 2007).

At presentation, IM patients shed high levels of virus from the oropharynx and have high loads within their peripheral B-cell compartment. Few studies have been able to access samples from patients preceding the development of symptoms to determine what immune responses may be occurring at this time. Recent careful studies following EBV-negative university students who subsequently develop IM have shown that prior to the development of symptoms there is no obvious disturbance of the CD4+ or CD8+ lymphocyte compartment (Balfour et al. 2013). However, once symptoms develop in the patient there is a major disturbance, principally driven by the expansion of highly activated CD8+ T cells. Historically, the nature of these T cells was enigmatic, with initial studies analysing TCR usage indicating that these were monoclonal or polyclonal expansions suggestive of antigen-driven proliferation (Callan et al. 1996). Ex vivo analysis of these populations by either cytotoxicity or cytokine secretion assays showed that they contained EBV specificities (White et al. 1996; Steven et al. 1996, 1997; Hoshino et al. 1999). The development of MHC class I tetramer reagents that could identify EBV reactive CD8+ T cells clearly showed that significant proportions of the expanded CD8+ cell population were in fact EBV-specific (Callan et al. 1998). Further studies by numerous groups have now shown that in IM patients the expanded CD8+ population is mostly EBV-specific (Catalina et al. 2001; Hislop et al. 2002, 2005) although evidence has been presented for the activation of other virus specificities during acute infection (Odumade et al. 2012; Clute et al. 2005).

2.1.1 CD8+ T-Cell Response in Infectious Mononucleosis

Analysis of the specificities within the expanded CD8+ T-cell population has shown that it is dominated by T cells specific for epitopes derived from the

immediate early (IE) gene products with lesser, yet still substantial, frequencies to some early (E) gene products and apparently few T cells specific for late (L) gene products (Pudney et al. 2005; Woodberry et al. 2005b). In some cases, individual lytic epitope-specific responses can constitute between 1 and 50 % of the expanded CD8+ repertoire in the peripheral circulation (Hislop et al. 2005). What drives the IE > E >> L immunodominance hierarchy is not clear. Two proposals have been suggested: firstly, as IE antigens are expressed in the first wave of viral protein synthesis IE-specific T cells may clear infected cells before other antigens are produced, reducing the supply of antigen to restimulate other later specificities (Pudney et al. 2005). Secondly, as immune evasion proteins are expressed predominantly in the E phase of lytic cycle, these reduce the presentation of epitopes on infected B cells that are derived from other E- and L-expressed proteins, limiting the ability of E- and L-phase epitope-specific T cells to be restimulated by infected cells (Hislop et al. 2007a).

Latent antigen-specific responses are also substantially expanded although generally not to the same degree as lytic epitope-specific T cells with individual epitope specificities representing from 0.1 to 5 % of the peripheral CD8+ T-cell population (Catalina et al. 2001; Hislop et al. 2002). In these cases, T cells are specific for epitopes predominantly drawn from the EBNA3 family of proteins and to a much lesser extent LMP2. EBNA1-specific responses are seen less frequently but in the context of certain HLA types strong responses have been observed (Blake et al. 2000) while CD8 specificities to LMP1 are rarely if ever seen (Hislop et al. 2002; Catalina et al. 2001; Woodberry et al. 2005b).

Although comprehensive studies of T-cell frequencies in the peripheral circulation of IM patients have demonstrated the high-frequency responses, a more relevant site to measure T-cell responses is the site of virus replication, namely the oropharyngeal lymphoid tissue making up Waldeyer's ring. Estimation of EBV-specific CD8+ T-cell frequencies in homogenised tonsillar preparations taken from IM patients showed substantial frequencies of EBV-specific CD8+ T cells in this anatomical compartment, with up to 25 % and 1.5 % of the CD8 population being specific for EBV lytic or latent epitopes, respectively (Hislop et al. 2005). However, comparing these frequencies to matched peripheral blood mononuclear cell (PBMC) preparations collected at the same time showed that these EBV-specific CD8+ T-cell frequencies were far lower in the tonsil than what was detected in periphery, particularly for lytic epitope-specific CD8+ T cells. This was despite very high virus genome loads being detected in these tonsillar preparations.

MHC class I tetramer-based analysis of the CD8+ T-cell response has yielded valuable information as to the phenotypic characteristics of EBV-specific T cells. These cells show evidence of being highly activated, expressing HLA-DR, CD38, CD69 and are in cycle as judged by the expression of ki-67(Callan et al. 1998). Historically, it has been known that PBMC from IM patients are highly susceptible to apoptosis when manipulated in vitro (Moss et al. 1985) and that this is likely a consequence of the low level of expression of the anti-apoptotic protein bcl-2 (Callan et al. 2000; Soares et al. 2004). Consistent with their highly activated

status, these EBV-specific T cells express little in the way of lymphoid homing markers such as CCR7 or CD62-L (Catalina et al. 2002; Hislop et al. 2002). This lack of expression may give some clue as to why relatively lower frequencies of EBV-specific T cells are seen in the tonsils compared to the peripheral circulation. Entry of T cells into the tonsils requires expression of lymphoid homing markers, particularly since tonsils do not have afferent lymphatics and lymphocytes cannot therefore drain into this lymphoid tissue from peripheral sites. Nevertheless, EBV-specific T cells are found at this site and other mechanisms of recruitment may be relevant. Thus, activated T cells are known to express CXCR3, which binds the IFN-γ inducible chemokines CXCL9 and CXCL10, which may direct effectors to this site. Conceivably, the inefficient recruitment of EBV-specific T cells to the tonsil during IM may explain why such high levels of virus are found at this location.

Within weeks, the symptoms of IM begin to resolve. During this time, there is a steep decline in virus genome loads found in the peripheral circulation although virus loads shed from the oropharynx remain high for several months (Fafi-Kremer et al. 2005; Hislop et al. 2005; Balfour et al. 2005). During this time, the EBV-specific T cells in the periphery are culled and the frequency and absolute number decline rapidly. Lytic epitope-specific populations are dramatically reduced, particularly those that are highly expanded (Catalina et al. 2001; Hislop et al. 2002) leading to a distribution of epitope specificities which does not necessarily reflect the frequencies seen during acute infection. Although frequencies of latent epitope-specific cells change less, with the contraction of the entire CD8 compartment numerically fewer latent epitope-specific cells will be present. Despite the dramatic impact, IM has on the T-cell compartment, when disease resolves there does not appear to be any attrition of pre-existing memory T cells, with absolute numbers of T cells specific to other viral epitopes broadly comparable before and after acute EBV infection (Odumade et al. 2012).

After resolution of disease, EBV-specific CD8+ T cells begin to return to a resting state, downregulate activation markers, come out of cycle and upregulate expression of anti-apoptotic proteins such as bcl-2 (Dunne et al. 2002). Latent, but not lytic, epitope-specific T cells begin to express CCR7 and CD62-L, and this is associated with recruitment of these T cells to the tonsil at a time when the control of growth transformation of B cells at this site is known to be controlled. Lytic epitope-specific cells remain poorly represented in this tissue, consistent with the continued high-level shedding of virus in saliva.

2.1.2 CD4+ T-Cell Response in Infectious Mononucleosis

Turning to the CD4+ T-cell response to EBV during IM, less is known about the response mediated by these cells due to the low frequency of specific responses and the fact that until very recently assays to measure CD4 T-cell responses were relatively insensitive. However, there is now a better appreciation of these cells as potential effectors against MHC class II targets, such as EBV-infected B

cells. During IM there is little, if any, expansion of the global CD4 compartment (Balfour et al. 2013) and in contrast to the CD8 compartment, TCR analysis shows there is no evidence of antigen-driven monoclonal or oligoclonal expansions of CD4 + T cells (Maini et al. 2000). Initial attempts to study CD4+ T-cell responses used cytokine secretion assays to measure responses from CD4+ T cells stimulated with recombinant antigens or lysates of EBV-infected cells. These detected relatively weak responses to lytic and latent antigens with lytic antigens eliciting responses more frequently (Precopio et al. 2003; Amyes et al. 2003).

However, an increasing range of MHC class II tetramer reagents have recently become available and these have been employed to follow responses in IM patients from acute infection through resolution of disease (Long et al. 2013). With the epitope-specific reagents used in this work, particular latent responses appeared to dominate lytic responses with up to 1.5 % of CD4+ T cells specific for individual latent epitopes, compared to a maximum of 0.5 % of CD4+ T cells for lytic responses. These measurements were substantially higher than those estimated by cytokine secretion analysis in this and previous studies. The EBV-specific CD4 T cells, like CD8+ T cells, were highly activated and low frequencies expressed lymphoid homing markers.

With resolution of acute symptoms, tracking the CD4+ T-cell response shows that the frequencies of EBV-specific cells drops precipitously over a short period of time (Long et al. 2013; Precopio et al. 2003). In contrast to other latent epitope specificities, EBNA1-specific CD4+ T-cell responses are not readily detected during acute infection but emerge with delayed kinetics over several months. This delayed appearance has been attributed to the restricted release of EBNA1 from infected cells, thereby reducing antigen available for priming CD4+ T-cell responses (Long et al. 2013).

2.2 T-Cell Response in Asymptomatic Primary Infection

Although IM has taught us much about the immune responses made during primary EBV infection, these responses likely do not represent the situation in the majority of primary infections which occur asymptomatically in children. Determining the host response to the virus in this situation is extremely difficult since, by definition, the infection occurs without obvious evidence.

Nevertheless, careful methodical studies following EBV-seronegative individuals over time have allowed cases of asymptomatic EBV infections to be identified. Early studies of infants monitored monthly for seroconversion indicated that they showed none of the features seen in IM patients in terms of disruption to the lymphocyte compartment or febrile illness (Biggar et al. 1978; Fleisher et al. 1979). More contemporary studies have shown that young adults undergoing asymptomatic infection can have high virus loads in the peripheral circulation, equivalent to what is seen in IM patients; however, there is no lymphocytosis and unlike IM patients, most showed no disruption within the T-cell compartment of

the clonality of their TCR Vβ repertoire (Silins et al. 2001). A recent study examining African children undergoing asymptomatic infection has suggested that, like the young adults in the earlier work, children can have genome loads equivalent to IM patients. Moreover, they can have substantial frequencies of activated EBV-specific CD8+ T cells as detected using MHC class I tetramers, up to 16 % of CD8+ T cells. Despite these high-frequency responses, there is no significant global expansion of CD8+ T-cell compartment (Jayasooriya et al. 2015).

Collectively these studies suggest that it is the global expansion of activated CD8+ T cells that is driving the pathology seen in IM. Although these studies are helpful for characterising the virus–host balance in asymptomatic infection, they do not explain the mechanism of control in the absence of pathology. Studies using the humanised mouse model of EBV infection have shed some light on this however, suggesting that specific subsets of natural killer cells may play a role in preventing the development of an IM like disease in this model (Chijioke et al. 2013) indicating that analysis of NK subsets in asymptomatic infected donors may be helpful.

3 T-Cell Response in Established Infection

The T-cell response seen in people with established EBV infections shows that there are substantial frequencies of EBV-specific CD8+ and CD4+ T cells present, although at a much reduced frequency and absolute number than what is seen in IM patients. In healthy donors, low frequencies of infected cells are detected in the memory B-cell compartment of the peripheral circulation (Babcock et al. 1998) and there is intermittent shedding of virus from the oropharynx (Fafi-Kremer et al. 2005) indicating that these responses are maintained in the presence of low-level antigen expression.

3.1 CD8+ T-Cell Response in Established Infection

Individual lytic antigen-specific CD8 responses can account for up to 2 % of the CD8+ population, while latent antigen-specific responses are smaller constituting up to 1 % of the CD8 population. As seen in IM patients, CD8+ T cells specific for immediate early-expressed epitopes are the dominant specificities with lower responses to a subset of early-expressed epitopes and rare responses to late-expressed epitopes (Abbott et al. 2013). Although responses to late epitopes are of low frequency, a diverse range of late antigens are targeted and these responses are thought to increase with age (Orlova et al. 2011; Stowe et al. 2007). Latent responses are mostly made to epitopes derived from the EBNA3 family of proteins and to a lesser extent LMP2, EBNA1 and EBNA2, while infrequent responses are

detected against EBNA-LP and LMP1. Interestingly, alterations to this hierarchy can be observed dependent on the HLA type of the donors. Thus, donors who are HLA B38 make strong responses to an epitope derived from EBNA2 (Chapman et al. 2001) while donors who are HLA-A*02.03 make a strong response to an EBNA-LP-derived epitope (unpublished observations).

Although there is evidence of chronic EBV shedding and thus antigen production, EBV-specific T cells circulating in the periphery show little evidence of activation. In contrast to the picture of IM, these cells appear mostly as resting antigen-experienced T cells expressing LFA-1 (Faint et al. 2001), they are not activated, are not in cycle and have relatively high levels of bcl2 (Callan et al. 2000; Hislop et al. 2001).

These T cells now also express variable levels of markers associated with homing to lymphoid tissues such as CCR7 and CD62L. The frequency of expression of these markers is higher on latent compared to lytic epitope-specific T cells for reasons that are unclear but perhaps related to the environment in which antigen is presented to the T cell. Analysis of the frequency of EBV-specific T cells in matched blood and lymph node specimens has, however, shown no obvious enrichment of EBV specificities in this latter compartment (Remmerswaal et al. 2012). Some enrichment of lytic but not latent epitope specificities in bone marrow specimens has been described although what is driving this enrichment of lytic specificities is unclear as there is no obvious increase in EBV genome loads in this compartment compared to blood (Palendira et al. 2008). However, an obvious enrichment of EBV-specific T cells is seen in the tonsil, where an approximate threefold and tenfold increase in lytic and latent specificities, respectively, is seen (Hislop et al. 2005; Woodberry et al. 2005a). In some tonsils then, at least 20 % of the CD8+ T-cell population is specific for EBV. Correlating with this enrichment is the surface expression of CD103 ($\alpha E\beta 7$) by these EBV-specific T cells. This integrin binds to E-cadherin which is expressed by epithelial cells, thereby retaining the T cells at these sites. Furthermore, this molecule is now recognised as marker of resident memory T cells, a population of cells poised to reactivate at a site of previous antigen expression (Gebhardt et al. 2009).

In most healthy donors, the virus appears to establish a stable balance with the immune response although occasional fluctuations in the size of the response are seen, possibly due to subclinical reactivation (Crough et al. 2005). However, evidence for disruption of this balance and dysregulation of the T-cell response is emerging in older donors. Thus, elevated virus loads and CD8+ T-cell responses have been described in the elderly with some EBV-specific responses constituting up to 15 % of the CD8+ T-cell population in individuals over 60 years of age, although some loss of T-cell function was seen in these donors (Stowe et al. 2007; Khan et al. 2004). Interestingly, these expansions were not seen or were less marked in elderly donors co-infected with cytomegalovirus (CMV), suggesting the presence of CMV may suppress immunity to other viruses (Khan et al. 2004; Stowe et al. 2007; Colonna-Romano et al. 2007; Vescovini et al. 2004).

3.2 CD4+ T-Cell Response in Established Infection

Analysis of people with established EBV infections shows the EBV-specific CD4+ T-cell response differs from the CD8+ responses in several respects. First, the size of the memory CD4+ T-cell response to individual epitopes is much smaller (Leen et al. 2001; Amyes et al. 2003; Long et al. 2005, 2011, 2013). Second, the antigen targets of the CD4 T-cell response are also different, with latent reactivities tending to outnumber lytic reactivities (Long et al. 2013). Third, the proportion of central and effector memory CD4+ T cells is the same regardless of whether the cognate epitopes are from lytic or latent antigens (Long et al. 2013); CD8+ T-cell responses to the former are concentrated in the effector memory pool. Fourth, the CD4 T-cell response against lytic antigens is spread equally between IE, E and L viral proteins (Long et al. 2011), whereas the CD8 T-cell response is heavily skewed towards the former (Pudney et al. 2005).

The fact that EBV infects and persists in B cells, which constitutively express MHC class II, raises the possibility that CD4+ T cells may be able to act as direct effector cells in their own right. Reports from several groups clearly demonstrate that CD4+ T-cell clones against a wide range of EBV lytic and latent cycle antigens are able to recognise and kill newly infected B cells or established EBV-transformed lymphoblastoid cell lines (LCLs) (Adhikary et al. 2006; Kobayashi et al. 2008; Long et al. 2005; Munz et al. 2000; Sun et al. 2002b; Haigh et al. 2008; Khanna et al. 1997; Landais et al. 2004; Omiya et al. 2002; Demachi-Okamura et al. 2006; Rajnavolgyi et al. 2000). Although only a minority of cells in such lines are lytically infected, structural and non-structural lytic cycle proteins are efficiently transferred to neighbouring LCLs sensitising them to recognition by lytic antigen-specific CD4 T cells (Adhikary et al. 2006; Landais et al. 2004; Long et al. 2011). Receptor-mediated uptake of virions by LCLs likely explains the efficient transfer of structural proteins. The mechanism responsible for efficient uptake, processing and presentation of non-structural lytic proteins is currently unknown, but it appears to be part of a general phenomenon since the latent cycle proteins EBNA2, EBNA3A, EBNA3B and EBNA3C are also efficiently transferred from antigen-positive to antigen-negative B cells (Taylor et al. 2006; Mackay et al. 2009).

As described earlier, EBNA1 does not appear to be transferred between cells in culture (Leung et al. 2010; Long et al. 2013). Instead, EBNA1 is able to access the MHC-II pathway within the infected cell itself via macroautophagy (Paludan et al. 2005) a catabolic pathway in which cytoplasmic contents are enveloped by double-membrane vesicles that in turn fuse with lysosomes. However, EBNA1's normal nuclear localisation limits its processing by macroautophagy (Leung et al. 2010) and such 'nuclear shelter' from macroautophagy means only a subset of EBNA1 CD4+ T-cell epitopes are presented by LCLs (Paludan et al. 2005; Khanna et al. 1995; Mautner et al. 2004; Leung et al. 2010). These observations may resolve the paradox that although EBNA1 is essential for viral persistence (Humme et al. 2003), it nevertheless contains the largest number of CD4+ T-cell

epitopes of any latent cycle protein and most EBV-infected individuals possess good CD4+ T-cell responses against the protein (Leen et al. 2001; Long et al. 2013; Munz et al. 2000).

3.3 Other T-Cell Subsets in Established Infection

Turning to other subsets of T cells, little so far is known about the relevance of more specialised T cells such as Th9, Th17 or Th21 T cells in EBV infection. Some studies have suggested a role for invariant natural killer T cells (iNKT) in control of EBV. Thus, patients deficient in the SLAM-associated protein (SAP) encoded by *SH2D1A* have no NKT cells and are exquisitely sensitive to EBV infection and may develop a life-threatening lymphoproliferative disease upon EBV infection (Nichols et al. 2005). However, such mutations also affect conventional T-cell and natural killer (NK) cell function making it unclear whether this disease is solely due to lack of iNKT cells (Tangye 2014). Similarly, patients with mutations in the *BIRC4* gene, which encodes the X-linked inhibitor of apoptosis protein (XIAP), show sensitivity to EBV infection and have low numbers of iNKT cells (Rigaud et al. 2006). These patients have normal numbers of T cells; however, these are more sensitive to apoptotic stimuli, again making it unclear whether iNKT numbers are the solely responsible for controlling disease (Lopez-Granados et al. 2014). Other models and observations hint to a role of iNKT cells: patients with EBV-associated malignancies have lower circulating numbers of these cells, while iNKT cells adoptively transferred into immunodeficient mice then challenged with EBV-related malignant cells show reduced tumour formation (Yuling et al. 2009). Similarly, in vitro studies of resting B cells challenged with EBV showed higher frequencies of transformation when NKT cells are depleted from such cultures (Chung et al. 2013).

Some 1–10 % of the total T-cell population is comprised of γδ T cells that recognise a distinct range of antigenic targets and have a broad functional phenotype upon activation (Vantourout and Hayday 2013). The importance of these cells in controlling natural EBV infection is not known, but several observations suggest they could play a role. The Vδ1 subset of γδ T cells can directly recognise and lyse EBV-transformed LCLs in vitro (Hacker et al. 1992) and high frequencies of these cells have been described in transplant recipients who have previously experienced EBV reactivation (Fujishima et al. 2007; Farnault et al. 2013). The Vγ2 Vδ9 subset of γδ T cells can also recognise and lyse LCLs in vitro, but efficiency is low unless the cells are activated with pamidronate and then positively selected using anti-γδ-TCR-specific beads (thus delivering a TCR signal to the cells) (Xiang et al. 2014). However in mice reconstituted with human immune system components, pamidronate administration was sufficient to significantly reduce EBV-positive lymphoproliferative disease and this control was dependent upon Vγ2 Vδ2 T cells (Xiang et al. 2014). The antigens that allow selective recognition of EBV-infected LCLs by γδ T cells are currently unknown.

4 T-Cell Responses in Patients with EBV-Associated Malignancy

The EBV-specific T-cell response generated by natural infection is important to control the growth transforming activity of the virus for the lifetime of the host. Loss of this control, as occurs in patients receiving immunosuppression, can lead to post-transplant lymphoproliferative disease (PTLD). Tumours that occur in the first year of transplantation, when immunosuppression is greatest, typically express the full range of EBV latency proteins including the EBNA3A, EBNA3B and EBNA3C proteins that are immunodominant targets of CD8 T-cell immunity. Furthermore, these tumours display high levels of HLA class I and II molecules. Accordingly, restoring immunological control by adoptive transfer of EBV-specific T-cell preparations generated in vitro has been used by several groups for prophylaxis or treatment of PTLD following solid organ or haematopoetic stem cell transplantation (Rooney et al. 1995; Khanna et al. 1999; Sun et al. 2002a; Barker et al. 2010; Haque et al. 2007) with an excellent track record of safety and efficacy (Heslop et al. 2010).

Until recently, the T cells used to treat patients with PTLD were generated using EBV-transformed LCLs as the antigen source. The key T-cell effectors mediating clinical responses in patients were therefore thought to be the EBNA3A-, EBNA3B- and EBNA3C-specific CD8+ T cells that tend to dominate LCL-stimulated T-cell preparations. Undoubtedly, these T cells are important (Gottschalk et al. 2001), but recent data suggests that other specificities may also contribute to tumour control. Thus, LCL-stimulated T-cell preparations containing a higher level of CD4+ T cells were associated with better outcome in a multicentre phase II trial of adoptive therapy (Haque et al. 2007). The antigenic specificity and function of these CD4 T cells were not characterised, and it is possible that their importance reflects the provision of CD4-mediated T-cell help rather than that of a role as direct effectors. Nevertheless, this result is intriguing given the multiple reports of CD4 T-cell clones specific for a range of EBV latent and lytic cycle proteins directly recognising and killing LCLs (Adhikary et al. 2006; Kobayashi et al. 2008; Long et al. 2005; Munz et al. 2000; Sun et al. 2002b; Haigh et al. 2008; Khanna et al. 1997; Landais et al. 2004; Omiya et al. 2002; Demachi-Okamura et al. 2006; Rajnavolgyi et al. 2000). Not all T-cell clones are capable of such recognition and it is clear that the abundance of different CD4+ T-cell epitopes on the target cell surface can vary markedly, even for epitopes derived from the same protein (Long et al. 2005; Leung et al. 2010). CD4+ T cells incapable of direct recognition of EBV-positive cells could still be of value, however, by providing T-cell help to the overall immune response. Cultures of T cells prepared using LCLs as stimulators also include CD4+ T cells specific for non-viral antigens upregulated in B cells by EBV transformation (Gudgeon et al. 2005; Long et al. 2009). These cellular-antigen-specific CD4+ T cells can also control LCL outgrowth and may therefore enhance the anti-tumour effect, but do not appear to be essential since T-cell lines prepared without the use of LCLs, presumably

Table 1 EBV-associated malignancies and their expression of EBV antigens

Tumour	Subtype	% EBV positive	EBV proteins expressed
Burkitt Lymphoma	Endemic AIDS-related	100 30–40	EBNA1[a]
T/NK Lymphoma	Extranodal	100	EBNA1, LMP2B[b]
Diffuse large B-cell lymphoma	Late PT-DLBCL Elderly DLBCL AIDS-related	>50 >50 ~50	EBNA1, LMP1, LMP2[c]
Hodgkin lymphoma	Classical AIDS-related	30 100	EBNA1, LMP1, LMP2
Lympho-proliferative disease	Post-transplant, AIDS-related	100	EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2
Nasopharyngeal carcinoma	Undifferentiated	100	EBNA1, LMP1, LMP2[d]
Gastric carcinoma		5–15	EBNA1, LMP2[d]

[a]Some 10–15 % of endemic BLs express EBNA1, EBNA3A, EBNA3B EBNA3C, EBNA-LP and BHRF-1

[b] LMP2B is expressed from a novel mRNA transcript in the absence of LMP2A

[c]Reports of wider range of EBV latency genes reported in some cases

[d]BARF1 expression is reported in a proportion of these tumours

lacking such responses, yield clinical responses in transplant recipients with PTLD or undergoing EBV reactivation (Gerdemann et al. 2012; Icheva et al. 2013).

Most cases of EBV-associated malignancy, however, develop in people who are not iatrogenically immunosuppressed and express a smaller number EBV antigens (Table 1). These malignancies include lymphomas of B-cell origin such as Burkitt lymphoma (BL), Hodgkin lymphoma (HL) and diffuse large B-cell lymphoma and a smaller number of cases of non-B-cell origin such as extranodal NK/T-cell lymphoma (ENKTL). EBV is also linked to almost all cases of undifferentiated nasopharyngeal carcinoma (NPC) and a proportion of gastric carcinomas (GCa). The immunodominant EBNA3A, EBNA3B and EBNA3C proteins are absent in these tumours, but the EBV antigens that are expressed are still *bona fide* T-cell targets (Hislop et al. 2007b). Given that loss of EBV immune control underpins post-transplant lymphoma, an important question is whether the EBV-specific T-cell response is perturbed in patients with these other malignancies. This appears to be the case for endemic Burkitt lymphoma, which occurs in areas of holoendemic *Plasmodium falciparum* malaria. Recurrent malarial infection of young children adversely affects the EBV-specific T-cell response as measured using regression assays (Moss et al. 1983; Whittle et al. 1984). Studies of children in high- and low-incidence malaria areas show the former have high EBV viral loads (Moormann et al. 2005). Subsequent studies have shown that children living in endemic malarial areas have reduced CD8 T-cell responses to EBV lytic and latent antigens (Moormann et al. 2007) or phenotypic changes in these responses consistent with greater differentiation (Chattopadhyay et al. 2013); each could conceivably alter the virus–host balance to favour the development of Burkitt lymphoma.

An alterative, but not necessarily mutually exclusive explanation for the development of Burkitt lymphoma is that the tumours are able to develop because they escape immune control. The focus of attention here has been EBNA1 since this is the only EBV protein expressed in the majority of Burkitt lymphoma cases (Kelly et al. 2002). Although it is now known that the glycine/alanine repeat domain within EBNA1 does not afford complete protection from the MHC class I processing pathway (Tellam et al. 2004; Voo et al. 2004; Lee et al. 2004), Burkitt lymphoma cells show reduced expression of HLA class I molecules as well as the TAP-1 and TAP-2 proteins required for transport of antigenic peptides into the endoplasmic reticulum for HLA binding. Together, these defects contribute to a profound impairment of the ability of CD8+ T cells to recognise BL cells (Rowe et al. 1995; Khanna et al. 1994) even in cases when the tumours express strong immune targets (Kelly et al. 2002). Burkitt lymphoma cells do, however, express HLA class II and have normal HLA class II processing function (Khanna et al. 1997; Taylor et al. 2006). EBNA1-specific CD4+ T cells can recognise and lyse Burkitt lymphoma cells in vitro (Paludan et al. 2002) and can control tumours in a murine model (Fu et al. 2004). It is therefore interesting that fewer children with endemic Burkitt lymphoma had detectable EBNA1 T-cell responses in the one study published to date (Moormann et al. 2009).

The immunological situation in NPC and HL is quite different. In both cases, the tumours express a wider range of EBV proteins: EBNA1, LMP2 and, in a proportion of cases, LMP1 as well. Furthermore, cell lines derived from NPC (Lee et al. 2000; Khanna et al. 1998) and HL (Lee et al. 1998) have functional HLA class I processing capacity in vitro, and HLA class I, TAP-1 and TAP-2 are frequently detected in biopsies from patients with these diseases (Lee et al. 1998; Murray et al. 1998; Khanna et al. 1998; Yao et al. 2000). Indeed, compared to EBV-negative cases expression of HLA class I is more frequently detected and is present at higher levels in EBV-positive HL (Huang et al. 2010; Liu et al. 2013). HLA class II is also detected in over half of EBV-positive HL and NPC biopsies tested (Huang et al. 2010; Liu et al. 2013). These observations suggest that HL and NPC tumours could be susceptible to CD8 and CD4 T cells effectors of appropriate specificity and this certainly seems to be the case in the clinic with several groups reporting clinical responses in NPC and HL patients treated with EBV-specific T cells (Comoli et al. 2005; Louis et al. 2010; Chia et al. 2014; Smith et al. 2012; Bollard et al. 2014).

The fact that adoptively transferred EBV-specific T cells can control a proportion of NPC and HL cases raises the question whether T-cell responses are compromised in these patients in the first place. Screening of Chinese NPC patients and healthy donors using a panel of defined HLA class I and II epitope peptides has found that the T-cell response in patients is generally unimpaired apart from a single HLA-B*40.01 restricted LMP2 epitope that was absent in patients (Lin et al. 2008). This work, however, examined only a single EBNA1 CD8 T-cell epitope. A subsequent study focusing on the CD8+ T-cell response to EBNA1 reported that the frequency of such cells in patients was lower (Fogg et al. 2009). In some cases, T-cell responses could be rescued from patients by in vitro culture,

suggesting that EBNA1-specific T cells may have become unresponsive rather than being lost. Similarly, the EBNA1-specific CD4 T-cell response is decreased in patients with Hodgkin lymphoma and AIDS–non-Hodgkin lymphoma (Heller et al. 2008; Piriou et al. 2005). The recent observation that adoptively transferred EBNA1-specific effectors can yield clinical benefit in patients with post-transplant lymphoma (Icheva et al. 2013) suggests that EBNA1-specific effectors could be candidates for the treatment of NPC and HL and that the above-described defects may therefore have clinical relevance.

In contrast to T-cell responses to EBNA1, LMP2-specific T cells appear less impaired in patients and CD8 T-cell responses are frequently detected in patients with HL and NPC (Lee et al. 2000; Fogg et al. 2009; Chapman et al. 2001; Lin et al. 2008) although the frequency of these cells may be lower in some cases (Gandhi et al. 2006). The increasing evidence linking HLA polymorphism with the risk of developing different EBV-associated malignancies is therefore intriguing. For Hodgkin lymphoma, HLA-A*01 increases and HLA-A*02 decreases the risk of developing EBV-positive but not EBV-negative disease (Niens et al. 2007). For NPC, a similar pattern is observed, although different HLA alleles are involved. Thus, a particular subtype of the HLA-A2 allele, A*02.07, increases disease risk, whereas the HLA-A*11.01 allele reduces risk (Su et al. 2013). Note that in most racial groups, the HLA-A*02.07 subtype is rare apart from the Chinese population, in whom NPC is a common malignancy and this subtype also increases the risk of developing HL in this population (Huang et al. 2012). For both diseases, it is notable that no EBV T-cell epitopes have yet to be identified as being presented by the HLA-A*01 or HLA-A*02.07 risk alleles, whereas the protective alleles can present multiple epitopes from a range of EBV proteins including LMP2 which is expressed in these malignancies. These observations are consistent with the hypothesis that a deficit in T-cell immunity may underpin the development of these malignancies (Niens et al. 2007; Brennan and Burrows 2008). However as noted earlier, patients with NPC and HL often possess detectable LMP2-specific T-cell responses at the time of their diagnosis. The in vivo situation is therefore likely to be more complex than a simple deficit in tumour surveillance.

Recent evidence suggests that some tumours may express additional viral antigens that could be exploited therapeutically. Although no LMP2 protein or mRNA could be detected in ENKTL cell lines, these cells were nevertheless efficiently recognised and killed by LMP2-specific CD8+ T-cell clones (Fox et al. 2010). This apparent paradox was resolved by the identification of a novel LMP2 mRNA transcript expressed from a different promoter that could not be detected by the standard molecular assays in use at the time but still contained the majority of T-cell epitopes. Although described as a lytic cycle protein, BARF1 is detected in many of the cases of the EBV-positive epithelial malignancies apparently in the absence of lytic replication (Decaussin et al. 2000; Seto et al. 2005; Stevens et al. 2006). Little is known about the immune response against BARF1. Several HLA-A2-restricted epitopes have been identified and T-cell responses are present at greater frequencies in NPC patients (Martorelli et al. 2008). The existence of

other immune responses to BARF1 is possible since a systematic analysis of the immune response against the protein has not yet been performed.

Very little is known about the EBV-specific immune response in patients with other EBV-positive cancers such as GCa, ENKTL and DLBCL. EBV-positive DLBCL of the elderly is now recognised as a provisional entity in the World Health Organisation classification and is defined as a clonal B-cell lymphoid proliferation occurring in patients older than 50 years without immunodeficiency or prior lymphoma. The detection of EBNA3 expression in a proportion of cases (Nguyen-Van et al. 2011; Cohen et al. 2013) and the fact that the disease is associated with ageing has led to the suggestion that the disease arises in a background of lowered EBV-specific immunity caused by ageing (Dojcinov et al. 2011). However, paediatric EBV-positive DLBLCL cases have been reported and although these occur at higher frequency in immunocompromised children at least some cases arise in apparently immunocompetent children (Cohen et al. 2013). Careful analysis of the antigen-processing phenotype and pattern of EBV gene expression in the tumour and the corresponding EBV-specific immune response in the blood of the patient will be required to provide a complete picture of whether virus-specific immunity is compromised in patients with EBV-positive GCa, ENKTL or DLBCL.

5 Suppression of EBV-Specific T-Cell Responses in Patients with EBV-Associated Malignancy

Evading immune destruction is a recognised hallmark of cancer (Hanahan and Weinberg 2011). In this regard, several mechanisms are employed by EBV-associated malignancies to suppress T-cell responses. Although HL tumours are heavily infiltrated by immune cells, these infiltrates are dominated by CD4+ regulatory and CD4+ Th2 cells. Many reports do not differentiate between EBV-positive and EBV-negative cases, but this distinction is important to make because, although the two subtypes appear superficially similar, several important differences exist between them, namely the tumour immune microenvironment. Firstly, EBV-positive HL has a distinct gene signature with markers indicating cytotoxic and Th1 responses being increased (Chetaille et al. 2009; Barros et al. 2012) although markers of suppression such as LAG-3 and IL-10 are also raised (Morales et al. 2014). Secondly, while the frequency of regulatory T cells is increased in the blood and particularly the tumour infiltrates of HL patients (Marshall et al. 2004), the presence of EBV correlates with higher numbers of both natural and induced regulatory T cells (Assis et al. 2012; Morales et al. 2014). The increased numbers of the former in EBV-positive disease may stem from EBNA1-mediated upregulation of CCL20 in the malignant Hodgkin/Reed–Sternberg cells (Baumforth et al. 2008). The immunoregulatory molecule PD-L1 is also expressed by HRS cells and in the case of EBV-positive disease, this may

result from LMP1- and LMP2-mediated upregulation of an AP-1-dependent pathway rather than an increase in PD-L1 gene dosage through chromosome 9p24.1 amplification (Yamamoto et al. 2008; Juszczynski et al. 2007; Green et al. 2012).

Less is known about the microenvironment of NPC. The most common subtype, undifferentiated NPC, is always EBV-positive and the tumours contain a sizeable infiltrate of lymphoid cells recruited to the tumour via CXCR6 and CCR5 (Parsonage et al. 2012). Regulatory CD4 + T-cell numbers are increased in the blood of some patients and these cells are also consistently detected in tumours (Lau et al. 2007; Yip et al. 2009). The presence in tumours of CD8+ FoxP3+ lymphocytes with suppressive function has also been reported (Li et al. 2011). In certain respects, HL and NPC tumour cells use similar strategies to evade immune responses. For example, an immunomodulatory galectin (galectin-1 in HL, galectin-9 in NPC) (Juszczynski et al. 2007; Gandhi et al. 2007; Klibi et al. 2009) is expressed by a proportion of cases of each disease as is Fas ligand, which may act as a tumour defence molecule (Dutton et al. 2004). However, important differences exist between HL and NPC. For example, few cases of EBV+ve HL express HLA-G, an inhibitor of T- and NK-cell function, whereas it is expressed by 80 % of NPC tumours with high expression predicting poor survival (Cai et al. 2012).

Expression of the immunoregulatory molecule PD-L1 was detected in 90 % of EBV-positive HL cases and NPC cases as well as a wide range of EBV-associated malignancies including extranodal NK/T-cell lymphoma, diffuse large B-cell lymphoma and PTLD (Chen et al. 2013). These diseases may therefore be amenable to immune checkpoint inhibitors that target the PD1/PD-L1 axis (Pardoll 2012). Several of these inhibitors are being tested in late-stage trials for melanoma and non-small cell lung cancer, and they have yielded impressive clinical outcomes. Whether PD1/PDL1 inhibition will be similarly effective in the context of EBV-associated malignancies is currently unknown. They may be effective when used as single agents or could be combined with existing adoptive T-cell therapy or therapeutic vaccination strategies (Smith et al. 2012; Chia et al. 2014; Bollard et al. 2014; Taylor et al. 2014; Hui et al. 2013) since they clearly have synergistic potential (Wolchok et al. 2013). Rational combination approaches may be of particular value in cases of advanced disease which currently represents a challenging clinical problem.

6 Future Directions

1. Defining the immunological factors influencing whether primary EBV infection is asymptomatic or leads to infectious mononucleosis.
2. Determining what sort of immunity is important in determining the viral load set point and how control over this is lost in elderly populations.
3. Understanding the role of innate immune cells in limiting primary EBV infection and whether such cells can be harnessed for therapy of EBV-associated malignancies.

4. Characterising the repertoire of immunomodulatory mechanisms operating in the different EBV-associated malignancies and whether perturbing those mechanisms can unleash EBV-specific T-cell immunity to attack the tumour.
5. Understanding the immunology of emerging EBV-associated malignancies such as EBV-positive gastric carcinoma and DLBCL, and how these causes of morbidity and mortality can be targeted immunologically.
6. Improving the efficacy of immunotherapies to treat EBV-associated malignancies, particularly in cases of advanced disease, and developing ways to apply immunotherapies to patients in low-resource countries where many cases of EBV-associated malignancy occur.

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