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Adoptive immunotherapy for Epstein-Barr virus-associated lymphoproliferative disorders complicating marrow allografts

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Introduction

Most individuals first encounter the herpes virus, Epstein-Barr virus (EBV) in early childhood or late adolescence [114]. For infants and children, this exposure is usually asymptomatic or manifests as a nonspecific upper respiratory infection. On the other hand, a primary infection in an adolescent or an adult causes infectious mononucleosis (IM), characterized by fever, pharyngitis, lymphadenopathy, and an atypical lymphocytosis in over 50% of cases [62]. Following initial infection, latent infection of the parotid and salivary glands persists for life. Latent salivary gland infection is periodically interrupted by lytic virus production, shedding of EBV in saliva, and occasional secondary viremias [50, 116]. EBV also establishes a latent infection in B lymphocytes. These EBV-transformed B cells express only a limited profile of EBV-encoded antigens and rarely produce lytic virus [223]. In EBV-seropositive individuals, small numbers of these latently infected B cells, which can spontaneously transform in vitro, can be detected in the bloodstream throughout life [201].

Several studies in the mid-1970s demonstrated that patients with IM developed specific cell-mediated immune responses against EBV [122, 151, 186, 198]. Analysis of the function of the EBV-specific T cells generated demonstrated that they could both lyse EBV-infected targets and, at low effector to target ratios, inhibit the growth of clonigenic EBV-transformed B cells [122, 151, 186, 198]. Subsequent analyses revealed that following IM, immunocompetent individuals generate and sustain high frequencies of circulating EBV-specific cytotoxic T cells [13, 92, 105]. On the other hand, patients with acquired or inherited deficiencies of T cell-mediated immunity were found to be at risk for severe or fatal EBV-induced infections as well as for the development of EBV-driven polyclonal or monoclonal B cell lymphoproliferations [77, 135, 141, 172, 227]. These findings led to the hypothesis that EBV-specific T cell-

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mediated immunity played a crucial role in controlling circulating EBV-transformed B cells [90].

In 1994, our group first reported that oligoclonal or monoclonal EBV-associated lymphoproliferative disorders (EBV-LPD) which occurred after bone marrow transplantation (BMT) could be eradicated by infusing small numbers of unfractionated peripheral blood mononuclear cells (PBMC) derived from the HLA-matched EBV-seropositive bone marrow donor, directly substantiating the hypothesized importance of cell-mediated immune responses in controlling EBV-induced LPD [131]. The benefit of adoptive immunotherapy in the treatment of EBV-LPD has since been confirmed by many other transplant centers utilizing either donor-derived PBMC or EBV-specific T cell lines [65, 161, 171]. These clinical studies, combined with experiments evaluating the effects of different human effector cell populations on the growth and dissemination of human EBV-LPD generated in xenografted immunodeficient mice, have identified EBV-specific T cells as the principal effectors responsible for the eradication of these tumors. Over the last 4 years, much new information has been generated as to the nature and magnitude of the T cell responses generated in the course of primary infection and the types of responses developed in patients with LPD following adoptive cell transfer. In addition, several biological processes which may potentiate the growth of EBV lymphomas in certain immunodeficient patients and, in rare instances, allow them to elude the cytotoxic effects of transferred cells, have been identified. In this brief review, we will summarize current evidence regarding the molecular and cellular events contributing to EBV-induced B cell transformation, latency, and in immunocompromised hosts, lymphomagenesis. Current preclinical and clinical studies utilizing adoptive immunotherapy to treat EBV-induced LPD will also be reviewed in the light of recent advances in research into the cellular interactions contributing to effective resistance against EBV in normal and immunocompromised patients.

EBV-induced transformation and immortalization of normal B lymphocytes

The mechanisms by which EBV infects, transforms, and immortalizes normal B cells are extraordinarily complex, and are the subjects of several excellent recent reviews [38, 87, 159]. Because the products of several genes critical to the establishment and maintenance of latency and the generation of lytic virus also constitute major targets for the immune response, the basic features of EBV infection, B cell transformation and latency will be briefly described.

Infection of a human B cell is initiated by the binding of EBV to the B cell membrane through the interaction of the viral membrane glycoprotein gp350/220 and the B cell surface antigen CD21, the receptor for the complement fragment CD3 [32]. Following internalization of the virus, B cell activation occurs, resulting in the induction of p56lck, the heat shock proteins (hsp) [29, 30, 122], and the viral BCRF-1 gene. This latter gene encodes a protein, BCRF-1, which is homologous to interleukin-10 (IL-10) [119]. The BCRF-1 gene product induces the activation and proliferation of EBV-infected cells. Strikingly, its inhibition with antisense oligonucleotides also prevents B cell transformation [119].

EBV infection of normal human B cells rarely results in lytic virus production. Instead, a latent infection is produced in which linear viral DNA forms a circular episomal plasmid which associates with but does not integrate into the host DNA [58, 119,

140]. The EBV genome contained in this episomal form is thought to replicate utilizing host DNA polymerases, since, in this form, the virus is resistant to antiviral agents which inhibit viral DNA polymerases [176]. Within 24 h of latent infection, the viral DNA directs the production of the EBV nuclear antigens, EBNA-1–6 and the latent membrane proteins, LMP-1, 2A, 2B [38, 87, 159]. Although the EBV genome can encode approximately 80–100 proteins, only 10, including these nuclear antigens and latent membrane proteins, are known to be produced during latent infection [38, 87, 159].

EBNA-1, is essential for the maintenance of latent infection [145, 224]. By binding to the oriP nucleotide sequence on the BamC fragment of EBV DNA, EBNA-1 controls the initiation and termination of episomal viral DNA replication [47]. It also activates an enhancer which regulates Cp, a promoter gene which controls the expression of other EBNA proteins [184].

EBNA-2 protein is a transcription activator essential to B cell immortalization. The essential role of EBNA-2 in B cell transformation is demonstrated by the failure of EBNA-2 deletion mutants to immortalize B cells and by co-infection experiments demonstrating that when an immortalization-incompetent strain of EBV lacking the EBNA-2 coding region (P3HR) is combined with a helper virus encoding native EBNA-2 (but not truncated EBNA-2), B cell immortalization is achieved [31, 54, 74]. Although the specific mechanism by which EBNA-2 contributes to B cell immortalization is not known, recent studies have shown that EBNA-2-induced transcriptional activation is mediated by the recombinant signal binding protein RBPJ-K [61]. This latter protein also interacts with the activated notch protein which induces T cell leukemias [73]. Because of this feature of RBPJ-K, it has been hypothesized that EBNA-2 through its interaction with RBPJ-K might affect the function of several genes in the notch gene signaling pathway, thereby inducing B cell immortalization. EBNA-2 also transactivates LMP-1 which may be essential to its transforming activity [31]. It also induces CD21 and CD23 expression [33, 215] which enhances the cell's sensitivity to B cell growth factors.

The critical roles of EBNA-3, EBNA-6 and EBNA-4 in B cell transformation have also been elucidated by analysis of EBV deletion mutants [108, 196]. EBNA-3, EBNA-4 and EBNA-6, similar to EBNA-2, interact with RBPJ-K to induce or alter transcription of genes at RBPJ-K-sensitive sites [157]. These three proteins can also compete with EBNA-2 for binding with RBPJ-K, thereby down-regulating EBNA-2-mediated transactivation of the LMP-1 and LMP-2 promotors. The EBNA-4 protein, potentially by up-regulating expression of bcl_2, can also reduce the sensitivity of transformed B cells to apoptosis [174].

The role played by EBNA-5 in the transformation of B cells is less clear. However, Szekely et al. [188] have recently observed that EBNA-5 can bind and form complexes with the retinoblastoma and p53 tumor suppressor gene products. Based on these findings, these authors have hypothesized that EBNA-5 may promote B cell transformation and immortalization by binding and inactivating the genes encoded by these suppressor genes.

The latent membrane protein, LMP-1 is essential for B cell transformation [78]. In cell culture and in animal models, expression of this protein can induce transformation of several cell types, including fibroblasts and epithelial cells [6, 35, 44, 212, 219]. Its function may also be crucial to the pathogenesis of EBV-induced lymphomas since certain mutants of LMP-1 which promote transformation have been detected at strikingly high frequency in EBV-positive lymphomas occurring in solid-organ recipients

and in AIDS patients [88]. LMP-1 can also directly induce the expression of the *bcl*$_2$ gene, thereby inhibiting apoptosis of infected cells [59]. Recently, it has been recognized that LMP-1 is a viral analogue of human tumor necrosis factor (TNF) receptor proteins. The cytoplasmic tail of LMP-1, like the cytoplasmic tail of TNF receptors, can interact with LMP-1-associated protein (LAP-1) and EB16, twoTNF receptor-associated factors (TRAF), which are hypothesized to be the effectors of activated TNF receptor-induced intracellular signaling of growth and the transduction of NF-κB [37, 79, 121]. In addition, LAP-1 binds to CD40 and to the lymphotoxin receptor [121]. It is postulated that the cytoplasmic domain of LMP-1, by associating with LAP-1 and EB16, may provide an alternate signaling pathway for the B cell activation and proliferation normally induced by CD40 and the TNF receptor [37, 79, 121]. Indeed, deletion mutants of LMP-1 which alter its capacity to interact with these TRAF prevent transformation [72, 104]. Indirect evidence supporting these events in the pathogenesis of posttransplant EBV lymphomas has recently been provided by Liebowitz [103] who has demonstrated the colocalization and binding of TRAF-1 and TRAF-3 with LMP-1 and the associated activation of NF-κB in eight out of eight LMP-1 positive EBV lymphomas. In contrast, activated NF-κB was not detected in EBNA-1-positive, but LMP-1 negative Burkitt's lymphoma cells.

In addition to these effects, LMP-1 also increases B cell expression of CD23, CD54 (ICAM-1), CD11a/CD18 (LFA-1) and CD58 (LFA-3) [213, 216] as well as the expression of CD44, a receptor for hyaluronate [75]. Up-regulating CD44 expression in LMP-1-transduced Burkitt's Lymphoma cells increases the dissemination of these lymphoma cells in xenogeneic transplant models [75].

During latency, EBV induces endogenous cellular IL-10 [9, 10], IL-6 [40], and encodes BCRF-1, a homologue of IL-10. EBV lymphomas derived from patients with AIDS or solid-organ allograft recipients also produce high levels of IL-10 and IL-6 [9, 43], as do SCID mice which develop EBV lymphomas following inoculation with tonsillar lymphocytes from EBV-seropositive donors [126]. Cellular IL-10 and EBV-induced BCRF-1 [206] act as autocrine growth factors which stimulate the proliferation of EBV-transformed B cells and inhibit their susceptibility to programmed cell death [8]. IL-10 suppresses CD4 TH_1 cell production of the cytokines IL-2 and IFN-α [142, 189] and promotes IL-4 and IL-5 secretion by TH_2 T cells [45]. In murine models, the EBV-encoded BCRF-1 protein has been found to induce local anergy, preventing rejection of several tumor types [185]. The IL-6 produced by EBV lymphomas also potentiates tumor growth. For example, in SCID mice, IL-6 has been shown to promote the tumorigenicity of EBV-transformed B cells. This effect can be reversed by anti-IL-6 antibodies [40, 166]. This increased tumorigenicity may reflect not only the B cell-stimulatory effects of IL-6 but also its ability to inhibit the function of NK cells which otherwise would suppress EBV-induced tumor cell growth [192].

Periodically, a small number of latently EBV-infected B cells may be activated to produce infectious virus particles. Initiation of this lytic cycle is associated with the transcription of the EBV BZLF1 gene which encodes the ZEBRA protein (Z EBV replication activator) [115] which triggers viral replication. The ZEBRA protein encoded by BZLF-1 can also interact directly with p53. Overexpression of p53 can prevent the ZEBRA protein from disrupting latency [226]. Conversely, the ZEBRA protein by interfering with p53 functions would be expected to prevent apoptosis. BZLF-1 also up-regulates BHRF-1, a viral protein encoded early in the reproductive cycle, that has been shown to be a homologue of human *bcl*$_2$ and may also protect infected cells from apoptosis during lytic viral infection [60, 133]. Parenthetically, EBV-in-

duced lymphomas, in which the expression of endogenous host cell *bcl*$_2$ activity is up-regulated by EBV-encoded LMP-1, do not express BHRF-1 [124]. The replication and production of intact EBV is initiated when the ZEBRA protein induces the expression of immediate early antigen (EA) genes which, in turn, up-regulate viral DNA polymerase and thymidine kinase [87]. Subsequent to replication of the complete viral genome, late viral genes are activated, including those encoding the viral capsid antigen (VCA), gp350, which mediates binding to uninfected bystander cells.

Cellular immune responses to acute and latent EBV infection in the normal host

In normal immunocompetent individuals, a primary EBV infection, exemplified by IM, elicits a marked T lymphocytosis. It was early recognized that EBV-specific T cells were included in these populations [63, 111, 169, 186]. However, until recently, it was thought that the proportion of EBV-specific T cells in this reactive T lymphocytosis was small. Estimates of the frequencies of EBV-specific T cells generated during the acute stages of infection by limiting dilution analysis have ranged from 1/100 to 1/500 of the circulatory T cells reactive against latent cycle antigens such as EBNA-3 [181], and equivalent or slightly higher frequencies against the immediate EA BZLF-1 generated in a lytic infection [182]. However, evaluation of the T cell receptor repertoire of CD8$^+$ T cells generated during acute IM has revealed large populations of T cells expressing a limited spectrum of T cell receptors, suggesting that these cells represent a response to a specific antigenic challenge [20]. Recently, the development of techniques by Altman et al. [1] for constructing fluorochrome labeled tetramers of HLA enfolding specific peptide antigens has permitted a more direct method for quantitating HLA-restricted T cells with receptors for a given epitope. Callan et al. [21] have recently used this technique to quantitate HLA-restricted responses to two dominant epitopes of the lytic cycle proteins BMLF-1 and BZLF-1, restricted by HLA A2 and HLA B8, respectively, as well as a dominant HLA B8-restricted epitope of the EBNA-3 protein produced during latency. In three HLA B8-bearing patients with IM, T cells restricted to the BZLF-1 peptide epitope constituted 29–44% of the T cells isolated from the blood. In addition, 1–2% of the circulating T cells were reactive against EBNA-3. Similarly, in HLA A2-bearing hosts with IM, T cells binding and reactive to the BMLF-1 epitope ranged from 0.5% to 6.6% of the circulatory pool. Thus, acute infection with EBV elicits an EBV-specific T-cell response which is of striking magnitude.

In addition to these virus-specific CD8$^+$ T cell responses, acute EBV infection also stimulates other EBV-reactive effector cells which may also contribute to the control of infection [111, 169, 191]. For example, NK cells derived from patients with IM are capable of lysing EBV-transformed T cells, although they do so at effector to target ratios exceeding those required by T cells [111, 169]. In addition, CD8$^+$ T cells are generated which are EBV reactive but do not appear to be restricted to targets sharing HLA-A, B or D antigens [169, 186, 191]. Some of these EBV-reactive T cells previously suggested to be HLA unrestricted may actually be restricted by HLA-C determinants shared by individuals who differ in HLA-A, B and D determinants [26]. Others may be T cells with broadly reactive NK-like activity [197]. In addition, CD3$^+$ T cells have been detected which are capable of preferentially lysing autologous EBV-transformed B cells but are also able to lyse allogeneic targets. For example, Shendel et al. [168] have described EBV-specific, HLA-C-restricted T cells which are also capable

of recognizing certain allogeneic EBV-negative cell lines. They have hypothesized that these T cells are reactive against self peptides preferentially expressed on EBV-infected cells which may mimic alloantigens expressed in allogeneic cells [168]. Similarly, Burrows et al. [18] have described T cell clones reactive against the EBNA-3 peptide epitope FLRGRAYGL presented by HLA B8, which lyse uninfected allogeneic targets expressing HLA B 4402. Other T cell clones recognizing this peptide on HLA $B8^+$ targets also cross-react with unrelated cell-derived self peptides in association with the alleles HLA B14 and HLA B35 [19]. These findings suggest that certain EBV peptides when bound to specific HLA antigens form complexes capable of mimicking certain other HLA alloantigens either alone or in complex with cell-derived peptides.

Suppressor T cells capable of inhibiting immunoglobulin production by mitogen-stimulated B cells have also been detected in patients with IM [57, 199, 200, 214]. These suppressor T cells are broadly inhibitory in their action, displaying neither HLA restriction nor EBV specificity. Despite this unrestricted activity, these $CD8^+$ T cells may limit the proliferation of EBV-infected B cells, thereby potentially helping to abrogate the acute infection.

As the clinical manifestations of IM abate, the predominant anti-viral T cell population consists of EBV-specific HLA class I-restricted $CD8^+$ cytotoxic T cells [117, 122, 149, 150, 186, 195, 209]. Smaller numbers of HLA class II-restricted $CD4^+$ T cells can also be detected [12, 113, 118]. Healthy EBV-seropositive individuals maintain circulating HLA-restricted EBV-specific CTL precursors (CTLp) at frequencies of 1/400 to 1/42,000 T cells [13, 92, 105] throughout their lifetime. These EBV-specific CTLp frequencies are 10- to 100-fold higher than the frequencies of CTLp against other viruses, but similar to those against major alloantigens [76]. Again, these quantitations of CTLp frequencies likely greatly underestimate the actual proportion of T cells reactive against EBV, reflecting as they do, only the clonigenic precursor population rather than the fully differentiated T-cell which may not be capable of multiple further divisions. Indeed, the studies of Callan et al. [21] suggest that while the frequencies of T cells capable of specifically binding dominant epitopes of BZLF-1 or BMF-1 fall following resolution of acute infection, up to 18% of the circulatory T cells specifically bind these epitopes late into convalescence [21].

EBV is a unique latent infection, for unlike herpes simplex virus (HSV), herpes zoster or cytomegalovirus (CMV) which latently infect non-dividing cells such as sensory nerve cells or salivary gland epithelial cells, latently infected B cells divide indefinitely in the host, constantly presenting EBV-encoded antigens, resulting in continuous T cell stimulation [50, 116, 203]. At any one time in an asymptomatic EBV-seropositive individual, between 1 and 10 out of every 10^6 B cells in the blood are EBV-transformed B cells capable of spontaneous outgrowth [158, 201, 202]. This frequency is only 1–2 Log_{10} orders lower than the frequencies of EBV-transformed B cells usually detected during acute IM. The prevalence of these EBV-transformed B cells is further demonstrated by the fact that infusion of as few as $1 \times 10^7 - 5 \times 10^7$ unfractionated circulating mononuclear cells from the majority of asymptomatic seropositive individuals into SCID mice will result in lethal EBV-LPD [136, 162].

Following an acute infection with EBV, the virus-specific HLA-restricted T cells generated and sustained in the immunocompetent host target a strikingly limited spectrum of EBV antigens. Although the EBV-transformed B cells express EBNA 1–6, LMP-1 and LMP-2, several studies have demonstrated that T cell responses are primarily directed against EBNA-3, 4 and 6, and occasionally directed against LMP-2 [82, 125, 153, 190]. Although EBNA-1, EBNA-2, and LMP-1 contain peptide epitopes

capable of binding to HLA antigens, EBNA-1-reactive T cells are not observed, and CTL against EBNA-2 or LMP-1 are rarely detected [183, 190, 221].

The absence of HLA class I-restricted, CD8+ CTL responses against EBNA-1 may be due in part to the glycine-alanine repeat region encoded by EBNA-1, which blocks the processing of EBNA-1. This 200-residue region prevents the complexing of EBNA-1 epitopes with HLA antigens. As a consequence, EBNA-1 peptides are not presented in association with HLA class I alleles on the cell surface [100]. The inhibition of formation of peptide complexes with HLA mediated by the glycine-alanine repeat regions within EBNA-1 is likely not a special adaptation of EBV to its human host. When genes encoding EBNA-1, EBNA-4, EBNA-5, LMP2A or LMP-1 have been transfected and expressed in non-immunogenic murine mammary carcinoma cells, tumors expressing each of the EBV proteins except EBNA-1 have been rejected by syngeneic murine hosts [205]. Thus, the non-immunogenic features of EBNA-1 are reiterated even in species in which EBV is not a natural pathogen. Although CD4+ cytotoxic T cell clones which recognize an EBNA-1 peptide epitope TSLYNRRGTALA in the context of HLA-DR1 have been isolated from the blood of an EBV-seropositive individual, these clones were unable to lyse EBV-transformed B cells or cells transduced with a vaccinia vector carrying a truncated EBNA-1 which lacked the glycine-alanine repeat sequences [83], demonstrating that additional mechanisms must be invoked to fully explain the lack of EBNA-1-reactive cytotoxic cells.

The dominance of CTL responses directed against specific EBNA-3, 4 and 6 epitopes and LMP-2 may be related to altered antigen processing resulting in up-regulated antigen expression. For example, studies comparing HLA A-11-restricted T cell responses to different EBNA-4 peptide epitopes reveal that the dominant epitope is expressed at higher density than the other HLA-A-11 binding EBNA-4 epitopes [97]. The selection of a particular viral epitope complexed with HLA as a dominant antigen may also be influenced by the efficiency with which the epitope is bound to HLA and transported to the cell surface. For example, unlike many other HLA alleles exhibiting multiple microvariant polymorphisms, several microvariants of HLA B44, including HLA B4402, 4403 and 4405 can bind the EBNA-6 peptide, EENLLDVFRM, and present it to HLA B4402, 4403 or 4405 restricted T cells specific for this peptide. Strikingly, peptide-specific T cells restricted by any of these HLA B44 microvariants preferentially kill targets presenting this epitope complexed with HLA B4405. Studies of the transport kinetics of the HLA microvariants indicate that HLA B4405 is more rapidly assembled and transported to the trans-Golgi compartment than the other microvariants. As a result, fully assembled HLA B4405-epitope complexes are more rapidly transferred to and more densely expressed on the cell membrane, thereby enhancing their sensitivity to peptide-specific HLA B4402, 4403 or 4405 restricted T cells [85]. In addition, while EBNA-3, LMP-2 and other viral antigens can be processed and transferred to the endoplasmic reticulum by TAP1 and TAP2 peptide transport proteins, certain dominant epitopes of LMP-2 encoded in the transmembrane domain can also be processed and presented by cells lacking TAP1 and TAP2 [84, 98]. Thus, alternative pathways of antigen processing can potentially augment LMP-2 presentation in the context of several HLA alleles, thereby fostering selection of LMP-2-specific responses.

A feature of T cell responses directed against the EBV latent proteins which may affect the pathogenesis of EBV-LPD and the efficacy of adoptive immunotherapy is the fact that the hierarchy of peptide epitopes recognized by T cells is dependent upon and possibly limited by the HLA alleles or microvariants polymorphisms thereof ex-

Table 1. Defined immunogenic epitopes of EBV-encoded proteins and presenting HLA-alleles

EBV protein	Presenting and restricting HLA allele	Residues	Epitope sequence
LMP-2	A0201	426-434	C L G G L L T M V
	A0201	329-337	L L W T L V V L L
	A0206	453-461	L T A G F L I F L
	A1101	340-349	S S C S S C P L S K
	A2402	419-427	T Y G P V F M C L
	B2704	236-244	R R R W R R L T V
	B40011 (B60)	200-208	I E D P P F N S L
EBNA-6 (3C)	B4405 > 03 > 02	281-290	E E N L L D V F R M
	B37	285-293	L D F V R F M G V
	A2	284-293	L L D F V R F M G V
	B62	213-222	Q N G A L A I N T F
	B7	881-889	Q P R A P I R P I
EBNA-4 (3B)	A11	399-408	A V F D R K S V A K
	A11	416-424	I V T D F S V I K
EBNA-3 (3A)	B35	458-466	Y P L H E Q H G M
	B8	325-333	F L R G R A Y G I
	B8	158-166	Q A K W R L Q T L
	A2	596-604	S V R D R L A R L
	A3	603-611	R L R A E A G V K
	B7	379-387	R P P I F I R R L
	A24	246-253	R Y S I F F D Y
BZLF1 (ZEBRA)	B8		R A K F K Q L L Q
BMLF1	A2		G L C T L V A M L

LMP, Latent membrane protein; EBNA, Epstein-Bar virus nuclear antigen; ZEBRA, z EBV replication activator

pressed by the target cells to which these epitopes bind. Individuals of a given HLA genotype may generate T cells which react predominantly to one or two epitopes of an EBV protein, such as EBNA-4, and be restricted by only one (or two) HLA class I alleles, such as HLA A11 [49]. The selection of a dominant antigenic epitope in the context of a dominant HLA-restricting element generally occurs early after a primary EBV infection and persists throughout life [181]. Several of these EBV epitopes have been identified and a list of some of these epitopes and the HLA alleles by which they are preferentially presented is provided in Table 1.

Various epitopes of the latent proteins EBNA-3, 4 and 6 can be recognized in the context of a broad range of HLA class I alleles [82, 125]. However, only certain of the epitopes encoded by these genes, when bound to a specific HLA allele, preferentially stimulate T cell responses. For example, individuals expressing the HLA B8 allele preferentially generate HLA B8-restricted T cells reactive against the EBNA3 peptide sequence FLRGRAYGL [184]. Individuals bearing the HLA microvariant HLA B35.01 generate T cells restricted to this allele, which are directed against YPLHEQHGM epitope encoded by EBNA-3 [101]. Two epitopes of EBNA-4 preferentially stimulate T cell responses restricted by HLA A11 [36, 48, 49, 125] as do certain EBNA-6 epitopes [48]. In contrast to the responses generated against EBNA-3, EBNA-4 and EBNA-6 peptide epitopes, potent responses to LMP-2 and EBNA-2 antigens are generally not

elicited at all, unless the host expresses a specific HLA allele, (i.e., HLA A2.1 for LMP-2, HLA B27.02 for EBNA-2) [16, 96].

As noted previously, normal individuals also maintain significant frequencies of HLA-restricted $CD8^+$ cytotoxic T cells directed against the immediate early transactivator ZEBRA [41] and the bcl_2 homologue BHRF-1 [218]. These antigen-specific responses may limit the percentage of transformed B cells which are capable of producing infectious virus.

It is still not fully understood how the equilibrium between EBV-transformed B cells and circulating EBV-specific cytotoxic T cells is maintained. It is well recognized that Burkitt's lymphoma cells are capable of suppressing the expression of all latent EBV antigens except EBNA-1, which is the one latent viral protein that cannot be recognized by T cells because it is not presented in complex with HLA on the surface of the lymphoma cells [100, 148, 205]. It is also clear that other EBV-associated malignancies, such as nasopharyngeal carcinoma, may express only a limited spectrum of EBV antigen for T cell surveillance [86]. However, the degree to which EBV-infected and transformed normal B cells in normal individuals invoke the modification of expression of latent EBV proteins or their presenting HLA antigens to maintain their populations is unknown. Latently infected B cells are also capable of up-regulating bcl_2, thereby resisting apoptosis. Furthermore, they can express BCRF-1, and can induce endogenous IL-10 and IL-6, thereby inhibiting the production or activity of EBV-specific T cells [9, 40, 59, 110]. Whatever the mechanism, an equilibrium is established in the immunocompetent host which is sustained through periodic sub-clinical reactivations of virus production throughout the life of the infected individual.

EBV lymphoproliferative disorders in patients with acquired or genetic disorders of T cells immunity

A pivotal role for T cell-mediated immunity in the control of EBV infections was suggested early by reports of lethal EBV-induced LPD or lymphomas in children afflicted with congenital T cell immunodeficiencies such as severe combined immunodeficiency, Wiskott Aldrich Syndrome [179, 186], or the X-linked lymphoproliferative disease, a disorder associated with a unique incapacity of T cells to respond to EBV [141]. Patients with AIDS are also susceptible to EBV-associated malignant lymphomas, particularly those with CD4 counts less than 200 cells/μl [3, 99, 170, 227]. Indeed, 40–60% of the non-Hodgkin's lymphomas which develop in AIDS patients contain EBV DNA [170]. That EBV contributes to lymphomagenesis in AIDS patients is suggested by the detection of EBV DNA in over 40% of lymph nodes isolated from patients with persistent lymphadenopathy, by its close correlation with subsequent lymphoma development, and by the detection of only a single episomal form of EBV in these lymphomas, implying that EBV infection precedes the development of lymphoma [173].

EBV-induced LPD complicating BMT and solid-organ transplantation are well documented. However, the LPD arising in these two clinical settings, although similar in immunophenotype, histology, and EBV antigen expression, vary markedly in their time of onset, degree of clonality, donor/host origin, and response to therapy.

The frequency of EBV-LPD following solid-organ allografts varies depending on the organ transplanted and the intensity and duration of immunosuppression utilized. EBV-LPD occur in approximately 1–3% of kidney or liver allograft recipients. In contrast, up to 15% of heart and/or lung transplant recipients may develop EBV lym-

phomas. These malignancies are usually of recipient origin [56, 134, 143, 217, 220]. In a large multi-institutional study of cardiac and renal transplant recipients, immunosuppression with OKT3, anti-lymphocyte globulin or anti-thymocyte globulin (ATG) was associated with a significantly higher incidence of EBV-LPD, particularly when combined with post-transplant Azathioprine and/or cyclosporine A (CyA) [129]. The time of onset EBV-LPD following a solid-organ transplant is also related to the organ transplanted and the regimen utilized to prevent organ rejection. When conventional long-term immunosuppression with Azathioprine and Prednisone is utilized, these lymphomas occur at an average of 20 months post transplant compared to 11 months when additional immunosuppression with CyA is invoked. Lymphomas following heart and/or lung transplants generally occur even earlier (2–9 months), most likely due to the aggressive graft rejection prophylaxis utilized in these organ recipients. Patients treated with the monoclonal antibody OKT3 are particularly at risk for early emergence of EBV-LPD [22, 123, 187].

The clinical presentation, pathology, and degree of clonality of EBV-LPD following solid-organ transplantation also varies depending on the organ transplanted and graft rejection prophylaxis utilized. EBV-LPD following solid-organ allografts generally present with fever, adenopathy, and tonsillar involvement [56, 127, 194]. Recipients of solid organ grafts given CyA have a higher incidence of isolated gastrointestinal tract involvement. On the other hand, while central nervous system (CNS) involvement is unusual in patients given CyA, it has been reported in up to 40% of those given Azothioprine [127].

At least three types of B cell pathology have been described in EBV-LPD complicating organ allografts. Most commonly, they present as diffuse hyperplasias of reactive EBV^+ plasmacytes which are usually polyclonal, frequently involve cervical lymph nodes and usually resolve with cessation of immunosuppression. Alternatively, they may present as diffuse polymorphic B cell proliferations with lymphomatous features which are commonly monoclonal, as evidenced both by Ig gene rearrangements and clonality of EBV episomal DNA. These proliferations are not associated with alterations in cellular proto-oncogenes or suppressor genes, and may arise in both nodes and extranodal sites. They are often refractory to alterations in immunosuppression or treatment with antiviral or anti-neoplastic agents. A third, extremely uncommon presentation is a monomorphic immunoblastic B cell lymphoma which is consistently monoclonal and may bear mutations in the ras proto-oncogene or the p53 tumor suppressor gene, or rearrangements of c-myc [91]. In addition to these B cell disorders, rare T cell lymphomas that contain EBV DNA, and are clonal, have also been described as a complication of organ allografts [210].

EBV-LPD which develop following allogeneic BMT differ from those emerging after solid-organ transplants in that they are almost invariably of donor origin and usually emerge within 2–6 months post BMT [105]. EBV-LPD are extremely rare following non-T cell-depleted (TCD) BMT [77, 228], except among patients given OKT3 to treat graft-versus-host disease (GvHD), in whom an incidence as high as 16% has been reported [109]. Following TCD BMT, the probability of developing an EBV-LPD depends on the method of TCD, the degree of HLA mis-matching between donor and host, and the type and intensity of additional immunosuppression given to prevent graft rejection. For example, in patients transplanted with marrow depleted with certain T cell-specific monoclonal antibodies, the incidence of EBV-LPD has been as high as 11–26% [4, 147, 177]. In contrast, T cell depletion of HLA-matched related bone marrow utilizing methods which remove B cells as well as T cells from the graft

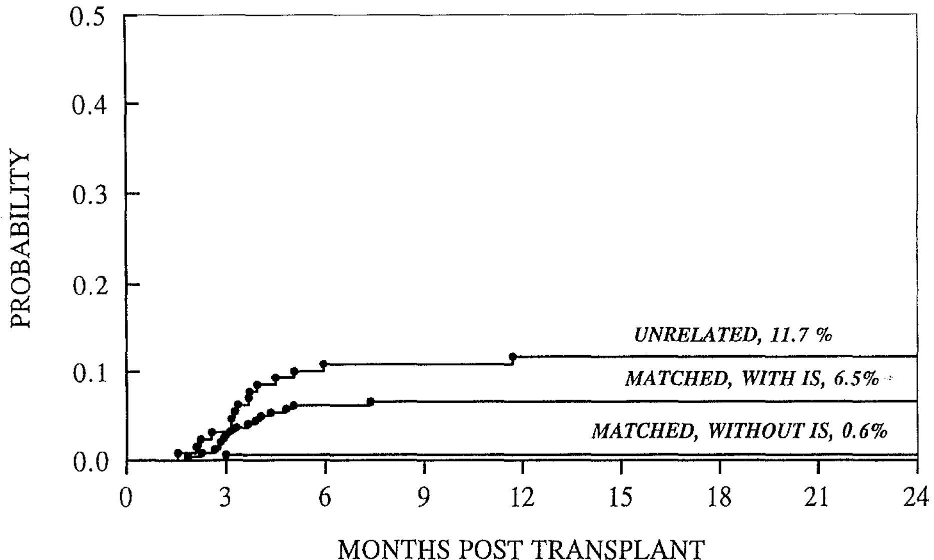

Fig. 1. Probability of Epstein-Barr virus lymphoproliferative disorders (EBV-LPD) in patients transplanted with T cell-depleted bone marrow with or without added immunosuppression with ATG and steroids (*IS* immunosuppressives)

(lectin agglutination or Campath-1) have had a low incidence of lymphoma unless other immunosuppressives, such as certain preparations of ATG, have also been administered [52, 53]. No lymphomas were reported in an analysis of 400 recipients of Campath-1 TCD marrow grafts [52]. Similarly, in our early experience, only 0.6% of recipients of HLA-matched sibling grafts depleted of T cells by soybean agglutination followed by rosetting with sheep red blood cells (SBA-E-BMT) developed an EBV-LPD [130]. However, when equine ATG and steroids were administered to patients receiving these HLA-matched TCD grafts to prevent graft rejection, the incidence of EBV lymphomas increased from 0.6% in the absence of this prophylaxis to 6.5% [130] (Fig. 1). Other groups using similar cytoreduction and the same method of TCD have not seen EBV-LPD when rabbit rather than horse ATG has been used [5]. The varying risk of EBV-LPD in patients who receive different anti-T lymphocyte globulins or T cell-specific monoclonal antibodies before or after BMT, may be due to differences in the ability of these preparations to induce B cell proliferation or apoptosis [11]. The type of donor used for the marrow allograft also affects the incidence of post-transplant EBV-LPD. For example, following unrelated SBA-E-BMT, the risk of developing an EBV-LPD is increased to 11.7% [130]. This is similar to the 12% incidence of EBV-LPD reported in children following transplantation of unrelated marrow TCD with monoclonal antibody T10B9 [23]. The basis for the increased risk of EBV-LPD associated with unrelated TCD grafts is unknown, but may, in part, reflect the high incidence of molecular microvariant disparities of HLA class I genes detected in these donor/recipient pairs [139], which may inhibit recovery of virus-specific immune responses.

EBV-LPD observed in marrow allograft recipients consistently present as malignant monomorphic, diffuse large B cell lymphomas which are usually monoclonal and of donor type [77, 131, 132, 147]. These lymphomas, in our experience, have not exhibited alterations in the cellular proto-oncogenes c-myc or ras, or suppressor genes, such as p53 [131, 132]. Of the EBV-LPD diagnosed at our institution, more than 80% of patients presented with fever and 50% with palpable adenopathy, predominantly of the cervical nodes; 33% had exudative tonsillitis. However, 65% presented with hepatic or gastro-intestinal involvement, 30% with pulmonary lesions, and 9% with multifocal tumors involving the CNS. Thus, involvement of extranodal sites is very common. Furthermore, of 16 lymphomas that could be adequately assessed 14 were monoclonal, as assessed by analysis of immunoglobulin gene rearrangements and/or the lengths of the termini of EBV episomes.

EBV-LPD arising following either an organ allograft or a marrow transplant may express the full array of latent EBV antigens, including EBNA-1, EBNA-2, EBNA-3, EBNA-4, EBNA-5, EBNA-6 and LMP-1 [225], but in many cases, only a limited spectrum of these antigens are detected [25, 146, 193]. While karyotypic analyses have often identified cytogenetic abnormalities in EBV-LPD occurring post BMT, no consistent pattern of chromosomal alteration has been identified. In particular, the characteristic translocations of Burkitt's lymphoma have not been observed in lymphoproliferations developing after either a marrow or organ allograft [88, 131, 132, 225].

The intensity and duration of the T cell immunodeficiency induced by the immunoablative therapy administered prior to a marrow transplant, which is also reflected by reductions in the frequency of EBV-specific cytotoxic T cells in the blood, is strongly correlated with risk for the development of EBV-LPD. Sequential analyses of the reconstitution of EBV-specific cytotoxic T cell populations following allogeneic unmodified or TCD marrow grafts have identified low to undetectable frequencies of EBV-specific CTL precursors extending from the time of transplant through the first 3–4 months post transplantation [105]. By 6 months post transplantation, a majority of marrow allograft recipients develop frequencies of EBV-specific cytotoxic T cells comparable to those detected in normal seropositive adults [105]. Thus, the period of risk for EBV-LPD (Fig. 1) matches that during which EBV-specific immune T cells are at their nadir. On the other hand, in our studies [105], there were no differences observed in the frequency of EBV-specific CTLp or their virus-specific cytotoxic activity at 3 or 6 months post transplant, which would explain the differential susceptibility to EBV-LPD observed in the recipients of TCD versus T cell-replete grafts. However, following TCD grafts, total clonable T cell doses administered are very small, ranging between $2 \times 10^4 - 10 \times 10^4$ T cells/kg as measured by limiting dilution analysis [80]. In contrast, doses which are 500- to 1000-fold higher are transferred in an unmodified graft. Based on the frequencies of EBV-CTLp detected in normal seropositive donors, the number of EBV-specific CTLp infused with a TCD graft of the type used in these studies would be expected to range from 10^5 to fewer than 10^3 EBV CTLp. As a result, the capacity of such patients to generate an effective response against clones of proliferating EBV-transformed cells early after transplant may be markedly inferior to that of a recipient of an unmodified graft. This response may be further compromised if additional immunosuppression is administered to prevent graft rejection. Despite this, by 6 months post transplant, over 70% of the recipients of the TCD grafts in our series developed frequencies of EBV-CTLp, which were comparable to those maintained by normal seropositive adults [105]. These findings coupled with the striking reduction in the risk of EBV-LPD that is observed thereafter (Fig. 1) suggests that despite the lim-

ited number of EBV CTLp transferred in the graft, most patients are able to recover effective and durable EBV-specific responses within the first 8 months post transplant.

It is also increasingly clear that the EBV-specific CTLp frequency, which estimates populations of CD8+ EBV-specific CTL, does not provide an accurate measure of the effectiveness of EBV-reactive T cells in the host [21]. In certain donor/host pairings, small numbers of donor T cells reactive against a dominant epitope expressed on the EBV-transformed cells might be adequate to prevent its emergence. Furthermore, the ability of some patients to suppress the expansion of EBV-transformed donor cells may be related not just to their reserve of virus-specific CTLp, but also to their capacity to generate sufficient T cell help. Indeed, among patients with the acquired immunodeficiency disease, EBV-LPD predominantly occur in patients with CD4 cell counts less than 200 cells/μl [34, 55]. Similarly, in our series 86% of patients who developed an EBV-LPD following related or unrelated SBA-E- BMT, had a CD4 cell count of less than 200 cells/μl [178].

The increased probability of EBV-LPD observed among recipients of identically treated unrelated TCD BMT may be partially ascribed to the more profound and prolonged deficiencies of CD3 and CD4 lymphocytes observed in these patients [178]. The basis of the more profound and protracted deficiencies of T cell immunity observed in these patients is unclear but may, in part, reflect an impaired capacity of lymphoid progenitors derived from an unrelated donor, who may frequently inherit allelic microvariants of major alloantigens and multiple minor alloantigens not shared by the host [139, 164], to develop within the host thymus. Such alterations in early T cell development could result if the homing of HLA disparate lymphoid precursors to the host thymus or their development within the host thymus is impaired, an hypothesis supported by the slow and quantitatively limited [178] recovery of T lymphocyte populations and their function in these patients. Furthermore, since EBV-specific CTL restricted by certain HLA alleles may be incapable of recognizing viral antigens in the context of a disparate molecular variant of that HLA-restricting element [49, 181], it could also be hypothesized that lymphoid progenitor cells derived from an unrelated TCD marrow graft developing within the host's thymus might be initially restricted by host unique HLA determinants and be relatively incapable of recognizing EBV antigens presented on donor-derived EBV-transformed lymphocytes. Conversely, because the number of T cells administered in an unmodified marrow graft may be 200- to 1000-fold higher than that contained in a TCD graft, a recipient of such a transplant from an unrelated donor might still have sufficient numbers of mature EBV-reactive T cells restricted to donor-unique HLA determinants to regenerate populations of T cells capable of controlling outgrowths of donor-derived EBV-transformed B cell populations.

Given the profound deficiencies of T cell function induced by the immunoablative regimens used to prepare patients for marrow transplants, particularly those used for TCD marrow grafts, the fact that only a small proportion of these transplant recipients develop EBV-LPD suggests that other features of the donor, the host environment or the clones of EBV-transformed donor B cells that develop into EBV-LPD must contribute significantly to permit or foster the emergence of this complication in a fraction of these transplant recipients. Among other possible characteristics of host and virus which might distinguish patients at particular risk for developing EBV-LPD, individuals with high concentrations of EBV-infected cells in the circulation, as measured by frequency of spontaneously transformable B cells or by measurement of cell-associated EBV DNA, have been highly correlated with the risk of EBV-LPD [106, 156, 160, 165]. Among organ allograft recipients, the highest virus loads are usually

observed in patients experiencing a primary infection rather than reactivation of latent EBV. Indeed, primary infection has been identified by several groups as the dominant risk factor for development of EBV-LPD in organ allograft recipients [68, 69, 156, 207, 208]. For example, among patients developing a primary infection following renal or heart transplants, incidences of EBV-LPD ranging from 14% to 50% have been recorded [68, 208], compared to 1–4% among seropositive transplant recipients. Furthermore, in one study, analyses of restriction length polymorphisms of the EBV DNA in these tumors indicated that, while the EBV-LPD developing in organ allograft recipients with a primary infection were of host type, they were caused by virus transmitted via the donor organ [24].

The characteristics of the EBV detected in EBV-associated lymphomas and phenotypic features of the virus-transformed cell may also contribute to lymphomagenesis. For example, it has been shown that type A strains of EBV are more lymphomagenic than type B strains [46, 152]. Furthermore, Picchio et al. [136], have shown that circulating B cells in PBMC of healthy seropositive individuals bearing the A strain of EBV have a greater capacity to induce lymphomas after inoculation into SCID mice. Furthermore, in large series, a very high proportion of EBV-LPD have been found to contain type A EBV [46, 71].

A deletion in the 3′ end of the LMP-1 gene, first detected in a nasopharyngeal cell carcinoma line [71] and subsequently detected in Burkitt's lymphoma cells [28], has also been detected in 38% to as high as 100% of EBV lymphomas emerging post organ allografts [88, 89, 167]. This deletion prolongs the half-life of LMP-1 [120] thereby potentially enhancing its transforming activity. Indeed, epithelial cells bearing this deletion are more tumorigenic in SCID and nude mice [27, 70]. Because of these findings, it has been hypothesized that B cells transformed by EBV strains bearing this deletion would be more likely to induce monoclonal EBV lymphomas [88]. However, evidence from recent studies by Smir et al. [180] and by Scheinfeld et al. [167] indicates that EBV strains bearing this LMP-1 deletion are not disproportionately represented in clonal EBV-associated malignant lymphomas emerging after organ allografts. Thus, the contribution of LMP-1 deletion to post-transplant EBV lymphomas is still unclear. Whether and to what degree this or other mutations in EBV contribute to the distinctive pathogenesis of EBV-LPD complicating marrow allografts is unknown.

Another mechanism whereby certain clones of EBV-transformed cells might elude residual T cell surveillance would be to alter the display of EBV-encoded peptides on the cell surface. The capacity of EBV to modulate antigen expression is well recognized, and the patterns of expression of the proteins synthesized in different EBV-associated diseases have also been characterized [81]. In Burkitt's lymphoma (type 1 latency) only EBNA-1 peptides are expressed. Because these EBNA-1 peptides are not presented in complex with HLA class I, they are not recognized by CTL [2, 81, 183, 190, 221]. In other EBV-associated malignancies, such as nasopharyngeal carcinoma and subsets of Hodgkin's disease and leiomyosarcoma, the EBV antigens expressed are limited to EBNA-1 and LMP-1 (type 2 latency) [2, 81]. While EBV-associated lymphomas emerging as complications of organ or marrow allografts may, like EBV-transformed normal B lymphocytes, express antigens encoded by each of the nuclear antigens, EBNA-1–6, as well as LMP-1 and LMP-2 (type 3 latency) [51, 193, 225], recent evidence indicates that individual tumors may vary strikingly in their display of these antigens, some expressing only EBNA-1 and EBNA-2 or LMP-1 [25, 146, 193]. Recent studies also indicate that certain natural strains of EBV may encode variants of normally dominant peptide epitopes of certain EBV antigens, such as EBNA-4, which

either fail to bind their restricting HLA allele or form complexes with HLA that are prematurely degraded intracellularly. As a consequence, they are not immunogenic and may not be recognized by T cells in most normal EBV-seropositive donors [102]. In transplant recipients, infection with such a strain might lead to malignant expansions of transformed clones in certain individuals which could not be detected by residual T cells in the host or the marrow graft.

Adoptive immunotherapy of EBV-associated lymphomas in human marrow allograft recipients with donor-derived lymphocytes or EBV-specific T cell lines

Recently, evidence directly demonstrating that immune T cells can control life-threatening monoclonal EBV lymphomas has been provided by clinical studies in which PBMC or expanded virus-specific T cells derived from the transplant donor have been used to treat EBV lymphomas developing early after a marrow allograft [10, 65, 66, 131, 161, 171]. In 1994, our group reported complete and sustained remissions of EBV^{+} lymphomas in a series of five patients treated with infusions of small doses of PBMC from their EBV-seropositive marrow donors [131]. In three of the five cases, these lymphomas were monoclonal. Each of the lymphomas was of donor origin. Since that report, we have treated EBV lymphomas in an additional 18 marrow allograft recipients, using either donor PBMC, or, in one case, in vitro-generated donor-derived EBV-specific T cell lines [53]. This experience can be briefly summarized.

Each of the patients in our series received marrow transplants for high risk forms of leukemia. The transplants were depleted of T cells by either lectin agglutination and E-rosetting or by treatment with monoclonal antibody $T_{10}B9$. The EBV-LPD presented as diffuse large cell lymphomas of B cell phenotype, which were of donor origin in each of 11 cases adequately evaluated. These lymphomas have emerged within the first 8 months post transplant (median 3 months). EBV DNA has been detected by polymerase chain reaction in each of the 23 cases tested. Evidence of clonality was demonstrated in 14 of 16 cases adequately studied, either by demonstration of a clonal rearrangement of the immunoglobulin genes or by showing uniformity in the size of genomic termini of EBV episomal DNA isolated from the lymphoma cells.

All but one of the patients in our series were treated with single infusions of PBMC from their normal seropositive marrow donor. The $CD3^{+}$ T cell populations in the donor PBMC were measured by cytofluorometry. The dose of PBMC to be administered was then calculated to provide a specific number of $CD3^{+}$ donor T cells. Doses of $2.1 \times 10^{5} - 5.0 \times 10^{5}$ T cells/kg recipient weight were initially used for unrelated marrow graft recipients and 10×10^{5} T cells/kg for matched related recipients. The doses administered were selected so as to provide a dose of T cells 10-fold higher than the dose of 10^{5} clonable T cells/kg that we have previously shown to be the threshold dose for acute GvHD in HLA-matched sibling recipients [80], but still 10-fold lower than that provided by an unmodified marrow graft. The infusions were well tolerated. Complete clinical and/or pathological resolution of the EBV lymphomas has been observed in 20 of 22 patients treated with donor PBMC. Clinical evidence of lymphoma regression has been observed within 14–30 days, the first signs being resolution of fever and reduction in the size of involved nodes or tumor masses. Two patients died 8 and 16 days post-infusion of idiopathic or CMV-induced interstitial pneumonias which developed prior to treatment with donor leukocytes. At autopsy, there was no

microscopic evidence of residual lymphoma in the lymph nodes. B cell populations had regressed and the nodes were infiltrated with T cells. A third patient succumbed 1 week post donor leukocyte infusion of multi-organ system failure, including severe adult respiratory distress syndrome. At autopsy, nodes and tumor masses still exhibited microscopic foci of residual lymphoma and focal necrosis. In our series, three patients developed acute GvHD (two grade I, and one grade II acute GvHD) which responded to topical or systemic steroids not initiated until at least 4 weeks post resolution of the lymphomas. Four patients developed limited and five patients extensive chronic GvHD, of whom one succumbed late after treatment. Three patients relapsed with their own leukemia. No patient has experienced a recurrence of the EBV lymphoma. Of the 22 patients, 13 are still in sustained remission with no further treatment, for more than 3–42 months since leukocyte infusion.

In our series, infusions of donor-derived PBMC have induced striking alterations in the lymphoid populations of the recipient [105, 179]. Increments in the number of $CD3^+$ T cells are observed as early as 14–21 days post infusion. Both $CD4^+$ and $CD8^+$ populations of T cells are expanded [178, 179]. Concurrently, lymphocyte responses to mitogens (PHA) and to antigens to which the donor had been exposed, also increase. This alteration in general immune function may also have effects on viral infections other than EBV-LPD. For example, the administration of donor leukocytes to two patients with persistent CMV infections which were unresponsive to antiviral drugs led to their resolution. Using limiting dilution techniques, we [105] have also demonstrated marked increments in the frequency of EBV-specific CTLp within 14–18 days following donor-leukocyte infusions. For example, one patient who developed a pathologically confirmed monoclonal EBV-LPD 4 months post transplant had a low frequency of EBV-specific CTLp (1/119,250) prior to treatment. However, within 2 weeks of receiving an infusion of donor PBMC providing 0.5×10^6 T cells/kg, the frequency of EBV-specific CTL in the blood rose to 1/10,970, which is in the range of frequencies detected in seropositive normal individuals. By 8 weeks post-infusion, the EBV CTLp frequency had increased to 1/1,580. At both time points, both $CD8^+$ and $CD4^+$ T cells were detected, with $CD8^+$ $CD3^+$ T cells predominating. The EBV-specific reactivity of isolated CD8 and CD4 T cells was HLA restricted, and could be blocked by antibodies to HLA class I or HLA class II, respectively [105]. In this case, approximately 800 EBV-specific CTLp were infused in the dose of donor leukocytes administered. If the expansion of EBV CTLp observed in the patient were exclusively due to replication of these cells alone, the frequencies attained within 2 weeks would amount to almost a 3,000-fold increase in the number of these T cells This rate of proliferation is comparable to maximal rates of expansion attainable with cloned virus-specific T cell in vitro ([154, 211] and S. Riddell, personal communication).

Our observation that monoclonal EBV^+ lymphomas emerging in marrow allograft recipients can be induced into durable remission following treatment with small doses of PBMC derived from a seropositive marrow transplant donor has now been confirmed by several transplant centers ([10, 137, 138, 171] and F. Falkenburg, T. Gordon-Smith, D. Emanuel, M. Cairo, personal communications). As noted above, 20 of our 22 patients treated with donor PBMC have achieved remission, including patients with large, multifocal tumors of the liver, lung and brain. However, in this series, recipients of TCD marrow grafts do not receive immunosuppressive drugs post transplant to prevent GvDH. In other centers using T cell-specific monoclonal antibodies for depletion coupled with drug prophylaxis to prevent GvHD, treatment of EBV lymphomas with donor leukocytes has been less consistently effective. Thus, in a preliminary com-

pendium of the results of donor-leukocyte infusions applied to the treatment of EBV lymphomas in recipients of unrelated marrow transplants reported to the National Marrow Donor Program, only 6 of 13 patients with EBV^+ lymphomas achieved complete remissions [137]. The reasons for treatment failures have not been specified. However, such factors as intervention late in disease course, continuation of immunosuppressive drug prophylaxis post PBMC infusion, or the development of an EBV lymphoma as a complication of an EBV infection latent in the host or acquired by third party-derived blood transfusion following a marrow transplant from a seronegative donor could prevent or fatally delay an effective response by donor-derived EBV-specific T cells.

The rapidity of the clinical and pathological responses observed in marrow allograft recipients with EBV lymphomas following treatment with marrow donor-derived PBMC has stimulated several transplant centers to explore the potential of leukocytes derived from normal, seropositive HLA-matched siblings to induce regressions of EBV lymphomas complicating organ allografts. The hypothesis being tested is whether such leukocyte infusions could provide the host with populations of EBV-reactive effector cells in sufficient numbers and for periods long enough to induce remissions of disease. In the study of Emanuel et al. [42], a lung allograft recipient who developed a monoclonal EBV^+ B cell lymphoma of host origin involving multiple sites in the brain was treated with PBMC from his HLA-matched sibling providing 1×10^6 $CD3^+$ T cells/kg recipient weight. This dose produced significant reductions in the size of the CNS lesions and marked neurological improvement; 5 months and 8 months later, the patient required two additional infusions at this dose to treat exacerbations of a temporal lobe lesion. Immunosuppression with steroids and cyclosporine was interrupted for only 5-day periods after each dose of cells. Prior to each infusion, EBV-specific CTLp frequencies in the blood were almost undetectable. At 5 days after the second and 28 days after the third infusion, the frequency of EBV-specific CTLp was documented to be in the range of normal seropositive adults. However, donor cells were not detected in these populations. After the third infusion, progressive neurological improvement and reduction of enhancing lesions were observed. A biopsy of the temporal lobe lesion 6 weeks after this third infusion, revealed no residual lymphoma. Unfortunately, the origin of the T cells infiltrating the brain lesion could not be determined. The site was replaced with gliosis and an infiltrate of $CD4^+$ and $CD8^+$ T cells. Nine months later, the patient was stable, on immunosuppression, but with significant bronchiolitis obliterans of the transplant lung.

More recently, Nalesnik et al. [128] have harvested autologous PBMC from four patients with EBV-LPD complicating organ allografts, who did not respond to discontinuation of immunosuppressive drugs and treatment with antiviral agents. These PBMC were activated in vitro for 10–11 days with IL-2. These cells contained a predominance of T cells and, in addition, a large fraction of $CD56^+CD3^+$ and $CD56^+CD3^-$ cells. Functional assays revealed that the cells exhibited preferential cytotoxicity against autologous EBV-transformed cells, with some activity against allogeneic EBV^+ targets and strong cytotoxicity against the NK cell-sensitive K562 line. The IL-2-activated autologous PBMC were then infused at doses of 0.7×10^{10}–5.6×10^{10} into the patients. All four achieved durable regressions of their lymphomas. While the IL-2-activated cells induced rejection reactions, these were controlled with steroids, without recurrence of the EBV lymphoma. Thus, in these patients, in vitro expanded autologous T and NK cell populations were effective in controlling the otherwise unresponsive EBV-LPD.

The applicability of allogeneic PBMC containing virus-specific T cells in marrow transplant recipients and other comparably immunosuppressed individuals is limited

by the fact that such populations may also contain alloreactive T cells capable of initiating severe GvHD. While restricting the dose of T cells administered may limit the incidence and severity of GvHD in HLA-matched recipients [107, 131], even doses as low as 10^5–10^6 T cells/kg may induce GvHD if administered early post transplant [131]. Furthermore, since precursors of both cytotoxic and helper T cells reactive against HLA alloantigens or molecular variants thereof are maintained at high frequency in the blood of most normal individuals [76], small doses of PBMC-derived T cells (10^5–10^6 T cells/kg) may precipitate severe GvH reactions in recipients of HLA-disparate marrow grafts. Indeed, Heslop et al. [65] observed grade IV acute GvHD in a TCD marrow graft recipient who was successfully treated for an EBV^+ lymphoma with PBMC containing 10^6 T cell/kg derived from his two HLA allele-mismatched sibling donor.

To circumvent the problem of severe GvHD, several groups are exploring the use of virus antigen-specific T cell clones or T cell lines which, after sensitization to virus antigens presented on autologous cells and subsequent culture in vitro for 28–35 days, are depleted of alloreactive T cells capable of inducing severe GvHD. Initial clinical trials exploring the use of such virus-specific T cell clones and lines for the prevention or treatment of CMV and EBV infections has been highly encouraging [66, 93, 154, 161, 211]. Furthermore, by clearly demonstrating the potential of virus-specific T cells to eradicate these lymphomas, these studies have provided compelling evidence that such cells are the principal effectors of resistance against these malignant tumors.

The successful application of EBV-specific T cell lines for the treatment or prevention of EBV lymphomas developing after TCD HLA-nonidentical marrow grafts was first reported by Rooney et al. [161]. The EBV-specific T cell lines used for adoptive therapy were generated in vitro by culturing T cells from the marrow donor in the presence of autologous B cells transformed with the EBV strain 95.8. The lines were expanded in vitro for at least 21–28 days to enrich for EBV-reactive T cells and to deplete alloreactive T cells. Thereafter, these T cell cultures were transduced with the G1NA recombinant retroviral vector, an MLV-based vector which directs the constitutive expression of a neomycin resistance gene, an enzyme which permits selection of transvected T cells and provides a marker for analyzing the fate of the T cells following adoptive transfer. In their initial studies, three patients with EBV-LPD of donor origin, two with a monoclonal EBV lymphoma and one with a polyclonal EBV proliferation, were treated with these lines. Regressions were observed in each case following infusions of CTL at total doses of 1.1×10^8 T cells/m^2. Subsequently, 25 more patients have received doses of 0.4×10^8–1.2×10^8 T cells/m^2 as prophylaxis against EBV lymphoproliferations [67]. No EBV lymphoproliferations have been observed in the patients who received the T cells as prophylaxis. In contrast, 5 of 27 nonprophylaxed historical controls developed EBV-LPD in the post-transplant period. The infusions of EBV-specific T cell lines increased the frequency of circulating EBV-specific CTL 5- to 100-fold over the first 4–8 weeks post infusion. Furthermore, neo^R-marked CTL persisted for periods exceeding 18 months post infusion. Similarly, Riddell and colleagues [154, 211] have demonstrated that infusions of marrow donor-derived, CMV-specific T cell clones can induce high levels of CMV-specific T cell-mediated cytotoxicity in marrow transplant recipients, thereby protecting them from CMV infection. Furthermore, T cells with clone-specific T cell receptor rearrangements have been detectable in the circulation of these patients for at least 12 weeks post-infusion [211].

Recently, our group has extended trials of EBV-specific T cell lines to the treatment of patients developing EBV^+ lymphomas as a complication of AIDS, congenital im-

munodeficiency or the immunosuppression induced to sustain organ transplants. Donor-derived EBV-specific T cell lines have been generated by culturing isolated T cells with EBV-transformed autologous B cell lines transformed with EBV strain 95.8. These lines have been characterized as to their EBV antigen specificity, HLA-restriction pattern, and EBV CTLp frequency and tested for the presence or absence of reactivity against normal or EBV-transformed allogeneic cells at different times in their expansion. Within the first 3 weeks of culture after sensitization, EBV-specific T cells expand 10- to 50-fold. By day 28, T cells reactive against allogeneic cells are usually, but not always, deleted. Under the conditions currently used, $CD8^+$ HLA class I-restricted T cells are preferentially expanded, although small populations of $CD4^+$ class II-restricted EBV-specific T cells can still be detected. As early as 10–14 days into their expansion, these lines usually select one or, maximally, two dominant HLA-restricting alleles. The pattern of HLA restriction has been ascertained by examining EBV-specific cytotoxic responses against a panel of HLA-homozygous EBV BLCL developed by Yang et al. [222], which share single or multiple HLA alleles expressed on the donor's T cells or by their reactivity against HLA null EBV-transformed cell lines transduced with vectors inducing expression of a single HLA allele. This approach has also allowed us to assess the significance of molecular variants of specific HLA alleles for T cell recognition of EBV antigens. For example, EBV-specific T cells generated from donors bearing HLA B3502 in our experience rarely recognize EBV in the context of other B35 microvariants. As would be predicted from the studies of Rickinson et al. [153] and others [181, 190], we have also observed rapid selection of T cells exhibiting EBV-specific responses which are restricted by a single microvariant of several other HLA alleles, including HLA B44, HLA B57, HLA A2 and HLA A11.

Once the in vitro expanded EBV-specific T cells have been ascertained to be depleted of alloreactive cells and able to recognize EBV in the context of HLA determinants expressed on the patient's lymphoma, such cells generated from normal HLA-compatible or haplotype-disparate donors can be safely used for the treatment of EBV-LPD in severely immunocompromised hosts, including marrow or organ allograft recipients. In our initial case, EBV-specific HLA-restricted T cell lines, generated from the PBMC of a parental renal allograft donor, induced a partial regression and stabilization of a host-type monoclonal EBV lymphoma involving the CNS and liver arising in her HLA haploidentical child. Two courses of three weekly infusions of $1 \times 10^6 - 3 \times 10^6$ EBV-specific T cells/kg recipient weight induced significant increments in the levels of circulating CTLp detectable 7 days after each dose (Table 2). However, as expected, these increments were transient, persisting for only 10–14 days. There was no evidence of durable engraftment or replication of these HLA-haplotype disparate cells in the host. Unfortunately, after the first two courses of T cell infusions, the T cell line progressively exhibited preferential reactivity against donor EBV BLCL, and ultimately selectively killed donor and not host EBV BLCL. The patient subsequently received autologous EBV-specific T cells generated ex vivo over 12 weeks of culture, and thereafter, IL-2. This patient ultimately achieved complete regression of disease and has had no recurrence of the lymphoma over 2 years of follow-up. In a second patient, a recipient of an unrelated marrow transplant who also developed a monoclonal EBV^+ lymphoma presenting with multifocal lesions in the CNS, treatment with an EBV-specific T cell line induced complete regression of disease. However, transient reactivation of GvHD was observed necessitating reinstitution of steroids. In this case, the pace of tumor regression was slow and the alterations in the circulating numbers of EBV-specific CTLp minimal. Indeed, the amplification of CTLp levels in recipients of

Table 2. EBV-specific CTLp frequencies among circulating T cells following infusions of HLA-haplotype disparate donor-derived EBV-specific T cells into a renal allograft recipient with an EBV lymphoma

		Dose	Donor PHA blasts	Donor BLCL (donor)	Recipient BLCL	Third party BLCL (allo)
First course	Day 0 (3/17/95)	1 × 10⁶/kg	NA	1/784,830	1/585,550	1/00
	Day 2		NA	1/109.260	1/161,584	1/176,040
	Day7	1 × 10⁶/kg	< 1/10⁶	< 1/10⁶	< 1/10⁶	< 1/10⁶
	Day 14	1 × 10⁶/kg	NA	1/00	1/00	< 1/10⁶
	Day 21		1/17,870	1/29,310	1/10,160	1/44,000
Observation period: 21 days						
Second course	Day 0 (4/25/95)	2 × 10⁶/kg	< 1/10⁶	1/00	1/00	< 1/10⁶
	Day 2		< 1/10⁶	1/55,550	1/147,820	< 1/10⁶
	Day 7	3 × 10⁶/kg	1/877,900	1/45,780	1/70,020	1/749,570
	Day 14	3.3 × 10⁶/kg	1/1,117,120	1/12,940	1/23,230	1/118,520
	Day 21		1/00	1/66,770	1/264,030	1/891,790

CTLp, Cytotoxic T lymphocyte precursor; PHA, phytohemagglutinin; BLCL, B lymphoblastoid cell line; NA, not applicable

EBV-specific T cell lines has been extremely limited (5–10-fold), largely reflecting frequencies which would be expected from the doses of CTLp administered, rather than the exponential amplifications in EBV CTLp frequency which we have documented following leukocyte infusions. In part, this may reflect the fact that the T cell line infused consisted predominantly of $CD8^+$ $CD3^+$ T cells which may have limited capacity to further expand in vivo in the absence of help provided by antigen-specific $CD4^+$ T cells.

To characterize the effects of different classes of EBV-reactive human effector cells on EBV-LPD, and explore the mechanisms whereby such effectors induce tumor regressions, we [95] and others [14, 17, 39, 144, 147] have established models of human adoptive cell immunotherapy in SCID mice bearing xenografted human EBV BLCL-induced lymphomas. In these studies our group has utilized C.B.-17 SCID/scid mice treated with anti-asialo GM1 [94]. In our studies and in those of Picchio et al. [136], treatment with anti-asialo GM1 markedly enhances the susceptibility of SCID mice to EBV-LPD by suppressing endogenous NK cell activity. Treatment with anti-asialo GM1 also eliminates the NK-mediated resistance to EBV-LPD that can be induced in SCID mice by treatment with human IL-2 [7].

In our experience, SCID mice inoculated intraperitoneally with 10^7 EBV-transformed B cells regularly develop EBV lymphomas involving lymph nodes in the mesentery liver, spleen and mediastinum, which are lethal by 28–30 days post inoculation [94]. However, when we treated these animals 5 days after EBV BLCL inoculation with an intraperitoneal infusion of 10^7 expanded autologous EBV-specific T cells, approximately 40% did not develop lymphomas and in those lymphomas that did develop, their growth was markedly delayed [95]. Recently, DiMaio et al. [39] have confirmed and extended these findings, demonstrating that SCID mice inoculated in-

traperitoneally with 5×10^6 cells derived from biopsy specimens of an EBV lymphoma from a heart allograft recipient also develop lethal disseminated lymphoma which can be prevented if the animals are treated at 7 days post tumor inoculation with an intraperitoneal infusion of 2.5×10^7 autologous EBV-reactive T cells generated in vitro after sensitization with autologous EBV-transformed B cells. These T cells had no effect against xenografts of an EBV negative allogeneic Burkitt's lymphoma line.

Our group has also evaluated the capacity of EBV-reactive T cells to migrate to distal sites of disease. When 10^7 effector T cells were administered intravenously to animals previously inoculated with EBV BLCL intraperitoneally, lymphomagenesis was delayed and could be prevented in 40% of animals if they were also treated with 2,500 IU IL-2 twice a day for 14 days. IL-2 alone had no significant effect. Similar to the findings of Rencher et al. [147], who evaluated the activity of EBV-specific $CD8^+$ and $CD4^+$ T cell clones, and Lieberman et al. [17], who used an HLA-restricted LMP-2-specific $CD8^+$ T cell line, we have observed that EBV-specific HLA-restricted $CD8^+$ T cells are able to protect mice inoculated with large doses (10^7 cells) of transformed EBV LCL from developing lethal EBV lymphomas. However, our studies contrast with the findings of Rencher et al. [147] in that EBV-specific HLA class II-restricted $CD4^+$ CTL have also induced protection in a proportion of animals.

We have also used this model to explore the capacity of different effector cells to migrate to and induce remissions of established EBV-induced lymphomas. In these experiments, SCID mice bearing large subcutaneous EBV^+ lymphomas, which are treated intravenously with autologous 10^7 EBV-specific CTL 35 days after initial inoculation of 10^7 EBV BLCL, can achieve complete tumor regressions which may be sustained for the life of the animal in 40–60% of the individuals [95]. In contrast, single infusions of PBMC derived from seropositive individuals stimulated either with IL-2 or OKT3 alone were unable to prevent the emergence of EBV-LPD or to induce regressions in tumor-bearing animals [95]. Thus, in this xenograft model in which expansion of the adoptively transferred EBV-reactive T cells is likely limited and recruitment of additional EBV-reactive murine effector cells is inhibited, high doses of specifically immune cytotoxic T cells were required to induce the ablation of the EBV-induced lymphoproliferation. This finding contrasts markedly with our own and others' observations of consistent durable regressions of EBV-induced lymphomas following infusions of donor leukocytes containing fewer than 1000 EBV-specific CTL in human marrow transplant recipients bearing donor-type EBV lymphomas. However, it is quite similar to our findings in kidney and lung allograft recipients treated with HLA-haplotype-disparate EBV-specific T cells, in whom the survival of the T cells was limited to 10–14 days. The high frequencies of EBV-specific T cells transiently achieved could be accounted for by the doses administered. Little or no detectable expansion of these cells could be detected after adoptive transfer into the host.

This mouse model also permits analysis of the mechanisms whereby HLA-restricted EBV-specific T cells induce tumor regressions. For example, our studies have shown that intraperitoneal infusions of 10^7 HLA-restricted EBV-reactive T cells into mice previously inoculated with EBV LCL will prolong survival and can prevent development of autologous EBV lymphomas but have no effect on the emergence of lymphomas in mice inoculated with allogeneic EBV LCL [95]. Furthermore, when we infused 10^7 EBV-specific T cell intravenously into SCID mice bearing two subcutaneous EBV^+ tumors, one autologous and the other HLA mismatched to the EBV-specific CTL donor, regressions only of the autologous tumor and not the allogeneic tumor were observed [95]. We were also able to demonstrate preferential homing of

PKH26 labeled EBV-specific CTL to autologous but not to the HLA-mismatched EBV^+ tumor in SCID mice as early as 24 h after intravenous adoptive transfer. Immunophenotypic analyses of the two tumors 14 days later also demonstrated preferential infiltration and expansion of $CD3^+CD8^+$ cells in the autologous EBV^+ tumor in SCID mice bearing both the autologous and a fully HLA-mismatched EBV^+ tumor [95]. We have recently repeated this experiment, using EBV-specific T cells infected with a retroviral vector, termed NTP, which encodes a mutated nerve growth factor receptor expressed on the surface of transduced cells. Again, the NTP^+ T cells, also labeled with PKH26, were preferentially retained in the autologous EBV lymphoma 48 h post infusion. In animals killed 21 days later, NTP^+ cells were selectively detected in autologous EBV lymphomas, representing 23% of the mononuclear cells in the tumor. In contrast, only 3% of the mononuclear cells detected in the allogeneic tumor were NTP^+. Such targeted migration and expansion of specific T cells within tumors has also been observed following adoptive transfer of specific T cells into SCID mice bearing xenografts of Daudi lymphoma cells [11].

In our own experiments and those of Lieberman et al. [17], IL-2-activated LAK cells from seropositive donors have had little effect on the lethality of EBV lymphomas. Furthermore, while infusions of highly cytotoxic NK cell clones have delayed mortality, they have been ineffective in eradicating established tumors or preventing the emergence of EBV-LPD. Potentially contrasting with these results are the studies of Randhawa et al. [144] who generated IL-2-activated killer cells (LAK cells) from autologous or allogeneic donors and used these cells to treat EBV-transformed BLCL-induced lymphomas 10 days after xenografting into beige-SCID mice. In these studies, control animals inoculated with 10^7 LCL intraperitoneally died of disease 29–32 days post inoculation, while all animals given autologous or HLA-disparate third party LAK cells survived for the 40-day observation period. At sacrifice, the autologous and allogeneic LAK cell-treated mice exhibited residual tumors which were significantly smaller only in the animals treated with allogeneic LAK cells. However, the cells infiltrating the tumors were $CD3^+$ T cells, potentially representing EBV-specific or allospecific T cell populations in the IL-2-activated preparations. Randhawa et al. [144] have ascribed the differences between their results and those of Lacerda et al. [95] either to the increased proportion of $CD56^+$ NK cells in their LAK preparations or to enhanced engraftment and growth of the LAK cell effectors in the beige-SCID mice which they have used as xenografted hosts. An alternative possibility is that differences in the numbers of EBV-reactive T cells in the LAK cell preparations could explain the results, since the frequencies of EBV-reactive T cells detected in the LAK cell preparations used by Lacerda et al. [95] were very low.

The above, very limited preclinical and clinical experience with the use of virus-specific T cell lines or clones, highlights the striking clinical advantages as well as some of the disadvantages of this approach. Clearly, adoptive transfers of T cell lines have induced durable remissions of autologous, donor-derived EBV lymphomas in marrow allograft recipients and have provided populations of EBV-specific T cells that, in this group of patients, can grow and persist to respond to secondary expansions of EBV-infected cells for at least 18 months post infusion [67]. In addition, their use for prophylaxis or treatment has thus far not been associated with severe GvHD, despite the fact that EBV-reactive T cell lines propagated for 4 weeks or more may still contain small populations of alloreactive T cells [67]. There are also disadvantages. First, the generation of virus-specific T cell lines or clones requires time. At least 2 weeks are required to establish infected donor-derived EBV-transformed B cell lines

for in vitro sensitization of the donor's T cells and at least an additional 3–4 weeks of T cell culture to produce virus-specific effector cells adequately depleted of alloreactive T cells. Since EBV lymphomas generally have a rapid, lethal course, this approach is practicable only if the T cell lines are established before or at the time of transplant, so that they can be ready at the time of maximal risk (2–6 months post transplant). Since only a small proportion of transplant recipients will develop EBV lymphomas, this can be an expensive and logistically difficult approach.

A second limitation arises from the fact that, while T cells in the donor's blood responding to autologous EBV-transformed B cells in vitro in the first 1–4 days after sensitization may include many T cell clones reactive against a number of EBV antigens and restricted by multiple donor HLA alleles, T cell lines generated from seropositive donors, and propagated for 2 weeks or more, almost invariably select for clones reactive against a single dominant EBV antigen presented in the context of one or at most two HLA alleles expressed on the donor B cells. If the lymphomas do not adequately express that targeted antigen and that restricting HLA allele, or the expression of the specific HLA-restricting element is down-regulated, adoptive transfer of the T cell line will not be effective.

The selective expansion of T cells reactive against a dominant EBV antigen preferentially bound to a single HLA-restricting element particularly limits the effectiveness of such cells if the lymphoma and the effector cell differ in origin and are not HLA identical. Since the lymphomas which have been treated in marrow transplant recipients have thus far been only of marrow donor origin, the problem of HLA disparity between effector and target has not arisen. However, lymphomas of host rather than donor type are common following transplants of HLA-haplotype disparate related or HLA-microvariant disparate unrelated TCD marrow transplants administered to patients with Wiskott Aldrich Syndrome [204], and may also arise in a proportion of patients transplanted for SCID or leukemia. Furthermore, EBV^+ lymphomas are usually of host origin in organ allograft recipients. In such cases, EBV-reactive T cell lines or clones derived from a donor other than an HLA-matched relative might be ineffective if these lines or clones are restricted by an HLA allele or microvariant thereof that is not shared by the lymphoma cells. This problem is compounded if the lymphoma emerging in a liver, heart or lung recipient is of donor rather than host origin, since the donor of the allograft is deceased and is usually not HLA-matched to the recipient. Such limitations, imposed by the emergence of cells reactive against a single dominant epitope and presented by single HLA alleles, may also be a problem for adoptive cell therapy of other infections, such as CMV infections which affect host cells when the donor and host are HLA disparate (S. Riddell, personal communication).

A third limitation which may be imposed by the use of EBV-specific T cell lines or clones rather than donor PBMC is expected: their activity is highly focused and specific, and has little effect on the general immune function of the host. In our series, infusions of donor PBMC induced striking increments in the number of host $CD4^+$ and $CD8^+$ T cells and in their responses to mitogens and non-EBV antigens including CMV, reflecting the multiple reactivities of the cells infused. In contrast, infusions of EBV-specific T cell lines, which are usually predominantly $CD8^+$ T cells, induce little or no alterations in circulating lymphocyte populations or their responses to antigens other than those induced by EBV [132]. As a result, the host's susceptibility to other viruses such as CMV or adenovirus is unchanged. Furthermore, if the EBV lymphoma differs from the standard EBV strain 95.8 in its expression of antigenically dominant EBV-encoded peptides, T cell lines generated in response to autologous B cells trans-

formed with EBV strain 95.8 may not lyse the lymphoma cells. Indeed, two marrow allograft recipients have recently been identified whose EBV+ lymphomas were not recognized by donor T cells generated in response to EBV strain 95.8 transformed autologous B cells (D. Emanuel and H. Heslop, personal communications). In one of these cases, this was due to the fact that the lymphoma had a deletion mutation of EBNA-3, whereas EBNA-3 was the dominant specificity of the T cell line raised against EBV strain 95.8 transformed donor type BLCLS which was used for therapy (H. Heslop, personal communication).

The use of donor T cells genetically modified to express a selectable marker and a suicide gene has been proposed as an alternative strategy by Sadelain and Mulligan [163], and Mavilio et al. [112] and Servida et al. [171] of C. Bordignon's group in Milan. PBMC are isolated from the blood of the marrow donor and are either nonspecifically activated with IL-2, or primed with irradiated EBV-transformed autologous B cells. As the cells begin to divide, they are transduced with the retroviral vector encoding the selectable marker and the suicide gene. The transduced cells are then isolated and administered to the patient. This approach has several attributes. First, the vector-modified cells are similar to unmodified donor leukocytes in that they contain a broad array of T cells capable of interacting with multiple pathogens, and, in the case of EBV, multiple EBV antigens presented by multiple different HLA alleles on the lymphoma cells. Secondly, the vector-modified cells, like the T cells in PBMC, would be expected to respond to antigens presented on HLA alleles presented in vivo rather than in vitro. As a result, the donor T cells can respond in vivo to EBV antigens presented by HLA alleles adequately expressed on the lymphoma cells, be they lymphoma cells of donor origin or host-type lymphoma cells sharing specific HLA alleles with the donor. A third, critical, feature of this strategy is that the genetically modified T cells infused can be controlled, in that they can theoretically be eliminated at will by treating the host with the drug to which they have been engineered to be sensitive. Thus, were GvHD to develop from expansion of alloreactive T cells, it could be reversed. This feature has an added advantage in that it potentially provides an approach whereby a controlled donor anti-host reaction or GvHD could be induced to foster engraftment or to eradicate residual host leukemic cells. A final, practical advantage of this approach is that the time required for generation of such cells for adoptive transfer is short, 3–4 days, a major attribute given the rapid and generally fatal course of EBV lymphomas.

In the studies of Servida et al. [171], recently updated by Bonini et al. [15], a Moloney leukemia virus-based recombinant retroviral vector, termed SFCMM-2, which encodes a mutant nerve growth factor receptor promoted by the 5′ LTR and an HSV thymidine kinase-neomycin (HSV TK-neo) resistance fusion gene promoted by an internal thymidine kinase promoter, is used to transvect PBMC isolated from the blood of the marrow transplant donor after 48 h activation in medium containing IL-2. After co-culturing these cells for an additional 48 h with irradiated monolayers of the packaging cell line producing the vector, the transduced cells expressing the mutant LNGFR product on their surface are isolated by immunoabsorption to magnetic beads coated with a monoclonal antibody specific for LNGFR. The isolated cells were then administered to a series of eight patients. Seven of these patients were evaluable for response, including one patient with an EBV lymphoma and six patients with leukemic relapse developing post transplant. Of these patients, three achieved a complete remission and two a partial remission of disease. The doses required to induce a response ranged from $1.5 \times 10^6 - 38.6 \times 10^6$ marked T cells/kg. The patient treated for an EBV lymphoma achieved a complete and durable remission following an infusion

of 1.5×10^6 isolated transduced cell/kg. As in our series, infusions of these transduced donor leukocytes also induced marked increases in the overall number of circulating T lymphocytes, particularly the $CD8^+$ T cells in each patient. In the patient treated for the EBV lymphoma, $NGFR^+$ cells constituted 13% of the circulating T cells when lymphocytosis was at its peak. Because the T cells were selected after non-specific activation with IL-2, it was anticipated that alloreactive T cells would also be present in the genetically modified T cell populations. In fact, three patients in this series developed GvHD. Acute GvHD was reversed in two of the patients and ameliorated in the third by treating the patients for 4–7 days with ganciclovir (GCV). Following this treatment, genetically marked T cells in the blood were dramatically reduced. One of the patients treated with GCV subsequently developed a cell-mediated response against HSV-TK, which limited the life span of infusions of cells administered after GvHD had resolved. However, no other patient developed an immune response to HSV-TK-neo and no responses to the mutant NGFR were detected [15]. In those patients who achieved a remission, including two patients with leukemia and the patient treated for an EBV-LPD, the lymphoma or leukemia did not recur after GCV treatment. However, in patients who had achieved a clinical, but not a cytogenetic remission of chronic myelogenous leukemia, treatment with GCV ultimately was followed by disease relapse.

As suggested by these cases, the administration of such genetically modified effector cells provides a novel and potentially safe approach for adoptive transfer of T cells isolated early in the course of in vitro sensitization for the treatment of EBV lymphomas or other viral infections when these T cell populations may still contain significant numbers of potentially alloreactive T cells. However, a meaningful assessment of the potential and limitations of this approach cannot be made until several features of the transfected cells are clarified. First, the levels of expression of introduced genes in effector cells sustained over extended periods in vivo must be defined. If expression of transduced drug sensitivity genes like TK is not sustained, the cells may not be susceptible to elimination by drug treatment if acute or chronic GvHD develops after a T cell infusion. Indeed, alterations in the expression of HSV-TK might explain the failure of Bonini et al. [10] to completely reverse the GvHD which evolved in one of their patients months after T cell infusion. It will also be essential to evaluate, in larger patient groups, the immunogenicity of vector-modified T cells. Existing data on this point are fragmentary and contradictory. On the one hand, the studies of Heslop et al. [66] have documented that neo-marked EBV-specific T cells can be detected for up to 18 months post transfer, and can re-expand at times when EBV is reactivated. These findings are similar to those recorded in all but one of the patients treated by Bonini et al. [10] with T cells transduced and expressing the mutant NGFR and TK. Similarly, Brenner et al. [15] have not detected immune responses against autologous hematopoietic cells transduced with the vector in myeloablated patients transplanted with such cells as treatment for acute myelogenous leukemia (AML) or neuroblastoma. On the other hand, Riddell et al. [155] who have used a vector encoding a hygromycin phosphotransferase TK fusion gene to transduce $CD8^+$ HIV-specific T cells for treatment of patients with AIDS have detected CTL responses directed against the fusion proteins which have limited the survival of adoptively transferred cells in each of six patients treated. Whether this reflects differences in the level or characteristics of the immunodeficiency exhibited by the AIDS patients as compared to the patients with neuroblastoma and leukemia, who have received intensive alkylator-based chemotherapy prior to cell transfer, or in the relative immunogenicity of the vector-encoded proteins remains to be determined.

In addition to these studies of the transduced effector cell populations it will be important to ascertain the relative contributions of transferred effector cells and other recruited cell populations to sustained anti-tumor responses and the period of engraftment of EBV-specific effector cells required to induce durable regressions of EBV^+ lymphomas. If EBV lymphomas can be truly eradicated by exposures to T cells lasting only 2–3 weeks, or, if the adoptively transferred effector cells can recruit other cells in the host to mount a response against EBV antigens, sustained engraftment of the EBV-specific effector cells may not be required. However, our own and other single case experiences with infusions of HLA-matched EBV-specific T cells into non-conditioned immunodeficient hosts with EBV lymphoma suggest that, while such infusions may have significant effects, those salutary responses have been short lived. Thus, elimination of transduced cells too early after transfer may lead to recurrence. Conversely, the safe use of preparations potentially containing alloreactive T cells expressing a drug sensitivity gene hinges on the reversibility of induced alloreactions once these cells are eliminated by drug treatment. If non-transvected effector cells can be recruited to participate in alloreactions initiated by transvected cells and can sustain this reaction at some stage in its development, the risks of severe uncontrolled GvH reactions would be formidable.

Extension of adoptive immunotherapy to other EBV-associated diseases

It is well recognized that several malignancies other than the EBV^+ lymphomas emerging in immunocompromised hosts may also contain EBV DNA and express EBV-encoded antigens. Prominent among these are African Burkitt's lymphomas, which selectively expresses EBNA-1, and subsets of nasopharyngeal carcinoma, Hodgkin's disease and leiomyosarcoma which express EBNA-1 and LMP-1 [2, 81, 153]. CTL responses to EBNA-1 capable of killing Burkitt's lymphoma have not been identified, likely reflecting the incapacity of transformed cells to express EBNA-1 in complex with HLA on the cell surface [2, 81, 183, 190, 221]. However, recent studies have demonstrated that LMP-1 can be appropriately presented by nasopharyngeal carcinoma cells and by EBV^+ Reed-Sternberg cells in Hodgkin's disease [86, 175]. Thus, these cells can be recognized and lysed by LMP-1-specific CTL. Unfortunately, as previously discussed, LMP-1-specific responses usually represent only a minor constituent of EBV-specific responses in most individuals, being superceded by the dominant responses elicited by EBNA-3 peptides. However, recent progress in the isolation and characterization of potentially immunogenic LMP-1 peptides [85, 86] suggests that strategies involving in vivo sensitization and/or selective in vitro expansion of LMP-1 specific T cells could soon be practicable. Preclinical evaluations of these approaches are in progress and may soon be extended into clinical trials (D. Moss, personal communication). In addition, clinical trials of genetically marked EBV-specific T cells generated in response to EBV-transformed B cells are being conducted to evaluate their activity in EBV^+ Hodgkin's disease [64].

Conclusions

In summary, EBV, which is carried as a latent infection in over 90% of normal adults, induces an intensive T cell-mediated host immune response that is maintained for the

life of normal healthy adults. Profound and sustained suppression of this T cell response is permissive for unchecked proliferations of endogenous EBV-transformed B cells which may clinically evolve into monoclonal EBV+ lymphomas that are refractory to conventional antiviral and antineoplastic agents. The adoptive transfer of small numbers of donor-derived EBV-specific CTL can induce durable and complete remissions of EBV-LPD in severely immunocompromised allogeneic marrow transplant recipients. The principal effectors detected in regressions of EBV-LPD in human marrow allograft recipients are CD8+ HLA class-I-restricted, EBV-specific T cells. The recent development of efficient and effective strategies for generating large numbers of EBV-specific CTL in vitro which minimize the risks of transferring alloreactive T cells in a donor-cell inoculum and for genetically modifying unselected T cells so that they express genes that render them susceptible to drug elimination may soon permit broader application of this approach to other immunocompromised hosts, including organ allograft recipients and patients with genetic or acquired immunodeficiency at risk for EBV-LPD. Ultimately, further modifications of these approaches which would foster the selective generation and expansion of T cells specific for LMP-1 and LMP-2 encoded by EBV may also provide useful strategies for adoptive immunotherapy of other EBV-associated malignancies, particularly nasopharyngeal carcinoma, EBV+ leiomyosarcomas and a subset of patients with Hodgkin's disease.

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