Immunotherapy for Epstein-Barr Virus-Associated Cancers in Children

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ABSTRACT

Latent Epstein-Barr virus (EBV) infection is associated with several malignancies, including Burkitt’s lymphoma, Hodgkin’s disease, nasopharyngeal carcinoma, and post-transplant lymphoproliferative disease (LPD). The presence of EBV antigens in these tumors provides a target for immunotherapy approaches, and immunotherapy with EBV-specific cytotoxic T cells (CTLs) has proved effective in post-transplant LPDs, which are highly immunogenic tumors expressing type III latency. The malignant cells in Hodgkin’s disease and nasopharyngeal carcinoma express type II latency and hence a more restricted pattern of EBV antigens. Trials with autologous EBV-specific CTL responses are under way in both of these diseases, and while some activity has been seen, no patient has yet been cured. This reduced CTL efficacy may reflect either downregulation of immunodominant EBV proteins, which are major CTL targets, or the ability of these tumors to evade the immune response by secreting inhibitory cytokines. Further improvement of EBV-specific CTL therapy for these type II latency tumors will require improved methods to activate and expand CTLs specific for the subdominant EBV genes expressed and to genetically modify the expanded CTLs to render them resistant to inhibitory cytokines. If these strategies to improve the therapeutic potential of immunotherapy for EBV-associated tumors prove successful, this type of treatment may be adapted to other tumors expressing known (viral) antigens. The Oncologist 2003;8:83-98

INTRODUCTION

Epstein-Barr Virus

Over 95% of the adult population worldwide is infected with the Epstein-Barr virus (EBV). Primary infection usually occurs during childhood and results in a mild, self-limiting illness. When infection is delayed until adolescence, infectious mononucleosis, characterized by fever, lymphadenopathy and pharyngitis, occurs in around 50% of individuals [1]. EBV enters via the oropharyngeal route and infects resting B cells
[2] and/or epithelial cells [3]. Virus released during this productive primary phase subsequently infects B cells circulating through the oropharynx, resulting in a latent infection. The infected B cells are highly immunogenic and therefore induce a massive expansion of virus-specific and nonspecific T cells that cause the clinical symptoms of infectious mononucleosis. This potent antiviral T-cell response results in regression of the infected B cells. A small number of B cells express only latent membrane protein (LMP)2 and small nonpolyadenylated viral RNAs (EBV-encoded RNA [EBER] 1 and 2) out of the almost 100 viral proteins. This limited viral antigen expression allows these EBV-infected B cells to evade the immune response [4, 5]. The virus can then persist in its latent state for the life of the individual. Periodically, the virus reactivates, resulting in shedding of virions and the potential of spreading the virus to new hosts.

### EBV-Associated Malignancies

Although self-limiting in the majority of cases, EBV is associated with a heterogeneous group of tumors, including lymphoproliferative disorders (LPDs), Hodgkin’s disease (HD), nasopharyngeal carcinoma (NPC), and Burkitt’s lymphoma. All of these tumors are associated with the EBV latent cycle, and three distinct types of EBV latency have been characterized [6] (Fig. 1). All are EBER positive, but the EBV latent protein expression varies. B cells infected in vitro become immortalized EBV-transformed lymphoblastoid B-cell lines (LCLs). These cells manifest type III latency with the entire array of EBV latency proteins: EBNA1, 2A, 2B, 3A, 3B, 3C, LP, BARFO, and the two viral membrane proteins LMP1 and LMP2. This pattern of EBV gene expression characterizes the EBV-associated LPD that occurs in individuals who are severely immunocompromised after...

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**Figure 1. EBV antigen expression in different tumor types.** Type I latency as seen in Burkitt’s lymphoma is defined by the presence of EBNA1 without expression of other latent antigens, which makes the tumor “invisible” for EBV-directed immunosurveillance. A restricted expression pattern including the subdominant EBV antigens LMP1 and LMP2 is a hallmark for HD and NPC tumor cells (type II latency). Although these tumors express MHC class I/II and costimulatory molecules and can thus be recognized by the immune system, the restricted antigen expression pattern and the active immune evasion strategies employed by these tumors are thought to enable the tumor to develop in immunocompetent hosts. Finally, in vitro-generated LCLs and lymphoproliferative tumors developing in immunosuppressed hosts express a full array of latent EBV antigens (type III latency). Moreover, expression of both MHC class I/II and costimulatory molecules makes these tumor cells highly immunogenic.
solid organ or stem cell transplantation, or who have congenital immunodeficiency or human immunodeficiency virus (HIV) infection. A more restricted EBV gene expression pattern including only EBV nuclear antigen (EBNA)1, BARFO, LMP1, and LMP2 called type II latency is the hallmark of EBV-positive HD and NPC. In type I latency found in EBV-positive Burkitt’s lymphoma, only EBNA1 and BARFO are expressed. The EBV antigens expressed on the different tumor types are potential targets for immunotherapy, but the number and type of EBV antigens expressed have important implications for the strategy and the expected therapeutic effect.

**LPD POST-HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)**

**Pathogenesis**

In normal EBV-seropositive individuals, an ongoing balance exists between virus-driven B-cell proliferation and cellular immune defense mechanisms. However, in individuals with compromised cellular immunity, increased virus reactivation and an increase in the number of latently infected B cells in the peripheral blood (PB) may be seen [7, 8]. Either or both of these factors may account for elevated levels of EBV DNA detected in PB and plasma by polymerase chain reaction [9-11]. In some patients, uncontrolled EBV-driven B-cell lymphoproliferation may then occur, leading to overt lymphoma. The highest incidence of LPD is in the first 6-12 months after transplant, indicating an important role of T-cell dysfunction in the pathogenesis of this disease. However, the incidence of post-transplant LPD (PTLD) is variable within patients receiving similar immunosuppressive regimens, and not all patients with severe T-cell dysfunction develop LPD. This observation suggests that, although T-cell dysfunction is unequivocally linked to PTLD, other immunologic factors such as cytokine imbalance likely play a role in the outgrowth of EBV-transformed B cells.

Most cases of PTLD probably arise as polyclonal proliferation, with some lesions progressing to oligoclonal or monoclonal tumors [12]. In general, monoclonal tumors are more aggressive than polyclonal tumors, possibly due to additional genetic mutation [13]. However, since no clear correlation between clinical behavior and clonality has been shown, assessment of tumor cell clonality is of limited use in directing therapeutic decisions.

**Incidence and Risk Factors**

In HSCT recipients, the overall cumulative incidence of PTLD is 1% at 10 years [14], but the risk varies with different sources of stem cells and manipulation (Table 1). For allogeneic transplants, the risk is significantly increased for recipients of stem cells from unrelated or HLA-mismatched donors (relative risk [RR] = 4.1). The risk of PTLD after unrelated umbilical cord blood transplantation is lower, with a reported incidence of 2% at 2 years [15]. Methods used for prophylaxis or treatment of graft-versus-host-disease (GVHD) also influence the risk, with higher incidences seen with selective T-cell depletion of the graft (RR = 12.7) or administration of anti-CD3 monoclonal antibody (RR = 43.2) or anti-thymocyte globulin (RR = 6.4) [14, 16, 17]. However, depletion methods such as CAMPATH-1 antibodies and counter flow elutriation that remove both T and B cells from the graft, and therefore less severely disrupt the equilibrium between latent virus and cellular immune system, are associated with a lower incidence of PTLD [14, 18].

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<th>Table 1: Risk factors for PTLD</th>
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For this reason, addition of monoclonal antibodies for B-cell depletion to the T-cell-depletion regimen can be used as prevention of EBV LPD [19]. Finally, underlying immunodeficiency, such as X-linked agammaglobulinemia and ataxia-telangiectasia, represents an independent risk factor in HSCT recipients. Thus, in patients with one or more of the above described risk factors, the index of suspicion for PTLD should be high, and close monitoring of early indicators of disease is advisable.

**IMMUNOTHERAPY APPROACHES**

**Anti-B-Cell Antibodies**

Immunotherapeutic approaches prevent and treat PTLD either by removal of EBV-infected B cells or enhancement of the compromised EBV-specific cell-mediated immunity. Reduction of the EBV-infected cells can be achieved by administration of monoclonal anti-B-cell antibodies. Initially, antibodies directed against CD21, the receptor used by EBV to enter B cells, and CD24, an antigen expressed by B-cell lineage and granulocytes, were used to target EBV-infected B cells. Complete remission was achieved in 57% of patients treated with a combination of these antibodies, with a long-term survival of 35% (follow-up, 35-72 months) [20, 21]. Risk factors for partial or no response to treatment included multivisceral disease, central nervous system (CNS) involvement, monoclonality, and late-onset PTLD. Although these results were promising, the murine origin of the antibodies used in these studies caused side effects including fever, pain, and more importantly, alloimmunization. Hence, effects of subsequent therapy were short lived and B cells reappeared rapidly after completion of treatment.

More recently, a mouse/human chimeric anti-CD20 antibody that had been successfully used as treatment for follicular lymphoma was introduced in the PTLD setting. This antibody is capable of direct lysis of B cells and has reduced immunogenicity and a longer half-life in comparison with the murine antibodies described above. So far, the results of 16 children treated with humanized anti-CD20 (rituximab) for LPD post HSCT have been reported [22-24]. Twelve patients treated in these studies entered complete remission. One patient developed fever and shivering at the first dose of rituximab, but subsequent infusions did not induce side effects [24]. In another patient, treatment was accompanied by hypogammaglobulinemia and B-cell deficiency for over 7 months [22]. Six of these patients were treated preemptively based on elevated EBV viral load with or without additional symptoms associated with LPD. In all of these patients, EBV DNA levels normalized and symptoms resolved, indicating the benefits of early or preemptive treatment.

**Adoptive Transfer of EBV-Specific Cytotoxic T Cells (CTLs)**

An alternative to abrogation of the B-cell compartment and a more physiological approach to restore the balance between EBV and the immune system is adoptive transfer of virus-specific CTLs. Papadopoulos et al. first adopted this strategy to treat stem cell recipients with established PTLD using unmanipulated T cells from the bone marrow donor [25]. Although donor lymphocyte infusions were proven to be effective in a number of patients [25], the infused product also contains alloreactive cells and can therefore induce GVHD. This risk can be circumvented by transduction of the donor T cells with a suicide gene that can be switched on in case of alloreactivity. The most commonly used suicide gene is herpes simplex virus thymidine kinase (HSV-tk), which renders transduced T cells sensitive to the cytotoxic effects of gancyclovir. HSV-tk-transduced T cells have been administrated to over 20 bone marrow recipients so far [26, 27]. Six of them developed GVHD, which was effectively controlled by gancyclovir-induced elimination of the transduced T cells in four patients. One drawback of this tk suicide gene is that it is virus derived, and therefore, CTL-mediated immune responses against the genetically modified cells can lead to selective elimination of these cells [28, 29]. New nonimmunogenic suicide genes based on an inducible Fas receptor are now being developed, and the first in vitro data show that this a promising alternative approach [28]. An additional concern is that the activation required ex vivo for retroviral transduction can downregulate EBV-specific T-cell function [30].

A second strategy to circumvent alloreactivity and to provide sufficient numbers of tumor-specific T cells is to selectively expand donor-derived EBV-specific CTLs ex vivo. First, donor lymphocytes are infected with a laboratory strain of EBV to initiate an EBV-transformed LCL that can be used as antigen-presenting cells of viral antigens. Then, irradiated LCLs are used to stimulate peripheral blood mononuclear cells (PBMCs) and expand EBV-specific CTLs (Fig. 2). Our group has used donor-derived EBV-specific T-cell lines as prophylaxis for EBV-induced lymphoma in over 60 patients post HSCT. None of the patients treated with this approach developed PTLD, compared with an incidence of 11.5% in a historical nontreated control group. Gene marking of donor CTLs allowed us to show persistence of infused CTLs for as long as 7 years [31, 32]. Furthermore, high EBV genome loads that existed prior to CTL administration rapidly decreased to normal levels with an increase of EBV-specific cytotoxicity [33]. More recently, another group treated six T-cell-depleted allogeneic bone marrow transplant recipients prophylactically with EBV-specific CTLs. One patient, who received a T-cell line lacking a major EBV-specific component, progressed to fatal EBV-positive lymphoma. However, in the other five patients, treatment resulted in reduction of the viral load,
thereby confirming the efficacy of this approach [34].

Immunotherapy using EBV CTLs was also used to treat three patients with overt lymphoma. Two of them were successfully treated, and gene-marked CTLs were shown to accumulate at sites of disease. One of the responders required temporary mechanical ventilation due to airway compromise from the inflammatory response at the disease site. A third patient who received CTLs as treatment died with progressive disease 24 days after infusion [35]. In this patient, the cytolytic activity of the generated CTL line was directed mainly against two HLA-11-restricted epitopes in the EBNA3b gene. However, after CTL infusion, only virus with an EBNA3b deletion could be detected, allowing the tumor to evade the immune response [35].

LYMPHOPROLIFERATIVE DISEASE POST-SOLID ORGAN TRANSPLANTATION (SOT)

Pathogenesis

In SOT recipients, the severe impairment of T-cell function as a result of the required immunosuppressive regimen post transplantation places these patients at risk for the development of PTLD. As with HSCT recipients, the highest incidence of PTLD is in the first 6-12 months after transplant. However in SOT recipients, PTLD can still develop several years after transplant, since the majority of these patients receive life-long immunosuppression. Early-onset PTLD is invariably associated with EBV, whereas the late-onset form is probably multifactorial, with a significant number of patients being EBV negative. These late-onset lymphomas have a poor prognosis and require aggressive therapy. Whereas PTLD is usually donor derived in the HSCT setting, in SOT recipients the tumor arises from recipient lymphocytes. The source of the virus is usually donor B cells within the graft, but blood transfusion transmissions have also been described [36].

Incidence and Risk Factors

Prolonged and profound immunosuppression and occurrence of primary infection after transplant are the two major
risk factors for the development of PTLD in SOT recipients (Table 1). The type and degree of immunosuppression varies with the type of organ transplanted. While the overall incidence is estimated at 1%-15%, PTLD is more common after lung and small bowel transplants than after renal, heart, and liver transplants because of the more intense suppression required and the lymphoid tissue present in the graft [37-40]. Up to 50% of EBV-naïve individuals receiving a transplant from an EBV-seropositive donor experience a symptomatic primary infection, which is then frequently followed by PTLD. The immunosuppressive environment during primary immune response may prevent the development of a fully protective immune response to EBV and is therefore thought to be the cause of this increased risk. Since a significant number of young children are EBV seronegative at the time of transplant, this risk factor accounts for the higher incidence of PTLD within pediatric transplant recipients (7.3%) as compared with adults (2%) [41].

TREATMENT

Withdrawal of Immunosuppression and Surgery

Reduction or withdrawal of immunosuppression is often used as first-line treatment in SOT recipients when PTLD is suspected or diagnosed. While a decrease in the level of immunosuppression can allow recovery of immunocompetence resulting in regression of localized or polyclonal disease, graft toxicity or even rejection may occur as a consequence. Therefore, particular caution must be taken for recipients of heart, lung, or liver transplant for whom rejection is fatal. In patients in whom PTLD presents as a local lesion, surgical resection or irradiation can be considered.

Chemotherapy

Chemotherapy has commonly been the treatment of choice for aggressive disease [42]; however, results are conflicting. Complete remissions are reported, but in general, survival of patients treated with chemotherapy is poor because of the severe toxic effects, especially for the organ graft, and increased susceptibility to life-threatening episodes of infection [43]. Since low-dose chemotherapy regimens have proven to be well tolerated in children in terms of treatment-related toxicity and infections, reduced-dose regimens may be the preferred option [44].

Antiviral Therapy

Since antiviral agents such as acyclovir or gancyclovir are only capable of interfering with lytic replication of the virus and cannot inhibit proliferation of latently infected cells, no therapeutic effect of these drugs is expected on established PTLD. However, antiviral agents may reduce symptomatic primary infection in EBV-seronegative transplant recipients when treatment is initiated immediately after EBV seroconversion, as detected by monitoring of EBV viral load. Such preemptive treatment resulted in a decrease in the incidence of PTLD from 40% to 10% among seronegative SOT recipients [45].

IMMUNOTHERAPY APPROACHES

Interferon-α and Interleukin (IL)-6

Interferon-γ can potentially enhance CTL activity against the tumor by upregulating the expression of major histocompatibility complex (MHC) class I and II on the tumor [46] and by activating a subset of natural killer cells [47]. The efficacy of interferon-α as treatment for PTLD has been studied in a limited number of patients in whom complete tumor responses were induced in 40%-50% of patients treated [48]. Side effects including neutropenia and flu-like symptoms were common [49], and episodes of acute graft rejection occurred in approximately 30% of patients [48]. Recently, IL-6 has been investigated as target for treatment of PTLD, since IL-6 plays an important role in the proliferation and maturation of virus-infected B cells. In vivo blocking of IL-6 with a murine anti-IL-6 monoclonal antibody resulted in complete remission in five patients and partial remission in 3 of 12 SOT patients with PTLD refractory to the reduction of immunosuppression [50]. Four patients did not respond and one patient relapsed after initial remission. The treatment was well tolerated and although preliminary, the experience with anti-IL-6 is encouraging.

Anti-B-Cell Monoclonal Antibodies

Initial results of treatment with murine anti-CD21 and anti-CD24 in SOT recipients were promising; complete responses were obtained in 64% of 31 patients, with a long-term survival of 55% [20, 21, 51]. More recently, several reports have described experience with rituximab as treatment for pediatric SOT recipients. A complete remission after rituximab treatment in combination with reduction of immunosuppression is described in one bilateral lung transplant recipient [52] and two out of three small bowel transplant recipients [53]. In addition, six pediatric liver transplant recipients were treated with 3-4 doses of rituximab after withdrawal of tacrolimus or cyclosporine therapy. In all patients, rituximab treatment was associated with decreased EBV load and disappearance of tumoral masses [54]. However, despite rituximab therapy, one patient was diagnosed subsequently with a cerebral tumor. Moreover, five patients experienced acute liver graft rejection episodes that were consolidated by reintroduction of immunosuppression in two patients but
were fatal in three other children. These results indicate, once more, the risk of withdrawal of immunosuppression, and since this type of treatment was combined with rituximab in these studies, the additional effect of anti-CD20 on response and outcome is difficult to interpret.

Adoptive Immunotherapy

In SOT recipients, reduction of immunosuppression allows for restoration of cellular immunocompetence, including EBV-specific cellular immunity in about 50% of cases. However, since T cells recognizing alloantigen may also recover, with risk for graft rejection, selective restoration of EBV-specific cellular immune responses should allow for safer treatment of disease. Therefore, the concept of adoptive transfer of EBV-specific CTLs that has been successful in HSCT recipients was extended to the SOT setting. However, since PTLD in the SOT setting is generally of host origin, autologous CTLs are required to target the EBV-expressing tumor cells. These autologous EBV-specific CTLs can be generated from PB of SOT recipients collected before transplantation [55]; if this approach is also feasible for patients receiving immunosuppression is an important question to be asked.

Indeed, several groups have shown that EBV-specific CTLs can also be expanded from SOT recipients who are being treated with immunosuppression [56-58]. So far, the results of 10 SOT patients treated with CTL therapy have been published. In eight patients with high viral load, infusion of EBV-specific CTLs resulted in a normalization of EBV DNA viral load [55, 58], and in all patients treated, an increase in EBV-specific CTL-precursor frequency was detected. In addition, Khanna et al. treated one patient with established PTLD with multiple infusions of ex vivo expanded autologous EBV-specific CTLs. PTLD regressed and the frequency of EBV-specific precursors increased, without signs of graft rejection [56]. However, the patient developed a secondary PTLD and died despite further CTL infusions. Thus, initial concerns that EBV-specific CTLs might have alloreactivity and cause graft rejection have been allayed, and the results obtained so far confirm that EBV-specific immunity can at least be temporarily restored in these patients. However, the persistence of CTLs, so far reported to be up to 4 months, requires further investigation. Furthermore, a significant number of the CTL lines generated from PBMCs of SOT patients consist mainly of CD4+ T cells [57, 58], and the protective and therapeutic effects of CD4+ T cells require further evaluation.

Particularly within the pediatric population, the application of CTL therapy is limited by the failure of standard techniques to generate CTLs from EBV-seronegative recipients. Generation of EBV-specific CTLs after seroconversion can circumvent this problem [56]. However, since rapid intervention after diagnosis of PTLD is often necessary, this approach may not allow enough time to expand the required number of CTLs. Though preliminary, successful CTL generation from naïve individuals has been reported after addition of IL-12 to cell culture or by selectively expanding antigen-specific CTL precursors after in vitro exposure to EBV-infected cells [59, 60].

As an alternative, the use of CTLs generated from healthy HLA-matched donors has been proposed. Of the nine patients who have been treated using this approach in combination with reduction of immunosuppression, complete remission was induced in four patients and a partial response was seen in one patient [55, 61, 62]. However, this approach will likely be limited by the short-term persistence of the allogeneic cells. In addition, administration of allogeneic CTLs may pose a risk of the induction of GVHD and graft rejection. In one lung transplant patient with significant lung rejection prior to T-cell therapy, grade 2 graft rejection responding to methylprednisolone was reported after treatment [61]. However, cytotoxicity to recipient and donor phytohemagglutinin (PHA) blasts can be used to screen for CTL alloreactivity before infusion. None of the patients treated with CTL lines with less than 10% cytolytic activity against patient PHA blast developed GVHD or enhanced graft rejection [62].

Finally, vaccination of EBV-seronegative recipients before transplantation could potentially simplify the expansion of EBV-specific CTLs and might reduce the risk of PTLD. Vaccination with a recombinant vaccinia virus expressing an EBV glycoprotein was not protective for primary EBV infection [63] but could reduce the viremia and symptoms associated with primary infection after SOT. Further research on the potential of dendritic cells pulsed with LCL lysates [64], irradiated LCLs, or EBV peptides as EBV vaccine is required in an attempt to prevent PTLD in high-risk seronegative patients.

Anti-B-Cell Antibodies versus EBV-Specific CTLs

Both rituximab and the adoptive transfer of EBV-specific CTLs are promising strategies to treat PTLD after both HSCT and SOT. However, both types of treatment have advantages and drawbacks, which might play an important role when weighing the expected benefit of treatment against the risk of adverse effects (Table 2). First, the profound B-cell depletion induced by treatment with rituximab may further exacerbate immunodeficiency in transplant patients [65]. Second, in tumors with heterogeneous expression of CD20, rituximab may cause selection of a CD20+ population of proliferating B cells, as has been reported in a few patients with lymphoma [66]. Third, it is unclear if the recovery of EBV-specific immunity will be delayed during anti-CD20 treatment as a
result of lack of exposure of the developing T-cell compartment to EBV-infected B cells. Although this problem has not yet been reported, it might result in development of PTLD later in the post-transplant course or primary infection in the future.

Elimination of the mature B-cell compartment does not seem to completely eliminate EBV, based on our observation that virus DNA reappeared with B-cell recovery in three of our four patients treated with rituximab. Although rituximab can cross the blood-brain barrier and antitumor responses in the eye and the CNS have been reported, local antibody concentrations might be too low to result in a complete response [67, 68]. In HSCT recipients, elimination of the B-cell reservoir for about 6 months is likely to allow enough time for the immune system to reconstitute. In contrast, in SOT recipients, no improvement of the immune response can be expected since immunosuppressive drugs are indicated for life. In those patients, recovery of B cells after anti-CD20 treatment can be accompanied by an increase in EBV load, placing these patients once more into the high-risk category. However, initial treatment with rituximab does allow the extra time required to generate EBV-specific CTLs for these patients.

Adoptive transfer of EBV-specific CTLs is a highly tumor-specific treatment, and therefore, side effects are expected to be limited. However, bulky disease and/or diffuse organ infiltration infusion of CTLs may cause morbidity to inflammation at the tumor site [32]. Therefore, this strategy appears to be safer and most effective when used as prophylaxis or for treatment of non-bulky disease. A drawback of the CTL approach is the time, generally 2-3 months, and the facilities required for generation of EBV-specific CTL lines in contrast to rituximab, which is readily available. Therefore, predictive parameters that can be used to select patients who might benefit from CTL therapy and allow early initiation of CTL lines will improve the efficiency of this strategy.

Risk Assessment in Transplant Recipients

Patients at risk of developing PTLD can be identified using known risk factors such as type of transplant, EBV serology, immunodeficiency syndromes, and immunosuppression regimens. However, in particular for the high-risk group, sensitive indicators of EBV reactivation are desirable so that appropriate therapy can be initiated at an early stage of disease and progression to overt clinical disease can be prevented. PCR-based assays that measure viral load in PB or serum are powerful aids to monitor EBV reactivation. EBV viral load in PBMCs of patients post-HSCT is strongly correlated to the histopathological diagnosis and, more importantly, often precedes the clinical diagnosis [69-71]. For example, in a group of 26 pediatric T-cell-depleted allogeneic stem cell recipients, at least 2 weeks prior to clinical diagnosis a rapid increase in EBV viral load indicated developing PTLD (sensitivity 100%, specificity 95%) [72]. However, some patients have normal EBV levels prior to diagnosis, while others with elevated levels do not develop PTLD [70]. In addition, the specificity of this type of monitoring varies between patient groups (nonmanipulated versus T-cell-depleted graft [73]) and applied PCR techniques. In SOT recipients, low positive predictive values of EBV DNA measurements are reported. While a low virus load identifies patients at low risk, only in a minority of patients is a high virus load followed by PTLD [74, 75].

Despite these concerns, EBV DNA measured in serum has been used to select recipients of a T-cell-depleted SCT for preemptive treatment with a single infusion of rituximab. Of 49 patients monitored in this study, EBV reactivation was detected in 17 patients, 15 of whom were treated with rituximab. Fourteen patients experienced clearance of EBV DNA from plasma. The remaining patient progressed to PTLD, which resolved after a second infusion with rituximab and

| Table 2. Advantages and disadvantages of anti-B-cell therapy and adoptive transfer of EBV-specific CTLs |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **Advantages** | **Disadvantages** |
| Anti-B-cell antibodies | • Readily available | • Exacerbation of immunodeficiency during profound B-cell depletion |
| | | • Selection of CD20+ population |
| | | • B-cell depletion and thus therapeutic effect lasts about 6 months |
| | | • Concentration in brain might be below therapeutic level |
| Adoptive transfer of EBV-specific CTLs | • Specific for EBV infected cells | • Available in a limited number of centers |
| | | • Labor intensive |
| | | • Time required to generate sufficient cell numbers |
| | | • Can cause severe side effects in cases of bulky disease inflammation at the tumor site |
| | | • Escape mutants of tumor cells |
| | | • Persistence in presence of continuing immunosuppressive treatment post SOT unknown |

• Well tolerated when used as prophylaxis or for treatment of non-bulky disease
donor lymphocyte infusion. Overall, this preemptive treatment reduced the incidence of PTLD to 18% as compared with 49% in a historical control cohort with the same risk profile [76]. These promising results show the high therapeutic potential of rituximab when used as first-line treatment and indicate the need to study the predictive value of EBV viral load in a prospective study within pediatric transplant recipients.

In addition to risk evaluation, early markers of response to therapy are needed in order to select and adjust treatment with minimal delay. EBV DNA levels in plasma were monitored in a group of 14 patients that developed LPD after T-cell-depleted allogeneic SCT from the time of diagnosis and was correlated with clinical response. A rapid decline in the plasma EBV DNA levels was observed in the seven patients who clinically responded to therapy consisting of discontinued immunosuppression, along with other modalities including acyclovir, chemotherapy, anti-CD20, and donor lymphocyte infusions. In contrast, nonresponders showed an increase in EBV DNA levels [77]. Similarly, Lankester et al. reported a decrease in EBV load of at least 1 log of magnitude in the first week of treatment in pediatric HSCT patients [72]. In the SOT group, measurements of EBV DNA load have been used as an indicator to adjust the degree of immunosuppression and evaluate the effect of treatment [75]. A decrease in viral load is generally observed after intervention with chemotherapy, rituximab, or EBV-specific CTL in SOT recipients. However, the decline in EBV DNA after administration of rituximab is not always accompanied by resolution of disease, which suggests that PB leukocytes may be more sensitive to rituximab than PTLD cells in tissue [78].

Thus, while virus load is generally a sensitive measure of risk and response, this test only indirectly reflects the impairment of the host’s immune status and is not specific enough to initiate treatments with associated toxicities. We hypothesize that EBV-specific immune response analysis could provide increased specificity. Detection of interferon-γ release by patient PBMCs after incubation with autologous LCLs, using intracellular cytokine staining [79] or the more sensitive enzyme-linked immunospot technique, might be a useful alternative to indicate T-cell reconstitution [80]. Since early polyclonal disease is more likely to respond to treatment than aggressive monoclonal lymphoma, attempts to improve the methods for screening of patients at risk for PTLD are essential to begin therapy as early as possible in order to reduce morbidity and mortality.

**HODGKIN’S DISEASE**

**Incidence and Current Treatment**

Hodgkin’s disease has an annual incidence of 7 cases per 1,000,000 children in the U.S. [81]. In up to 50% of cases, HD tumors are associated with expression of EBV-derived antigens in malignant Reed-Sternberg cells. EBV association is more common in the patients from Hispanic origin as compared with Caucasians (odds ratio = 4.1) [82, 83]. Early-stage HD has an excellent prognosis, with a disease-free survival rate of 80%-90% [84]. In advanced disease, 60%-85% can be expected to be cured with conventional chemotherapy and/or radiotherapy [85-88]. However, of the 10%-15% of patients who relapse after initial combined-modality therapy, only 50% will enter a second remission. Similarly, the prognosis is poor for patients who fail salvage chemotherapy or who relapse a second time. In addition, long-term follow-up studies of HD survivors show increased risk of treatment-related morbidity and mortality such as second malignancy for up to 20 years beyond diagnosis [89, 90]. Nonfatal sequelae of therapy, such as altered somatic growth, infertility, and restrictive lung disease, can seriously affect the quality of life of survivors [91]. It is therefore desirable to develop novel therapies that could improve disease-free survival in relapsed/refractory patients and might ultimately reduce the incidence of long-term treatment-related complications in all patients.

**Anti-CD25 and Anti-CD30 Antibodies**

The human lymphocyte activation markers CD25 and CD30 are present on nearly all HD Reed-Sternberg cells and on a small subset of activated lymphocytes and, therefore, represent attractive targets for selective immunotherapy [92]. Monoclonal antibodies against CD25 and CD30 can be chemically linked to an active toxin such as *Pseudomonas* endotoxin A or deglycosylated ricin A. The efficacy of anti-CD25 immunotoxin has been studied in 15 advanced-stage HD patients. Clinical responses included two partial remissions, one minor response, three with stable disease, and nine with progressive disease [93]. Side effects were related to vascular leak syndrome and were tolerable at doses of 15 mg/m². Although, anti-CD30 immunotoxin has been shown to have in vivo antitumor activity in a severe combined immunodeficient mouse model [94], in a phase I/II clinical trial, only 4 of 17 relapsed HD patients responded to this type of treatment. Moreover, this type of immunotoxin was less well tolerated [95]. A modification of this approach involves the use of bispecific antibodies that simultaneously bind to CD30 on the tumor cell and CD16 on natural killer cells to induce tumor-directed cytotoxicity. In a phase I/II clinical trial, 16 heavily treated HD patients with refractory disease have been treated with anti-CD16/CD30 with or without prestimulation with IL-2. This resulted in one complete remission, three partial remissions, and four cases of stable disease. Mild toxic effects including fever in six patients accompanied treatment. Coadministration of IL-2 seemed to augment the antitumor
activity of this bispecific antibody by recruiting natural killer cells [96]. The major obstacle for broader study of efficacy of this approach is the immune response against murine antibodies and the toxin component. Therefore, although anti-CD25 and anti-CD30 are promising therapeutic strategies, further research is required to generate humanized antibodies and reduce the side effects of the linked toxin.

Adoptive Transfer of EBV-Specific CTLs

Having shown that the EBV-positive cells in PTLD, which express a wide range of EBV-encoded antigens, are susceptible to immunotherapy, our group is now evaluating if the malignant cells of HD, which express a more restricted pattern of antigens, are also targets for this approach. In a phase I dose escalation study, we are evaluating the use of autologous EBV-specific CTLs for patients with EBV-positive HD [97]. We have treated eight patients with relapsed HD with two infusions (2 \times 10^7/m^2 – 1.2 \times 10^6/m^2) of EBV-specific CTLs. In seven of these patients, the CTLs were retrospectively marked. One patient progressed rapidly and came off study within 1 week. Another patient had erosion of tumor through the left upper lobe bronchus and died 2 months after CTL infusion. In situ PCR for the retroviral integrant revealed gene-marked CTLs in part of the tumor but not at the site of the left upper lobe bronchus erosion. Four patients with aggressive disease at the time of CTL infusion survived 10-18 months and one patient is alive 28 months after infusion. Gene-marked CTLs were detected in the PB up to 9 months following infusion [98]. In addition, five patients with minimal residual disease post autologous bone marrow transplant for relapsed HD have been treated with nonmarked autologous EBV-specific T cells (4 \times 10^7/m^2 – 1.2 \times 10^6/m^2). One patient died of progressive disease and four patients remain well 9-23 months post CTL infusion [91].

Although these results are promising, the antitumor responses have been transient and no patient with aggressive relapsed HD has been cured. This may be due to a lack of specificity of the EBV-specific CTL for the immunosubdominant LMP1 and LMP2 antigens present on the HD tumor. In addition, the tumor produces inhibitory factors such as tumor growth factor (TGF)-\(\beta\), thymus and activation-regulated chemokine, IL-10, and IL-13, which affect CTL and antigen-presenting cell activity [99-102]. This cytokine is secreted by the most devastating effects on CTL proliferation and function [105, 106]. This cytokine is secreted by a wide variety of childhood tumors and is a powerful mechanism by which the tumor cells can escape the immune response [107]. To overcome this inhibition, CTLs from four normal donors and four patients with relapsed EBV-positive HD were transduced with a retrovirus vector expressing the dominant-negative TGF-\(\beta\) type II receptor (DNR). Cytotoxicity, proliferation, and cytokine release assays were performed to compare the effects of exogenous TGF-\(\beta\) on DNR-transduced and GFP-transduced CTLs. Unlike untransduced or GFP-transduced CTLs, DNR-transduced CTLs were resistant to the antiproliferative effects of recombinant TGF-\(\beta\). In addition, while transduced-CTLs were protected from the negative effects of TGF-\(\beta\), long-term expression of this construct had no deleterious effects on the function, phenotype, or growth characteristics of the transduced CTL lines [108]. We therefore concluded that CTLs expressing the DNR may have a selective advantage in vivo in patients with TGF-\(\beta\)-secreting tumors such as HD, and after safety testing in an animal model, we plan to assess the use of TGF-\(\beta\)-resistant LMP2 CTLs in a clinical trial.

NASOPHARYNGEAL CARCINOMA

Incidence and Current Treatment

Nasopharyngeal carcinoma is a rare malignancy in most populations, with an annual incidence of nearly 1 case per 100,000 children (<21 years) [109]. However, incidence rates are very high in populations in southern China and moderate in Greenland and northern Africa as a result of environmental factors such as the consumption of salted fish. In addition, nonkeratinizing NPCs are uniformly associated with EBV, implicating EBV as a causative factor in its etiology [110]. Despite the good overall survival rates, particularly in children, current NPC treatment regimens including radiotherapy and chemotherapy are still far from ideal. Follow-up reports have shown increased risks for treatment-related morbidity and mortality. Late medical complications after treatment for NPC include growth hormone deficiency, hypothyroidism, pulmonary fibrosis, and secondary malignancies [111, 112]. It is therefore desirable to develop novel therapies that could improve disease-free survival in relapsed/refractory patients and might ultimately reduce the incidence of long-term treatment-related complications in all patients.

Adoptive Transfer of EBV-Specific CTLs

Chua et al. treated four patients with advanced NPC with 5 \times 10^7 – 3 \times 10^6 autologous EBV CTLs [113]. Although it was difficult to confirm improved tumor control in these
patients who had significantly bulky disease, the treatment was safe and elevations in CTL precursor frequency were seen. In three patients, host surveillance of EBV replication was restored, resulting in a reduction in the plasma EBV burden. Taken together, these results provide a rationale to further explore EBV as a target for immunotherapy of NPC, and we are currently generating EBV-specific CTLs as treatment for refractory or relapsed NPC confirmed to be EBV positive on immunohistochemistry.

**Burkitt’s Lymphoma**

Burkitt’s lymphoma is a small noncleaved-cell lymphoma with significant geographic differences in distribution and incidence. It represents approximately 50% of childhood cancers in equatorial Africa, with an incidence of approximately 100 per 1 million children under 15 years of age, which is in strong contrast to an incidence of 2 per million under the age of 15 years in North America [114]. Over 90% of Burkitt’s lymphomas in Africans are associated with EBV, compared with only about 20% in the U.S. [115]. The current treatment of Burkitt’s lymphoma consists of aggressive chemotherapeutic programs. Since Burkitt’s lymphoma cells evade the immune system by downregulating the expression of EBV latency antigens, cell adhesion molecules, and MHC class I molecules, EBV-specific CTL therapy for Burkitt’s lymphoma is problematic. Recent reports show that although EBNA1 is not presented to HLA class I-restricted CD8+ CTLs due to blocking of its processing by an internal glycine-alanine repeat [116], EBNA1-specific CD4+ CTLs can be generated [117, 118]. Further exploration of the role of CD4+ CTL in the control of Burkitt’s lymphoma might open new possibilities for immunotherapy approaches for this tumor. In addition, upregulation of the MHC class I presentation pathway in Burkitt’s lymphoma cells may provide an alternative strategy for targeting these tumor cells with immunotherapy.

**EBV-Associated Malignancies in HIV Patients**

Children infected with HIV have an increased risk of developing HD (RR = 8) or non-Hodgkin’s lymphoma (NHL) (RR > 100), including systemic, primary CNS, and body cavity lymphomas [119]. Because HIV infection and the occurrence of a malignancy can be associated with similar symptoms, it is not uncommon that the diagnosis of such a tumor is delayed in a child with HIV. On the other hand, a malignancy can be the first symptom of HIV infection [120]. Based on histology, the EBV association of HIV NHL ranges from 30% in systemic HIV-related Burkitt’s lymphoma to 70%-80% in HIV-related immunoblastic lymphoma to almost 100% of primary CNS lymphomas [121, 122]. In systemic HIV NHL, the risk of CNS involvement is significantly increased when the tumor is EBV positive and can be preceded by detection of EBV DNA in the cerebrospinal fluid [123].

The lower numbers of CD4+ T cells in HIV patients are shown to account for the loss of function of CD8+ EBV-specific T cells in patients with acquired immune deficiency syndrome (AIDS)-related NHL [124]. An increase in CD4+ cells induced by highly active antiretroviral therapy (HAART) was shown to improve the antigen responsiveness of both HIV- and EBV-specific T cells in NHL patients [125]. However, the incidence of HIV NHL has not declined after the introduction of HAART. In contrast, LPD resolves in most cases after restoration of cellular immunity following HAART. Thus, in HIV NHL patients, more active strategies to boost both the CD4+ and CD8+ EBV-specific T-cell response might be required. Within this patient group, monitoring of EBV DNA might help to identify patients at risk for the development of HIV LPD. These patients may benefit from adoptive transfer of EBV-specific CTLs generated from PBMCs that were obtained early in the clinical course before CD4 cells were affected by disease or when disease is controlled by HAART.

In addition, the risk of HIV NHL can potentially be reduced by long-term antitherapy with acyclovir, gancyclovir, or foscarnet [126]. Moreover, partial and complete responses have been observed with anti-herpes virus therapy for established HIV NHL [127, 128]. Recently, treatment with low-dose hydroxyurea that is capable of removing extra chromosomal DNA elements of tumor cells, thereby disrupting their drug-resistant or growth-transformed phenotype, has been shown effective in two AIDS patients with primary CNS lymphoma [129]. Thus, a combination of treatment strategies that target EBV is potentially effective as prevention or treatment of EBV-positive tumors in HIV patients.

**Conclusions**

The introduction of immunotherapeutic strategies including anti-B-cell antibodies and adoptive transfer of EBV-specific CTLs is expected to improve the outcome of PTLD among pediatric HSCT and SOT recipients. In HSCT, disruption of the B-cell compartment for approximately 6 months as induced by rituximab is likely to allow for T-cell recovery and thereby immunosurveillance against EBV. In the SOT recipients, this same treatment will allow for the time required to generate sufficient numbers of EBV-specific CTLs ex vivo, which can then be used in case PTLD develops after reoccurrence of EBV-infected B cells. However, although the first results are promising, the experience with rituximab in the pediatric population is limited, and thus further evaluation is
required to demonstrate safety and to study the implications of potential side effects such as the recovery of EBV-specific immunity in the absence of EBV-infected cells. So far, a number of transplant recipients with an elevated EBV viral load but without tumor involvement have been treated successfully, indicating that the beneficial effect of immunotherapy can be enhanced when used preemptively. A remaining challenge is to define the indications for intervention for patients at risk for LPD but without active disease. Monitoring EBV viral load can be used for this purpose, but to improve predictive value, additional parameters that reflect the T-cell function are desirable. After encouraging results were obtained in the PTLD setting, the effect of CTL therapy on other EBV-associated tumors such as HD and NPC are now being evaluated. The first results in HD patients are promising but indicate that more potent strategies to stimulate and expand T cells with tumor-specific antigens are required. In addition, genetic modification of tumor-specific CTLs might be needed to overcome the immune evasion strategies employed by the tumor cells.

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