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Review Article

Strategies to harness immunity against infectious pathogens after haploidentical stem cell transplantation

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Abstract: Viral and fungal infections account for significant morbidity and mortality, particularly in pediatric patients with profound immune suppression resulting from allogeneic hematopoietic stem cell transplantation (HSCT). Therapies with anti-viral and anti-fungal drugs are often associated with significant toxicity, are of limited efficacy and can induce drug resistance. One innovative approach to prevent and/or treat viral and fungal infections involves the adoptive transfer of in vitro-expanded or in vitro-generated pathogen-specific T cells. This review summarizes the clinical trials that have been run to date with virus- and fungus-specific T cells, with special emphasis on the clinical context of haploidentical HSCT for pediatric malignancies. It will also discuss initiatives and strategies to overcome the hurdles associated with time-consuming and complex GMP-grade laboratory procedures required to generate pathogen-specific T cells.

Keywords: Cytomegalovirus, adenovirus, Epstein-Barr virus, Aspergillus, Candida, immune reconstitution, stem cell transplantation, adoptive cell therapy

Introduction

Hematopoietic stem cell transplantation (HSCT), either autologous or allogeneic, is a potentially corrective therapy for both malignant and non-malignant disorders of children and adults. In particular, allogeneic HSCT has been used for pediatric patients with acute lymphoblastic leukemia (ALL) in second or subsequent complete remission (CR) after marrow relapse, as well as in patients in first CR but with high-risk characteristics. However, HLA-identical sibling donors are not available for approximately 75% of the patients, and unrelated donors, matched at the allelic level, cannot be found in time for all patients who are in need of an allograft. For patients lacking a matched donor, transplantations using alternative donor sources, such as unrelated umbilical cord blood (UCB) or haploidentical stem cells, are increasingly invoked [1]. In adult patients with hematological malignancies who receive a transplant from an HLA-disparate relative, the infusion of a large number of extensively T cell–depleted $CD34^+$ cells ensures sustained engraftment of donor hematopoiesis and minimizes the risk of both acute and chronic graft-versus-host disease (GVHD) [2]. The feasibility of haploidentical HSCT was demonstrated also in children, in particular in patients with ALL lacking a HLA-identical sibling donor [3]. As the infusion of bone marrow cells from an HLA-haploidentical relative may be associated with a high incidence of graft failure, a megadose of granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells is required to overcome histocompatibility barriers in the donor-recipient pair and to elude residual anti-donor cytotoxic T lymphocyte (CTL)-precursor activity [3].

It has been recommended that haploidentical HSCT be reserved to highly specialized Centers who run specific programs for this type of allograft [4]. The reported probability of survival at 3-4 years after the allograft ranged from 18 to 48%, was influenced by many factors, the most important being the state of remission at the time of transplantation, and seemed to be poorer in children with myeloid leukemia [5]. Both transplant-related mortality (TRM), mainly attributable to infectious complications, and leukemia recurrence in patients with malignan-

cies may contribute to treatment failure. Current approaches of adoptive immunotherapy with pathogen specific T-cell lines/clones for the prevention and/or treatment of infectious complications are promising to improve post-transplant outcome and will be the specific focus of this review.

Immune reconstitution after HSCT

The kinetics of recovery of immune cell number and function after autologous HSCT and allogeneic HSCT from sibling donors has been the focus of previous studies [6-8] and will not be further discussed. Although haploidentical HSCT in children given a myeloablative conditioning regimen is feasible without significant GVHD or disease relapse, it translates into delayed immune recovery, with risk of severe and often fatal viral and fungal infections [9]. In general, the degree of post-transplant immune suppression is dictated by the degree of tissue mismatch between donor and recipient. The depletion of mature T cells from the G-CSF-mobilized grafts, either direct or indirect in the form of $CD34^+$ positive selection, is necessary for preventing the occurrence of GVHD in the context of great immune genetic disparity. This implies that recipients cannot benefit from the adoptive transfer of memory T lymphocytes that, through their peripheral expansion, are the main source of protection from infections in the first months after transplantation.

Alloreactive NK cells play a crucial role in preventing not only infectious complications but also disease recurrence. The graft-versus-leukemia (GVL) effect is provided primarily by NK cells, if killer immunoglobulin receptor (KIR) incompatibility in the graft-versus-host direction is present. KIR are in fact specific for allotypic determinants that are shared by different HLA class I alleles (referred to as KIR ligands). In the context of $CD34^+$ selection and myeloablation, the repertoire of NK cells expressing KIR is re-established after approximately 3 months from transplantation, whereas NK cells without KIR or with a skewed KIR repertoire are the predominant cell type during the first months post-transplant [10].

The state of profound immune deficiency in the recipients lasts for at least 4-6 months after haploidentical HSCT [11]. In addition, immunodominant virus-specific T cells in the graft may fail to recognize virus-infected cells of the host, if such T cells are restricted by an HLA allele not shared by the host. After this period of immune fragility, immune recovery in children given a haploidentical HSCT is substantially superimposable to that of patients given the allograft from other alternative donors.

Several pathogens may cause severe infections in immunocompromised patients. Viral infections, which are mainly controlled by T-cell immunity, are an important cause of morbidity and mortality in recipients of HSCT [6, 12]. A growing number of viral pathogens have been implicated in infectious complications after HSCT. This may result from more intensive screening with improved detection methods and from the extension of HSCT to higher-risk patients receiving manipulated products and/or requiring intense post-transplant immunosuppression [13]. Reactivation of latent viruses such as CMV, EBV, herpes simplex and herpes zoster commonly causes symptomatic disease. Herpes viruses such as CMV and EBV may cause pneumonia and post-transplantation lymphoproliferative disease (PTLD), respectively. In addition, respiratory viruses such as human adenovirus (HAdV), influenza and respiratory syncytial virus (RSV) have been reported to cause post-transplant infections. Emerging opportunistic pathogens include HAdV and polyomaviruses such as JCV or BKV. GMP-compatible antigens have been recently produced to select and expand pathogen-specific T cells. In addition, multimers and cytokine-capture methods have been used to positively select antigen-specific T cells. Patients receiving HSCT are also at high risk of life-threatening invasive fungal infections. Aspergillus, Cryptococcus or Candida may cause pneumonia or other signs and symptoms dependent on their tissue localization. Therapeutic approaches with granulocyte transfusions and IFN-γ have suggested some benefit for outcome [14, 15]. Preliminary results of transferring fungus-specific T-cell responses after HSCT are promising, as will be highlighted below.

Cell-based therapeutic approaches for viral infections

Pharmacologic agents can be considered standard therapy for post-transplant infections. However, most drugs have substantial toxicity and are ineffective against all viruses, driving the outgrowth of resistant viral variants. In addition,

anti-viral drugs have no impact on antiviral immunity, and infections often recur after drug discontinuation. These considerations have prompted the development of adoptive immunotherapy for disseminated infections, which is based on the premise that recovery of virus-specific T cells is a prerequisite to obtain protection from infection. Importantly, adoptive immunotherapy to reconstitute antiviral immunity is a non-toxic strategy, both immediately and in the long-term. Both unmanipulated T cells and in vitro-generated, virus-specific T cells have been tested in clinical trials to prevent and/or treat viral infections [12].

Unmanipulated donor lymphocytes

To date, donor lymphocyte infusions (DLI) have been given to provide anti-tumor and, to a lesser extent, antiviral immunity. Theoretically, DLI should contain T cells reactive against latent viruses, such as CMV and EBV, at particularly high frequency. Unselected DLI were initially used to treat PTLD [16]. In the first MSKCC study, 5 patients with PTLD received unirradiated donor leukocytes containing $1x10^6$ $CD3^+$ T cells/kg of recipient's body weight. No drug prophylaxis against GVHD was given. Of the 3 patients who survived and in whom the EBV-related disorder resolved, 2 had acute GVHD of the skin (grade II) [16]. An update published in 1997 reported the clinical results with 18/19 patients treated with single infusions of PBMC from their seropositive bone marrow donors [17]. Complete clinical and/or pathological resolution of the EBV lymphomas was observed in 16 patients within 14-30 days. Three patients developed acute GVHD and 8 patients experienced chronic GVHD. No recurrence of the EBV lymphoma occurred and 10 of the 18 patients survived in sustained remission with no further treatment for 3 to 42 months since PBMC infusion [17]. Patients exhibited an accelerated recovery of $CD3^+$ T cells, beginning as early as 14-21 days post-infusion, associated with an increase of T-cell responses to TCR activation and to challenge with mitogens and antigens such as CMV [17]. Notably, the frequencies of CTL precursors (CTLp) specific for EBV increased over 10-fold within 2 weeks of receiving an infusion of donor PBMC. One of the limitations of this approach is that it may carry a significant risk of GVHD due to the high frequency of alloreactive T cells contained in the DLI. The high ratio of alloreactive to virus-specific T cells is particularly problematic in the haploidentical HSCT setting, where the higher incidence of GVHD lowers the tolerable DLI dose. Secondly, the efficacy of DLI may be limited by the very low frequency of T cells specific for common viral pathogens, such as HAdV, RSV and parainfluenza virus.

Depletion of alloreactive T cells

Protocols for the physical depletion of alloreactive T cells have been investigated as a means to prevent GVHD without compromising anti-pathogen immunity. For instance, donor T cells have been cultured with recipient-type APC to activate the alloreactive component, followed by treatment with antimetabolite drugs, photodepletion or immunotoxins and magnetic beads to target differentially-expressed surface molecules, such as CD25, CD69, CD134 and CD137 that are up-regulated on donor T cells in response to challenge with recipient cells (reviewed in ref. [18]). A 4,5-dibromorhodamine methyl ester (TH9402)-based photodepletion technique termed photodynamic purging has been recently developed with the aim at targeting activation-induced changes in P-glycoprotein that result in an altered efflux of the photosensitizer [19]. The experimental conditions required to achieve optimal T-cell allodepletion have been recently published [20]. This methodology allows for a remarkable reduction of alloantigen-specific T cells, paralleled by modest decreases in the frequency of T cells specific for a wide array of viral and fungal pathogens [20]. Kiadis Pharma has recently commercialized ATIR™ ('Add-back of donor T cells to accelerate Immune Reconstitution'), a cell therapy product for mismatched HSCT that relies on the depletion of alloreactive T cells to reduce transplant-related mortality due to GVHD and infectious complications and that has been granted the Drug Orphan Designation by the FDA. A phase I clinical trial in 19 patients treated with haploidentical HSCT for very-high risk hematological malignancies has shown that DLI depleted of alloreactive T cells through this approach mediate both decrease of post-transplant infections and acceleration of immune recovery, without causing severe GVHD [21]. Increasing doses of donor cells were administered after photodynamic depletion, with a T-cell content ranging from $1x10^4$ to $5.0x10^6$ $CD3^+$ cells/kg. Whereas 7 patients in the low-T-cell cohort developed infectious complications, only 6 of the following

12 patients who received $3.2x10^5$ to $5.0x10^6$ allodepleted T cells developed infections, none resulting in a fatal event. The same approach has been used in 17 patients with high-risk hematological malignancies who received low-dose cyclosporine as sole immune suppression in the absence of GVHD [22]. Overall survival was 73% and relapse-free survival was 65%, with a relapse probability of 21% at a median follow-up of 385 days. Collectively, these studies suggest that photodynamic depletion of alloreactive T cells in the context of haploidentical HSCT translates into an enhanced graft-versus-malignancy effect and a reduced incidence of fatal infectious complications. However, photodynamic purging may be complicated by late non-relapse mortality associated with chronic GVHD and with infections, indicating that this strategy needs to be optimized [23]. An update available on the Company's website (www.kiadis.com; accessed July, 2011) reports no transplant-related mortality in a group of 10 high-risk leukemia patients treated with ATIR™, with an overall survival of 70%. This estimate favorably compares with outcomes of HSCT from fully-matched donors. ATIR™ is currently in a multinational, registration clinical study, with sites in Europe and Canada open for enrollment.

Alloreactive T cells have also been depleted ex vivo using a CD25 immunotoxin. The allodepleted T cells infused after haploidentical HSCT [24, 25] or HLA-matched sibling donor HSCT [26] produced reconstitution of antiviral immunity without causing GVHD, indicating that this strategy is feasible. A first clinical trial in 15 pediatric patients given either haploidentical HSCT or MUD transplants has shown that immunotoxin-treated PBMC retained < 1% alloreactivity and induced no severe GVHD when infused at $1\text{-}8x10^5$/kg, between days 15 and 47 after transplant [25]. In the 3 patients with active CMV infection at time of HSCT, a remarkable peripheral expansion of memory $CD3^+$ T cells was observed. In vitro, patients' PBMC proliferated to CMV and displayed strong cytolitic activity against CMV-infected target cells [25]. Other infections reported in this patient cohort included HAdV infection in 1 patient and diffuse EBV-related PTLD in 1 patient (both of which resolved after the infusion of allodepleted T cells), transient EBV re-activation in 1 patient and pulmonary aspergillosis in 2 patients, one of whom died of infection together with relapse of acute myeloid leukemia [25]. Another study explored the efficacy of 2 dose levels of immunotoxin-depleted T cells (10^4 cells/kg/dose and 10^5 cells/kg/dose) after haploidentical HSCT [24]. Patients assigned to the higher cell dose had improved T-cell recovery as a result of preferential expansion of memory T cells derived from the infused, allodepleted cells. In 4 of 6 evaluable patients allocated to the higher cell dose, CMV-specific and EBV-specific responses were detected early (2 to 4 months) after HSCT, whereas such responses were not documented in patients receiving the lower cell doses, even at late tempos after transplantation and despite the fact that some of these patients had viral re-activations [24]. Interestingly, in 2 cases tetramer-positive cells were detected in the patients that were not detected in the donors, suggesting that both the expansion of pre-existing CMV-specific T cells and the emergence of de novo CMV-specific T cells occurred after the adoptive transfer of immunotoxin-depleted cells. However, only 5 of 16 patients survived at a median follow-up of 33 months, leading the authors to conclude that any advantage offered by this approach in terms of accelerated recovery of immune responses to infections may be offset by the high incidence of leukemia relapse [24].

Antigen-specific T cells for CMV infection

CMV is a persistent β-herpesvirus causing an asymptomatic infection in immunocompetent individuals. CMV is frequently re-activated from recipient or host tissues after allogeneic HSCT. The manifestations of CMV reactivation in transplant recipients may include interstitial pneumonitis, gastroenteritis, fever, hepatitis, encephalitis and retinitis. CMV infection is controlled by T-cell immunity after both primary infection and virus reactivation. Although ganciclovir and foscarnet, the currently used drugs for CMV infection, can reduce mortality and prevent early CMV disease in combination with intravenous immunoglobulins, they have significant side effects, including neutropenia and nephrotoxicity.

CMV-specific T-cell clones have been successfully generated in culture and then transferred to 18 recipients of allogeneic bone marrow from HLA-matched sibling donors [27]. Patient PBMC were cultured with autologous skin fibroblasts

infected with the AD169 strain of CMV. CD8+ CTL clones were then isolated after depletion of the CD4+ T cells with an anti-CD4 monoclonal antibody [27]. The CMV-specific clones were injected in 4 escalating doses, each given one week apart starting more than 30 days after transplantation. This approach was safe and led to the early appearance of CMV-specific T cells in patients who lacked CTL responses against CMV post-transplantation [27]. The expanded cells persisted in vivo for at least 8 weeks but progressively declined in patients who failed to develop concomitant endogenous CMV-specific CD4+ T-cell responses. All the patients maintained CTL responses specific for CMV for at least 8 weeks after the completion of T-cell therapy. In another clinical trial, polyclonal CMV-specific CTL containing both CD4+ and CD8+ T cells were administered at 10^7 cells/m^2 in patients with CMV viremia, leading to significant reduction of the viral load in the 7 evaluable cases [28]. This effect was sustained in 5 patients and transient in the remaining 2 patients who had the highest viral load. An increase in CMV-specific CD4+ T cells was demonstrated in 5 of 7 patients. Clinical trials were also conducted with CTL lines generated with other CMV antigen sources. For instance, inactivated CMV antigens produced from human fibroblasts infected with human CMV can be used to stimulate PBMC from allogeneic donors. The CTL generated with this strategy were infused in 16 patients after the first episode of CMV viremia, proving to be safe and effective without causing GVHD [29]. CMV-specific T cells have also been obtained after the stimulation of polyclonal CD4+ and CD8+ T cells with dendritic cells (DC) transduced with a chimerical adenoviral vector encoding the immunodominant CMV pp65 antigen. The CMV-reactive T cells were infused as prophylaxis of CMV infection in 12 adult patients treated with HSCT, with no infusion-related toxicity [30]. Although 4 patients reactivated CMV at low titer, antiviral therapy was not needed to achieve viral control [30].

CMV-specific CD4+ and CD8+ T cells for adoptive transfer can also be generated from seropositive donors from a single 500 ml blood donation in 10 days, using autologous cells and humoral components for T-cell expansion and the IFN-γ secretion assay for isolation [31]. CMV lysate, HLA-A*0201 binding peptides derived from the CMV pp65 and the CMV_{IE} antigen, HLA-B*0702-binding peptides derived from the CMV pp65 and exogenous IL-2 were used to generate functional CMV-specific T-cell lines, with the ability to lyse CMV-infected fibroblasts and with abolished alloreactivity against third-party DC despite the presence of IL-2 as a growth and survival factor for T cells [31]. It is known that CMV-positive recipients of stem cell grafts from CMV-negative donors are at particular risk to develop severe CMV-related disease, because the reconstitution of CMV-specific immunity is delayed. Intriguingly, CMV-specific reactivity can be conferred to primary T cells from CMV-negative donors through the transfer of CMV-specific T-cell receptors (TCR; [32]). The TCR-transgenic T cells can be expanded up to 672-fold in an antigen-specific manner. The TCR-transduced T cells release both IFN-γ and IL-2 after challenge with the pp65 antigen and exert cytotoxicity against pp65-positive targets [32].

Antigen-specific T cells for EBV infection and PTLD

EBV is a oncogenic γ1 herpesvirus that infects more than 95% of the world's population early in adolescence. Primary infection is usually asymptomatic and self-limited, and is followed by latent infection in B cells and productive viral replication (lytic cycle) both in B cells and in parietal gland ductal cells and nasopharyngeal epithelial cells [17]. The lytic cycle correlates with the expression of immediate early (IE), early (E) and late (L) lytic antigens and culminates in infectious virus production. The B cells can be immortalized by EBV through a complex set of molecular interactions, although spontaneously transformed B cells are detected at very low frequency among the circulating B cells of seropositive individuals throughout their lifetime [17]. Four types of viral latency have been described, with type 4 being found in the majority of circulating B cells of healthy individuals. In acutely T-cell-compromised patients, the uncontrolled growth of B cells expressing type 3 latency, which are highly susceptible to virus-specific T cells, may lead to the development of PTLD. Type 3 latency is characterized by the expression of latent antigens including EBV nuclear antigens (EBNA) 1, 2, 3, 4, and 6. EBNA-1 is the only EBV-latent protein universally expressed in PTLD. In the hierarchy of EBV-specific CD8+ T-cell immunity, EBNA3A, 3B and 3C proteins contain the most frequently recognized epitopes. EBNA-1 was initially viewed as an antigen capable of evading immune recognition.

Currently, EBNA-1 is considered an important target for $CD4^+$ and $CD8^+$ EBV-specific T-cell responses. Interestingly, EBNA-1-specific $CD8^+$ T cells can be generated in vitro both from patients with PTLD and from healthy controls [33]. In these assays, stimulation with 17-mer peptide pools derived from EBNA-1 and cytokine release were used as a read-out. The magnitude of CD8 responses was greater for HLA-$B35^+$ individuals compared with persons expressing other HLA alleles. HLA-B35 is known to preferentially bind atypically long $CD8^+$ T-cell target peptides. This has important implications for the choice of proper peptide pools for the stimulation of EBNA-1-specific $CD8^+$ T cells [33]. EBNA-1 also generated a relatively robust $CD4^+$ T-cell response that was greater in magnitude in controls than in patients with PTLD [33]. In this respect, $CD4^+$ T cells are required to inhibit tumor growth in EBV-induced PTLD both by direct mechanisms and indirectly by providing T-cell help to $CD8^+$ T cells.

As reviewed elsewhere [34], the risk factors for PTLD include myeloablative conditioning, UCB as the stem cell source, T-cell depletion with ATG for prophylaxis or treatment of GVHD, and haploidentical HSCT. Rituximab is a viable option for prophylaxis and treatment of PTLD, having led to response rates between 55% and 100%. However, rituximab produces depletion of normal B cells for more than 6 months, an undesired event in a patient population that is already immunosuppressed. The lesions of PTLD express the full spectrum of EBV latent proteins and contain a subpopulation of cells that switched into the lytic cycle. The lytic cycle-specific CD8 response is skewed towards IE and E proteins, whereas the CD4 response is widely spread across IE, E and L antigen targets [35]. A combined T-cell depletion (TCD) and B-cell depletion method that allows the retention of potentially alloreactive NK cells, while eliminating B cells to avoid PTLD, has been recently developed. Clinical trials at St. Jude Children's Research Hospital in Memphis used either a myeloablative, TBI-containing preparative regimen or reduced intensity conditioning (RIC), coupled with rituximab for in vivo B-cell depletion [36]. Patients who received the RIC regimen had a faster de novo thymic generation of T cells, as documented by increased TREC numbers and by Vβ spectratyping scores, and an accelerated $CD4^+$ and NK-cell reconstitution, compared with patients given a myeloablative conditioning [37]. This was associated with less CMV and HAdV reactivation and, in case reactivation occurred, with lower copy numbers of the viral pathogen [37]. A retrospective analysis of the incidence of lethal viral infections after haploidentical HSCT has shown major differences when comparing pediatric patients receiving CD34-selected cells with myeloablative conditioning and those given CD3/CD19-depleted stem cells with RIC. As of June 2007, approximately 20% of patients treated with myeloablative drugs died of infections, whereas none of the 37 patients given CD3/CD19-depleted stem cells after RIC died from this complication [36].

Autologous B cells transformed with the EBV strain 95.8 (lymphoblastoid cell lines or LCL) have been widely used as stimulators to sensitize donor-derived T cells and generate EBV-specific T cells. T-cell lines were derived from 20 ml of donor peripheral blood, and were expanded with irradiated LCL for at least 21 to 28 days to allow proliferation of EBV-reactive T cells and to deplete alloreactive T cells [38]. T cells were marked with the neo^R-containing G1Na retroviral vector to directly determine the fate of the infused cells. The EBV-reactive T cells were infused weekly in 3 patients with evidence of EBV disease after bone marrow transplantation. No T-cell lines had discernible reactivity against patient-derived PHA blasts or fibroblasts. Each patient received 4 infusions of the gene-marked cells for a total of $4x10^7$ cells/m^2 in 2 patients and $1.2x10^8$ cells in the third patient. The infused EBV-specific, donor-type T cells, containing variable proportions of $CD4^+$ and $CD8^+$ T cells, persisted for 10 weeks and produced clinically significant activity against the PTLD lesions. The gene-marked cells were also infused in 7 other recipients of bone marrow grafts as prophylaxis against EBV disease [38]. The cells persisted for a median of 10 weeks after infusion and no patient developed GVHD or EBV disease 2-11 months later. In another clinical trial, EBV-specific T cells were administered at $0.4\text{-}1.2x10^8$ T cells/m^2 as prophylaxis of EBV-induced PTLD in 25 patients [39]. Compared with 27 historical controls not given T-cell-based prophylaxis, 5 of whom developed PTLD, patients treated with the EBV-specific T cells did not develop PTLD. The frequency of circulating, EBV-specific T cells increased by 5-100 fold and the T cells persisted in vivo for more than 18 months post-infusion.

The preemptive/early treatment of PTLD with EBV-specific T cells is another viable option that

has been applied to 46 pediatric patients enrolled in a haploidentical HSCT trial. This EBV surveillance program has shown that patients with EBV DNA levels >1,000 copies/10^5 PBMC who do not respond to rituximab can be successfully treated with EBV-specific CTL, with viral clearance and remission of established PTLD in all patients [40]. Recently, the EBV-specific T cells have been rendered resistant to the immunosuppressive drug tacrolimus (FK506), which is widely used to prevent rejection of solid organ transplants [41]. Tacrolimus inhibits immune responses by binding to the 12-kDa FK506-binding protein (FKBP12) and leading to the inhibition of calcineurin. EBV-CTL were transduced with a retroviral vector encoding a small interfering RNA, designated siRNA4, that targets and stably knocks-down the human FKBP12 mRNA [41]. The transduced EBV-CTL were predominantly $CD3^+CD8^+$, retained antigen-specific expansion in the presence of tacrolimus, efficiently lysed EBV^+ targets in vitro and exerted anti-tumor activity against EBV^+ lymphomas that were implanted into SCID mice [41]. This approach could be exploited for the generation of virus-specific CTL for recipients of HSCT who receive immunosuppressive treatments that are expected to impair the expansion and long-term persistence of the adoptively transferred CTL.

Finally, it should be noted that in exceptional cases therapy with EBV-specific CTL has been unsuccessful. The EBV-LCL behave as antigen presenting cells, as they express a range of viral latent and early lytic antigens. However, immunogenicity of EBV-LCL is hierarchical and HLA-dependent, and some CTL lines are specific for a limited number of epitopes from 1 or 2 viral proteins. This implies that efforts to control PTLD may fail if the tumor mutates an immunodominant viral target antigen that is the major specificity of the CTL line [42]. Thus, the potential for tumor immune evasion should be minimized through the infusion of a CTL product that is polyclonal (both $CD4^+$ and $CD8^+$) and has broad antigen and epitope specificity.

Antigen-specific T cells for HAdV infection

HAdV is a non-enveloped lytic DNA virus. Fifty-one serotypes of HAdV can infect human mucosal epithelium. HAdV has developed complex strategies to interfere with innate and adaptive immune responses and may persist within the monocyte-macrophage system of infected hosts, being shed for months or years and then acquired by horizontal transmission. HAdV elicits both $CD4^+$ and $CD8^+$ T-cell responses. The HAdV-activated $CD4^+$ T cells also release IFN-γ and exert cytotoxic functions. Antibodies against capsid and fiber proteins may offer life-long protection in immunocompetent individuals. The viral circulation among children accounts for the preferential occurrence of HAdV infection in pediatric patients (20-26%) rather than adult patients (9%) undergoing HSCT. Although acute infection is rarely fatal in immunocompetent hosts, it may cause life-threatening infections in immunocompromised patients [43], being associated with respiratory disease, hepatitis and colitis, but also hemorrhagic cystitis and keratoconjunctivitis. Patients with primary infection or with disease re-activation can be divided into patients with sub-clinical viremia, patients with viremia and disease symptoms, and patients with disseminated disease [43]. As recently reviewed [43], the incidence of disseminated disease is 1-7%, with a reported mortality of 8-26%. Infections with HAdV that develop early post-transplant are primarily due to re-activation or de novo exposure to the virus in the peri-transplant period [44]. This is suggested by the observation that HAdV DNA is not routinely detected in stored aliquots of donor stem cell products [44]. Notably, HAdV DNA has been found at higher levels and earlier in the post-transplantation period in the blood of patients receiving stem cell transplants from haploidentical or MUD, compared with patients given stem cells from matched related donors (MRD) [44]. In line with this, the recovery of HAdV-specific T cells is faster in MRD recipients, with T-cell levels approaching those of normal donors within 30 days post-HSCT compared with 3-12 months in MUD and haploidentical stem cell recipients [44].

The currently available treatment options for HAdV include antiviral drugs, such as ribavirin and cidofovir, and immunotherapy, in the form of either DLI or adoptive immunotherapy. Guidelines for prophylaxis, preemptive treatment and therapeutic treatment of HAdV infections in pediatric HSCT recipients have been recently published [43]. The major risk factors for HAdV infection are related to lack of cellular antiviral activity, which occurs in the first 100 days after transplantation. The number of $CD3^+$ T cells is viewed as a valuable tool to identify patients at

high risk of developing HAdV infection. Absence of T cells ($CD3^+$ < 25/μL) or the failure of an HAdV response shortly after HAdV detection ($CD3^+$ < 300/μL within 2 weeks of HAdV detection) have been associated with a poor clinical outcome [43].

Additional risk factors include GVHD, use of anti-thymocyte globulin (ATG) and anti-CD52 antibodies in the conditioning regimen, and extensive T-cell depletion of the graft. DLI have been successfully administered to patients with HAdV infection, leading to clearance of the virus [45, 46]. As already discussed, the efficacy of DLI is often limited by the very low frequency of HAdV-specific T cells (usually < 0.01% of vital T cells) and by the relatively high frequency of alloreactive T cells with the potential to induce GVH reactions. Some groups are currently investigating the role of adoptive immunotherapy with HAdV-specific T cells, either directly isolated from the peripheral blood or expanded in vitro [47]. The HAdV hexon protein is a conserved and an immunodominant T-cell target antigen. Efforts at identifying a broad panel of hexon epitopes have considerably simplified the current immunotherapy approaches to HAdV infections. A relevant number of CTL lines against HAdV have been recently screened, using a library of 20-mer peptides covering the entire hexon protein and overlapping by 15 amino acids [48]. This has led to the identification of 5 new hexon-derived HLA class I epitopes and 28 new hexon-derived class II-restricted epitopes [48]. Five of these epitopes stimulated $CD4^+$ and $CD8^+$ T cells simultaneously, thus being suitable vaccine antigens.

HAdV-specific T cells were initially generated after culturing donor PBMC with HAdV antigen type C for 16 hours, followed by magnetic enrichment of the IFN-γ^+ HAdV-specific T cells with the Cytokine Secretion System® and the CliniMACS® device from Miltenyi Biotec (Germany) [49]. On average, 1.1% of total donor T cells expressed surface membrane IFN-γ prior to the isolation of HAdV-specific T cells, and the magnetic separation yielded an average 45.7% of HAdV-specific T cells, containing both $CD4^+$ and $CD8^+$ T cells [49]. The T-cell product was infused in 9 patients with either benign or malignant disorders. The HAdV-specific T cells expanded in vivo, and this was paralleled by a reduction or clearance of the viral load and by a clinical improvement of HAdV disease. Two patients with HAdV type A infection successfully cleared the virus, although the specific T cells were generated with HAdV type C, presumably because of cross-reactivity [49]. In addition, low numbers of HAdV-specific T cells, ranging from 1,200 to 6,000 T cells/kg of recipient body weight, were sufficient to induce an immune response in vivo, re-assuring about the feasibility of this approach.

The exon protein of HAdV capsid contains most immunogenic epitopes which are conserved across several HAdV subtypes. Some epitopes are particularly important for the development of HAdV-targeting T-cell immunotherapy, as they trigger both $CD4^+$ and $CD8^+$ T cells. The exon protein from type 5 HAdV can be used to generate HAdV-specific T cells under GMP conditions [50]. T-cell responses against the HAdV exon protein were preliminarily tested in 76 healthy individuals using intracellular IFN-γ detection. The majority of donors (72.4%) had detectable T-cell responses that were mainly due to $CD4^+$ cells, whereas 17% of donors showed no response. The magnetic isolation of IFN-γ-expressing T cells translated into a 3-log enrichment of HAdV-specific T cells [50]. The isolated T cells were ~40% viable, and were characterized by an intermediate effector phenotype, consisting of CD27, CD62L and low CD45RA expression, in the absence of CCR7 and CTLA-4. The T cells were fully functional after their in vitro expansion, as shown by the ability to lyse HAdV-infected skin fibroblasts [50]. Another study focused on the ability of a pool of 30-mer peptides derived from 5 HAdV exon protein to expand virus-specific T cells [51]. This protocol led to the generation of 21 T-cell lines starting from PBMC of healthy controls elected to donate stem cells to a haploidentical pediatric relative. The established cell lines contained a majority of $CD4^+$ T cells and were reactive against the HAdV peptides. Importantly, the HAdV-specific T cells had a low reactivity against recipient's PBMC and maintained a broad spectrum of specificity against the peptides used to stimulate their in vitro expansion [51]. Collectively, this strategy yielded sufficient numbers of HAdV-specific T cells to cryopreserve at least 2 aliquots of $0.5x10^6$ cells/kg of body weight for clinical use.

Multivirus-specific T cells

Bivirus-specific CTL lines have been generated with APC transduced with a $Ad5f35^{null}$ vector [52]. The CTL lines were predominantly $CD4^+$

and acquired cytotoxic activity against EBV-LCL and Ad5f35^{null}-transduced EBV-LCL. The safety of the bispecific T-cell lines was assessed in 13 patients with leukemia or congenital diseases, at 40 to 150 days after transplants from haploidentical or unrelated donors. No toxicity was recorded and the majority of patients had a marked rise in EBV-specific T cells within 2 weeks of CTL infusion. By contrast, an increase in the precursor frequency of anti-HAdV T cells was measured only in the 2 patients with evidence of HAdV infection/disease [52]. However, none of the remaining 11 patients developed HAdV infection or disease, compared with an expected incidence of 68% in pediatric patients receiving similar transplants in the absence of CTL administration [44].

CTL lines that simultaneously target EBV, CMV and HAdV can also be obtained in the clinical laboratory. APC were produced by expressing CMV-pp65 antigen in activated monocytes and EBV-LCL. The APC activated CTL specific for each virus in a single culture, although the dominant specificity was for CMV with a smaller number of EBV and HAdV-reactive T cells [48, 53]. The trivirus-specific CTL were infused in recipients of HLA-matched related or unrelated donors and they showed activity against all 3 viruses in vivo. Intriguingly, only the CTL directed to EBV and CMV expanded and persisted in vivo. Although HAdV-specific T cells were detected in vivo only in patients with positive HAdV cultures, none of the trivirus-specific CTL recipients developed HAdV infection, suggesting that HAdV-specific CTL survived and expanded, likely residing in the spleen and circulating only in periods of infection [13].

Regulatory T cell (Treg)-based approaches

Naturally occurring Treg cells are currently identified by their co-expression of membrane CD25, the α chain of the IL-2 receptor molecule, and intracellular FoxP3, a master transcription factor controlling their development and function [54]. Although broadly implicated in the maintenance of tolerance to self-antigens [55], Treg cells may be a double-edged sword, given their role also in suppressing immune responses against tumor-associated antigens (reviewed in [56, 57]). Intriguingly, Treg infusions followed by conventional T cells can improve T-cell reconstitution, as suggested by a recent clinical trial of haploidentical HSCT in 28 patients with high-risk hematological malignancies [58]. Escalating numbers of conventional T cells were given, ranging from $0.5x10^6$/kg in 4 patients to $1.0x10^6$/kg in 17 patients, whereas the Treg cell number was kept at $2.0x10^6$/kg. Another cohort of 5 patients received $1.0x10^6$/kg conventional T cells and $4.0x10^6$/kg Treg cells. Only 2 out of 26 evaluable patients developed ≥ grade II GVHD, whereas chronic GVHD was not observed at a median follow-up of 11.2 months [58]. Compared with standard haploidentical transplants performed at the same clinical centre [2], $CD4^+$ and $CD8^+$ T cells specific for opportunistic pathogens, such as CMV, HAdV, herpes simplex virus and varicella zoster virus, emerged significantly earlier. In line with this observation, fewer episodes of CMV reactivation were documented and no patient developed CMV disease [58]. In addition, 7 patients were vaccinated against pandemic influenza with 2 doses of MF59-H1N1 California. Five of them developed protective antibody titers, in association with marked increases in H1N1 California-specific $CD4^+$ T cells [58]. Although the long-term impact of Treg infusions on immune control of the underlying malignancy is unknown, this study provides convincing proof-of-principle in favor of Treg effects on the reconstitution of immunity against opportunistic pathogens.

Immunity to fungal infections

A stable host-fungal relationship requires a fine balance between pro- and anti-inflammatory signals. Immunity to fungi comprises two main components, resistance and tolerance, with the former limiting fungal burden and the latter being essential to limit host damage caused by the immune response [59]. The mechanisms of fungal recognition by the innate immune system, which are used by the host to respond in a rapid and conserved manner to a range of fungal pathogens, have been reviewed elsewhere [60]. Innate sensing circuits centered around DC with a specific transcriptional program activate distinct $CD4^+$ T-cell subsets with protective and non-protective functions. A dominant Th1 response correlates with protective immunity against fungi and fungal vaccines, through the release of IFN-γ and the provision of T-cell help for the production of opsonizing antibodies [59]. Conversely, Th2 responses dampen protective Th1 responses and promote the alternative pathway macrophage activation, thus favoring fungal infections and disease relapse. Although

generally associated with autoimmune and pathogenic responses, Th17 cells are present within the fungus-specific T-cell memory repertoire in humans and play a protective role in fungal infections [59]. IL-17A mobilizes neutrophils and induces the production of defensins, greatly contributing to prompt and efficient control of an infection at different body sites. However, Th17 cells activated by fungal components may contribute to defective fungal clearance in experimental aspergillosis and mucosal candidiasis [61]. In addition, neutralization of IL-17A reduced fungal burden and corrected inflammation by reducing neutrophil recruitment [61]. This indicates that neutrophils are required to control systemic fungal infections (see section below) and that a failure to restrain IL-17-induced inflammation may lead to chronic fungal diseases, fungal persistence and non-resolving inflammation [61]. The indoleamine 2, 3-dioxygenase 1 (IDO1)-driven breakdown of tryptophan into immune suppressive kynurenines and the activation of Treg cells may be additional mechanisms to limit the host damage in response to fungi and potentially leading to commensalisms or chronic infection [59]. This complex network of interactions among different modules of immunity should be taken into account when designing immune therapies for invasive fungal infections.

Invasive fungal infections remain a life-threatening complication of allogeneic HSCT [62]. Approximately 60% of invasive fungal infections can be attributed to Aspergillus species, followed by invasive candidiasis, zygomycoses and infections caused by other molds. Mortality due to invasive fungal infections can be as high as 80% in invasive aspergillosis, 50% in invasive candidiasis and 70% in zygomycosis [62]. In general, the frequency of antifungal T cells is significantly lower than that of antiviral T cells. This poses a major challenge to the large-scale generation and characterization of anti-fungal T cells for adoptive immunotherapy. At variance with virus-specific T cells that can be detected only in seropositive donors, T cells against Candida albicans or Aspergillus fumigatus have been described in all healthy individuals tested thus far [62]. Anti-Aspergillus T cells have been reproducibly detected by means of flow cytometry in healthy controls, as IFN-γ-producing cells after in vitro challenge with cellular extracts of A. fumigatus consisting of multiple fungal antigens [63, 64]. The same study also showed that anti-Aspergillus T cells cannot be detected during the first 4 months after allogeneic HSCT, and that their numbers at 1 year after transplant are lower compared with those measured in healthy controls [64]. T cells generated as above detailed not only respond to in vitro restimulation with A. fumigatus but also with other Aspergillus species, such as A. flavus and A. niger, and potentially reflect the overall cellular immunity against invasive aspergillosis [64].

Cell-based therapeutic approaches for fungal infections

Protocols that have been established for the generation of anti-Candida, anti-Aspergillus and anti-Zygomycetes T cells rely on the IFN-γ secretion assays using a cellular fungal extract for stimulation. Anti-fungal T cells homogeneously express CD3, CD4, CD45RO and HLA-DR, and release IFN-γ and TNF-α, but not IL-4 or IL-10, after re-stimulation, indicating a memory, activated Th1 phenotype [64-66]. The anti-fungal T cells maintain their ability to proliferate in vitro if challenged with autologous APC loaded with cellular fungal extracts, suggesting that they can also expand in vivo when stimulated by a fungal antigen. In vitro data also indicate that anti-fungal T cells may be less likely to induce GVHD compared with $CD4^+$ T cells of the original cell fraction, being less efficient in proliferation and IFN-γ release. Another important issue pertains to the ability of the in vitro-generated T cells to induce damage of fungal hyphae. For instance, the water-soluble cellular extract of Aspergillus fumigatus can induce anti-Aspergillus T cells in PBMC from healthy individuals [64]. The anti-fungal T cells obtained after 10-14 days of culture are enriched with IFN-γ/IL-2-secreting $CD3^+CD4^+$ cells, are expanded up to 500-fold and also retain viability and functionality after cryopreservation in liquid nitrogen [64]. Interestingly, expanded anti-fungal T cells induce maximal damage of fungal hyphae in vitro when co-incubated with neutrophils and APC, tentatively attributed to the enhancement of phagocyte anti-fungal activity by cytokines released from anti-Aspergillus T cells [64]. Hyphal damage by the anti-fungal T cells alone was less powerful compared with that induced by neutrophils. These data suggest that the adoptive transfer of anti-Aspergillus T cells mostly benefits non-neutropenic patients with impaired cellular immunity after HSCT or that the anti-fungal T cells

should be infused together with granulocyte concentrates in order to take advantage of the above indirect mechanism of fungal damage [64, 67]. Similar to anti-viral T cells, anti-Aspergillus T cells can be generated under GMP conditions, also based on IFN-γ secretion after incubation with Aspergillus antigens, starting from a single, unstimulated leukapheresis product containing $1.1x10^9$ white blood cells [66]. This approach yielded a median number of $12x10^7$ anti-Aspergillus Th1 cells within 1-13 days.

Hematopoietic growth factors such as G-CSF may adversely affect anti-pathogen immune responses through their ability to skew both T-cell and DC function [68, 69]. The administration of G-CSF to 43 patients with acute leukemia after haploidentical HSCT impaired anti-fungal T-cell responses, an effect mainly attributable to G-CSF-driven down-regulation of IL-12p40 production by DC and to differentiation of IL-4/IL-10-producing $CD4^+$ cells not expressing the IL-12 receptor β2 chain [70]. The authors also showed that the elimination of post-grafting G-CSF in a subsequent series of 36 patients with acute leukemia translated into the anticipated appearance of IL-12-producing DC (1-3 months after transplantation versus > 12 months in transplant recipients given G-CSF), of $CD4^+$ cells of a mixed Th0/Th1 phenotype, and of antifungal T-cell reactivity in vitro [70].

A recent immunotherapy trial with virus- and fungus-specific T cells in haploidentical HSCT has provided the first evidence of in vivo efficacy of anti-fungal T cells [71]. Ten patients with invasive aspergillosis were assigned to receive anti-Aspergillus T cells in addition to liposomal amphotericin. Within the same trial, CMV-specific T cells were administered to 25 patients receiving haploidentical HSCT who were at risk for CMV re-activation. In the 46 haploidentical transplant recipients who were not given adoptive T-cell therapy, the spontaneous recovery of $CD4^+$ and $CD8^+$ T cells occurred at very low frequency as late as 9-12 months after HSCT. The T cells had a non-protective type 2 cytokine profile [71]. Conversely, both Aspergillus-specific and CMV-specific T-cell clones were detected at high frequency within 3 weeks of the immunotherapy infusion. The 13 controls for the anti-Aspergillus trial displayed positive galactomannan antigenemia and/or signs of active aspergillosis. Six of the 13 patients died of invasive aspergillosis and 7 patients cleared the disease in 5 weeks of diagnosis. The 10 recipients of one single infusion of anti-Aspergillus T cells, although affected by Aspergillus pneumonia at time of adoptive infusion, experienced a reduction of galactomannan antigenemia to the normal range within 6 weeks. Nine of the 10 patients cleared the infection in a mean of 7.8 weeks, whereas 1 patient died of Aspergillus pneumonia within 2 weeks of diagnosis. Mechanistically, in vivo expansion of the Aspergillus-specific donor T cells may potentially have occurred as a result of exposure to fungal epitopes presented by the reconstituting donor-type APC, particularly in a T-cell-depleted environment. In addition, a large fraction (approximately 50%) of donor anti-fungal T cells may have responded to recipient-type APC remaining after conditioning and recognizing epitopes presented by the shared HLA haplotype.

A similar laboratory approach has been used to generate anti-Candida T cells [65]. The $CD4^+$ T cells expanded with C. albicans antigens produced IFN-γ and TNF-α, and were skewed from a polyclonal to an oligoclonal pattern, indicating restriction of the T-cell repertoire [65]. Purified anti-Candida T cells proliferated poorly to third-party APC compared with unselected $CD4^+$ T cells, suggesting loss of alloreactivity. In line with the data on anti-Aspergillus T cells [66], anti-Candida T cells induced the highest fungal damage in vitro when co-incubated with APC and polymorphonuclear cells [65].

Other approaches

Exogenous cytokines

Studies in the allogeneic HSCT setting have suggested that exogenous cytokines can enhance immune recovery and boost the emergence of pathogen-specific T cells in the post-transplantation period [7]. For instance, IL-2 has been given as consolidating immunotherapeutic agent early after HSCT, at a time of minimal residual disease, leading to enhanced GVL responses [72]. An ongoing clinical trial of haploidentical donor-derived NK cells, epratuzumab and low-dose IL-2 in relapsed/refractory $CD22^+$ acute lymphoblastic leukemia will help define the use of IL-2 also in the haploidentical setting (http: // ClinicalTrials. govidentifier: NCT 0094 1928). Clinical trials with IL-15, another member of the IL-2 family of γ-chain signaling cyto-

kines, have not been conducted in the HSCT setting thus far. In this respect, it was previously shown that in vitro treatment with exogenous IL-15 of peripheral T cells isolated at different tempos after autologous HSCT antagonizes T-cell spontaneous apoptosis and restores T-cell responses to a CMV lysate and to nominal antigens such as tetanus toxoid [73]. Both IL-2 and IL-15 have been used to enhance the expansion of EBV-CTL [74]. In this study, the transgenic expression of IL-2 or IL-15 increased the antitumor activity of the EBV-CTL that maintained their antigen specificity. The incorporation of an inducible caspase-9 suicide gene allowed the efficient elimination of the transgenic CTL after exposure to a chemical inducer of dimerization, thereby increasing the safety and feasibility of this approach [74].

Chimeric antigen receptor (CAR)$^+$ CTL

T cells with concomitant anti-viral and anti-leukemic specificity have been generated from peripheral blood and UCB units that recognize multiple viruses and provide anti-leukemic activity by transgenic expression of a CAR. CAR are fusion proteins between single-chain variable fragments from monoclonal antibodies recognizing tumor-associated antigens and intracellular signaling domains such as the CD3 ζ-chain cytoplasmic tail [75]. CAR-transduced T cells recognize targets in MHC unrestricted manner, thus being applicable to a wider group of patients. The transduction of peripheral blood T cells with these targeting moieties confers specific cytotoxicity against cells that express the target epitope [75]. The combination product was engineered to recognize CD19 on B-ALL through the introduced CAR, while being capable of responding to viral antigens through the native αβ TCR [74]. The CAR$^+$ CTL produced IFN-γ in response to CMV_{pp65}, HAdV exon protein, and EBV peptide mixes and lysed primary B-ALL cells. This approach may have advantages over activated but otherwise poorly defined cell populations obtained with viral/fungal antigen mixes, insofar the CAR$^+$ CTL products do not contain undesired cell populations such as alloreactive T cells or Treg cells.

Current selection protocols to enrich for pathogen-specific T cells

The time required for the generation of anti-pathogen CTL limits a broader application of strategies to reconstitute antiviral immunity. For instance, the generation of EBV-specific, bivirus-specific and trivirus-specific CTL requires 4-6 weeks to obtain EBV-LCL to be used as APC, followed by 4-6 weeks to expand sufficient numbers of CTL for infusion, sterility testing, and functional analyses. Other protocols are based on the differentiation of DC as APC and thus require large volumes of blood to procure sufficient numbers of monocytes that must be matured into immunocompetent DC. Each reagent needed for the generation of CTL lines must be available as a clinical-grade product. The length of time required for the generation of CTL implies that most CTL are available as prophylaxis rather than therapy of established infections, which are frequently evident in patients early post-transplantation. Several groups have focused on the development and validation of simpler and faster methods to obtain pathogen-specific CTL. Clinically, both tetramer selection and IFN-γ selection have been used, providing evidence that even small numbers of pathogen-specific T cells expand substantially in vivo in the presence of the relevant antigen(s) and offer protection from the targeted pathogen. CMV-specific CD8$^+$ T cells, that typically account for 0.5-4% of the CD8$^+$ T-cell pool, have been selected directly from the peripheral blood of HSCT donors after staining with HLA-peptide tetramers containing peptides from CMV pp65 or IE-1, followed by selection with magnetic beads [76]. The recovery of CMV-specific T cells averaged 61% and the purity of the selected fraction ranged from 97.8 to 99.9% [76]. The adoptive transfer of 1.2×10^4 to 2×10^6 tetramer-selected CMV-specific CTL in 9 patients with hematological malignancies translated into the reconstitution of virus-specific immunity with reduction of CMV viremia. The lack of significant GVHD in this study reflects transfer of a very low number of T cells with antigen specificity other than that for CMV. However, multimers have not been produced to GMP standards. In addition, this methodology would be limited to patients expressing HLA alleles for which viral peptides are available and for which the frequency of reactive T cells in the peripheral blood is above the detection threshold of multimer staining. Conversely, the streptamer technology may have less stringent regulatory requirements, because streptamers are considered as adjuvants. CMVpp65-specific T cells have been isolated with streptamers under GMP-conditions and were adoptively transferred in 2 patients

who developed high-grade CMV antigenemia [77]. An increase of CMV-specific $CD8^+$ T cells and long-lasting CD8 responses were observed, allowing for the discontinuation of antiviral drugs without CMV reactivation.

The IFN-γ capture assay has been designed to isolate pathogen-specific T cells from leukapheresis products. This approach is GMP-compatible and can be applied to pathogens where a source of clinical-grade antigen is available. The number of cells that can be isolated with this procedure is limited and a thorough characterization of the infused products may be difficult. GMP-grade peptide pools consisting of 15-mer peptides with 11 amino acid overlap are commercially available (Peptivator®, Miltenyi Biotec, Germany). The peptides cover the complete sequence of the respective antigen, including EBV LMP2A, EBV BZLF1, EBV EBNA-1, CMV pp65, CMV IE-1 and HAdV5 hexon (http://www.miltenyibiotec.com/en/NN_700_Peptide_pools.aspx; accessed July, 2011).

An isolation method for virus- and fungus-specific T cells based on the activation-dependent expression of CD154 has been recently proposed [78]. Although the isolation of $CD154^+$ cells favored $CD4^+$ T cells, a small number of $CD4^+$ T cells specific for EBV, HAdV, C. albicans and A. fumigatus were generated. The pathogen-specific, CD154-expressing T cells did not react against third-party DC, suggesting their clinical applicability for the treatment of viral and/or fungal infections [78].

Concluding remarks

The currently available clinical trials show that the adoptive immunotherapy of viral and fungal infections occurring after haploidentical HSCT is safe and efficacious. In spite of the undeniable enthusiasm for the administration of ex vivo-activated pathogen-specific CTL, important limitations to a broad implementation of this technique exist and should be taken into account [13]. They include time to manufacture CTL lines under GMP conditions, costs and complexities associated with CTL production, competition among viral antigens for HLA molecules and wide spectrum of emerging viruses detected post-transplantation that require coverage. Some of the above constraints will hopefully be overcome in the near future through the design of protocols for the rapid and simplified production of CTL.

In principle, banks of CTL lines may be created to provide partly HLA-matched allogeneic CTL as an "off-the-shelf" product for immediate clinical use. Some studies have confirmed the feasibility of this approach and also reported clinical benefit in patients with EBV-related PTLD arising after HSCT or solid-organ transplantation [79, 80]. In one multi-center trial, responses to 3rd party EBV-specific CTL were documented in 64% and 52% of patients at 5 weeks and 6 months, respectively, after the infusion of 4 doses of $4x10^6$ CTL/kg at weekly intervals [75]. Not surprisingly, outcomes at 6 months were more favorable with closer HLA matching between donor and recipient. Overall, only 1 patient of the 33 treated developed detectable anti-alloantibodies directed against non-shared HLA antigens, but no patient developed GVHD. Another approach under investigation is based on the use of alternative sources of antigen, such as DNA plasmids that encode antigens from CMV, EBV and HAdV and that might be introduced into APC, such as monocytes and DC, with the clinically applicable AMAXA nucleofection system [13]. This option, although appealing, may introduce additional GMP-related issues, such as vectors and manipulations that render such in vitro protocols more complex to perform and approve [81].

It must be emphasized that the establishment of pathogen-specific T cells from non-immune donors and/or from UCB units may be a major challenge for adoptive therapy, due to the antigen-inexperienced or naïve status of the T cells. CMV and EBV-specific T cells have been generated from non-immune donors or from UCB lymphocytes using DC as APC and cytokine cocktails including IL-5, IL-12 and IL-15 [82, 83]. Although more complex and time-consuming than the seropositive setting, this approach should lead to effective strategies to treat infections in the UCB transplant setting [83]. Differences may also exist in the ability to generate EBV-specific T cells with LCL when comparing EBV seronegative adults and children, with the latter requiring an additional CD25 selection step to enrich for EBV-specific precursor T cells [80].

In view of the different methods of generating pathogen-specific T cells, future studies will have to address the questions which are the

preferred T-cell subpopulations to be used for antiviral and antifungal therapy and what is the optimal cell dose in relation with pathogen load and immunosuppression of the patients. Methodological advances to simplify the process of CTL generation are eagerly awaited and should render this approach broadly applicable.

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