Treatment of CMV infection after allogeneic hematopoietic stem cell transplantation

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Abstract: Despite a remarkable reduction in the past decades, cytomegalovirus (CMV) disease in allogeneic hematopoietic stem cell transplant (HSCT) recipients remains a feared complication, still associated with significant morbidity and mortality. **Today first line treatment of CMV infection/reactivation is still based on dated antiviral compounds Ganciclovir (GCV), Foscarnet (FOS) and Cidofovir (CDF) with their burdensome weight of side effects. Maribavir (MBV), Letermovir (LMV) and Brincidofovir (BDF) are three new promising anti-CMV drugs without myelosuppressive properties or renal toxic effects that are under investigation in randomized phase II and III trials. Adoptive T-cell therapy (ATCT) in CMV infection possesses a strong rationale, demonstrated by several proof of concept studies; its feasibility is currently under investigation by clinical trials. ATCT from third-party and naïve donors could meet the needs of HSCT recipients of seronegative donors and cord blood grafts. In selected patients such as recipients of T-cell depleted grafts, ATCT, based on CMV-specific host T-cells reconstitution kinetics, would be of value in the prophylactic and/or preemptive CMV treatment.** Vaccine-immunotherapy has the difficult task to reduce the incidence of CMV reactivation/infection in highly immunocompromised HSCT patients. **Newer notions on CMV biology may represent the base to flush out the Troll of transplantation.**

Key words: cytomegalovirus (CMV), immune response, CMV-vaccine, hematopoietic stem cell transplantation (HSCT), adoptive T-cell therapy (ATCT).

1.Introduction

Over thirty years ago, Henry Balfour [1] nicknamed cytomegalovirus (CMV) the troll of transplantation and since then it has remained a problem for clinicians and patients in the setting of solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT) [2]. Indeed, CMV specific viral load detecting methods and pre-emptive therapy with current antiviral drugs remarkably reduced the incidence of CMV disease in allogeneic HSCT recipients over the past twenty years. Estimated incidences of early CMV disease (before day 100 post-transplant) and late CMV disease (after day 100) in CMV-seropositive allogeneic recipients are currently around 5% and 15% [3]. Major risk factors for CMV infection in HSCT recipients, particularly refractory CMV reactivation, include a transplant from a CMV seronegative donor into a seropositive recipient [4]; the intensity of the immunosuppression; the degree of T-cell depletion including the use of T-cell depleting agents such as alemtuzumab, antithymocyte globulin [5], and mycophenolate mofetil [6]; a transplant from unrelated or human leucocyte antigen (HLA)-mismatched donors including haploidentical donors [7]; umbilical cord blood transplantation; acute and chronic graft versus host disease (GvHD) and its treatment [8]; low CD3+ content in the graft [9]. Both myeloablative and non-myeloablative conditionings are associated with relative risk of CMV infection [10, 11]. Conflicting results have been reported regarding the relative risk of CMV infection in bone marrow recipient as compared with peripheral blood stem cells (PBSC) recipients [12]. Interestingly, recipient hepatitis B virus serostatus has been associated with the development of CMV-DNAemia in a recent retrospective study on haploidentical HSCT [13]. The switch from prophylactic to pre-emptive therapy has substantially spared organ toxicities and costs [14]. Myelosuppression and nephrotoxicity are the most important side effects of the anti-CMV agents readily available today – ganciclovir (GCV), valganciclovir (VGC), foscarnet (FOS) and cidofovir (CDF). Antiviral resistance could be a difficult phenomenon to overcome and management options still have important limitations [15]. The need for newer agents with better toxicity profiles have led to develop several promising antiviral compounds [16, 17]. Most interesting agents are maribavir (MBV), letermovir (LMV) and brincidofovir (BDF). Phase II/III studies are in progress to evaluate their safety and efficacy in the clinical setting. As newer and deeper insights into immune reconstitution of transplant recipients and CMV biology are obtained, immunological strategies are drawing much more attention. Optimal reconstitution of CMV-specific cytotoxic T lymphocytes (CMV-CTL) after allogeneic HSCT is essential for immune control of

CMV infection. Its deficiency is a major factor for the development of CMV infection and disease and represents the major concept to set the base of adoptive T-cell therapy (ATCT) [18]. Moreover, vaccine immunotherapy appears to play a new role in CMV-prophylaxis [19].

2.1 The old drugs: Ganciclovir (GCV), Valganciclovir (VGC), Foscarnet (FOS), Cidofovir (CDF).

The drugs currently approved by the Food and Drug Administration (FDA) for the prophylaxis or treatment of systemic CMV infections are GCV and its oral prodrug VGC; FOS, and CDF. All of them target viral DNA polymerase.

2.1.1 Ganciclovir – the first choice is a myelosuppressive one.

It is still the first choice in CMV reactivation/infection and disease treatment [20]. GCV is phosphorylated intracellularly to ganciclovir monophosphate by a viral kinase encoded by the CMV gene UL97. It inhibits viral DNA polymerase. The most relevant side effect of GCV is bone marrow depression, particularly neutropenia with an absolute neutrophil count of less than 750/mL occurring in 30% of GCV recipients. The development of neutropenia has been associated with risk factors such as impaired renal function, high baseline viral load and low-level neutrophil counts prior to CMV-therapy [21]. As prophylaxis and pre-emptive therapy, GCV is usually infused at an induction dose of 5 mg/kg twice daily intravenously for two weeks, followed by 5 mg/kg/day for 7-14 days as maintenance. As mentioned above, a not negligible portion of transplanted patients cannot receive GCV because of persistent neutropenia after HSCT. A randomized study conducted on 68 HSCT recipients with documented CMV infection between standard dose (10 mg/kg/day) (n=32) versus low-dose (5 mg/kg/day) GCV (n=36) as pre-emptive therapy did not show any significant difference in CMV disease incidence [22]. Park et al. explored pre-emptive low-dose (5 mg/kg/day) versus conventional dose GCV in a prospective observational study on 97 allogeneic HSCT patients. By using a logistic regression model to reduce selection bias in the treatment assignment, the

Authors reported no significant differences in viral clearance, secondary episodes of CMV infection, CMV disease and overall mortality between the two groups [23]. In the setting of CMV disease, the addition of intravenous immunoglobulins, either CMV-specific or not, is not supported by clear efficacy in treatment outcomes. A recent retrospective study on over four hundred allogeneic HSCT patients with CMV-pneumonia clearly showed improved overall survival with both GCV and FOS, whereas the addition of intravenous pooled or CMV-specific immunoglobulins did not appear to improve overall or CMV-attributable mortality [24]. In case of progressive CMV disease despite first line therapy with GCV, it is mandatory to consider the presence of GCV-resistant CMV strains and look for alternative therapies including the increase of GCV dosage [25] or the use of other antiviral agents [26].

2.1.2 Valganciclovir – oral versus intravenous.

It is the valine ester of GCV and it is hydrolyzed to GCV after oral absorption. Since 85% of GCV delivered orally is excreted by the kidneys, VGC dosage should be recommended in the light of patient creatinine clearance (CrCl). Some Authors however suggest that the currently recommended renal adjustments based on their CrCl calculated using the ideal body weight may not be sufficient to prevent viral replication in overweight patients given the possible underestimation of the renal function in such patients [27, 28**]**. Large prospective randomized trials comparing VGC and GCV in HSCT patients are lacking. Though small, some interesting studies compared the efficacy of VGC therapy versus non-VGC therapy - primarily GCV - in the pre-emptive setting on allogeneic HSCT patients. In a pilot prospective randomized clinical trial conducted on 37 HSCT patients, VGC (n=19) was not inferior to intravenous GCV (n=18) as pre-emptive therapy with rates of viral clearance at 28 days after the start of therapy of 89.5% and 83% respectively. Similar toxicities were reported between the two arms [29]. A retrospective single center German study conducted on 118 allogeneic HSCT recipients demonstrated superiority of VGC (n=48) versus non-VGC (n=70) pre-emptive therapy in terms of viraemia clearance and mean duration of hospitalization, without neutropenia episodes during twice weekly neutrophil count monitoring [30]. Similar results emerged from a two-year prospective, comparative cohort study of CMV infection on 166 allogeneic HSCT recipients in which intravenous GCV,

FOS or oral VGC were given as first-line pre-emptive CMV treatment. VGC was as efficacious and safe as non-VGC treatment without requiring hospitalization [31]. To reduce myelotoxicity, a small retrospective study on 68 allogeneic HSCT recipients, comparing efficacy and safety of standard intravenous GCV (n=24) versus low-dose oral VGC (n=44) in pre-emptive treatment of CMV infection**,** demonstrated lower incidence of low-grade neutropenia and high-grade thrombocytopenia without any significant differences in viral titers between the two groups [32]. Conversely, Takahata et al. in a recent non-inferiority study on 38 HSCT patients, observed that by reducing the standard dosage of VGC from 900 mg twice daily (n=18) to 900 mg once a day (n=20) there was no statistically significant difference in myelotoxicity [33].

2.1.3 Foscarnet (FOS) – neutropenia-free but nephrotoxic.

Neutropenic patients prior engraftment or during GCV/VGC therapy or infected with UL-97- resistant CMV strains are commonly treated with FOS [20, 34]. A virustatic agent that inhibits viral DNA polymerase, FOS does not cause myelotoxicity [35] but primarily causes renal side effects with renal impairment and electrolytes imbalance. Seizures and local genital irritation/ulceration [36] are also occasionally seen. FOS has been compared to GCV for pre-emptive treatment of CMV infections in a randomized trial with satisfactory results in terms of control of antigenaemia and survival rates with remarkably less neutropenia episodes though renal toxicities were relevant [37]. Though rare, FOS resistance is mediated by mutation of the UL54 gene and it might also develop after a short drug exposure [38].

2.1.4 Cidofovir (CDF) – the last resort.

CDF is a nucleotide analogue that does not require viral phosphorylation for its activation with a favorable pharmacokinetic profile. It is considered a third line drug, because of its important nephrotoxicity, gastrointestinal and ocular morbidities such as uveitis, retinal detachment and chronic ocular hypotension. To decrease nephrotoxicity, CDF is usually infused with Probenecid (PBC), an inhibitor of organic anion transport. Administered orally with each dose of CDF, PBC blocks active renal tubular secretion of CDF. Side effects like nausea and vomiting are generally mild; Hypersensitivity reactions can also occur. The Infectious

Disease Working Party of the European Society for Bone and Marrow Transplantation reported the largest recipient series of allogeneic HSCT treated with CDF. Mostly given at 5 mg/kg/week in both primary or secondary pre-emptive therapy for CMV infection with response rates of 50% for CMV disease and 62% to 66% for CMV infection, significant renal toxicities were reported [39]. Several applications of CDF against different viral pathogens in pediatric patients [40] have been reported in recent years, in particular against adenovirus [41], BK virus-associated hemorrhagic cystitis [42, 43] and poxvirus [44].

2.2 Antiviral drug resistance

CMV antiviral drug resistance among allogeneic HSCT recipients is an uncommon but feared complication because it is associated with poor clinical outcomes and potential graft-loss. Its incidence is estimated around 2-8% of allograft recipients [45] and it is generally associated with prior prolonged exposure, at least six weeks, to the antiviral drug and persistent reactivation episodes which usually lead to the identification of resistant strains [46]. Antiviral drug resistance should be suspected in the presence of poor clinical response and progressive rise in viral load for more than fourteen days or with stable serum CMV-DNA levels despite antiviral therapy. In this case, genotypic testing for sequence analysis of UL97 – phosphotransferase - and UL54 - polymerase - genes and switch to an alternative antiviral drug should be recommended. UL97 kinase mutations, that alter the phosphorylation process, are the most represented cause of CMV-antiviral resistance, Seven most common UL97 mutations account for over 80% of GCV-resistant CMV strains whereas UL54 mutations are generally associated with FOS and CDF- resistance though cross-resistance to GCV may be also observed [47]. Newer technologies such as next-generation sequencing are being explored for the detection of genotypic resistance [48]. CMV multidrug-resistance with mutations in both UL97 and UL54 genes usually results from UL54 cross-resistance [49, 50]. At present, there are no standardized therapeutic approaches and new antiviral drugs targeting alternative CMV key structures are needed [51].

2.3 The others: Artesunate and Leflunomide

2.3.1 Leflunomide (LFN) – not only for rheumatic arthritis.

An FDA-approved drug for rheumatoid arthritis, LFN is an oral compound with good bioavailability immunosuppressive properties (its active metabolite, teriflunomide, is a strong lymphocyte proliferation inhibitor) and antiviral activity against a number of viruses [52]. Its mechanism of action resides in the ability to interfere with the assembly of the virionic capside without cross-resistance activity with current anti-CMV compounds. Its side effects includes gastrointestinal toxicity with diarrhea, liver toxicity and polyneuropathy. Given the relatively slow onset of complete antiviral action, the drug is not however ideal for infections with rapidly increasing viral loads [53]. Its proper dosage, timing and duration of treatment for CMV need further investigations in future randomized studies [54].

2.3.2 Artesunate – the antimalarial.

It is an artemisinin-derived monomer able to inhibit *in vitro* CMV replication in human foreskin fibroblasts at micromolar concentrations through cell cycle modulation with an early arrest in G1 phase [55]. Cell cycle modulation via cyclin-dependent kinases and retinoblastoma protein appears to play an important role in artemisinins activities [56]. Unfortunately, there is not yet a thorough evaluation of its efficacy but only sporadic reports of its *in vivo* anti CMV activity with contrasting results [57]. Interesting insights come from artemisinin-derived dimers, novel compounds with more potent *in vitro* anti-CMV effects [58, 59] the efficacy of which remains to be addressed.

2.4 The new drugs: Maribavir (MBV) , Letermovir (LMV) and Brincidofovir (BDF).

Three multicenter, randomized, placebo-controlled phase II, proof of concept studies [60, 61, 62] led to the identification of three new compounds with anti-CMV activity (Table I).

2.4.1 Maribavir – a false start.

A benzimidazole riboside compound that, unlike GCV, is a direct inhibitor of the UL97 protein kinase. It is active against GCV- and CDF-resistant CMV strains. Its bioavailability is greater than that of oral GCV, but less than that of VGC. It is not associated with hematological or renal toxicities. Its most relevant side effects are dysgeusia and nausea. Strengthened by encouraging results from a phase II study [60] and granted fast track status drug, MBV was tested in a subsequent multicenter, randomized, double blind, phase III study, conducted on 681 HSCT patients. Rather surprisingly, it failed to demonstrate superior efficacy to prevent CMV disease as compared with to placebo [63]. Explanations may partly be due to the choice to adopt CMV end organ disease as the primary study endpoint and the exclusions of parameters such as viral load or start of pre-emptive therapy, the exclusion of high risk patients, the possibly too low dose of MBV (100 mg twice daily) employed [64, 65]. Not conclusive results were reported in a randomized, multicenter, double-blind study on a cohort of 303 CMV-seropositive liver transplant recipients with CMV-seropositive donors where prophylaxis with MBV at 100 mg twice daily was compared to oral GCV. The non-inferiority endpoint of MBV in preventing CMV disease was not reached. Moreover, significantly fewer CMV events – both as overt disease or increased viral load – were registered in the GCV arm both at day +100 and at six months post transplant [66]. Opposite results came from a recent French report on a cohort of 12 transplant recipients – including three HSCT – with resistant CMV strains. Treatment with MBV at a minimum daily dose of 800 mg was successful in seven patients without significant toxicities [67]. To address the issue as whether there is still a role today for MBV in CMV disease prevention and treatment in HSC and SOT patients, a phase II (NCT01611974), double blind, randomized, dose-ranging study (with high dose MBV at 400 mg BID, 800 mg BID and 1200 mg BID) was recently completed and final results are eagerly awaited. Furthermore, warnings on a rapid rise of CMV strains resistant to high MBV doses have recently been published [68]. Different isoforms of kinase pUL97 greatly affect susceptibility of CMV to MBV efficacy [69]. There is generally no overlap between the kinase ATP binding site mutations and the UL97 mutations, that respectively confer MBV and GCV resistance, with the exception of a single p-loop

mutation (F342S) involved in dual resistance to both drugs, even though it has not yet been observed *in vivo* [70, 71].

2.4.2 Letermovir - the terminaSEtor.

Member of the new antiviral class of quinazolines, it acts after viral DNA synthesis by inhibiting the subunit protein pUL56 that, together with pUL89, is a key element of the enzyme complex named terminase,[72, 73] directly involved in the cleavage and package of viral DNA chains in the virionic capside [74, 75]. Because of its distinct mechanism of action, it does not show cross-resistance with other antiviral drugs and there are reports of clinically relevant activity against GCV-, FOS- and CDF-resistant CMV strains [76, 77]. Given its virus-specific, human-cell sparing mechanism of action, it has not been associated with any clinical significant side effects. LMV was studied as CMV prophylaxis in a randomized, placebo-controlled phase II study on 131 CMV-seropositive allogeneic HSCT recipients, across various US and German transplant centers, at three different dosages - 60, 120 or 240 mg once a day. It showed higher efficacy in reducing plasma CMV-DNA levels as compared with placebo, with the greatest anti-CMV effect at the 240 mg dose. Failures were recorded only in 21%, 19% and 6% of patients, at the 60, the 120 and the 240 mg dose, respectively, as compared with 36% in the placebo arm. Of note, the tolerability profile of the drug was reported to be excellent, with no hematological or renal toxicity [61]. Efficacy and safety of this compound were similarly investigated in a recent phase II study performed on 27 kidney transplant recipients, with LMV at 40 mg BID or 80 mg once daily in comparison with the current standard of care (SOC). Viral clearance was reached in 50% of treated patients as compared with 29% in those receiving SOC [78]. A phase III randomized, placebo-controlled, multicenter study conducted by Merck (that acquired the rights to develop and market the drug in 2012) on the prevention of CMV infection and CMV disease in allogeneic HSCT recipients, is currently recruiting patients. Primary endpoint of this study is to compare the CMV infection rate between the two arms through week 24 after transplant. The study is expected to

complete the accrual in January 2017 for primary outcome (NCT02137772). Overall, *in vitro* LMV resistant CMV strains have been reported [79, 80, 81].

2.4.3 Brincidofovir (CMX001) – the fatty one.

Originally developed as an agent against the hypothetical biological warfare with smallpox virus, this drug has potent *in vitro* activity against a wide range of double-stranded viruses, including CMV. This is a lipid pro-drug of CDF, which is intracellularly converted into CDF diphosphate and inhibits DNA polymerase of adenoviruses, polyomaviruses, orthopoxviruses and herpesviruses. Given its high oral bioavailability and long half-life, it can be administered twice weekly by oral route. Its lipophilic nature, obtained with the addition of a lipidic side chain to the parent compound, allows the absorption through plasma membranes, reducing the amount of circulating drug and avoiding damage to renal tubules [82]. In a multicenter double blind, placebo-controlled, phase II dose-escalation study on a cohort of 230 allogeneic HSCT patients, BDF reduced incidence of CMV infection and CMV disease in those patients who received BDF at doses of 100 mg weekly or higher as compared with those who received placebo [62]. Of 15 patients in the study cohort who developed GvHD and required systemic steroid treatment, only one developed CMV reactivation. Overall, at 100 mg twice weekly, the drug was well tolerated and efficacious, without increasing myelosuppression or nephrotoxicity. Its principal side effect was significant diarrhea in over 50% of the patients treated with 100 mg twice weekly which became dose-limiting at 200 mg twice weekly. This toxicity was likely determined by an excessive concentration of CDF in enterocytes as reported in animal models [83]. The SUPPRESS trial, a phase III randomized multicenter, placebo-controlled (ratio 2:1), on 450 CMV seropositive allogeneic HSCT recipients treated with BDF at a dose of 100 mg twice weekly has recently stopped recruiting patients. Results are expected in 2016 (NCT01769170). BDF is also being evaluated in two randomized, double-blind, multicenter, phase III registered clinical trials for the prevention of CMV-disease in CMV seropositive (SURPASS trial - NCT02439957) and seronegative (SUSTAIN trial - NCT02439970) kidney transplant recipients. Furthermore, BDF is under investigation for its potential

antiviral activity against other types of viral pathogens such as adenovirus and herpes simplex virus [84], BK polyomavirus in immunocompromised, mostly transplant, patients [85]. Preliminary data from transplant patients who received BDF for adenovirus infections in an ongoing multicenter open-label phase III trial are encouraging in terms of efficacy and safety (NCT02087306).

3. Adoptive T-cell therapy

In the HSCT setting, several factors weaken the host immunologic defense. The intensity of the conditioning regimens, prevention/treatment of GvHD, often with high dose steroids, and the long process of immune-reconstitution are all associated with the risk of viral infections. *In vivo* expansion and persistence of CMV-CTL is crucial for an appropriate immune response both in the early and late post-transplant phases until the establishment of stem cell-derived immunity. Soon after transplant, most CMV-CTLs are of donor origin, while newly "educated" endogenous T cells from thymic output appear only later [86]. In haploidentical HSCT, early T-cell recovery is primarily based on peripheral expansion of naïve T cells and it appears delayed when compared with that of HSCT from HLA-identical siblings [87-90] Long-term immune reconstitution, however, mostly thymus-dependent, appears appropriate to maintain an adequate naïve T cell pool [91-94]. Adoptively transferred CMV-CTL can be detected long after HSCT and up to 2 years after infusion [95]. There are two main ways to obtain virus-specific T cells (VSTCs): a) *in vitro* expansion and b) *ex vivo* separation and *in vivo* expansion (Fig.1).

3.1 Ex vivo culture

The first is a culture-based technique with many amplification procedures to increase the specificity of T cells after repeated *in vitro* expansions. It is a time-consuming and costly procedure but with the advantage of generating a large number of T cells (> 10^7/kg) with the desired antiviral specificity regardless of the

host immunity. Given the long *in vitro* process, the expansion should be carried out prior to the transplant in selected all high-risk patients as only a few of these patients will eventually need this treatment.

One of the most significative experience on HSCT patients who received donor CMV-VSTCs and compared with a control group was reported in 2013 [96]. The endpoint was to evaluate if prophylaxis with CMV-specific T cells could provide short- and long-term protection against CMV infection. VSTCs were generated through dendritic cells genetically modified with an adenoviral vector encoding the full pp65 antigen or pulsed with a HLA2-restricted immunodominant peptide from the CMV pp65 antigen. One major limitation was however the risk of escape mutants among the VSTCs. Overall, there was a reduction in the percentage of patients who required CMV antiviral therapy and in a number the duration was shorter. Importantly, there was not an increased rate of acute or chronic GvHD attributable to VSTCs as compared to the control arm - two cases of acute grade III-IV GvHD , and an overall incidence of chronic GvHD of 42%.

3.2 Direct ex vivo selection techniques.

Techniques for direct selection of VSTCs include the use of peptide pools derived from viral antigens to expand T cells with multiple antigen specificities [97], the selection of VSTCs based upon the secretion of interferon-gamma (IFN-gamma) [98, 99] or the binding to class I HLA-multimers [100] or immunomagnetic beads [101]. The multimer selection method requires HLA-specific elements for every viral epitope and is actually restricted to CD8+ T-cells, while the IFN-gamma secretion technique is based upon a HLA-unrestricted selection of CD4+ and CD8+. Both techniques require a considerable volume of donor blood and imply the fact that only viruses with a high frequency of circulating T-cell precursors can be targeted. The number of recovered cells is usually small. Multimer selection technique was first exploited by Cobbold [102] with a direct selection technique by using a panel of CMV IE1 and pp65 tetramers to select specific T cells from HSCT donors. Later, Uhlin and colleagues [101] described a separation technique based upon positive selection with HLA-pentamers and magnetic beads that bind to CD8+T-cells reactive to CMV, EBV and adenovirus from donor lymphocyte infusion and peripheral blood of haploidentical donors. A total of 8

patients with infections caused by different viruses were treated. Six out of 8 showed a decrease in viral titers within two weeks post-VSTCs infusion. Overall, the study emphasized the need for preemptive rather than therapeutic use of immune therapy after allogeneic HSCT. Concerns have been raised about the potential clonal exhaustion of the multimer-bound T cells after a prolonged HLA-peptide/T-cell receptor interaction. In an attempt to overcome this problem, Schmitt and colleagues [103] utilized streptamers that could be dissociated from T cells by the addition of a competitor molecule and injected CD8+T-cells isolated with this technique into two allogeneic HSCT patients with CMV refractory disease. In both cases there was an increase in reactive cells and CMV viral load clearance, without GvHD. Another method to select VSTCs is based upon the ability of memory T cells to secrete IFN-gamma in an antigen-dependent manner. Feuchtinger first reported this method in severe adenovirus infections in pediatric patients and later in CMV refractory infection and disease which included two cases of CMV-encephalitis, in T-cell depleted allogeneic HSCT [98]. In 2011, in a phase I/II study, Peggs and colleagues described the use of IFN-gamma captured CMV pp65-specific T cells as prophylaxis and preemptive CMV-treatment with increase of both CD4+ and CD8+ T-cells. However, 8 cases of acute GvHD in the 18 patients treated and 3 cases of chronic extensive GvHD [99] that posed relevant safety issues were reported. IFN-gamma selection technique was also used in ATCT for clinically relevant adenovirus infection in allogeneic HSCT patients, with promising results [104]. Two randomized studies, recently completed, exploited direct selection of VSTCs through the streptamer and IFN-gamma CMV-CTL selection methods in T-cell depleted HSCT from CMV-seropositive donors: the IMPACT (NCT01077908) and the ASPECT trials (NCT01220895). The first is a multicenter, prospective, controlled, open-label phase III study of CMV-prophylaxis in T cell depleted HSCT from sibling donors with VSTCs selected by both multimer and IFN-gamma selection. T-cells were administered on day 27 post transplant and primary objectives of the study were the number of CMV reactivation episodes and GvHD incidence. The ASPECT trial is a randomized, multicenter open label phase II study of pre-emptive adoptive CMV cellular therapy where T cells were collected by multimer selection technique in recipients of unrelated donor transplants. In this study, the VSTCs selection occurred during the stem cell collection procedures. Primary end point was to establish the efficacy of pre-emptive VSTCs with CMV-specific T cells and their post infusion *in vivo* expansion. Results of both studies are expected early in 2016.

3.3 Recent developments: Third-party VSTCs and naïve donors T-cells.

The ability to isolate and expand VSTCs from seronegative donors or cord blood units may represent a clinical issue. Potential solution in this scenario is the so called “third party bio-banks" of VSTCs where they can be selected by HLA haplotypes [105]. The first multicenter trial of ATCT with cells obtained from banked third-party VSTCs for the treatment of refractory viral infections after HSCT was reported by Leen et al. in 2013 [106]. The Authors developed a bio-bank of 32 virus-specific lines from volunteers with common HLA polymorphisms against Epstein-Barr virus, CMV, or adenovirus. Eighteen lines were administered to 50 HSCT patients with refractory viral disease. Cumulative incidence of complete or partial responses was overall 74%: 73.9% for CMV (n = 23), 77.8% for adenovirus (n = 18) and 66.7% for EBV (n = 9). Only four responders had recurrence or progression. GvHD developed in eight patients (two of them showed *de novo* GvHD, and in only one of grade 3) confirming previous observations [107, 108]. Most interestingly, the VSTCs used in this study were "off-the-shelf" and only partially HLA-matched. If the low rate of GvHD is confirmed in further studies, this treatment option will have the potential to be widely employed given its rapid action and immediate efficacy. Nevertheless, it is important to point out that the results of this trial must be validated also in more compromised patients with active acute GvHD; or treated with T-cell–depleting monoclonal agents such as anti-thymocyte globulin or alemtuzumab, or more than 0.5 mg/kg/day of prednisone. T-cells restricted by specific HLA alleles exhibited different clinical activity and certain HLA alleles were more capable of inducing clearance of CMV disease/infection as recently reported by O’Reilly et al. [109]. Notably, the same group also proposed the use of artificial antigen-presenting cells (AAPCs) to more easily generate VSTCs for the treatment of infections in HSCT recipients. AAPCs consist of genetically modified murine cells, expressing human molecules required for T-cell stimulation such as ICAM-1 and LFA-3. VSTCs sensitized with AAPCs not only recognize well known immunogenic HLA epitopes but also subdominant epitopes, generally not recognized by autologous APCs [109]. There are two interesting phase II studies currently recruiting - one conducted by Prockop and colleagues at Memorial Sloan Kettering Cancer Center, the other by Betul and colleagues at MD Anderson Cancer Center (NCT02136797 and

NCT02210078) - on the role of third party donor derived T-cells in CMV disease/infection in allogeneic HSCT recipients. These studies are expected to be completed in 2017- 2018. T-cell products able to recognize unusual epitopes of different pathogens would potentially be a valid tool to avoid immune escape when donor and recipient T-cells are not fully HLA matched. The naïve donor T-cell compartment may be of interest in this setting given its propensity to generate a broad spectrum of immune control over several pathogens [110]. In 2011, a study made by Jedema first described a method of *in vitro* generation of antigen-specific CD8+ T cells obtained from a naïve T-cell donor repertoire. This method implied the depletion of CD45R0+ T cells that resulted in increased antigen-specific naïve T cells, but its poor reproducibility hampered its wide application [111]. In 2012 Hanley et al. demonstrated that multivirus-specific T-cells (against Adenovirus, EBV and CMV) from naïve T-cell populations, from both cord blood and peripheral blood of seronegative donors, are protective *in vivo* despite their unusual atypical epitope repertoire. VSTCs, mostly CD8+, were obtained with the use of a protocol based on both professional antigen presenting cells and activating Th1-polarizing cytokines [112, 113]. A recently published phase I feasibility study demonstrated the feasibility of generating CMV pp65-specific T-cells from CMV-negative individuals and cord blood units, and the ability of naïve-origin T-cells to recognize atypical epitopes of pp65. Given its phase I design, definitive conclusions cannot be drawn. The preliminary results are however encouraging and should be confirmed in larger studies [114]. Preliminary results of the currently ongoing clinical trial MUSTAT (Multivirus-Specific Cytotoxic T-Lymphocytes for the Prophylaxis and Treatment of EBV, CMV, and Adenovirus Infections post Allogeneic Stem Cell Transplant; NCT01945814) that compares clinical efficacy of CTLs derived from CMV-seropositive versus CMV-naïve donors are eagerly awaited.

4. CMV vaccines

Vaccine-induced immunity with safe and immunogenic compounds represents a feasible way to reduce the rate of CMV reactivation/disease in high risk patients. However, the precarious state of immuno-

competence of HSCT recipients with altered lymphocyte repertoires and antigen-presenting cells remains a barrier that hampers the efficacy of this strategy [115]. It is clear that robust protection against CMV relies on both cellular and humoral immunity and the ideal vaccine should be able to elicit a strong stimulation of both the adaptive and the natural immunity compartments [116]. Interesting clinical experiences came from two studies. In a randomized, placebo-controlled phase II clinical trial on SOT - 140 kidney or liver transplant patients - a subunit vaccine made up of purified glycoprotein B protein coupled with MF59 adjuvant led to a significantly shorter duration of viraemia, defined as viral loads higher than 200 genome/mL of blood, and a shorter duration of anti-viral therapy as compared with the placebo group. A strong antibody activity was seen without, however, a T cell-immunity involvement that determined a short durability of the immune response [117]. A second study employed a vectored vaccine, also known as ASP0013 or TransVax, with plasmids encoding CMV glycoprotein B and phosphoprotein pp65. This vaccine is employed in the only approved phase III study of vaccine-immunotherapy against CMV in the setting of hematopoietic stem cell transplantation. This trial is currently in progress and its completion is expected in 2017. A previous phase II, multicenter, randomized, placebo-controlled, double-blind study, was developed to test efficacy, safety and immunogenicity of ASP0113. Patients enrolled in the study were CMV-seropositive recipients of a myeloablative or a reduced-intensity HSCT for hematological malignancies. They were randomly assigned with a 1:1 ratio to receive CMV therapeutic vaccine (n=48) or placebo (n=46) on a four times schedule injections – the first before, the other three after the transplant. The vaccine was safe and only one patient discontinued it because of a minor allergic reaction. Though the primary endpoint of the study, the reduction of CMV viraemia requiring CMV-specific antiviral therapy, was not reached, there was a reduction in CMV viraemia episodes, defined as CMV copies >500/mL in the blood, in the vaccine arm. The immunogenicity analysis showed a statistically not significant increased rate of pp65, IFN-gamma producing T-cells in the vaccine cohort without a clear involvement of the B-cell compartment [118, 119]. An interesting, currently on going, multicenter phase II randomized, double-blind, placebo-controlled study in HSCT recipients has been designed to evaluate efficacy of another vectored vaccine, PepVax, containing plasmids encoding for pp65 combined with a toll-like receptor 9-agonist (NCT02396134). Primary endpoint of the study is the incidence rate of CMV reactivation/infection or CMV disease up to day 100 after HSCT.

The estimated completion date is 2019. Further progress in the scientific knowledge on CMV cell cycle and its biology currently offers potential novel approaches in vaccine-immunotherapy. CMV enters the host cells through two different pathways: CMV enters fibroblast by employing glycoproteins gB and gH/gL, whereas to enter epithelial and endothelial cells an additional five-member protein complex, composed of gH, gL, UL128, UL130, and UL131A, referred to as the gH/gL-pentamer complex, is required. Neutralizing antibodies that prevent gH/gL-pentamer complex mediated CMV entry into epithelial cells (AbNEIs) are putative candidates for an *in vivo* protective role against CMV infection [120, 121]. Recently, Gimenez et al. addressed the potential role of CMV-specific AbNEIs in CMV infection control in allogeneic HSCT patients by using a neutralization assay. The results did not confirm the protective role of AbNEIs both in prevention and clearance of CMV-DNAemia. AbNEIs levels did not correlate with CMV-DNAemia nor with viral load kinetics. The observation that patients with high baseline and peak AbNEIs levels were more likely to develop CMV-DNAemia was of interest. Possible explanations include a major role played by memory B cells of donor and recipient origins [122]. The gH/gL-pentamer complex represents the platform for prophylactic Pentamer-based vaccines of absolute interest [123, 124].

5. Expert commentary

The development of new tools against CMV infection/disease in HSCT recipients has been very active in recent years. Clinical trials now in progress have been designed to define the role of new antiviral compounds and to replace the currently broadly employed antiviral drugs associated with important toxicities and growing inefficacy due to mechanisms of drug-resistance. New anti-viral drugs such as BDF and LMV showed promising results in phase II studies and are being evaluated in phase III clinical trials. ATCT may play a role in the future because of its promising mechanism of action, able to sunder specific antiviral T-cell response from significant alloreactivity. Different techniques for isolation and expansion of CMV-specific T-cells are emerging as the knowledge on immune-reconstitution post allogeneic HSCT and CMV biology is expanding. The ability to determine and monitor CMV-CTL levels in the blood of HSCT

recipients would be a useful tool to identify patients with poor CMV-specific immune reconstitution at higher risk of developing CMV infection/reactivation and overt disease [125, 126]. In selected patients such as recipients of T-cell depleted grafts, ATCT, based on CMV-CTL reconstitution kinetics, would be of value in the prophylactic and/or preemptive setting. Third party and pathogen-naïve donor CTLs could represent an alternative option for patients who undergo cord blood transplants or receive grafts from CMV-seronegative donor [127]. Vaccine immunotherapy may soon become a clinical reality although concerns about its real efficacy in highly immuno-compromised patients such as HSCT recipients raise legitimate doubts.

6. Five-year view

A future paradigm may include first line antiviral therapy with one of the current investigated agents (LMV, BDF or MBV) and, in case of treatment failure and/or progressive CMV disease, ATCT may play a role as salvage strategy. ATCT may allow shorter courses of antiviral drug prophylaxis to stimulate cellular immune response. The creation of “bio-banks” with HLA-typed T-cells from peripheral blood of healthy donors specifically "armed" against different viruses that could readily be used in HSCT recipients without seropositive donors appear a promising approach. The use of VSTCs in HSCT patients with GvHD is currently under investigation with some promising reports. Menger recently elaborated a proof of concept study [128] based upon the genetic disruption of steroid cellular receptor in streptamer-selected CMV-specific CD8+ T cells, adopting the technique of transcription activator-like effectors nuclease (TALEN) messenger RNA, through electropermeabilization. The modified VSTCs showed resistance to steroid-induced apoptosis whereas they preserved their CMV-specific killing ability. However, there are concerns about the possibility of conferring steroid-resistance to CD8+ T-cells responsible of GvHD. The possibility of inserting suicide genes capable to interrupt VSTCs activity by inducing apoptosis has not been explored. In another recently published, phase I study in the setting of haploidentical HSCT, Zhou X et al. [129] employed haploidentical T-cells with inducible human caspase 9 (iC9) suicide gene to delete the alloreactive T-cells responsible of

GvHD, sparing the virus-reactive T cells even during GvHD treatment. The authors demonstrated that alloreplete iC9-T cells provide protection against EBV, CMV, human herpesvirus-6, Varicella zoster virus and BKV infections, with a possible role played by the recovery of endogenous T cells. This hallmark study may highly contribute to the future management of viral infections/disease in HSCT patients with life-threatening GvHD.

7. Key Issues

- The use of current antiviral drugs for CMV is hampered by potentially severe side effects and by the growing issue of drug-resistance.
- New antiviral compounds, currently under investigation, appear promising for their efficacy against CMV-resistant strains and for their low toxicity profile both in the prophylactic/pre-emptive and therapeutic settings.
- In the next future, ATCT, possibly combined with shorter courses of new antiviral compounds in the prophylactic/pre-emptive setting will be likely to play an important role especially in high-risk patients.
- Third-party virus-specific T-cells, possibly with the creation of VSTCs “bio-banks”, will be crucial for the treatment of overt CMV disease in critically ill patients.
- CMV-vaccine strategies may become key factors to reduce incidence rate of CMV reactivation/infection in immuno-compromised patients such as HSCT recipients.

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Appendix

2. Table I. Principal characteristics of the newer anti-CMV compounds.

Drug	Route of administration	Mechanism of action	Side efffects	Dosing	Main publications
Maribavir	Per os	Inhibition of CMV protein-kinase UL97	Gastrointestinal: dysgeusia and nausea, vomiting.	From 400 mg to 1200 mg twice daily	.Winston et al. Blood. 2008 (60) .Marty et al. Lancet Infect Dis. 2011 (63) .Winston et al. American Journal of transpl. (66) .clinicaltrialsgov: NCT01611974
Letermovir	Per os	Inhibition of terminase complex subunit UL56	Gastroenteritis, nasopharyngitis, dyspnea, and elevation in serum creatinine	120 mg or 240 mg once-daily	. Chemaly et al. NEJM. 2014 (61) .clinicaltrialsgov NCT02137772)
Brincidofovir	Per os	Inhibition of DNA polymerase	Gastrointestinal: diarrhea (dose limiting at 200 mg twice weekly)	100 mg twice weekly	. Marty et al. NEJM. 2013 (62) .clinicaltrialsgov NCT02137772

Figure 1.

Notes: A) Ex-vivo expansion. In the classic process antigen presenting cells (APC) are transduced with viral vectors or plasmids encoding antigens of interest. APCs are then combined with T cells to stimulate them until a sufficient number have been expanded. B) Direct selection techniques: multimer selection. In this case T cells are incubated with HLA multimers (tetramers, pentamers or, more recently, streptamers) that resembles the peptide binding HLA-mediated. The complex multimer-T cells is then isolated with magnetic beads or cell sorting. C) T cells are stimulated utilizing virus-derived overlapping peptides. Activated T cells secrete interferon gamma (IFN-gamma). Virus-specific T-cells are then immune-magnetically selected.