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Chapter 1

General introduction and aim of the thesis

Allogeneic stem cell transplantation

Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment for various malignant and non-malignant hematological diseases[1]. The goal of alloSCT is to replace the recipient hematopoietic cells with hematopoietic cells derived from stem cells from a human-leukocyte-antigen (HLA) matched donor. Immune suppressive conditioning of the recipient prior to the alloSCT is necessary to allow engraftment of the donor stem cells. This conditioning leads to a period of profound pancytopenia prior to engraftment of donor hematopoietic cells. The duration of this pre-engraftment period is correlated with the occurrence of infectious complications[2]. Conventional myeloablative conditioning (MAC) regimens aim to fully eradicate the hematopoietic cells of the recipient. Reduced intensity conditioning (RIC) regimens aim to allow engraftment of donor hematopoietic cells without full elimination of recipient derived hematopoietic stem cells. RIC regimens are less toxic, but additional immune suppression is necessary to allow engraftment of donor hematopoietic cells leading to additional immune deficiency[3,4]. The mere ablation of recipient hematopoietic cells by chemotherapy and/or irradiation in the conditioning regimen is not sufficient to prevent disease relapse after transplantation, as demonstrated by the high risk of disease relapse after autologous stem cell transplantation in acute leukemia. Long-term control of the disease can be achieved by donor derived alloreactive T cells. These alloreactive T cells can eradicate residual malignant cells by inducing a graft versus leukemia (GVL) response when the immune response is directed against the hematopoietic cells of the patient[5,6]. However, when alloreactive T cells also target non-hematopoietic cells in the tissues and organs of the patient, potentially fatal graft versus host disease (GVHD) can occur. GVL is part of a spectrum of GVHD, as illustrated by the increased risk of disease relapse in the absence of GVHD in alloSCT using stem cells from an HLA identical syngeneic twin[7].

Prevention of GVHD

GVHD can be prevented or reduced by long-term immune suppression or by depleting donor T cells from the graft (T cell depletion, TCD)[8-10]. In non-TCD alloSCT, recipients are treated with long-term immune suppression, which may be tapered in the months or years after alloSCT. Immune suppression is not selective and suppresses not only alloreactive immune responses causing GVHD, but also potentially beneficial immune responses causing GVL or immune responses needed for protection against infectious diseases. In TCD alloSCT strategies, donor T cells are depleted from the stem cell graft or depleted in-vivo by infusion of T cell specific antibodies (e.g. ATG, alemtuzumab). Various methods are used to deplete T cells from the graft such as $CD34^+$ selection or the use of lymphocyte-depleting antibodies such as alemtuzumab (anti CD52)[11,12]. TCD strategies are effective in preventing GVHD and long-term post-transplant immune suppression is generally not required. The absence of immune suppression makes TCD alloSCT suitable as a platform for cellular therapy such as donor lymphocyte infusion (DLI) or adoptive cell transfer (ACT) of selected or manipulated T cell populations. Postponed application of DLI several months after TCD alloSCT to induce

a GVL effect is associated with an acceptable risk of GVHD[13-15]. In ACT strategies, in-vitro selected T cell populations are adoptively transferred to restore (anti-viral) immunity. By selecting specific T cells with a defined antigen specificity (e.g. targeting viral epitopes), the risk for inducing GVHD is lower compared to unmodified DLI containing T cells of unknown specificity[16, 17].

Inherently to the effect of TCD on the prevention of GVHD, a period of profound and prolonged T cell deficiency follows TCD. During this period patients are at risk for developing infectious complications, especially for reactivations of endogenous herpes viruses.

Herpes virus reactivations after alloSCT

Infections with herpes viruses are common in the general population. These infections usually occur during childhood and the clinical symptoms are often mild or even absent. The infection is controlled by virus-specific memory T cells, which develop following a primary immune response. Although virus-specific T cells control these viruses, herpes viruses are not completely cleared and lead to latent infections in their hosts. This latency results in an equilibrium between these viruses and the virus-specific T cells. The most common herpes viruses complicating alloSCT are cytomegalovirus (CMV), Epstein-Barr virus (EBV) and varicella zoster virus (VZV)[18-20]. These viruses share the ability for lifelong persistence and reactivation when T cell immunity fades. T cell immunity is provided by $CD4^+$ and $CD8^+$ T cells, where $CD4^+$ T cells regulate immune responses and $CD8^+$ T cells eliminate the pathogens. $CD4^+$ T cells recognize peptides presented in HLA class II molecules, that are primarily expressed by antigen presenting cells (APCs), whereas $CD8^+$ T cells recognize peptides presented in HLA class I molecules that are ubiquitous expressed on all human tissues. Professional APCs are required for the induction of a primary T cell response leading to a rapid increase of effector T cells and the formation of memory T cells that can react directly upon re-encounter with the pathogen. In CMV and EBV infections, repeated stimulation of memory T cells by reactivation of the virus can result in frequencies of up to 40% of these virus-specific T cells within the T cell compartment in peripheral blood in immune competent individual[21, 22]. VZV resides in an immune privileged site and does not reactivate as often as CMV and EBV. VZV-specific memory T cells are therefore not stimulated repeatedly leading to decreasing frequencies of circulating VZV-specific memory T cells in time[23].

In the period of profound and prolonged T cell deficiency after (TCD) alloSCT the equilibrium between the T cells and the virus is lost and control of reactivation of CMV and EBV infection is impaired. The impaired control may lead to potentially fatal CMV disease in case of CMV reactivation or Post Transplantation Lymphoproliferative Disease (PTLD) after EBV reactivation, caused by uncontrolled proliferation of EBV infected B cells. The decline in VZV-specific memory T cells is accelerated by the conditioning and/or TCD leading to an increased risk for reactivation of VZV leading to herpes zoster. Uncontrolled herpes zoster due to insufficient VZV-specific T cells may lead to potentially fatal disseminated herpes zoster.

Cytomegalovirus

Cytomegalovirus, a double stranded DNA virus, can infect a broad range of cell types upon primary infection. Primary infection is followed by a lifelong persistence with monocytes and vascular endothelial cells as important sites for latency[24]. The clinical course of CMV infection in immune competent individuals is generally asymptomatic or mild and self-limiting with the exemption of congenital neurological disease by maternal transfer of the virus in primary CMV infection during pregnancy. In immune competent individuals CMV reactivation is controlled by CMV-specific memory T cells. In immune compromised patients, lack of CMV-specific T cells and consequential absence of immune control of CMV reactivation can lead to potentially fatal CMV disease, such as CMV pneumonitis, CMV colitis or CMV encephalitis following CMV infection or reactivation[25]. Reactivation of endogenous CMV is the most frequently occurring herpes virus reactivation following alloSCT with an incidence of 80% in CMV seropositive recipients[19]. Approximately 60% of alloSCT recipients are seropositive for CMV and are therefore at risk for endogenous reactivation of latent CMV virus[26]. CMV infection of a CMV seronegative recipient via a stem cell graft from a CMV seropositive donor occurs, but less frequently because endothelial cells and monocytes, the most important sites for CMV latency and persistence, are not an elementary components of the stem cell graft[27]. $CD8^+$ T cells can be analyzed and monitored using artificial HLA class I constructs loaded with a specific antigen. These constructs consist of multiple HLA molecules (tetramers or pentamers depending on the number of HLA molecules used) combined with a fluorescent label, allowing direct detection using flow cytometry. For CMV several HLA constructs have been developed and studies have demonstrated that presence of CMV-specific $tetramer^+$ $CD8^+$ T cells is directly related with control of CMV reactivation[28, 29].

Epstein-Barr Virus

Epstein-Barr Virus (EBV) is a herpes virus, which infects more than 90% of the population. After primary infection, which may lead to the clinical syndrome of infectious mononucleosis, the virus latently resides in the B cell population[30]. Infectious mononucleosis is caused by a massive expansion of EBV specific T cells upon recognition of an EBV antigen presented in HLA molecules with the goal to control the EBV infection. After alloSCT, reactivation of EBV may occur in the absence of sufficient EBV specific T cell immunity. With failing T cell control, EBV infected B cells can expand massively leading to potentially fatal PTLD. Although the incidence of EBV associated PTLD is low following alloSCT (4%)[31], the risk correlates with the level of TCD. TCD strategies deleting only T cells, the risk increases because B cells, the principle site for EBV latency are not depleted. In TCD strategies using depleting antibodies targeting both T and B cells, such as alemtuzumab, the risk is not increased[32]. Analogous to CMV, also or EBV several HLA constructs have been developed and studies have also demonstrated increased control of EBV reactivation by EBV specific $tetramer^+$ $CD8^+$ T cells[28, 29, 33, 34].

Varicella zoster virus

Varicella zoster virus (VZV) is a herpes virus, which infects about 95% of the population. The primary infection with VZV leads to the clinical entity of varicella (chickenpox). After the primary infection VZV resides latently in neurons and reactivation leads to herpes zoster (shingles). Similar to CMV, cellular immunity is essential for preventing reactivation of VZV. After alloSCT, reactivation of the virus causes considerable morbidity and is potentially fatal in disseminated reactivation[18]. Most frequent complications are post-herpetic neuralgia and peripheral neuropathy. In contrast to CMV little is known about VZV-specific $CD8^+$ T cell immunity because validated VZV-derived immunodominant peptides for HLA class I are lacking[35]. Previous studies demonstrated VZV-specific memory $CD4^+$ T cells but VZV-specific $CD8^+$ T cells were only detectable after in-vitro expansion. The inability to directly detect VZV-specific $CD8^+$ T cells directly ex-vivo may be due to the low frequencies of VZV-specific $CD8^+$ T cells or to the low sensitivity of the screening methods used to detect $CD8^+$ T cells[36-38]. Identification of a VZV derived immunodominant peptide and the construction of VZV-specific peptide-HLA complexes is important to ex vivo analyze the role of $CD8^+$ T cells in the immune responses to VZV infection and reactivation after alloSCT.

Prevention of CMV disease by antiviral medication

In order to prevent CMV disease, a period of profound T cell deficiency after (TCD) alloSCT must be bridged to allow CMV-specific T cell immunity to restore and prevent CMV disease. Bridging this period is possible using antiviral medication. Ganciclovir is a synthetic nucleoside that inhibits DNA viruses, such as herpes viruses and especially CMV, by inhibiting viral DNA polymerase and viral DNA elongation. Ganciclovir is the golden standard for treating CMV disease but has considerable side effects, the most important being bone marrow suppression[39]. Furthermore ganciclovir has poor bioavailability, which precludes oral administration and often necessitates hospitalization for intravenous treatment[40]. Prophylactic use of ganciclovir to prevent CMV disease is therefore not feasible. However, because high viral loads precede the development of CMV disease when patients are still asymptomatic, prevention of CMV disease is possible by pre-emptive administration of ganciclovir[40, 41]. In a pre-emptive treatment strategy, antiviral therapy is initiated when the viral load is above a predetermined threshold. CMV viral load can be detected and monitored by using quantitative PCR[42]. Valganciclovir is an orally administered pre-drug of ganciclovir and suitable for pre-emptive outpatient clinical treatment to prevent CMV disease[43-45]. However, similar to ganciclovir, prolonged usage of valganciclovir is not appropriate for long-term prevention of CMV disease due to adverse effects and possible development of resistance[46].

CMV-specific T cell reconstitution

Restoration of immune control by reconstitution of CMV-specific T cells is required for long-term control of viral replication and prevention of CMV disease[28, 29, 47, 48]. Reconstitution of

CMV-specific T cells can be the result of expansion of recipient memory T cells that survived the conditioning regimen prior to alloSCT or donor memory T cells transferred with the graft. Various factors can influence CMV-specific T cell reconstitution. Immune suppression for prevention of GVHD after transplantation with an unrelated or partially matched donor or treatment of GVHD can impair T cell reconstitution. CMV-specific T cell reconstitution may also be impaired by more intensive conditioning regimens prior to alloSCT due to more profound eradication of residual recipient T cell immunity.

Following transplantation with a CMV *seronegative* donor, the CMV-specific T cells reconstituting after alloSCT are expected to be of recipient origin, because a primary immune response by donor T cells is not likely to occur shortly after alloSCT. Residual CMV-specific T cells of the recipient can be eradicated by alloreactive donor T cells when an immune response is induced after alloSCT and/or Donor Lymphocyte Infusion (DLI), leaving the patient at risk for developing CMV disease. In these patients, development of a primary donor derived CMV-specific T cell response from donor origin would be essential to prevent CMV disease. For a primary CMV-specific immune response, naive T cells recognizing CMV antigens are required. Naive T cells need thymic education, and because the function of the thymus is impaired in (adult) alloSCT patients[49], a primary donor derived CMV-specific T cell response is not expected shortly after alloSCT.

Following transplantation with a CMV *seropositive* donor, CMV-specific T cells can be of recipient and/or donor origin, possibly at the same time leading to a state of mixed CMV-specific T cell chimerism. CMV-specific T cell reconstitution can originate from donor memory T cells transferred with the graft from CMV seropositive donors. Manipulation of the graft by TCD may abrogate this transfer of CMV-specific T cells and increase the risk of developing CMV disease. Eradication of recipient lymphopoietic cells in patients with mixed CMV-specific T cell chimerism by an alloreactive donor T cell response is not expected to be harmful as protection by donor CMV-specific T cells is still present or transferred with the DLI.

Despite pre-emptive antiviral medication, persistent CMV reactivation or CMV disease can occur when CMV-specific T cell reconstitution is not sufficient. Adoptive transfer of donor T cells may be an elegant strategy to enhance T cell reconstitution after alloSCT. However, although this approach may be effective in reconstituting antiviral T cell immunity, it may induce potentially fatal GVHD. To enhance CMV-specific T cell reconstitution and to minimize the risk of inducing GVHD, donor derived CMV-specific T cells can be transferred to the recipient after alloSCT (CMV-specific adoptive cell transfer (ACT)) [50-52]. CMV-specific ACT can be used either as a prophylactic or pre-emptive treatment to prevent CMV disease or as treatment for overt CMV disease. Adoptive transfer of T cells is most effective in the absence of immune suppression, as is in general the case in TCD alloSCT. However, the use of adoptive transfer is not commonplace, as questions regarding safety and efficacy still need answering.

Aim of the thesis

Profound T cell deficiency can lead to reactivation of endogenous herpes viruses after TCD alloSCT. Inadequate control of these viruses by virus-specific T cells can lead to significant complications. Long-term immunity depends on virus-specific T cell reconstitution. In case of CMV reactivation, antiviral medication can bridge the period of T cell deficiency, at the expense of potential toxic side effects. The aim of this thesis is to evaluate several options for preventing CMV disease after T cell depleted (TCD) allogeneic stem cell transplantation (alloSCT). These options include a choice in conditioning regimen and in donor prior to alloSCT, pharmacological intervention following alloSCT and adoptive cell transfer in treatment of refractory CMV reactivation or CMV disease.

In **chapter 2** we aimed to determine the efficacy and safety of oral valganciclovir compared to intravenous ganciclovir to prevent CMV disease after TCD alloSCT in a pre-emptive outpatient strategy. Ganciclovir is associated with hematological toxicity and intravenous administration necessitates hospital admission. Oral valganciclovir is considered to be less toxic compared to intravenous ganciclovir and does not necessitate hospital admission. Efficacy and safety of valganciclovir was already demonstrated in other high-risk populations such as renal- and heart-transplant patients. In this chapter we evaluated the use of oral valganciclovir in preventing CMV disease in 107 consecutive patients following TCD alloSCT. Reduced intensity conditioning (RIC) relatively spares residual recipient hematopoietic cells compared to conventional myeloablative conditioning (MAC). Therefore, reconstitution of CMV-specific T cells may be improved after RIC by sparing residual recipient CMV-specific T cell immunity. In **chapter 3** our aim was to determine whether the incidence and severity of CMV reactivation was affected by the intensity of the conditioning regimen. To determine whether a less toxic conditioning regimen would lead to differences in incidence of CMV reactivation and disease, we compared the frequency and severity of CMV reactivation and the incidence of CMV disease in 107 consecutive patients following RIC or MAC TCD alloSCT. Transplantation with a CMV seropositive donor implies that the donor graft may confer donor derived CMV-specific T cells in contrast to the graft from a CMV negative donor. CMV-specific T cells may be transferred with the graft from CMV seropositive donors and provide protection for CMV disease, but profound TCD can eradicate this transfer of CMV-specific T cells. In **chapter 4** our aim was to determine the effect of donor CMV serostatus on the incidence of CMV disease and T cell reconstitution after TCD alloSCT. We analyzed the incidence of CMV disease after TCD alloSCT in CMV positive recipients transplanted with either a CMV seropositive or seronegative donor. Furthermore we investigated if and when a primary donor derived CMV-specific T cell response could be detected following TCD alloSCT. Demonstrating CMV-specific T cells of donor origin after transplantation with a CMV seronegative donor who lacks CMV-specific memory T cells would be illustrative of the induction of a primary CMV specific T cell response. Therefore, we determined the origin of

CMV-specific T cells in CMV seropositive recipients transplanted with a CMV seronegative donor.

The risk for potentially fatal CMV disease increases if pre-emptive treatment fails to control CMV reactivation and rapid reconstitution of CMV-specific T cells is then pivotal for preventing CMV disease. Adoptive transfer of CMV-specific T cells may be a treatment option in patients failing preemptive anti-viral treatment, although routine application of adoptive cellular immunotherapy is hampered by questions regarding safety and efficacy. Therefore, in **chapter 5** we aimed to analyze the safety and efficacy of adoptive transfer of CMV pp65-specific $CD8^+$ T cell lines to restore CMV-specific T cell immunity in patients with persistent CMV reactivation failing anti-viral therapy. CMV-specific T cells from donor or patient were isolated using an IFNg-based isolation technique, cultured for 1–2 weeks to generate CMV-specific T cell lines, which were transferred to patients with refractory CMV reactivation. Adverse events, clinical effects and CMV-specific T cell reconstitution were monitored to assess the safety and efficacy of adoptive transfer of CMV-specific T cells.

In contrast to CMV-specific $CD8^+$ T cell reconstitution little is known about VZV-specific $CD8^+$ T cell reconstitution. Identification of VZV-derived immunodominant peptides binding in HLA class I and development of VZV-specific peptide-HLA complexes could facilitate analysis of VZV-specific T cell reconstitution. In **chapter 6** we searched for immunogenic antigens for VZV to develop VZV-specific pentamers using a new pentamer-based epitope discovery method. This method has the potential to quickly assess whether part of a protein can be immunogenic by determining the binding affinity with the HLA molecule. Development of VZV-specific peptide-HLA complexes is important to ex vivo analyze VZV-specific $CD8^+$ T cell reconstitution and the immune response to VZV infection, reactivation, and possibly VZV vaccination.

In **chapter 7** we summarized and reviewed recent studies on prevalence and treatment of CMV disease after alloSCT in the era of pre-emptive antiviral treatment. We reviewed literature on the influence of Graft versus Host Disease, unrelated or HLA mismatched donors and TCD on the prevalence of CMV disease. We reviewed studies on the influence of donor CMV status on CMV-specific T cell reconstitution and CMV disease. Recent studies on the safety and efficacy of adoptive transfer of donor CMV-specific T cells for the prevention and treatment of CMV disease following alloSCT are discussed, including studies on adoptive transfer of third-party CMV-specific T cells as a possible alternative when donor T cells are not available.

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Chapter 2

Oral valganciclovir as pre-emptive therapy has similar efficacy on cytomegalovirus DNA load reduction as intravenous ganciclovir in allogeneic stem cell transplantation recipients

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Abstract

The efficacy and safety of oral valganciclovir was compared to ganciclovir i.v. in pre-emptive treatment of cytomegalovirus (CMV) in T cell depleted allogeneic stem cell transplant (alloSCT) recipients. A therapeutic guideline was developed to allow the safe application of valganciclovir in alloSCT recipients requiring CMV therapy. In total, 107 consecutive transplant recipients were evaluated. Cytomegalovirus DNA load in plasma was monitored longitudinally; details on antiviral therapy and treatment responses were analyzed retrospectively. Fifty-seven CMV treatment episodes were recorded in 34 patients: 20 with valganciclovir (900 mg twice-daily) and 37 with ganciclovir (5 mg/kg twice-daily). Median CMV DNA load reduction was 0.079 and 0.069 $\log_{10}$copies/ml/ day in the ganciclovir and valganciclovir group, respectively. Good response on CMV DNA load (reduction below 3.0 $\log_{10}$ copies/ml) was observed in 75.7% of ganciclovir and 80.0% of valganciclovir treatment episodes. Severe adverse effects were not observed and CMV-related disease did not occur. However, the percentage of patients receiving erythrocyte transfusion was higher in the group of patients receiving ganciclovir as compared to valganciclovir (41 versus 20%, P=0.116). In conclusion, pre-emptive treatment with valganciclovir and ganciclovir, led to similar reduction of CMV DNA load. Oral valganciclovir is an attractive and safe alternative for pre-emptive CMV treatment in T cell depleted allo- SCT recipients.

Introduction

In myeloablative (MA) allogeneic stem cell transplant (alloSCT) recipients, cytomegalovirus (CMV) infection contributes significantly to morbidity and mortality.[1] Primary infection results in a lifelong persistence of the virus with reactivation and potentially fatal disease when immunity fails. Cytomegalovirus seropositivity in a patient before transplantation is associated with the highest risk of CMV disease.[2] Furthermore, graft-versus-host disease (GVHD) and T cell depletion (TCD) of the transplant are important contributing factors.[3] Current strategies for the prevention of CMV disease aim at preventing end-organ disease by using ganciclovir or valganciclovir prophylaxis[4,5] or ganciclovir pre-emptive therapy, initiated upon early detection of CMV infection by antigenemia or CMV DNA in plasma.[5,6] The relative merits of both strategies have been debated extensively in the literature.[7,8] The major drawback limiting the use of oral ganciclovir is its poor bioavailability, which precludes therapeutic use by oral administration.[6] This has now changed with the introduction of valganciclovir, which is an orally administered prodrug of ganciclovir with good bioavailability. Previous pharmacokinetic studies showed similar drug exposure to ganciclovir after a single oral dose of 900 mg valganciclovir as compared to an intravenous dose of 5mg/kg ganciclovir.[9–11] Recently, oral valganciclovir and intravenous ganciclovir were shown to have similar efficacy in pre-emptive CMV treatment in solid organ transplant recipients.[12–14] As a consequence, the prevention of CMV disease in high-risk renal, renal–pancreas and heart transplant patients was added as another indication to the original approval of valganciclovir for the treatment of CMV retinitis in AIDS patients. So far, no data are available on the efficacy of 900 mg valganciclovir twice daily as compared to intravenous 5 mg/kg ganciclovir twice daily in the pre-emptive therapy of CMV infection in stem cell transplant recipients and therefore valganciclovir is not licensed for use in alloSCT patients. A comparison with intravenous ganciclovir in alloSCT patients is warranted, as hematological toxicity is a common side effect of ganciclovir and of particular significance in this population. In this observational prospective study, we compared the efficacy and safety of CMV DNA load-guided pre-emptive therapy with valganciclovir to ganciclovir intravenously in alloSCT recipients.

Patients and methods

Patients

All consecutive patients undergoing MA and reduced-intensity allogeneic stem cell transplantation at the Leiden University Medical Center between January 2001 and December 2004 were included in this analysis. All patients at risk for CMV infection (i.e. CMV seropositivity in either the recipient (R^+), the donor (D^+) or both (D^+R^+)) were routinely monitored by CMV DNA load detection in plasma. Data were available on demographic characteristics, underlying diseases, donor and recipient CMV serostatus, occurrence

of GVHD and treatment (i.e. initiation, duration, type and dosage of drugs used) and the ganciclovir formulation (i.e. valganciclovir or ganciclovir), CMV DNA load measurements and general laboratory parameters.

Transplantation

T cell depleted transplantation was performed either according to a reduced-intensity conditioning (RIC) protocol or a conventional MA regimen as described previously.[15,16] The RIC regimen consisted of fludarabine (30 mg/m^2 , intravenously, days -10 to -6), busulphan (3.2 mg/kg, intravenously, days -6 and -5) and ATG (10 mg/kg/day intravenously, days -4 to -1), for both sibling and matched unrelated donor (MUD) grafts. The MA conditioning regimen consisted of cyclophosphamide (60 mg/kg/day intravenously for 2 consecutive days) followed by single dose of total body irradiation (TBI, 9 Gy, day -1) in patients receiving sibling donor grafts. Recipients of MUD grafts, in the MA regimen, received additional Campath- 1G or -1H (days -8 and -4) and cyclosporine (3mg/kg intravenously, starting on day -1) and TBI (6 Gy, days -8 and -7). The stem cell product was infused on day 0. In all conditioning regimens, TCD of the graft was performed by in vitro incubation of the graft with Campath-1H (20 mg). Assessment of acute and chronic GVHD was performed using the Glucksberg and Shulman criteria.[17,18] In the absence of GVHD or graft failure, patients received donor lymphocyte infusion (DLI) after RIC transplantation or in mixed chimerism or relapsed disease after MA transplantation. Donor lymphocyte infusion was administered at least 6 months following transplantation. Donor lymphocyte infusion was not used as a therapeutic modality for CMV infection.

Cytomegalovirus monitoring and treatment

CMV DNA load was measured at weekly intervals for at least 180 days following transplantation, until death occurred or beyond day 180 until CMV DNA became undetectable. The real-time quantitative PCR for detection of CMV DNA in plasma was performed according to the method described previously.[19] Cytomegalovirus DNA load-guided pre-emptive therapy was initiated according to a guideline as described previously.[13] In short, any symptomatic CMV infection would be treated with intravenous 5 mg/kg ganciclovir twice daily. In case of a first reactivation or a significant viraemia (CMV DNA load >10^4 copies/ml, or CMV load >10^3 copies/ml and more than 1.0 $\log_{10}$ increase as compared to preceding measurement) without clinical symptoms of CMV disease, either 900 mg valganciclovir twice daily or intravenous 5 mg/kg ganciclovir twice daily was administered for 2 weeks. Until 2003 intravenous ganciclovir was used as primary pre-emptive treatment. From 2003 onwards, as soon as it became available for clinical use, valganciclovir was used as preferred primary treatment of outpatients, only limited to approval by the patients' medical insurance. When such approval was not granted, or if hospital admission was indicated for other reasons, intravenous ganciclovir was administered. Ganciclovir and valganciclovir dosages were adjusted to renal function as described previously.[20] During (val)ganciclovir treatment, CMV

DNA load and hematological parameters were monitored at least weekly; G-CSF prophylaxis was not routinely used. Donor lymphocyte infusion was not used as a therapeutic modality for CMV infection.

End points and statistical analysis

The effect of CMV treatment on CMV DNA load in plasma, following a full course of either ganciclovir or valganciclovir, was defined as good response (CMV DNA load reduction of more than 0.5 $\log_{10}$ and to a level below 3.0 $\log_{10}$ copies/ml), moderate response (reduction of CMV DNA load of more than 0.5 $\log_{10}$, but not to a level below 3.0 $\log_{10}$ copies/ml) and no response (equal DNA load (i.e. reduction of less than 0.5 $\log_{10}$) or an increase). The levels of 3.0 $\log_{10}$ and 0.5 $\log_{10}$ were chosen as reference values based on a previous report on pre-emptive CMV treatment in SCT recipients.[19] In addition, absolute reduction in number of CMV DNA copies/ml was calculated to compensate for differences in baseline CMV load before treatment. To avoid bias owing to possible differences in CMV reduction rate in first episodes as compared to subsequent episodes, the effect of antiviral medication in first and subsequent episodes was analyzed separately. Cytomegalovirus load reduction per day was calculated by dividing the difference in pre- and post-treatment CMV DNA load by the number of treatment days.

Hematological toxicity was assessed by comparing the number of erythrocyte and thrombocyte transfusion units administered during and following antiviral treatment and by comparing leucocyte ratios (calculated by dividing the leucocyte count before treatment by the count at the end of treatment). Criteria for erythrocyte and thrombocyte transfusion were hemoglobin concentration below 6.0 mmol/l and platelet count below 10 x 10^{10}/l, respectively. Definitions of CMV infection, CMV disease and CMV detection in blood were consistent with internationally accepted criteria.[21]

All statistical analyses were performed using SPSS version 12.0.1. Differences in the distribution of categorical data were tested using χ^2 test. For comparison of the antiviral effect between the two treatments (i.e. ganciclovir or valganciclovir) and comparison of baseline non-categorical data we used Mann–Whitney U-test. Paired observations (e.g., pre-treatment versus post treatment measurements) were analyzed non-parametrically using the Wilcoxon signed ranks test for paired observations.

Results

A total of 107 patients were included in this study. The demographic and disease characteristics for both CMV treatment groups are shown in **Table 1**. Distribution of the characteristics across the two groups was similar. Briefly, 48 patients received a transplantation following an RIC protocol, whereas 59 patients received their transplants following an MA conditioning regimen. With regard to donor and recipient CMV serostatus, 40 D^+R^+ (37.4%), eight D^+R^- (7.5%), 30 D^-R^+ (28.0%) and 29 D^-R^- (27.1%) combinations were observed. The D^-R^- patients were excluded from further analysis, as they are not considered

to be at risk for CMV infection. The median follow-up period following transplantation was 200 days (range: 30–611). During the follow-up period, CMV DNA load became detectable in 42 out of 78 (54%) patients at risk for CMV infection, resulting in 57 CMV treatment episodes with either ganciclovir or valganciclovir in 34 patients. The incidence of GVHD and the percentage of patients treated for GVHD were similar in the two CMV treatment groups. In none of the patients DLI was administered during treatment episodes.

The CMV treatment results are shown in **Table 2**. Intravenous ganciclovir was used in 37 episodes. A good response was observed in 28 episodes (76%). A moderate response was observed in five episodes (14%) occurring in four separate patients. One of these patients died as a result of extensive GVHD without signs of CMV disease. The remaining three patients reached a good response following a second course of intravenous ganciclovir. In four ganciclovir treatment episodes (11%), occurring in four individual patients, no response on CMV load was observed. In three of these four non-responding patients, CMV DNA load decreased below undetectable levels within 2 weeks after cessation of ganciclovir. In the remaining patient, CMV DNA load increased from 3.5 to 4.8 $\log_{10}$ copies/ml, despite 4 weeks of ganciclovir treatment, and subsequently foscarnet was administered, resulting in a CMV DNA load below detectable levels within 14 days of treatment. Treatment with valganciclovir was administered in 20 of the 57 episodes, resulting in a good response in 16 out of these 20 episodes (80%). Moderate response was observed in three out of these 20 episodes (15%) occurring in three individual patients. One of these patients died as a result of extensive GVHD without signs of CMV disease, and the remaining two patients showed a good response following a second course of valganciclovir. In one out of the 20 valganciclovir treatment episodes (5%), no response on CMV DNA load was observed; this patient showed a good response upon a second course of valganciclovir.

Table 1. Characteristics of the study population in both treatment groups. In total, 57 CMV treatment episodes were observed in 34 patients. No statistically significant differences were observed between the two treatment groups. Systemic treatment of GVHD consisted of oral prednisone, intravenous methylprednisolone and/or oral cyclosporine. (CLL: chronic lymphocytic leukemia; CML: chronic myelogenous leukemia; MM: multiple myeloma; NHL: non-Hodgkin lymphoma).

Parameter		ValGCV	GCV
Treatment episodes, n		20	37
Number of patients, n		14	26
Median age in years (range)		51 (41-62)	50 (24-62)
Male gender, n (%)		9 (64)	17 (65)
Type of conditioning, n (%)	Reduced intensity	6 (40)	14 (54)
	Myeloablative	8 (60)	12 (46)
Type of donor, n (%)	Related	11 (80)	20 (76)
	Unrelated	3 (20)	6 (24)
Underlying disease, n (%)	Acute leukemia	5 (38)	9 (35)
	CML	2 (14)	3 (12)
	CLL	1 (7)	1 (4)
	MM	1 (7)	6 (23)
	NHL	4 (29)	1 (4)
	Other	1 (7)	6 (23)
GvHD, n (%)	No GVHD	10 (70)	19 (73)
	Grade I/II	4 (25)	6 (24)
	Grade III/IV	1 (5)	1 (3)
	Treatment	3 (20)	5 (19)
CMV serostatus, n (%)	D^+R^-	0 (0)	1 (3)
	D^+R^+	7 (50)	13 (51)
	D^-R^+	7 (50)	12 (46)
Median duration of treatment in days (range)		14 (7-36)	14 (7-28)
Hematological parameters at start of treatment [Median values (range)]	Hemoglobin (mmol/L)	7.3 (5.1-8.3)	6.9 (4.5-10.6)
	Leucocyte count (x109/L)	5.0 (1.9-8.0)	3.1 (0.7-11.5)
	Thrombocyte count (x109/ L)	88.0 (62.0-264.0)	100 (12.0-206.0)

Table 2. Characteristics of 57 CMV treatment episodes in 34 patients and response on CMV DNA load according to treatment group (valGCV: valganciclovir; GCV: ganciclovir). No statistically significant differences were observed between the two treatment groups.

Parameter		valGCV (n = 20)	GCV (n = 37)
First treatment episodes, n (%)		8 (40)	26 (70)
Subsequent treatment episodes, n (%)		12 (60)	11 (30)
Response on CMV DNA load	Good response, n (%)	16 (80)	28 (76)
	Moderate response, n (%)	3 (15)	5 (14)
	No response, n (%)	1 (5)	4 (11)
Erythrocyte transfusion, n (%)		4 (20)	15 (41)
Thrombocyte transfusion, n (%)		3 (15)	5 (14)
Leucocyte ratio* (median, range between parenthesis)		1.6 (0.6-27.1)	1.2 (0.2-11.0)
Leucocyte count x109/l (median, range between parenthesis)	Pre-treatment	5.0 (1.9-8.0)	3.1 (0.7-11.5)
	Post-treatment	3.6 (0.1-9.7)	3.0 (0.4-8.6)

*Calculated by dividing leucocyte count before treatment by the count and the end of treatment.

The effect of anti-CMV treatment with ganciclovir and valganciclovir was further assessed by comparing the CMV DNA load at the start and at the completion of the treatment episode. When first treatment episodes as well as all subsequent episodes were evaluated, CMV DNA load at start of therapy in the ganciclovir and the valganciclovir group was similar (median 4.3 (range: 3.3–6.2) and 4.2 $\log_{10}$ copies/ml (range: 3.1–5.7), P>0.4, respectively, Figure 1b). The kinetics of CMV DNA following treatment with ganciclovir and valganciclovir for individual patients are shown in **Figure 1a**. A median reduction of 1.20 and 1.10 $\log_{10}$ DNA copies/ml was reached in the ganciclovir- (n = 37) and the valganciclovir- (n = 20) treated patients, respectively (P<0.0001 for both groups). No difference in the magnitude of CMV DNA load reduction/treatment day was observed between the ganciclovir and valganciclovir groups (median 0.0786 (range: -0.0464–0.767) and 0.0690 $\log_{10}$ copies/ml/day (range: 0.0182–0.171), P>0.8, respectively; **Figure 1b**). Cytomegalovirus treatment episodes were further subdivided into 34 first episodes (26 ganciclovir, eight valganciclovir) and 23 subsequent episodes (11 ganciclovir, 12 valganciclovir) (**Figure 2a**). Cytomegalovirus DNA load at start of therapy, according to treatment episode, was similar in the ganciclovir and valganciclovir groups (median 4.4 (range: 3.3–5.6) versus 4.1 $\log_{10}$ copies/ml (range: 3.1–5.1) in first episodes, P>0.3, respectively and 4.3 (range: 3.5–5.7) versus 4.3 $\log_{10}$ copies/ ml (range: 3.5–5.7) in subsequent episodes, P>0.7, respectively). The magnitude of CMV load reduction/ treatment day in first treatment episodes was similar for the ganciclovir and valganciclovir group (median 0.0941 (range: 0.000–0.767) and 0.0833 $\log_{10}$ copies/ml/day (range: 0.0381–0.171), P>0.6, respectively, Figure 2b). For subsequent episodes, the same

result was obtained (median 0.0786 (range: -0.0464–0.260) and 0.0685 log_{10} copies/ml/ day (range: 0.0182–0.150), P>0.4, for ganciclovir and valganciclovir, respectively; **Figure 2b**).
Erythrocyte transfusions were administered in 15 out of the 37 (41%) ganciclovir treatment episodes (median number of units: 2, range 2–6 units) as compared to four out of the 20 (20%) (median number of units: 2, range 2–6 units) of the valganciclovir treatment episodes (P = 0.116). The percentage of patients receiving thrombocyte transfusions was similar in the ganciclovir- and valganciclovir- treated groups (15.0 and 13.5%, P40.8, respectively). Furthermore, the leucocyte ratio was not significantly different between ganciclovir and valganciclovir treatment episodes (median 1.16 and 1.55, P > 0.1, respectively).
No signs of CMV disease and no severe adverse reaction (NCI grade 3–4) of (val)ganciclovir treatment were observed.

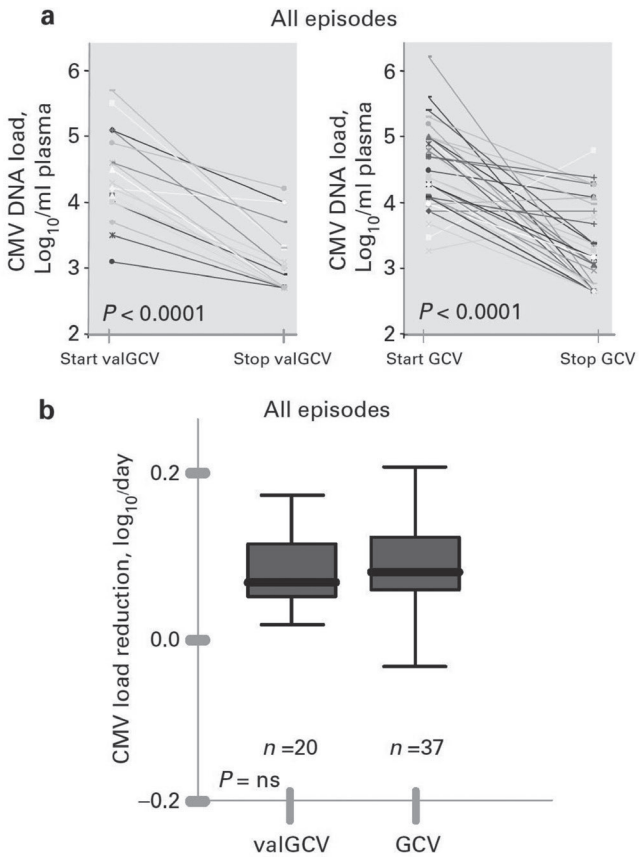

Figure 1.
In panel A, the course of CMV DNA load before and after treatment with valganciclovir or ganciclovir for individual patients is shown (all treatment episodes).
In panel B, the CMV DNA load reduction per treatment day with valganciclovir (ValGCV) and intravenous ganciclovir (GCV) is shown (all treatment episodes). The box plots display the median, the 25th and 75th percentiles (box), and the smallest and largest values (whiskers).

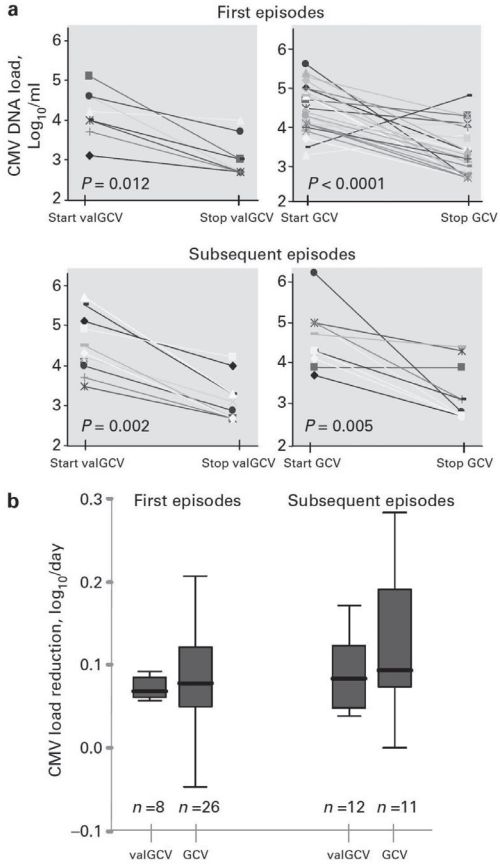

Figure 2.
In panel A, the courses of CMV DNA load before and after treatment with valganciclovir or ganciclovir for individual patients are shown. First (upper panel) and subsequent (lower panel) treatment episodes are plotted separately. In panel B, the CMV DNA load reduction per day during treatment with valganciclovir (ValGCV) and intravenous ganciclovir (GCV). First (left box plots) and subsequent (right box plots) episodes are shown separately. The box plots display the median, the 25th and 75th percentiles (box), and the smallest and largest values (whiskers). No significant differences are present.

Discussion

This study demonstrates that pre-emptive treatments with oral valganciclovir and intravenous ganciclovir are equally effective in reducing CMV DNA load in allogeneic stem cell recipients. Pre-emptive treatment of CMV viraemia episodes in allogeneic stem cell recipients with either valganciclovir or ganciclovir led to a similar median CMV DNA load reduction in plasma of approximately 0.1 $\log_{10}$ copies/ml/day, which is in accordance with our previous report on renal and renal/pancreas transplant recipients.[13]

Although initially no response was seen upon treatment with intravenous ganciclovir in four patients, CMV DNA load spontaneously declined in three of these whereas in only one patient a switch to foscarnet was made. Furthermore, in four other patients (five treatment

episodes), treatment with intravenous ganciclovir for 14 days did not reduce the CMV DNA load below the level of 3.0 $\log_{10}$ copies/ml and a subsequent course was needed to further reduce CMV DNA load. Similarly, in four patients treated with valganciclovir, either a subsequent course or a switch to foscarnet was needed to reduce CMV DNA load beyond detectable levels. Reasons for these failures are not clear and this study was not designed to identify factors associated with antiviral treatment failure. Therefore, further investigation with regard to these treatment failures is warranted.

As soon as valganciclovir became available in our institution in 2003, it was used as preferred primary treatment of asymptomatic patients, only limited to approval by the patient's medical insurance. In case such an approval was not granted or in case of co-morbidity leading to hospitalization, intravenous ganciclovir was administered. Patient selection might therefore have occurred, as co-morbidity was more likely to be present in admitted patients treated with ganciclovir. However, we do not expect that this possible bias has influenced our results to such an extent that the conclusions drawn might be incorrect. The baseline CMV loads in the ganciclovir- and valganciclovir-treated groups were similar, indicating similar CMV activity. Furthermore, the magnitude of CMV decline in all analyzed subgroups was similar, substantiating our conclusion on the equal efficacy of both drugs in CMV infection.

In our study, the hematological toxicity of oral valganciclovir in alloSCT patients was similar as compared to ganciclovir intravenously. The slightly higher, although not statistically significant, percentage of patients receiving erythrocyte transfusions in the intravenous ganciclovir group might be the result of co-morbidity in the admitted patients treated with ganciclovir intravenously. Mainly owing to the retrospective nature of this study, differences in non-hematological toxicity, such as gastrointestinal and neurological complications, between the two treatment groups could not be assessed adequately and further evaluation in a prospective study is warranted.

So far, no other studies have been reported on the use of valganciclovir compared to intravenous ganciclovir in stem cell recipients. In conclusion, based on our findings, oral valganciclovir (900 mg, twice daily) is equally effective and safe as intravenous ganciclovir (5 mg/kg, twice daily) in the pre-emptive treatment of CMV disease following alloSCT. There is an urgent need for an effective oral treatment for pre-emptive CMV therapy, which would enable prevention and treatment of CMV in an outpatient setting leading to reduced patient burden and health-care cost. The finding of the therapeutic equivalence of oral valganciclovir and intravenous ganciclovir is a confirmation of previous reports with respect to pre-emptive[12–14, 22] and prophylactic treatment[20] in solid organ transplant recipients.

The large majority of alloSCT recipients, without any signs and symptoms of CMV disease when the first laboratory signs of CMV infection are detected, can benefit from treatment with an oral drug, without the need of hospitalization. Based on rational precautions, intravenously administered ganciclovir remains the first choice drug for patients with suspected symptomatic CMV infections, as the course of CMV disease can be serious, rapidly progressive and ultimately fatal.

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Chapter 3

Comparable incidence and severity of cytomegalovirus infections following T cell depleted allogeneic stem cell transplantation preceded by reduced intensity or myeloablative conditioning

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Abstract

Reports on infectious complications following reduced intensity conditioning (RIC) before allogeneic stem cell transplantation (alloSCT) are equivocal. This prospective follow-up study compared the impact of cytomegalovirus (CMV) infections following RIC with fludarabine, ATG and busulphan or conventional myeloablative conditioning (MAC). Forty-eight RIC and 59 MAC patients were enrolled. The occurrence and severity of CMV infections within 100 days following alloSCT were assessed, using plasma CMV DNA load kinetics. CMV DNAemia was observed in 21 RIC (60%) and in 19 MAC (44%) patients at risk for CMV. The mean CMV DNAemia free survival time was comparable following RIC and MAC: 70 days (95% (confidence interval) CI: 59–80 days) and 77 days (95% CI: 68–86 days), respectively (P¼0.24). Parameters indicative for the level of CMV reactivation, including the area under the curve of CMV DNA load over time as well as the onset, the peak values and duration of CMV infection episodes, the numbers and duration of CMV treatment episodes and recurrent infections, were not different in both groups. During follow-up, none of the patients developed CMV disease. RIC with fludarabine, ATG and busulphan demonstrated safety comparable to conventional MAC with regard to frequency and severity of CMV infections within 100 days following T cell depleted alloSCT.

Introduction

Allogeneic stem cell transplantation (alloSCT) is increasingly used to treat hematological and non-hematological malignancies. Recently, conditioning regimens have been designed to exploit the graft-versus-tumor effects while reducing the intensity of the conditioning to minimize toxicities[1-3]. Results of studies demonstrate rapid allogeneic engraftment with minimal non-hematological toxicity and a significant antitumor effect. Despite the lower toxicity of the reduced intensity conditioning (RIC), acute and chronic graft-versus-host disease (GvHD) remains a significant cause of morbidity and mortality with a reported incidence of severe GvHD of 30–60%[1].

Recently, an in vitro T cell depleted alloSCT protocol following non-myeloablative conditioning with fludarabine, antithymocyte globulin (ATG), busulphan and Campath-in-the-bag was reported as a suitable platform for subsequent cellular immunotherapy[4]. It was shown that this protocol leads to durable donor engraftment, favorable response of the disease and minimal GvHD. Still, infections remain a prominent cause of transplant-related mortality following RIC[5]. As in myeloablative SCT recipients, risk factors for infections include the degree of myeloablation, GvHD and organ toxicities. However, as the timing and types of infections may differ[5], information regarding infectious risks and outcomes are important to develop preventative strategies in alloSCT recipients following RIC.

Cytomegalovirus (CMV) is one of the major causes of infectious complications following alloSCT[6], and the strategy of viral load guided pre-emptive antiviral therapy has been shown to reduce the risk of CMV disease[7, 8]. Viral load kinetics has been reported to be predictive for the development of CMV disease, with the initial viral load and the initial rate of increase in viral load being independent risk factors[9] and as such this method can also be applied to assess the incidence and severity of CMV reactivation following transplantation. However, in this context, it should be considered that an episode of CMV viremia is characterized not only by its level (for example, peak load), but also by its duration[9, 10]; as a consequence, long-term viremia at lower levels may have the same clinical significance as shorter episodes of high-level viremia. A novel approach has been devised previously to assess both quantities (level and duration of viremia) with a single parameter, which is based on calculating the area under the curve (AUC) of viral load over time[10]. Hence, the AUC approach is a universal means of assessing interrelated determinants, including peak viral load, initial viral load and rate of increase of viral load, parameters that have been described as independent risk factors for CMV disease[9].

In the current prospective follow-up study, viral load kinetics were used to assess the incidence and the level of CMV reactivation in patients receiving in vitro T cell depleted alloSCT following either non-myeloablative conditioning with fludarabine, ATG and busulphan or after myeloablative conditioning (MAC).

Patients and methods

Patients

Forty-eight consecutive patients who received alloSCT following RIC between January 2001 and December 2004 were analyzed for CMV reactivation. Patients eligible for alloSCT were selected to receive RIC either when MAC was contraindicated (due to comorbidity or age) or in patients with an HLA identical donor who failed to respond on conventional treatment for lymphoma, multiple myeloma or chronic lymphocytic leukemia, or in patients with solid tumors such as metastatic renal cell carcinoma or breast carcinoma. Forty-three RIC patients had hematological malignancies, four had renal cell carcinoma and one had breast carcinoma. Additionally, 59 consecutive patients who received alloSCT using conventional MAC regimens between August 2001 and December 2004 were included in this analysis. All conventional MAC patients had hematological malignancies. General institutional policy with respect to patients' informed consent for inclusion into the study, approved by the ethical institutional board, was applied.

Transplantation

T cell depleted transplantation was performed either according to a RIC protocol or a MAC regimen as described previously[4, 11]. The RIC regimen consisted of fludarabine (30mg/m^2, intravenously, day -10 to -6), busulphan (3.2 mg/kg, intravenously, day -6 and -5) and ATG (10mg/kg/day intravenously, day -4 to -1), for both sibling and matched unrelated donor (MUD) grafts. The MAC regimen consisted of cyclophosphamide (60mg/kg/day intravenously for 2 consecutive days) followed by single dose of total body irradiation (TBI, 9 Gy, day -1) in patients receiving sibling donor grafts. Recipients of MUD grafts, in the myeloablative regimen, received additional Campath-1G or -1H (day -8 and -4) and cyclosporine (3 mg/kg intravenously, starting on day -1) and TBI (6 Gy, day -8 and -7). The stem cell product was infused on day 0. In all conditioning regimens, T cell depletion of the graft was performed by in vitro incubation of the graft with Campath-1H (20mg). Prophylaxis for GvHD was not administered. Assessment of acute and chronic GvHD was performed using the Glucksberg and Shulman criteria[12, 13]. In the absence of GvHD or graft failure, patients received donor lymphocyte infusion (DLI) after RIC transplantation or in mixed chimerism or relapsed disease after MAC transplantation. DLI was never administered before 6 months following transplantation.

CMV monitoring and pre-emptive treatment

CMV DNA load was measured at least once a week for up to 100 days following transplantation. The real-time quantitative PCR for detection of CMV DNA in plasma was performed according to the method described previously[14]. The course of CMV DNA load in plasma was documented longitudinally for each patient during follow-up. Individual areas under the CMV DNAemia curve post-transplant were calculated using the trapezoidal rule as described previously[10, 15].

CMV DNA load guided pre-emptive therapy was initiated according to a protocol based on criteria established in a previous study[14]. In short, CMV DNAemia episodes following transplantation treatment was initiated at a CMV DNA load level of $>10^4$ copies/ml or at a level of $>10^3$ copies/ml and more than one $_{10}$log increase as compared to previous measurement, without clinical symptoms of CMV disease[14]. Pre-emptive treatment consisted of 900 mg valganciclovir b.i.d. or intravenous 5 mg/kg ganciclovir b.i.d for an average duration of 2 weeks. CMV disease would be treated with intravenous 5 mg/kg ganciclovir b.i.d. Ganciclovir and valganciclovir dose were adjusted to renal function as described previously[16]. Serum creatinine levels and hematological parameters (that is, hemoglobin, leucocyte and thrombocyte counts) were monitored throughout treatment episodes.

Study end points and statistical analysis

The primary end point for this study was CMV infection, defined as 'detection of two consecutive positive CMV DNA loads (more than $\log_{10}$ 2.7 (=500) copies/ml plasma) within 100 days following alloSCT transplantation'. The level of $\log_{10}$ 2.7 copies/ml plasma as the lower detection limit of the 'real-time' quantitative CMV DNA PCR was established by earlier assessments with respect to the sensitivity and reproducibility of the assay[14]. The number of two consecutive detections of $\log_{10}$ 2.7 copies/ml as the definition of CMV infection was arbitrarily chosen to exclude incidental single positive findings. Secondary end points were CMV DNA load requiring antiviral treatment and recurrent infections. Definitions for CMV infection, CMV disease, CMV detection in blood and recurrent infection were adopted from internationally accepted criteria[17].

All database entries and statistical analyses were performed with SPSS version 12.0.1. Differences in age at transplantation, time to the first CMV DNA load detection, CMV DNA peak load, the duration of the CMV infection and the area under the DNAemia curve (AUC) were compared between groups using Mann–Whitney U-test and analyses of variance. For all measurements, the median and range or the 25th and 75th percentiles are presented. Differences in the distribution of CMV serostatus, underlying disease, GvHD and gender were tested using χ^2 and Fisher exact-test statistics. Kaplan–Meier analysis was performed to detect differences in CMV DNAemia free survival between groups during the first 100 days following transplantation and a Cox regression analysis was used to adjust for the possible confounders age and donor type. Relative risks for occurrence of CMV disease are presented with 95% confidence interval (95% CI).

Results

Patient characteristics

A total of 107 patients were included in this study. The demographic and disease characteristics for patients in both conditioning groups are shown in Table 1. Distribution of

the characteristics across the two groups was similar with respect to risk for CMV infections (based on donor and recipients CMV serostatus), underlying disease, GvHD and gender. However, significant differences were noted with regard to mean age at transplantation and donor type (**Table 1**). The mean age at transplantation was 54.5 years in the RIC patients compared with 44.0 years in the MAC patient group ($P<0.01$). In the reduced intensity group, 31 patients were transplanted with hematopoietic stem cells from an HLA identical donor and 17 patients had mismatched unrelated donors (in the myeloablative group, 52 and 7, respectively) ($P=0.004$). Further analyses were restricted to 78 patients who were considered to be at risk for CMV infection/reactivation (based on donor and receptor serostatus: 8 D^+R^-, 40 D^+R^+ and 30 D^-R^+). This selection did not introduce significant change in the patients' characteristics.

Table 1. Relevant characteristics of the study population in both conditioning groups. No significant differences were present between the two groups, with the exception of age and donor type. Systemic treatment of GvHD consisted of oral prednisone, intravenous methylprednisolone and/or oral cyclosporine. RIC: reduced intensity conditioning, MAC: myeloablative conditioning, ns: not significant. CLL: chronic lymphocytic leukaemia; CML: chronic myelogenous leukaemia; CMV: cytomegalovirus; GvHD: graft-versus-host disease; MAC: myeloablative conditioning; MM: multiple myeloma; NHL: non-Hodgkin lymphoma; NS: not significant; RIC: reduced intensity conditioning.

Characteristics	RIC (n = 48)	MAC (n = 59)	Statistical relevance
Age (median/range)	54.5 (26-76)	44.0 (21-62)	p<0.01
Male gender (%)	(34) 71	(43) 73	*ns*
Serostatus: (%)			
D^+R^+	20 (42)	20 (34)	*ns*
D^+R^-	4 (8)	4 (7)	
D^-R^+	11 (23)	19 (32)	
D^-R^-	13 (27)	16 (27)	
Donor type (%)			
Related	31 (65)	52 (88)	p<0.01
Unrelated	17 (35)	7 (12)	
Underlying disease (%)			*ns*
Acute leukemia	10 (21)	33 (56)	
CML	5 (10)	10 (17)	
CLL	5 (10)	1 (2)	
MM	5 (10)	7 (12)	
NHL	10 (21)	7 (12)	
Other	13 (27)	1 (2)	
T cell depletion (%)	48 (100)	59 (100)	*ns*
Acute GvHD (%)			p=0.07
Grade I/II	4 (8)	13 (22)	
Grade III/IV	0	0	
Chronic GvHD (%)	0	5 (9)	*p=0.07*
GvHD treatment (%) (systemic)	0	5 (8.5)	*ns*

Incidence of CMV DNAemia

CMV DNAemia occurred in 40 patients within 100 days following transplantation, which accounts for 37% of all 107 patients and 51% of patients at risk for CMV (n=78). The first signs of CMV DNAemia were observed at a median of 27 days (range: 8–81) and all first episodes occurred within 90 days following transplantation. None of the patients developed CMV disease during the follow-up of 100 days following alloSCT. Among the 78 patients at risk for CMV DNAemia, the highest incidence of CMV DNAemia was observed in R^+ cases; 21 (53%) D^+R^+ and 18 (60%) D^+R^+ compared with 1 (12.5%) D^+R^- patients within 100 days following transplantation. Within the group of patients at risk for CMV (35 and 43 receiving RIC and MAC, respectively), CMV DNAemia was observed in 21 (60%) patients receiving RIC and in 19 (44%) patients receiving MAC. Although the mean CMV DNAemia free survival time was shorter in RIC patients (70 days, 95% CI: 59–80) then in MAC patients (77 days, 95% CI: 68–86), this difference was not statistically significant (P=0.24; **Figure 1**). This was not different when a multivariate Cox regression analysis was performed to control for the possible confounders age, GvHD and donor type.

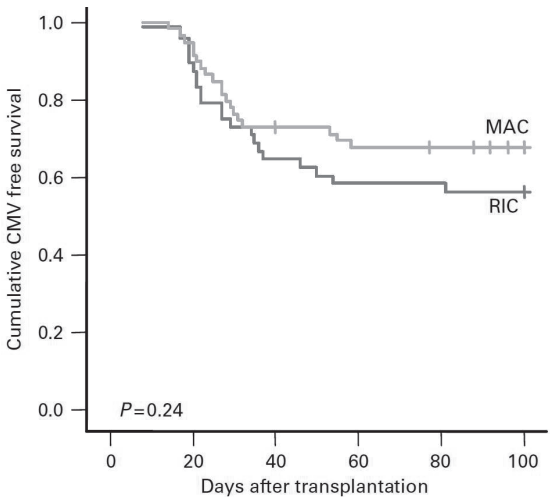

Figure 1.
Pattern of CMV-free survival (Kaplan-Meier) during the first 100 days following allo-SCT in patients receiving reduced intensity (RIC) or myeloablative conditioning (MAC). CMV viraemia was observed in 21 (60%) and 19 (44%) of the RIC and MAC patients respectively. The mean CMV free survival time in RIC patients was 70 days, (95% CI: 59-80 days) compared to 77 days (95% CI: 68-86 days) in MAC patients (p= 0.24).

Level of CMV reactivation following RIC and MAC

To assess the level of CMV reactivation, the onset of the first positive CMV PCR following transplantation, the peak load of the first episodes following alloSCT and the duration of the first CMV DNAemia episodes were evaluated in patients receiving RIC or MAC. There was no difference in the onset of the first CMV DNAemia episodes following RIC or MAC; median of 27 days (range: 8–81) and 27 days (range: 14–58) following transplant in recipients of RIC and MAC, respectively (P=0.36). Also the median peak loads of the first CMV episodes following alloSCT were comparable between the RIC and MAC patients: $\log_{10}$ 4.7 copies/ml (range: $\log_{10}$ 3.2–$\log_{10}$ 5.6) and $\log_{10}$ 4.7 copies/ml (range: $\log_{10}$ 3.5–$\log_{10}$ 6.2), respectively (P=0.74). The median duration of the first CMV DNAemia episode was longer in RIC patients (42 days (range: 7–73)) compared with MAC patients (28 days (range: 2–83)). However, this difference was not statistically different (P=0.72). These findings did not change after correcting for the possible confounders age, GvHD and donor type. Alternatively, the level of CMV reactivation was evaluated by calculating the time-adjusted area under the DNAemia curve (assessing both, the level and the duration of CMV DNAemia in mentioned time period). Although the median area under the DNAemia curve over time during the first 100 days following alloSCT was higher in RIC patients (0.61 (range: 0.08–1.68)) compared with MAC patients (0.49 [range: 0.10–1.42]), this difference was not statistically significant (P=0.41). These findings did not change after correcting for differences in age, GvHD and donor type between the two induction groups. Another approach to assess the level of CMV reactivation in both groups was to evaluate CMV load episodes requiring antiviral treatment. (Val)ganciclovir was administered to an equal amount of RIC and MAC patients with CMV DNAemia: 17 out of 21 (81%) and 16 out of 19 (84%), respectively (P=0.45). The total duration of CMV treatment was also comparable in both groups: median duration of 14 days (range: 7–53) in RIC patients and 14 days (range: 11–29) in MAC patients (P=0.279). Multiple treatment episodes (with a maximum of 2) within 100 days following alloSCT were seen in 7 patients (41%) following RIC and in 4 patients (25%) following MAC. This difference did not reach statistical significance (P=0.458), also not after correction for the possible confounders age, GvHD and donor type. Foscarnet was never administered within 100 days following alloSCT. These findings also indicate equal levels of CMV reactivation in both conditioning groups.

Recurrent CMV infections following RIC and MAC

CMV infection recurred within 100 days following transplantation in 3 out of 21 patients (14.3%) receiving RIC and also in 3 out of 19 (15.8%) with MAC. None of the six patients with recurrent CMV infections developed more than 2 CMV DNAemia episodes within 100 days following transplant.

Influence of donor and recipient CMV serostatus on CMV infections

In a univariate analysis, serological status of recipient and donor appeared to be associated with the occurrence of CMV infection within 100 days following alloSCT,

when D^-R^- patients were included (P=0.071). Among patients at risk for CMV (donor and/or recipient seropositive), seropositive recipients were at higher risk for CMV infections compared with seronegative recipients, whereas no significant difference was observed between seropositive and seronegative donors (**Table 2**). Within the high-risk CMV patients (seropositive recipients), the relative risk for CMV reactivation was 1.1 for D^-R^+ patients compared with D^+R^+ patients; this difference was not statistically significant (P=0.65; **Figure 2a**). Also, the level of CMV reactivation was comparable (**Figure 2b**). These findings did not change after stratification for conditioning therapy (**Figure 2c–f**). Donor type and recipients' age did not have significant impact on the occurrence of CMV within 100 days following transplantation.

Table 2. Univariate analysis of risk factors for CMV within 100 days following alloSCT in patients at risk for CMV infection (n=78)

Risk factors		Crude RR (95% CI)	P-value
Conditioning (RIC vs MAC)		1.50 (0.81-2.79)	0.20
Recipient age (years) (>45 vs <45)		1.40 (0.69-2.78)	0.35
CMV serostatus	D^- vs D^+	1.40 (0.75-2.62)	0.29
	R^+ vs R^-	6.10 (0.84-45.50)	0.07
Donor type unrelated vs related		1.40 (0.67-2.80)	0.39

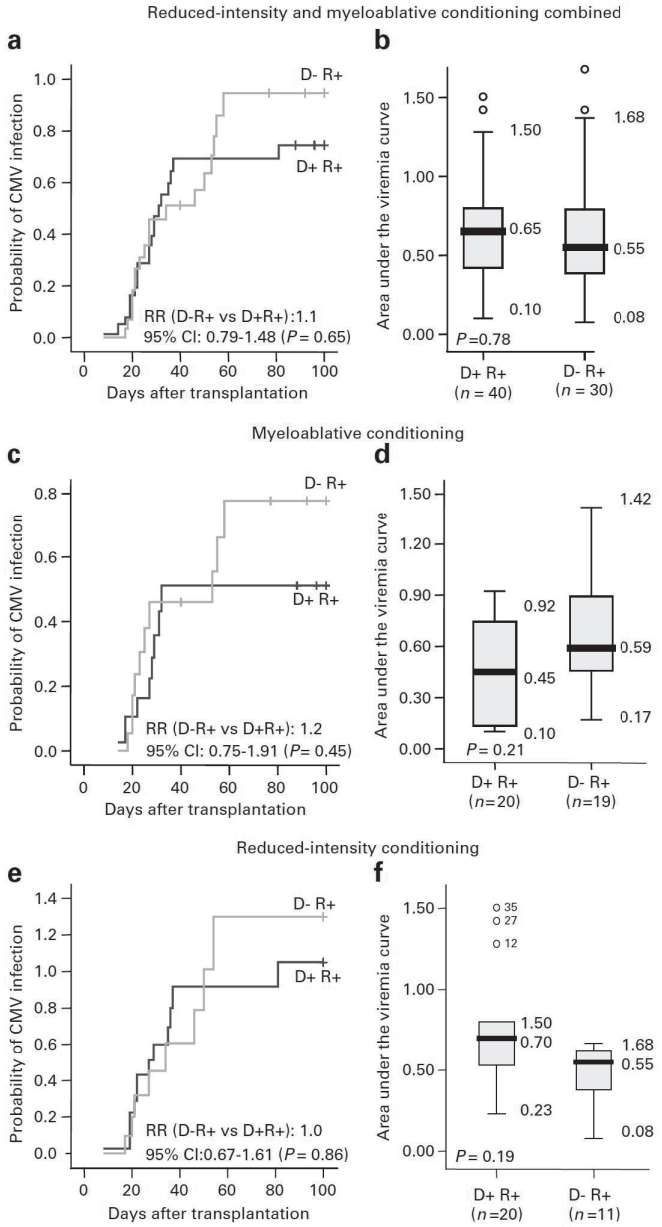

Figure 2.
The probability (left panels) and severity (right panels) of CMV infection in high-risk patients (i.e. CMV seropositive recipients) within 100 days following alloSCT, according to CMV serostatus of the donor and conditioning regimens. The probability and severity of CMV infection were comparable between seropositive and seronegative donors (panels **A** and **B**). This did not change after stratification for induction therapy (**C** and **D**, and **E** and **F**). The box plots display the median (horizontal bars), the 25th and 75th percentiles (box), and the smallest and largest values (whiskers). Open circles depict the outliers (values between 1.5 and 3 box lengths from the upper or lower edge of the box).

Discussion

It has been established that allogeneic transplantation with RIC can be successfully performed in individuals with a wide variety of different diseases and with reduced risk of transplant-related mortality[5, 18]. Previously, an in vitro T cell depleted alloSCT protocol following RIC with fludarabine, ATG, busulphan and Campath-in-the-bag was reported to lead to durable donor engraftment and favorable response of the disease with no GvHD[4]. The current analysis demonstrates that there was no significant difference in incidence and severity of CMV infections within 100 days following alloSCT preceded by RIC compared to a conventional MAC. A limitation in the current study concerns its non-randomized nature. Patients were allocated to the RIC or MAC group on clinical grounds, rather than by random selection. Therefore the possibility of confounding by indication could not be entirely excluded.

Although there was a trend towards a shorter CMV DNAemia free survival following RIC, this difference was not statistically significant. Furthermore, various parameters related to the severity of CMV infections (that is, the onset of CMV DNA detection in plasma following alloSCT, the duration of a CMV infection, the peak load, the area under the DNAemia curve, the number and duration of pre-emptive CMV treatment episodes as well as the number of recurrent infections within 100 days following alloSCT) were not different after RIC and MAC, supporting the conclusion of comparable severity of CMV infections in both groups.

In this study, both patient groups received T cell depleted grafts. By itself, T cell depletion of the graft is associated with an increased risk for CMV infections[19], which seems to be reflected by the high overall incidence of CMV infections (51%) within 100 days following alloSCT in this study.

Previous studies have reported variable outcomes with regard to CMV infections following RIC[20-22]. Such differences can be explained by the variable immune suppressive potentials of the RIC regimens investigated at different centers, presumably reflecting a balance between more residual immunity in the host and a higher risk for opportunistic infections either due to more persisting intracellular pathogens or an increased incidence of GvHD following RIC. A high rate of CMV infections was observed in alemtuzumab-based RIC regimen[20]. Recent reports with respect to CMV infections following fludarabine, busulphan and ATG-based RIC regimens compared to MAC have either reported no influence of conditioning protocols[23] or a significant increase of CMV infection following RIC[22]. However, limitations in these studies included analysis of CMV infections mainly using CMV antigenemia detection rather than the more sensitive and accurate CMV DNA PCR in plasma[14]. Another difference is the use of GvHD prophylaxis in these previous studies, which may be of major importance with respect to CMV infections.

The association of CMV positive serostatus of the recipients (R^+) and an increased risk for CMV infections following alloSCT is well established[24]. Recently, it has been demonstrated that a CMV seronegative donor for a seropositive patient (D^-R^+) in particular was found to be a risk factor for CMV infections following alloSCT in an study including both reduced

intensity as well as MAC regimens. Although the previous report did not show a difference between conditioning regimens[25], we observed increased frequency and severity of CMV infections in seropositive patients receiving a graft from seronegative donors (D^-R^+) compared to seropositive donor and recipient combination (D^+R^+) only following MAC, presumably reflecting residual immunity following RIC. However, this difference was not statistically significant and the clinical relevance of this observation is questionable.

Another relevant conclusion resulting from the current study was that irrespective of the conditioning regimen, monitoring of CMV DNA in plasma and pre-emptive therapy proved highly effective in preventing CMV disease following alloSCT, as CMV disease was not seen in any patient.

In conclusion, RIC with busulphan, fludarabine and ATG demonstrated comparable safety to conventional MAC with regard to the frequency and severity of CMV infections within 100 days following T cell depleted alloSCT. Moreover, with RIC, pre-emptive CMV treatment guided by CMV DNA load monitoring in plasma is highly effective in preventing CMV disease following T cell depleted alloSCT.

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Chapter 4

CMV seronegative donors: effect on clinical severity of CMV infection and reconstitution of CMV-specific immunity

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Abstract

Cytomegalovirus (CMV)-specific T cells are crucial to prevent CMV disease. CMV seropositive recipients transplanted with stem cells from a CMV seronegative allogeneic donor (R^+D^-) may be at risk for CMV disease due to absence of donor CMV-specific memory T cells in the graft. We analyzed the duration of CMV reactivations and the incidence of CMV disease in R^+D^- and R^+D^+ patients after alemtuzumab-based T cell depleted allogeneic stem cell transplantation (TCD alloSCT). To determine the presence of donor-derived primary CMV-specific T cell responses we analyzed the origin of CMV-specific T cells in R^+D^- patients. The duration of CMV reactivations (54 versus 38 days, respectively, p=0.048) and the incidence of CMV disease (0.14 versus 0.02, p=0.003 at 1 year after alloSCT) were higher in R^+D^- patients compared to R^+D^+ patients. In R^+D^- patients, CMV-specific $CD4^+$ and $CD8^+$ T cells were mainly of recipient origin. However, in 53% of R^+D^- patients donor-derived CMV-specific T cells were detected within the first year. In R^+D^- patients, immunity against CMV was predominantly mediated by recipient T cells. Nevertheless, donor CMV serostatus significantly influenced the clinical severity of CMV reactivations indicating the role of CMV-specific memory T cells transferred with the graft, despite the ultimate formation of primary donor-derived CMV-specific T cell responses in R^+D^- patients.

Introduction

The presence of anti-viral T cell immunity is crucial for effective and sustained protection against cytomegalovirus (CMV) following allogeneic stem cell transplantation (alloSCT)[1]. In vitro and in vivo T cell depletion (TCD) via addition of the anti-CD52 monoclonal antibody alemtuzumab to the stem cell graft (alemtuzumab "in the bag") is used to reduce the incidence of acute Graft versus Host Disease (GVHD) following alloSCT[2-4]. Alemtuzumab does not exclusively eliminate alloreactive T cells, but affects presumably all T cells, including donor-derived CMV-specific T cells in the graft and residual CMV-specific T cells of the recipient. Despite the profound TCD, protection against CMV is observed early after TCD alloSCT in CMV seropositive recipients (R^+) transplanted with a CMV seropositive donor (R^+D^+) mediated by CMV-specific T cells that can either originate from the donor via transfer with the graft or from the recipient as residual memory T cells. In CMV seropositive recipients (R^+) transplanted with a CMV seronegative donor (R^+D^-) donor-derived CMV-specific memory T cells are not present in the graft and R^+D^- patients must therefore rely on residual CMV-specific T cells of recipient origin and/or a donor-derived primary CMV-specific T cell response to control CMV reactivations. If despite the in vivo T cell depletion mediated by the free alemtuzumab transferred with the graft, recipient-derived T cell immunity predominates in the protection against CMV, the incidence and severity of CMV reactivation and disease would not differ between R^+D^+ and R^+D^- patients. Because the function of the thymus is likely to be impaired after TCD alloSCT[5], it is not known if or when to expect a donor-derived primary immune response after TCD alloSCT. Demonstrating donor derived CMV-specific T cells after transplantation with a CMV seronegative donor (R^+D^-) would be indicative of a newly developed CMV-specific primary T cell response.

In this study we analyzed the effect of donor CMV serostatus on the incidence of CMV reactivation and CMV disease in R^+D^- patients versus R^+D^+ patients following TCD alloSCT using alemtuzumab in the bag (20 mg). Furthermore we analyzed the origin of circulating CMV-specific $CD4^+$ and $CD8^+$ T cell populations in R^+D^- patients by chimerism analysis to detect donor derived CMV-specific T cells indicative of a donor derived primary CMV-specific T cell response.

Objectives

The objectives of this studies were to analyze the effect of donor CMV serostatus on the incidence of CMV reactivation and CMV disease following T cell depleted allogeneic stem cell transplantation and to detect CMV-specific primary T cell responses by demonstrating donor derived CMV-specific $CD4^+$ and $CD8^+$ T cell populations in seropositive recipients transplanted with stem cells from a CMV seronegative allogeneic donor.

Material and Methods

Patients and CMV monitoring

General institutional policy with respect to patients' informed consent for inclusion into the study, approved by the ethical institutional board, was applied. Consecutive patients transplanted in the period 2004-2010 were included. Patients with haplo-identical or cord blood transplantation were excluded from the analysis. We retrospectively analyzed CMV PCR loads, determined as part of regular post transplantation monitoring. The real-time quantitative PCR for detection of CMV DNA in plasma was performed according to the method described previously[6]. CMV DNA load guided pre-emptive therapy was initiated according to a protocol based on criteria established in a previous study[7]. CMV reactivation was defined as previously described by the detection of two consecutive positive CMV DNA loads (>log10 2.7 (>500)/ml copies plasma) and CMV disease was defined as previously published[8]. Post transplantation sampling for T cell analysis was scheduled every 3 months and continued for 1 year after alloSCT or longer if deemed necessary.

T cell depletion and transplantation

T cell depletion of the graft was performed by *in vitro* incubation of the graft with alemtuzumab (20 mg). The stem cell product was infused on day 0. Pre-transplantation conditioning was performed either according to a myeloablative (MA) conditioning protocol or a nonmyeloablative (NMA) conditioning (RIC) protocol as described previously[9, 10].

Detection and isolation of CMV-specific $CD4^+$ and $CD8^+$ T cells based on CD137 expression

CMV-specific $CD4^+$ or $CD8^+$ T cells were detected by flow cytometric analysis of expression of the activation marker CD137 upon stimulation of PBMC with protein spanning overlapping peptide pools of the CMV-derived proteins pp65 and IE1[11-13]. A cluster of ≥5 $CD137^+$ events on FACS analysis within a total of 10.000 acquired events was considered positive based on the low level of background seen in CMV seronegative individuals. The isolation of CMV-specific $CD4^+$ or $CD8^+$ $CD137^+$ T cells was performed as described previously[13]. In short, after thawing, PBMCs at a concentration of $10*10^6$/ml were stimulated with 10^{-6} M CMV-derived pp65 and IE1 protein spanning peptide pools in culture medium supplemented with 10 IU/mL IL-2 (Chiron, Amsterdam, The Netherlands) for 24 hours at 37°C and 5% CO_2. Viability after thawing was consistently >75%. After stimulation the cells were stained with CD137-allophycocyanin (APC, BD Pharmingen, Franklin Lakes, USA), fluorescein isothiocyanate-labelled CD16 (BD, Franklin Lakes, USA), CD14 (BD Pharmingen), CD19 (BD) and TCRγδ (BD) (dump gate), phycoerythrin (PE labelled CD4, BD Pharmingen), Alexa fluor 700 labelled $CD8^+$ (Invitrogen, Waltham, MA, USA) and PE Texas Red labelled CD3 (Invitrogen) for 30 minutes at 4°C. Isolation was performed by Fluorescence Activated Cell Sorting using the FACS Aria (BD). CD16, CD14, CD19 and TCRγδ negative and CD3/CD4/CD137 triple positive and CD3/$CD8^+$/CD137 triple positive cells were sorted in bulk for chimerism analysis.

Chimerism analysis

Chimerism analysis on sorted CMV-specific $CD4^+$ and $CD8^+$ $CD137^+$ T cells was performed as described previously[14]. In short, we performed PCR analysis with primers specific for patient and donor selected polymorphic short tandem repeats using the AmpFLSTR Profiler Plus ID amplification kit (Applied Biosystems, Waltham, MA, USA) and a GeneAmp 9700 thermocycler (Applied Biosystems) using AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR products were analyzed using the ABI PRISM 3100 Genetic Analyzer and Genemapper V3.5 analysis software (Applied Biosystems). Maximum sensitivity of the markers was set at 2% for all markers.

Statistical analysis

Analysis of CMV reactivation and CMV disease was performed using competing risk analysis as described earlier[15]. Factors taken into account as competing risks were death, non-engraftment, rejection, systemic immune suppression, DLI and relapse. Additional analyses were performed using Student T-test IBM SPSS Statistics version 22.

Results

CMV reactivation and disease in CMV seropositive patients following TCD alloSCT

From the cohort of 157 CMV seropositive patients, 51 were transplanted with a CMV seronegative donor (R^+D^-) and 106 were transplanted with a CMV seropositive donor (R^+D^+). The donor and patient demographics (age, gender, type of conditioning regimen, unrelated/related donor) did not significantly differ between the two patient groups (**table 1**). The cumulative incidences of CMV reactivations and CMV disease were compared by separate competing risks analyses, taking non-engraftment, rejection, immune suppression, DLI, relapse and death of the patient without any of these events into account as competing risks. Non-engraftment did not occur and the cumulative incidence of rejection was very low in both groups (cumulative incidence 0.02 and 0.03 in R^+D^- and R^+D^+ respectively). The cumulative incidence of CMV reactivation did not differ between the R^+D^- cohort and the R^+D^+ cohort (0.80 versus 0.74 at 1 year after alloSCT, respectively; Gray's test p=0.91), nor did the moment of onset of CMV reactivation after alloSCT (27 days versus 22 days, range 4-129 vs. 4-271, respectively; p=0.7). In the patients who developed at least one CMV reactivation, the mean number of episodes of CMV reactivation was found to be similar in both groups (1.4 versus 1.4 CMV reactivations per patient in the R^+D^- (n=44) and R^+D^+ (n=84) group, respectively). However, the median duration of individual CMV reactivations was significantly longer in the R^+D^- cohort compared to the R^+D^+ cohort (54 versus 38 days, respectively, p=0.048). The cumulative incidence of CMV disease was significantly higher in the R^+D^- cohort compared to the R^+D^+ cohort (0.14 versus 0.02 at 1 year after alloSCT, respectively; Gray's test p=0.003; **table 1**). The cumulative incidences of the competing

events non-engraftment, rejection, immune suppression, DLI, relapse and death did not differ significantly between the two groups.

Table 1. Outcome of CMV reactivation and disease in CMV seropositive recipients transplanted with a CMV seronegative donor (R^+D^-) compared to CMV seropositive recipients transplanted with a CMV seropositive donor (R^+D^+) patients up to one year after TCD alloSCT.

	R^+D^-	R^+D^+	P
Total number of patients	51	106	
Male/Female	29/22	62/44	NS
Median age (years)	52	51	NS
Myelo-ablative conditioning	25 (49%)	49 (46%)	NS
Nonmyelo-ablative conditioning	26 (51%)	57 (54%)	NS
Matched related donor	24 (47%)	54 (51%)	NS
Matched unrelated donor	27 (53%)	52 (49%)	NS
CI Relapse	0.33	0.33	NS
CI Non relapse mortality	0.33	0.18	NS
Onset CMV reactivation (days after TX, range)	27 (4-129)	22 (4-271)	NS
Mean number of CMV reactivations	1.4	1.4	NS
Median days of CMV reactivation	54	38	0.048
CI CMV reactivation*	0.80	0.74	NS
CI CMV disease*	0.14	0.02	0.003
CI Systemic immune suppression	0.22	0.23	NS
CI Donor Lymphocyte Infusion	0.24	0.31	NS

* Competing risks analyses taking non-engraftment, rejection, systemic immune suppression, Donor Lymphocyte Infusion, relapse and death of the patient without CMV reactivation or CMV disease, respectively, into account as competing risks.
CMV = cytomegalovirus; TCD = T cell depleted; alloSCT = allogeneic stem cell transplantation; NS = not significant; CI = cumulative incidence; NRM = non-relapse mortality; CMV reactivation = defined by the detection of two consecutive positive CMV DNA loads (>$\log_{10}$ 2.7 (>500)/ml copies plasma); Days of CMV reactivation = number of days between first positive CMV DNA load ($\log_{10}$>2.7) and first negative CMV DNA load ($\log_{10}$<2.7). CMV disease = defined as previously published[8].

Origin of CMV-specific T cells in R^+D^- patients following TCD alloSCT

Chimerism analysis of circulating CMV-specific T cells in R^+D^+ patients demonstrated recipient and donor origin, ranging from mixed donor/recipient chimerism to full donor chimerism or full recipient chimerism (n=6, donor origin in 5/6, data not shown). To investigate the presence and origin of anti-viral immunity in R^+D^- patients, we performed in-depth analyses in the cohort of R^+D^- patients, allowing the discrimination of pre-existing patient-derived memory T cells and the possible induction of a primary CMV-directed immune response mediated by donor T cells. CMV-specific $CD4^+$ or $CD8^+$ T cells were detected by flow

cytometric analysis of expression of the activation marker CD137 upon stimulation of PBMC with protein spanning overlapping peptide pools of the CMV-derived proteins pp65 and IE1[11-13]. A representative example of CD137 expression on T cells following stimulation with CMV-derived pp65 and IE1 protein spanning peptide pools and the corresponding negative control without peptide stimulation is shown in **Figure 1**.

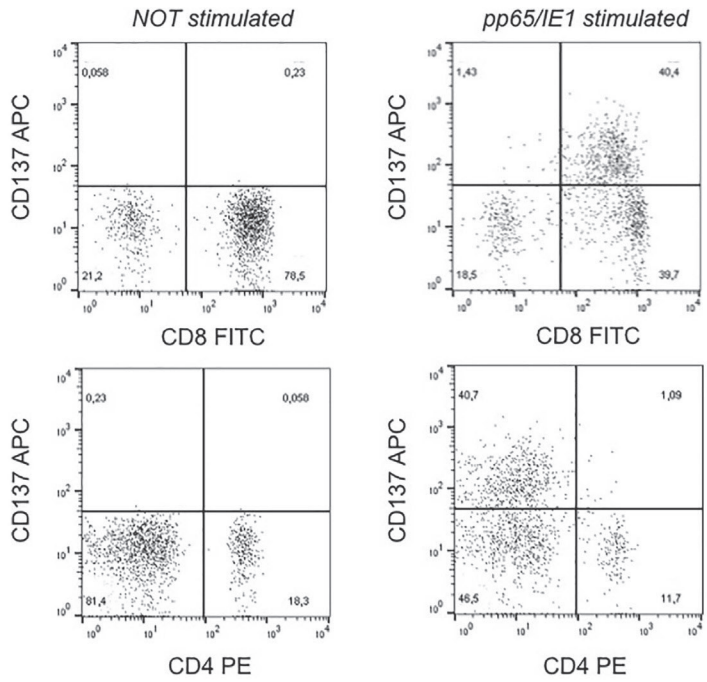

Figure 1.
Representative example for CD137 expression on unstimulated T cells and following stimulation of PBMC from R^+D^- patient with 10^{-6} M CMV-derived pp65 and IE1 protein spanning peptide pools for 24 hrs. Left panels show CD137 expression of unstimulated T cells ($CD8^+$ T cells on top panels) and $CD4^+$ T cells on bottom panels) and right panels demonstrate CD137 expression of stimulated T cells. Additional staining allowed for a gating strategy for bulk sorting of CD16, CD14, CD19 and TCRγδ negative and CD3/CD4/CD137 triple positive and CD3/$CD8^+$/CD137 triple positive cells.

From the cohort of 51 R^+D^- patients, 26 patients were excluded from this analysis due to graft failure, early disease relapse, therapeutic use of systemic immune suppression, early death or lack of cryopreserved samples for analysis. Twenty-five patients were eligible for analysis of the presence of CMV-specific $CD4^+$ and/or $CD8^+$ T cells. Samples were cryopreserved as part of routine follow-up after alloSCT (irrespective of viral load). In 19/25 (76%) of the analyzed patients of the R^+D^- cohort, visible frequencies of CMV-specific $CD4^+$ and/or $CD8^+$ T cells were detected (median of 198 (range 85-361) days after TCD alloSCT). The median frequency of CD4 CMV-specific T cells in the CD4 compartment was 3.3%, (range 0.4 to 5.1%; n=8) and the median $CD8^+$ CMV-specific T cells in the $CD8^+$ compartment was 6.2 (range 0.4 to 26.2%; n=18) **(Figure 2A)**. To analyze the origin of these CMV-specific $CD4^+$ and/or $CD8^+$

T cells, chimerism analysis was performed on CMV-specific T cells purified from peripheral blood of the 19 patients with detectable frequencies of circulating CMV-specific T cells. Of these 19 patients, 17 had developed a CMV reactivation within the first year following TCD alloSCT. As expected, in most patients the majority of these CMV-specific T cells were of recipient origin (median 95.5%,range 0-100; n=8) in CMV-specific $CD4^+$ T cells versus 100% (range 0-100; n=18) in CMV-specific $CD8^+$ T cells. However, although in varying frequencies, in 10/19 (53%) of patients in this R^+D^- cohort CMV-specific CD4 and/or $CD8^+$ T cells of donor origin were detected within the first year following TCD alloSCT **(Figure 2B)**. In the 2 patients without detectable CMV reactivation within the first year following TCD alloSCT (marked in green in Figure 2), unexpected high numbers of $CD4^+$ and $CD8^+$ CMV-specific T cells were detected (4.1% and 5.1 in $CD4^+$ compartment and 1.3% and 5.9% in $CD8^+$ compartment in both patients, respectively, analysis on day 85 and day 99). Part of these CMV-specific T cells was even found to be of donor origin in both patients (4% and 5% within $CD4^+$, and 0% and 9% in $CD8^+$ CMV-specific T cells, respectively).

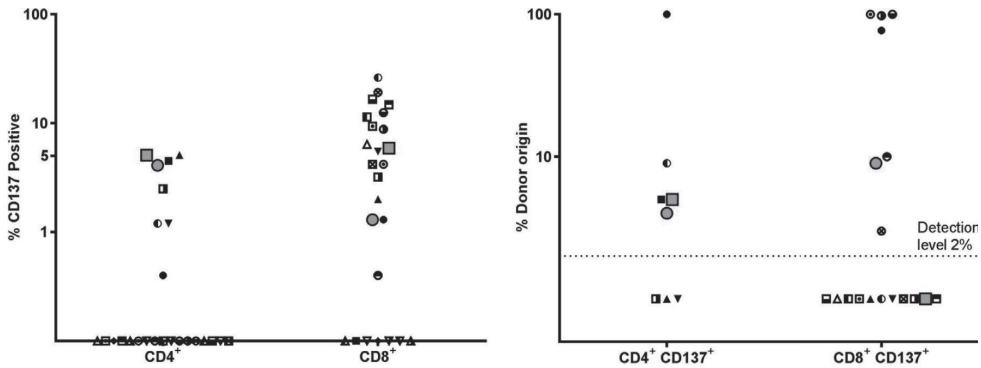

Figure 2.
Frequencies and origin of CMV-specific T cells in CMV seropositive patients after TCD alloSCT with a CMV seronegative donor (R^+D^-). (A) Frequencies of CMV-specific $CD4^+$ and $CD8^+$ T cells following TCD alloSCT were detected by flow cytometric analysis of CD137 expression upon stimulation with CMV-derived pp65 and IE1 protein spanning peptide pools in 19/25 R^+D^- patients. Frequencies of CMV-specific T cells in individual patients are depicted as unique symbols. The symbols in green represent 2 patients without detectable CMV reactivation in the first year following alloSCT. (B) Chimerism analysis of isolated CMV-specific $CD4^+$ $CD137^+$ and $CD8^+$ $CD137^+$ T cells. Samples were cryopreserved as part of routine follow-up after alloSCT (irrespective of viral load). The dotted line represents the detection level of 2% in chimerism analysis. Donor origin of CMV-specific T cells in individual patients is depicted as unique symbols. The symbols in green represent 2 patients without detectable CMV reactivation following alloSCT.

Discussion

The observed effect of the donor serostatus on the course of CMV reactivations in CMV seropositive patients suggests that in vitro TCD by addition of 20 mg of alemtuzumab to the bag is not 100% effective in fully depleting grafts from T cells**.** This importance of donor-derived CMV-specific memory T cells for sustained control of CMV reactivation has been demonstrated in previous studies[5]. Our clinical data on CMV reactivation are in agreement with these studies and suggest that donor-derived CMV-specific memory T cells are able to survive profound TCD and provide protective immunity. Indeed, chimerism analysis to assess the origin of CMV-specific T cells circulating in R^+D^+ patients demonstrated that CMV-specific immunity in these patients can be mediated by CMV-specific T cells of donor origin, patient origin or a mixture of these. A recent study[16] described loss of expression of the Alemtuzumab target antigen CD52 as a possible escape mechanism allowing survival of T cells (including virus-specific donor T cells) following alemtuzumab based TCD alloSCT.

The data in our manuscript confirm previous data on the origin of CMV-specific T cells following TCD alloSCT in CMV seropositive patients transplanted with a CMV seronegative donor (R^+D^-) and demonstrate that also recipient CMV-specific memory T cells are able to survive alemtuzumab based TCD and are the main actors supplying protective immunity to prevent CMV disease in these patients[5, 17]. However, the demonstration of donor-derived CMV-specific T cells, as indicator of the development of a donor-derived primary immune response after TCD alloSCT in R^+D^- patients, adds an important novel insight to the findings made in previous studies. It may provide a rationale for adoptive cell transfer (ACT) of CMV-specific T cells from healthy third party donors or autologous CMV-specific T cells harvested prior to the transplant for bridging the period of severe T cell deficiency prior to development of the primary T cell response[18-20]. Although these strategies imply a risk of rapid rejection, a short-term protective effect may be sufficient to prevent CMV disease while allowing the development of donor-derived CMV-specific T cells.

In previous studies, analysis of CMV-specific T cells after alloSCT consistently demonstrated mainly cells of patient origin[5, 17]. A possible explanation for the better detection of donor-derived CMV-specific T cells in our study may be the utilized methodology for detection and purification of virus-specific T cells. Whereas previous studies were focused only on CMV-specific $CD8^+$ T cells, isolated using peptide/MHC multimers, in our study we analyzed both CMV-specific $CD4^+$ and $CD8^+$ T cells, isolated based on expression of the activation marker CD137 upon stimulation with CMV-derived pp65 and IE1 protein spanning peptides, which allows the analysis of a broader repertoire of CMV-specific T cells[11-13].

In conclusion, we demonstrated a significantly increased duration of CMV reactivation and a significantly increased incidence of CMV disease in CMV seropositive patients transplanted with a CMV seronegative donor (R^+D^-) compared to CMV seropositive patients transplanted with a CMV seropositive donor (R^+D^+) following TCD alloSCT, illustrating that despite alemtuzumab-based TCD, memory T cells can be transferred from the graft to provide protective anti-viral immunity. Furthermore, we demonstrated that protective immunity

against CMV was predominantly mediated by T cells from recipient origin in patients transplanted with a CMV seronegative donor (R^+D^-) within the first year after TCD alloSCT, but that a primary donor-derived CMV-specific T cell response was frequently observed within the first year following TCD alloSCT, even as early as 3 months following TCD alloSCT.

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Chapter 5

Effective treatment of refractory CMV reactivation after allogeneic stem cell transplantation with in vitro-generated CMV pp65-specific $CD8^+$ T cell lines

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Abstract

To treat patients with refractory cytomegalovirus (CMV) reactivation after allogeneic stem cell transplantation, a phase I/II clinical study on adoptive transfer of in vitro-generated donor-derived or patient-derived CMV pp65-specific $CD8^+$ T cell lines was performed. Peripheral blood mononuclear cells from CMV seropositive donors or patients were stimulated with HLA-A*0201-restricted and/ or HLA-B*0702-restricted CMV pp65 peptides (NLV/TPR) and 1 day after stimulation interferon-γ)-producing cells were enriched using the CliniMACS Cytokine Capture System (interferon-γ), and cultured with autologous feeders and low-dose interleukin-2. After 7–14 days of culture, quality controls were performed and the CMV-specific T cell lines were administered or cryopreserved. The T cell lines generated contained 0.6–17 x 10^6 cells, comprising 54%–96% CMV pp65-specific $CD8^+$ T cells, and showed CMV-specific lysis of target cells. Fifteen CMV-specific T cell lines were generated of which 8 were administered to patients with refractory CMV reactivation. After administration, no acute adverse events and no graft versus host disease were observed and CMV load disappeared. In several patients, a direct relation between administration of the T cell line and the in vivo appearance of CMV pp65-specific T cells could be documented. In conclusion, administration of CMV pp65-specific $CD8^+$ T cell lines was found to be feasible and safe, and enduring efficacy of administered CMV pp65-specific $CD8^+$ T cell lines could be demonstrated.

Introduction

Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment for a variety of hematological malignancies and inherited hematopoietic disorders. A significant complication of alloSCT is the development of severe graft versus host disease (GvHD), which can be treated by immune suppression. Depletion of T cells from the stem cell graft can be performed to prevent the development of severe GvHD. However, T cell depletion and immune suppression significantly delay immune reconstitution in patients after alloSCT, which is associated with an increased risk of opportunistic infections including cytomegalovirus (CMV) infection.[1-3] Reactivation of CMV can cause serious morbidity and mortality during the prolonged period of immune deficiency after alloSCT, which is also illustrated by increased CMV-related morbidity in patients receiving a graft from a CMV seronegative donor.[4-5] The availability of antiviral agents like ganciclovir and foscarnet has contributed to a significant reduction of CMV-related morbidity and mortality after alloSCT. However, if appropriate T cell responses against CMV do not develop after alloSCT, subsequent viral reactivations and refractory disease are commonly observed. Reconstitution of the CMV-specific T cell repertoire directed against immunodominant proteins in the first year after alloSCT has been demonstrated to confer sustained protection from CMV disease. Furthermore, for the long-term protection against CMV, the development of CMV-specific T cell immunity has been found to be essential.[6-9] Therefore, adoptive transfer of donor-derived virus-specific T cells is an attractive strategy for treatment and prevention of viral disease in alloSCT recipients. Clinical studies have demonstrated that the adoptive transfer of donor-derived CMV-specific memory T cells can be a safe and effective treatment for patients with refractory CMV reactivation.[10-18]

In most clinical studies, CMV-specific $CD8^+$ T cell lines were generated by repetitive stimulation of the T cells in vitro for several weeks to obtain highly pure T cell lines to minimize the risk of the induction of GvHD.[11,13-18] However, isolation of CMV-specific T cells from peripheral blood also allows the generation of highly specific T cell lines without prolonged culture and repetitive stimulation. As it has been demonstrated that adoptive transfer of interferon-γ (IFNγ)-producing memory T cells can provide long-lived functional memory T cell reconstitution and that the presence of IFNγ-producing CMV-specific T cells is associated with protection from CMV reactivation, isolation of CMV-specific memory $CD8^+$ T cells based on their IFNγ production is an attractive strategy for generating effective CMV-specific $CD8^+$ T cell lines.[4,6,8,9,12,13,19] Although clinical studies have shown that adoptive transfer of CMV-specific T cells is safe and effective, in these studies the clinical effect could not always be directly correlated to the infusion of CMV-specific $CD8^+$ T cells.

In this study, a phase I/II clinical protocol for the adoptive transfer of donor or patient-derived CMV pp65- specific $CD8^+$ T cell lines for patients with refractory CMV reactivation after alloSCT was conducted. CMV- specific T cells were isolated using an IFNγ-based isolation technique and cultured for 1–2 weeks. Six patients with CMV reactivation failing antiviral therapy were treated with these CMV pp65-specific $CD8^+$ T cell lines. In all the 6 patients,

CMV DNA load turned negative and CMV- specific T cells could be detected in the peripheral blood. In 2 patients, a direct relation was demonstrated between the administration of CMV-specific T cells, the appearance of CMV-specific T cells in the peripheral blood, and the clearance of CMV reactivation.

Materials and methods

Study design

Adult and pediatric patients with refractory CMV reactivation after HLA-matched alloSCT were eligible. Patients were weekly monitored for CMV reactivation after receiving a stem cell transplant by measuring CMV DNA copy numbers with quantitative polymerase chain reaction. Refractory CMV reactivation was defined as persisting CMV viremia for >2 weeks while receiving pharmacotherapy or as early relapse (within 2 wk) after therapy. As only peptides binding in HLA-A*0201 or HLA-B*0702 were available for clinical use and therefore only T cells specific for these epitopes could be isolated, HLA-A*0201 and/or HLA-B*0702^{+} patients were eligible. In case of a CMV seropositive donor, a donor-derived T cell line was generated. When the donor was CMV seronegative, a patient-derived T cell line was generated from cryopreserved peripheral blood mononuclear cells (PBMNCs) harvested before transplantation. When the CMV pp65-specific T cell line met the release criteria and CMV was still detectable, the T cell line was administered to the patient. Antiviral treatment could be continued conform standard protocol. The procedure was repeated in case of stable or progressive CMV reactivation and no severe toxicity. Patients or their parents (in case of children) gave written informed consent, and the study was performed in accordance with the regulations of the institutional ethics committee.

The aim of this phase I/II study was to investigate the feasibility of generating CMV-specific T cell lines for clinical application, of which at least half should be administrated. Endpoints of the study were feasibility and toxicity of the treatment. Feasibility was defined as ≥80% success rate in generating a T cell line and toxicity was defined as ≥30% of patients developing GvHD after administration of the T cell line. In addition, the efficacy of the treatment was analyzed; however, this was not a primary endpoint.

Collection of peripheral blood cells

After informed consent, at least 2 x 10^9 PBMNCs were harvested by leukapheresis from each donor or patient. Red blood cells were lysed using an NH_4Cl (8.4g/L) and $KHCO_3$ (1 g/L) buffer (pH = 7.4) (LUMC Pharmacy, Leiden, The Netherlands). PBMNC were directly used or cryopreserved in the vapor phase of liquid nitrogen until further use. Cells were cryopreserved in IMDM (Lonza, Basel, Switzerland) containing 20 g/L human albumin (CeAlb; Sanquin, Amsterdam, The Netherlands) and 10% dimethyl sulphoxide (LUMC Pharmacy).

Generation and administration of clinical grade CMV-specific CD8$^+$ T cell lines

PBMNC were resuspended in culture medium consisting of IMDM supplemented with 3mmol/L L-glutamine (Lonza) and 100 U/mL penicillin and 100 mg/mL streptomycin (Lonza) plus 10% heat-inactivated pooled prescreened AB serum from healthy blood bank donors (Sanquin). Cells were cultured at a cell concentration of 10 x 10^6 cells/mL and 1 mg/mL of peptide was added. Peptides used were the HLA- A*0201-binding peptide NLVPMVATV (NLV) and the HLA-B*0702-binding peptide TPRVTGGGAM (TPR) both purchased from Bachem (Bubendorf, Switzerland).

After overnight stimulation, IFNγ-secreting cells were isolated using the CliniMACS Cytokine Capture System (IFNγ) (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. After washing, the positive fraction was resuspended in culture medium containing 10 IU/mL interleukin (IL)-2 (Proleukin; Novartis Pharma B.V., Arnhem, The Netherlands). Cells were cultured at a 1:10 ratio with 30 Gy-irradiated feeder cells, which were obtained from the negative fraction after isolation. Cultures were refreshed every 3–4 days, and after 7–14 days the T cell lines were analyzed. Cultures were evaluated for the presence of CMV NLV-specific and/or TPR-specific CD8$^+$ T cells by tetramer staining, and quality control was performed. Quality assurance specifications included: (1) no microbiological contamination; (2) confirmation of the origin of the material (donor or patient); (3) the presence of minimally 50% of CMV-specific CD8$^+$ T cells as measured by tetramer staining; and (4) the presence of maximally 10% CD19$^+$ B cells. Functionality was tested in a ^{51}Cr release assay and defined as minimally 20% lysis of positive target cells and maximally 10% lysis of negative target cells. When released for administration, the CMV-specific T cell lines were resuspended in 100 mL NaCl 0.9% (LUMC Pharmacy) supplemented with 2% human albumin (Sanquin) in a 500 mL cryocyte freezing container (Baxter, Deerfield, IL).

Phenotypic analysis

To determine the composition and phenotype of the starting material, the fractions after isolation, the T cell line, and the PBMC fractions from the peripheral blood cells were stained using fluorescein isothiocyanate-labeled TCRab, CD14, CD4, HLA-DR, CD27 (BD Biosciences, San Jose, CA) and CD197 (R&D systems, Minneapolis, MN) antibodies, phycoerythrin-labeled TCRgd, CD56, CD25 (BD) and IFNγ (Miltenyi Biotec) antibodies, PerCP-labeled CD8$^+$, CD3 (BD) and CD45RO (Invitrogen, Frederick, MD) antibodies, allophycocyanin (APC)-labeled CD19, CD33, CD45RA, CD28 (BD), and CD4 (Beckman Coulter, Fullerton, CA) antibodies, and PE-labeled or APC-labeled tetramers of the NLVPMVATV peptide bound to HLA-A*0201 or the TPRVTGGGAM peptide bound to HLA-B*0702, which were prepared as described previously.[20] After labeling, cells were analyzed on a FACSCalibur (BD).

Cytotoxicity assay

To determine the cytotoxicity of the T cell lines, standard ^{51}Cr release assays were performed as described previously.[21] Target cells (donor and patient phytohemagglutinin (PHA) blasts,

HLA-A*0201 and HLA-B*0702^{+} EBV-LCL (JY), or pp65-transduced JY (JY-pp65)[22]) were loaded with 100 mCi of $Na^{51}Cr_2O_4$ (Amersham, Roosendaal, The Netherlands) and when necessary with 1mM of peptide (NLV or TPR) for 1 hour. Effector/target ratios ranged from 30:1 to 1:1 and after 4 hours of incubation ^{51}Cr release was determined.

Ex vivo analysis of cytokine production of CMV-specific T cells

After thawing, PBMNC from the patients were resuspended in culture medium and with 10^{-6} M NLV or TPR peptides for 5 hours at 371C and 5% CO_2. After 1 hour of peptide stimulation, 10mg/mL of brefeldin A (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added for the remaining 4 hours of incubation. After stimulation, cell-surface staining with monoclonal antibodies was performed, followed by intracellular staining as described previously.[23] Cells were stained using fluorescein isothiocyanate-labeled IFNγ (BD), PE-labeled tumor necrosis factor (TNFα) (BD), PerCP-labeled CD4 and CD8^{+} (BD), and APC-labeled IL-2 (BD).

Ex vivo identification of CMV-specific CD8^{+} T cells

To identify donor or recipient origin of CMV-specific T cells reconstituting in peripheral blood after infusion of the CMV pp65-specific CD8^{+} T cell lines, PBMNC from the patient were stained using HLA class I tetramers specific for NLV or TPR. Subsequently, the tetramer^{+} CD8^{+} T cells were selected by flow cytometric sorting using a FACSDiva and Cellquest software (BD). The DNA profile of the sorted cells was established by chimerism analysis based on short tandem repeats polymorphism as previously described.[24]

Results

Generation of clinical grade CMV-specific CD8^{+} T cell lines

A total of 12 donor-derived and 3 patient-derived CMV-specific T cell lines specific for the HLA-A*0201-restricted NLV epitope and/or for the HLA-B*0702-restricted TPR epitope were generated for patients failing antiviral therapy after alloSCT. Donor-derived T cell lines were generated when the donor was CMV seropositive, whereas patient-derived T cell lines were generated when the donor was CMV seronegative and the patient CMV seropositive. **Figure 1** shows the data of a representative donor-derived (Figs. 1A–D) and patient-derived (Figs. 1E–H) CMV-specific T cell line. The frequencies of the NLV-specific T cells in the PBMNC starting material were 0.4% in the donor (Figure 1A) and 1.45% in the patient (Figure 1E). After overnight stimulation with the HLA-A*0201-binding NLV peptide, IFNγ-producing cells were isolated using the CliniMACS Cytokine Capture System (IFNγ). The positive fraction contained between 20% and 25% IFNγ^{+} CD8^{+} T cells within the lymphocyte gate (Figure 1B, F), other T cells present in the positive fraction were CD4^{+} T cells and IFNγ-negative CD8^{+} T cells. After 10 days and 7 days of culture, respectively (Figs. 1C, G), specific proliferation of the NLV-specific CD8^{+} T cells resulted in a donor-derived T cell line containing 83% NLV-

specific $CD8^+$ T cells and in a patient-derived T cell line containing 66% NLV-specific $CD8^+$ T cells. Less than 1% of $CD19^+$ B cells were detected in the T cell lines (data not shown). As is shown in Figures 1D, H both T cell lines showed reactivity against NLV-loaded target cells and no reactivity against donor or patient cells. Furthermore, both T cell lines also showed recognition of endogenous presented antigen using CMV pp65-transduced EBV-LCL (JY-pp65).

Table 1 shows the results of all the CMV-specific T cell lines generated. Frequencies of NLV-specific and/or TPR- specific $CD8^+$ T cells in the donor or patient starting material used for the generation of the T cell lines ranged from 0.02% to 1.45% of total PBMNC. Cell numbers obtained directly after isolation ranged from 0.25 to 14.4 x 10^6 cells and contained between 3% and 45% $IFN\gamma^+$ $CD8^+$ T cells within the lymphocyte gate. Total cell numbers obtained after culture ranged from 0.6–17.1 x 10^6 cells. During culture, no increase in total cell numbers was observed, but further enrichment of CMV-specific T cells was observed resulting in T cell lines containing 54%–96% NLV-specific and/or TPR-specific T cells.

All donor-derived and patient-derived CMV-specific T cell lines generated met the quality control criteria. No microbiological contamination was detected in any of the T cell lines, and chimerism analysis showed the correct origin of the material. As is shown in Table 1, all T cell lines contained >50% of NLV-specific and/or TPR-specific T cells and showed recognition of peptide-loaded PHA blasts and no reactivity against donor-derived or patient- derived PHA blasts.

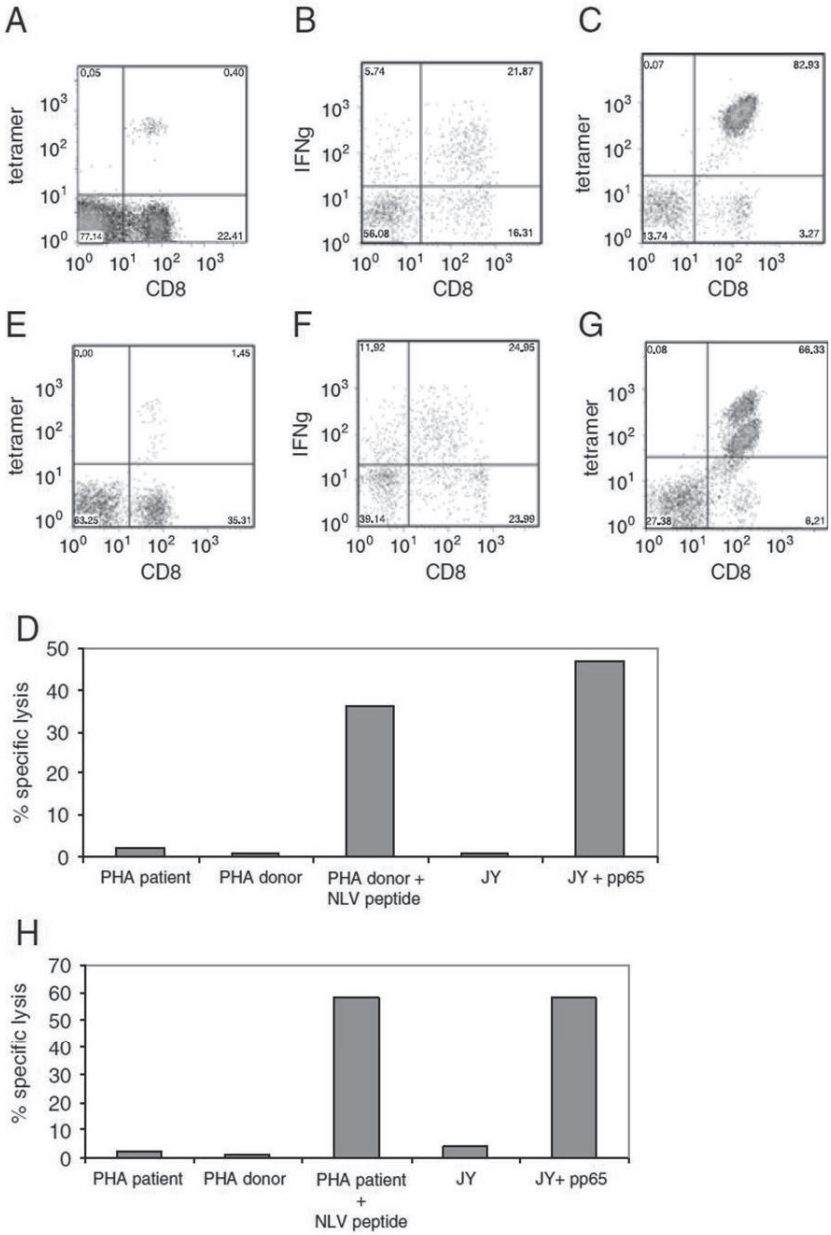

Figure 1
Composition and functionality of donor-derived (A-D) and patient-derived (E-H) CMV-specific T cell lines. **(A, E)** the percentage of CMV NLV (A) and CMV TPR (E) -specific T cells in the starting material (day 0); **(B, F)** the percentage of IFN positive cells in the positive fraction direct after isolation; **(C, G)** the percentage of CMV NLV (C) or CMV TPR (G) in the T cell line after culture (day 7); **(D, H)** functionality of the T cell lines in a ^{51}Cr release assay. Dot plots are shown and events shown are gated on the lymphocyte gate and PI-negative gate. Percentage lysis after 4 hours in a ^{51}Cr release assay at an E:T ratio of 3:1 is depicted. JY = EBV-LCL; JY-pp65 = CMV pp65 transduced JY.

Table 1. Characteristics of the CMV-specific T cell lines

CTL Line	Specificity	freq in PBMC	# cells (x 10^6)[2]	Phenotype (% positive cells)[2, 3]			# cells (x 10^6)[2]	Cytotoxicity[2,4]		
								PHA donor		PHA
				CD4	$CD8^+$	$CD8^+Tet^+$	$CD8^+Tet^+$	- peptide	+ peptide	patient
P1-a	TPR	0.28	3.4	26	71	68	2.3	1	53	4
P1-b	TPR	0.28	3.8	10	88	72	2.7	2	70	0
P2-a	TPR	0.3	7.5	9	91	81	6.1	2	56	2
P3-a	TPR	0.02	5.0	20	80	72	3.6	1	41	4
P4-a	NLV+TPR	<0.01 / 0.25	5.9	11	85	5 / 69	3.8	3	66	0
P4-b	NLV+TPR	<0.01 / 0.25	17.1	5	73	14 / 50	12.7	1	39	0
P5-a	TPR	0.14	4.8	4	95	95	3.1	0	26	1
P7-a	NLV+TPR	0.04 / 0.52	10	4	96	4 / 92	9.6	9	65	0
P8-a[1]	TPR	0.7	3.0	11	77	66	2.1	1	62	1
P9-a	NLV	0.48	1.0	22	77	65	0.7	1	80[5]	0
P9-b	NLV	0.98	3.3	7	93	83	2.7	2	30	1
P10-a[1]	NLV	1.45	6.0	8	90	79	4.7	1	58	2
P11-a[1]	NLV	1.1	6.7	27	61	54	3.6	2	25	4
P12-a	NLV	0.4	16.8	9	85	82	13.8	1	36	2
P14-a	NLV	0.17	0.6	n.d.	88	71	0.43	n.d.	n.d.	n.d.

[1]patient derived T cell line; [2] after 7-14 days culture; [3] within lymphocyte gate; [4] percentage lysis after 4 hours in a 51Cr release assay, E:T ratio of 3:1;
[5] measured in a CFSE-based cytotoxicity assay (29); Tet = tetramer; PHA = PHA blasts; TPR = TPRVTGGGAM peptide; NLV = NLVPMVATV; n.d. = not done.

Administration of CMV-specific CD8$^+$ T cell lines

In a phase I/II clinical study, the toxicity and the potential antiviral effect of treatment with CMV pp65- specific T cell lines for refractory CMV reactivation after alloSCT was investigated. Eight of the 15 T cell lines generated were administrated. Six patients with refractory CMV reactivation after alloSCT received donor-derived CMV pp65-specific T cell line(s). Four patients (patient 2, 3, 4, and 14) received 1 CMV pp65-specific T cell line and 2 patients (patient 1 and 9) received 2 CMV pp65-specific T cell lines. The other T cell lines generated were not administrated, as patients were CMV load negative (n = 4), had relapsing malignant disease (n = 1), or deceased because of CMV disease (n = 2) at the time the production of the CMV pp65-specific T cell line was completed.

Despite prior treatment with antiviral agents, all 6 patients who received a CMV pp65-specific T cell line had ongoing positive CMV DNA loads (log 2.5–log 3.2) at the time of administration of the CMV-specific T cell lines. The patients received a total cell dose ranging from 0.6 to 7.5 x 10^6 cells (analogous to 0.9 x 10^4–3.1 x 10^5 T cells/kg), corresponding to 0.4–6.1 x 10^6 NLV-specific and/or TPR- specific T cells. In 5 out of 6 patients, antiviral therapy was discontinued before infusion of the CMV-specific T cell line. Patient 9 was treated until 2 weeks after infusion of the second CMV-specific T cell line. None of the 6 patients developed GvHD or any other complications during infusion of the T cell lines, and all patients cleared CMV reactivation within weeks after administration of the CMV pp65-specific T cell line.

Reconstitution of CMV-specific T cells after administration of the CMV-specific CD8$^+$ T cell lines

In all patients CMV-specific, NLV-specific, and/or TPR-specific CD8$^+$ T cells could be detected after administration of the T cell lines. To determine a possible correlation between the infusion of the CMV-specific T cell lines and the appearance of CMV-specific T cells in peripheral blood of the patients, the presence of CMV- specific T cells was analyzed at different time points before and after administration of the T cell lines. Before infusion of the CMV-specific T cell lines in 4 of the 6 patients, CMV pp65-specific T cells with the same specificity as the T cell line could already be detected in the peripheral blood. In 3 of these patients, a rise in CMV-specific T cells in the peripheral blood was observed after infusion of the T cell line. In the other patient, the numbers of CMV-specific T cells was stably high after infusion. In 2 of the 6 patients (patient 4 and 9), no CMV-specific T cells were detected before infusion of the T cell line, and a direct relation was observed between infusion of the CMV pp65-specific T cell line, the appearance of CMV pp65-specific T cells in the peripheral blood.

From 3 patients showing CMV-specific T cells already before infusion, we compared the phenotype and functionality of the CMV-specific T cells in peripheral blood before and after infusion. As illustrated in **Figure 2A**, both before and after infusion the CMV-specific T cells in peripheral blood of the patients predominantly produced IFNγ and TNFα after antigenic stimulation; only a minority produced IL-2 after restimulation with the specific peptide. Both

effector memory (CD45RO$^+$, CD45RA$^-$, CD27$^-$, CD28$^-$) and effector (CD45RO$^-$, CD45RA$^+$, CD27$^-$, CD28$^-$) CMV-specific T cells were similarly found before and after infusion (data not shown). In addition, the tetramer-binding capacity of the CMV pp65 NLV-specific and/or TPR-specific T cells was not different before and after adoptive transfer, as is shown in **Figure 2B**. Thus, the contribution of the infused CMV-specific T cells to the clearance of CMV could not be determined in these patients.

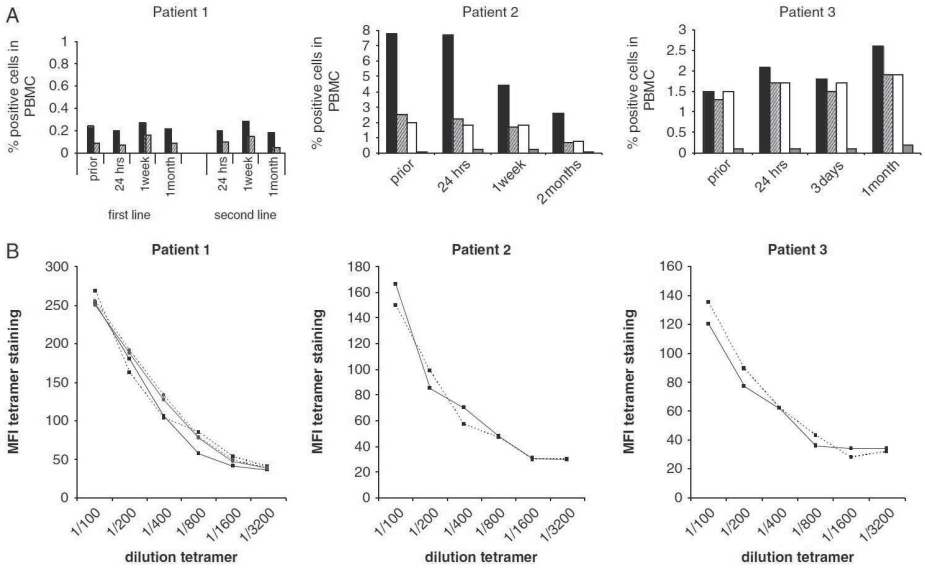

Figure 2.
A, Cytokine production after antigen-specific restimulation of cytomegalovirus (CMV)-specific T cells in PBMNC from patient 1 to 3 obtained prior and at different time points after infusion of the CMV-specific T cell line (depicted on the x-ax). The percentage of positive cells within the peripheral blood mononuclear cells (PBMNC) population is shown. Black bars represent tetramer positive cells; dashed bars represent interferon (IFN)γ-producing T cells; white bars represent TNFα-producing T cells; and gray bars represent interleukin-2- producing T cells. B, Tetramer-binding capacity of CMV TPR-specific T cells of patients 1, 2, and 3 in the peripheral blood before and after infusion of the CMV-specific T cell line. The mean fluorescence intensity of the tetramer staining and the dilution of the tetramer is depicted. Solid lines are before infusion and the broken lines are after infusion of the T cell lines; ■, first infusion; •, second infusion.

Patient 4 developed CMV reactivation within the first month after alloSCT, which was treated with antiviral drugs (**Figure 3**). The rise in CMV load coincided with a rise in CMV TPR-specific T cells. Four months after alloSCT, the patient received unmanipulated donor lymphocyte infusion for the treatment of progressive disease, and 6 months after alloSCT the patient developed GvHD for which he was treated. The CMV-specific T cells disappeared, and the patient developed refractory CMV reactivation. A CMV TPR-specific T cell line was administered 8 months after alloSCT (7.6 x 10^4 T cells/kg) and within 2 weeks CMV TPR-specific CD8$^+$ T cells appeared in peripheral blood, and the CMV load turned negative and remained negative (follow-up >4 y). To asses why no persistence of the CMV TPR-specific

T cells from the first peak response was observed and to determine whether the CMV TPR-specific T cells appearing in the second peak response were the same as the T cells in the first peak response, the patient or donor origin of the CMV-specific T cells from both peak responses (day 33 and day 289 after alloSCT, respectively) was determined. The CMV TPR-specific T cells were isolated from both peak responses by fluorescence-activated cell sorting, and DNA profiling showed that the CMV-specific T cells during the first response (day 33) were of patient origin, and that the CMV-specific T cells that appeared after administration of the T cell line (day 289) were completely donor-derived, illustrating the correlation between the infusion of the CMV TPR-specific T cell line and the in vivo appearance of the donor-derived CMV TPR-specific T cells. Ex vivo stimulation of PBMNC isolated from the patient after adoptive transfer of the T cell line with the specific CMV peptide illustrated high IFNγ and TNFα production by the CMV-specific T cells.

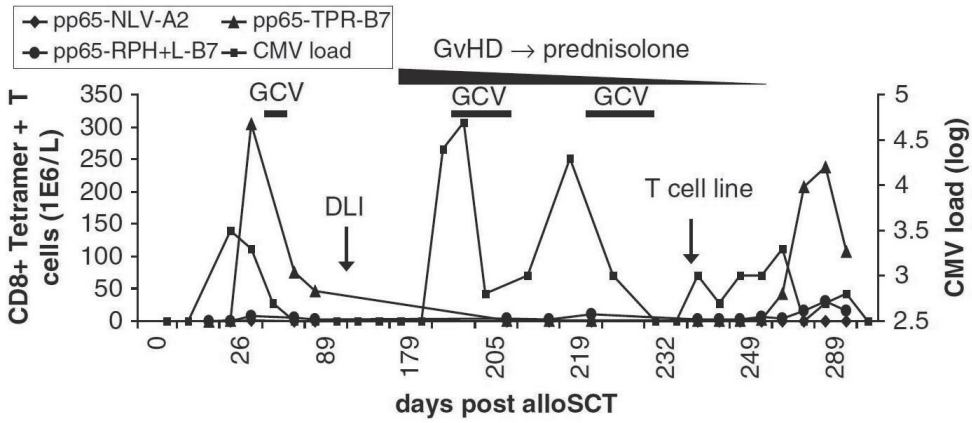

Figure 3
CMV pp65-specific T cells in peripheral blood from patient 4 in relation to the CMV DNA load in peripheral blood. CMV-specific T cells ($CD8^+$/tetramer+) are expressed as absolute numbers per liter in peripheral blood (left axis). ▲, CMV TPR-specific T cells; ◆, CMV NLV-specific T cells; ●, CMV RPH-specific T cells. CMV DNA load (■) is depicted as log CMV DNA load in peripheral blood (right axis).The lower level of detection is log 2.5. GCV = ganciclovir; GvHD = graft versus host disease.

Patient 9 developed refractory CMV reactivation post- alloSCT in the absence of CMV-specific T cells (**Figure 4**). A CMV NLV-specific T cell line was administrated at day 72 (1 x 10^5 T cells/kg), but no CMV NLV-specific T cells appeared in peripheral blood and the CMV load remained positive. The quality of the T cells in this T cell line was poor because of poor quality, as reflected by the low cell viability, of the starting material, explaining the absence of in vivo persistence of the infused T cells. A second CMV NLV-specific T cell line was generated from new starting material, which was administered at day 116 after alloSCT (3.1 x 10^5 T cells/kg). After 2 weeks, CMV NLV-specific T cells appeared in peripheral blood, which was coincided with a disappearance of CMV DNA load (follow-up >2 y).

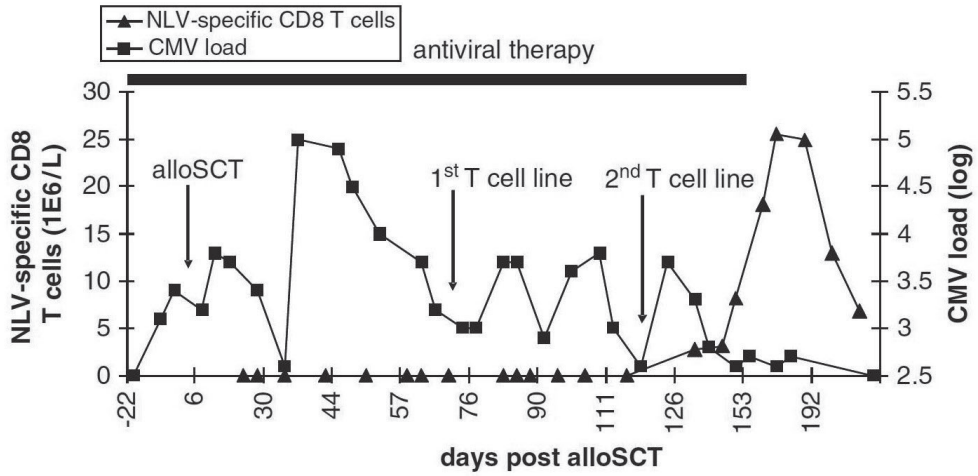

Figure 4.
CMV pp65 (NLV)-specific T cells in peripheral blood from patient 9 in relation to the CMV DNA load in peripheral blood. CMV-specific T cells (▲, $CD8^+$/CMV NLV-tetramer+) are expressed as absolute numbers per liter in peripheral blood (left axis) and CMV DNA load (■) is depicted as log CMV DNA load in peripheral blood (right axis). The lower level of detection is log 2.5.

Discussion

In this study, a phase I/II clinical protocol for the adoptive transfer of donor-derived or patient-derived CMV pp65-specific $CD8^+$ T cell lines for patients with refractory CMV reactivation after alloSCT was conducted. No toxicity was observed after administration of the T cell lines, and all patients who received a CMV-specific T cell line cleared CMV reactivation. A direct relation between administration of donor-derived CMV pp65-specific T cell lines and clinical effect could be demonstrated in 2 patients. Limited culture time in vitro using low doses of IL-2 apparently does not impair the ability of T cells to expand in vivo and to be effective. This study illustrates that clinical efficacy of adoptively transferred T cells can be achieved with low cell numbers.

Both donor-derived and patient-derived CMV pp65-specific $CD8^+$ T cell lines could be reproducibly generated, even when frequencies of CMV pp65-specific $CD8^+$ T cells were very low (< 0.01%). Directly after isolation, the purity of the positive fraction (percentage $CD8^+$ / $IFN\gamma^+$ T cells) appeared to be low for some isolation, which was also observed in other studies using the IFNγ-based isolation method.[12,26] This can be due to nonspecific co-isolation of other cells, what preponderates especially in those cases where the starting frequency of CMV-specific T cells is very low. Preferential expansion of the CMV-specific $CD8^+$ T cells during the subsequent culture period led to T cell lines with a high purity of CMV-specific $CD8^+$ T cells, reducing the risk of the induction of GvHD after administration.

In the current study, 6 patients with refractory CMV reactivation received 1 or 2 donor-derived CMV-specific CD8$^+$ T cell line(s). Administration was safe, no toxicity was observed after infusion, and all patients cleared CMV reactivation after treatment with the CMV-specific CD8$^+$ T cell lines. In 4 patients, T cells with the same reactivity as the T cell line could already be detected in peripheral blood of the patients before administration of the T cell lines. Although it is likely that the infused T cells contributed to the clearance of CMV reactivation in these patients, direct evidence for this cannot be provided. No differences in the phenotype, cytokine profile, or tetramer-binding capacity of the NLV-specific or TPR-specific T cells in the peripheral blood before and after adoptive transfer could be determined.

However, in 2 of the 6 patients a direct time correlation between the administration of donor-derived CMV pp65-specific T cell lines, the appearance of CMV-specific CD8$^+$ T cells, and clearance of CMV reactivation was demonstrated. In these patients, no CMV-specific T cells were present before the administration of the T cell lines, and within 2 weeks after administration of the T cell line increasing numbers of CMV NLV-specific or CMV TPR- specific CD8$^+$ T cells could be detected in peripheral blood of the patients, illustrating persistence and in vivo proliferation of the infused T cells. The appearance of the CMV-specific CD8$^+$ T cells coincided with the clearance of CMV reactivation. Although CMV-specific CD8$^+$ T cells have been detected in peripheral blood after the adoptive transfer of CD8$^+$ T cell lines in previous studies, this study shows a direct correlation between the administration of a CD8$^+$ T cell line and the clinical effect.[10,11,13-18]

Two patients eligible for the study deceased because of CMV disease. CMV-specific T cell lines could be generated for these patients, however, both patients deceased before the T cell line could be administered. Decreasing the time interval between the appearance of the clinical need and administration of the T cell product by direct administration of the isolated CMV pp65-specific T cells may improve the efficacy, although in vivo proliferation is also likely to be necessary in these patients to allow a clinical effect. It has been shown that direct administration of CMV-specific T cells isolated based on their IFNγ production can be effective and does not lead to GvHD induction[12]

In the current protocol, T cell lines could only be generated for patients who were HLA-A*0201 and HLA- B*0702$^+$. To increase the potential clinical use of CMV- specific T cells, a CMV pp65 whole protein overlapping 15-mer (11-mer overlapping) peptide pool may be used for the generation of CMV pp65-specific T cell lines. It has been shown by us and others that using this peptide pool CMV pp65-specific T cells can be isolated irrespective of HLA restriction of the donor and patient.[23,27-29] Furthermore, using these peptide pools also CMV-specific CD4$^+$ T cells will be isolated and studies have indicated that adoptively transferred CMV-specific CD4$^+$ T cells may promote development of a CMV-specific CD8$^+$ T cell response in vivo and that adoptively transferred CMV-specific CD8$^+$ T cells may show better persistence in the presence of CMV-specific CD4$^+$T cells.[12,13,15-18,27]

In conclusion, we have shown that the clinical grade generation of donor-derived and patient-derived CMV pp65-specific $CD8^+$ T cell lines is feasible, and that administration of CMV-specific T cell lines to patients with refractory CMV reactivation after alloSCT was safe. A direct relation between infusion of CMV-specific $CD8^+$ T cells and clinical effect could be demonstrated.

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Chapter 6

Identification of varicella zoster virus-specific CD8$^+$ T cells in patients after T cell depleted allogeneic stem cell transplantation

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Abstract

To study the role of $CD8^+$ T cells in the control of varicella zoster virus (VZV) reactivation, we developed multimeric major histocompatibility complexes to identify VZV-specific $CD8^+$ T cells. Potential HLA-A2 binding peptides from the putative immediate-early 62 protein (IE62) of VZV were tested for binding, and peptides with sufficient binding capacity were used to generate pentamers. Patients with VZV reactivation following stem cell transplantation were screened with these pentamers, leading to the identification of the first validated class I-restricted epitope of VZV. In 42% of HLA-A2 patients following VZV reactivation, these IE62-ALW-A2 T cells could be detected ex vivo.

Varicella zoster virus (VZV) infects about 95% of the population, persists throughout life, and may lead to herpes zoster when the virus reactivates. After T cell depleted allogeneic stem cell transplantation (TCD alloSCT), reactivation of the virus leads to considerable morbidity[10]. Primary infection elicits both humoral and cellular responses, but cellular immunity is essential for preventing herpes zoster. The VZV genome comprises more than 70 unique open reading frames that encode proteins that are coordinately expressed during replication. The product of open reading frame 62, the immediate-early 62 (IE62) protein, is required for the initiation of VZV replication[9] and is expressed at high levels before viral replication has occurred[8]. Previous research has demonstrated that IE62-specific T cells were detected after primary VZV infection and in immune subjects[2, 4]. In addition, T cells recognizing various other IE proteins and glycoproteins of VZV, as demonstrated by gamma interferon (IFN-γ) production upon stimulation with peptides or lysate derived from these proteins, have been described[1, 6, 13]. The VZV- specific memory T cells found in these studies were predominantly CD4 T cells, while no VZV-specific $CD8^+$ T cells were demonstrated without prior in vitro expansion, possibly due to the low frequency of VZV-specific $CD8^+$ T cells or to the low sensitivity of the screening methods used to detect $CD8^+$ T cells by IFN- γ production upon stimulation. Frey et al. described $CD8^+$ epitopes of IE62 detected following in vitro restimulation. However, the HLA restriction and specificity of these T cells were not confirmed[4]. Due to the lack of validated VZV-derived immunodominant peptides for major histocompatibility complex (MHC) class I, the analysis of VZV-specific $CD8^+$ T cell responses is hampered[14]. To be able to analyze the role of $CD8^+$ T cells in VZV reactivation, we therefore set out to identify epitopes for VZV by using VZV-IE62-specific MHC class I peptide complexes.

The predictive algorithms BIMAS[11] and SYFPEITHI[12] were used to select potential HLA-A2 binding peptides from the IE62 protein. Peptides with a score of ≥ 3 (BIMAS) or ≥ 20 (SYFPEITHI) were considered to have potentially significant binding affinity. The 81 resulting 9-mer peptides were synthesized and tested for binding affinity with the REVEAL MHC-peptide binding assay (ProImmune, Oxford, United Kingdom). HLA-A2 binding affinity was determined by the ability of the peptides to stabilize the HLA-peptide complex. Based on the binding affinity measurements, 34 high- to medium-affinity HLA-A2 binding peptides were selected and used to generate ProVE MHC pentamers (ProImmune, Ox- ford, United Kingdom).

To enable screening of this large number of pentamers, the pentamers were divided into five pools, each containing six or seven pentamers. In the initial screening with pooled pentamers, four HLA-A2-positive patients were screened after a clinical diagnosis of VZV reactivation after TCD alloSCT. The presence of viral DNA in plasma at the time of clinical observations of VZV reactivation was confirmed by real-time PCR on plasma samples as previously described[7]. After informed consent was obtained, peripheral blood mononuclear cells (PBMCs) were cryopreserved and thawed and 0.5×10^6 cells were incubated with pentamers at a concentration of 0.03 mg/ml for 10 min at room temperature in RPMI

medium supplemented with 2% fetal bovine serum. After the cells were washed twice, 8 μl of FluoroTag-phycoerythrin (PE) was added for 20 min of incubation at 4°C and the cells were counterstained with CD4, CD40, and CD19- fluorescein isothiocyanate (FITC). Flow cytometric analysis was performed on a FACScalibur fluorescence-activated cell sorter (FACS; Becton-Dickinson [BD], San Jose, CA). In one of four patients, pentamer pool 6, containing pentamers 61, 62, 64, 65, 66, and 67, was positive (0.06% of $CD8^+$ T cells); no other positive signals were observed. Staining with the individual pentamers revealed that pentamer 66, containing the epitope ALWALPHAA derived from the IE62 protein of VZV (IE62-ALW-A2) was responsible for the positive signal (0.06% of $CD8^+$ T cells, **Figure 1B**).

To confirm the specificity of the IE62-ALW-A2-specific T cells, the pentamer-positive T cells were sorted into a single cell per well with a FACSDiva (BD) and expanded as previously described[5]. The expanded T cell clones were labeled specifically with the IE62-ALW-A2 PE-conjugated tetramer that was constructed as previously described[3] **(Figure 1D)**, and Vβ analysis with the T cell receptor Vβ repertoire kit (BD) showed that at least two different T cell clones were isolated, demonstrating the oligoclonal origin of IE62-ALW-A2-positive T cells **(Figure 1E and F)**.

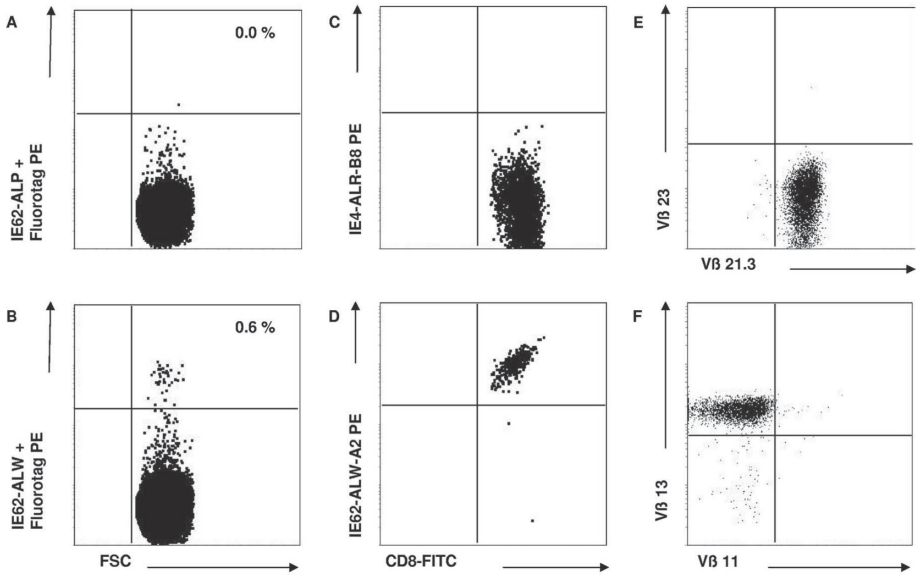

Figure 1.Screening with Pentamers containing VZV derived immunogenic epitopes.
PBMCs of a patient after VZV reactivation following a TCD alloSCT were incubated with Pentamers and then stained with Fluorotag-PE to detect the Pentamer positive cells (A and B), and counterstained with CD4-, CD40- and CD19FITC. Pentamer staining is shown of the CD4, CD40, CD19 negative cells. (A) PBMCs stained with Pentamer 67 containing the epitope ALPHAAAAV, showing no specific staining. (B) PBMCs stained with Pentamer 66 containing the epitope ALWALPHAA, showing specific staining. IE62-ALW-A2-specific T cell clones were sorted single cell per well and expanded non-specifically. The clones were stained with an irrelevant tetramer (C) and the IE62-ALW-A2 tetramer (D) in combination with $CD8^+$ FITC. Clones 1 and 2 were stained with Vbeta kit (BD) to demonstrate that clone 1 (E) and clone 2 (F) express different TCR. The results demonstrate that we have isolated different T cell clones that specifically stain with the IE62-ALW-A2 tetramer.

To assess the cytolytic capacity of IE62-ALW-A2 T cells, chromium release assays were performed as described earlier[5]. ^{51}Cr-labeled Epstein-Barr virus (EBV) lymphoblastoid cell lines (LCLs) loaded with the IE62-ALW peptide were incubated with IE62-ALW-A2 T cells for 4 h. As demonstrated in Figure 2A, HLA-A2-positive EBV LCLs loaded with the IE62-ALW-A2 peptide were lysed by both T cell clones, whereas unloaded EBV LCLs were not lysed. To determine the avidity of the T cell clones, the IE62- ALW-A2 peptide was titrated on EBV LCLs, and after 24 h of coculture, supernatants were harvested and used to determine the IFN-γ production of the stimulated T cells by standard enzyme-linked immunosorbent assay. Half-maximum IFN-γ production of the T cell clones was observed when the stimulator cells were loaded with 10 ng/ml peptide, indicative of high-avidity T cell clones **(Figure 2B)**. To determine whether the T cells recognized cells endogenously expressing the IE-62-encoding gene, COS-A2 cells were transfected with Lipofectamine (Invitrogen, Carlsbad, CA) by using pcDNA vectors coding for different VZV genes, which were kindly provided by E. Wiertz (Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands). The transfected COS-A2 cells were used 24 h after transfection

as stimulator cells in this assay. After 24 h of coculture, supernatants were harvested and used to determine the IFN-γ production of the stimulated T cells. IE62-ALW-A2 T cell clones produced IFN-γ in response to COS-A2 cells endogenously expressing the IE62 protein, as well as COS-A2 cells pulsed with the IE62-ALW-A2 peptide. No IFN-γ was produced when the COS-A2 cells were transfected with the IE63-encoding gene of VZV or pulsed with an irrelevant peptide **(Figure 2C)**.

To determine whether IE62-ALW-A2-specific T cells were present in healthy individuals, cryopreserved PBMCs from 18 healthy, VZV-seropositive, HLA-A2-positive individuals were screened with the PE-conjugated VZV tetramer. PBMCs were labeled with tetramers for 15 min at 37°C in RPMI medium without phenol supplemented with 2% fetal bovine serum, washed, and analyzed with a FACScalibur. In 3 of these 18 serologically VZV-positive individuals, IE62-ALW-A2 tetramer-positive T cells could be detected (range, 0.01 to 0.02% of $CD8^+$ T cells). These data demonstrate that IE62-ALW-A2- specific T cells can be observed and that the frequency of these T cells is low under steady-state conditions in immunocompetent persons.

To assess the frequency of IE62-ALW-A2-specific T cells in a cohort of patient who suffered from VZV reactivation following TCD alloSCT, 19 HLA-A2-positive patients after VZV reactivation following TCD alloSCT were screened by using the IE62-ALW-A2 tetramer. We screened these patients at a median of 47 days after the clinical diagnosis of VZV reactivation. In 8 of these 19 patients, IE62-ALW-A2-specific T cells could be directly detected ex vivo (mean, 0.04% [range, 0.01 to 0.11%] of $CD8^+$ T cells), indicating that this epitope is recognized in 42% of the HLA-A2-positive patients during VZV reactivation **(Table 1)**. In VZV-seronegative patients (six screened), no IE62-ALW-A2 tetramer-positive cells could be detected.

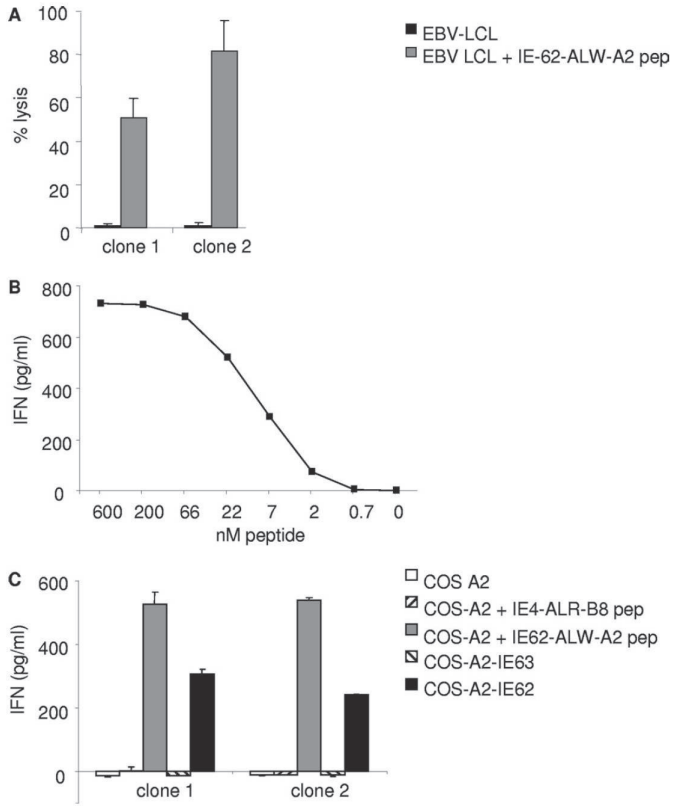

Figure 2. IE62-ALW-A2 T cells recognize IE62-ALW-A2 peptide loaded target cells and target cells endogenously expressing IE62.

(A) Cytolytic activity of the IE62-ALW-A2 positive T cell clones 1 and 2 was analyzed using the 51Cr release assay. T cells were incubated for 4 h with IE62-ALW-A2 peptide loaded or unloaded HLA-A2 positive EBV-LCLs at an effector to target ratio of 10:1. (B) IE62-ALW-A2 T cell clone 1 was stimulated with HLA-A2 positive EBV-LCLs loaded with different concentrations of the IE62-ALW-A2 peptide. Release of IFN-γ (pg/ml) after 24 hours of stimulation is shown. (C) IE62-ALW-A2 T cell clones 1 and 2 were stimulated with HLA-A2 positive COS-A2 cells, untreated, or loaded with the IE62-ALW-A2 peptide or with the IE4-ALR-B8 peptide as an irrelevant peptide or transfected with the IE63 gene (COS-A2-IE63) or the IE62 gene (COS-A2-IE62). Release of IFN-γ (pg/ml) after 24 hours of stimulation is shown.

Table 1. The presence of IE62-ALW-A2-specific T cells in HLA-A2 patients after VZV reactivation following TCD alloSCT

Patient	days after alloSCT	days after VZV	IE62-ALW-A2+ T cells %* (SD)	after IVS# % (SD)
1	180	46	neg	**0.22 (0.15)**
2	190	38	**0.03 (0.01)**	**0.51 (0.21)**
3	545	31	neg	neg
4	294	52	neg	**0.12 (0.06)**
5	82	38	neg	neg
6	183	16	neg	**0.01 (0.01)**
7	176	81	**0.02 (0.01)**	**0.44 (0.06)**
8	99	35	**0.11 (0.02)**	**0.22 (0.04)**
9	601	88	neg	**0.01 (0.01)**
10	95	63	neg	neg
11	90	83	neg	neg
12	179	48	neg	neg
13	1224	62	neg	neg
14	173	20	**0.03 (0.01)**	**0.22 (0.12)**
15	514	21	**0.03 (0.01)**	nd$
16	635	40	**0.02 (0.01)**	nd
17	161	8	neg	neg
18	174	48	**0.01 (0.00)**	**0.02 (0.01)**
19	92	49	**0.04 (0.01)**	**0.06 (0.02)**

*Mean percentages of IE62-ALW-A2 tetramer positive cells of $CD8^+$ T cells of 3 tetramer stainings performed on different days are indicated. #PBMCs were in vitro stimulated (IVS) for 7 days with IE62-ALW-A2 peptide, the mean percentages of tetramer positive cells of 3 to 6 stimulations are indicated. A negative result was determined as < 0.01% of $CD8^+$ T cells. $No PBMCs were available to do the analysis.

To verify the presence of the IE62-ALW-A2-specific T cells in the patient and donor cohort and to investigate whether individuals negative for IE62-ALW-A2-specific T cells were unable to mount a response against the epitope or whether the frequency of IE62-ALW-A2-specific T cells was too low to detect by FACS, the presence of these T cells was further measured after in vitro stimulation. PBMCs were cultured at a concentration of 1×10^6/ml in 24-well plates in Iscove's modified Dulbecco's medium supplemented with 10% human serum in the presence of IE62-ALW peptide (1 µg/ml), interleukin-2 (IL-2; 50 IU/ml), and IL-15 (10 ng/ml). After stimulation for 7 days, the presence of IE62-ALW-A2-specific T cells was reassessed by tetramer labeling. These in vitro stimulations demonstrated that IE62-ALW-A2 $CD8^+$

T cells were detectable in another four patients and confirmed the presence of IE62-ALW-A2-specific T cells in eight patients and three healthy, VZV-seropositive individuals with ex vivo-detectable IE62-ALW-A2-specific T cells **(Table 1; Figure 3A to D)**. Thus, in 12 (63%) of 19 patients, IE62- ALW-A2 $CD8^+$ T cells could be detected either by direct tetramer labeling or after in vitro expansion, indicating that this HLA-A2-restricted epitope is commonly used in HLA- A2-positive individuals.

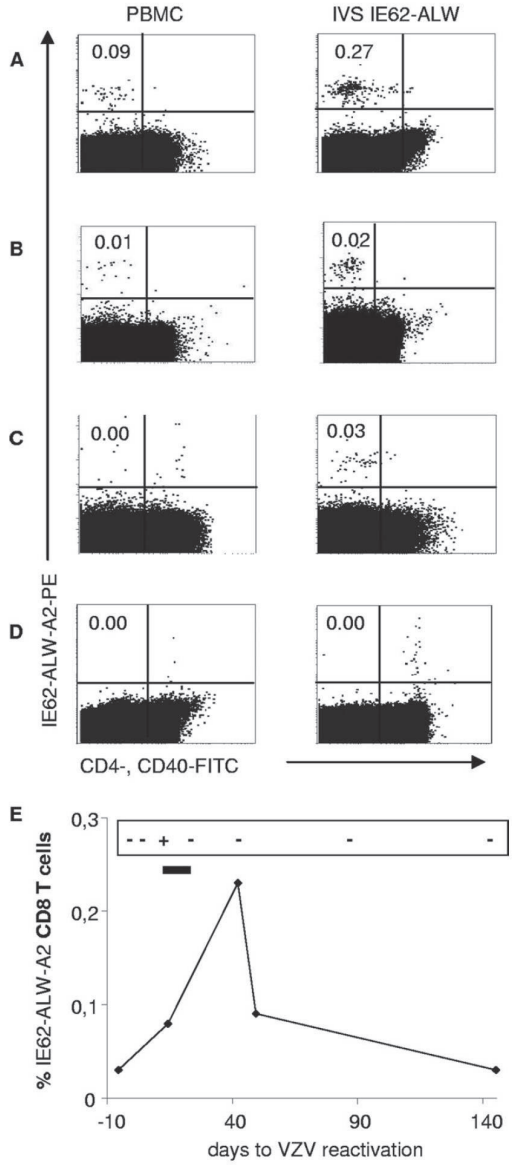

Figure 3. Detection and kinetics of IE62-ALW-A2-specific T cells.
PBMCs with detectable IE62-ALW-A2 T cells (A, left figure), low (B, left figure), or without detectable tetramer positive cells (C and D, left figures) were in-vitro stimulated (IVS) for 7 days with IE62-ALW-A2 peptide (I μg/ml), in the presence of IL-2 and IL-15 (A, B, C, D right figures). Cells were stained with CD4 FITC, CD40 FITC and IE62-ALW-A2 tetramer, and percentages IE62-ALW-A2 tetramer positive cells of $CD8^+$ T cells are indicated. $CD8^+$ T cells are defined as CD4- CD40- lymphocytes. E) PBMCs of a patient during the course of a VZV reactivation following TCD alloSCT were stained with the IE62-ALW-A2 tetramer in combination with $CD8^+$ FITC. The percentages IE62-ALW-A2-specific $CD8^+$ T cells before, during and after VZV reactivation are shown. In the box, the presence of viral DNA in peripheral blood is shown as measured by real time PCR at various time points. The bold line illustrates the use of valaciclovir for treatment of the VZV reactivation.

To study whether the immune response against the IE62- ALW-A2 epitope correlated with clinical reactivation, the percentage of IE62-ALW-A2-positive T cells was analyzed during the course of VZV reactivation in one patient. To determine the presence of viral DNA in plasma before and during the course of VZV reactivation, real-time PCR was performed on plasma samples derived at different time points. Six days prior to clinical signs of VZV reactivation, only 0.03% of the $CD8^+$ T cells were IE62-ALW-A2-specific. At 42 days after the onset of VZV reactivation, 0.23% of the $CD8^+$ T cells were IE62- ALW-A2-specific. After the VZV infection resolved, the percentage of IE62-ALW-A2-specific $CD8^+$ T cells declined to 0.09% at day 49 and 0.03% at day 145 after reactivation **(Figure 3D)**. The T cells present at the peak of the response were predominantly HLA-DR positive, CD45RA negative, CCR7 negative, CD28 negative, and CD27 positive, consistent with an activated effector memory phenotype.

In this study, we demonstrate that $CD8^+$ T cells specific for VZV are detectable without prior in vitro stimulation in patients with VZV reactivation following TCD alloSCT. We identified the ALWALPHAA peptide derived from the IE62- encoding gene of VZV as the first validated VZV-specific HLA class I-restricted immunogenic epitope by a pentamer- based epitope discovery method. The detection of the IE62-ALW peptide as an immunogenic peptide for VZV-specific $CD8^+$ T cells demonstrates the usefulness of this procedure for discovering new immunogenic virus- or tumor-specific epitopes. We demonstrated that, despite the low frequency, it is possible to detect VZV-specific $CD8^+$ T cells, allowing ex vivo analysis of the immune response to VZV infection, reactivation, and possibly VZV vaccination.

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Chapter 7

Control of cytomegalovirus viremia after allogeneic stem cell transplantation; a review on CMV-specific T cell reconstitution

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Abstract

Recipients of allogeneic stem cell transplantation (alloSCT) are at risk for reactivation of endogenous herpesviruses due to profound and prolonged T cell deficiency following conditions such as GVHD, immunosuppression and/or T cell depletion. Reactivation of endogenous CMV is the most frequently occurring herpesvirus reactivation following alloSCT. Antiviral medication is often used in pre-emptive treatment strategies initiated when increases in CMV viral loads are detected as a result of active reactivation of the virus. Despite pre-emptive antiviral treatment, the incidence of CMV disease in CMV seropositive alloSCT patients is still 10% at 1 year following alloSCT. This illustrates the necessity for adequate CMV-specific T cell immunity for long-term control of CMV and prevention of CMV disease. In this review, we analyzed the available studies on the influence of donor CMV status on CMV-specific T cell reconstitution and CMV disease. Furthermore, we reviewed the available studies on the safety and efficacy of adoptive transfer of donor CMV-specific T cells for the prevention and treatment of CMV disease following alloSCT, including studies on adoptive transfer of third-party CMV-specific T cells as a possible alternative when donor T cells are not available.

Introduction

Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment for various hematological diseases[1]. Following alloSCT, patients experience a period of profound and prolonged T cell deficiency in which they are at risk for developing infectious complications, including reactivations of endogenous herpesviruses like cytomegalovirus (CMV)[2], Epstein-Barr virus (EBV)[3] and varicella zoster virus (VZV)[4]. Infections with herpesviruses usually occur during childhood, and are controlled via the development of virus-specific T cell responses and ultimate formation of immunological memory. Despite virus-specific T cells control, herpesviruses are not completely cleared resulting in latent infections with equilibrium between the viruses and the virus-specific T cells. The frequencies of circulating memory T cells directed against these latent viruses are relatively high in immune competent, CMV or EBV infected hosts and can comprise up to 40% of the complete T cell repertoire[5, 6]. These high frequencies are presumably the result of repeated stimulation by frequent reactivations of CMV or EBV during life. Reactivation of endogenous CMV is the most frequently occurring herpes virus reactivation following alloSCT. In Western Europe and the United states 45-60% of alloSCT recipients is seropositive for CMV and therefore at risk for endogenous reactivation of latent CMV infection[7, 8].

CMV-specific T cells are essential for long-term control of CMV reactivation following alloSCT[9-11]. Failing CMV-specific T cell immunity, either quantitatively due to eradication by the conditioning regimen or qualitatively due to immune suppression or exhaustion by chronic antigen stimulation[12, 13] leads to impaired control of CMV reactivation, and may result in CMV disease, such as CMV pneumonitis, CMV colitis or CMV encephalitis[14]. Antiviral medication used in a pre-emptive treatment strategy can prevent CMV disease during this period of impaired CMV-specific T cell immunity. In a pre-emptive treatment strategy, viral load is routinely monitored using quantitative PCR and antiviral therapy is initiated when the viral load is above a predetermined PCR threshold. Multiple trials have demonstrated the safety and efficacy of pre-emptive strategies using oral valganciclovir[15]. However, despite the use of a pre-emptive strategy, the incidence of CMV disease is still 10% at 1 year following alloSCT in CMV seropositive patients[16, 17], illustrating the need for effective CMV-specific T cell immunity.

Several circumstances can increase the risk for CMV disease despite pre-emptive antiviral treatment (**table 1**). Graft versus Host Disease (GVHD) is associated with an increased risk of CMV disease despite pre-emptive antiviral treatment[18-22]. The use of an unrelated or HLA mismatched donor also implies an increased risk of developing CMV disease[20-22]. This may be caused by a higher risk of GVHD due to HLA mismatches requiring prolonged systemic immune suppression for prevention or treatment. Donor derived alloreactive T cells cause GVHD by targeting non-hematopoietic cells in the tissues and organs of the patient. Treatment of GVHD with systemic immune suppression not only suppresses alloreactive T cells responsible for the GVHD but also CMV-specific T cells. The increased risk of developing CMV disease in the presence of GVHD can also be caused by eradication

of recipient derived CMV-specific memory T cells by the profound alloreactive donor T cell response mediating the GVHD[23, 24]. If adequate reconstitution of donor-derived CMV-specific T cells is not yet sufficiently in place, these patients suffer from impaired anti-viral immune control.

GVHD can be prevented by immunosuppression after alloSCT or by T cell depleted (TCD) alloSCT[25-27]. In TCD alloSCT strategies, mature donor T cells are depleted from the stem cell graft. Various methods are used to deplete T cells from the graft resulting in different levels of TCD including $CD34^+$ selection resulting in almost complete T cell depletion or lymphocyte-depleting antibodies such as anti-thymocyte globulin (ATG)[28] or alemtuzumab[29, 30]. Although TCD strategies are effective in preventing GVHD and long-term post-transplant immune suppression is rarely needed, TCD also contributes to T cell impairment. Several studies demonstrate a higher incidence of CMV *reactivation* following TCD alloSCT. However, with the exception of $CD34^+$ selection, the incidence of CMV *disease* is not increased compared to non-TCD alloSCT[18, 19, 21, 31, 32]. The avoidance of immunosuppression after TCD alloSCT strategies, may allow even small numbers of CMV-specific T cells to reconstitute effective CMV-specific T cell immunity controlling CMV reactivation. This is confirmed by the finding that the high incidences of CMV disease after $CD34^+$ selected alloSCT can be reduced by adding back small numbers of T cells to the $CD34^+$ selected stem cell graft[33, 34]. This positive effect of even minimal numbers of donor T cells in the graft on the prevention of CMV disease leads to a preference of selecting a CMV seropositive donor for a CMV seropositive recipient.

Table 1. Overview of evidence for factors potentially associated with increased risk for CMV disease after allogeneic stem cell transplantation

Factor	Number of patients	Outcome on CMV Disease	Reference
GVHD	117 R^+ and R^-	Increased incidence CMV disease at day 100 in grade II–IV acute GVHD vs grade 0–I acute GVHD (17.1% vs 1.3%)	18
	162 R^+or D^+	Increased risk for CMV disease in acute GVHD (OR 9.7)	19
	1571 R^+	Increased risk for CMV disease in acute or chronic GVHD (Adjusted HR 4.1)	20
	186 R^+or D^+	Increased risk for CMV disease in steroid therapy for moderate- to-severe GVHD (HR 4.7)	21
	488 recipients with CMV reactivation after alloSCT	Increased risk for refractory CMV reactivation in acute GVHD (HR 1.9)	22
Unrelated/HLA mismatched donor	1571 R^+	Increased risk for CMV disease with unrelated or HLA mismatched donor (Adjusted HR 2.1)	20
	186 R^+or D^+	Increased risk for CMV disease with non-HLA-identical donors (HR 2.7)	21
	488 recipients with CMV reactivation after alloSCT	Increased risk for refractory CMV reactivation with HLA mismatched family donors (HR 2.0)	22
TCD	117 R^+ and R^- (15 (12.8%) received ATG for TCD)	No difference in CMV disease in conventional and ATG based TCD	18
	162 R^+or D^+	No difference in CMV disease in patients treated with ATG and not treated with ATG	19
	186 R^+ and R^- (23 alemtuzumab/ATG for T cell depletion)	No difference in CMV disease following RIC in conventional and alemtuzumab/ ATG based TCD	21
	73 R^+ and R^-	No CMV disease following Alemtuzumab based TCD; all matched related donor	31
	107 R^+ and R^-	No CMV disease within 100 days following Alemtuzumab based TCD, additional ATG in transplantation unrelated donor	32

Abbreviations: CMV = cytomegalovirus; R^+ = recipient CMV seropositive; R^- = recipient CMV seronegative; D^+ = Donor CMV seropositive; GVHD = graft vs host disease; OR = odds ratio; HR = hazard ratio; HLA = human leucocyte antigen; ATG = antithymocyte globulin; TCD = T cell depletion

Influence of donor CMV serostatus on CMV specific T cell reconstitution and CMV disease

Donor CMV serostatus and the associated presence or absence of donor CMV-specific memory T cells in the graft impact on the incidence of CMV related complications especially in CMV seropositive recipients undergoing alloSCT. In different studies the incidence of CMV related complications in CMV seropositive recipients transplanted with a CMV seropositive donor (R^+D^+) versus CMV seropositive recipients transplanted with a CMV seronegative donor (R^+D^-) has been investigated for TCD and non-TCD alloSCT programs (**table 2**). These studies demonstrate better CMV-specific T cell reconstitution[35-37] and less CMV related complications and deaths in R^+D^+ patients compared to R^+D^- patients[19, 36-41].

In R^+D^- patients CMV-specific memory T cells are not present in the graft and as a result the short term anti-viral immunity depends on residual recipient-derived CMV-specific memory T cells[42] and/or on the ultimate formation of a donor-derived primary CMV-specific T cell response. Recipient-derived T cells may be affected by the conditioning regimen prior to the alloSCT. Furthermore, all residual recipient derived lymphopoietic cells, including the T cells, may be attacked after the alloSCT by an alloreactive T cell response mounted by donor T cells. This is demonstrated by a study in which absence of CMV-specific T cells and CMV disease was seen in R^+D^- patients following T cell replete and not in T cell depleted alloSCT, indicating the eradication of residual CMV-specific T cells due to an alloreactive T cell response[43]. Consequently, adequate development of a donor derived primary T cell response is warranted for long-term anti-viral immune protection. Donor derived primary T cells can originate post transplant from donor stem cells via thymic development or from mature naive T cells present in the graft. Because the function of the thymus is anticipated to be greatly impaired in mature recipients following alloSCT, a primary T cell response derived from donor stem cells via thymic development cannot be expected shortly after alloSCT[43, 44]. High numbers of naive mature donor T cells present in the graft as seen in umbilical cord blood transplantation (UCBSCT) could hypothetically prevent CMV disease via the development of a primary CMV-specific immune response. However, despite the high number of naive T cells in the graft, delayed immune reconstitution and a high incidence of CMV disease is observed following UCBSCT[45-47].

Although it may take a period of 6 months to even several years, eventually most R^+D^- patients develop CMV-specific immunity. If time is allowed for successful immune reconstitution and primary CMV-specific T cell responses develop, CMV reactivation is controlled and CMV disease is prevented. This paves the way for strategies to bridge the period of impaired immunity via adoptive T cell transfer (ACT) to prevent CMV disease.

Table 2. Effect of donor CMV serology on survival, clinical endpoints and CMV-specific T cell reconstitution in CMV positive recipients

Endpoint	No of CMV^+ recipients (R^+D^+ vs R^+D^-)	Outcome	Reference
Survival	298; TCD in all patients (177 vs 121)	Lower mortality in R^+D^+ (42% versus 56%)	36
	531; TCD in all patients (331 vs 200)	Lower mortality in R^+D^+; even in R^+D^+ unrelated donor vs R^+D^- sibling donor	38
	29349 (19385 vs 9964)	Superior overall survival R^+D^+ in MAC, not in RIC	39
	10638 (7008 vs 3630)	Superior overall survival R^+D^+ in MAC and RIC	40
CMV disease or treatment	298 (177 vs 121)	Lower incidence of CMV disease (3% versus 13%) in R^+D^+	36
	178; T cell repletion (128 vs 50)	More recurrent need for antiviral therapy in R^+D^- (16% vs 0.8)	37
	147 (78 vs 69)	Increased failure of pre-emptive treatment in R^+D^-	41
CMV-specific T cell reconstitution	69 (55 vs 14)	More CMV-specific T cell responses detectable at day 100 in R^+D^+ compared to R^+D^- (82% vs 42%)	35
	298; TCD in all patients (177 vs 121)	CMV-specific $CD4^+$ and $CD8^+$ T cell counts higher in R^+D^+	36
	178; T cell repletion (128 vs 50)	CMV-specific $CD8^+$ T cell counts higher in R^+D^+ compared to R^+D^-	37

Abbreviations: CMV = cytomegalovirus; R^+ = recipient CMV seropositive; D^+ = donor CMV seropositive; D^- = donor CMV seronegative; TCD = T cell depletion; MAC = myeloablative conditioning; RIC = reduced intensity conditioning

Adoptive cell transfer for prevention and treatment of CMV disease following alloSCT

Several trials have been performed using ACT of purified populations of CMV-specific T cells isolated via different in-vitro strategies (**table 3**)[48-59]. ACT in these trials was either prophylactic or pre-emptive or intended for treatment of persistent CMV reactivation or CMV disease. All trials published thus far are phase-1/2 trials with relatively small numbers of patients. These studies suggest safety, proof of concept, and an association between ACT and viral clearance, but no formal phase-3 efficacy trials have been performed yet. Restoration of anti-viral immunity after CMV-specific ACT was demonstrated, however it remained unclear whether all immune responses seen following ACT were causally related to the ACT or that CMV-specific T cell responses developed irrespective of the ACT.

In general, 2 different approaches are used to produce CMV-specific T cell products for adoptive transfer. CMV-specific T cell products can consist of in vitro selected and expanded CMV-specific T cell lines or non-expanded CMV-specific T cells. T cell lines consisting of expanded CMV-specific T cells can be produced by repeated stimulation of peripheral blood derived mononuclear cells (PBMCs) with CMV derived antigens. After repeated stimulation in the course of several weeks, T cells specific for the antigens used for stimulation will be the main component of the cultures due to preferential outgrowth. The advantage of this technique is than combined $CD4^+$ and $CD8^+$ T cell lines are usually generated, depending on the antigens used for stimulation. The antigens used for stimulation also determine the broadness of specificity of the T cells lines, ranging from specificity to a single peptide to protein spanning peptide pools or viral proteins. Although expansion techniques will lead to large numbers of CMV-specific T cells, the down side is that repeated stimulation may lead to exhaustion of the expanded T cells leading to reduced persistence following ACT[54, 60]. However, if the goal of ACT is to temporarily overcome persistent CMV reactivation or CMV disease, transfusing large numbers of CMV-specific effector T cells may be sufficient to bridge and allow CMV-specific T cell reconstitution to develop.

CMV-specific T cell lines can also be generated from isolated CMV-specific T cells without or after only minimal expansion, aiming to prevent T cell exhaustion. In vivo, naive T cells differentiate to effector T cells upon first activation by antigen encounter and into memory T cells, which can mount another response after re-exposure to the antigen. Memory T cells can either be less differentiated central memory T cells or more differentiated effector memory T cells[61]. Stem cell characteristics such as multi-potency and self-renewal capacity have been demonstrated within the less differentiated central memory T cell compartment [62]. Experimental studies demonstrated the capacity of a single naive $CD8^+$ T cells to repopulate and develop into various memory and effector subsets and transferring even very low numbers of less differentiated T cells may be effective for successful T cell reconstitution[63-65]. Therefore, adoptively transferring less differentiated CMV-specific T cells may lead to more effective CMV-specific T cell reconstitution and persistence than transferring in vitro expanded effector T cells. CMV-specific T cell products generated with no or very limited expansion can be made by selection of T cells producing interferon gamma (IFN γ) upon in-vitro stimulation with

CMV derived antigens using the cytokine capture assay and a magnetic bead isolation system (MACS)[66, 67]. Using stimulation with CMV antigens as basis for isolation allows isolation of both $CD4^+$ and $CD8^+$ CMV-specific T cells. Another method is the highly specific isolation based on interaction of the T cell receptor with CMV peptide-HLA-multimer complexes (e.g. tetramers and streptamers) loaded on isolation beads[68, 69]. In this technique however, isolation is only possibly for $CD8^+$ T cells, specific for the peptide used and with known HLA restriction pattern. In non-expansion techniques the number of CMV-specific T cells generated is lower compared to techniques based on T cell expansion, however with lower risk of culture-induced T cell exhaustion and better proliferative capacity and persistence. If time for in vivo proliferation is granted, i.e. in the absence of persistent CMV viremia or CMV disease as in prophylactic ACT and in the absence of immunosuppression or GVHD, using non-expanded CMV-specific T cell lines may be best suited for reconstituting CMV-specific T cell immunity.

A major limitation for interpreting the clinical relevance of ACT following alloSCT has been the exclusion of active GVHD treated with systemic immune suppression in all trials. However, GVHD and treatment with systemic immune suppression are major risk factors for CMV disease and these patients may benefit the most from CMV-specific ACT. Considering the body of evidence that ACT with in-vitro selected CMV-specific T cells is safe with minimal risk of inducing concurrent GVHD, future trials may consider including patients with active GVHD, especially when using ACT products with high purity. In case of ongoing immune suppressive therapy the numbers of CMV-specific T cells used for ACT may need to be higher to overcome the immune suppression, e.g. derived from an expansion-based approach to generate sufficient number of CMV-specific T cells. A potential future approach for patients with uncontrolled CMV viremia and active GVHD may be treatment with CMV-specific T cells rendered resistant to corticosteroids by gene editing techniques[70]. In this approach the glucocorticoid receptor gene of CMV-specific T cells is disrupted leading to corticosteroid resistance. Adoptively transferring these cells for ACT may be effective in treating CMV disease during corticosteroid treatment for GVHD. This technique is still in a preclinical stage, and safety and efficacy have to be determined in clinical trials.

A major limitation of clinical applicability of CMV-specific ACT is that isolation of CMV-specific memory T cells from the donor is restricted to CMV seropositive donors. However, especially CMV seropositive patients transplanted with a graft from a CMV seronegative donor (R^+D^-) are at greatest risk of developing CMV disease, due to delayed reconstitution of virus-specific immunity. In theory, CMV-specific T cells from the CMV seropositive recipient harvested prior to the alloSCT procedure (autologous CMV-specific T cells) could be used for ACT post-transplant for prevention or treatment of CMV disease in R^+D^- patients. However, transfusing autologous CMV-specific T cells poses a risk of inducing graft rejection and would be eradicated by alloreactive donor T cells in case of GVHD. Autologous CMV-specific ACT has not yet been studied in a clinical trial. A different solution for R^+D^- patients may be the use of CMV-specific T cells isolated from CMV seropositive third-party donors (TPD).

Table 3. Overview of trials using Adoptive Cell Transfer to prevent or treat CMV disease

Trial phase	Purpose	Method of generating T cell line	Number of patients	Clinical outcome	Reference
2	Pre-emptive treatment of CMV viraemia	Stimulation with donor DCs pulsed with HLA-A2 restricted CMV pp65 peptide NLV or transduced with adenoviral vector encoding CMV protein pp65 ; cultured for 21 days	50; D^+ recipients compared to contempory controls	No increase in GVHD Reduction of patients who required CMV directed antiviral therapy (17% vs 36%)	48
1-2	Treatment of refractory CMV reactivation or CMV disease	Isolation of IFN γ -producing cells after stimulation with pp65protein; infused without further in vitro expansion	18; 16 D^+ recipients, 2 D^- recipients received third-party T cells	1 case of GVHD 15/18 cases CMV infection cleared or viral burden reduced	49
1-2	Pre-emptive treatment of CMV, EBV or AdV reactivation	Stimulation with donor DCs transduced with immunodominant viral antigens from EBV, CMV and AdV; cultured for 2–3-weeks	10 D^+ recipients	Complete virological responses in 80% Decrease in viral load correlated with an increase in the frequency of virus-specific T cells	50
1	Pre-emptive treatment of CMV viraemia or treatment of CMV disease	Stimulated with 15-mer peptide pool spanning CMV derived protein pp65; cultured for 28 days	17; 16 D^+ recipients , 1 D^- recipient received third-party T cells	No de novo GHVD 15/17 cases cleared CMV viraemia, including 3/5 cases with CMV disease	51
1-2	Prophylactic treatment CMV, EBV, AdV and VZV related complications	Stimulated with donor DCs transduced with adenoviral vector encoding CMV protein pp65 and EBV epitopes or pulsed with a VZV vaccine; cultured for 21 days	10 D^+ recipients	3/10 grade II-IV de novo GVHD No CMV disease (7 CMV reactivation, 1 required antiviral therapy)	52

1-2	Treatment of refractory CMV reactivation	Stimulation with HLA-A2 and/ or HLA-B7 CMV pp65 peptides (NLV/TPR), enrichment IFN γ -producing T cells after 1 day; cultured for 7–14 days	6 HLA-A2$^+$ or B7$^+$ D$^+$ recipients	No GVHD Efficacy of administered CMV pp65-specific CD8$^+$ T cell lines demonstrated in several patients	53
1	Prophylactic treatment	Stimulation with donor DCs pulsed with CMV derived pp65 NLV peptide; cultured for 21 days	9 HLA-A2$^+$ D$^+$ recipients with a HLA-A2$^+$ donor	3/9 cases Grade 3 acute GVHD 1 patient died due to gastrointestinal GVHD	54
1-2	Pre-emptive treatment EBV, AdV, CMV, BK, and HHV-6 reactivation	Stimulation with overlapping peptide libraries of EBV, CMV, AdV, BK virus and HHV; cultured for 9-11 days	11 patients No generation of CMV-specific T cell lines possible in R$^+$D$^-$ patients	1/11 grade II de novo GVHD 94% virological and clinical response rate	55
1-2	Prophylactic and pre-emptive treatment	Stimulated with recombinant pp65 or pool of overlapping peptides from CMVpp65 and after 1 day isolation and storage IFN γ -producing T cells	25 D$^+$ recipients	8/25 cases GVHD (5 grade I) Expansions of CD4$^+$ and CD8$^+$ CMV-specific T cells shortly after adoptive transfer	56
1-2	Prophylactic and pre-emptive treatment	Stimulation with donor DCs and CMV viral lysate; cultured for 2 weeks	30 D$^+$ recipients	4/30 developed Grade II-III GvHD Massive expansions of CMV -specific T cells	57
1-2	pre-emptive treatment	Stimulation with donor DCs and CMV antigen and cultured for 14-21 days	16 D$^+$ recipients	3/16 cases Grade I acute GVHD Massive expansions of CMV -specific T cells after adoptive transfer	58
1-2	Prophylactic treatment	Stimulation with autologous CMV infected fibroblasts; cultured for 7-14 days. CMV-specific CD8$^+$ T cells cloned from cultures by limiting-dilution method	14 D$^+$ recipients	No toxic effects Increased in vivo cytotoxic activity to CMV after infusions	59

Abbreviations: DC = dendritic cell; D$^+$ = donor CMV seropositive; GVHD = graft versus host disease; IFN = interferon; HLA = human leucocyte antigens; UCB = umbilical cord blood transplantation; EBV = Epstein Barr virus; AdV = adenovirus

Adoptive cell transfer using third-party CMV specific T cells

Using CMV-specific T cells from third-party donors (TPD) allows the formation of a bank of stored T cell lines from CMV seropositive donors with different HLA types. TPD CMV-specific T cell lines can be used "off the shelf" for treating persistent CMV viremia or CMV disease which eliminates delays caused by obtaining fresh cells from the donor, T cell isolation, processing and quality control. The potential efficacy as well as the potential toxicity of this approach is likely to be associated with the level of HLA matching between the third party donor and the respective patient and stem cell donor. Potential toxicity risks include the risk for graft rejection by an alloreactive response to donor hematopoietic cells or induction of GVHD by an alloreactive response to recipient tissue antigens by the adoptively transferred TPD T cells. It has been demonstrated that T cells, including CMV-specific T cells, harbor the capacity to cross-react to one or more allo-HLA molecules, thereby potentially inducing GVHD[71]. Vice versa, rejection of the adoptively transferred TPD T cells by anti-HLA/alloreactive donor derived T cells may also occur, consequently hampering efficacy.

In one recent multicenter trial 50 patients with severe refractory CMV, EBV or adenovirus infections were treated with banked TPD virus-specific T cells[72]. The cell lines used for adoptive transfer in this trial were generated by expansion, generating large numbers of CMV-specific T cells. The cumulative rate of complete responses (decrease of viral load below limit of detection and resolution of symptoms) or partial responses (decrease of viral load of at least 50% and alleviation of symptoms) was 74%. Development of de novo GVHD was seen in only 2/50 patients. Another recent study described ACT with TPD CMV-specific T cells in 8 R^+D^- patients with persistent CMV reactivation and no visible frequencies of circulating CMV-specific T cells[73]. The cell products used in this trial were generated without expansion, resulting in low numbers of CMV-specific T cells. In all but one patient survival/persistence of TPD CMV-specific T cells could not be demonstrated in peripheral blood of the patients after infusion. In one patient TPD T cells were found back at detectable frequencies after the adoptive transfer. In contrast to the other patients in this study, in this single case there was a complete HLA match between the patient, the stem cell donor and the TPD T cells.

These trials indicate that ACT with TPD virus-specific T cells is feasible, probably safe and may be effective in treating persistent CMV reactivation and CMV disease. However, long-term persistence of these T cells is unlikely. The level of HLA matching between the TPD and the respective patient and stem cell donor most likely impacts on the rejection of the adoptively transferred virus-specific T cells. However, the induction of an alloreactive response to reject the TPD takes time to develop and will depend on a functional T cell compartment in the patient. Therefore, the use of a third party T cell product with confers immediate protection is probably preferred over a product in which the T cells depend on profound in vivo proliferation for protection because by the time the third party T cells may have sufficiently proliferated, these cells may be eradicated by a developing alloreactive response by patient T cells. Thus, for immediate short-term protection in R^+D^- patients

with refractory CMV viremia or CMV disease, large numbers of TPD CMV-specific T cells produced by expansion techniques may be more effective than non-expanded T cells. A short-term effect as demonstrated in the recent clinical trials may be sufficient for bridging a period of severe CMV-specific T cell deficiency, thereby preventing or treating CMV disease and allowing for the development of subsequent CMV-specific immunity from the stem cell donor T cell repertoire for long-term control of CMV viremia.

Although the clinical results so far suggest that ACT with TPD CMV-specific T cells is safe, the induction of GVHD is still a major concern when using partially HLA matched TPD T cells and may correlate with in vivo persistence of the TPD T cells. The low incidence of GVHD after TPD ACT observed in the clinical studies may at least in part be explained by the rapid rejection and/or limited persistence of TPD T cells. If TPD were to persist and proliferate, GVHD may manifest as collateral damage.

A potential future alternative to TPD T cells is CMV-specific T cell Receptor (TCR) transfer to T cells from the CMV negative donor[74]. If stem cell donor derived T cells will be used, they will be likely to persist and expand after infusion. However, a potential danger is still the induction of GVHD due to a co-expressed endogenous alloreactive TCR. A clinical trial is currently undertaken to determine to the incidence of GVHD and the efficacy to generate CMV-specific T cells responses by infusing CMV TCR transduced T cells (ClinicalTrials.gov NCT02988258). Another potential future approach for R^+D^- patients is adoptively transferring CMV-specific T cells developed from the naive repertoire of the CMV seronegative donor. Although the procedure is experimental and the procedure is time-consuming, two studies demonstrated proof of principle that generation of CMV-specific T cells from a naive T cell repertoire is possible[75, 76].

Conclusion

Despite the use of pre-emptive strategies to control CMV viremia, CMV disease is not prevented in all patients. Patients with impaired CMV-specific T cell immunity due to GVHD, systemic immune suppression or absence of CMV-specific memory T cells in the graft have the greatest risk of developing CMV disease. The selection of a CMV seropositive donor for a CMV seropositive patient will potentiate efficient CMV-specific T cell reconstitution and significantly reduces the incidence of CMV disease. Multiple trials demonstrated that ACT with CMV-specific donor T cells is feasible and safe. However, the ultimate proof of efficacy of these strategies must come from future placebo controlled phase-3 clinical trials with significant patient numbers. In patients with GVHD treated with immune suppression, larger numbers of T cells may be required for ACT to overcome the effect of the immune suppression. ACT using third-party off the shelf donor derived CMV-specific T cell lines may be applied to provide short-term protection and temporary control of persistent CMV reactivation and disease in alloSCT patients. However, no long-term survival/persistence of partially HLA matched third-party donor derived CMV-specific T cells is likely to occur. The use of larger numbers of third-party CMV-specific T cells may be effective in bridging the period

of impaired immunity, despite the eradication by an alloreactive response. The execution of a large trial evaluating the effect of third party T cell products on CMV reactivation and -disease, the relevance of HLA-matching between third party donor and recipient, and the persistence of third party donor T cells in relation to the occurrence of GVHD would greatly enhance our knowledge to prevent and treat CMV disease in immunocompromised patients.

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Chapter 8

Summary

Following allogeneic stem cell transplantation (alloSCT), most patients experience a period of profound and prolonged T cell deficiency due to the immune suppression and/or T cell depletion (TCD). In this period the patients are at risk for developing infectious complications by reactivation of endogenous herpes like viruses cytomegalovirus (CMV), Epstein-Barr virus (EBV) and varicella zoster virus (VZV). Reactivation of endogenous CMV is the most frequently occurring herpes virus reactivation following alloSCT. Approximately 60% of alloSCT recipients are seropositive for CMV and are therefore at risk for endogenous reactivation of latent CMV. CMV reactivation can lead to potentially fatal CMV disease, comprising CMV pneumonitis, CMV colitis or CMV encephalitis. The aim of this thesis was to evaluate factors that influence the incidence of CMV disease after TCD alloSCT. These factors include the conditioning regimen, serostatus of the donor, pharmacological intervention following alloSCT and adoptive T cell transfer for treatment of refractory CMV reactivation or CMV disease.

CMV disease can be prevented by pre-emptively treating CMV reactivation using ganciclovir intravenously. In a pre-emptive treatment strategy, antiviral therapy is initiated when the viral load is above a predetermined PCR threshold. An effective oral treatment for pre-emptive CMV therapy would enable prevention and treatment of CMV in an outpatient setting and would lead to reduced patient burden and health-care costs. In **chapter 2** we demonstrate that pre-emptive treatment with oral valganciclovir is equally effective in reducing CMV DNA load in allogeneic stem cell recipients compared to intravenous ganciclovir. Severe adverse effects were not observed and CMV disease did not occur. The percentage of patients receiving erythrocyte transfusions was higher in the group of patients receiving ganciclovir, which is possibly the result of co-morbidity in the admitted patients treated with ganciclovir intravenously. Pre-emptive treatment of CMV viremia episodes in allogeneic stem cell recipients with either valganciclovir or ganciclovir led to a similar median CMV DNA load reduction in plasma of approximately 0.1 $\log_{10}$ copies/ml/day. We concluded that oral valganciclovir (900 mg, twice daily) is equally effective and safe as intravenous ganciclovir (5 mg/kg, twice daily) in the treatment of CMV reactivation aiming to prevent CMV disease following alloSCT. The vast majority of alloSCT recipients, without any clinical signs and symptoms of CMV disease when the first laboratory signs of CMV infection are detected, can benefit from treatment with an oral drug, without the need for hospitalization. For patients with suspected symptomatic CMV infections intravenously administered ganciclovir remains the first choice drug, as the course of CMV disease can be rapidly progressive and ultimately fatal.

It has been established that alloSCT with reduced intensity conditioning (RIC) can be successfully performed in individuals with a wide variety of different diseases and results in reduced risk of transplant-related mortality. Durable donor engraftment and favorable response of the disease with no graft versus host disease (GVHD) was reported for the in vitro TCD alloSCT protocol using RIC with fludarabine, anti-thymocyte globulin (ATG), busulphan and Campath-in-the-bag. It can be hypothesized that following RIC more residual

recipient T cells survive the conditioning regimen and can confer protective immunity following alloSCT. In **chapter 3** we demonstrate that there was no significant difference in incidence and severity of CMV infections within 100 days following alloSCT preceded by RIC compared to a conventional MAC. The onset of CMV DNA detection in plasma following alloSCT, the duration of a CMV infection, the peak load, the area under the DNAemia curve, the number and duration of pre-emptive CMV treatment episodes, as well as the number of recurrent infections within 100 days following alloSCT were comparable after RIC and MAC. This comparable severity after RIC and MAC may be explained by TCD as both patient groups received TCD grafts. By itself, TCD of the graft is associated with an increased risk of CMV infections, which seems to be reflected by the high overall incidence of CMV infections (51%) within 100 days following alloSCT in this study. As RIC relatively spares recipient hematopoietic cells, recipients who depend on recipient CMV-specific T cells were expected to benefit most from RIC in control of CMV reactivation. CMV seropositive recipients (R^+) transplanted with a CMV seronegative donor (R^+D^-) depend on residual recipient CMV-specific T cells, as the graft of the donor does not contain memory CMV-specific T cells. In this study no statistical difference in frequency and severity of CMV reactivation was present R^+D^- patients compared to CMV seropositive recipients transplanted with a CMV seropositive donor (R^+D^+). However, a non-significant increase of frequency and severity of CMV reactivation was observed in R^+D^- patients compared to R^+D^+ and as expected this difference was more pronounced in MAC compared to RIC. This difference did not reach statistical significance presumably due to small numbers of patients and a short follow-up of 100 days following alloSCT.

In **chapter 4** we specifically investigated the effect of donor CMV serostatus on the incidence of CMV disease after TCD alloSCT in a larger cohort of CMV seropositive patients. CMV-specific T cells may be transferred with the graft from CMV seropositive donors and provide protection against CMV disease. However, profound T cell depletion may eradicate these CMV-specific T cells. To determine the effect of donor CMV serostatus, we analyzed the incidence of CMV disease after TCD alloSCT in 157 CMV seropositive recipients, comprising 51 R^+D^- patients and 106 R^+D^+ patients. Furthermore, we determined the origin of CMV-specific T cells in a selection of 25 R^+D^- patients to determine whether primary donor-derived CMV-specific T cell responses could be demonstrated. The duration of CMV reactivations and the incidence of CMV disease were higher in R^+D^- patients compared to R^+D^+ patients. In R^+D^- patients, CMV-specific $CD4^+$ and $CD8^+$ T cells were mainly of recipient origin. However, in 53% of R^+D^- patients donor-derived CMV-specific T cells were detected within the first year, even as early as 3 months following TCD alloSCT. We conclude that donor CMV serostatus significantly influenced the clinical severity of CMV reactivations indicating the role of CMV-specific memory T cells transferred with the graft, despite the ultimate formation of primary donor-derived CMV-specific T cell responses in R^+D^- patients. Considering the pivotal role of CMV-specific T cells in preventing CMV disease, improving CMV-specific T cell reconstitution in patients by adoptive transfer of CMV-specific T cells

can be an attractive treatment modality. However, the use of adoptive T cell transfer is not commonplace, as questions regarding safety and efficacy still need answering. In **chapter 5** we analyzed the safety and efficacy of adoptive transfer of CMV pp65-specific $CD8^+$ T cell lines to restore CMV-specific T cell immunity by performing a phase I/II clinical study on adoptive transfer of in vitro-generated donor-derived or patient-derived CMV pp65-specific $CD8^+$ T cell lines. Peripheral blood mononuclear cells from CMV seropositive donors or patients were stimulated with HLA-A*0201-restricted and/ or HLA-B*0702-restricted CMV pp65 peptides (NLV/TPR) and 1 day after stimulation interferon-gamma producing T cells were enriched using the CliniMACS Cytokine Capture System, and cultured with autologous feeders and low-dose interleukin-2. After 7–14 days of culture, quality controls were performed and the CMV-specific T cell lines were administered or cryopreserved. The T cell lines generated contained 0.6–17 x 10^6 cells, comprising 54%–96% CMV pp65-specific $CD8^+$ T cells, and showed CMV-specific lysis of target cells. Fifteen CMV-specific T cell lines were generated, of which 8 were administered to patients with refractory CMV reactivation. Seven cell lines were generated but not administered because patients had cleared the CMV reactivation by the time the cell line was generated (n=4), due to a relapse of the malignant disease (n=1), or patients died due to the progressive CMV disease before infusion of the CMV-specific T cells (n=2). After administration, no acute adverse events and no graft versus host disease were observed and CMV loads disappeared. In several patients, a direct relation between administration of the T cell line and the in vivo appearance of CMV pp65-specific T cells could be documented. In conclusion, administration of CMV pp65-specific $CD8^+$ T cell lines was found to be feasible and safe.

In contrast to CMV, little is known about VZV-specific $CD8^+$ T cell immunity because validated VZV-derived immunodominant peptides for Human Leucocyte Antigen (HLA) class I are lacking. Because of this lack of validated VZV-derived immunodominant peptides for HLA class I, the analysis of VZV-specific $CD8^+$ T cell responses is hampered. To be able to analyze the role of $CD8^+$ T cells in VZV reactivation, we set out to identify epitopes for VZV by a new pentamer-based epitope discovery method. In **chapter 6** we describe our search for immunogenic antigens for VZV to develop VZV-specific pentamers and the development of multimeric HLA complexes to identify VZV-specific $CD8^+$ T cells. Potential HLA-A2 binding peptides from the putative immediate-early (IE) 62 protein of VZV were tested for binding, and peptides with sufficient binding capacity were used to generate pentamers. Patients with VZV reactivation following TCD alloSCT were screened with these pentamers, leading to the identification of the first validated HLA class I-restricted epitope of VZV. In 42% of HLA-A2 positive patients following VZV reactivation, these IE62-ALW-HLA-A2-specific T cells could be detected ex vivo. We demonstrated that despite the low frequencies, it is possible to detect VZV-specific $CD8^+$ T cells, allowing ex vivo analysis of immune responses to VZV infection, reactivation, and possibly VZV vaccination.

Despite pre-emptive antiviral treatment the incidence of CMV disease in CMV seropositive alloSCT patients is still 10% at 1 year following alloSCT. This illustrates the necessity for

adequate CMV-specific T cell immunity for long-term control of CMV and prevention of CMV disease. In **chapter 7** we provide an overview of factors relevant for prevention of CMV disease after alloSCT. GVHD and the use of an unrelated or HLA mismatched donor was found to be associated with an increased risk of developing CMV disease despite pre-emptive antiviral treatment, either due to systemic immune suppression needed to prevent or treat GVHD or due to eradication of recipient derived CMV-specific memory T cells by the alloreactive donor T cell response mediating GVHD. T cell depletion was found to be associated with an increased risk for CMV reactivation but not with an increased risk for CMV disease. It can be hypothesized that because immune suppression is in general not needed after TCD alloSCT, CMV-specific T cells are not hampered by immune suppression to provide protective immunity in case of CMV reactivation. Donor CMV serostatus significantly appears to influence CMV-specific T cell reconstitution and the risk of developing CMV disease. The incidence of CMV related complications and mortality is lower in R^+D^+ patients compared to R^+D^- patients. In R^+D^- patients memory CMV-specific T cells are not present in the donor graft and recipient-derived virus-specific T cells can be (partially) eradicated by the conditioning regimen and/or by an alloreactive T cell response mounted by donor T cells. Finally, we reviewed the available studies on the safety and efficacy of adoptive transfer of donor CMV-specific T cells for the prevention and treatment of CMV disease following alloSCT. All trials published thus far are phase-1/2 trials, demonstrating safety, proof of concept and association between adoptive T cell therapy (ACT) and viral clearance. A major limitation for ACT following alloSCT is that treatment of active GVHD with systemic immune suppression was an exclusion criterion for administration of CMV-specific ACT in all trials. Another major limitation of clinical applicability of CMV-specific ACT is that isolation of CMV-specific memory T cells from the donor is restricted to CMV seropositive donors. Adoptive transfer of T cells isolated from healthy third-party donors may be a solution for R^+D^- patients as donor CMV-specific T cells are not readily available for these patients. Trials demonstrate that ACT with third-party donor derived virus-specific T cells is feasible, safe and may be effective in treating persistent CMV reactivation and CMV disease. However long-term persistence of these T cells is uncertain.

In conclusion, the aim of this thesis was to evaluate the factors that influence the incidence of CMV disease after TCD alloSCT. We determined that prevention of CMV disease is safe and feasible using pre-emptive treatment with oral valganciclovir. We did not demonstrate a reduced risk for CMV reactivation or disease in patients treated with a RIC regimen and TCD alloSCT. GVHD and the use of immune suppression following alloSCT were found to be important risk factors for the development of CMV disease. T cell reconstitution was found to be improved in CMV seropositive patients transplanted with a CMV seropositive donor, which leads to a decreased risk of developing CMV disease. Donor derived CMV-specific primary T cell responses were detected in the majority of R^+D^- patients within one year after transplantation.

Chapter 9

General discussion

Developments in CMV-specific antiviral medication

We investigated the safety and efficacy of pre-emptive therapy using (val)ganciclovir following TCD alloSCT and demonstrated that valganciclovir was equally effective to ganciclovir in preventing CMV disease[1]. In our study of 107 patients following TCD alloSCT, CMV disease did not occur following pre-emptive therapy with (val)ganciclovir. Based on results obtained by us and others[2-4], pre-emptive strategies using valganciclovir are now the golden standard for prevention of CMV disease following alloSCT despite considerable side effects such as myelotoxicity and nephrotoxicity[5]. Although in our study on the safety and efficacy of pre-emptive therapy using (val)ganciclovir no CMV disease was observed, 13 patients had no or only moderate response to (val)ganciclovir. Larger studies demonstrate that despite a pre-emptive therapy strategy, the incidence of CMV disease is still 10% at 1 year following alloSCT[6, 7]. Our study was not designed to identify factors associated with an increased risk of treatment failure. In the review of literature that we performed, it is demonstrated that GVHD and the use of an unrelated or HLA-mismatched donor are important risk factors for developing CMV disease despite pre-emptive therapy[8]. In these patients prolonged use or prophylactic use of antiviral medication may be beneficial to allow more time for CMV-specific T cell reconstitution to develop while suppressing CMV reactivation and preventing CMV disease. However, due to the side effects, prolonged treatment with ganciclovir or valganciclovir as prophylaxis is not feasible[9-12]. Foscarnet and cidofovir are alternatives to (val)ganciclovir, but are also not suitable for prophylaxis due to considerable side effects.

Maribavir, brincidofovir and letermovir have been described as promising new anti-CMV drugs[13], possibly suitable for prophylaxis. Maribavir was not beneficial over placebo in a phase II trial[14] and is therefore not recommended for CMV prophylaxis after alloSCT. Phase III trials with maribavir are ongoing (NCT02927067 and NCT02931539) but for pre-emptive treatment of CMV reactivation rather than prophylaxis. Brincidofovir (also known as CMX001) was effective in decreasing the incidence of CMV reactivation in a phase II study as prophylaxis following alloSCT. However, efficacy to prevent CMV reactivation was only achieved in a dosage which was associated with increased gastrointestinal symptoms[15]. A phase III trial was performed (SUPPRESS, NCT01769170) and although the results of this trial have not yet been published, a manufacturer statement reported that prophylaxis with brincidofovir did not prevent CMV reactivation. In contrast to maribavir and brincidofovir, prophylaxis with Letermovir was demonstrated to be safe and effective to prevent CMV disease in a double blinded randomized control trial in CMV seropositive patients following alloSCT[16]. However, after cessation of the prophylaxis (predetermined at 100 days after alloSCT), the incidence of clinically significant CMV reactivation increased. The incidence of CMV disease was low in the letermovir group and in the placebo group, 1.5% and 1.8% at 24 weeks after alloSCT. The incidence and frequency of side effects was comparable to placebo with, most notably no increase in myelotoxic or nephrotoxic evens in the letermovir group. With the FDA approval based on this phase III trial, it can be concluded that prophylaxis for

CMV reactivation after alloSCT is possible using letermovir as an alternative for pre-emptive treatment with (val)ganciclovir.

Although Letermovir for CMV prophylaxis appears promising, trials directly comparing letermovir prophylaxes to pre-emptive therapy with (val)ganciclovir to prevent CMV disease following alloSCT have not been performed. Thus far no clinical superiority in efficacy of letermovir has been demonstrated compared to pre-emptive (val)ganciclovir. Besides efficacy, additional questions remain to be answered before CMV prophylaxis with Letermovir can be recommended over pre-emptive therapy with (val)ganciclovir. First, in order to prevent CMV disease, letermovir should provide protection during the period in which CMV-specific T cell immunity reconstitutes. It may take longer than the 100 days used in the trial to bridge this period especially in high-risk populations such as CMV seropositive recipients transplanted with CMV seronegative donors or during GHVD and treatment with immune suppression. However, prolonged use (i.e. longer than 14 weeks) of letermovir may eventually induce letermovir resistance. Second, it is not known whether CMV-specific T cell reconstitution is effective during letermovir prophylaxis, as Letermovir suppresses CMV viremia completely[17, 18]. By completely suppressing CMV reactivation, CMV-specific antigen presentation may also be suppressed, possibly hampering CMV-specific T cell reconstitution. Future studies should focus on development of Letermovir resistance and on the influence of letermovir use of T cell reconstitution before the place of letermovir prophylaxis for prevention of CMV disease following alloSCT is determined.

CMV-specific vaccination after alloSCT

Since CMV-specific T cells are essential for long-term control of CMV reactivation, interventions to accelerate CMV-specific T cell reconstitution may significantly contribute to the prevention of CMV disease. Traditionally, T cell immunity can be boosted by vaccination, an intervention in which antigen is presented in conjunction with a stimulatory adjuvant. A beneficial effect of CMV vaccination on CMV-specific T cell reconstitution may overcome the aforementioned possibly impaired CMV-specific antigen presentation when Letermovir prophylaxis is applied. It is not clear what the nature of the CMV-specific T cell reconstitution would be following vaccination, whether recipient or donor memory CMV-specific T cells are expanding on a recall response or whether naïve donor CMV-specific T cells develop into effector T cells by a primary immune response. We have demonstrated that a primary CMV-specific T cell response is possible shortly after alloSCT. However, the majority of patients depend on recipient CMV-specific T cells during the first year after alloSCT[19]. It can therefore be anticipated that CMV-specific vaccination early after alloSCT will primarily lead to a boosting of CMV-specific memory T cells. In CMV seropositive patients transplanted with a CMV seronegative donor (R^+D^-), no CMV-specific memory T cells of donor origin are present and CMV vaccination early after transplantation will boost memory CMV-specific T cell response from recipient origin. However, recipient CMV-specific memory T cells are at risk of being eradicated when an alloreactive immune response eradicates the recipient

hematopoietic cells. When this alloreactive immune response eradicates the boosted recipient CMV-specific memory T cells, the effect of vaccination will be abrogated. In that case, vaccination can be only effective when it induces a primary immune response from donor origin.

A commercially available CMV-specific vaccine has not yet been developed. Several phase I/II trials demonstrated that CMV-specific vaccination can boost pre-existing memory T cells[20-23]. Two randomized controlled trials have been performed to determine the clinical benefit of CMV-specific vaccination after alloSCT. The first trial was a randomized, double blind, placebo-controlled trial that investigated safety and efficacy of a CMV DNA vaccine (TransVax) in 108 patients following alloSCT[24]. Although the frequency of CMV reactivation did not differ between the vaccine recipients and the controls, the combined endpoint of clinically significant CMV viremia and initiation of antiviral therapy was significantly reduced in vaccine recipients. Despite this significant effect on the combined endpoint, no difference in occurrence of CMV disease and CMV-specific T cell reconstitution could be demonstrated. The second randomized trial investigated the safety and efficacy of a chimeric peptide vaccine containing a CMV pp65 derived $CD8^+$ T cell epitope combined with a tetanus T helper epitope (CMVPepVax) in 36 patients following alloSCT[25]. This study demonstrated a significant effect after CMVPepVax vaccination with a significant rise in pp65-specific $CD8^+$ T cells, reduced incidence of CMV reactivation and usage of antiviral treatment, and increased relapse free survival compared to patients with observation only. This trial provides proof of principle that vaccination can improve CMV-specific T cell reconstitution after alloSCT. However, in this trial only HLA-A2 positive patients could be vaccinated due to the HLA restriction of the peptide in the CMVPepVax vaccine. Vaccination of non-HLA-A2 patients would require multiple vaccines with different CMV peptides or a single vaccine with multiple CMV peptides. Currently, a clinical trial (#NCT02506933) is being performed to determine the efficacy to prevent CMV disease after alloSCT with an attenuated poxvirus Modified Vaccine Ankara (MVA) containing 3 immunodominant CMV antigens (pp65, IE1 and IE2, Triplex). This vaccine was demonstrated to be safe and effective in inducing CMV-specific T cell responses in CMV seronegative and CMV seropositive healthy adults[26]. The results of this phase 2 trial must be awaited.

It has thus far not been demonstrated that CMV-specific vaccination can induce primary immune responses from donor origin. The efficacy of vaccination after alloSCT to induce a primary immune response depends on the immune status of the alloSCT recipient. This immune status is influenced by T cell reconstitution, occurrence of GVHD and use of immune suppression[27]. The optimal timing of vaccination after alloSCT to induce primary T cell responses is not clear. In our study on the origin of CMV-specific T cells early after alloSCT, the majority of the analyzed R^+D^- patients developed a CMV-specific primary immune response within the first year after TCD alloSCT, some patients even as early as 3 months after TCD alloSCT[19]. In a T cell depended pneumococcal vaccination it was demonstrated that the primary immune response rate increased from 54% at 3 months to 94% at 9 months after

alloSCT, demonstrating a time dependent effect probably due to T cell reconstitution[28]. To induce a primary immune response, presentation of the antigen by antigen presenting cells is mandatory. Dendritic cells (DC) are professional antigen presenting cells, which can be pulsed with antigen and used for vaccination after alloSCT (DC vaccination). In DC vaccination, donor derived DC are pulsed with pathogen specific peptides and transferred to the patient [29]. This approach, although time consuming and laborious, may be more effective to induce a primary CMV-specific T cell response and prevent CMV disease compared to peptide only vaccination in R^+D^- patients[20, 30]. Analysis of efficacy of CMV-specific vaccination to induce a primary immune response should include chimerism analysis to determine the origin of the induced CMV-specific immunity to exclude the effect of boosting residual recipient CMV-specific T cells after vaccination.

Due to the increased risk for CMV disease in R^+D^- patients, accelerating CMV-specific T cell reconstitution is especially important for these patients to prevent CMV disease. Because vaccination after transplantation does not yet reliably induce primary CMV-specific immune responses, vaccinating the CMV seronegative donor prior to harvesting the stem cell graft may be an effective approach to avoid the R^+D^- serostatus combination. Thus far, CMV vaccination of the donor prior to transplantation was attempted in one trial, but was not feasible because the time between donor identification and transplantation was not enough to perform adequate vaccination[24]. CMV vaccination of CMV seronegative donor prior to alloSCT to avoid the R^+D^- combination has never been studied.

VZV-specific T cell reconstitution after alloSCT

Cellular immunity is essential for preventing reactivation of VZV leading to the clinical syndrome of herpes zoster. Ex vivo analysis of VZV-specific T cell reconstitution after TCD is hampered by the lack of clinically validated immunodominant peptides needed for artificial HLA class I constructs (tetramers or pentamers). Therefore, we developed and validated the first VZV-specific pentamer (IE62-ALW-A2) by determining immunogenic antigens for VZV using a pentamer-based epitope discovery method[31].

Using this VZV-specific pentamer it was possible to detect VZV-specific $CD8^+$ T cells upon VZV reactivation after TCD alloSCT in 63% of HLA-A2 patients after TCD alloSCT. Compared to CMV-specific T cells, the frequency of IE62-ALW-$CD8^+$ T cells in ex vivo analysis was low (mean 0.04%, range 0.01%-0.11%). This lower frequency may be explained by differences in viral tropism and replication between CMV and VZV. Whereas CMV resides and reactivates regularly in monocytes and vascular endothelial cells, VZV resides in neurons, which are immune privileged sites, and reactivates only sporadically. We also demonstrated that antigenic stimulation by VZV reactivation following alloSCT leads to an increase in IE62-ALW-A2-specific T cells. It is demonstrated that introduction of VZV antigens to T cells in this situation leads to a boost in VZV-specific memory T cells, providing protection when VZV reactivates[32]. VZV-specific vaccination provides antigenic stimulation to boost T cell immunity to prevent VZV reactivation. As discussed earlier, the efficacy of vaccination after alloSCT is

determined by the immune competence of the patient after alloSCT, which is influenced by factors like the occurrence of GVHD and/or treatment with immune suppression affecting T cell reconstitution. The efficacy of vaccination in general is defined by a predefined rise in antibody titer with at least partially recovered B and T cell immunity. However, a recent study in VZV vaccination in alloSCT recipients demonstrated VZV vaccination induced T cell responses in the absence of a B cell antibody response[33]. Therefore, to determine vaccination efficacy after alloSCT additional immunological assays are necessary[34]. The IE62-ALW-A2 pentamer can potentially be used for ex vivo analysis of efficacy of VZV vaccination of HLA A2 positive patients after TCD alloSCT. Future directions on vaccination after alloSCT should focus on determining the individual immune competence to allow for optimal vaccination and protection from preventable diseases such as herpes zoster.

Origin of CMV-specific T cells

Understanding the mechanisms leading to successful CMV-specific T cell reconstitution is important for future attempts to improve CMV-specific T cell reconstitution and prevent CMV disease after alloSCT. We demonstrated that in CMV positive recipients (R^+) transplanted with a CMV seronegative donor (R^+D^-) CMV-specific T cells are mainly of recipient origin and that in time primary CMV-specific T cell responses can develop from donor origin[19]. Selecting a CMV negative donor will exclude the possibility of donor derived CMV-specific memory T cells to provide protection in the first months following alloSCT. Protection in that period depends solely on residual recipient CMV-specific T cells until the development of a primary CMV-specific T cell response of donor origin.

As discussed earlier, residual recipient CMV-specific T cells may be the target of an alloreactive response and may therefore be eradicated. It can therefore be hypothesized that prevention of an alloreactive T cell response can help to preserve recipient CMV-specific T cells. T cell depletion is used to prevent GVHD after alloSCT by preventing alloreactive T cell responses. T cell *re*pletion by adding back small numbers of T cells to the graft is performed with the intent to induce an alloreactive T cell response to eradicate the residual malignant cells (GVL) with lower risk of inducing GVHD. In a study by Chalandon the origin of CMV-specific T cells was compared after T cell *replete* and T cell *depleted* alloSCT[35]. CMV-specific T cells could be demonstrated in only 1/6 R^+D^- patients following T cell replete alloSCT compared to 2/2 R^+D^- patients following T cell depleted alloSCT. In the R^+D^- patients following T cell depleted alloSCT, the CMV-specific T cells were of recipient origin. This study demonstrates that lowering the chance to induce an alloreactive T cell response by TCD may allow persistence of residual CMV-specific T cells providing long-term control of CMV reactivation.

Donor Lymphocyte Infusion is used to induce an alloreactive immune response targeting minimal residual disease (MRD) and residual hematopoietic cells, aiming at conversion to full donor chimerism. It could be hypothesized that the application of DLI poses a potential risk of developing CMV disease in R^+D^- patients because of the eradication of recipient CMV-specific T cells by the induced alloreactive immune response. However, apart from developing CMV

disease in the setting of acute GVHD and subsequent treatment with immune suppression following DLI, it is unclear whether the incidence of CMV disease is increased after DLI in R^+D^- patients. It has been demonstrated that following DLI in R^+D^- patients, the recipient T cells were indeed eradicated. However, these cells were directly replaced by donor derived CMV-specific T cells, indicating a donor derived primary T cell response[36].

Whether or not recipient CMV-specific T cells are present in the patient following TCD alloSCT presumably also depends on the conditioning strategy used prior to alloSCT. In the study by Grimaldi and Sellars, demonstrating persistence of recipient CMV-specific T cells, patients received reduced intensity conditioning (RIC). In RIC a less toxic condition regimen is used with relative sparing of recipient hematopoietic cells including recipient CMV-specific T cells. In our study, the incidence and severity of CMV reactivation following alloSCT was comparable after RIC and myeloablative condition (MAC)[37]. This may be explained by the additional Anti Thymocyte Globulin (ATG) used in RIC conditioning in our study. This additional T cell depletion, used to avoid graft rejection by recipient T cells may not only eradicate alloreactive T cells from recipient origin, but also residual CMV-specific T cells. Also in our study the observation period was short, only 100 days following alloSCT.

Monitoring MRD by measuring total leucocyte chimerism (TLC) is important to predict relapse of the malignant disease for which alloSCT was indicated. Upon a rise of recipient TLC, interventions such as DLI are performed. However, It has been shown that following CMV reactivation the TLC demonstrated more recipient origin, especially in R^+D^- patients[36, 38]. A recent trial of 45 recipient of TCD alloSCT for severe aplastic anemia confirmed this positive correlation between recipient chimerism and CMV reactivation[39]. CMV reactivation caused a massive expansion of CMV-specific T cells from recipient, thereby influencing the TLC. Unlike following TCD alloSCT for hematologic malignancies, full donor chimerism was not promoted in these patients and mixed chimerism was not treated with DLI. This state of mixed T cell chimerism persisted for years even after stopping immune suppression, indicating a state of mutual tolerance of donor and recipient T cells. In this case TLC may not be a marker of minimal residual disease and DLI should not be performed to achieve full donor chimerism.

Adoptive cell transfer for prevention and treatment of CMV disease following alloSCT

Although it may take a period of several months to even years, eventually most R^+D^- patients develop CMV-specific immunity. If time is allowed for successful immune reconstitution and primary CMV-specific T cell responses develop, CMV reactivation will be controlled and CMV disease will be prevented. This paves the way for strategies to bridge the period of impaired immunity via adoptive T cell transfer (ACT) to prevent CMV disease. Although the rationale for ACT is clear, thus far no evidence for efficacy has been demonstrated in formal phase 3 trials. As discussed in our review on prevention of CMV disease following alloSCT, all trials published thus far are phase-1/2 trials with relatively small numbers of patients[8]. These trials suggest safety, proof of concept, and an association between ACT and viral clearance. Although restoration of anti-viral immunity after CMV-specific ACT was demonstrated, it

remains unclear whether all immune responses seen following ACT were causally related to the ACT, or that CMV-specific T cell responses developed irrespective of the ACT. This should be the focus of a formal randomized controlled clinical trial.

The purpose of ACT can be prophylactic (e.g. early administration to prevent CMV related complications) or therapeutic when administered in case of persistent CMV reactivation or overt CMV disease. The purpose of ACT may affect the choice of the techniques used for generating the T cell. In general, 2 different approaches are used to produce CMV-specific T cell products for adoptive transfer. Techniques without expansion or only minimal expansion generate less differentiated CMV-specific T cells. Stem cell characteristics such as multi-potency and self-renewal capacity have been demonstrated within these less differentiated T cells[40]. Adoptive transfer of these cells may lead to more effective CMV-specific T cell reconstitution and persistence than transfer of in vitro expanded effector T cells. If time for in vivo proliferation is granted, i.e. in the absence of persistent CMV reactivation or CMV disease as in prophylactic ACT and in the absence of immunosuppression or GVHD, the use of non-expanded CMV-specific T cell lines may be best suited for reconstitution of CMV-specific T cell immunity. Techniques using expansion by repeated stimulation generate large numbers of more differentiated CMV-specific effector T cells. However, repeated stimulation may lead to exhaustion and reduced persistence following ACT[41, 42]. If the goal of ACT is to temporarily overcome persistent CMV reactivation or CMV disease, transfusing large numbers of CMV-specific effector T cells may be sufficient to bridge and allow CMV-specific T cell reconstitution to develop. Future research to demonstrate efficacy of ACT should tailor the technique for isolating CMV-specific T cells and generating the T cell lines to the purpose of ACT.

Two factors hamper proper assessment of the clinical relevance of ACT following alloSCT, the exclusion of patients with active GVHD treated with systemic immune suppression and the exclusion of R^+D^- patients. GVHD and treatment with systemic immune suppression are major risk factors for CMV disease and these patients may benefit the most from CMV-specific ACT. Considering the body of evidence that ACT with in-vitro selected CMV-specific T cells is safe with minimal risk of inducing concurrent GVHD, future trials may consider including patients with active GVHD, especially when using cell products with high purity. In case of ongoing immune suppressive therapy the numbers of CMV-specific T cells used for ACT may need to be higher to overcome the immune suppression. R^+D^- patients are at greatest risk of developing CMV disease, due to delayed reconstitution of virus-specific immunity and may benefit greatly from CMV-specific ACT. However, most trials thus far isolate CMV-specific T cells from CMV seropositive donors. In theory, CMV-specific T cells from the CMV seropositive recipient harvested prior to the alloSCT procedure (autologous CMV-specific T cells) could be used for ACT in R^+D^- patients. Autologous CMV-specific ACT has not been extensively studied in a clinical trial. Successful treatment of CMV disease in one R^+D^- patient with ACT using autologous CMV-specific T cells is described in one case report[43]. In our study on CMV-specific ACT, autologous CMV-specific T cell lines were generated for 3 R^+D^- patients,

but these cell lines were never administered[44]. One patient died due to CMV disease during the cell production period and two patients cleared the CMV reactivation before the CMV-specific T cell product could be administered. In theory, transfusing autologous CMV-specific T cells could pose a risk for inducing graft rejection. In addition, the infused recipient CMV-specific T cells could be eradicated by alloreactive donor T cells in case of an alloreactive response from donor T cells, either as part of the desired GVL effect or as part of GVHD. Despite these considerations, the use of autologous CMV-specific ACT should be studied, as the treatment options for R^+D^- patients with CMV disease are limited.

A different solution for R^+D^- patients may be the use of CMV-specific T cells isolated from CMV seropositive third-party donors (TPD). Using TPD CMV-specific T cells allows the formation of a bank of stored T cell lines from CMV seropositive donors. In such a bank CMV-specific T cell lines from donors partially HLA matched with the ACT recipient can be stored, for example an HLA–A2 restricted CMV-specific T cell line for HLA-A2 positive alloSCT recipients. TPD CMV-specific T cell lines can be used "off the shelf" for the treatment of persistent CMV viremia or CMV disease which eliminates delays caused by obtaining fresh cells from the donor, T cell isolation, processing and quality control. It has been demonstrated that all T cells have the potential to cross-react to allo-HLA molecules, thereby inducing GVHD[45]. Therefore, potential toxicity risks include the risk for graft rejection by an alloreactive response to donor hematopoietic cells or induction of GVHD by an alloreactive response to recipient tissue antigens by the adoptively transferred TPD T cells. Trials indicate that ACT with TPD virus-specific T cells is feasible, probably safe and may be effective in treating persistent CMV reactivation and CMV disease[46, 47]. However, long-term persistence of these T cells is unlikely. The level of HLA matching between the TPD and the respective patient and stem cell donor impacts on the persistence of the adoptively transferred virus-specific T cells. Despite the concerns regarding the persistence of TPD T cells, a short-term effect as demonstrated in the recent clinical trials may be sufficient for bridging a period of severe CMV-specific T cell deficiency, thereby preventing or treating CMV disease and allowing for the development of subsequent CMV-specific immunity from the stem cell donor T cell repertoire for long-term control of CMV viremia.

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Chapter 10

Nederlandse samenvatting

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Allogene stamceltransplantatie

De cellulaire componenten die samen het bloed vormen (erytrocyten, leukocyten en trombocyten) ontwikkelen zich uit stamcellen, een proces dat hematopoïese wordt genoemd. Bij verschillende kwaadaardige en niet-kwaadaardige hematologische aandoeningen is een hematopoïetische stamceltransplantatie dan ook een potentieel curatieve behandeling. Een hematopoïetische stamceltransplantatie kan worden verricht met lichaamseigen stamcellen (autologe stamceltransplantatie) of met lichaamsvreemde stamcellen (allogene stamceltransplantatie). In het geval van autologe stamceltransplantatie worden eigen stamcellen geoogst en gebruikt om te kunnen herstellen na intensieve chemotherapie. Het doel van allogene stamceltransplantatie (alloSCT) is om hematopoïetische cellen van de patiënt inclusief de cellen die de ziekte dragen, te vervangen door hematopoïetische cellen van een gezonde donor.

Een alloSCT voor maligne hematologische aandoeningen kan worden overwogen indien de ziekte met chemotherapie onder controle is gebracht. Vervolgens moet er een geschikte donor gezocht worden. Om na alloSCT afstoting van het donor transplantaat te voorkomen is het van belang dat het Human Leucocyte Antigen (HLA) van donor en ontvanger voldoende overeenkomen. Een donor kan verwant of onverwant zijn aan de patiënt maar bij voorkeur zoveel mogelijk HLA gematcht. Om het aanslaan (engraftment) van donor stamcellen na alloSCT mogelijk te maken, is conditionering van de ontvanger met immuunsuppressiva en eventueel bestraling voorafgaand aan de alloSCT noodzakelijk. Deze conditionering leidt tot een periode van diepe beenmergdepressie met een hoge infectiegevoeligheid en transfusiebehoefte tot gevolg.

Hoewel de conditionering de hematopoïetische cellen van de ontvanger door chemotherapie en/of bestraling zoveel mogelijk verwijdert en onderdrukt om engraftment te faciliteren, is dit onvoldoende om een recidief van de ziekte na transplantatie te voorkomen. Dit blijkt uit het hoge risico op een recidief na autologe stamceltransplantatie bij acute leukemie. Genezing van de ziekte kan worden bereikt door een immunologische respons door alloreactieve T cellen van de donor. Deze alloreactieve T cellen kunnen resterende maligne cellen uitroeien wanneer de respons gericht is tegen hematopoïetische cellen van de ontvanger (graft versus leukemia, GVL). Echter wanneer de respons ook gericht is tegen niet-hematopoïetische cellen in weefsels en organen, kan potentieel fatale graft-versus-host ziekte (GVHD) ontstaan. GVL en GVHD worden beide veroorzaakt door alloreactieve T cel responsen. Deze alloreactiviteit kan ontstaan door verschillen tussen donor en ontvanger in HLA-moleculen maar ook door genetische verschillen die leiden tot andere presentatie van delen van eiwitten in HLA-moleculen. Indien een donor en ontvanger geheel genetisch identiek zijn, zoals bij HLA-identieke tweelingen, is er geen risico op GVHD maar kan er ook geen GVL effect ontstaan.

Preventie van GVHD door T cel depletie

GVHD is de belangrijkste complicatie na alloSCT en kan leiden tot aanzienlijke morbiditeit en mortaliteit na alloSCT. De frequentie en ernst van GVHD kan worden verminderd door het gebruik van immuunsuppressiva of door het verwijderen van donor T cellen uit het transplantaat (T cel depletie). Zonder T cel depletie (TCD) moeten ontvangers langdurig behandeld worden met immuunsuppressie om GVHD te voorkomen. Immuunsuppressie is ook niet selectief en onderdrukt niet alleen de alloreactieve immuunresponsen die GVHD veroorzaken, maar ook potentieel gunstige immuunreacties zoals GVL of immuunreacties die nodig zijn voor bescherming tegen infecties.

Bij T cel depletie worden donor T cellen, waaronder alloreactieve T cellen verantwoordelijk voor het ontstaan van GVHD uit het transplantaat verwijderd. T cel depletie is effectief in het voorkomen van GVHD waardoor GVHD profylaxe met immuunsuppressie veelal niet nodig is. Het voorkomen van GVHD door TCD gaat echter gepaard met een verminderde kans op het ontstaan van een GVL respons. Om toch een GVL respons te ontwikkelen worden, in een later stadium, donor T cellen toegediend. Deze uitgestelde donor lymfocyten infusie (DLI) kan leiden tot controle van de ziekte met een kleiner risico op het ontstaan van GVHD.

TCD kan op verschillende manieren bereikt worden en de volledigheid van de depletie hangt af van de gekozen techniek. Bij $CD34^+$ selectie worden alleen stamcellen geselecteerd, leidend tot diepe T cel depletie. TCD kan ook verricht worden door depleterende antistoffen aan het transplantaat toe te voegen. Alemtuzumab (Campath) is een monoclonaal antilichaam gericht tegen CD52, een marker die tot expressie komt op lymfocyten. Het toedienen van alemtuzumab aan het transplantaat dat daarna direct aan de patiënt toegediend wordt ("campath in de zak") is een goed toepasbare en tijdbesparende methode om TCD uit te voeren met een aangetoond laag risico op het ontwikkelen van GVHD. Hoewel TCD effectief is in het voorkomen van GVHD en langdurige immuunsuppressie na transplantatie over het algemeen niet nodig is, leidt TCD tot een periode van diepe en langdurige T cel deficiëntie. Gedurende deze periode lopen patiënten risico op het ontwikkelen van infectieuze complicaties, vooral door reactivaties van eerder doorgemaakte endogene herpesvirussen. Deze virussen worden in de normale situatie na infectie voortdurend onderdrukt door T cel immuniteit maar kunnen leiden tot klinische problemen bij reactivatie door het wegvallen van T cel controle door immuunsuppressie of TCD.

T cel immuniteit

T cel immuniteit ontstaat door de ontwikkeling van antigeen-specifieke memory T cellen, die zich na een primaire immuunrespons ontwikkelen vanuit naïeve T cellen. T cellen worden onderverdeeld in $CD4^+$ en $CD8^+$ T cellen, waarbij $CD4^+$ T cellen belangrijk zijn voor het ontwikkelen en reguleren van immuunresponsen en $CD8^+$ T cellen belangrijk zijn in het elimineren van de pathogenen. $CD4^+$ T cellen herkennen delen van eiwitten (peptiden) gepresenteerd in HLA klasse II moleculen, die voornamelijk tot expressie worden gebracht door Antigeen Presenterende Cellen (APC), terwijl $CD8^+$ T cellen peptiden herkennen die

worden gepresenteerd in HLA klasse I moleculen welke tot expressie worden gebracht in alle weefsels. De inductie van een primaire T cel reactie leidt tot een snelle toename van effector $CD4^+$ en $CD8^+$ T cellen en de vorming van memory $CD4^+$ en $CD8^+$ T cellen die direct kunnen reageren op een nieuwe blootstelling aan het pathogeen. Voor het ontwikkelen van naïeve T cellen uit hematopoïetische stamcellen is de functie van de thymus van groot belang. Na de kindertijd raakt de thymus echter in regressie en neemt de functie sterk af. Het is niet duidelijk in welke mate na alloSCT de functie van de thymus noodzakelijk is om T cel immuniteit te herstellen.
Antigeen-specifieke T cellen kunnen op verschillende manieren geanalyseerd worden. $CD8^+$ T cellen specifiek voor een bepaald antigeen kunnen direct gevisualiseerd worden door binding met een kunstmatig HLA klasse I/peptide complex. Deze HLA/peptide complexen (tetrameren of pentameren, afhankelijk van het aantal gebruikte HLA moleculen) zijn voorzien van een fluorescerend label wat met flowcytometrie te detecteren is. T cellen kunnen ook geanalyseerd worden op basis van activatie. Na herkenning van een specifiek antigeen, produceren T cellen cytokinen, zoals interferon-gamma of interleukinen. De productie van deze cytokinen kan gemeten worden of direct gevisualiseerd worden. Functionele assays zijn geschikt om $CD4^+$ en $CD8^+$ T cellen te detecteren na antigene stimulatie. Deze antigene stimulatie kan een enkel peptide zijn, maar ook een combinatie van peptiden. Op deze manier kan een brede T cel respons tegen antigenen zoals virussen geanalyseerd worden.

Herpesvirus reactivaties na alloSCT

T cel immuniteit is cruciaal om reactivatie van herpesvirussen zoals cytomegalovirus (CMV), Epstein-Barr-virus (EBV) en varicella-zoster-virus (VZV) te controleren. Infecties met herpesvirussen komen veel voor in de algemene bevolking. Primaire infecties treden meestal op in de kindertijd en klinische symptomen zijn vaak mild of zelfs afwezig. Hoewel virus-specifieke T cellen massale replicatie kunnen voorkomen, worden herpesvirussen niet volledig geklaard en leiden ze tot latente infecties in hun gastheer. Deze latentie is het gevolg van een evenwicht tussen het virus en de virus-specifieke T cellen. In immuuncompetente individuen zonder klinische symptomen kan het percentage van herpesvirus-specifieke T cellen oplopen tot wel 40% van het totaal aantal T cellen in het bloed. Dit hoge percentage ontstaat waarschijnlijk door recidiverende stimulatie van memory T cellen door subklinische reactivaties van het virus. Diepe en langdurige T cel deficiëntie na TCD alloSCT of immuunsuppressie verstoort de balans tussen T cel immuniteit en de herpes virussen en kan leiden tot mogelijk fatale CMV ziekte in geval van CMV reactivatie, Post Transplantatie Lymfoproliferatieve ziekte (PTLD) na EBV reactivatie of gedissemineerde herpes zoster bij VZV reactivatie.

Cytomegalovirus

Ongeveer 60% van de westerse bevolking is geïnfecteerd met het cytomegalovirus. De infectie vindt meestal plaats in de kinderjaren. Het klinische beloop van CMV-infectie bij

immuuncompetente personen is mild en soms zelf geheel asymptomatisch. Een uitzondering is een primaire CMV infectie bij een zwangere vrouw waarbij de infectie tot neurologische afwijkingen bij het kind kan leiden. Na de primaire infectie persisteert CMV latent met name in monocyten en endotheelcellen. Bij immuungecompromitteerde patiënten kan door het ontbreken of disfunctioneren van CMV-specifieke T cellen de CMV reactivatie leiden tot mogelijk fatale CMV-ziekte, zoals CMV pneumonitis, CMV colitis of CMV encefalitis.
Reactivatie van CMV is de meest voorkomende reactivatie van herpesvirussen na alloSCT met een incidentie van 80% bij CMV seropositieve ontvangers. Primaire CMV infectie van een CMV seronegatieve ontvanger via een stamceltransplantaat van een CMV seropositieve donor is mogelijk maar in mindere mate (slechts 10% van de CMV seronegatieve ontvangers ontwikkelt een CMV infectie middels een stamceltransplantaat van een CMV seropositieve donor). Dit komt waarschijnlijk omdat met name endotheelcellen, de belangrijkste cellen voor CMV latentie en persistentie, niet in grote getalen in het stamceltransplantaat aanwezig zijn.
Met PCR technieken is virale replicatie van CMV aan te tonen en te kwantificeren tijdens primaire infectie en reactivatie. Dit maakt het mogelijk om het beloop in de hoeveelheid CMV (viral load) te analyseren en te relateren aan de respons van de CMV-specifieke T cellen. Deze analyses hebben aangetoond dat CMV-specifieke T cel immuniteit essentieel is om CMV reactivatie te controleren en om CMV ziekte te voorkomen.

Epstein-Barr Virus

Epstein-Barr Virus is een herpesvirus dat meer dan 90% van de westerse bevolking infecteert. Na primaire infectie, die kan leiden tot het klinische syndroom van infectieuze mononucleose, verblijft het virus latent in B cellen. Infectieuze mononucleose wordt veroorzaakt door een massale expansie van EBV-specifieke T cellen na herkenning van een deel van een EBV eiwit met als doel de EBV infectie te beheersen.
Na alloSCT kan reactivatie van EBV plaatsvinden door afwezigheid van voldoende EBV-specifieke T cellen. Met falende T cel controle kan het aantal EBV geïnfecteerde B cellen enorm toenemen, wat kan leiden tot potentieel fatale PTLD. Hoewel de incidentie van EBV-geassocieerde PTLD laag is na alloSCT (4%), correleert het risico met het niveau van TCD. Bij strategieën die alleen T cellen verwijderen neemt het risico toe omdat B cellen, de belangrijkste plaats voor EBV-latentie, niet zijn verwijderd. In TCD door middel van antilichamen die zich richten op het verwijderen van zowel T als B cellen, zoals alemtuzumab, is het risico op PTLD laag.

Varicella zoster virus

Varicella zoster virus (VZV) is een herpesvirus dat ongeveer 95% van de bevolking infecteert. De primaire infectie met VZV leidt tot varicella (waterpokken). Na de primaire infectie verblijft VZV latent in neuronen en reactivatie leidt tot herpes zoster (gordelroos). Doordat zenuwcellen minder blootgesteld worden aan het immuunsysteem dan andere weefsels in het lichaam en omdat VZV minder frequent reactiveert vergeleken met CMV en EBV

worden VZV-specifieke memory T cellen niet herhaaldelijk gestimuleerd. Hierdoor neemt de frequentie van circulerende VZV-specifieke memory T cellen langzaam af in de loop der tijd. De afname van VZV-specifieke memory T cellen wordt versneld door de conditionering en/of TCD in het kader van alloSCT.
Na alloSCT veroorzaakt reactivatie van het virus aanzienlijke morbiditeit en is mogelijk fataal bij gedissemineerde ziekte. Meest voorkomende complicaties zijn postherpetische neuralgie en perifere neuropathie. Net als bij CMV en EBV is cellulaire immuniteit essentieel voor het controleren van een reactivatie van VZV. In tegenstelling tot CMV en EBV is er weinig bekend over VZV-specifieke $CD8^+$ T cel immuniteit omdat er voor VZV geen immunodominante HLA klasse I peptiden bekend zijn en daardoor HLA constructen voor analyse niet beschikbaar zijn. Eerdere studies met functionele assays tonen wel VZV-specifieke memory $CD4^+$ T cellen aan, maar VZV-specifieke $CD8^+$ T cellen zijn alleen aantoonbaar na kweken. Het onvermogen om VZV-specifieke $CD8^+$ T cellen direct ex vivo te detecteren, kan het gevolg zijn van de lage frequenties van VZV-specifieke $CD8^+$ T cellen of van de lage gevoeligheid van de screeningsmethoden die worden gebruikt om $CD8^+$ T cellen te detecteren.

Preventie van CMV ziekte door antivirale medicatie
Om CMV ziekte te voorkomen, moet een periode van ernstige T cel deficiëntie na (TCD) alloSCT worden overbrugd tot CMV-specifieke T cel immuniteit hersteld is. Tijdens een CMV reactivatie, voorafgaand aan de ontwikkeling van CMV ziekte en wanneer patiënten nog asymptomatisch zijn, is CMV-DNA aantoonbaar in het bloed. Door deze viral load routinematig na alloSCT te vervolgen middels kwantitatieve PCR technieken, is preventie van CMV ziekte met preëmptieve behandeling mogelijk. Hierbij wordt antivirale therapie gestart wanneer de viral load boven een vooraf bepaalde drempel komt.
Ganciclovir is een synthetisch nucleoside dat DNA-virussen remt, zoals herpesvirussen en in het bijzonder CMV. Ganciclovir is de gouden standaard voor de behandeling van CMV gerelateerde complicaties, maar heeft aanzienlijke bijwerkingen, waarvan beenmergdepressie de belangrijkste is. Bovendien heeft ganciclovir een slechte biologische beschikbaarheid, waardoor orale toediening niet mogelijk is en ziekenhuisopname vaak vereist is. Deze factoren maken het profylactisch gebruik van ganciclovir om CMV ziekte te voorkomen niet haalbaar. Valganciclovir is afgeleid van ganciclovir en kan wel oraal toegediend worden en is daarom geschikt voor preëmptieve poliklinische behandeling om CMV ziekte te voorkomen. Langdurig gebruik van valganciclovir is echter, net als ganciclovir geassocieerd met ernstige bijwerkingen en het ontwikkelen van resistentie.

CMV-specifieke T cel reconstitutie
Ondanks preëmptieve antivirale medicatie is herstel van T cel immuniteit en reconstitutie van CMV-specifieke T cellen vereist voor de lange termijn controle van CMV reactivatie en preventie van CMV ziekte. Reconstitutie van CMV-specifieke T cellen kan het resultaat zijn van expansie van memory T cellen van de ontvanger die het conditioneringsregime

overleven of van donor memory T cellen die met het transplantaat worden overgedragen. Daarnaast kunnen CMV-specifieke T cellen ook ontstaan uit een primaire respons vanuit naïeve donor T cellen. Het is echter onduidelijk of deze naïeve donor T cellen direct uit het transplantaat komen of ontstaan uit hematopoïetische stamcellen gevolgd door verdere ontwikkeling in de thymus.
Verschillende factoren kunnen CMV-specifieke T cel reconstitutie beïnvloeden. Immuunsuppressie voor de preventie of behandeling van GVHD na transplantatie kan de T cel reconstitutie nadelig beïnvloeden. CMV-specifieke T cel reconstitutie kan ook worden verstoord door eradicatie van T cellen van ontvanger door intensieve conditioneringsregimes voorafgaand aan alloSCT. Hiernaast speelt de CMV serostatus van de donor mogelijk ook een rol in CMV-specifieke T cel reconstitutie. Als CMV-specifieke T cellen van een CMV seropositieve donor de TCD kunnen overleven, zouden deze na transplantatie kunnen reconstitueren en bescherming tegen CMV ziekte kunnen bieden. Het transplantaat van een CMV seronegatieve donor bevat geen CMV-specifieke memory T cellen. Hierdoor zou de bescherming tegen CMV ziekte na TCD alloSCT dus door residuale CMV-specifieke T cellen van ontvanger moeten komen. Deze overgebleven CMV-specifieke T cellen van de ontvanger lopen echter het risico om verwijderd te worden door alloreactieve donor T cellen na alloSCT en/of DLI, waarna de patiënt mogelijk het risico loopt om CMV ziekte te ontwikkelen.
Het toedienen van ongeselecteerde donor T cellen kan een strategie zijn om T cel reconstitutie na alloSCT te verbeteren. Hoewel deze benadering effectief kan zijn bij het herstellen van antivirale T cel immuniteit, kan deze mogelijk fatale GVHD veroorzaken. Om het risico op het induceren van GVHD te minimaliseren en om gericht de CMV-specifieke T cel reconstitutie te verbeteren, kunnen CMV-specifieke T-cellen uit bloed van de donor worden geïsoleerd en overgedragen naar de ontvanger na alloSCT (CMV-specifieke Adoptive Cell Transfer, ACT). CMV-specifieke ACT kan worden gebruikt als een profylactische of preventieve behandeling om CMV ziekte te voorkomen of als behandeling voor CMV ziekte. ACT is het meest effectief in afwezigheid van immuunsuppressie, zoals het geval is bij TCD alloSCT. ACT wordt nog niet standaard toegepast omdat er op dit moment nog onvoldoende gegevens beschikbaar zijn met betrekking op de veiligheid en de effectiviteit van deze behandeling.

Dit Proefschrift

Ernstige T cel deficiëntie kan leiden tot reactivatie van endogene herpesvirussen na TCD alloSCT. Onvoldoende controle van deze virussen door virus-specifieke T cellen kan tot aanzienlijke complicaties leiden. Immuniteit op lange termijn hangt af van virus-specifieke T cel reconstitutie. Reactivatie van CMV is de meest voorkomende herpesvirus reactivatie na alloSCT. Ongeveer 60% van alloSCT-ontvangers zijn seropositief voor CMV en lopen daarom risico op endogene reactivatie van latente CMV. CMV reactivatie kan leiden tot mogelijk fatale

CMV ziekte, bestaande uit CMV pneumonitis, CMV colitis of CMV encefalitis. Het doel van dit proefschrift is om factoren te evalueren die de incidentie van CMV ziekte na TCD alloSCT beïnvloeden. Deze factoren omvatten het conditioneringsregime, CMV serostatus van de donor, farmacologische interventie na alloSCT en ACT voor behandeling van refractaire CMV reactivatie of CMV ziekte.

CMV ziekte kan worden voorkomen door het preëmptief behandelen van CMV reactivatie met behulp van intraveneus ganciclovir. In een preëmptieve behandelingsstrategie wordt met antivirale therapie begonnen wanneer de viral load boven een vooraf bepaalde PCR drempelwaarde ligt. Een effectieve orale behandeling voor preëmptieve CMV therapie zou de preventie en behandeling van CMV poliklinisch mogelijk maken en zou leiden tot een lagere belasting voor de patiënt en lagere kosten voor de gezondheidszorg. In **hoofdstuk 2** laten we zien dat preëmptieve behandeling met oraal valganciclovir even effectief is bij het verminderen van de hoeveelheid circulerend virus (CMV viral load) na alloSCT in vergelijking met intraveneus ganciclovir. Ernstige bijwerkingen werden niet waargenomen en CMV ziekte trad niet op. Het percentage patiënten dat erytrocytentransfusies ontving, was hoger in de groep patiënten die ganciclovir kregen. Mogelijk was dit het resultaat van co-morbiditeit bij de opgenomen patiënten die intraveneus met ganciclovir werden behandeld. Preëmptieve behandeling van CMV reactivaties in allogene stamcelontvangers met valganciclovir of ganciclovir leidde tot een vergelijkbare mediane reductie van het CMV DNA in plasma van ongeveer 0,1 log10 kopieën / ml / dag. We concludeerden dat oraal valganciclovir (900 mg, tweemaal daags) even effectief en veilig is als intraveneus ganciclovir (5 mg / kg, tweemaal daags) bij de behandeling van CMV reactivatie gericht op het voorkomen van CMV-ziekte na alloSCT. De overgrote meerderheid van alloSCT-ontvangers zonder symptomen van CMV ziekte wanneer de CMV reactivatie wordt gedetecteerd, heeft baat bij behandeling met een oraal geneesmiddel, zonder dat opname in het ziekenhuis noodzakelijk is. Voor patiënten met verdenking op symptomatische CMV reactivatie blijft intraveneus toegediend ganciclovir het eerste keuzegeneesmiddel, omdat het beloop van de CMV ziekte snel progressief en uiteindelijk fataal kan zijn.

Het is vastgesteld dat alloSCT met verminderde intensiteitsconditionering (Reduced Intensity Conditioning, RIC) met succes kan worden uitgevoerd bij individuen met een grote verscheidenheid aan hematologische ziekten en kan leiden tot een verminderd risico op transplantatie gerelateerde sterfte. Duurzame donor engraftment en gunstige respons van de ziekte zonder graft-versus-host ziekte (Graft versus Host Disease, GVHD) werd gemeld voor het in vitro TCD RIC alloSCT-protocol met fludarabine, anti-thymocyten globuline (ATG), busulphan en “Campath in de zak”. Er kan worden verondersteld dat na RIC meer resterende T cellen van ontvanger het conditioneringsregime overleven en beschermende immuniteit na alloSCT kunnen verlenen. In **hoofdstuk 3** laten we zien dat er geen significant verschil was in incidentie en ernst van CMV reactivaties binnen 100 dagen na alloSCT voorafgegaan door RIC in vergelijking met een conventionele conditionering (myeloablatieve conditionering, MAC). Het begin van detectie van CMV DNA in plasma na alloSCT, de duur van een CMV

reactivatie, de DNA piek load, het gebied onder de DNAemia-curve, het aantal en de duur van pre-emotieve CMV behandelingsperioden, evenals het aantal recidiverende infecties binnen 100 dagen na alloSCT waren vergelijkbaar na RIC en MAC. Deze vergelijkbare ernst van CMV reactivaties na RIC en MAC kan worden verklaard door de TCD aangezien dit in beide patiëntengroepen toegepast werd. Op zichzelf is TCD van het transplantaat geassocieerd met een verhoogd risico op CMV reactivaties, wat lijkt te worden weerspiegeld door de hoge incidentie van CMV reactivaties (51%) binnen 100 dagen na alloSCT in dit onderzoek.
Omdat RIC de hematopoïetische cellen van de ontvanger relatief spaart, kan verwacht worden dat ontvangers die afhankelijk zijn van ontvanger CMV-specifieke T cellen het meeste baat hebben bij RIC in de controle van CMV reactivatie. CMV seropositieve ontvangers (R^+) die getransplanteerd zijn met een CMV seronegatieve donor (R^+D^-) zijn afhankelijk van resterende CMV-specifieke T cellen van de ontvanger, omdat het transplantaat van de donor geen memory CMV-specifieke T cellen bevat. In deze studie was er geen statistisch verschil in frequentie en ernst van CMV reactivatie aanwezig in R^+D^- patiënten in vergelijking met CMV seropositieve ontvangers getransplanteerd met een CMV seropositieve donor (R^+D^+). Een niet-significante toename in frequentie en ernst van CMV reactivatie werd echter waargenomen bij R^+D^- patiënten in vergelijking met R^+D^+ en zoals verwacht was dit verschil meer uitgesproken in MAC in vergelijking met RIC. Dit verschil bereikte echter geen statistische significantie vermoedelijk als gevolg van het kleine aantal patiënten en een korte follow-up van 100 dagen na alloSCT.
In **hoofdstuk 4** hebben we het effect onderzocht van de CMV serostatus van de donor op de incidentie van CMV ziekte na TCD alloSCT in een groter cohort van CMV seropositieve patiënten. CMV-specifieke T cellen kunnen met het transplantaat van CMV seropositieve donoren worden overgedragen en op die manier bescherming bieden tegen CMV ziekte. T cel depletie kan echter deze CMV-specifieke T cellen elimineren. Om het effect van CMV serostatus bij TCD alloSCT te bepalen, analyseerden we de incidentie van CMV ziekte bij 157 CMV seropositieve ontvangers, bestaande uit 51 R^+D^- en 106 R^+D^+ patiënten. Daarnaast bepaalden we de oorsprong van CMV-specifieke T cellen in een selectie van 25 R^+D^- patiënten om te bepalen of primaire CMV-specifieke T cel responsen konden worden aangetoond. De duur van CMV reactivaties en de incidentie van CMV ziekte waren hoger bij R^+D^- patiënten in vergelijking met R^+D^+ patiënten. Bij R^+D^- patiënten waren CMV-specifieke $CD4^+$ en $CD8^+$ T cellen voornamelijk van ontvanger origine. Daar staat tegenover dat in 53% van de R^+D^- patiënten in het eerste jaar CMV-specifieke T cellen van donor origine werden gedetecteerd, zelfs al na 3 maanden na TCD alloSCT. We concluderen dat CMV serostatus de klinische ernst van CMV reactivaties significant beïnvloedde, wat de rol van CMV-specifieke memory T cellen die met het transplantaat werden overgedragen aangeeft, ondanks de uiteindelijke vorming van primaire CMV-specifieke T cel responsen bij R^+D^- patiënten.
Gezien de cruciale rol van CMV-specifieke T cellen bij het voorkomen van CMV ziekte, kan het verbeteren van CMV-specifieke T cel reconstitutie bij patiënten door ACT van CMV-specifieke T cellen een aantrekkelijke behandelingsmodaliteit zijn. Echter, vragen

met betrekking tot veiligheid en effectiviteit van het gebruik van ACT moeten nog steeds beantwoord worden. In **hoofdstuk 5** hebben we de veiligheid en effectiviteit van ACT om CMV-specifieke T cel immuniteit te herstellen geanalyseerd door een fase I/II klinisch onderzoek uit te voeren naar ACT van in vitro-gegenereerde CMV pp65-specifieke $CD8^+$ T cellijnen. Deze cellijnen werden gegenereerd door perifere mononucleaire cellen van CMV seropositieve donoren of patiënten te stimuleren met HLA-A*0201 en/of HLA-B*0702 gerestricteerde CMV-pp65 peptiden (NLV / TPR). Op dag 1 na stimulatie werd verrijkt door interferon-gamma producerende T cellen te isoleren met het CliniMACS Cytokine Capture System en te kweken met autologe feeders en lage dosis interleukine-2. Na 7-14 dagen kweken werden kwaliteitscontroles uitgevoerd en werden de CMV-specifieke T cellijnen toegediend of ingevroren. Vijftien CMV-specifieke T cellijnen werden gegenereerd, waarvan er 8 werden toegediend aan patiënten met refractaire CMV reactivatie. Zeven cellijnen werden gegenereerd maar niet toegediend omdat patiënten de CMV reactivatie inmiddels hadden geklaard tegen de tijd dat de cellijn geschikt was voor infusie (n = 4) of patiënten overleden waren door een recidief van de kwaadaardige ziekte (n = 1) of als gevolg van de progressieve CMV ziekte vóór infusie van de CMV-specifieke T cellen (n = 2). Na toediening werden geen acute bijwerkingen en geen GVHD waargenomen en daalden de CMV PCR loads. Bij verschillende patiënten was er een directe relatie tussen toediening van de T cellijn en het in vivo voorkomen van CMV-pp65-specifieke T cellen. Concluderend werd gevonden dat toediening van CMV-pp65-specifieke $CD8^+$ T cellijnen mogelijk en veilig was.

In tegenstelling tot CMV is er weinig bekend over VZV-specifieke $CD8^+$ T cel immuniteit. Dit komt omdat er, anders dan bij CMV, geen gevalideerde van VZV afgeleide immunodominante peptiden voor HLA klasse I bekend zijn. Hierdoor wordt de analyse van VZV-specifieke $CD8^+$ T cel responsen belemmerd. In **hoofdstuk 6** beschrijven we onze zoektocht naar immunogene antigenen voor VZV om VZV-specifieke pentameren te ontwikkelen om VZV-specifieke $CD8^+$ T cellen te identificeren. Potentiële HLA-A2 bindende peptiden van het Immediate Early (IE)-62 eiwit van VZV werden getest op binding met HLA-A2 en peptiden met voldoende bindingscapaciteit werden gebruikt voor het genereren van pentameren. Patiënten met VZV-reactivatie na TCD alloSCT werden gescreend met deze pentameren, wat leidde tot de identificatie van het eerste gevalideerde HLA klasse I gerestricteerde epitoop van VZV. Bij 42% van de HLA-A2 positieve patiënten na VZV reactivatie konden deze IE62-ALW-HLA-A2-specifieke T cellen ex vivo worden gedetecteerd. We hebben aangetoond dat het ondanks de lage frequenties mogelijk is om VZV-specifieke $CD8^+$ T cellen te detecteren, waardoor ex vivo analyse van T cel responsen op VZV infectie en reactivatie en wellicht VZV vaccinatie mogelijk wordt.

Ondanks preëmptieve antivirale behandeling is de incidentie van CMV ziekte bij CMV seropositieve alloSCT patiënten nog steeds 10% 1 jaar na alloSCT. Dit illustreert de noodzaak van adequate CMV-specifieke T cel immuniteit voor langdurige controle van CMV en preventie van CMV ziekte. In **hoofdstuk 7** geven we een overzicht van factoren die relevant zijn voor de preventie van CMV ziekte na alloSCT. GVHD en het gebruik van een

onverwante of mismatch HLA donor bleken geassocieerd te zijn met een verhoogd risico op het ontwikkelen van CMV ziekte ondanks pre-emotieve antivirale behandeling, hetzij als gevolg van systemische immuunsuppressie die nodig is om GVHD te voorkomen of te behandelen of als gevolg van eradicatie van memory CMV-specifieke T cellen van patiënt origine door de alloreactieve T cel respons van donor. T cel depletie bleek geassocieerd te zijn met een verhoogd risico op CMV reactivatie, maar niet met een verhoogd risico op CMV ziekte. Er kan worden verondersteld dat omdat immuunsuppressie over het algemeen niet nodig is na TCD alloSCT, CMV-specifieke T cellen niet worden belemmerd om beschermende immuniteit te bieden in het geval van CMV-reactivatie. De CMV serostatus van de donor beïnvloedt de CMV-specifieke T cel reconstitutie en het risico op het ontwikkelen van CMV ziekte significant. De incidentie van aan CMV gerelateerde complicaties en mortaliteit is lager bij R^+D^+ patiënten in vergelijking met R^+D^- patiënten. Bij R^+D^- patiënten zijn memory CMV-specifieke T cellen niet aanwezig in het donortransplantaat en de van ontvanger afkomstige virus-specifieke T cellen kunnen (gedeeltelijk) worden verwijderd door het conditioneringsregime en/of door een alloreactieve donor T cel reactie. Ten slotte hebben we de beschikbare onderzoeken naar de veiligheid en werkzaamheid van ACT van donor CMV-specifieke T cellen voor de preventie en behandeling van CMV ziekte na alloSCT onderzocht. Alle tot nu toe gepubliceerde trials zijn fase 1/2 trials, waarbij veiligheid, proof of concept en een associatie tussen ACT en klaring van CMV worden aangetoond. Een belangrijke beperking voor ACT na alloSCT is dat de behandeling van actieve GVHD met systemische immuunsuppressie een uitsluitingscriterium was voor toediening van CMV-specifieke ACT in alle onderzoeken. Een andere belangrijke beperking van klinische toepasbaarheid van CMV-specifieke ACT is dat isolatie van CMV-specifieke memory T cellen van de donor beperkt is tot CMV seropositieve donoren. ACT van T cellen geïsoleerd van een andere donor dan de stamceldonor (third-party donor) kan een oplossing zijn voor R^+D^- patiënten. Trials tonen aan dat ACT met van een third-party donor afkomstige virus-specifieke T cellen mogelijk, veilig en effectief kan zijn bij de behandeling van persistente CMV-reactivatie en CMV-ziekte. Het persisteren van deze T cellen is echter onzeker.

Het doel van dit proefschrift was om factoren te evalueren die de incidentie van CMV ziekte na TCD alloSCT beïnvloeden. We hebben vastgesteld dat preventie van CMV ziekte veilig is met behulp van een preëmptieve behandeling met oraal valganciclovir. We hebben geen effect op het risico aangetoond voor CMV reactivatie of CMV ziekte bij patiënten die werden behandeld met een RIC-regime en TCD alloSCT. GVHD en het gebruik van immuunsuppressie na alloSCT bleken belangrijke risicofactoren te zijn voor de ontwikkeling van CMV ziekte. CMV specifieke T cel reconstitutie was efficiënter bij R^+D^+ patiënten, wat leidt tot een verlaagd risico op het ontwikkelen van CMV ziekte. Van donor afkomstige CMV-specifieke primaire T cellen werden gedetecteerd in de meerderheid van de R^+D^- patiënten binnen een jaar na transplantatie.

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gewerkt aan het proefschrift. Het voelde heel fijn om het schrijven te combineren met ons samenzijn en het kopje thee was altijd een welkome afleiding.
Mariëlle, mijn lieve vrouw, is mijn voortdurende motivator. Zij kent mij van haver tot gort, kan altijd relativeren en mij weer opbeuren. Wat we samen doen, dat doen we samen goed. Mijn promotie zit er nu op, nu is zij aan de beurt! Mijn lieve dochters Lotte en Sanne maken elke dag weer tot een avontuur. Ik geniet van hun energie en hun liefde. Ze zijn trots op het boekje van Papa, al zagen zij liever een roze omslag en is het geen leuk voorleesboekje. Ik zal het ze over een tijdje wel uitleggen waar het over gaat!

Curriculum vitae

Pim van der Heiden is geboren op 10 juni 1976 te Rijnsburg. In 1994 behaalde hij zijn Diploma van het Voortgezet Wetenschappelijk Onderwijs aan het College Leeuwenhorst te Noordwijkerhout. Bij de loting vanwege de numerus fixus is hij drie keer uitgeloot voor de studie Geneeskunde. Na drie jaar waarin hij de studies Psychologie en Biomedische Wetenschappen aan de Universiteit Leiden volgde, kon hij in 1997 beginnen aan de studie geneeskunde van de Universiteit Leiden.
Gedurende zijn studie geneeskunde heeft hij in 1999 meegewerkt aan de studie van dr. R.E. Brouwer naar een manier van leukemische cellen om te ontsnappen aan het immuunsysteem. Dit was zijn eerste kennismaking met het Afdeling Hematologie en het Laboratorium voor Experimentele Hematologie van het LUMC. Zijn afstudeeronderzoek voor geneeskunde deed hij samen met Prof. Dr. M.V. Huisman en beschreef het voorkomen van longembolie als eerste klinisch teken van een maligniteit. In 2002 is hij begonnen met zijn coschappen die hij afsloot met een keuze coschap op de afdeling Hematologie en Beenmergtransplantatie onder supervisie van dr. R.M.Y. Barge. In 2004 behaalde hij zijn artsexamen. In 2004 en 2005 was hij werkzaam als “arts-assistent niet in opleiding tot specialist” op de afdeling Interne Geneeskunde van het Bronovo Ziekenhuis in Den Haag.
In 2005 startte hij met zijn promotie welke gecombineerd werd met de opleiding tot internist in het LUMC. De promotie begon onder begeleiding van dr. R.M.Y. Barge, dr. W.A.F Marijt en prof. dr. J.H.F. Falkenburg. Na twee jaar werken “in het lab” en na een begin gemaakt te hebben met het proces waarvan het eindresultaat nu voor u ligt, startte hij met de opleiding tot internist, wederom in het Bronovo Ziekenhuis in Den Haag (opleider Dr. J. W. Van ’t Wout). Van 2010 tot 2012 werd de opleiding tot internist onderbroken om weer verder te gaan met het promotieonderzoek. In 2012 zette hij de opleiding tot internist voort met achtereenvolgend de opleiders prof. dr. J.W.A. Smit, prof. dr. J.T. van Dissel en prof. dr. J.W. de Fijter. Het afronden van de promotie gedurende de periode 2016 tot 2018 vond plaats onder begeleiding van dr. I. Jedema, dr. W.A.F. Marijt en prof. dr. J.H.F. Falkenburg.
In 2012 startte hij met de opleiding voor het deelspecialisme Intensive Care (opleider dr. M.S. Arbous) die hij in 2014 afrondde met registratie tot internist-intensivist. Gedurende zijn opleiding tot intensivist heeft hij onderzoek gedaan naar de overleving van patiënten na allogene stamceltransplantatie die opgenomen zijn op de intensive care. Van 2014 tot 2015 was hij werkzaam als internist-intensivist in het HAGA Ziekenhuis in Den Haag en van 2015 tot 2016 in het Alrijne Ziekenhuis in Leiderdorp. Gedurende deze periode heeft hij deelgenomen aan de commissie verantwoordelijk voor het schrijven van de landelijke richtlijn voor de behandeling van patiënten met een hematologische aandoening op de intensive care. Sinds 2016 is hij werkzaam op de afdeling Intensive Care van het Reinier de Graaf Gasthuis in Delft waar hij in 2018 een vaste positie in de vakgroep verkreeg.

List of publications

Publications included in this thesis

Oral valganciclovir as pre-emptive therapy has similar efficacy on cytomegalovirus DNA load reduction as intravenous ganciclovir in allogeneic stem cell transplantation recipients. **P.L.J. Van der Heiden***, J.S. Kalpoe*, R.M.Y. Barge, R. Willemze, A.C. Kroes, E.F. Schippers. Bone Marrow Transplant. 2006 Apr;37(7):693-8.
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