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Immune Response against human Cytomegalovirus in the Context of Hematopoietic Stem Cell Transplantation (HSCT)

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Summary

T cells play an important role after hematopoietic stem cell transplantation (HSCT) in enhancing graft-versus-leukemia effect (GvL) and controlling infections of latent viruses (such as Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Adenoviruses (ADV)). Immunosuppression after HSCT leads to reactivation of latent viruses, mainly due to the lack of T cells. Pre-emptive therapy with e.g. ganciclovir (GCV) reduces the risk of CMV-disease but recurrent CMV reactivation (CMV-R) remains a major factor for increased morbidity and prolonged hospitalization. In addition, long-term use of antiviral drugs can lead to the development of drug resistant virus strains and has side effects on the haematopoiesis as well as the kidneys and other organ toxicities. While T cell reconstitution is beneficial for GvL and protection from viral infections / reactivations, early or fast reconstitution of T cells after HSCT may contribute to severe acute graft-versus-host disease (aGvHD), thus contributing to reduced overall survival (OS) after allo-HSCT. To control CMV-R, restoration of $CD4^+$ CMV-specific helper cells and $CD8^+$ CMV-specific cytotoxic cells (CMV-CTLs) is essential. Adoptive transfer of virus-specific T cells (CD8+ T cells (CMV-CTL)) is an elegant and efficient method to restore CMV-specific immunity, but is very expensive, thus we analyzed patient samples after HSCT to investigate the presence of either donor or recipient derived CMV-CTL to identify patients who would really benefit from CMV-CTL transfer. Close monitoring (day +25, +50, +100, +200 and +365 after HSCT) of the reconstitution of the virus-specific immunity helped to identify patients at risk for prolonged or multiple reactivations of latent CMV. To contribute to current research, we investigated methods to produce suitable virus-specific T cells from CMV-seropositive healthy donors (D+) and explored different sources for CMV-CTL generation. Healthy donor whole blood with and without in vitro stimulation with granulocyte-colony stimulating factor (G-CSF) and the

apheresis product from the stem cell donors (after G-CSF-stimulation) were used to analyze the frequency and functionality of CMV-CTL. We observed that the G-CSF treatment in vitro or in vivo did not interfere with the quantity of CMV-CTL but influenced the quality by leading to functional impairment of the T cells in all samples analyzed. Both, CD3+/CD4$^+$ and CD3+/CD8$^+$ cells showed reduced cytokine secretion and degranulation when stimulated with the CMV-immuno-dominant protein (phosphoprotein 65; pp65). Next, we used lentiviral vectors expressing CMV-pp65 to transduce self-differentiated myeloid-derived antigen presenting dendritic cells (SMART-DC) to expand CMV-CTL in vitro. Conventional dendritic cells (ConvDCs) loaded with CMVpp65 peptide pools served as controls to expand CMV-CTLs in vitro. CMV-CTL expanded well on both antigen-presenting cells exhibiting only few signs of senescence and exhaustion.

T cell reconstitution and CMV-CTL have a possible influence on overall survival (OS), cumulative incidence of relapse (CIR) and non-relapse mortality (NRM). This relationship was analyzed in samples collected longitudinal after HSCT from 103 patients, 91 with acute leukemia or myelodysplastic syndrome (n=12) following CMV-seropositive recipient/donor (R+/D+) allo-HSCT. Patients were subdivided based on the presence or absence of CMV-CTLs at 3 months after allo-HSCT. Interestingly, we observed that CMV-R led to the rapid engraftment of CD8$^+$ T cells, CMV-CTLs and secretion of inflammatory cytokines that could foster the progression of aGvHD, but also lead to an increased GvL-effect and a decreased risk of leukemic relapse. Better understanding of the control of relapse and the involvement of CMV-CTL will lead to a better OS. Understanding the involvement of CMV-R in the control of aGvHD (contributing about 25% to overall mortality after HSCT) will make the application of HSCT safer by reducing NRM.

Zusammenfassung

T-Zellen spielen nach einer hämatopoetischen Stammzelltransplantation (HSZT) eine zentrale Rolle für den sogenannten Transplantat-gegen-Leukämie (engl. graft versus leukemia, GvL) Effekt und bei der Kontrolle von Reaktivierungen latenter Viren wie z. B. des Cytomegalievirus (CMV), Epstein-Barr-Virus (EBV) und Adenovirus (ADV). Die Reaktivierung von latenten Vires nach HSZT durch die Immunsuppression ist hauptsächlich im Mangel an spezifischen T-Zellen begründet. Zwar lässt sich durch präventive Therapie mit z. B. Ganciclovir (GCV) das Risiko von CMV-Erkrankungen reduzieren, aber wiederkehrende CMV-Reaktivierungen (CMV-R) bleiben einer der Hauptrisikofaktoren für erhöhte Morbidität und verlängerte Hospitalisation. Außerdem kann der langfristige Einsatz von antiviralen Medikamenten zur Entwicklung resistenter Virusstämme führen und hat Nebenwirkungen, die die Hämatopoese stören und Toxizität für die Nieren und andere Organe auslösen. Während die Rekonstitution der T-Zellen für den GvL-Effekt und den Schutz vor Virusreaktivierungen von Vorteil ist, kann eine frühzeitige oder schnelle T-Zell-Rekonstitution auch den Ausbruch einer akuten Transplantat-gegem-Wirt Krankheit (engl. acute graft versus host disease, GvHD) begünstigen, was wiederum zu einem reduzierten Gesamtüberleben (engl. overall survival, OS) nach HSZT beiträgt. Um CMV-R zu kontrollieren ist die Wiederherstellung CD4-positiver CMV-spezifische T-Helferzellen und CD8-positiver CMV-spezifischer zytotoxischer T-Zellen essentiell. Der adoptive Transfer von virusspezifischen T-Zellen (CD8-positive CMV-spezifische zytotoxische T-Lymphozyten, CMV-CTL) ist eine elegante und effiziente Methode um die CMV-spezifische Immunität zu regenerieren, aber auch sehr kostenintensiv – daher haben wir im Rahmen dieser Arbeit Analysen an Patientenmaterial von Patienten nach HSZT durchgeführt, um die Anwesenheit von CMV-CTL von Spender oder Empfänger nachzuweisen und so die

Patienten identifizieren zu können, die am ehesten von einem adoptiven T-Zell-Transfer profitieren könnten. Engmaschiges Monitoring (Tag +25, +50, +100, +200 und +365 nach HSZT) der Rekonstitution der antiviralen Immunanwort ermöglichte die Identifikation von Patienten mit einem erhöhten Risiko für längere oder wiederholte Reaktivierung latenten CMV. Als Beitrag zu aktuellen Forschungsbemühungen suchten wir nach geeigneten Methoden zur Generierung CMV-spezifischer T-Zellen aus gesunden seropositiven Spendern und betrachteten verschiedene Quellen für die Herstellung von CMV-CTL. Vollblut gesunder Spender mit und ohne in vitro Stimulation mit G-CSF und das Aphereseprodukt von Stammzellspendern (nach G-CSF-Stimulation) wurden zur Analyse der Frequenz und Funktionalität von CMV-CTL verwendet. Dabei zeigte sich, das G-CSF weder in vitro noch in vivo die Quantität der CMV-CTL beeinflusste, jedoch die Qualität – in alle getesteten G-CSF-behandelten Proben zeigte sich eine Beeinträchtigung der Funktionalität. Sowohl CD3CD8-positive, als auch CD3CD4-positive Zellen zeigten reduzierte Zytokinsekretion und Degranulation nach Stimulation mit dem immundominanten CMV-Protein pp65 (Phosphoprotein 65). Als nächstes untersuchten wir ein System zur Generierung selbst-differenzierender antigenpräsentierender Dendritischer Zellen, bei dem durch Transduktion mit lentiviralen Vektoren die Expression von pp65 erreicht wird (engl. self-differentiated myeloid-derived antigen presenting dendritic cells – SMART-DC), für die in vitro Expansion von CMV-CTL. Dabei dienten konventionelle mit pp65-Antigen beladene DC (ConvDC) als Kontrolle. Die CMV-CTL expandierten bei beiden Typen antigenpräsentierender Zellen gut und zeigten nur geringe Anzeichen von Seneszenz und Exhaustion („Ermüdung“).

Die T-Zell-Rekonstitution und CMV-CTL haben möglicherweise einen Einfluss auf Gesamtüberleben (OS), kumultative Inzidenz von Rezidiven (CIR) und nicht-rezidiv-bedingte Mortalität (NRM). Dieser Zusammenhang wurde basierend auf allen nach HSCT-gesammelten Proben von 103 Patienten analysiert, die Kohorte enthielt 91 Patienten mit

akuter Leukämie und 12 mit Myeodysplastischem Syndrom nach HSZT von CMV-seropositiven Spendern in seropositive Empfänger (R+/D+). Die Patienten wurden basierend auf der Anwesenheit von CMV-CTL 3 Monate nach HSZT gruppiert. Interessanterweise beobachteten wir, dass CMV-Reaktivierung zu einem schnellen Engraftment von CD8-positiven T-Zellen und CMV-CTL führte und dass die Sekretion inflammatorischer Zytokine die Progression einer GvHD begünstigte / bedingte, allerdings führte sie auch zu einem stärkeren GvL-Effekt und einem geringer Rezidivrisiko. Ein besseres Verständnis der Rezidivkontrolle und der Rolle, die CMV-CTL dabei spielen könnten, würde das OS verbessern. Weiterhin könnte sich Sicherheit von HSZT durch Verminderung der NRM erhöht werden, wenn die Rolle von CMV-Reaktivierungen bei der Kontrolle von aGvHD, diese trägt ca. 25% zur Gesamtsterblichkeit nach HSZT bei, geklärt wäre.

List of Abbreviations

ADCC	antibody dependent cellular cytotoxicity
ADV	adenovirus
aGvHD	acute graft-versus-host disease
AIDS	acquired immune-deficiency syndrome
ATG	anti thymocyte globulin
BM	bone marrow
CB	cord blood
CD34	cluster of differentiation 34
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
cGvHD	chronic graft-versus-host disease
CM	central memory
CMV-CTLs	cytomegalovirus cytotoxic T lymphocytes
CMV-R	cytomegalovirus reactivation
ConvDC	conventional dendritic cells
DE	delayed early phase
DLI	donor lymphocyte infusion
dsDNA	double stranded deoxyribonucleic acid
EBV	Epstein - Barr virus
EM	effector memory
FACS	fluorescence activated cell sorting
G-CSF	granulocyte-colony stimulating factor
gp	glycoprotein
GvL	graft-versus-leukemia
GvHD	graft-versus-host disease
HCMV	human cytomegalovirus
HHV-5	human herpesvirus 5

HSCT	hematopoietic stem cell transplantation
HSV1	herpes simplex virus 1
HSV2	herpes simplex virus 2
TRAIL	tumour necrosis factor related apoptosis inducing ligand
GM-CSF	human granulocyte macrophage-colony stimulating factor
IL-2	human interleukin-2
IL-4	human interleukin-4
IE	immediate early phase
IFN-α	interferon-alpha
IgG	immunoglobulin G
IgM	immunoglobulin M
IL10	interleukin-10
IL-6	interleukin-6
IP-10	interferon-γ inducible protein 10
IR	immune reconstitution
LV	lentivirus
MHC I	major histocompatibility complex I
MHC II	major histocompatibility complex II
MIP-1β	macrophage inflammatory protein-1 beta
NRM	non-relapse mortality
OS	overall survival
PBSC	peripheral blood stem cells
PD1	programmed cell death receptor 1
PDC	plasmacytoid dendritic cells
pp65	phosphoprotein 65
pp71	phosphoprotein 71
RI	relapse incidence
Smart DCs	self-differentiated myeloid-derived antigen presenting cells
SOT	solid organ transplantation

TCD	T cell depletion
TemRA	terminally differentiated effector memory cells
TRM	transplant related mortality
Tim3	T cell immunoglobulin domain mucin 3
Tk	thymidine kinase
TNF-α	tumor necrosis factor-alpha
TREC	T cell receptor gene rearrangement excision circles
VZV	varicella zoster virus

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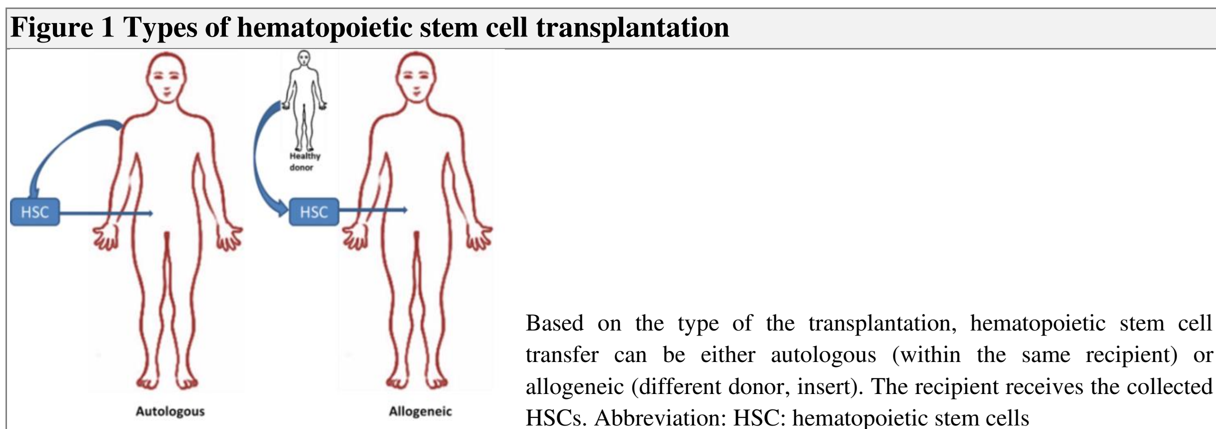
1. Introduction

Hematopoietic stem cell transplantation (HSCT) is the transfer of multipotent progenitor stem cells ($CD34^+$ cells) to the recipient. These stem cells can be obtained from various sources such as bone marrow (BM), peripheral blood (PB, after G-CSF mobilization), or umbilical cord blood (CB).[1] The first bone marrow transplantation (BMT) was successfully performed in lethally irradiated acute leukemia patients in 1956.[2] Since then allogeneic HSCT has become a the only curative treatment for adults for treating leukemia as well as non-malignant disease.[3, 4] The initial success was followed by several severe complications such as graft rejection, graft-versus-host disease (GvHD) and of course relapse which led to death of most of the early patients.[5] The major breakthrough in transplantation came after the identification of human leukocyte antigens (HLA), which made it possible to match the HLA-loci of donors and recipients. This led to the improvement of the therapy by reducing the graft rejection.[6-8] The severity of GvHD was reduced by the inclusion of antithymocyte globulin (ATG® (Fresenius), Thymoglobulin® (Genzyme)) into the conditioning regimen.

Figure 1 Types of hematopoietic stem cell transplantation



Based on the type of the transplantation, hematopoietic stem cell transfer can be either autologous (within the same recipient) or allogeneic (different donor, insert). The recipient receives the collected HSCs. Abbreviation: HSC: hematopoietic stem cells

HSCT can be autologous (**Figure 1**) – meaning the transplantation of the recipient's own stem cells – or as also described above allogeneic – meaning transplantation of a family or an unrelated mainly HLA-matched donor's stem cells.[9] The selection of autologous or

allogeneic transplantation mostly depends on disease (multiple myeloma is still transplanted by autologous HSCT) while all other malignancies are treated by allogeneic HSCT.[10]

1.1 Autologous HSCT

Autologous HSCT (auto-HSCT) is mostly performed for patients who do not have allogeneic donors or for malignancies (e.g. multiple myeloma and other lymphomas) treated with high dose chemotherapy, which eradicates the bone marrow of the patient.[11, 12] Peripheral blood pluripotent stem cells (PBPCs) collection for auto-HSCT is usually done in remission, but malignant cells are still contaminating the graft.[10] Immune reconstitution is fast after auto-HSCT and therefore there is a very low risk of infectious complications.[10-12] In spite of these advantages, auto-HSCT is associated with a very high relapse rate especially in patients with leukemia or lymphomas.[10] This is mainly due to lack of the graft-versus-leukemia effect as well as the contamination of the grafts with malignant cells.[10]

1.2 Allogeneic HSCT

Allogeneic HSCT (allo-HSCT) requires the search for a suitable donor (HLA-identical, matched or mismatched) and is the main treatment for hematologic malignancies and benign dysfunctions of the immune system in adults. It involves transferring immunocompetent cells of the donor together with HSCs. This leads to an increase of GvHD , but also to the desired graft-versus-leukemia effect (GvL) and thus to decreased relapse rates.[10] The major limitation to the broad application of HSCT is the toxicity mediated by the conditioning regimens, acute graft-versus-host disease (aGvHD), infections or very rarely graft rejections. Immunosuppressive treatment is given as a prophylaxis of GvHD, but this is contributing to infectious complications and delayed immune reconstitution which further contributes to non-relapse mortality (NRM).[11]

1.2.1 Choice of allogeneic donors

Initially the major donor source was HLA-identical sibling donors. In early years of HSCT, donor and recipients were matched using antibodies directed against HLA, also known as major histocompatibility complex (MHC).[5] HLA comprises of highly clustered genes encoding for HLA class I (HLA-A, -B and -C), HLA class II (HLA-DR, -DP and -DQ) and HLA class III molecules (complementary factors and tumor necrosis factor family-TNF).[6] The establishment of polymerase chain reactions (PCR) in the early 1980's allowed typing of patient and donor HLA loci (HLA-A, -B, -C, -DRB1 and DPB1) with low, intermediate and high resolution.[13] Low resolution PCR is HLA typing up to two digits (e.g. HLA A*01), while high resolution is depicting four or more digits (e.g. B8*1302).[14] These typing methods allowed to identify the HLA match between donor and recipient with much higher resolution, thus allowing for the use of unrelated donors by matching at least 5 loci in both recipient and donor: HLA-A, -B, -C, -DRB1 and -DQB1, resulting in a 10/10 match. [13, 14 15, 16]

HLA disparities between patient and donor cause T cell activation leading to several complications after allo-HSCT.[14] Multimer studies using tetra-, penta, deca or streptamers relay on high resolution HLA-typing and has enabled understanding of the virus-specific immune reconstitution. For example, cytomegalovirus-specific reconstitution of cytotoxic T lymphocytes (CMV-CTLs) could be monitored with suitable multimers.[17] Patients without HLA-identical donors available but with an urgent need of HSCT are transplanted from HLA-mismatched donors. This can lead to severe complications such as engraftment failure, increased infectious complications as well as increased severity of aGvHD and cGvHD.[6, 8, 14, 18-20]

1.2.2 Conditioning regimens prior a-HSCT

Conditioning (chemo- and/or radiotherapy) is an essential treatment prior to HSCT to eradicate the underlying disease and to make room for the transplanted HSCs.[5] Conditioning can be myeloablative or non-myeloablative based on the intensity of chemo- or radiotherapy.

a. Myeloablative conditioning

Myeloablative conditioning (MAC) leads to the complete eradication of the patient's immune system and bone marrow and consists of usually of either total body irradiation (TBI, 12 Gy to 8Gy) or busulfane (Bu) and derivatives or other alkylating chemotherapy mostly in combination with cyclophosphamide (CY; 120mg/kg body weight; BW).[21] MAC leads to rapid engraftment of the donor cells, but is also associated with aGvHD especially after transplantation of peripheral blood stem cells (PBSC), due to the cytokine storm initiated by high dose conditioning regimen **(GvHD-prophylaxis see Chapter 1.2.3)**.[21, 22] Older patients (above age 60 to 65) or patients with increased comorbidities are prone to succumb to transplant related mortality (TRM) also called non-relapse mortality (NRM). [21, 23] This requires reduced intensity conditioning (RIC) prior to HSCT.[21]

b. Reduced intensity conditioning regimen (RIC)

RIC uses lower doses of chemo- and/or radiotherapy.[21] RIC-protocols are often associated with an increased risk of GvHD, because patients require an increased GvL-effect, due to the residual host hematopoiesis and leukemic cells.[24, 25] RIC is also followed by an increased immunosuppression with or without immunosuppressive antibodies to enable engraftment and to reduce the incidence and severity of GvHD.[5]

1.2.3 GvHD prophylaxis

In order to enable engraftment and to control the severity of acute and chronic GvHD, several prophylactic methods are in use:

a. Immunosuppression with medication

In Europe, immunosuppressive antibodies (antithymocyte globulin, ATG; Fresenius, now Neovii; or thymoglobulin, Genzyme USA) are administered prior to HSCT (day -3 to -1). ATG thymoglobulin or alemtuzomab (campath) reduce or impair T cell functions in vivo*,* thus leading to higher incidence of opportunistic infections, e.g. CMV.[26] The type of GVHD prophylaxis is independent of underlying disease. GvHD-prophylaxis consist of a calcineurin-inhibitor (mainly cyclophosphamide; CsA) based immunosuppression in combination with either methotrexate (MTX) or mycophenol motefil (MMF). Post-transplantation immunosuppression with CsA is 2.5 mg/kg starting on day −1 twice a day and then reduced to twice-daily 1.5 mg to maintain blood levels of 100 to 300 mg/dl. MTX is given at 15 mg/m^2 on day 1 and 10 mg/m^2 on days +3, +6, +9 and +11. [27-29] MTX can lead to severe mucositis and MTX is switched to prednisolone (0.5 mg/kg on days +8 to +14 and 1 mg/kg from day +14 to +28, followed by a taper as indicated). The combination of CsA with mycophenolate mofetil (MMF) is most commonly used together with RIC. MMF is initiated at 15–45mg/kg body weight (BW) twice or thrice from day 0 for 27–40 days, and then is either stopped or tapered through day 96 up to180. Since GvHD prophylaxis is a prolonged immunosuppression after HSCT, it contributes to an increase in opportunistic infections.[30]

b. T cell depletion (TCD) of the graft

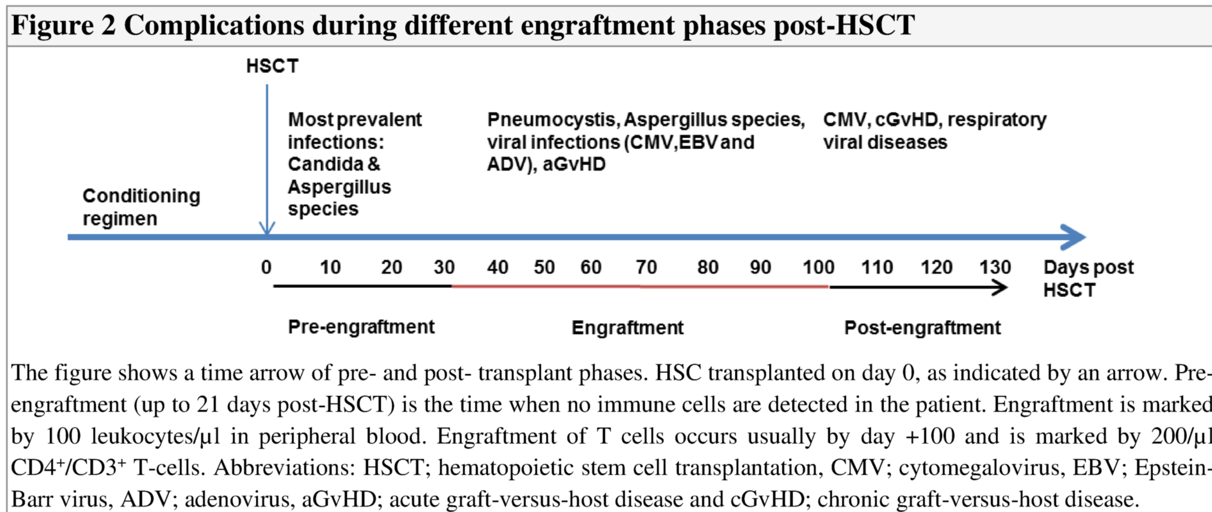
Ex vivo T cell depletion (TCD) of the graft by either enriching CD34$^+$ cells or depleting T cells reduces the incidence of GvHD to almost none occurring, but is associated with a delayed immune reconstitution, increased infectious complications and high relapse rates.[26]

Thus, *ex vivo* T cell-depleted grafts are used sparingly for patients with malignancies and T cell repletion protocols are implemented (e.g. HSV-Tk- transduced T cell transfer, other DLI-based protocols).[31, 32]

1.3 Complications after allo-HSCT

Relapse of the underlying disease and severe aGvHD are the most feared life-threatening complications after HSCT. In addition, in the first weeks after HSCT opportunistic bacterial or fungal infections can complicate the early phases of HSCT. Infection or reactivation of latent viruses like CMV, EBV and ADV contribute to morbidity and even mortality of transplanted patients.[5, 33, 34] Recovery of the immune system after transplantation is shown in **Figure 2**: it is divided in the pre-engraftment (leukocytes below 500/µl, 14-21 days), engraftment (leukocytes above 500/µl, until day +100) and post engraftment phase (fully engrafted bone marrow). [5]

Figure 2 Complications during different engraftment phases post-HSCT



The figure shows a time arrow of pre- and post- transplant phases. HSC transplanted on day 0, as indicated by an arrow. Pre-engraftment (up to 21 days post-HSCT) is the time when no immune cells are detected in the patient. Engraftment is marked by 100 leukocytes/µl in peripheral blood. Engraftment of T cells occurs usually by day +100 and is marked by 200/µl $CD4^+/CD3^+$ T-cells. Abbreviations: HSCT; hematopoietic stem cell transplantation, CMV; cytomegalovirus, EBV; Epstein-Barr virus, ADV; adenovirus, aGvHD; acute graft-versus-host disease and cGvHD; chronic graft-versus-host disease.

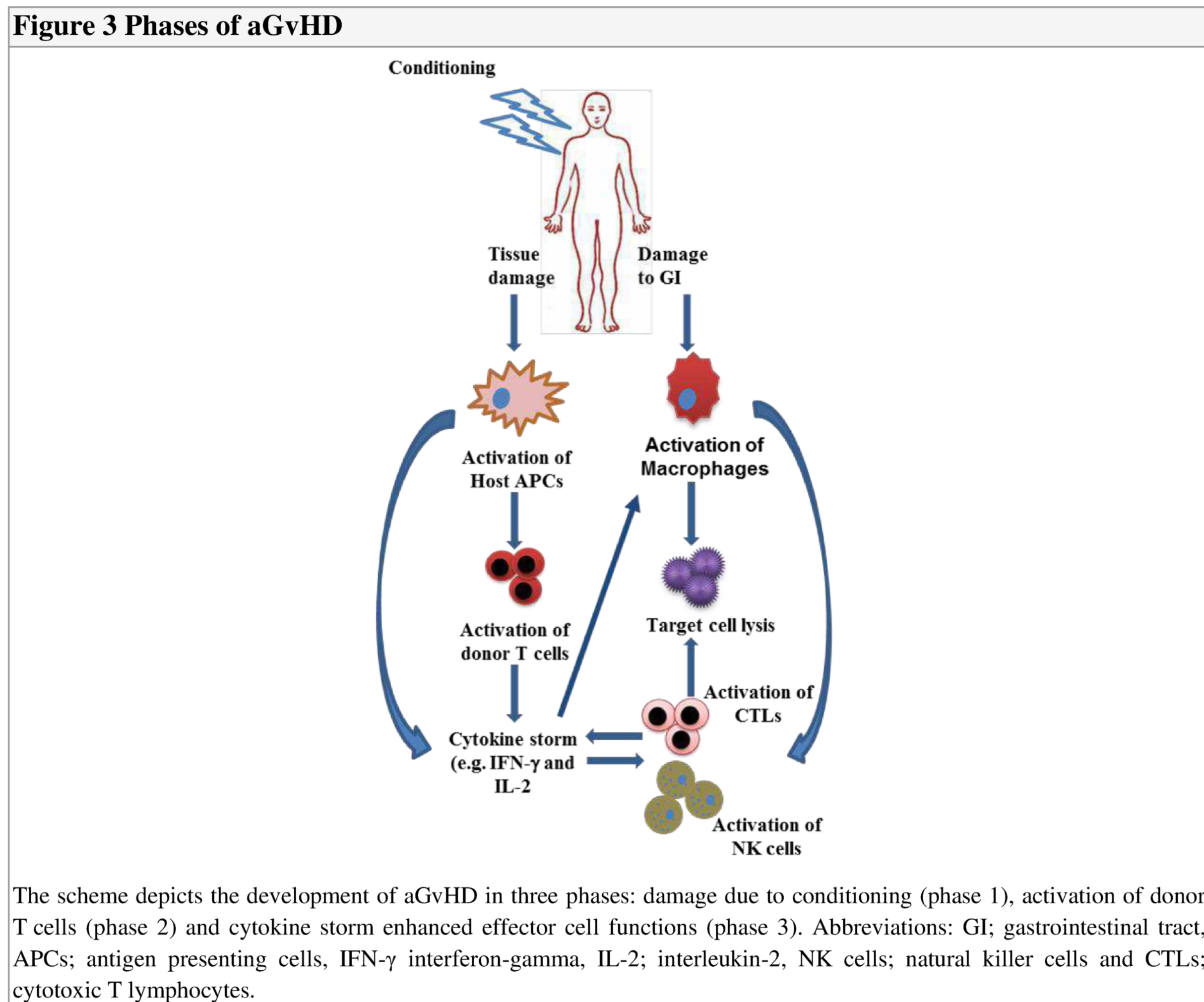
1.3.1 Acute graft-versus-host disease

Allo-reactive T cells of the donor directed against the recipient antigens expressed on tissues and organs (e.g. skin, liver and gastro intestinal tract) mediate (severe) acute graft-versus-host disease (aGvHD).[35-37] Several studies have suggested that pro-inflammatory cytokines (e.g.

interleukin-1alpha; IL-1α, tumor necrosis factor-alpha; TNF-α and Th1 response associated cyto- and chemokines) trigger severe aGvDH.[36, 37] The incidence of aGvHD is about 35-50% , severity is graded in four different stages of aGvHD. aGvHD grade I and even grade II respond well to treatment with prednisolone or other corticosteroids, but aGvHD grades III to IV contribute to the overall mortality in approximately 25% of patients.[35, 38] The risk to develop aGvHD increases with recipient's age, source of the graft (e.g. peripheral blood stem cells), HLA-mismatching, recipient's gender (male) and low intensity of conditioning regimen.[38 39, 40]

The pathophysiology of aGvHD can be divided in three phases **(Figure 3)**:[41, 42]

Figure 3 Phases of aGvHD



The scheme depicts the development of aGvHD in three phases: damage due to conditioning (phase 1), activation of donor T cells (phase 2) and cytokine storm enhanced effector cell functions (phase 3). Abbreviations: GI; gastrointestinal tract, APCs; antigen presenting cells, IFN-γ interferon-gamma, IL-2; interleukin-2, NK cells; natural killer cells and CTLs; cytotoxic T lymphocytes.

Phase 1: Conditioning regimens cause tissue damage followed by the release of inflammatory cytokines or even intestinal bacteria or fragments, which in turn activate host macrophages and other antigen presenting cells (APCs) and ultimately natural killer cells (NK) which further increases the production of cytokines;[5, 35]

Phase 2: The stimulated host APCs activate the donor T cells which recognize host-molecules expressed on health organs.[35, 43,44]

Phase 3: This increase in inflammatory cyto-/chemokines lead to a further activation of macrophages, APC, effector NK cells and stimulate cytotoxic T lymphocytes (CTLs). Both effector NK cells and CTLs lyse the target cells and this leads to further increases in cytokines secretion, the so-called cytokine storm.

1.3.1. a. Severity of acute GvHD

The classification of aGvHD goes back to Glucksberg et al. in 1974 [5, 45] and is divided into four grades shown in **Table 1** and based on severity of organ damage (stage) summarized in in **Table 2**.[5, 45]

Table 1 Grading of aGvHD

Grade	Skin	Liver	Gastrointestinal tract
I	Stage 1–2	0	0
II	Stage 3 or	Stage 1 or	Stage 1
III	-	Stage 2–3 or	Stage 2–4
IV	Stage 4 or	Stage 4	Stage 3-4

aGvHD grades are defined based on the severity of the damage to the tissue or organ. Table adapted from Gluckmann et al.,[5, 45]

Table 2 Stages of tissue or organ damage

Stage	Skin	Liver	Gastrointestinal tract
+ (1)	+ <25% of body surface	34-50 µmol/L	> 500 mL
++(2)	25-50% of body surface	51-102 µmol/L	> 1000 mL
+++(3)	>50% erythroderma	103-255 µmol/L	> 1500 mL
++++(4)	erythroderma with bullae formation and desquamation	>255 µmol/L	Severe abdominal pain with or without ileus

The severity of damage is assessed by the organ function. Increase in the severity of tissue or organ damage is shown form (+, ++, +++ and ++++). Table adapted from Gluckmann et al[5, 45]

1.3.1b. Treatment of acute GvHD

The first line therapy for aGvHD therapy is prednisolone in addition to the GvHD-prophylaxis.[5] About 70% of patients with aGvHD grade I and II respond to the primary treatment. The non-responders receive second line therapy with a variety medications, such as ATG/Thymoglobulin or monoclonal immunosuppressive antibodies like directed anti-interleukin-2 receptor, anti-CD3, anti-tumor necrosis factor-α, anti-CD52 and anti-CD147), extra corporal photopheresis (ECP) or mesenchymal stem cell transfer are also used or under investigation.[5, 46, 47]

1.3.1c. Acute GvHD treatment mediates effects on T cells

Acute GvHD and its treatment leads to substantial delay in the recovery of T lymphocytes and skewed maturation of T cell repertoire ($CD4^+$, $CD8^+$ T cells and antigen specific T cells).[43,33] Moreover, Ozdemir and colleagues reported that steroids induced a significant impairment of $CD8^+$ T cells in the production of tumor necrosis factor-α (TNF-α), rather than a decrease in the frequency or absolute numbers of CMV-specific $CD8^+$ T cells.[48] This delay in T cell reconstitution and loss of functional properties increases the likelihood for reactivation of latent viruses (CMV, EBV and ADV).

1.3. Cytomegalovirus (CMV) and it's reactivation after allo-HSCT

CMV is a complex double stranded deoxyribonucleic acid (dsDNA) virus of the herpes virus family encoding more than 200 viral proteins.[49, 50] CMV-infection occurs in about 50-70% of adult individuals in developed countries and 90-100% in developing countries. CMV infects and replicates in wide variety of cells such as neutrophils, epithelial-, smooth muscle- and endothelial cells.[50, 51] After primary infection, CMV enters into a latent state and stays lifelong in his host's infected cells. Immune competent cells, mainly T cells protect healthy individuals from CMV-disease. In the immunocompromised host (like patients after

allogeneic HSCT) changes in the microenvironment can lead to the reactivation of latent CMV and may lead to CMV-disease in untreated individuals. CMV-R is one of the major complications after HSCT contributing to morbidity and long-term hospitalization. About 60-80% of seropositive-patients after HSCT have at least one CMV-R.[50, 52] The biggest problem present recurrent/multiple CMV-R mainly when CMV-seropositive patients (R+) are transplanted from CMV-seronegative donors (D-). [44, 53]

a. Mode of Transmission

CMV is transmitted via aerosol droplets, urine, faeces, transfusion of blood products and during intercourse.[54] The most frequent mode of infection (61%) is aerosol (droplet) transmission of saliva followed by urine (31%).[54-56]

b. Clinical diagnosis and treatment

During the lytic phase, CMV can be isolated from a wide range of body fluids and secretions such as blood, milk, urine, saliva and faeces.[54, 57] Over the last decade, several methods have been developed to diagnose CMV-R earlier. These methods include the detection of CMV-specific IgM and/or IgG antibodies in serum, the immunohistochemistry of CMV-infected cells (leukocytes) to detect the expression of the phosphoprotein 65 (CMVpp65), and qPCR (quantitative real time polymerase chain reaction to detect and quantify CMV-DNA). CMV-R monitoring is performed with the qPCR for CMV-DNA detection in serum/plasma during the time of aplasia, while CMVpp65 immunohistochemistry assay detects CMV-R after engraftment.[49, 50]

Upon CMV-R, ganciclovir or valganciclovir compose the first line therapy and are given as preemptive treatment, second line treatment is usually done with foscarnet or cidofovir.[58] All these antiviral drugs have shown to decrease the incidence of CMV-disease post

allo-HSCT.[59, 60] Several other approaches such as CMV-vaccination or adoptive therapy are currently investigated.

c. CMV vaccination

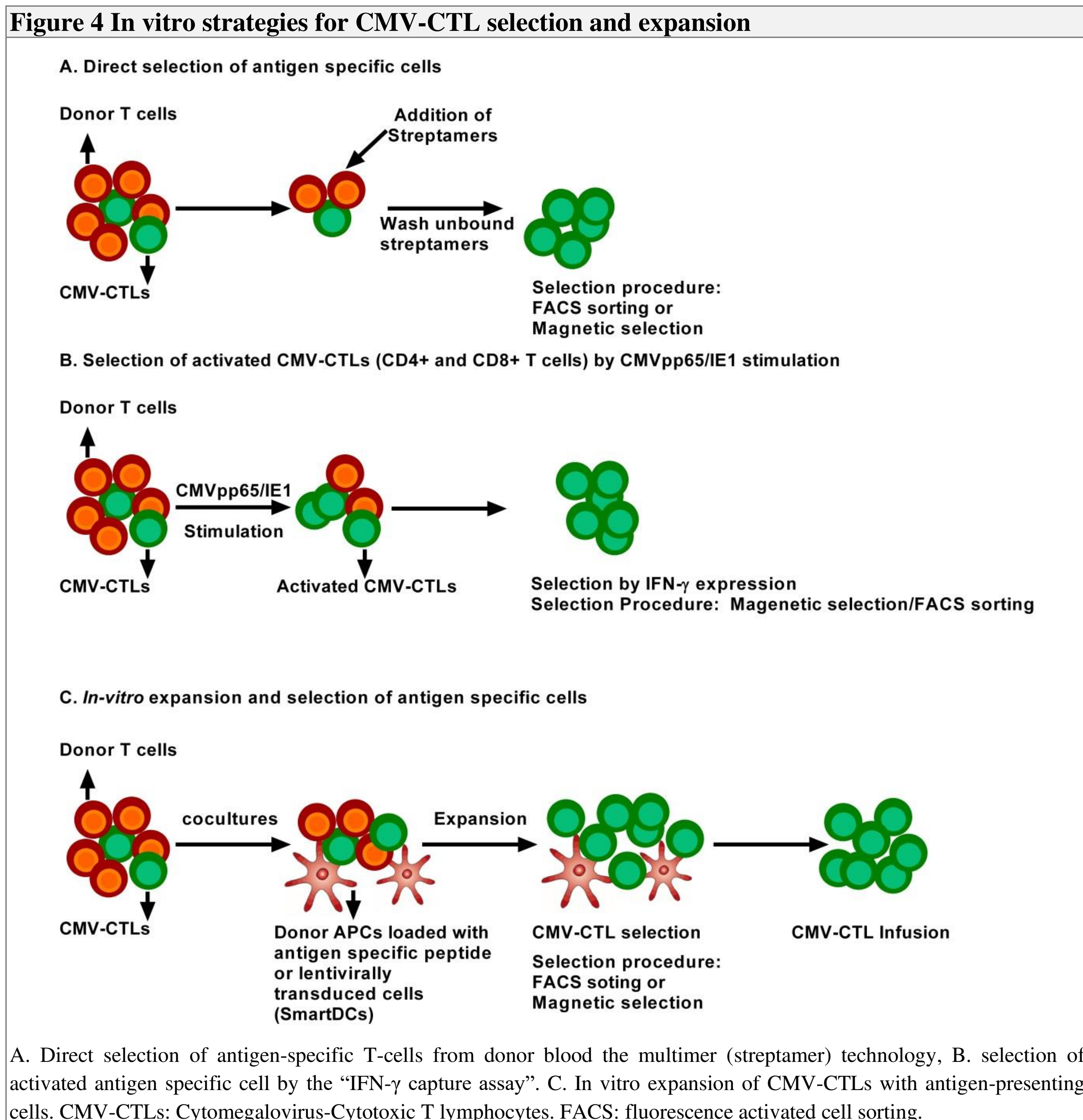
Although pre-emptive therapy decreased the incidence of early CMV-R or CMV-disease post-HSCT (day 0-100),[59, 61] it still remains a major challenge in patients with poor immune reconstitution (IR) or in patients transplanted from CMV-seronegative donors.[62] Vaccination of CMV-seronegative individuals against CMV is currently under investigation especially in solid organ transplantation patients and several clinical trials are ongoing. In the early 90's, live attenuated vaccines for CMV elicited good short term responses of B and T cells, but failed to provide CMV-specific immunity in vaccinated patients.[56, 63] Despite several advances to develop a CMV vaccines (DNA vaccines, live attenuated and subunit vaccines), the approval for a potential vaccine candidate remains pending due to suboptimal performances.

d. In vitro production of CMV-CTLs and adoptive therapy and

Clinical studies in the early 90s showed that the adoptive transfer of in vitro expanded (doses ranges from $3.3x10^6$ to $1x10^{11}$) CMV-specific T cell-clones ($CD8^+$ T cells) showed low toxicity and a possible reduction of CMV-R after HSCT.[64, 65] The production and cloning of CMV-specific cells is expensive and has several regulatory pitfalls. Therefore, CMV-specific T cell lines were produced for pre-emptive treatment to control CMV-R. Many groups have started to investigate the possibility of adoptive transfer of CMV-specific T cell lines (CMV-CTLs) and showed that very low numbers of CMV-CTLs (about $3x10^4$ CMV-specific cells/kg BW) are sufficient to control CMV-R.[64, 66, 67] CMV-CTLs were generated using different protocols for selection and expansion.[66, 68] In order to provide strong anti-CMV responses, restoration of both $CD4^+$ and $CD8^+$ CMV-specific T cells is mandatory. In order to

improve CD4^{+} and CD8^{+} antigen-specific cell selection, overlapping peptide pools (15mer sequence) of CMVpp65 and immediate early protein-1 (IE1) have been extensively used to activate and expand CMV-CTL in vitro with or without dendritic cells.[67] The selection of stimulated CMV-specific CD4^{+} and/or CD8^{+} T cells from directly from donor blood samples using magnetic beads to capture cytokines such as IFN-γ or by multimer-based selection improved CMV-CTL production.[64, 69]

Figure 4 In vitro strategies for CMV-CTL selection and expansion



A. Direct selection of antigen-specific T-cells from donor blood the multimer (streptamer) technology, B. selection of activated antigen specific cell by the "IFN-γ capture assay". C. In vitro expansion of CMV-CTLs with antigen-presenting cells. CMV-CTLs: Cytomegalovirus-Cytotoxic T lymphocytes. FACS: fluorescence activated cell sorting.

Both, multimer-based selection and the IFN-γ capture assay techniques allowed to select a wide range of antigen specific cells with high purity. For clinical transfer of CMV-CTL streptamers, which are biotinylated HLA-CMV-peptide complexes, are of major interest. This approach is extremely interesting, since streptavidin disconnects the streptamer molecules and allow transfer of untouched CMV-CTL to the patient.[70] All other methods require short/long-term stimulation and culture of CMV-CTLs.

1.3.3 Risk factors for CMV-Reactivation

The risk for (recurrent) CMV-R correlates with several clinical factors such as patients' age (higher risk in older patients), CMV-serostatus of recipient and donor, conditioning regimen, matched unrelated donor, mismatched donors, T cell depletion, immunosuppression after transplantation, aGvHD and cGvHD and the treatment with (high dose) steroids **(Table 3)**.[49, 71 49, 72]

Table 3 Risk factors for CMV-R post-HSCT

Risk factors for CMV-R	**Risk of CMV-R**	**Reference(s)**
ATG	+	73, 74
Recipient Age (above 40 years)	++	75
aGvHD grade II-IV	++	18, 72, 73, 76, 77
cGvHD	++	18, 78
aGvHD prophylaxis (MMF)	++	30
Matched unrelated donor (MUD)	+	75, 78, 79
Mismatched donor	++	72, 79
MHC I and MHC II mismatches	++	19, 80
Serostatus of recipient and donor (e.g. R^+D^-, R^+D^+)	++	18, 72, 77, 79, 81, 82
T cell depletion	+++	74

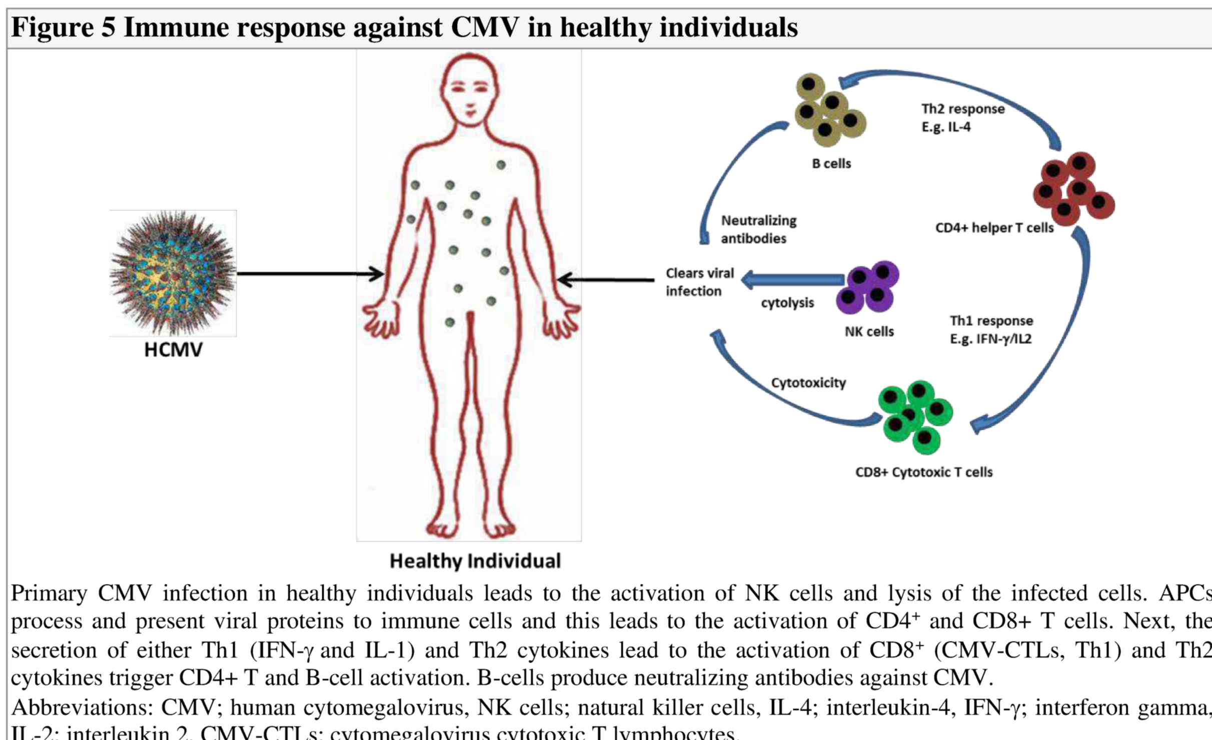
Donor positive for CMV serostatus, R+D-; Recipient positive, donor negative for CMV serostatus, R+D+; Recipient positive, donor positive for CMV serostatus, ++; high risk, +; moderate risk and +/-; low risk.

1.3.4 Immune response against CMV in the immunocompromised host

The host's innate and adaptive immune system plays a critical role in the clearance of CMV infection **(Figure 5)**.[50, 83] In the immunocompromised host the reconstitution of CMV specific immunity has been analyzed by monitoring for the restoration of the CMV-immune response and CMV-CTL were increased by either adoptive therapy or vaccination against CMV after transplantation **(refer section 1.3.2 c and d)** are currently investigated.

CMV-specific cellular responses are monitored by direct and indirect methods.[84] Indirect methods involve stimulation of whole blood or PBMCs with peptides and peptide pools followed by the detection of cytokines (e.g. interferon-gamma (IFN-γ)) by the cytokine capture assay, enzyme linked immunosorbent assay (ELISA), enzyme linked immunospot (ELISpot), intracellular staining and cytotoxicity by degranulation (CD107a) or release of perforin and granzyme-B.[84, 85]

Figure 5 Immune response against CMV in healthy individuals



Primary CMV infection in healthy individuals leads to the activation of NK cells and lysis of the infected cells. APCs process and present viral proteins to immune cells and this leads to the activation of $CD4^+$ and CD8+ T cells. Next, the secretion of either Th1 (IFN-γ and IL-1) and Th2 cytokines lead to the activation of $CD8^+$ (CMV-CTLs, Th1) and Th2 cytokines trigger CD4+ T and B-cell activation. B-cells produce neutralizing antibodies against CMV.
Abbreviations: CMV; human cytomegalovirus, NK cells; natural killer cells, IL-4; interleukin-4, IFN-γ; interferon gamma, IL-2; interleukin 2, CMV-CTLs; cytomegalovirus cytotoxic T lymphocytes.

Direct evaluation of $CD8^+$ CMV-specific T cells using multimers was established in the late 1990's.[85, 86] Peptide-MHC-complexes identify T cells which can bind to the peptide-antigens

presented within the HLA-grooves. Multimers consist of tetra-, penta,- hexa or streptamers, all of these are enzymatically biotinylated with fluorochrome labelled streptavidin and thus allow the detection of the CMV-CTL by flow cytometer (FACS). [86]

The introduction of CMV-HLA-specific multimer-fluorochrome-conjugates made it easier to study the reconstitution of CMV-CTLs.[85, 87, 88] It has been shown that reconstitution of antiviral immunity / virus-specific immune cells is necessary to control CMV-R [87, 89] **,** and it became possible to identify high-risk patients for recurrent CMV-R.[90] In addition, the dynamics of CMV-CTL reconstitution in CMV-seropositive recipients show a high degree of variation. CMV seropositive recipients (R^+) transplanted from CMV seropositive donors (D^+) reconstitute CMV-CTL between 50-75 days, while in CMV seropositive recipients transplanted from seronegative donors (D^-) CMV-CTL reconstitution occurs after 120 days or more.[90, 91] This again varies based on the extent of immuno suppression, mismatches in the HLA alleles and also the development of GvHD. In addition, some studies have shown that the source of stem cells (PBSC or BM) plays an important role in the reconstitution of CMV-specific T cells (CMV-CTL), showing that recipients of grafts from PBSC had better and faster CMV-CTL recovery (both $CD8^+$ and $CD4^+$ T cell reconstitution) than those of bone marrow. [33, 34]

1.3.5 Pro-inflammatory response mediated by CMV-R

Pro-inflammatory cytokines play an important role in the severity of aGvHD.[92] The number of naïve T cells, regulatory and effector cells as well as the excretion level of inflammatory cyto-/chemokines in the recipient determines the severity of aGvHD post-HSCT.[92] Conditioning regimen prior to HSCT lead to the activation of antigen presenting cells along with inflammatory cytokines (e.g. IL-4, IL-12, IFN-γ, TNF-α). This in turn leads to the expansion of host $CD4^+$ and $CD8^+$ T cells which could possibly result in graft rejection.[93] Thus, in Europe ATG or thymoglobulin or even campath is administered to avoid graft

rejection. The conditioning regimen and the activation of donor T-cells on the other hand cause tissue damage resulting in the development of aGvHD.[92] However, the role of these cytokines (Th1 and Th2) responses and their involvement in the development of aGvHD post CMV-R is not clear in allo-HSCT recipients.

2. Aims of the thesis

2.1. We aimed to assess the presence and functionality of antiviral CTL in stem cell donors and respective grafts

Multimer-monitoring of antigen-specific cytotoxic T cells (CTL) directed against latent viruses (e.g. CMV) quantifies the epitope-specific immune response in patients after allo-HSCT in several studies. However, antiviral CTLs were not analyzed in stem cell donors or their respective grafts. Since the number of specific CTL transferred with the graft could impact the reconstitution of antiviral immunity, quantification of CTL in the respective donors and/or grafts prior to transplantation may provide insight in the differing kinetics of immune reconstitution in different recipients. Therefore, we investigated the number of antiviral CTL using a set of multimers in stem cell donors. To explore whether the mobilization and the apheresis procedure itself did affect the functionality of CTL, donor samples from different sources were analyzed. These were: whole blood prior to G-CSF mobilization (WB), whole blood after G-CSF mobilization on the day of apheresis (WBM), blood from the apheresis tubing set (A) and an aliquot from the collected graft (G). All samples were compared for detection, functionality and collection antigen-specific-CTL.

2.2 We aimed to assess different in vitro expansion strategies for CMV-CTL.

CMV-CTL collection from the seropositive donors may yield only few cells for adoptive transfer, thus we study the in vitro expansion of CMV-CTL. Our protocol should avoid exhaustion of effector T-cells a common problem in earlier works. Thus, we aimed to compare different stimulation methods in DCs generated either by cytokine-induced differentiation from macrophages with lentiviral transduced self-differentiating so-called

SMART-DC expressing a full-length antigenic CMV protein or convention DCs loaded with overlapping peptide pools.

2.3. We aimed to identify patients in need of adoptive T cells transfer and to determine the influence of CMV-CTL and CMV-R on the outcome after HSCT.

Since CMV-CTL generation in vitro remains a cost-intensive therapy, we assessed patients who would most likely experience recurrent CMV-reactivations and would most likely profit from adoptive CTL-transfer for permanent control CMV-R. Early identification of these patients may lead to timely CMV-CTL generation/isolation and reduce repeated use of antiviral drugs and their side effects.

To understand the relationship of CMV-R and outcome after HSCT, we investigated the relationship between CMV-R, overall T cell and CMV-CTL expansion and cytokine secretion and correlated the results to overall survival (OS), cumulative relapse incidence (CRI) and non-relapse mortality (NRM) in CMV seropositive recipients (R+) transplanted from seropositive donors (D+). These data are summarized in the published manuscript attached in section 4.2 of this thesis.

3. Materials and Methods

3.1 Materials

3.1.1 Buffers and culture media

MACS buffer
1X phosphate buffer saline (PBS), 2 mM EDTA and 0.5 % HSA

Fixation buffer
Prepare 1% of paraformaldehyde (PFA) from a stock of 4% PFA (BioLegend; Cat No: 420801).

Thawing buffer
20% of HSA in X-Vivo15

Freezing medium
X-Vivo15, 10% HSA and 10% DMSO

HEPES buffer
1 M stock

FACS buffer
0.5 mM EDTA, 1% BSA/HAS and 1X PBS

Agarose gel running buffer
0.5X TBE

Whole blood lysis buffer
To 1ml of lysis solution (Beckmann Coulter; BC Ref no: PN IM3514) add 25 µl Fixative (BC, Ref no; A09777 IM3648)

D10 Medium
Dulbecco's modified eagle medium (DMEM) consisting of 10% heat inactivated FBS with 50 U/ml Penicillin and 50 U/ml Streptomycin.

Sample Diluent buffer for GM-CSF and IL-4 ELISA
PBS with 0.1% BSA and 0.05% Tween20.

Monocyte to DC differentiation medium
X-vivo15 medium (Lonza, Cat No: BE04-418F) with granulocyte macrophage-colony stimulating factor (GM-CSF; 25 ng/ml) and interleukin-4 (IL-4: 25 ng/ml).

Dendritic cell maturation medium
To the above monocyte to DC differentiation medium tumour necrosis factor-alpha (TNF-α: 200 IU/ml), interleukin-1 beta (IL-1β: 5 ng/ml), interleukin-6 (IL-6: 10 ng/ml) and prostaglandin E_2 (PGE_2; 1 µg/ml).

T cell culture medium
X-vivo15 medium (Lonza, Cat No: BE04-418F) with interleukin-2 (IL-2: 25 IU/ml), interleukin-7 (IL-7: 5 ng/ml) and interleukin-15 (IL-15: 5ng/ml).

3.1.2 Cytokines for cell culture

Cytokine	Catalogue number	Company
IL-1β	11340012	Immuno Tools
IL-2	11340025	Immuno Tools
IL-4	11340042	Immuno Tools
IL-6	11340060	Immuno Tools
IL-7	11340075	Immuno Tools
IL-15	11340155	Immuno Tools
GM-CSF	11343122	Immuno Tools
TNF-α	11343013	Immuno Tools
PGE_2	P6532	Sigma Aldrich

3.1.3 Antibodies for cell surface marker staining

Antibody	Conjugate	Catalogue number	Company
CD3	PeCy7	737657	Beckmann Coulter
CD3	V500	561416	Becton Dickinson
CD4	FITC	301039	BioLegend
CD4	PE	IM0449	Beckmann Coulter
CD8	BV605	301039	BioLegend
CD8	FITC	6603861	Beckmann Coulter
CD45RA	PerCP-Cy5.5	304122	BioLegend
CCR7	PE/Cy7	353226	BioLegend
CD57	FITC	322306	BioLegend
PD1	Alexa647	329910	BioLegend
PD1	APC	329908	BioLegend
Tim3	BV421	345008	BioLegend
CD14	PeCy7	325618	BioLegend
CD83	Alexa647	305316	BioLegend
CD86	BV421	305426	BioLegend
CD209	PE	330106	BioLegend
HLA-A/B/C	PerCP-Cy5.5	311420	BioLegend
HLA-DR	FITC	307604	BioLegend
L/D NEAR IR	Alexa750	A-20011	Life technologies
7AAD	PerCP	A07704	Beckmann Coulter

3.1.4 HLA specific antibodies to detect CMV antigen specific cells

Reagent	HLA-molecule	Virus, protein source	Provider	Peptide sequence	Conjugate
Tetramer	A*01:01	CMV, pp50	Beckmann Coulter	VTEHDTLLY	PE
Tetramer	A*02:01	CMV, pp65	Beckmann Coulter	NLVPMVATV	PE
Tetramer	A*24:02	CMV, pp65	Beckmann Coulter	QYDPVAALF	PE
Tetramer	B*07:02	CMV, pp65	Beckmann Coulter	TPRVTGGGAM	PE
Tetramer	B*08:01	CMV, IE1	Beckmann Coulter	ELRRKMMYM	PE
Pentamer	A*01:01	CMV, pp65	Proimmune	YSEHPTFTSQY	PE
Pentamer	A*02:01	CMV, IE1	Proimmune	VLAELVKQI	PE
Pentamer	B*07:02	CMV, pp65	Proimmune	RPHERNGFTVL	PE
Pentamer	B*08:01	CMV, IE1	Proimmune	QIKURVDMV	PE

3.1.5 Plasmids and vectors

Plasmid	Source
PMA_pp65 (ID: 12ABATQP)	Life technologies
Plasmid (RSV-REV)	Prof. Renata Stripecke (MHH)
Packaging Plasmid (pMDLGg/p)	Prof. Renata Stripecke (MHH)
Envelope Plasmid (PMD.G)	Prof. Renata Stripecke (MHH)

3.1.6 Other antibodies and chemicals

Reagents and kits	Catalogue number	Company
CD 8 Microbeads	130-045-201	Miltenyi Biotech
CD14 Microbeads	130-050-201	Miltenyi Biotech
Cytomegalovirus pp65 Monoclonal Antibody (1-L-11)	MA1-7597	Thermoscientific
Peptivator CMVpp65 peptide pool	130-093-438	Mitenyi Biotec
Human GM-CSF ELISA kit	3480-1H-20	Mabtech
Human IL-4 ELISA kit	3410-1H-6	Mabtech
P24 titre kit	VPK-107-CB	BioCat

Chemicals	**Catalogue number**	**Company**
Agar	6352.2	Roth
Ampicillin	10044-5G	Fluka
10X Tris/Buffered Saline	170-6435	Bio Rad
10X Tris/Glycine Buffer	161-0734	Bio Rad
2-Mercaptoethanol	M3148-25ML	Sigma Aldrich
Beta 2-Mercaptoethanol	M3148	Sigma
Bio-Plex Pro 21 cytokine and chemokine detection kit	MF0005KMII	Bio-Rad
BSA	A4503-50G	SIGMA
Cell dissociation buffer	13150-016	Gibco-BRL
ECL detection kit	34080	Pierce
DMSO	D2650	Sigma
Dulbecco’s Phosphate Buffered Saline	14190-169	Gibco
Dulbecco’s Phosphate Buffered Saline	17-512F	Bio-Whittaker
DMEM	41966-052	Invitrogen
EDTA 0,5M Steril	E177-100MLDB	VWR
Ethanol 99% denaturiert TechniSolv	84105.360DB	VWR
Gel extraction kit	28704	Qiagen
HEPES	H3537	Sigma
Laemmli Sample Buffer	161-0737	Bio Rad
Methanol	20903.368DB	VWR Chemicals
Milk powder	T8154	Sigma Aldrich
Penicillin/Streptomycin	A2213	Biochrom
Poly-L-Lysine	P-4832	Sigma
Protein ladder	SM0671	Fermentas
Plasmid purification kit	27104	Qiagen
SDS PAGE gels (10%)	161-1119	Bio-Rad
SuperSignal West Pico Chemiluminescent Substrate	34087	Thermoscientific
Tris ultra >99.9%	T145.3	Roth
Trypan blue	T8154	Sigma Aldrich
X-Vivo 15 cell culture media	BE04-418F	Lonza

3.1.7 Materials

Materials	Catalogue number	Company
1.5ml Eppendorf	56 15 000	Ratio labs
FACS tubes 12x75mm	352008	Becton Dickinson
15 ml tube	62.554.502	Sarstedt
50 ml tube	62.547.254	Sarstedt
6 Well Clear Flat Bottom TC-Treated	353046	Falcon
24 Well Clear Flat Bottom TC-Treated	353047	Falcon
48 Well Clear Flat Bottom TC-Treated	353078	Falcon
96 Well Clear Round Bottom TC-Product	353077	Becton Dickinson
96 Well Clear Flat Bottom TC-Treated	353936	Becton Dickinson
Filter Paper	1703932	Bio-Rad
Flow count beads	7547053	Beckman Coulter
Ficoll	L6115	Biochrom
MACS LS columns	130-042-401	Mitenyi Biotec
MACS MS columns	130-042-201	Mitenyi Biotec
Serological Pipette 5ml	86.1253.001	Sarstedt
Serological Pipette 10ml	86.1254.001	Sarstedt
Serological Pipette 25ml	86.1685.001	Sarstedt
PVDF Western Blotting Membrane	3010040001	Roche Applied Science
Pipet tip xl, 0.1-10ul	21 00 500	Ratio labs
Pipet tip E, volume 1-200ul	21 00 600	Ratio labs
Pipet tip, volume 100-1000ul	24 00 610	Ratio labs
T175 cell culture flask	159910	Nunc

3.1.8 Softwares used for analysis

Serial no.	Software	Company
1	CXP FC-500	BC
2	Diva6: FACS analysis software	BD
3	Endnote version 7	Thomson Reuters
4	Flowjo version 10: FACS analysis software	Treestar
5	Graph pad prism 6	Graphpad
6	SPSS version 20	IBM

3.2 Methods

3.2.1 Sample Collection:

a. Patient sample collection

Whole blood samples from allo-HSCT recipients were collected as a part of an extended monitoring at +30, +50 and +100 days post-HSCT approved by Ethics Committee at the Hannover Medical School (MHH) under #2906.

b. Healthy donor sample collection:

Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte reduction filters from healthy donors undergoing apheresis after informed consent in collaboration with transfusion medicine at the Hannover Medical School (MHH). All the experiments were performed with the approval of Ethics Committee at MHH.

3.2.2 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated from blood of healthy donors (leukocyte reduction filters) and from patients after allo-HSCT using ficoll by density gradient centrifugation.

Blood samples were diluted 1:2 with PBS and under layered with 10 ml ficoll per 35 ml diluted blood. Density gradient centrifugation was performed at 2000 x g for 20 min at room temperature (RT). The interphase ring was collected, washed twice with 50 ml of sterile PBS (1000 x g for 10 min) and frozen at a concentration of $2x10^7$ or $5x10^7$/ml in freezing medium and stored in liquid nitrogen until further use.

3.2.3 Selection of T cells and/or monocytes using magnetic bead sorting

Positive selection of T cells and monocytes was performed using anti-CD8 or anti-CD14 labelled microbeads, respectively. MACS-based isolation was done according to the manufacturer's instructions. Briefly, PBMCs were washed twice with MACS buffer, centrifuged at 1000 x g for 10 min) and suspended on ice MACS buffer (10^7 cells in 8 0µl). Microbeads (CD8+ or CD14+ 20µl each) were added and mixture was incubated for 15min at 4 ºC. After washing cells were layered on the pre-rinsed MACS-column (in magnetic holder) and washed 3 times. The column was removed from the magnetic field and bound cells were eluted by the addition of ice-cold MACS buffer. Purity of the selected cells was determined by flow cytometry. Cells ($2x10^5$) were suspended in 200 µl of PBS. Anti-CD3, anti-CD4, anti-CD8 or anti-CD14 were added as appropriate, incubated for 30 min at 4 °C, washed twice in PBS suspended in 200 µl PBS and analyzed by flow cytometry.

3.2.4 Monitoring of CMV-CTLs

WBC were collected in 2.5ml EDTA tubes at day +30, day +50, day +100 and day +300 post allo-HSCT. Reconstitution of CD3+/$CD4^+$ and -$CD8^+$ as well as CMV-CTL was analyzed with CMV HLA-specific tetramers **(materials 3.1.4)** by incubating 30 min in dark 4º C (**Table 4**). Then erythrocytes were lysed by adding lysis buffer **(materials 3.1.1**) and incubating for 15min at RT (in the dark). Subsequently, cells were washed twice with PBS centrifuged and resuspended in 200 µl of wash buffer. Flow count beads (100 µl) were added to be able to determine the absolute counts of $CD3^+$, $CD4^+$, $CD8^+$ T cells and CMV-CTLs.

Table 4 Staining procedure for whole blood samples

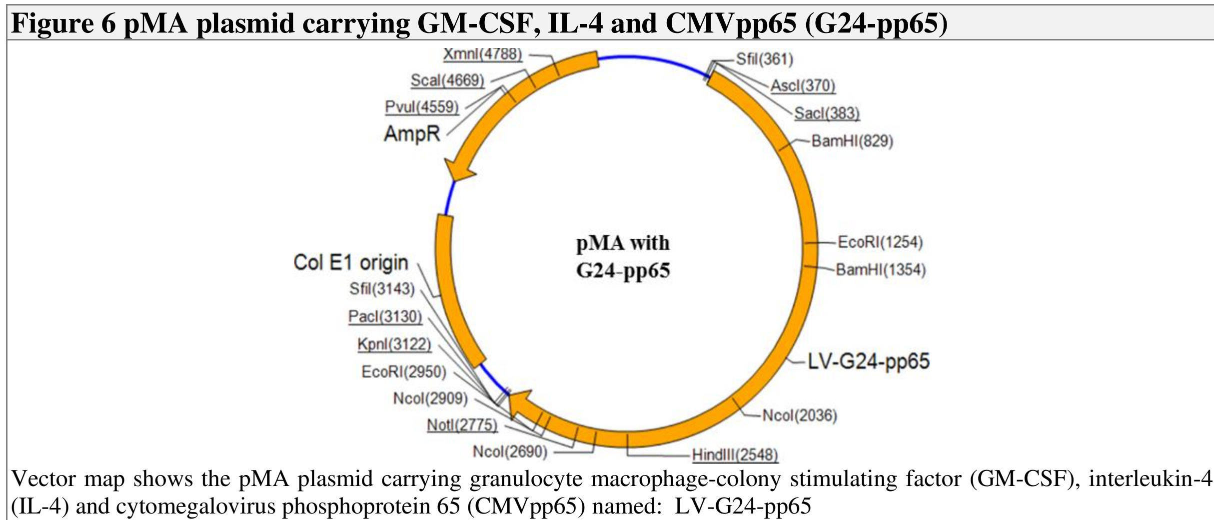
Test/tube	CD8-FITC	CD4-PE	Tetramer-PE	CD3-PeCy7	Sample	PBS	Lysis	Final volume
Count	5 µl	10 µl	---	5 µl	100 µl	---	1 ml	1 ml
Neg Tet	5 µl	---	5 µl	5 µl	200 µl	---	2 ml	200 µl
Multimers	5 µl	---	---	5 µl	200 µl	---	2 ml	200 µl

Staining procedure for quantification of CMV-CTLs using HLA specific tetramers (**Materials 3.1.5**). Abbreviations: Neg Tet; negative tetramer/unspecific tetramer, PBS; phosphate buffer saline.

3.2.5 Construction of a tricistronic Lentiviral Vector expressing pp65

A tricistronic vector expressing of human granulocyte macrophage-colony stimulating factor (GM-CSF), human interleukin-4 (IL-4) and CMV phosphoprotein65 (CMVpp65) was synthesized and inserted in the pMA plasmid **(Figure 6)** resulting in the LV-G24-pp65 plasmid.

Figure 6 pMA plasmid carrying GM-CSF, IL-4 and CMVpp65 (G24-pp65)



Vector map shows the pMA plasmid carrying granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-4 (IL-4) and cytomegalovirus phosphoprotein 65 (CMVpp65) named: LV-G24-pp65

The lyophilized pMA plasmid was dissolved in sterile distilled H_20 (5 µg/ 50 µl).) Two µl (200 ng) of LV-G24-pp65 plasmid and 200 µl of competent bacterial cells (E. coli DH5) were mixed, incubated on ice for 30 min and followed by heat shock at 42 ºC for 90 sec. Luria-Bertani (LB) medium (800 µl) was added to the bacteria were incubated for 1 hour at 37 ºC on a shaker. Subsequently, the incubated bacteria were plated onto ampicillin (100 µg/ml) supplemented agar plates and incubated overnight at 37 ºC. Ampicillin resistant colonies were picked and grown overnight in LB media containing 100µg/ml ampicillin. Mini and Maxipreparation's were performed to analyzed the LV-G24-pp65 in detail. Restriction enzyme analyzes were done with *XbaI*, *ClaI* and *ScaI* to create smaller fragments allowing the identification of the genes of interest on a preparative gel **(Table 5A).** To verify the orientation and proper expression of the genes of interest the fragments were separated a 0.8% agarose gel stained with Ethidium bromide (expected fragment size LV-G24-pp65:

2733 base pairs). Vectors in the correct orientation were purified by gel elution. Pure plasmid DNA was isolated from 3 ml of the overnight cultured ampicillin resistant colonies in LB media (100 µg/ml of ampicillin) using a Qiagen mini prep kit according to manufacturer's instructions. A glycerol stock was prepared from the remaining transformed bacteria and stored at -80 °C.

Table 5 Restriction digestion of pMA plasmid and SIN-LV backbone			
A. Restriction digestion of pMA		**B. Restriction digestion of SIN-LV**	
Reaction mixture	**Volume**	**Reaction mixture**	**Volume**
pMA DNA	15 µl (1 µg/µl)	#204 (SIN-LV)	10 µl (1 µg/µl)
Buffer	5 µl	Buffer	5 µl
BSA (100X)	0.5 µl	BSA (100X)	0.5 µl
Water	22.5 µl	Water	30.5 µl
Xba I	2 µl	*Xba I*	2 µl
ClaI	2 µl	*ClaI*	2 µl
ScaI	3 µl	NA	NA
Total	**50 µl**	**Total**	**50 µl**
The purified pMA plasmid (A) and SIN-LV backbone plasmid (B) were digested as shown above for 1 hour at 37 ºC.			

3.2.6 Sequence verification and transduction of SMART-DC with LV-G24-pp65

Sequencing of SIN-LV-G24-pp65 was performed at Seqlab (Göttingen) and verified using Clone Manager. After the verification, maxiprep (according to the manufacturer's instructions) of the cloned plasmid (SIN-LV-G24-pp65) was performed as per the manufacturer's instructions (Qiagen cat no: 12162) and stored at -80 °C for further experiments.

3.2.7 Lentivirus Production

The SIN-LV-G24-pp65 virus was produced by co-transfection of four plasmids (**Materials 3.1.5)** by calcium phosphate into 293 T cells **(Figure 7)**.

Briefly, 293T cells (1.3-1.5x10^7) were cultured overnight in D10 medium at 37 ºC in T175 cell culture flask pre-coated with Poly-L-Lysine. On the following day, cells were fed with 40 ml fresh D10 medium. Plasmids were resuspended in 2.7 ml of double distilled H_2O. The

plasmid mixture contained 60 µg of expression vector SIN-LV backbone vector, 39 µg of packaging plasmid pMDLGg/p, 15 µg of transcription plasmid RSV-REV and 21 µg of the envelope plasmid PMD.G. For transfection, 300 µl of 2.5M $CaCl_2$ was added and thoroughly mixed, filtered (0.2 µm filter) with 2.8 ml of 2xHBS, which was added drop-wise under constant vortexing for 2-3 min.

Figure 7 Large-scale production of lentivirus

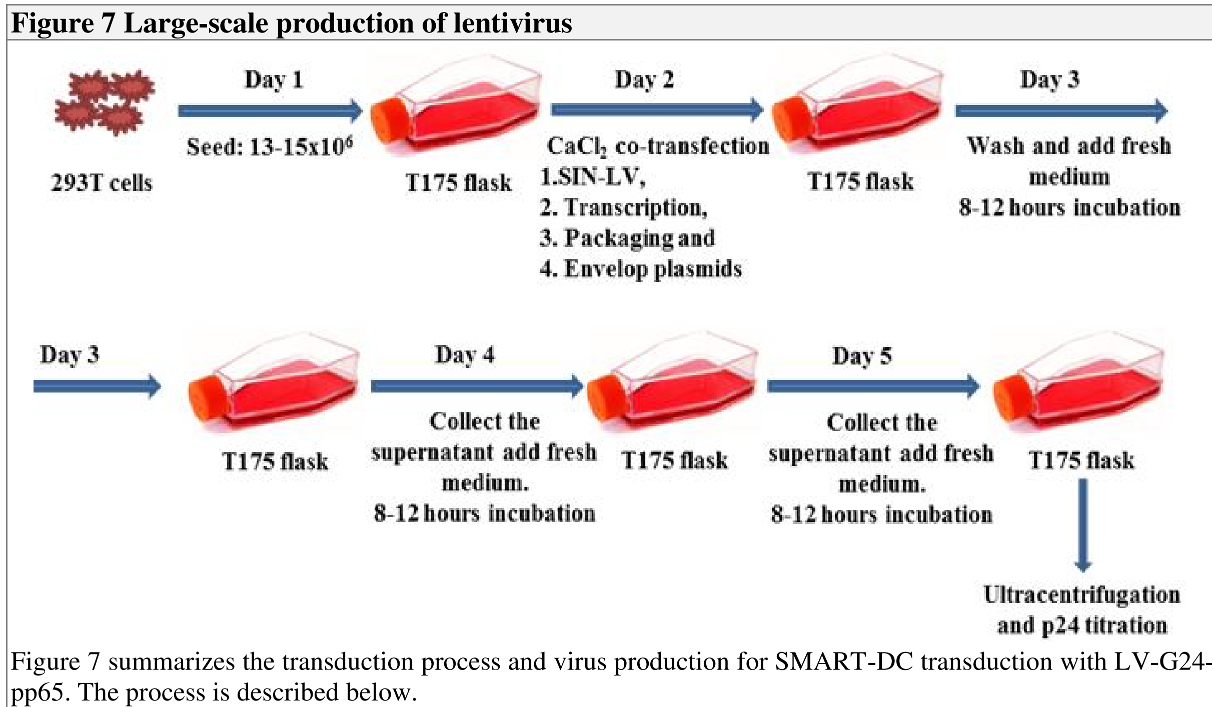


Figure 7 summarizes the transduction process and virus production for SMART-DC transduction with LV-G24-pp65. The process is described below.

Next, 293T cells were transfected with the mixture for 12-15 hours at 37^0C. Subsequently, cells were washed with DMEM and re-supplemented with 40 ml of fresh D10 medium and incubated for additional 8-12 hours at 37^0C. The viral particles were collected in 50 ml conical tubes. This virus production by transient expression of the genes of interest was repeated on the following day and virus particles were harvested. The supernatant was centrifuged at 2000 rpm for 5 min and filtered through a 0.45 µm membrane to remove cellular debris. After the first purification the virus particles were enriched by ultra-centrifugation at 19000 rpm for 2 hours and 20 min with a slow break. The viral pellet was collected and resuspended in 200-350 µl of X-vivo15 medium and left for one hour on a shaker. Subsequently, the enriched viral particles were again centrifuged at 3000 rpm for 10

min to remove any leftover debris. The concentrated viral supernatant was frozen at -80°C and a small aliquot of 30 µl was stored separately for quantifying the lentivirus titer by p24 ELISA.

3.2.8 p24 ELISA to verify correct expression of GM-CSF and IL-4

293T cells ($1x10^5$ per/well) were seeded in a 6-well culture plate with D10 medium and incubated overnight at 37°C supplemented with 5% CO_2. One µg/ml of the SIN-LV was added in the presence of 5 µg of protamine sulphate and incubated overnight at 37 ºC supplemented with 5 % CO_2. Transduced cells were washed twice (D10 medium; 2 x 10ml), supplemented with 1 ml D10 medium and expanded for 48 hours at 37 ºC with 5 % $C0_2$. Both, supernatant and cell lysates were analyzed for proper expression of GM-CSF and IL-4 by ELISA. The transduced cells were harvested and lysed using a lysis buffer (on ice for 15min, mixing thoroughly every 5 min). The cell lysate was centrifuged at1500 rpm for 10 min and the supernatant was collected and stored at -80°C for quantification of CMVpp65 protein expression by western blot.

3.2.9 Verification of GM-CSF and IL-4 by ELISA

The supernatant from 293 T cells was thawed and diluted 1:100 with sample diluent buffer (see section 3.1.1). The diluent (100 µl/well) as well as standards (100 µl/well) were added in duplicates to the primary antibody pre-coated ELISA plate (monoclonal antibody against GM-CSF-I or IL-4-I; 1 µg/ml) and incubated for one hour at room temperature (RT). After washing the plates for five times the secondary biotin-labelled antibody (monoclonal antibody anti-GM-CSF-II/IL-4-II; 1 µg/ml) was added and incubated for another hour at RT. After washing the plates 5 times the detection antibody (100 µl/well of streptavidin-HRP) diluted (1:1000) with diluent buffer was added and incubated for an hour at RT. After further washing steps TMB (100 µl per well) was added, and the plate was incubated for 15 minutes

at room temperature. Upon the color change, reaction was terminated with 100 µl per well of stop solution. The plate was read in an ELISA reader at 450 nm optimal density (OD). The total concentration of cytokines (GM-CSF/IL-4) secreted by the transduced 293T cells was calculated by comparing the standard curve generated by using defined amounts of GM-CSF/ IL-4 in ng/ml.

3.2.10 Verification of the expression of the CMVpp65 protein by western blot

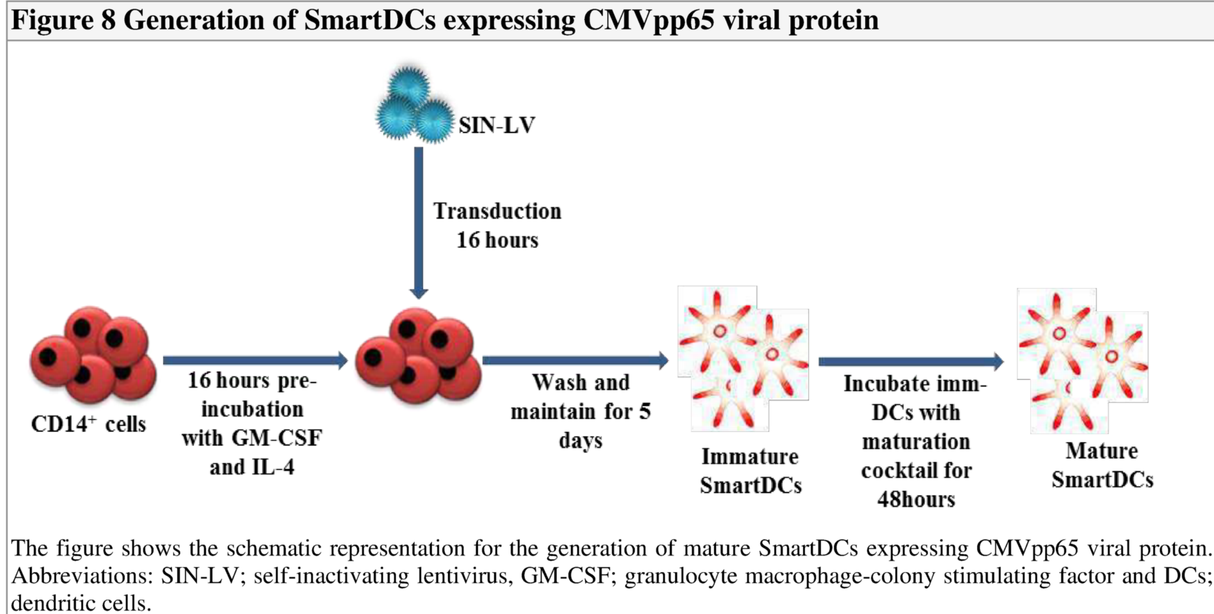
The lysate was thawed and mixed with an equal volume of gel loading dye and denatured at 95ºC for 10 min. The samples were loaded (30 µl) onto 10% SDS polyacrylamide gels and separated at 150 V. Western blotting was done for 1 hour at 350 MW and proteins were transferred to the polyvinylidene difluoride (PVDF) membrane by wet transfer. After blocking the with 5% dry milk (in PBS-0.1 % Tween20), the primary anti-CMVpp65 antibody was diluted was added after 1:1500 dilution and the membrane was incubated on a rocker overnight at 4°C. After washing three times in wash buffer (PBS-0.1 % Tween20) the secondary antibody conjugated to HRP (anti Mouse IgG antibody-HRP) was added (dilution: 1:2000) and incubated for 2hours on rocker at room temperature. The membrane was washed again four times and stained (solutions A and B from the ECL detection kit) and protein bands were detected using chemidoc.

3.2.11 Transduction of $CD14^+$ monocytes with SIN-LV-G24-pp65

$CD14^+$ monocytes were selected as described earlier **(Methods 3.2.3)**. $CD14^+$ cells ($1x10^6$) were expanded in the presence of GM-CSF and IL-4 in 6-well tissue culture plates overnight (8-12 hours at 37°C/5% CO_2 and were subsequently transduced overnight (16 hours at 37 ºC with 5% CO_2) with LV-G24-pp65 (3 µg/ml) in the presence of protamine sulphate (5 µg/ml). Cells were washed twice, replenished with fresh X-Vivo15 medium (1 ml) and cultured for 7 days at 37 ºC with 5 % $C0_2$. On day 5, a maturation cocktail (tumour necrosis factor-alpha;

TNF-α, interleukin-1; IL-1β, interleukin-6; IL-6 and prostaglandin E_2; PGE_2) [94, 95] was added and for another 2 days. On day 7, cells were harvested and used as antigen presenting cells (SMARTpp65) for in vitro expansion of CMV-CTL **(Figure 8)**.

Figure 8 Generation of SmartDCs expressing CMVpp65 viral protein



The figure shows the schematic representation for the generation of mature SmartDCs expressing CMVpp65 viral protein. Abbreviations: SIN-LV; self-inactivating lentivirus, GM-CSF; granulocyte macrophage-colony stimulating factor and DCs; dendritic cells.

To verify the DC phenotype the cells (at least $2x10^5$) were stained with antibodies (**as shown in table 6)** for 30 min at 4 ºC, washed twice with PBS (1500 rpm, 5 min) and resuspended in 200 µl of FACS buffer and analyzed by flow cytometry.

Table 6 Phenotyping of SMART-DCs and /or conv-DC

Antibody Mix 1	**Volume**
Live/Dead	0.2 µl
Note: incubate for 15min at 37˚C for	
Antibody Mix 2	Volume
HLA-DR	5 µl
CD209	8 µl
HLA-A/B/C	1.25 µl
CD14	1.25 µl
CD83	2.5 µl
CD86	2.5 µl
Note: add aliquot of mix 2 directly to the tube and incubate for 30 min at 4°C; wash twice (1500 rpm, 5 min) in 1ml of PBS and acquire.	

The above table shows the staining of dendritic cells with several surface markers.

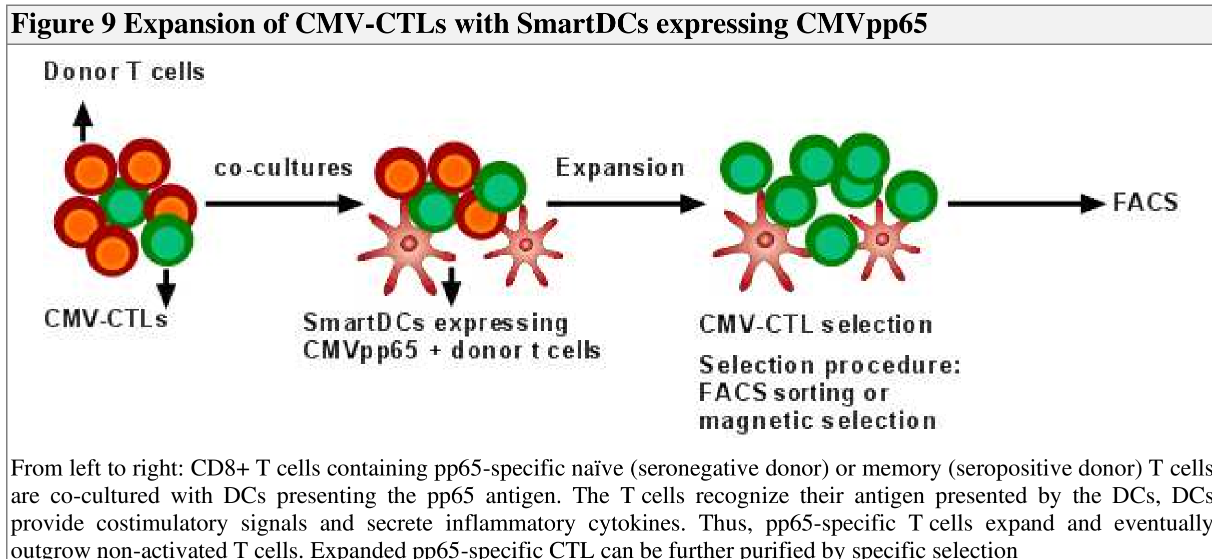
Untransduced $CD14^+$ monocytes cultured for 7 days in the presence of GM-CSF and IL-4 to generate conventional DCs (ConvDC) loaded with/without the CMVpp65 overlapping peptide pool were used as control.

3.2.8 In vitro expansion of CMV-CTLs using conventional or SmartDCs

$CD8^+$ T cells were isolated from PBMC as described earlier **(Methods 3.2.3)** and $CD8^-$ cells were irradiated at 100 Gy and used as feeder cells in 1:3 ratio ($CD8^+$ T cells: feeder cells). Conventional DCs (ConvDCs) and SmartDCs produced as shown in **3.2.7** were used in 1:50 ratio (DCs: T cells) to expand CMV-CTLs (**Figure 9**).

The cells were co-cultured for 7 days (37 °C with 5 % CO_2) along with the addition of IL-2, IL-7 and IL-15 (final cconcentration of cytokines/mL: IL-2; 25 IU, IL-7; 5 ng and IL-15; 5 ng). Fresh medium (X-Vivo15) with cytokines was supplemented every 2 days.

Figure 9 Expansion of CMV-CTLs with SmartDCs expressing CMVpp65



From left to right: CD8+ T cells containing pp65-specific naïve (seronegative donor) or memory (seropositive donor) T cells are co-cultured with DCs presenting the pp65 antigen. The T cells recognize their antigen presented by the DCs, DCs provide costimulatory signals and secrete inflammatory cytokines. Thus, pp65-specific T cells expand and eventually outgrow non-activated T cells. Expanded pp65-specific CTL can be further purified by specific selection

On day 7, $CD8^+$ T cells expanded with ConvDC (loaded with CMVpp65) and SmartDCs expressing pp65 were harvested, stained for T cell surface markers **(Table 7)** for 30 min at 4ºC washed twice with PBS (1500 rpm, 5 min) and resuspended in 200 µl of FACS buffer. Flow cytometry was performed to analyze these resuspended cells.

Table 7 Phenotyping of CMV-CTLs	
Antibody Mix 1	**Volume**
Live/Dead	0.2 µl
CCR7	1.25 µl
Tetramer	5 µl
Note: incubate for 15min at 37˚C for	
Antibody Mix 2	Volume
CD8	0.5 µl
CD3	1.25 µl
CD45RA	1.25 µl
CCR7	1.25 µl
Tim3	2.5 µl
PD1	2.5 µl
CD57	2.5 µl
Note: Add aliquot of mix 2 directly to the tube and incubate for 30 min at 4°C; wash twice (500 x g, 5 min) in 1 ml of PBS and acquire.	

3.2.9 Statistical analysis of patients after HSCT to assess the impact of CMV-R on outcome

For details on the statistical analyses performed on a set of 103 patients, please refer to the attached manuscript **"4.2.2"** in **section b**.

4. Results

In this thesis, we aimed to address the following points:

1. We aimed to assess the presence and functionality of antiviral CTL in stem cell donors and respective grafts. – The results are summarized in section 4.1.1-4.1.3

2. We aimed to assess different in vitro expansion strategies for CMV-CTL. – The results are presented in section 4.1.4-4.1.7

3. We aimed to identify patients in need of adoptive T cells transfer and to determine the influence of CMV-CTL and CMV-R on the outcome after HSCT. – The results of this part are subject of the published manuscript “Cytomegalovirus-specific CD8+ T cells are associated with a reduced incidence of early relapse after allogeneic stem cell transplantation”, attached in section 4.2.

4.1 Generation of CMV-CTLs for adoptive therapy

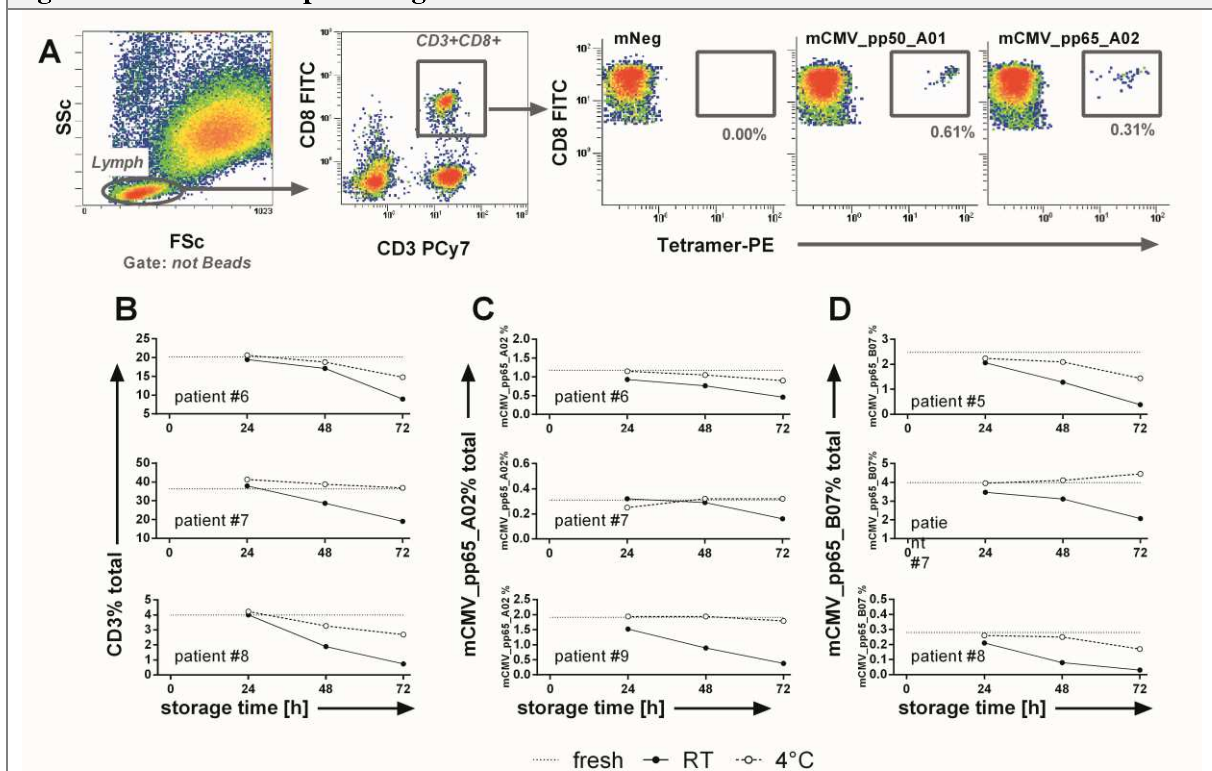
Adoptive therapy using CMV-CTLs to treat high-risk patients (R^+) for recurrent CMV-Rs is currently under investigation[68, 96, 97]. For this reason, the source of CMV-CTL and timing of collection from CMV seropositive (D^+) donors is an important question to date. Therefore we wanted to investigate whether the G-CSF mobilized stem cell donor could be an optimal source for the collection of CMV-CTLs. Thus, in collaboration with the Institute for Transfusion Medicine (Prof. Dr. Britta Eiz-Vesper), we studied the possibility to quantify and enrich CMV-CTLs from peripheral blood from granulocyte-colony stimulating factor (G-CSF) mobilized CMV-seropositive donors.[98]

4.1.1 Effect on CMV-CTLs based on sample storage time and temperature

Routine multimer analysis was performed within 24 hours after the sample acquisition (**Methods 3.2.4**). However, this process is occasionally delayed due to delays in graft

processing and collection of the sample after apheresis. To investigate the effect of storage time and temperature on CMV-CTLs, we selected 9 HSCT patients expressing immunodominant CMVpp65 epitopes on HLA-A02 (n=4) and B07 (n=5). These samples were analyzed for changes in the percentage of CD3 in lymphocytes, mNeg (non-specific background), total percentages of multimer CMV-pp65-HLA-A02 (BC; NLVPMVATV) and multimer CMV-pp65-HLA-B07 (BC; TPRVTGGGAM) over time. Samples stored at 4°C showed fewer variations over time in the percentage of $CD3^{+}$ T cells, and also in specific multimer percentages, up to 48 hours after sample collection **(Figure 10 B-D)**.

Figure 10 Effect of sample storage on CD3 and CMV-CTLs



Representative image of density plots depicting the gating strategy to detect CMV tetramer HLA specific CTLs (A). The course of changes in the percentage of CD3+ T cells (B), CD8+ tetramer CMV-pp65 HLA A02:01 (C) and CMV-pp65 HLA B07:02 (D) are shown at 24, 48, and 72 hours. The whole blood samples stored at 4ºC (open circle), room temperature (filled circle) and fresh sample (dotted horizontal line) of the respective individuals post-SCT are shown. The x-axis (B-D) shows the storage time in hours and the y-axis shows the percentage of CD3+ T cells or CD8+ tetramer CMV-CLs of HLA A02:01or CMV-pp65 HLA B07:02. Abbreviations: h, storage time in hours; Neg, tetramer negative staining; CMV_pp65_A02:01: CD8+ HLA A02:01 specific tetramer staining, and RT, room temperature. Graph taken from[98] Bunse C E, Borchers S, Varanasi PR et al, 2013, Impaired Functionality of Antiviral T Cells in G-CSF Mobilized Stem Cell Donors: Implications for the Selection of CTL Donor.

In comparison to a fresh sample, after 48 hours of storage at 4°C the samples showed a mean of 93% multimer CMV-pp65-A02 (n=4) and 96% multimer CMV-pp65-B07 (n=5). Similar analysis after 72 hours showed high variation in multimer percentages with a mean of 82% multimer CMV-pp65-A02 and 90% multimer CMV-pp65-B07 in comparison to a fresh sample. Moreover, this variation was more pronounced in the samples stored at room temperature for 48 hours (CMV-pp65-A02:57% and CMV-pp65-B07: 68%) and 72 hours (CMV-pp65-A02: 30% and CMV-pp65-B07: 37%) as compared to a fresh sample. In conclusion, we observed that samples stored at 4°C have similar values in the % of $CD3^+$ and CMV-CTLs compared to fresh samples, unlike samples stored at RT.

4.1.2 Influence of G-CSF mobilisation on CMV-CTLs numbers

Next, we studied the influence of G-CSF mobilization on the CMV-CTLs in healthy PBSC donors. These PBSC donors were characterised for HLA type and CMV serostatus. Whole blood, G-CSF mobilized whole blood, apheresis and graft samples were collected from the donors and analyzed to check for variations in multimer-binding **(Table 8).**

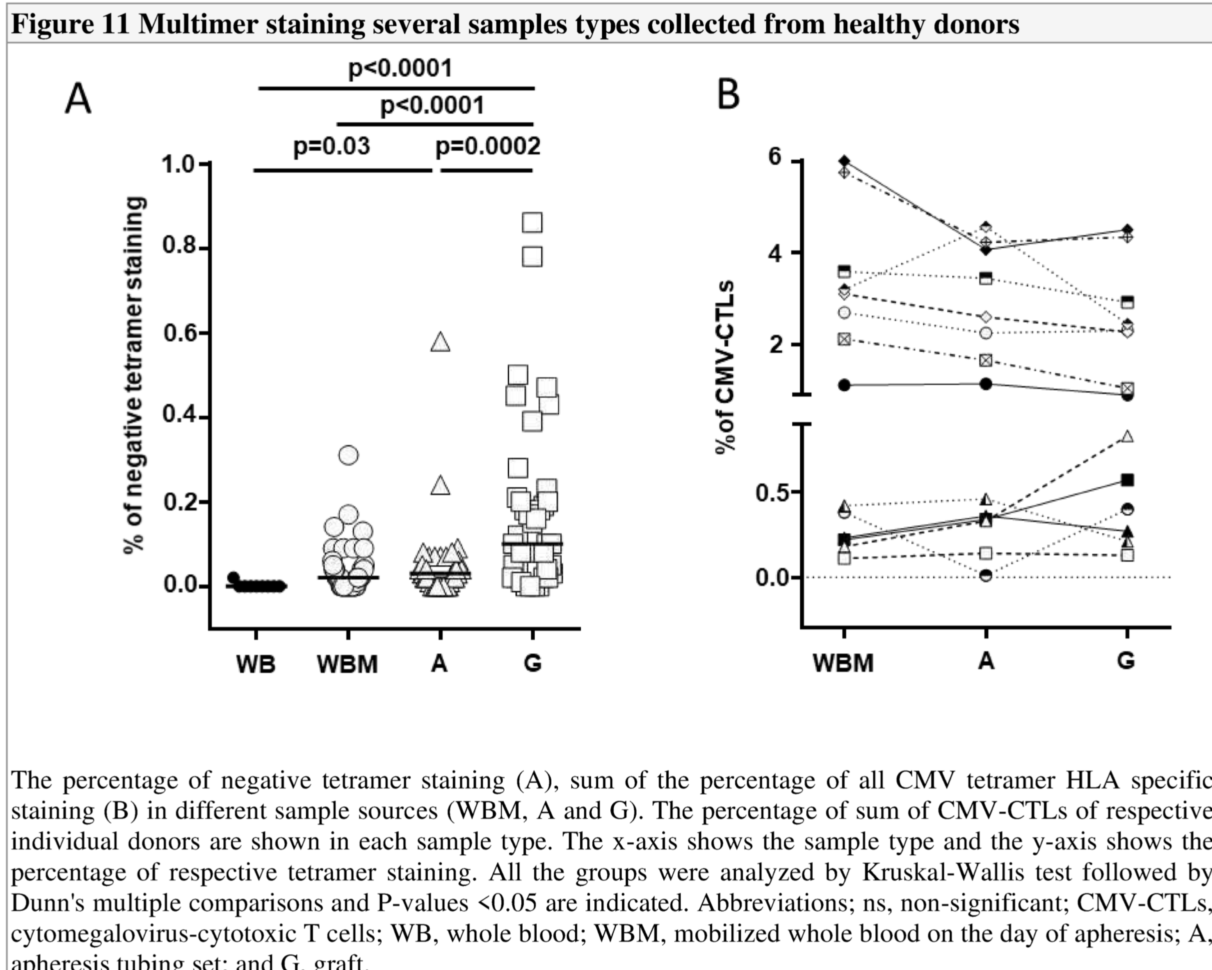
Table 8 Samples monitored for tetramers in healthy donors

Sample type	Total (n)	Tetramer staining	
		Yes	No
Whole blood (WB)	16	9	7
G-CSF Mobilized whole blood (WBM)	58	58	0
Apheresis (A)	157	52	105
Graft (G)	89	39	50
Total	320	158	162

Samples processed from apheresis showed a significant increase in unspecific background in comparison to whole blood samples ($p<0.03$). In a similar analysis, samples processed from grafts also showed a significant increase in the unspecific background in comparison to whole blood ($p<0.0001$), G-CSF mobilized whole blood ($p<0.0001$) and apheresis ($p=0.0002$.

However, after subtraction of background, the sum of percentages of multimer staining (CMV-CTLs) from each specific donor remained within the same range for all sample types **(Figure 11A & 11B).**

Figure 11 Multimer staining several samples types collected from healthy donors



The percentage of negative tetramer staining (A), sum of the percentage of all CMV tetramer HLA specific staining (B) in different sample sources (WBM, A and G). The percentage of sum of CMV-CTLs of respective individual donors are shown in each sample type. The x-axis shows the sample type and the y-axis shows the percentage of respective tetramer staining. All the groups were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons and P-values <0.05 are indicated. Abbreviations; ns, non-significant; CMV-CTLs, cytomegalovirus-cytotoxic T cells; WB, whole blood; WBM, mobilized whole blood on the day of apheresis; A, apheresis tubing set; and G, graft.

4.1.3 Tetramer staining differentiates CMV seronegative and seropositive donors

Additionally, we also validated the specificity of CMV HLA specific tetramers that are commercially available. The total number of donor samples stained for a specific HLA type are shown in table 9. As expected, median levels of tetramer staining in CMV-seropositive donors was significantly higher in tetCMV_pp50_A01:01 (0.52%, p<0.0001), pp65_A02:02 (0.53%, p<0.0001) and pp65_B07:02 (2.46%, p=0.007) than in CMV-seropositive donors **(Figure 12)**. No significant differences in tetramer staining were observed in CMV

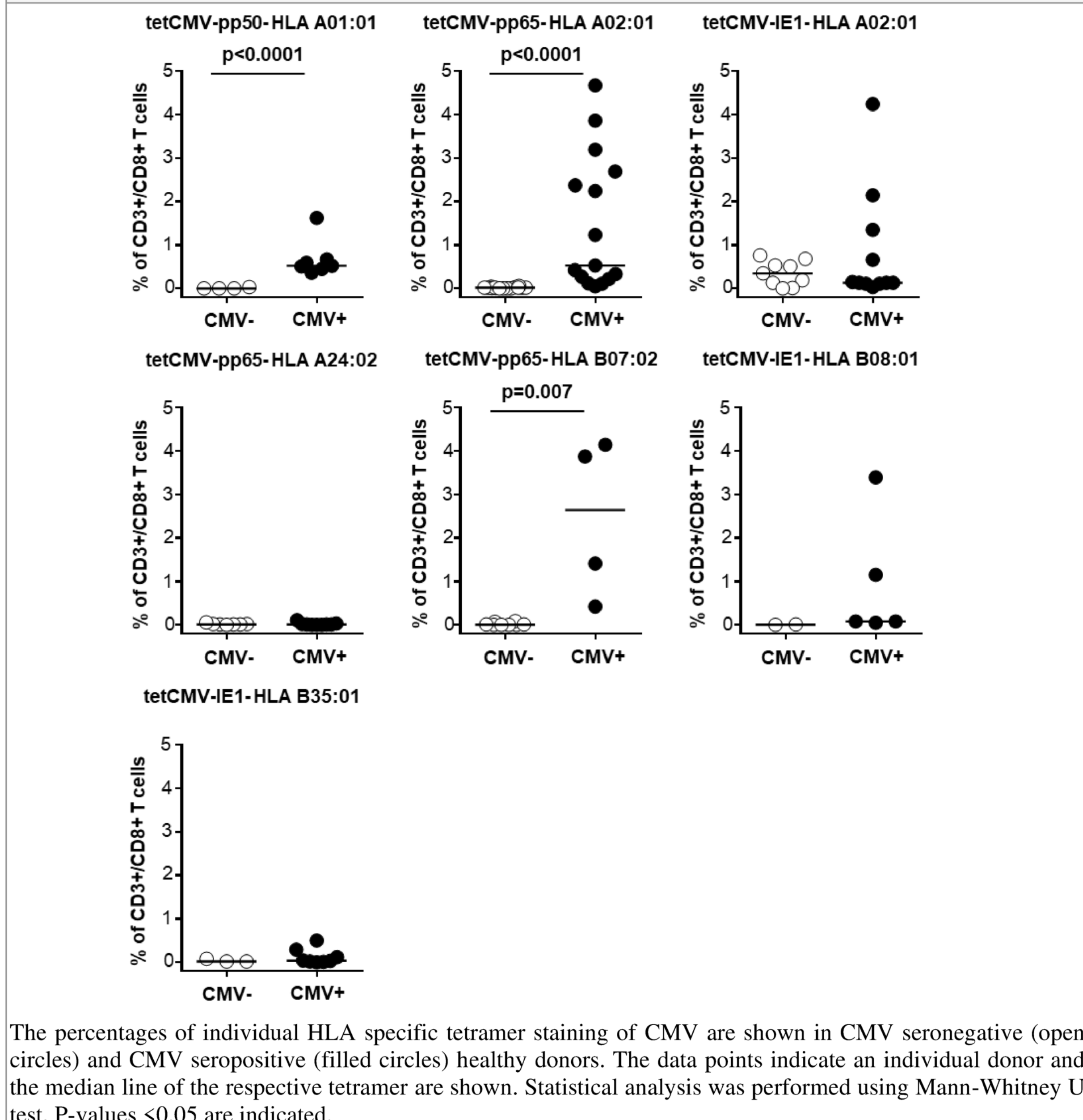
seropositive donors in tetCMV_IE1_A02:01, pp65_A24:02, IE1_B08:01 and pp65_35:01 in comparison to CMV seronegative donors.

Table 9 Tetramer staining in CMV seropositive and seronegative donors

Tetramer	CMV Serostatus	Mean	Median	Number of donors tested
tetCMV_pp50_A01:01	Total	0.43%	0.45%	11
	Positive	0.67%	0.52%	7
	Negative	0.01%	0.00%	4
tetCMV_pp65_A02:01	Total	0.73%	0.05%	31
	Positive	1.48%	0.53%	15
	Negative	0.02%	0.02%	16
tetCMV_IE1_A02:01	Total	0.62%	0.17%	20
	Positive	0.83%	0.13%	11
	Negative	0.35%	0.35%	9
tetCMV_pp65_A24:02	Total	0.02%	0.01%	16
	Positive	0.02%	0.01%	9
	Negative	0.02%	0.01%	7
tetCMV_pp65_B07:02	Total	0.83%	0.04%	12
	Positive	2.46%	2.64%	4
	Negative	0.02%	0.01%	8
tetCMV_IE1_B08:01	Total	0.68%	0.08%	7
	Positive	0.95%	0.08%	5
	Negative	0.01%	0.01%	2
tetCMV_pp65_B35:01	Total	0.10%	0.03%	11
	Positive	0.13%	0.04%	8
	Negative	0.04%	0.02%	3

We further investigated the efficiency of these CMV-CTLs with and without G-CSF treatment in collaboration with the group of Prof. Eiz-Vesper. Interestingly, we observed that G-CSF-treatment impaired the function of T cells, i. e. cytokine secretion and degranulation upon stimulation with CMV immunodominant proteins (pp65 and IE1). Due to this observation, we investigated in vitro methods to expand CMV-CTLs using professional antigen presenting cells; in vitro generated dendritic cells (DCs) loaded with CMV antigens (either in the form of single peptides, peptide mix, recombinant proteins or tumor cell lysates) successfully induced antigen-specific immune responses [99, 100].

Figure 12 Tetramer staining in CMV seropositive and seronegative healthy donors



The percentages of individual HLA specific tetramer staining of CMV are shown in CMV seronegative (open circles) and CMV seropositive (filled circles) healthy donors. The data points indicate an individual donor and the median line of the respective tetramer are shown. Statistical analysis was performed using Mann-Whitney U test, P-values <0.05 are indicated.

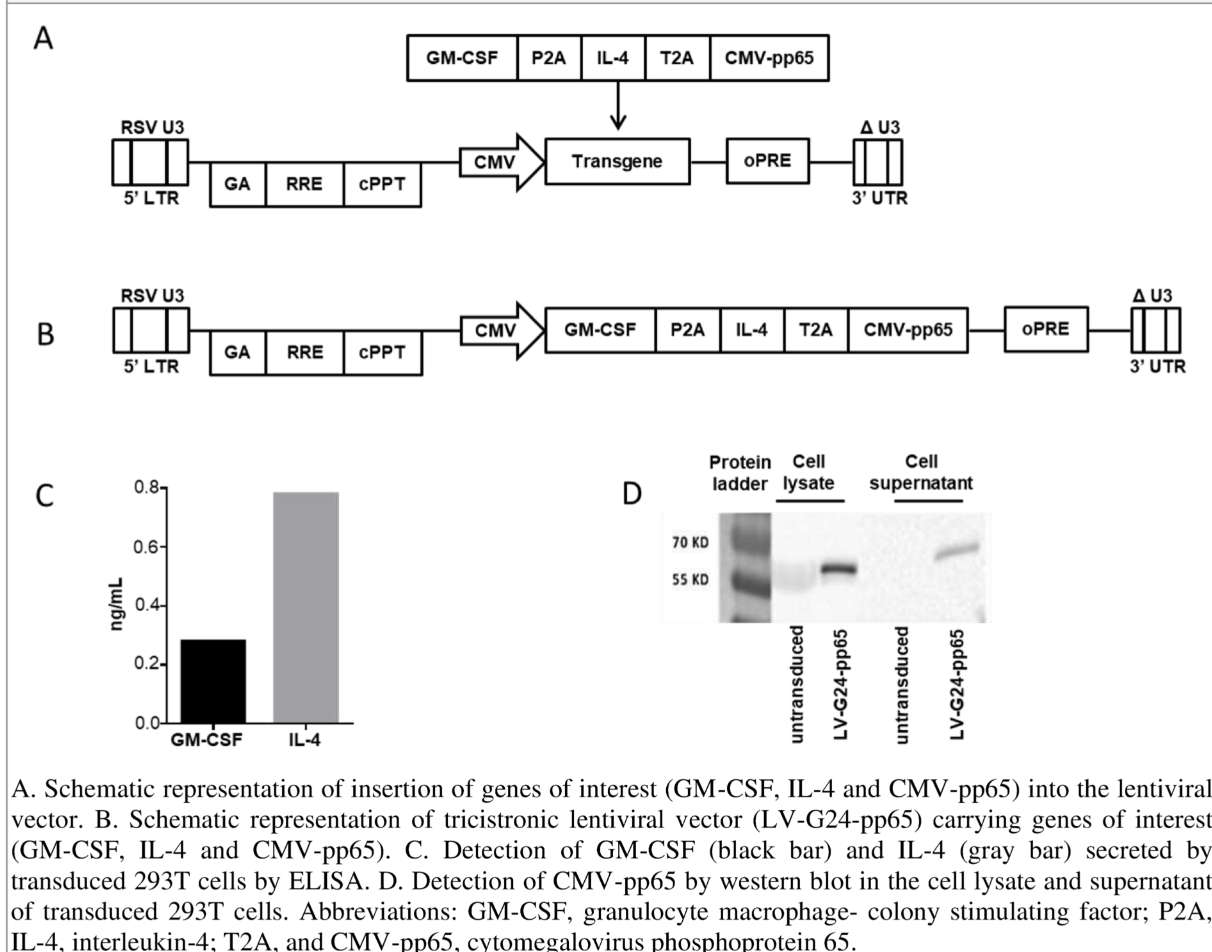
4.1.4 Construction and validation of tricistronic LV vector

A tricistronic lentiviral vector (GM-CSF-P2A-IL-4-T2A-CMVpp65; LV-G242pp65) containing human GM-CSF, IL-4 and pp65 was constructed with the respective 2A elements (Life Technologies®; pMA plasmid). The 2A elements are short peptide sequences of 20 amino acids, which are highly conserved and efficiently involved in the translation of transgenes. 2A elements allow the simultaneous expression of genes by a ‘ribosome

skipping' mechanism that leads to equimolar expression of three proteins [101, 102]. The pMA plasmid containing the three genes and the interspacing 2A elements was digested using restriction enzymes *XbaI*, *ClaI and ScaI to* yield the gene fragment 2733kb. This gene of interest was ligated into the lentiviral vector backbone between *XbaI* and *ClaI* restriction sites under the control of the constitutive CMV-promoter **(Figure 13A & B)**. This vector backbone contains a full-length operator mutated wPRE (Woodchuck hepatitis virus post-transcriptional regulatory element), oPRE which ensured the safety of 3rd generation SIN-LV[102]. The gene integration into the LV backbone was confirmed in transformants selected using ampicillin resistance, by examination of the DNA cleavage pattern following treatment with restriction enzymes to ensure the gene integration into the vector. Of the four clones selected, two clones showed the desired restriction pattern. These positive clones were sequenced by Seqlab (Göttingen) and were found to contain the insert in the correct orientation when analyzed using Clone Manager Software.

The validation of the transgene expression was performed in 293T cells. 50,000 293T cells were transduced (in duplicates) with LV-G242pp65 (1 µg/mL) in a six-well plate in the presence of protamine sulphate (5µg/mL) and incubated for 16 hours at 37ºC & 5% CO_2. After the incubation, cells were washed twice and supplemented with D10 medium and cultured for 3 days at 37ºC with 5% CO_2. Non-transduced 293T cells served as a negative control. After the stipulated incubation period, supernatants were harvested from both transduced and non-transduced 293T cells. Harvested supernatants showed high levels of secreted cytokines (GM-CSF: 0.28 ng/mL and IL-4: 0.78 ng/mL) compared to the negative controls and CMVpp65 transgene expression was detected in cell lysates by Western blot (**Figure 13C & D**).

Figure 13 Construction and validation of tricistronic lentiviral vector



A. Schematic representation of insertion of genes of interest (GM-CSF, IL-4 and CMV-pp65) into the lentiviral vector. B. Schematic representation of tricistronic lentiviral vector (LV-G24-pp65) carrying genes of interest (GM-CSF, IL-4 and CMV-pp65). C. Detection of GM-CSF (black bar) and IL-4 (gray bar) secreted by transduced 293T cells by ELISA. D. Detection of CMV-pp65 by western blot in the cell lysate and supernatant of transduced 293T cells. Abbreviations: GM-CSF, granulocyte macrophage- colony stimulating factor; P2A, IL-4, interleukin-4; T2A, and CMV-pp65, cytomegalovirus phosphoprotein 65.

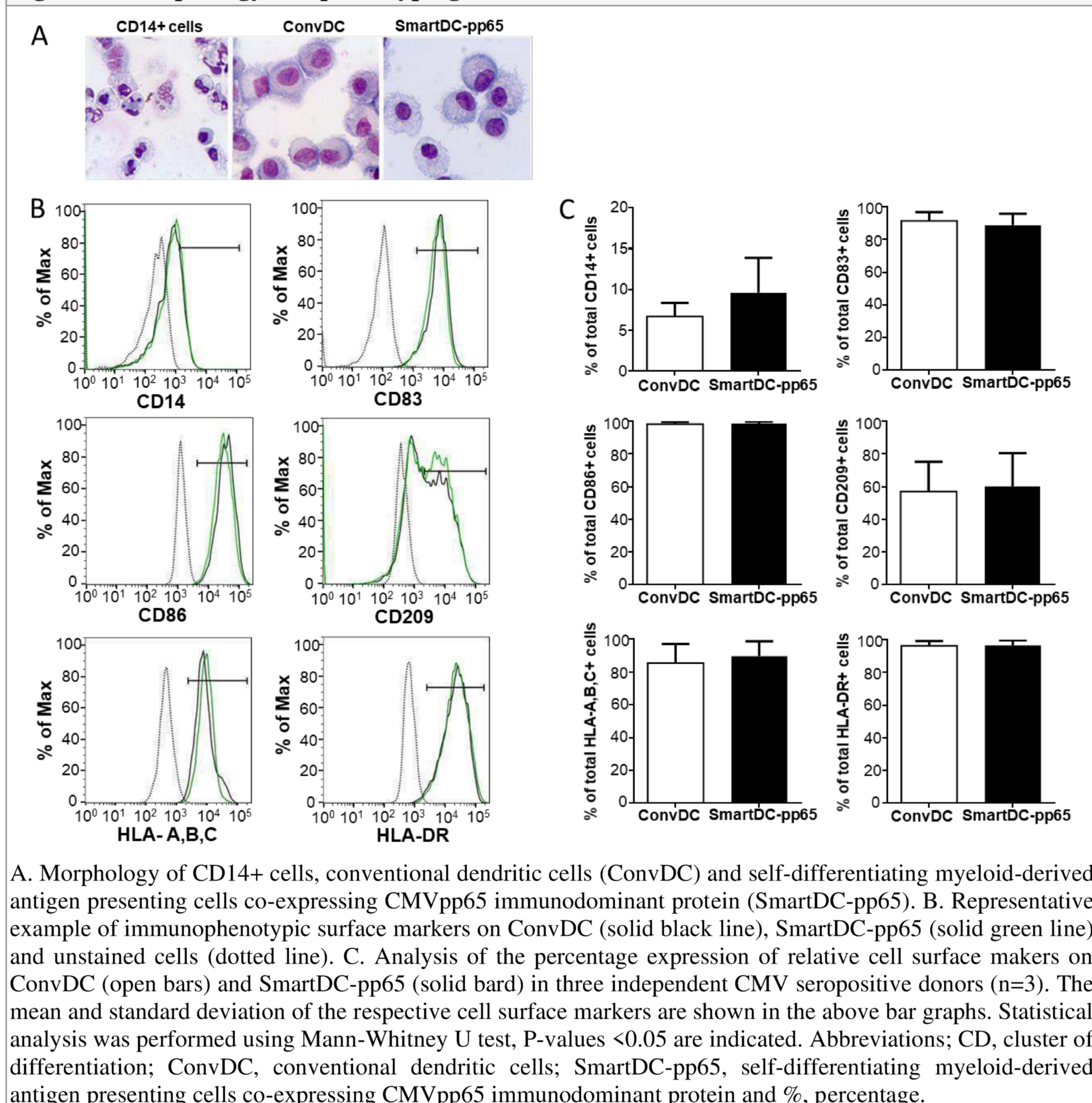
4.1.5 Characterization of SmartDCpp65 and in vitro expanded CMV-CTLs

To generate CMV-CTLs in vitro, we produced the LV-G242pp65 in large scale and transduced the hematopoietic precursors. $CD14^+$ monocytes were isolated by magnetic separation **(Methods 3.2.3)** from 3 healthy CMV-seropositive donors with high purity (>98 %). $5x10^6$ $CD14^+$ cells were pre-conditioned with GM-CSF, IL-4 for 8 hours (50 ng/mL of each cytokine) and transduced with 5 µg/mL of LV-G242pp65 for 16 hours in the presence of protamine sulphate.

After the incubation, virus was removed, and cells were washed twice with X-Vivo15 medium without detaching the cells from the well. Cells were then cultured for 7 days in X-Vivo 15 without exogenous addition of cytokines.

GM-CSF and IL-4 secretion from the transduced cells will lead to the self-differentiation of the CD14^{+} monocytes towards dendritic cells. These self-differentiated DCs will express CMV-pp65. The transgene expression was confirmed by ELISA and Western Blot **(Figure 13C & 13D)** in 293T cells.

Figure 14 Morphology and phenotyping of SmartDCs



A. Morphology of CD14+ cells, conventional dendritic cells (ConvDC) and self-differentiating myeloid-derived antigen presenting cells co-expressing CMVpp65 immunodominant protein (SmartDC-pp65). B. Representative example of immunophenotypic surface markers on ConvDC (solid black line), SmartDC-pp65 (solid green line) and unstained cells (dotted line). C. Analysis of the percentage expression of relative cell surface makers on ConvDC (open bars) and SmartDC-pp65 (solid bard) in three independent CMV seropositive donors (n=3). The mean and standard deviation of the respective cell surface markers are shown in the above bar graphs. Statistical analysis was performed using Mann-Whitney U test, P-values <0.05 are indicated. Abbreviations; CD, cluster of differentiation; ConvDC, conventional dendritic cells; SmartDC-pp65, self-differentiating myeloid-derived antigen presenting cells co-expressing CMVpp65 immunodominant protein and %, percentage.

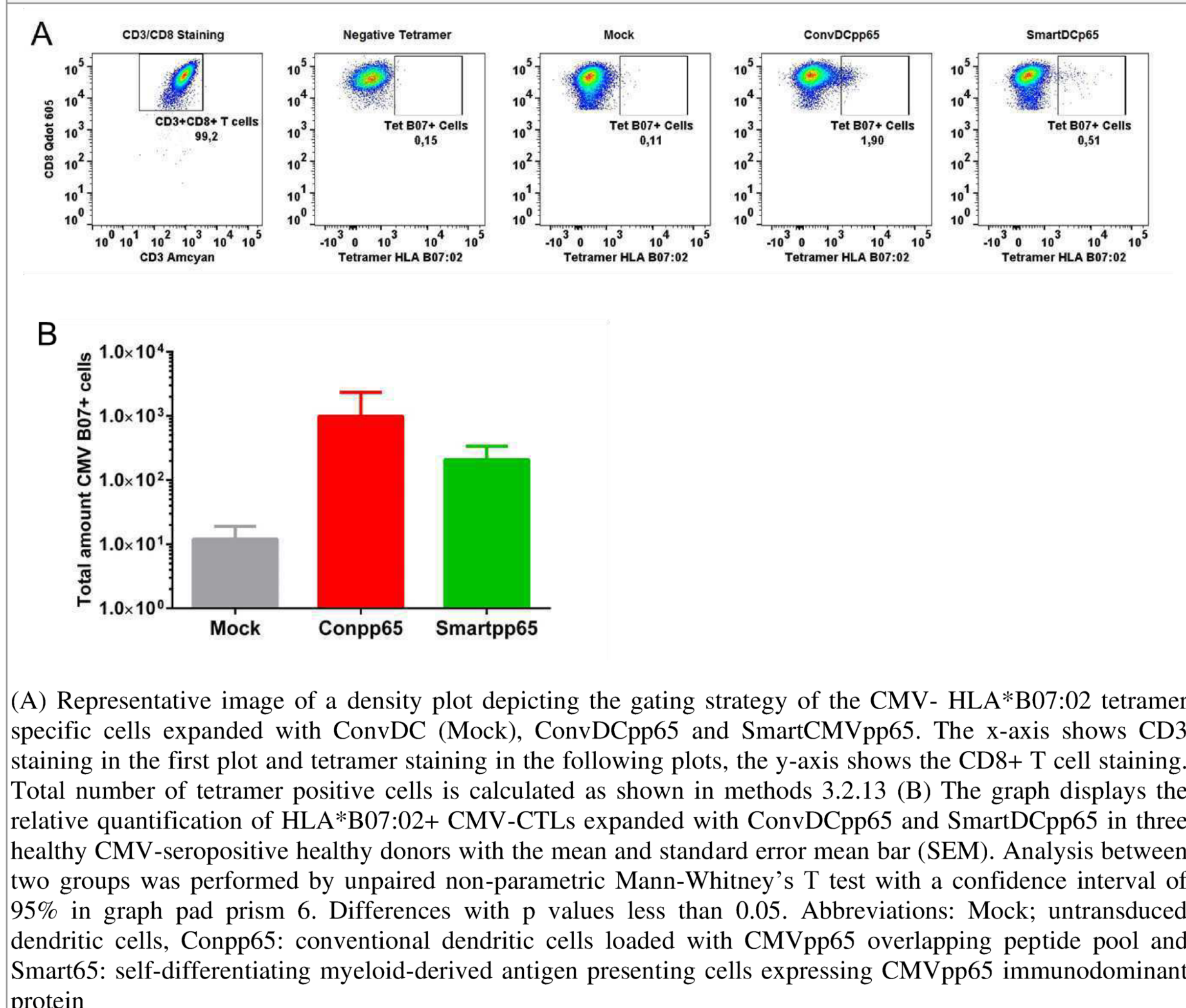
In parallel, conventional DCs (ConvDCs) were generated by culturing CD14^{+} monocytes in the presence of recombinant cytokines, GM-CSF and IL-4 (50 ng/mL each cytokine) for 7 days by supplementing the cytokines every 2-3 days. Both SmartDCs and the ConvDCs were evaluated by microscopy for the morphology and changes in the expression of dendritic cell

surface markers by flow cytometry. ConvDCs served as controls in these experiments. SmartDCpp65 without exogenous addition of cytokines showed typical DC cell morphology (dendrites) and were comparable to the ConvDCs cultured in the presence of recombinant cytokines GM-CSF and IL-4 **(Figure 14A)**. Moreover, both ConvDCs and SmartDCpp65 showed down-regulation of the monocytic marker CD14 and up-regulation of DC markers such as CD209, HLA-ABC (MHC-I) and HLA-DR (MHC-II) and upregulation of co-stimulatory markers CD83 and CD86. There was no significant difference observed in three independent experiments between the ConvDCs and SmartDCpp65 in the expression of DC surface markers and co-stimulatory markers **(Figure 14B & C)**, demonstrating that the DC generation using the LV-G242pp65 was efficient.

4.1.6 Comparison of CMV-CTL expansion using SmartDCs and ConvDCs

Having generated the SmartDCs efficiently, we next evaluated the expansion of CMV-specific CTLs in vitro and further characterised the in vitro generated CMV-CTLs. For this purpose, we co-cultured the SmartDCpp65 or ConvDCs with autologous $CD8^+$ T cells magnetically isolated from the PBMCs of healthy CMV-seropositive donors (n=3) HLA typed for HLA-B*07:02. One million autologous $CD8^+$ T cells were co-cultured with SmartDC co-expressing pp65, or with ConvDCs, either untreated or loaded with CMV-pp65 peptide mix, in a 50:1 ratio ($CD8^+$ T cells to DCs) for 7 days in X-Vivo 15 medium supplemented with recombinant IL-2 (25 U/mL), IL-7 (5 ng/mL) and IL-15 (5 ng/mL) in a 24 well plate. Irradiated $CD14^-CD8^-$ cells served as feeder cells for the co-cultures. Every two days, cultures were supplemented with fresh medium (X-vivo 15) containing the above-mentioned cytokines. After 7 days $CD8^+$ T cells were harvested and analyzed for the expansion of antigen specific T cells using a CMV-specific tetramer (HLA-B*07:02, Beckmann Coulter), memory phenotypes and the expression of markers associated with senescence (CD57) and T cell exhaustion (PD1 and Tim3).

Figure 15 Antigen-specific T cell expansion using ConvDCpp65 and SmartDCpp65



(A) Representative image of a density plot depicting the gating strategy of the CMV- HLA*B07:02 tetramer specific cells expanded with ConvDC (Mock), ConvDCpp65 and SmartCMVpp65. The x-axis shows CD3 staining in the first plot and tetramer staining in the following plots, the y-axis shows the CD8+ T cell staining. Total number of tetramer positive cells is calculated as shown in methods 3.2.13 (B) The graph displays the relative quantification of HLA*B07:02+ CMV-CTLs expanded with ConvDCpp65 and SmartDCpp65 in three healthy CMV-seropositive healthy donors with the mean and standard error mean bar (SEM). Analysis between two groups was performed by unpaired non-parametric Mann-Whitney's T test with a confidence interval of 95% in graph pad prism 6. Differences with p values less than 0.05. Abbreviations: Mock; untransduced dendritic cells, Conpp65: conventional dendritic cells loaded with CMVpp65 overlapping peptide pool and Smart65: self-differentiating myeloid-derived antigen presenting cells expressing CMVpp65 immunodominant protein

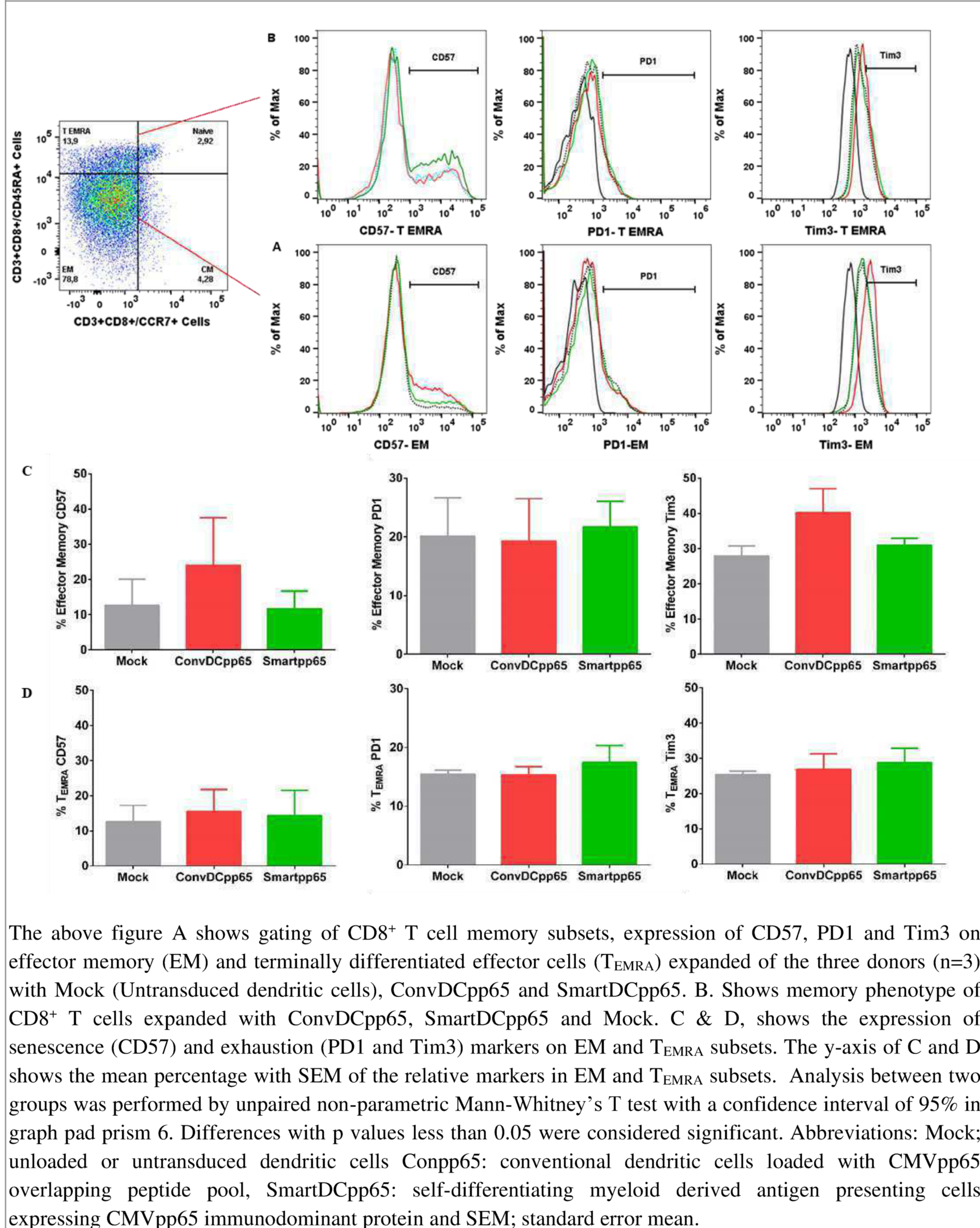
T cell expansion analyzed by quantification of relative cell numbers showed that SmartDCpp65 were comparable to ConvDCs loaded with pp65 peptide mix. Analyzing the CMV-CTL expansion, we observed that both ConvDCpp65 (peptide mix) and SmartDCpp65 were equally efficient, however both groups showed significantly higher CMV-CTL expansion after one-week stimulation compared to ConvDCs alone **(Figure 15B)**. In conclusion, the expansion of $CD8^+$/ HLA*B07:02 CMV-CTLs was similar in three healthy CMV-seropositive donors stimulated with ConvDCpp65 and SmartDCpp65.

4.1.7 Distribution of memory phenotyping and expression of markers associated with senescence (CD57) and exhaustion (PD1 and Tim3) on memory subsets.

Also, we investigated the memory phenotype of $CD8^+$ T cells expanded with ConvDCpp65 and Smartpp65. Expanded $CD8^+$ T cells were stained with CCR7 and CD45RA to distinguish naïve (N; $CD45RA^+$ $CCR7^+$), central memory (CM; $CD45RA^-CCR7^+$), effector memory (EM; $CD45RA^-CCR7^-$) and terminal effector memory (T_{EMRA}; $CD45RA^+CCR7^-$) cells. We observed no significant differences in the memory subsets of $CD8^+$ T cells (naïve, central memory, effector memory, and terminally differentiated effector cells) expanded with ConvDCpp65 and Smartpp65 **(Figure 16B)**.

Next, we investigated the expression of markers associated with senescence (CD57) and exhaustion (PD1 and Tim3) on EM and T_{EMRA} subsets of $CD8^+$ T cells expanded with Smartpp65 compared to ConvDCpp65 **(Figure 16C)**. No significant differences were observed in the expression of CD57, PD1, and Tim3 on EM and T_{EMRA} $CD8^+$ T cells expanded with ConvDCpp65 and SmartDCpp65 (**Figure 16D**). In conclusion, the expansion of $CD8^+$/ T cells lead to the expression of similar levels of senescence and exhaustion in three healthy CMV-seropositive donors expanded with ConvDCpp65 and SmartDCpp65.

Figure 16 Memory Phenotyping of CD8+ T cells expanded with ConvDCpp65 and SmartDCs



The above figure A shows gating of CD8⁺ T cell memory subsets, expression of CD57, PD1 and Tim3 on effector memory (EM) and terminally differentiated effector cells (T_{EMRA}) expanded of the three donors (n=3) with Mock (Untransduced dendritic cells), ConvDCpp65 and SmartDCpp65. B. Shows memory phenotype of $CD8^+$ T cells expanded with ConvDCpp65, SmartDCpp65 and Mock. C & D, shows the expression of senescence (CD57) and exhaustion (PD1 and Tim3) markers on EM and T_{EMRA} subsets. The y-axis of C and D shows the mean percentage with SEM of the relative markers in EM and T_{EMRA} subsets. Analysis between two groups was performed by unpaired non-parametric Mann-Whitney's T test with a confidence interval of 95% in graph pad prism 6. Differences with p values less than 0.05 were considered significant. Abbreviations: Mock; unloaded or untransduced dendritic cells Conpp65: conventional dendritic cells loaded with CMVpp65 overlapping peptide pool, SmartDCpp65: self-differentiating myeloid derived antigen presenting cells expressing CMVpp65 immunodominant protein and SEM; standard error mean.

4.2. Manuscript, PLoS One. 2019 Mar 19, PMID30889204:

Cytomegalovirus-specific CD8^{+} T cells are associated with a reduced incidence of early relapse after allogeneic stem cell transplantation

Short title: Reduced incidence of early relapse in patients with CMV-CTLs

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ABSTRACT

Leukemia relapse is the main cause for mortality after allogeneic stem cell transplantation (allo-SCT). Donor-derived allo-immune responses eliminate the residual host hematopoiesis and protect against relapse. Cytomegalovirus (CMV) reactivation (CMV-R) after allo-SCT may trigger anti-leukemic effects. The impact of CMV-specific CD8+ T-cells (CMV-CTLs) on the outcome after allo-SCT is currently unknown. Here, we studied the relationship between CMV-CTLs, overall T-cell reconstitution and relapse incidence in 103 patients with acute leukemia (n=91) or myelodysplastic syndrome (n=12) following CMV-seropositive recipient/donor (R+/D+) allo-SCT. Patients were subdivided based on the presence or absence of CMV-CTLs at 3 months after allo-SCT. Presence of CMV-CTLs was associated with preceding CMV-R and a fast T-cell reconstitution. Univariate analysis showed a significantly lower 1-, 2- and 5-year cumulative incidence of relapse (CIR) in patients with CMV-CTLs compared to those without CMV-CTLs. Multivariable regression analysis of the outcome performed with other relevant parameters chosen from univariate analysis revealed that presence of CMV-CTLs and chronic graft-versus-host disease (cGvHD) were the only

independent factors associated with a low CIR. Onset of relapse was significantly later in patients with CMV-CTLs (median 489 days) than in in those without (median 152 days, p=0.041) during a five-year follow-up. Presence of CMV-CTLs was associated with a lower incidence of early relapses (1 and 2-years), while cGvHD lead to a lower incidence of late relapses (2 to 5-years). In conclusion, our data show that CMV-CTLs indicate a functional immune-reconstitution protective against early relapse.

4.2.1 Introduction

Relapse is the main cause for mortality after allogeneic stem cell transplantation (allo-SCT) in patients with acute leukemia and myelodysplastic syndrome (MDS) [103]. An adverse disease status [104, 105], unfavorable cyto- and molecular-genetics [106, 107] or reduced intensity conditioning (RIC) [108] are major disease or transplant related risk factors for relapse after allo-SCT. The immune-mediated graft-versus-leukemia (GvL) effect after allo-SCT is often associated with the occurrence of graft-versus-host disease (GvHD) [103]. Chronic but not acute GvHD has been shown to be protective against relapse of acute leukemia [109] and myelodysplastic syndrome (MDS) [110]. The exact mechanisms driving the allo-immune responses responsible for the GvL effect and for GvHD are still unknown.

Recent studies provide increasing evidence that cytomegalovirus (CMV) influences allo-immune responses after allo-SCT. CMV reactivation (CMV-R) has been described to boost the overall T-cell reconstitution [111, 112] and to be associated with GvHD [113, 114]. However, the impact of CMV-R on the protection against relapse is still highly controversial. While some studies demonstrated a reduced leukemia relapse risk in patients after CMV-R [115-117], others reported no impact of CMV-R on the relapse incidence after allo-SCT [118-120]. Nevertheless, two recent studies showed that host chimerism is considerably influenced by the CMV specific donor immunity. CMV-seropositive patients showed lower host chimerism levels subsequent to reduced intensity conditioning (RIC) when transplanted from a CMV-

seropositive (R+/D+) as compared to CMV seronegative donors (R+/D-) [121]. Moreover, we have shown recently that patients transplanted in the CMV R+/D+ setting after RIC have a faster overall T-cell reconstitution and lower host chimerism levels in the presence of CMV-CTLs at 3 months after allo-SCT [122]. Persistence of complete donor chimerism is an important indicator for complete remission (CR) after allo-SCT [123, 124]. Since alloreactive T-cells are responsible for the conversion to complete donor chimerism [125], our data provided first evidence that CMV-CTLs may not only trigger the reconstitution of T-cells but also allo-immune responses in the CMV R+/D+ setting. To date, it is unclear whether this proposed effect of CMV-CTLs on allo-reactivity also translates in a reduced relapse incidence in the long-term follow-up of patients.

Here, we studied the relationship between CMV-CTLs, overall T-cell reconstitution and relapse incidence in patients with acute leukemia or MDS after allo-SCT in the CMV R+/D+ setting.

4.2.2 Patients, materials and methods

a. Patient cohort

All CMV-seropositive patients transplanted with a T-cell replete graft of a CMV-seropositive donors between May 2006 and December 2014 at the Hannover Medical School were eligible for this study. Myeloablative conditioning (MAC) regimens were based on busulfan (Bu, n=17) and total body irradiation (TBI, n=16). Reduced intensity conditioning (RIC) was preceded by additional anti-leukemic treatment in 40 patients using FLAMSA [126] (n=28) or ClArac [127] (n=12). RIC protocols comprised busulfan (n=27), melphalan (n=23) and TBI (n=20), based protocols. T-cell depletion was achieved either with antithymocyte globulin (ATG-F (n=68, Fresenius Biotech, Gräfelfing, Germany) or Thymoglobulin (n=24, Genzyme, Naarden, The Netherlands). Patients were typed for 10 HLA alleles on high

resolution level for exon 2+3 for HLA-A, B, C and for exon 2 for HLA-DRB1 and -DQB1 according to the current European Federation for Immunogenetics guidelines. Donors typed on high resolution level were considered HLA-matched, if they were identical for 10/10 HLA-alleles to the respective patients. Sibling donors were considered HLA-matched also when identical for HLA-DRB1 and -DQB1 typed on high resolution level and phenotypically identical for HLA-A and –B. Patients receiving haploidentical or cord blood ALLO-SCT were excluded from the study. ALLO-SCT protocols were approved by the Institutional Review Board of Hannover Medical School. Informed consent was obtained in writing from all patients in accordance with the Helsinki declaration. The analysis was approved by the Institutional Review Board of the Hannover Medical School (1886-2013 and 2934-2015).

b. T-cell monitoring

T-cell immune reconstitution was monitored in peripheral blood samples at 1, 2 and 3 months after allo-SCT as previously described [122]. Briefly, fresh whole blood was stained with anti-CD3, anti-CD8 and either anti-CD4 antibodies (all from Beckman Coulter, Marseille, France) or one of 6 commercially available HLA/CMV tetramers (HLA-A*01:01 pp50-VTEHDTLLY; HLA-A*02:01 pp65-NLVPMVATV; HLAA*24:02 pp65-QYDPVAALF; HLA-B*07:02 pp65-TPRVTGGGAM; HLA-B*08:01 IE1-ELRRKMMYM; HLA-B*35:01 pp65-IPSINVHHY, MBL International, Woburn, USA). The tetramers containing the A245V mutation in the HLA class I heavy chain α3 domain were selected due to their reduced background staining [128]. The HLA-A*02:01/negative tetramer loaded with a proprietary non-antigen related peptide (PE, MBL) was used as negative control. After tetramer staining at room temperature (RT) for 30 mins, erythrocyte lysis was performed as previously described[90]. After standardization using calibration beads, samples were acquired on a FC500 flow cytometer (Beckman Coulter). Fluorescent beads (FlowCount Fluorospheres™, Beckman Coulter) were used to determine absolute T-cell numbers. CMV-

CTL numbers for every tetramer were calculated: CMV-tetramer binding T-cells minus negative-control-tetramer binding T-cells. The CMV-CTL levels were calculated as mean of CMV-CTL counts obtained for each tetramer used. CXP software (Beckman Coulter) was used for FACS.

c. Clinical parameters and events

Advanced (in contrast to standard) disease status was defined as acute myeloid leukemia (AML) beyond first cytological remission or persistent disease after second induction therapy, acute lymphoblastic leukemia (ALL) beyond first cytological / molecular remission or persistent disease after second induction therapy, high-risk myelodysplastic syndrome (MDS) (IPSS higher than intermediate-2) and CML blast crisis. **Adverse (in contrast to standard) cyto- and molecular genetics** was defined for AML according to the ELN adverse risk [129], for ALL by the presence of t(9;22) or t(4;11) or a complex karyotype (≥ 3 anomalies), for MDS by the presence of a complex karyotype or chromosome 7 anomalies and for bcr-abl positive CML by the presence of additional molecular abnormalities. **Relapse** was defined as detection of leukemic blasts in the peripheral blood or of more than 5% blasts in the bone marrow, as detection of multi-lineage dysplasia in the bone marrow (in cases of MDS or unexplained by concurrent medication) or as extramedullary disease manifestation. None of the patients had relapse prior to the month 3 measurement of CMV-CTLs. **CMV reactivation** was detected by monitoring of peripheral blood samples for CMV-DNA during aplasia followed by measurement of CMV-pp65 antigen in leukocytes [90]. CMV reactivation was defined as 1) CMV-DNA load increase by more than 0.5 log levels above the baseline, 2) more than 5 pp65 antigen positive cells per $4x10^5$ leukocytes in a single test or more than 2 pp65 antigen positive cells per $4x10^5$ leukocytes in 2 consecutive tests. CMV reactivation was preemptively treated first line with ganciclovir and second line with foscarnet. **Acute GvHD**

(aGvHD) was graded according to the Glucksberg score [45]. **Chronic GvHD** (cGvHD) was diagnosed and staged according to the Seattle criteria [130].

d. Statistical analysis

Major study endpoints were overall survival (OS), disease-free survival (DFS), NRM (non-relapse mortality) or cumulative incidence of relapse (CIR). Kaplan-Meier curves were used to estimate the probability of OS, DFS and the curves were compared by the log-rank test [131]. Time to death after allo-SCT was considered as an event for OS and time to death or time to relapse was considered as an event for DFS. The CIR and NRM were compared by Gray´s test in a competing risk setting [132]. For relapse, NRM was considered as a competing risk factor and vice versa. The categories reaching a p-value below 0.05 were included in the multivariable Cox proportional hazards regression model. Potential factors affecting OS and DFS outcomes were identified by multivariable analyses using Cox proportional hazards regression models [133]. The NRM and CIR were estimated by the proportional sub distribution hazard regression model of Fine and Gray [134]. Continuous variables were analyzed by Mann-Whitney U test. Statistical analysis was performed using the Statistical Program for Social Science (SPSS version 23, IBM, New York, USA), and EZR [135] on Rcommander (R-software ver. 3.4.1, http://www.R-project.org). A p-value below 0.05 was considered statistically significant. Figures on T-cell reconstitution were prepared with GraphPad Prism 6 (California, USA).

4.2.3 Results

Patient cohort

Patients (n=103) without relapse until month 3, with data on CMV-CTL recovery at month 3 and a minimum follow-up of 2 years after allo-SCT were included in this analysis.

Manuscript Table 1. Patient characteristics.

	CMV-CTL neg. n=16	CMV-CTL pos. n=87	p value
	No. (%)	No. (%)	
Median patient age (range)	59 (22-72)	52 (19-70)	0.202
Median donor age (range)	44 (16-58)	39 (20-68)	0.588
Recipient gender			0.585
Male	11 (69)	52 (60)	
Female	5 (31)	35 (40)	
Diagnosis			0.330
AML	10 (62.5)	64 (73.6)	
ALL	2 (12.5)	13 (15)	
MDS	3 (19)	9 (10.3)	
Others*	1 (6)	1 (1.1)	
Disease status			0.588
standard	8 (50)	51 (59)	
advanced	8 (50)	36 (41)	
Cyto- and molecular genetics			0.782
standard	9 (56)	54 (62)	
high risk	7 (44)	33 (38)	
Stem cell source			0.497
PBSC	15 (94)	84 (97)	
BM	1 (6)	3 (3)	
Donor type			0.695
MRD	3 (19)	25 (29)	
MUD	11 (69)	48 (55)	
MMUD	2 (12)	14 (16)	
Conditioning			1.00
MAC	5 (31)	28 (32)	
RIC	11 (69)	59 (68)	
GvHD prophylaxis			0.755
CSA/MMF	13 (81)	64 (74)	
CSA/MTX	3 (19)	23 (26)	

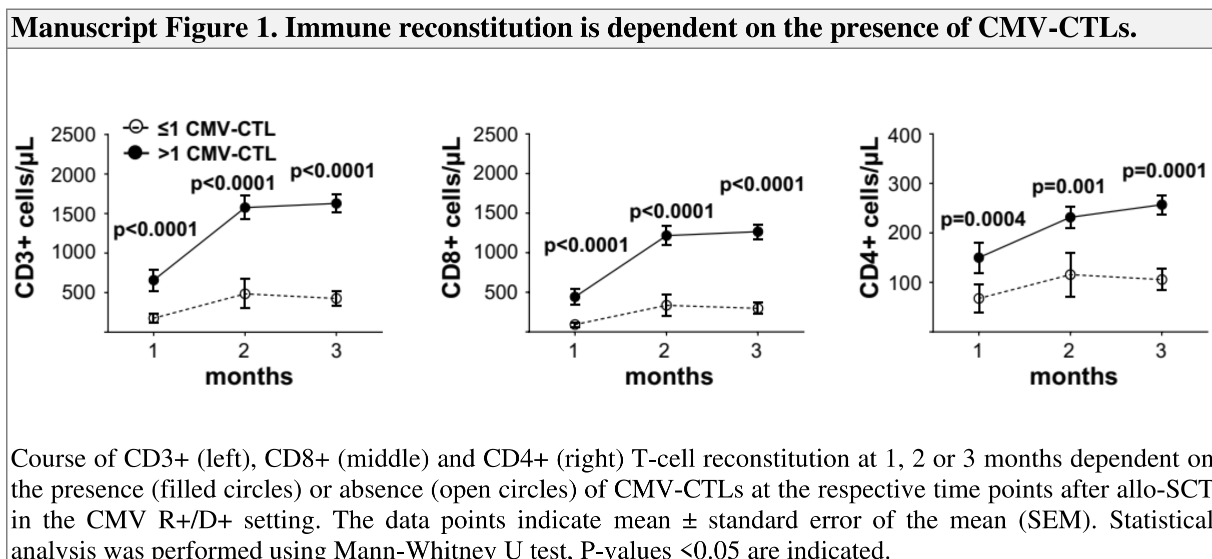
Table 1: Statistical analysis was performed to compare patient characteristics between CMV-CTL negative (neg.) and CMV-CTL positive (pos.) patients. Comparisons of patient and donor age were performed by Mann-Whitney U test. Comparisons of recipient gender, disease status, cyto- and molecular genetics, stem cell source, GvHD prophylaxis and conditioning regimen were performed using Fisher's exact test. Comparisons of diagnosis, donor and T-cell depleting antibodies were performed using chi-square test. Immunosuppressive antibodies (antithymocyte globulin (ATG, Fresenius ®) or Thymoglobulin, Genzyme ®) for in vivo depletion of T-cells were given to 91 patients (88%), while only 12 were not treated with in vivo T-cell depletion. Fifteen patients (93%) without CMV-CTL and 77 (88.5%) with CMV-CTL received ATG or Thymoglobulin. **Abbreviations:** No., number; %, percentage; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; PBSC, peripheral blood stem cells; BM, bone marrow; MRD, Matched related donor; MUD, Matched unrelated donor; MMUD, Mismatched unrelated donor; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; CSA, Cyclosporine A; MMF, mycophenolate mofetil; MTX, methotrexate; [a] : AL; biphenotypic acute leukemia (n=1); CML, chronic myeloid leukemia blast crisis (n=1).

Patients were subdivided in two groups based on the presence or absence of more than 1 CMV-CTLs per µL blood 3 months after allo-SCT. This threshold was based on our previous observation that more than one CMV-CTL/µl blood at 3 months after allo-SCT was associated with low host chimerism levels[122]. CMV-CTLs were detected in 87/103 patients (84%) 3 months after allo-SCT. Detailed patient characteristics are shown in **Table 1**. The demographic and clinical parameters were not significantly different in patients with or without CMV-CTLs **(Table 1)**.

Immune reconstitution, CMV reactivation, acute and chronic GvHD

Patients were monitored for the reconstitution of overall T-cells and CMV-CTLs at 1, 2 and 3 months after allo-SCT. Patients with CMV-CTLs had significantly more CD3+, CD8+ and CD4+ T-cells at these time points than patients without CMV-CTLs (**Figure 1**).

Manuscript Figure 1. Immune reconstitution is dependent on the presence of CMV-CTLs.



Course of CD3+ (left), CD8+ (middle) and CD4+ (right) T-cell reconstitution at 1, 2 or 3 months dependent on the presence (filled circles) or absence (open circles) of CMV-CTLs at the respective time points after allo-SCT in the CMV R+/D+ setting. The data points indicate mean ± standard error of the mean (SEM). Statistical analysis was performed using Mann-Whitney U test, P-values <0.05 are indicated.

Next, the incidences of CMV reactivation (CMV-R), clinically significant aGvHD grade II-IV and cGvHD were analyzed depending on the presence or absence of CMV-CTLs at 3 months after allo-SCT. CMV-R occurred in 63 patients (61%) on day 36 (median; range 8 to 68) after allo-SCT. The incidence of CMV-R was higher in patients with CMV-CTLs than in those without (67% vs. 31%; p=0.011). Acute GvHD grade II-IV occurred in 27 patients

(26%) on day 39 (median; range 18-123) after allo-SCT irrespective of the detection of CMV-CTLs (26% vs 25%, p=1.0). Chronic GvHD developed in 41 patients (40%) on day 167 (median; range 94-809) after allo-SCT independent of the presence of CMV-CTLs (42.5% vs 25%, p=0.268) **(Table 2)**.

Manuscript Table 2. Complications after allo-SCT.

	CMV-CTL neg. n=16	CMV-CTL pos. n=87	p value
	No. (%)	No. (%)	
CMV-R			**0.011**
no	11 (69)	29 (33)	
yes	5 (31)	58 (67)	
aGvHD			1.00
grade 0-I	12 (75)	64 (74)	
grade II-IV	4 (25)	23 (26)	
cGvHD			0.268
no	12 (75)	50 (58)	
yes	4 (25)	37 (42)	

Table 2: Statistical analysis was performed to compare complications between CMV-CTL negative (neg.) and CMV-CTL positive (pos.) patients. Comparisons of CMV-R and aGvHD were performed using Fisher's exact test. Comparisons of cGvHD were performed using chi-square test.
Abbreviations: No., number; %, percentage; CMV-R, CMV-reactivation; aGvHD, acute graft-versus-host disease; cGvHD: chronic GvHD.

Univariate analysis of parameters affecting the outcome

OS, DFS, NRM and CIR were determined at a follow-up of 1, 2 and 5 years after allo-SCT. The OS was 75% / 64% / 54%, the DFS was 72% / 59% / 50%, the NRM was 16% / 21% / 24% and the CIR was 13% / 19% / 26% until 1, 2 and 5 years, respectively, in the whole cohort. The potential impact of the presence of CMV-CTLs at 3 months after allo-SCT on the outcome at 1, 2 and 5 years after allo-SCT was studied in univariate analysis along with other potential prognostic factors including patient age, gender, diagnosis, disease status, cyto-/molecular genetics, stem cell source, donor type, conditioning, GvHD prophylaxis, CMV-R, aGvHD or cGvHD (**Table 3**). Table 3 summarizes parameters significantly correlated with OS, DFS, NRM and CIR in the univariate regression analysis. Factors not significant are shown in **supplemental Table 1**.

Manuscript Table 3. Univariate analysis of the parameters influencing the outcome after allo-SCT.

Para-meter	Variables	OS			DFS			NRM			CIR		
		HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
Disease status	**standard / advanced**												
	1 year	1.16	0.54-2.52	0.699	1.30	0.63-2.70	0.475	1.06	0.40-2.82	0.910	1.59	0.54-4.68	0.400
	2 years	1.31	0.69-2.50	0.410	1.59	0.87-2.91	0.135	0.94	0.40-2.18	0.880	2.64	1.05-6.62	**0.039**
	5 years	1.39	0.78-2.46	0.264	1.52	0.88-2.62	0.131	0.90	0.41-1.99	0.790	2.19	1.02-4.70	**0.044**
Donor type	**matched / mismatched**												
	1 year	2.19	0.92-5.21	0.077	1.93	0.82-4.52	0.131	1.86	0.62-5.55	0.260	1.75	0.48-6.38	0.400
	2 years	2.49	1.2-5.16	**0.014**	2.46	1.23-4.92	**0.011**	2.80	1.19-6.60	**0.019**	1.47	0.48-4.43	0.500
	5 years	1.96	0.97-3.95	0.061	2.00	1.02-3.91	**0.043**	2.41	1.03-5.63	**0.043**	1.02	0.34-3.07	0.980
GvHD prophylaxis	**CsA-MTX / CsA-MMF**												
	1 year	2.05	0.70-.5.94	0.188	2.37	0.82-6.81	0.109	2.53	0.59-10.87	0.210	1.94	0.44-8.57	0.380
	2 years	2.50	0.97-6.41	0.057	2.02	0.90-4.55	0.090	2.34	0.71-7.73	0.170	1.44	0.50-4.18	0.500
	5 years	2.34	1.05-5.22	**0.038**	1.99	0.97-4.09	0.060	1.49	0.58-3.81	0.410	2.10	0.73-6.05	0.170
aGvHD	**grade 0-I / II-IV**												
	1 year	3.69	1.71-7.97	**0.001**	3.08	1.48-6.41	**0.003**	4.57	1.73-12.06	**0.002**	1.29	0.40-4.14	0.670
	2 years	4.92	2.57-9.43	**<0.001**	3.90	2.12-7.18	**<0.001**	6.54	2.79-15.34	**<0.001**	1.26	0.48-3.26	0.640
	5 years	3.93	2.19-7.05	**<0.001**	3.24	1.82-5.68	**<0.001**	5.83	2.66-12.77	**<0.001**	0.83	0.33-2.11	0.700
cGvHD	**no / yes**												
	1 year	0.21	0.06-0.71	**0.012**	0.18	0.05-0.59	**0.005**	0.24	0.06-1.07	0.061	0.14	0.02-1.11	0.063
	2 years	0.21	0.08-0.55	**<0.001**	0.21	0.09-0.51	**<0.001**	0.34	0.12-0.99	**0.047**	0.17	0.04-0.72	**0.016**
	5 years	0.26	0.12-0.53	**<0.001**	0.27	0.14-0.52	**<0.001**	0.51	0.22-1.19	0.120	0.22	0.08-0.61	**0.004**
CMV-CTLs	**no / yes**												
	1 year	1.00	0.34-2.89	0.994	0.51	0.22-1.19	0.120	2.89	0.38-22.03	0.300	0.18	0.06-0.53	**0.002**
	2 years	0.64	0.29-1.39	0.257	**0.48**	**0.24-0.98**	**0.045**	1.23	0.38-3.98	0.730	0.27	0.11-0.68	**0.005**
	5 years	0.61	0.30-1.22	0.161	0.61	0.30-1.21	0.159	1.43	0.44-4.64	0.560	0.40	0.16-1.00	0.050

Univariate regression analysis of OS and DFS were performed by Cox-regression/cox proportional hazard regression analysis. Analysis of NRM and CIR were performed by the Fine and Gray test. The second column shows for each tested parameter two alternative variables. For the calculation of the hazard ratio, the first variable was set as 1.00.

Abbreviations: OS, overall survival; DFS, disease free survival; NRM, non-relapse mortality; CIR, cumulative incidence of relapse; HR, hazard ratio; CI, confidence interval; CSA, Cyclosporine A; MTX, methotrexate; MMF, mycophenolate mofetil; CMV-R, CMV reactivation; aGvHD, acute graft-versus-host disease; cGvHD: chronic GvHD.

The OS was not affected by the presence of CMV-CTLs at 3 months after allo-SCT, but OS at 2 years was significantly longer in patients receiving matched donor grafts (p=0.014) or at 5 years in patients receiving GvHD-prophylaxis with CsA/MTX (p=0.038). The OS at 1, 2 and 5 years was also prolonged by the absence of aGvHD grade II-IV (p=0.001, p<0.001 and p<0.001) and by the presence of cGvHD (p=0.012, p<0.001 and p<0.001).

Manuscript Supplemental Table 1. Univariate analysis of the parameters influencing the outcome after allo-SCT (not significant).

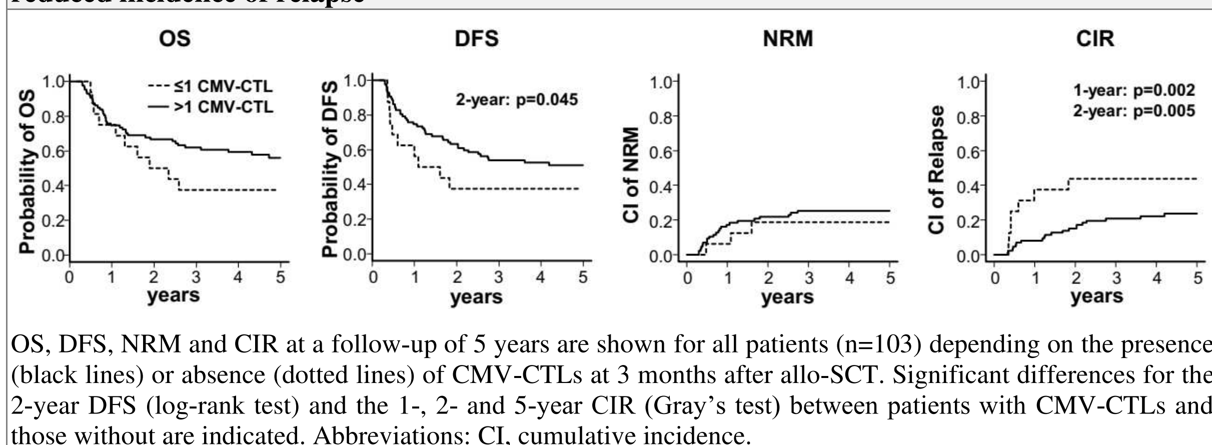
Parameter	OS			DFS			NRM			CIR		
	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
≤60y / >60y												
1 year	1.32	0.59-2.96	0.502	1.29	0.60-2.76	0.521	1.48	0.54-4.06	0.440	1.03	0.32-3.28	0.970
2 years	1.20	0.66-2.38	0.608	1.24	0.65-2.35	0.512	1.42	0.60-3.36	0.430	0.99	0.39-2.56	0.990
5 years	1.31	0.72-2.40	0.380	1.22	0.68-2.18	0.497	1.17	0.50-2.73	0.720	1.17	0.53-2.56	0.700
male/female												
1 year	1.39	0.64-3.01	0.400	1.48	0.72-3.07	0.289	1.61	0.61-4.25	0.340	1.33	0.45-3.90	0.600
2 years	1.11	0.58-2.14	0.751	1.10	0.60-2.05	0.752	1.36	0.59-3.13	0.470	0.84	0.34-2.08	0.700
5 years	0.92	0.51-1.66	0.775	1.02	0.58-1.79	0.937	1.30	0.59-2.83	0.520	0.77	0.35-1.71	0.520
Acute leukemia no / yes												
1 year	1.70	0.40-7.20	0.470	1.30	0.39-4.29	0.669	0.95	0.23-3.95	0.940	1.70	0.24-11.92	0.590
2 years	2.58	0.62-10.75	0.192	1.43	0.51-4.00	0.498	1.35	0.32-5.74	0.680	1.28	0.32-5.10	0.730
5 years	1.27	0.50-3.22	0.610	1.44	0.57-3.61	0.443	0.99	0.31-3.18	0.990	1.80	0.44-7.46	0.420
MDS no / yes												
1 year	0.59	0.14-2.49	0.470	0.77	0.23-2.55	0.669	1.05	0.25-4.37	0.940	0.59	0.08-4.14	0.590
2 years	0.39	0.09-1.61	0.192	0.70	0.25-1.96	0.498	0.74	0.17-3.13	0.680	0.78	0.20-3.13	0.730
5 years	0.79	0.31-1.99	0.610	0.70	0.28-1.75	0.443	1.01	0.31-3.24	0.990	0.55	0.13-2.29	0.420
Cytomolecular genetics standard / high-risk												
1 year	1.04	0.47-2.28	0.931	0.96	0.45-2.03	0.914	0.94	0.35-2.58	0.910	0.99	0.33-2.99	0.980
2 years	1.43	0.75-2.74	0.276	1.51	0.82-2.76	0.185	1.33	0.58-3.05	0.500	1.62	0.68-3.86	0.280
5 years	1.67	0.94-2.96	0.080	1.62	0.94-2.79	0.085	1.27	0.58-2.77	0.550	1.79	0.85-3.77	0.130
MAC/RIC												
1 year	1.26	0.56-2.83	0.572	1.23	0.57-2.64	0.599	2.00	0.65-6.13	0.230	0.73	0.25-2.14	0.560
2 years	1.24	0.63-2.43	0.534	1.20	0.64-2.26	0.570	1.45	0.60-3.49	0.410	0.94	0.39-2.29	0.890
5 years	1.47	0.79-2.71	0.221	1.41	0.79-2.51	0.248	1.20	0.54-2.67	0.650	1.52	0.66-3.50	0.330
No CMV-R / CMV-R												
1 year	2.43	0.97-6.05	0.057	1.87	0.88-4.22	0.133	2.07	0.69-6.23	0.200	1.44	0.44-4.65	0.550
2 years	2.04	0.99-4.21	0.054	1.48	0.78-2.82	0.230	1.87	0.75-4.66	0.180	0.97	0.40-2.35	0.950
5 years	1.65	0.89-3.05	0.108	1.56	0.87-2.79	0.132	1.82	0.78-4.25	0.170	1.11	0.51-2.39	0.800

Univariate regression analysis of the outcome was performed at 1, 2 or 5 years after allo-SCT. Univariate regression analysis of OS and DFS were performed by Cox-regression/cox proportional hazard regression analysis. Here, non-significant parameters are summarized. Analysis of CIR and NRM were performed by the Fine and Gray test. The first column shows the tested variables in the respective parameters and the hazard ratio (HR) are calculated using the first variable as a reference and set to 1. symbol: -, no events and results cannot be calculated

The DFS at 2 years was significantly longer in patients with CMV-CTLs (p=0.045, **Figure 2**). An improved DFS at 2 and 5 years was associated with matched donor grafts (p=0.011 and p=0.043). Additionally, an improved DFS at all time points analyzed was associated with the absence of aGvHD grade II-IV (p=0.003, p<0.001 and p<0.001) and the presence of cGvHD (p=0.005, p<0.001 and p<0.001). The NRM was not affected by the presence of CMV-CTLs by 3 months after allo-SCT. In contrast, an increased NRM at 2 and 5 years was associated with mismatched donor grafts (p=0.019 and p=0.043), at all-time points with the presence of aGvHD grade II-IV (p=0.002, p<0.001 and p<0.001) and at 2 years with the

absence of cGvHD (p=0.047). The CIR at 1 and 2 years was significantly lower in patients with CMV-CTLs (p=0.002; p=0.005; **Figure 2**). A reduced CIR at 2 and 5 years was also associated with a standard disease status (p=0.039 and p=0.044) and with cGvHD (p=0.016; p=0.004). There was a considerable difference in the time to relapse between patients with and without CMV-CTLs. At a 5-year follow up, only 65% of the relapses in CMV–CTL positive patients occurred until 2 years. In contrast, all relapses in CMV-CTL negative patients occurred until 2 years. Accordingly, onset of relapse was significantly later in CMV-CTL positive patients than in CMV-CTL negative patients (median 489 days, range 120-1532 vs. 152 days, range 129-668, p=0.041, **Figure 2**).

Manuscript Figure 2. Presence of CMV-CTLs at 3 months after allo-SCT is associated with a reduced incidence of relapse



OS, DFS, NRM and CIR at a follow-up of 5 years are shown for all patients (n=103) depending on the presence (black lines) or absence (dotted lines) of CMV-CTLs at 3 months after allo-SCT. Significant differences for the 2-year DFS (log-rank test) and the 1-, 2- and 5-year CIR (Gray's test) between patients with CMV-CTLs and those without are indicated. Abbreviations: CI, cumulative incidence.

Multivariate regression analysis of parameters affecting the outcome

Multivariate regression analysis of the outcome was only performed with parameters statistically significant in the univariate analysis at 1, 2 and 5 years after allo-SCT (significant parameters in **Table 4**). The influence of the disease status on CIR was lost in the multivariate analysis (supplemental Table 2).

Manuscript Supplemental Table 2. Factors not significant after multivariable analysis														
Para-meter	**Variables**	**OS**			**DFS**			**NRM**			**CIR**		
		HR	**95% CI**	**p value**	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
Disease status	**standard/ advanced**												
	1 year		-			-			-			-	
	2 years		-			-			-		2.14	0.82-5.53	0.120
	5 years		-			-			-		1.94	0.88-4.27	0.099

Multivariate regression analysis of the outcome was performed only with those parameters statistically significant in the univariate analysis at 1, 2 or 5 years after allo-SCT. Standard or advanced disease was significant in univariate analysis for CIR, but this was lost in the multivariate analyses.
Multivariate regression analysis of OS and DFS were performed by Cox-regression/cox proportional hazard regression analysis. Analysis of NRM and CIR were performed by the Fine and Gray test. The second column shows for each tested parameter two alternative variables.
For the calculation of the hazard ratio, the first variable was set as 1.00. Here, factors significant in univariate analysis, which lost significance in multivariable analysis are shown."-" indicates parameters not significant in univariate analysis.
Abbreviations: HR, hazard ratio; CI, confidence interval; -, not applicable; CSA, Cyclosporine A; MMF, mycophenolate mofetil; CMV-R, CMV reactivation; aGvHD, acute graft-versus-host disease; cGvHD: chronic GvHD.

The OS at 1, 2 and 5 years was reduced by aGvHD grade II-IV (p=0.001, p<0.001 and p<0.001). Chronic GvHD had a positive influence on OS at all time points analyzed (p=0.009, p=0.001 and p<0.001). The DFS at 2 and 5 years was significantly reduced in patients after mismatched transplantation (p=0.049 and p=0.038) and in patients with aGvHD grade II-IV (p=0.001, p<0.001 and p<0.001) at all time points. In contrast, cGvHD led to an increase of the DFS at 1, 2 and 5 years (p=0.003, p<0.001 p<0.001). The NRM at 1, 2 and 5 years was significantly increased by aGvHD grade II-IV (p=0.002, p<0.001 and p<0.001) and at 2 years by the lack of cGvHD (p=0.044). The CIR was only reduced at 1 and 2 years by the presence of CMV-CTLs (p=0.006 and p=0.039) and at 2 and 5 years by cGvHD (p=0.04 and p=0.02).

Manuscript Table 4. Multivariate regression analysis of the parameters influencing the outcome after allo-SCT.

Para-meter	Variables	OS			DFS			NRM			CIR		
		HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
Donor type	**matched/mismatched**												
	1 year		-			-			-			-	
	2 years	1.78	0.81-3.90	0.15	2.11	1.00-4.45	**0.049**	1.69	0.59-4.84	0.330		-	
	5 years				2.17	1.04-4.51	**0.038**	1.51	0.58-3.93	0.400		-	
aGvHD	**grade 0-I / II-IV**												
	1 year	3.95	1.82-8.55	**0.001**	3.34	1.59-6.99	**0.001**	4.69	1.79-12.27	**0.002**		**-**	
	2 years	4.66	2.32-9.36	**<0.001**	3.62	1.88-6.98	**<0.001**	5.71	2.16-15.12	**<0.001**		**-**	
	5 years	4.25	2.32-7.80	**<0.001**	3.14	1.71-5.76	**<0.001**	5.37	2.28-12.65	**<0.001**		**-**	
cGvHD	**no / yes**												
	1 year	0.20	0.06-0.66	**0.009**	0.17	0.05-0.55	**0.003**	0.24	0.05-1.09	0.065	0.17	0.02-1.31	0.088
	2 years	0.19	0.07-0.49	**0.001**	0.19	0.08-0.46	**<0.001**	0.34	0.12-0.97	0.044	0.21	0.05-0.93	**0.040**
	5 years	0.25	0.12-0.52	**<0.001**	0.22	0.11-0.44	**<0.001**		**-**		0.28	0.10-0.82	**0.020**
CMV-CTLs	**no / yes**												
	1 year		**-**			-			**-**		0.21	0.07-0.66	**0.006**
	2 years		**-**		0.82	0.39-1.73	0.600		-		0.35	0.14-0.95	**0.039**
	5 years		-			-			-			-	

Multivariate regression analysis of the outcome was performed only with those parameters statistically significant in the univariate analysis at 1, 2 or 5 years after allo-SCT. Multivariable regression analysis of OS and DFS were performed by Cox-regression/cox proportional hazard regression analysis. Analysis of NRM and CIR were performed by the Fine and Gray test. The second column shows for each tested parameter two alternative variables. Not significant data sets are indicated by "-" in the 95% CI column. For the calculation of the hazard ratio, the first variable was set as 1.00.
Abbreviations:"-": not significant in univariate analysis. OS, overall survival; DFS, disease free survival; NRM, non-relapse mortality; CIR, cumulative incidence of relapse; HR, hazard ratio; CI, confidence interval

4.2.4 Discussion

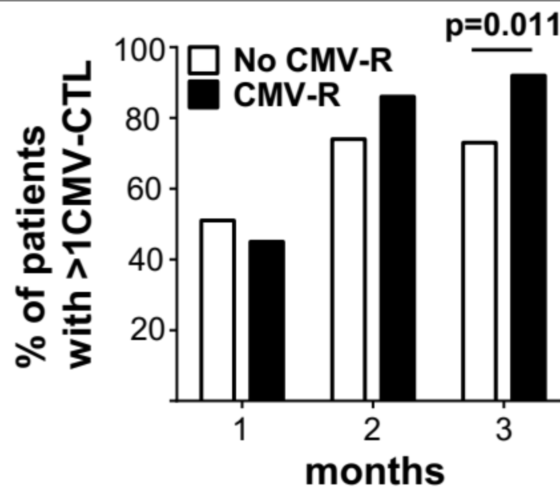
Our study is the first to show that the presence of CMV-CTLs at 3 months after allo-SCT in patients with hematological malignancies transplanted in the CMV R+/D+ setting is associated with a reduction of early relapses. The current study was prompted by our previous observations that the presence of CMV-CTLs patients in the CMV R+/D+ setting was associated with a fast T-cell reconstitution and elimination of the host hematopoiesis in patients with a broad spectrum of hematological diseases [122]. The latter data had suggested that CMV-CTLs might be a trigger for allo-immune responses reflected by the conversion to complete donor chimerism. The present study focused on the impact of CMV-CTLs in comparison to other demographic and clinical parameters on the outcome selectively in acute leukemia and MDS patients after allo-SCT. In accordance with previous reports [136], patients receiving a mismatched unrelated donor graft had a (borderline) reduced OS and a reduced

DFS in the multivariable regression analysis. Severe aGvHD had no impact on the CIR but was associated with a reduced OS and DFS and an increased NRM.[109, 110] In accordance with previous reports, [109, 110] cGvHD was associated with an improved OS and DFS and a reduced CIR in the multivariable regression analysis. The advanced disease status [104, 105] and high-risk cyto- and molecular-genetics [106, 107] were significantly or by trend, respectively, associated with an increased CIR in the univariate analysis. However, this effect was lost in the multivariate analyses, maybe due to the still small sample size in our cohort (Supplemental Table 2). Taken together, our cohort compares well with other publications on the impact of the major disease or transplant related factors on the outcome. The most important finding of the current study was the association of the presence of CMV-CTLs with a reduced 1- and 2-year CIR both in the uni- and multivariable analysis. These data suggest that the previously observed suppression of host chimerism at 3 months after allo-SCT in patients with CMV-CTLs [122] translates at a longer follow-up in an anti-leukemic effect. Of note, the presence of CMV-CTLs at 3 months after transplantation and cGvHD were the only independent parameters significantly associated with a reduced CIR. However, while cGvHD was protective against late relapses, the protective effect of CMV-CTLs was restricted to early relapses after allo-SCT.

The mechanisms for the relationship between the presence of CMV-CTLs and prevention of early relapse remain unclear. CMV-R alone had no impact on the relapse incidence in our study. This may be due to the fact that in the previous studies on the protective effect of CMV-R against relapse the cohorts were heterogeneous regarding the CMV serostatus of patients and donors[115-120]. Here we studied the role of CMV-R selectively in the CMV R+/D+ setting alone, thus the impact of CMV-R is possibly less pronounced. Nevertheless, presence of CMV-CTLs at 3 months after allo-SCT was associated with prior CMV-R (**Table 2 and Supplemental Figure 1)** thus boosting the emergence of CMV-CTLs in the CMV +/+

setting.[27] Therefore, the presence of CMV-CTLs after CMV-R may be the key of protection against early relapse. Similar to our previous report [122], we found a strong association between presence of CMV-CTLs and a fast recovery of CD3+, CD8+ and CD4+ T-cells after allo-SCT also in the current cohort. Nevertheless, whether the presence of CMV-CTLs is solely a marker for a functional donor immune system becoming effective enough already at early time points after allo-SCT to protect from relapse or whether CMV-CTLs themselves play a causal role in promoting an effective anti-leukemic immune response remains speculative at present.

Manuscript Supplemental Figure 1. CMV-R influences the presence of CMV CTLs until 3 months after allo-SCT.



Depicted is the relationship between the presence or absence of CMV-R and the positivity for CMV CTLs until 1, 2 or 3 months after allo-SCT. The bars indicate % patients with >1 CMV-CTL/µl in patients without (open bars) or with (filled bars) CMV-R. Statistical analysis between groups at the respective months was performed by Fisher's exact test.

Here, we analyzed the presence of CMV-CTLs in the context of clinical events. Functionality of the CMV-CTLs was not analyzed for this study, since tetramer staining alone does not give this information. Tetramer-staining of T-cells only indicates the presence of T-cells recognizing a CMV-peptide in context with a particular HLA-molecule, but is not sufficient to show the functionality of these T-cells. Additional tests such as intracellular cytokine staining or cytokine capture assay for IFN-gamma or IL-2 upon specific stimulation with CMV-peptides may quantify the functionality of the detected CMV-CTLs. Expansion of

CMV-CTLs after CMV-reactivation/infection is a good surrogate marker for functionality of CMV-CTLs [90]. Furthermore, adoptive CMV-CTL transfer also increases overall CD8+ and CD4+ T-cell counts, [66, 137] indicating that CMV-CTLs might be a cause and not simply the consequence of an enhanced T-cell recovery. Moreover, the associations between anti-viral immune responses, allo-reactivity and lower leukemia relapse risk are most likely not limited to CMV. Own preliminary data reported previously [122] showed - at least by trend - an association between EBV-CTLs in HLA-A02+ patients and T-cell reconstitution even after restricting the analysis to patients without CMV-CTLs as potential confounding factor. Additionally, presence of T-cell responses directed against a broad spectrum of herpes viruses was associated with alloreactive T-cell responses in pediatric patients after umbilical cord blood transplantation[138] and protective against leukemia relapse in high risk AML patients [139]. However, what links herpes viruses as immunological targets to antigens/peptides expressed on leukemia cells? Although cross-reactivity with antigens expressed on leukemia cells has been described [140], the targets of the virus-specific immune responses are most likely not expressed on leukemia cells. As discussed earlier [122], epithelial, myeloid and interstitial dendritic cells (DCs) [141, 142] are important reservoirs for latent CMV. Residual host-derived DCs are capable of presenting both host-alloantigens and CMV-antigens after allo-SCT. We hypothesize that the recognition of CMV on host DCs by CMV-CTLs may create a pro-inflammatory environment causing an enhanced presentation of host alloantigens.[143] Thereby, alloreactive donor T-cells might be boosted mediating the elimination of the residual leukemia cells [125]. This hypothesis is supported by the fact that the presence of CMV-CTL at 1, 2 and 3 months post-allo-SCT is associated with significantly higher counts of CD3+ T-cells in the patients (Figure 1). Alternatively, myeloid leukemia cells themselves may have been infected by CMV and CMV-antigens may directly be presented on the cell surface, thus direct elimination of leukemia cells by the CMV-CTLs

may be possible as well. Future studies are required to proof either the proposed association between CMV-CTL and allo-antigen specific CTLs[125] by combined immune monitoring studies after allo-SCT.

In addition, NK-cells, namely the subgroup of the $NKG2C^+CD57^+$ are reported to play important roles in reduction of CIR. We limited our studies to overall T cells and CMV-CTLs. A recent review by Litjens and colleagues[144] excellently summarizes several papers dealing with the impact CMV-R on CIR and the possible role of NK cells in both allo-SCT and solid organ transplantation. Interestingly, many studies describe an impact of early CMV-R on significantly reduced CIR in different cohorts. The reduction of relapse risk was associated with CMV-R, while pre-transplant CMV- seropositivity actually was associated with an increased relapse risk. The reduction of CIR in all previously published studies was correlated to patients with AML transplanted following MAC regimens and without in vivo depletion of T/NK-cells or immunosuppressive antibodies, like ATG or Thymoglobulin. These are major differences to our cohort. We included MDS and ALL patients to the AML patients (n = 74) for this paper to obtain larger patient numbers for the univariate and multivariable analysis. In the cohort (n = 103) described here CMV-reactivation had no influence in univariate analysis on CIR or any of the other parameters analyzed (Supplemental Table 1). To put our findings in perspective to data of other groups[144] we have analyzed patients with only AML (n = 74, 10 patients without CMV-CTL at 3 month) and found that in this cohort CMV-R was significantly correlated to OS at 2 and 5 years after SCT and with DFS at 5 years in the univariate regression analysis (Supplemental Table 3), but this significance was lost in the multivariable analysis (Supplemental Table 4). CMV-R did not correlate with CIR in our AML-cohort this may be due to the comparatively small numbers of patients but also other major differences of the cohort. In addition to the inclusion of other diseases as AML, diseases that have not shown the same correlation of CMV-R to

CIR reduction, our patients were mainly transplanted following RIC (60%). Furthermore, more than 88% of the patients received in vivo T cell depletion with either ATG or thymoglobulin, which may also interfere with the correlation of CMV-R to the reduction of CIR. While these factors may explain the differences between the studied cohorts, we none the less feel that we may have elucidated one possible mechanism of CIR reduction following CMV-R. CMV-R is a prerequisite for the development and expansion of CMV-CTL.[90] We have shown here that the expansion of overall T cells is fast and yields higher numbers in patients with CMV-CTL (Figure 1). We argue that CMV-R may provide the optimal environment for not only CMV-CTL expansion, but an increased expansion of overall, thus possibly allo-reactive T cells.

In conclusion, we have shown for the first time in a retrospective analysis that the presence of CMV-CTLs three months after allo-SCT is associated with a reduced incidence of early relapses in the CMV R+/D+ setting. Additional studies in larger and prospective cohorts are required to confirm the current observations and to unravel the mechanisms of the potential protective effects of CMV-CTLs against relapse.

Supplemental Table 3. Univariate analysis of the parameters influencing the outcome after allo-SCT in only AML patients.

Parameter	Variables	OS			DFS			NRM			CIR		
		HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
Patient Age	**≤60years/>60years**												
	1 year	2.47	0.89-6.79	0.082	2.13	0.80-5.67	0.132	2.04	0.60-6.91	0.250	1.88	0.39-9.09	0.430
	2 years	2.22	0.96-5.13	0.061	1.73	0.79-3.82	0.174	2.06	0.73-5.76	0.170	1.10	0.32-3.73	0.880
	5 years	1.87	0.90-3.90	0.093	1.57	0.79-3.11	0.195	1.62	0.61-4.30	0.340	1.22	0.48-3.12	0.680
Gender	**male/female**												
	1 year	1.30	0.47-3.60	0.609	1.49	0.56-3.98	0.423	0.97	0.28-3.39	0.970	2.96	0.55-16.09	0.210
	2 years	1.25	0.54-2.90	0.601	1.19	0.54-2.62	0.665	1.09	0.39-3.10	0.870	1.26	0.39-4.05	0.700
	5 years	0.77	0.36-1.65	0.500	0.94	0.47-1.88	0.857	0.88	0.32-2.39	0.800	0.95	0.37-2.45	0.920
Disease status	**standard/advanced**												
	1 year	1.38	0.50-3.79	0.536	1.60	0.60-4.30	0.351	1.19	0.35-4.02	0.780	2.40	0.45-12.91	0.310
	2 years	1.49	0.65-3.46	0.349	1.65	0.75-3.63	0.217	0.90	0.32-2.55	0.840	3.35	0.89-12.57	0.073
	5 years	1.38	0.66-2.85	0.390	1.52	0.78-2.99	0.223	0.92	0.35-2.45	0.870	2.11	0.83-5.34	0.120
Cyto-molecular genetics	**standard/high-risk**												
	1 year	0.55	0.16-1.96	0.357	0.50	0.14-1.75	0.275	0.54	0.12-2.56	0.440	0.44	0.05-3.79	0.450
	2 years	1.26	0.53-3.01	0.598	1.24	0.55-2.81	0.606	1.21	0.42-3.50	0.730	1.28	0.38-4.26	0.690
	5 years	1.37	0.65-2.90	0.414	1.44	0.72-2.87	0.306	1.00	0.36-2.82	1.000	1.85	0.75-4.56	0.180
Stem cell source	**BM/PBSC**												
	1 year	-	-	-	-	-	-	-	-	-	-	-	-
	2 years	0.94	0.13-6.99	0.951	1.08	0.15-7.98	0.940	0.56	0.09-3.39	0.530	-	-	-
	5 years	1.30	0.18-9.60	0.795	1.53	0.21-11.2	0.675	0.63	0.10-4.05	0.630	-	-	-
Donor type	**matched/mismatched donors**												
	1 year	1.95	0.55-6.92	0.301	1.86	0.53-6.55	0.332	1.83	0.42-7.91	0.420	1.55	0.17-13.86	0.690
	2 years	2.52	0.93-6.83	0.071	2.89	1.15-7.26	**0.024**	3.14	1.08-9.16	**0.036**	1.72	0.37-7.91	0.490
	5 years	1.99	0.75-5.25	0.165	2.21	0.91-5.36	0.081	2.71	0.93-7.92	0.068	0.98	0.21-4.52	0.980
Conditioning	**MAC/RIC**												
	1 year	3.24	0.73-14.36	0.122	3.49	0.80-15.4	0.098	4.28	0.54-33.94	0.170	2.32	0.29-19.35	0.440
	2 years	3.33	0.99-11.27	0.053	2.16	0.81-5.77	0.124	2.95	0.67-12.91	0.150	1.27	0.35-4.60	0.710
	5 years	3.54	1.23-10.18	**0.019**	2.67	1.10-6.45	**0.029**	2.17	0.64-7.33	0.210	2.44	0.70-8.47	0.160
T cell depleting antibodies**	**no/yes**												
	1 year	1.97	0.26-15.02	0.511	2.18	0.30-16.51	0.450	1.24	0.16-9.84	0.840	-	-	-
	2 years	3.13	0.42-23.31	0.265	3.74	0.51-27.65	0.196	1.82	0.23-14.53	0.570	-	-	-
	5 years	2.19	0.52-9.23	0.285	2.73	0.65-11.41	0.169	1.00	0.24-4.17	1.000	-	-	-
GvHD prophylaxis	**CsA.MTX/CsA.MMF**												
	1 year	2.62	0.59-11.62	0.205	2.83	0.64-12.46	0.169	3.48	0.44-27.64	0.240	1.90	0.23-15.78	0.550
	2 years	2.67	0.79-9.04	0.114	1.73	0.65-4.61	0.275	2.39	0.55-10.45	0.250	1.04	0.29-3.73	0.960
	5 years	2.81	0.98-8.07	0.056	2.10	0.87-5.07	0.100	1.75	0.52-5.91	0.370	1.97	0.57-6.84	0.280
CMV-R	**no/yes**												
	1 year	2.75	0.78-9.74	0.118	2.91	0.83-10.21	0.096	2.62	0.58-11.88	0.210	3.07	0.35-26.73	0.310
	2 years	3.24	1.10-9.57	**0.033**	2.31	0.92-5.78	0.075	2.45	0.70-8.52	0.160	1.72	0.47-6.31	0.410
	5 years	2.40	1.03-5.63	**0.044**	2.16	1.01-4.64	**0.048**	2.03	0.68-6.09	0.200	1.73	0.63-4.74	0.280
aGvHD	**grade 0-I/ II-IV**												
	1 year	3.92	1.42-10.82	**0.008**	3.43	1.28-9.16	**0.014**	4.99	1.46-17.05	**0.010**	1.37	0.26-7.26	0.720
	2 years	5.48	2.33-12.86	**<0.001**	4.20	1.90-9.24	**<0.001**	6.19	2.13-17.97	**0.001**	1.62	0.48-5.46	0.430
	5 years	4.11	1.96-8.60	**<0.001**	3.04	1.53-6.06	**0.002**	5.90	2.2-15.84	**<0.001**	0.81	0.26-2.53	0.720

Supplemental Table 3. (continued) Univariate analysis of the parameters influencing the outcome after allo-SCT in only AML patients.

Parameter	Variables	OS			DFS			NRM			CIR		
		HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
cGvHD	**no/yes**												
	1 year	0.10	0.01-0.73	**0.024**	0.09	0.01-0.66	**0.018**	0.16	0.02-1.22	0.077	-	-	-
	2 years	0.12	0.03-0.53	**0.005**	0.16	0.05-0.54	**0.003**	0.23	0.05-1.00	**0.050**	0.14	0.02-1.05	0.055
	5 years	0.24	0.09-0.64	**0.004**	0.28	0.12-0.64	**0.003**	0.45	0.15-1.33	0.150	0.26	0.08-0.87	**0.029**
CMV-CTLs	**negative/positive**												
	1 year	1.02	0.23-4.50	0.984	0.64	0.18-2.23	0.481	1.42	0.18-11.10	0.740	0.30	0.06-1.55	0.150
	2 years	0.51	0.19-1.38	0.184	0.55	0.21-1.46	0.228	0.57	0.17-1.89	0.360	0.65	0.14-3.07	0.590
	5 years	0.68	0.26-1.79	0.436	0.78	0.30-2.01	0.606	0.66	0.20-2.21	0.500	1.19	0.25-5.59	0.830

Univariate regression analysis of the outcome in the AML-only cohort was performed at 1, 2 or 5 years after allo-SCT. Univariate regression analysis of OS and DFS were performed by Cox-regression/cox proportional hazard regression analysis. Here, non-significant parameters are summarized. Analysis of CIR and NRM were performed by the Fine and Gray test. The first column shows the tested variables in the respective parameters and the hazard ratio (HR) are calculated using the first variable as a reference and set to 1. symbol: -, no events and results cannot be calculated.

Abbreviations: HR, hazard ratio; CI, confidence interval; -, not applicable; CSA, Cyclosporine A; MMF, mycophenolate mofetil; CMV-R, CMV reactivation; aGvHD, acute graft-versus-host disease; cGvHD: chronic GvHD. In S3 Table CMV-R is associated with OS at 2 and 5 years and with DFS at 5 years in the univariate analysis, this correlation was lost in the multivariate analysis (Supplemental table 4).

Supplemental Table 4. Multivariable analysis of the parameters influencing the outcome after allo-SCT in only AML patients.

Parameter	Variables	OS			DFS			NRM			CIR		
		HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
Donor type	matched/mismatched donors												
	1 year	-	-	-	-	-	-	-	-	-	-	-	-
	2 years	-	-	-	2.54	0.94-6.88	0.066	1.87	0.46-7.70	0.380	-	-	-
	5 years	-	-	-	-	-	-	-	-	-	-	-	-
Conditioning	MAC/RIC												
	1 year	-	-	-	-	-	-	-	-	-	-	-	-
	2 years	-	-	-	-	-	-	-	-	-	-	-	-
	5 years	2.92	0.92-9.25	0.069	2.41	0.92-6.33	0.075						
CMV-R	no/yes												
	1 year	-	-	-	-	-	-	-	-	-	-	-	-
	2 years	2.92	0.98-8.67	0.054	-	-	-	-	-	-	-	-	-
	5 years	1.46	0.58-3.71	0.425	1.38	0.60-3.19	0.451	-	-	-	-	-	-
aGvHD	grade 0-I/ II-IV												
	1 year	4.30	1.55-11.93	**0.005**	3.76	1.40-10.09	**0.009**	-	-	-	-	-	-
	2 years	6.92	2.90-16.53	**<0.001**	4.16	1.76-9.83	**0.001**	5.50	1.51-20.1	**0.010**	-	-	-
	5 years	5.99	2.69-13.34	**<0.001**	4.44	2.11-9.37	**<0.001**				-	-	-
cGvHD	no/yes												
	1 year	0.09	0.01-0.68	**0.020**	0.08	0.01-0.62	**0.015**	-	-	-	-	-	-
	2 years	0.11	0.03-0.48	**0.003**	0.13	0.04-0.44	**0.001**	0.22	0.05-0.97	**0.045**	-	-	-
	5 years	0.20	0.07-0.55	**0.002**	0.23	0.10-0.56	**0.001**	-	-	-	0.26	0.08-0.87	**0.029**

Multivariable regression analysis of the AML-only cohort for outcome was performed only with those parameters statistically significant in the univariate analysis at 1, 2 or 5 years after allo-SCT. Multivariate regression analysis of OS and DFS were performed by Cox-regression/cox proportional hazard regression analysis. Analysis of NRM and CIR were performed by the Fine and Gray test. The second column shows for each tested parameter two alternative variables. For the calculation of the hazard ratio, the first variable was set as 1.00. Here, factors significant in univariate analysis, which lost significance in multivariable analysis are shown."-" indicates parameters not significant in univariate analysis.

Abbreviations: HR, hazard ratio; CI, confidence interval; -, not applicable; CSA, Cyclosporine A; MMF, mycophenolate mofetil; CMV-R, CMV reactivation; aGvHD, acute graft-versus-host disease; cGvHD: chronic GvHD.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: PV, LH, EMW. Performed the experiments: PV, JO, SB, SL. All experiments performed by SB were done while employed at MHH. Analyzed the data: PV, LH, EMW. Collection of materials/provision of analysis tools: PV, JO, SL. Patient documentation: ED. Wrote the paper: PV, MS, AG, LH, EMW.

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COMPETING INTERESTS

SB is employed by MSD, **AG**: Advisory boards of Jazz Pharmaceuticals, Novartis, and Celgene, none of these had no influence on the data and analysis. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

All other authors have declared that no competing interests exist.

5. Discussion

5.1 Generation of CMV-CTLs for adoptive therapy

Faster or more efficient immune reconstitution and immune therapeutics have shown promising results in protection against CMV-R [66, 84, 96]. CMV-CTLs play an important role in controlling CMV-R and recurrent CMV-R [87, 89]. CMV-CTL immune reconstitution is delayed by conditioning, T cell depletion (TCD), mismatched graft and aGvHD post allo-HSCT [49, 71, 72]. GCV therapy reduces CMV viral load and controls CMV-R; however prolonged use of GCV leads to side effects (e.g. kidney damage) and development of drug-resistant strains [58]. For this reason, adoptive transfer of antigen-specific T cells (CMV-CTLs) is currently under investigation as an alternative therapy to control CMV-R [64-66, 68]. This approach currently is dependent on CMV-seropositive stem cell donors for isolation of CMV-CTLs. Although third-party CMV-CTLs have been used to overcome this limitation, the success of this treatment is under discussion [64, 66, 68]. The source of CMV-CTL is still an important question and the apheresis collected from CMV-seropositive stem cell donors could be an optimal source. Thus, in collaboration with the Institute for Transfusion Medicine (Prof. Dr. Britta Eiz-Vesper), we investigated the possibility of quantifying CMV-CTLs from peripheral blood from granulocyte-colony stimulating factor (G-CSF) mobilized CMV-seropositive donors, apheresis tubes, and grafts.

5.1.1 Influence of G-CSF mobilization on CMV-CTL numbers and function

The reconstitution of CMV-CTLs is mostly dependent on the donor-derived immune cells and total number of antigen-specific T cells (CMV-CTLs) transferred from CMV seropositive donor to control CMV-R post allo-HSCT [145, 146]. The majority of allo-HSCTs use peripheral blood stem cells (PBSC) collected from granulocyte-colony stimulating factor-mobilized donors [147]. Thus, we quantified CMV-CTLs from different sources of G-CSF mobilized whole blood samples with the aim of enriching CMV-CTLs for

adoptive therapy. The frequency of CMV-CTLs (sum total of all matched multimers) from the G-CSF mobilized donors does not differ between whole blood samples, apheresis tubes and grafts **(Figure 11).** Additionally, tetramers can be readily used to detect HLA specific immunodominant CMV-CTLs (HLA A01:02, HLA A02:01, HLA B07:02) in CMV seropositive donors **(Figure 12)**.

However, it is known that G-CSF mobilization leads to the reactivation of CMV in latently infected CMV humanized animal models [148]. Upon in vitro stimulation with CMVpp65 and CMV-IE1 proteins the G-CSF treated PBMCs showed an impairment of T cell functions as cytokine secretion (IFN-γ) was downregulated and degranulation (Granzyme B) was reduced (Performed by AG Prof. Dr. Britta Eiz-Vesper). Thus, the G-CSF mobilized blood materials could potentially also affect the generation of CMV-CTLs from the naïve donors and hence G-CSF mobilization may increase the risk of CMV-R in CMV-seropositive recipients post allo-HSCT.

Taken all together, these in vitro results demonstrated that the material collected from donors after G-CSF mobilization is not a viable option for collection of CMV-CTLs for adoptive therapy [98]. Thus, we aimed to optimize the in vitro expansion of CMV-CTLs to improve the availability of functional, unexhausted CMV-CTLs for patients whose donors have either low numbers or no CMV-CTLs (induction of CMV-CTLs from naive T cells in vitro).

5.1.2 In vitro generation of CMV-CTL using genetically reprogrammed dendritic cells (SmartDCpp65)

Recent studies have shown the potential of lentivirus (LV) induced DCs to trigger humoral and cellular immune responses against CMVpp65 [149, 150]. Dendritic cells (DCs) are the sentinels of the immune system and are professional antigen presenting cells (APCs). In vitro generated dendritic cells (DCs) loaded with antigens (in the form of single peptides, peptide mixes, recombinant proteins or tumor cell lysates) have a capability to induce antigen-

specific immune responses. Currently, these antigen-pulsed DCs are increasingly used as an adjuvant for immunotherapy [151, 152]. Difficulties in scaling up such as high costs for production, low viability, and sub-optimal antigen loading tend to limit their therapeutic application. Lentiviral reprogramming of hematopoietic precursors offers a novel approach to induce potent DCs directed against a variety of antigens to target cancer and infectious diseases [102].

5.1.3 Validation of transgenes and characterization of SmartDCs

The third generation of SIN LV (self-inactivating lentiviral vector) has been synthesized and constructed to manipulate hematopoietic precursors genetically **(Figure 13B)**. GM-CSF and IL-4 are consistently used in the generation of professional antigen presenting cells, where GM-CSF plays a major role in upregulation of co-stimulatory molecules [153]. IL-4 and GM-CSF help in the differentiation of $CD14^+$ monocytes into immature DCs [154]. Our transduction results showed that the LV system was functional with strong expression of cytokines (GM-CSF: 0.28 ng/mL and IL-4 0.78 ng/mL) in 293T transduced cells **(Figure 13D)**. As expected, the enriched $CD14^+$ cells transduced with SIN LV-G242pp65 in 3 healthy CMV-seropositive donors successfully differentiated into DCs without exogenous addition of GM-CSF and IL-4. Also, these SmartDCpp65 showed typical DC morphology (**Figure 14A**, dendrites) and were comparable to the ConvDCs cultured in the presence of recombinant cytokines (GM-CSF and IL-4) added exogenously. Both ConvDCs and SmartDCs displayed a decrease in expression of the monocytic marker CD14 and the induction of typical DC markers such as CD209, HLA-ABC, and HLA-DR **(Figure 14C)**. High expression of co-stimulatory molecules such as CD83 and CD86 in both SmartDCs and ConvDCs indicated maturation towards dendritic cells and pertinent immunophenotypic markers as described in previous studies [155, 156].

5.1.4 Characterisation of in vitro expanded T cells and CMV-CTLs using SmartDCpp65

Several studies have demonstrated that LV reprogrammed DCs can successfully prime and generate high numbers of CMV-CTLs both in vitro and in vivo (mouse models) [149, 150, 154]. Salguero et al. have shown that SmartDCs expressing CMVpp65 induce an increase in the expansion of effector memory cells upon in vitro T cell stimulation [154, 157]. In line with these findings, we observed higher HLA B07:02 CMV-CTL expansion **(Figure 15B)**, $CD8^+$ effector memory (EM; $CD45RA^-CCR7^-$) and terminal effector memory cells (T_{EMRA}; $CD45RA^+CCR7^-$) as compared to results for T cells treated with the untransduced or unloaded DCs.

5.1.5 Expression of markers associated with senescence and exhaustion on EM and T_{EMRA} subsets of expanded $CD8^+$ T cells

To produce sufficient numbers of CMV-CTLs in vitro, repetitive priming of T cells is necessary. This repetitive priming could lead to replicative senescence (expression of CD57) and exhaustion (expression of PD1 and Tim3) of T cells as well as antigen-specific T cells [158]. The CD57 expression on T lymphocytes is correlated with replicative senescence[159] which could lead to activation-induced cell death (AICD) and clonal exhaustion [160-163]. In line with this, we observed an increase in CD57 expression on EM and T_{EMRA} $CD8^+$ T cells expanded with ConvDCpp65 and SmartDCpp65 **(Figure 16C and 16D)**.

On the other hand, PD1 and Tim3 expression are implicated in inhibition of both adaptive and innate immune responses [164]. PD1 has an antagonistic function on the activation of $CD8^+$ T cells to minimize the tissue damage upon inflammation [165]. Moreover, PD1 ligands such as B7H1 and B7DC play a crucial role in the control of human T cell activation and in maintaining peripheral immune tolerance [161]. The increase in the expression of PD1 and Tim3 results in the secretion of anti-inflammatory cytokines (e.g. IL-10) which in turn can hinder

Th1 cytokines and inhibit pro-inflammatory cytokines [160, 162, 166-168]. An increase of PD1 expression (EM) and Tim3 expression (EM and T_{EMRA}) in T cells expanded with ConvDCpp65 and SmartDCpp65 shows a trend towards exhaustion **(Figure 16C and 16D)**. The expression of CD57, PD1, and Tim3 indicates that extensive *in-vitro* expansion could lead to the accumulation of senescence and exhaustion markers. This, in turn, could hamper the efficacy of CMV-CTLs.

In conclusion, our data suggests that the antigen presenting cells generated conventionally by using exogenous GM-CSF & IL-4 or by LV based-transduction showed no significant morphological differences. The expansion of CMV-CTLs by these cells revealed no difference and deleterious phenotypical changes in memory $CD8^+$ T cells. These results indicate that both ConvDCpp65 and SmartDCpp65 expanded T cells showed a comparable phenotype.

6. Conclusions and future work

In order to improve treatment of CMV-reactivation in the context of HSCT, several issues need to be addressed:

(1) First, the patients at highest risk for CMV-R need to be identified as early as possible to enable timely planning and implementation of alternative and advanced therapies like adoptive immuno-therapy. From our study cohort, we have understood that CMV-R prior to or post aGvHD grade II-IV and aGvHD grade II-IV alone are high risk factors for mortality of patients post allo-HSCT. Our data also suggests that CMV-R increases the risk of mortality in aGvHD-patients. Additionally, we also observed that CMV-R leads to the expansion or reconstitution of $CD8^+$ T cells that could potentially lead to the development of aGvHD by the secretion of several pro-inflammatory cytokines like IL1Rα, IL18, Hu TRAIL, MIP-1β and TNF-α. However, it is known that recurrent CMV-R could lead to the development of aGVHD.[44, 53, 77] Along with this several clinical parameters such as age of the patient, conditioning, steroids, matched unrelated donor, mismatched graft, and aGvHD leads to the reactivation of CMV-R.[49, 71, 81] But, so far the cellular mechanism that is underlying for the reactivation of latent CMV is not well understood.

However, $CD14^+$ monocytes carry the latent genes of CMV. Several genes such as, IE1, pp65 and transcription factors can be upregulated and involved that lead to the reactivation of CMV-R. Several other studies show that elevated levels of cytokines such as IL6, IL8 and TNF-a can reactivate CMV-R.[169] So far the mechanism and the interaction of these cytokines on $CD14^+$ cells is not well understood and still need to be further explored. This might provide insights in the mechanism that leads to CMV-R and may help in developing better strategies for CMV management post allo-HSCT.

(2) Next, a suitable donor for specific T cells needs to be identified. To this end, CMV-seropositivity alone might not suffice to deem a donor suitable: Our approach in exploring the

novel methods to produce/select CMV-CTLs revealed that G-CSF mobilised donor grafts to select CMV-CTLs would not be a good option. Our finding suggests that G-CSF mobilization impairs antiviral T cells characteristics such as proliferation, secretion of inflammatory cytokines and cytotoxicity. The ideal time for CMV-CTL collection should be further investigated with regard to donor mobilization.

(3) Finally, if the CMV-CTL number in the donor does not suffice or the donor is CMV-seronegative, effective techniques for in vitro generation and expansion of CMV-CTL need to be at hand. Additionally, the number of CMV-CTL in the donor and their functionality or it's impairment (due to exhaustion/senescence in elderly donors, effects of certain medication etc.) should also be taken into account. Our preliminary results showed that ConvDC loaded with CMVpp65 peptide pool and SmartDC co-expressing CMVpp65 expanded T cells induced CMV-CTL expansion in a comparable rate. In order to prove the efficiency of expanded T cells, the further examination of functional properties such as specificity (CMV-CTL yield), cell viability, senescence and exhaustion of these expanded T cells has to be further validated.

(4) Our recently published retrospective analysis (PMID 30889204) showed that the presence of CMV-CTLs three months after allo-SCT is associated with a reduced incidence of early relapses in the CMV R+/D+ setting. This observation should be further investigated and confirmed by studies in larger and prospective cohorts. Unravelling the mechanisms of the potential protective effects of CMV-CTLs against relapse might add further relevance to monitoring of CMV-CTL and also adoptive transfer of CMV-CTL and its timing following HSCT.

7. References

1. Cheuk DKL. Optimal stem cell source for allogeneic stem cell transplantation for hematological malignancies. *World Journal of Transplantation* 2013; **3**(4)**:** 99-112. doi: 10.5500/wjt.v3.i4.99

2. Thomas ED, Lochte HLJ, Lu WC, Ferrebee JW. Intravenous Infusion of Bone Marrow in Patients Receiving Radiation and Chemotherapy. *New England Journal of Medicine* 1957; **257**(11)**:** 491-496. doi: doi:10.1056/NEJM195709122571102

3. Appelbaum FR. Hematopoietic-Cell Transplantation at 50. *New England Journal of Medicine* 2007; **357**(15)**:** 1472-1475. doi: doi:10.1056/NEJMp078166

4. Gatti R, Meuwissen H, Allen H, Hong R, Good R. IMMUNOLOGICAL RECONSTITUTION OF SEX-LINKED LYMPHOPENIC IMMUNOLOGICAL DEFICIENCY. *The Lancet* 1968; **292**(7583)**:** 1366-1369. doi: http://dx.doi.org/10.1016/S0140-6736(68)92673-1

5. E G, A M, A S, C C, E M, A W-F *et al. Hematopoietic Stem Cell Transplantation*, 6th edn forum service editor: Genova, Italy, 2012.

6. Park M, Seo JJ. Role of HLA in Hematopoietic Stem Cell Transplantation. *Bone Marrow Research* 2012; **2012:** 7. doi: 10.1155/2012/680841

7. Morishima Y, Sasazuki T, Inoko H, Juji T, Akaza T, Yamamoto K *et al.* The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. *Blood* 2002; **99**(11)**:** 4200-4206. doi: 10.1182/blood.V99.11.4200

8. Petersdorf EW, Hansen JA, Martin PJ, Woolfrey A, Malkki M, Gooley T *et al.* Major-Histocompatibility-Complex Class I Alleles and Antigens in Hematopoietic-Cell Transplantation. *New England Journal of Medicine* 2001; **345**(25)**:** 1794-1800. doi: doi:10.1056/NEJMoa011826

9. Li HW, Sykes M. Emerging concepts in haematopoietic cell transplantation. *Nat Rev Immunol* 2012; **12**(6)**:** 403-416.

10. Kufe DW, Holland JF, et al. *Cancer medicine 6*, 6th edn Hamilton, Ont. ; Lewiston, NY: Canada, 2003.

11. Gonçalves TL, Benvegnú DM, Bonfanti G. Specific factors influence the success of autologous and allogeneic hematopoietic stem cell transplantation. *Oxidative Medicine and Cellular Longevity* 2009; **2**(2)**:** 82-87.

12. Miller CB, Piantadosi S, Vogelsang GB, Marcellus DC, Grochow L, Kennedy MJ *et al.* Impact of age on outcome of patients with cancer undergoing autologous bone marrow transplant. *Journal of Clinical Oncology* 1996; **14**(4)**:** 1327-1332.

13. Bartlett JM, Stirling D. A Short History of the Polymerase Chain Reaction. In, vol. 226, 2003, pp 3-6.

14. Nowak J. Role of HLA in hematopoietic SCT. *Bone Marrow Transplant* 2008; **42**(S2)**:** S71-S76.

15. van Rood JJ, Lagaaij EL, Doxiadis I, Roelen D, Persijn G, Claas F. Permissible mismatches, acceptable mismatches, and tolerance: new trends in decision making. *Clinical transplants* 1993**:** 285-292.

16. Myers GD, Bollard CM, Wu MF, Weiss H, Rooney CM, Heslop HE *et al.* Reconstitution of adenovirus-specific cell-mediated immunity in pediatric patients after hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2007; **39**(11)**:** 677-686.

17. Lechler RI, Lombardi G, Batchelor JR, Reinsmoen N, Bach FH. The molecular basis of alloreactivity. *Immunology today* 1990; **11**(3)**:** 83-88.

18. Parody R, Martino R, Rovira M, Vazquez L, Vázquez MJ, de la Cámara R *et al.* Severe Infections after Unrelated Donor Allogeneic Hematopoietic Stem Cell Transplantation in Adults: Comparison of Cord Blood Transplantation with Peripheral Blood and Bone Marrow Transplantation. *Biology of Blood and Marrow Transplantation* 2006; **12**(7)**:** 734-748. doi: http://dx.doi.org/10.1016/j.bbmt.2006.03.007

19. Du J, Liu J, Gu J, Zhu P. HLA-DRB1*09 Is Associated with Increased Incidence of Cytomegalovirus Infection and Disease after Allogeneic Hematopoietic Stem Cell Transplantation. *Biology of Blood and Marrow Transplantation* 2007; **13**(12)**:** 1417-1421. doi: http://dx.doi.org/10.1016/j.bbmt.2007.09.003

20. Petersdorf EW, Mickelson EM, Anasetti C, Martin PJ, Woolfrey AE, Hansen JA. Effect of HLA mismatches on the outcome of hematopoietic transplants. *Current Opinion in Immunology* 1999; **11**(5)**:** 521-526. doi: http://dx.doi.org/10.1016/S0952-7915(99)00016-3

21. Bacigalupo A, Ballen K, Rizzo D, Giralt S, Lazarus H, Ho V *et al.* DEFINING THE INTENSITY OF CONDITIONING REGIMENS : working definitions. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2009; **15**(12)**:** 1628-1633. doi: 10.1016/j.bbmt.2009.07.004

22. Pérez-Simón JA, Díez-Campelo M, Martino R, Brunet S, Urbano Á, Caballero MD *et al.* Influence of the intensity of the conditioning regimen on the characteristics of acute and chronic graft-versus-host disease after allogeneic transplantation. *British Journal of Haematology* 2005; **130**(3)**:** 394-403. doi: 10.1111/j.1365-2141.2005.05614.x

23. Clift RA, Buckner CD, Appelbaum FR, Bearman SI, Petersen FB, Fisher LD *et al.* Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens. *Blood* 1990; **76**.

24. Schmid C, Schleuning M, Schwerdtfeger R, Hertenstein B, Mischak-Weissinger E, Bunjes D *et al.* Long-term survival in refractory acute myeloid leukemia after sequential treatment with chemotherapy and reduced-intensity conditioning for allogeneic stem cell transplantation. *Blood* 2006; **108**(3)**:** 1092-1099. doi: 10.1182/blood-2005-10-4165

25. Schmid C, Schleuning M, Hentrich M, Markl GE, Gerbitz A, Tischer J *et al.* High antileukemic efficacy of an intermediate intensity conditioning regimen for allogeneic stem cell transplantation in patients with high-risk acute myeloid leukemia in first complete remission. *Bone Marrow Transplant* 2008; **41**(8)**:** 721-727.

26. Marek A, Stern M, Chalandon Y, Ansari M, Ozsahin H, Gungor T *et al.* The impact of T-cell depletion techniques on the outcome after haploidentical hematopoietic SCT. *Bone Marrow Transplant* 2014; **49**(1)**:** 55-61. doi: 10.1038/bmt.2013.132

27. Storb R, Deeg HJ, Fisher L, Appelbaum F, Buckner CD, Bensinger W *et al.* Cyclosporine v methotrexate for graft-v-host disease prevention in patients given marrow grafts for leukemia: long-term follow-up of three controlled trials. *Blood* 1988; **71**.

28. Storb R, Deeg HJ, Whitehead J, Appelbaum F, Beatty P, Bensinger W *et al.* Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. *N Engl J Med* 1986; **314**. doi: 10.1056/nejm198603203141201

29. Rubio MT, Labopin M, Blaise D, Socié G, Contreras RR, Chevallier P *et al.* The impact of graft-versus-host disease prophylaxis in reduced-intensity conditioning allogeneic stem cell transplant in acute myeloid leukemia: a study from the Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. *Haematologica* 2015; **100**(5)**:** 683-689. doi: 10.3324/haematol.2014.119339

30. Hambach L, Stadler M, Dammann E, Ganser A, Hertenstein B. Increased risk of complicated CMV infection with the use of mycophenolate in allogeneic stem cell transplantation. *Bone Marrow Transplant* 2002; **29:** 903-906. e-pub ahead of print 7 march 2002;

31. Weissinger EM, Borchers S, Silvani A, Provasi E, Radrizzani M, Beckmann IK *et al.* Long term follow up of patients after allogeneic stem cell transplantation and transfusion of HSV-TK transduced T-cells. *Frontiers in Pharmacology* 2015; **6:** 76. doi: 10.3389/fphar.2015.00076

32. Ciceri F, Bonini C, Stanghellini MTL, Bondanza A, Traversari C, Salomoni M *et al.* Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I–II study. *The Lancet Oncology*; **10**(5)**:** 489-500. doi: 10.1016/S1470-2045(09)70074-9

33. Hakki M, Riddell SR, Storek J, Carter RA, Stevens-Ayers T, Sudour P *et al.* Immune reconstitution to cytomegalovirus after allogeneic hematopoietic stem cell transplantation: impact of host factors, drug therapy, and subclinical reactivation. *Blood* 2003; **102**(8)**:** 3060-3067. doi: 10.1182/blood-2002-11-3472

34. Cwynarski K, Ainsworth J, Cobbold M, Wagner S, Mahendra P, Apperley J *et al.* Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. *Blood* 2001; **97**(5)**:** 1232-1240. doi: 10.1182/blood.V97.5.1232

35. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol* 2012; **12**(6)**:** 443-458.

36. Subramaniam DS, Fowler DH, Pavletic SZ. Chronic graft-versus-host disease in the era of reduced-intensity conditioning. *Leukemia* 2007; **21**(5)**:** 853-859.

37. Krenger W, Ferrara JL. Dysregulation of cytokines during graft-versus-host disease. *Journal of hematotherapy* 1996; **5**(1)**:** 3-14. doi: 10.1089/scd.1.1996.5.3

38. Jacobsohn DA, Vogelsang GB. Acute graft versus host disease. *Orphanet Journal of Rare Diseases* 2007; **2**(1)**:** 1-9. doi: 10.1186/1750-1172-2-35

39. Podgorny PJ, Liu Y, Dharmani-Khan P, Pratt LM, Jamani K, Luider J *et al.* Immune Cell Subset Counts Associated with Graft-versus-Host Disease. *Biology of Blood and Marrow Transplantation* 2014; **20**(4)**:** 450-462. doi: http://dx.doi.org/10.1016/j.bbmt.2014.01.002

40. Huttunen P, Taskinen M, Siitonen S, Saarinen-Pihkala UM. Impact of very early CD4+/CD8+ T cell counts on the occurrence of acute graft-versus-host disease and NK cell counts on outcome after pediatric allogeneic hematopoietic stem cell transplantation. *Pediatric Blood & Cancer* 2015; **62**(3)**:** 522-528. doi: 10.1002/pbc.25347

41. Hill GR, Ferrara JL. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood* 2000; **95**.

42. Ferrara JL, Cooke KR, Teshima T. The pathophysiology of acute graft-versus-host disease. *International journal of hematology* 2003; **78**(3)**:** 181-187.

43. Auletta JJ, Lazarus HM. Immune restoration following hematopoietic stem cell transplantation: an evolving target. *Bone Marrow Transplant* 2005; **35**(9)**:** 835-857.

44. Cantoni N, Hirsch HH, Khanna N, Gerull S, Buser A, Bucher C *et al.* Evidence for a Bidirectional Relationship between Cytomegalovirus Replication and acute Graft-versus-Host Disease. *Biology of Blood and Marrow Transplantation* 2010; **16**(9)**:** 1309-1314. doi: http://dx.doi.org/10.1016/j.bbmt.2010.03.020

45. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA *et al.* Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation* 1974; **18**(4)**:** 295-304.

46. Garnett C, Apperley JF, Pavlů J. Treatment and management of graft-versus-host disease: improving response and survival. *Therapeutic Advances in Hematology* 2013; **4**(6)**:** 366-378. doi: 10.1177/2040620713489842

47. Hill GR, Krenger W, Ferrara JL. The role of cytokines in acute graft-versus-host disease. *Cytokines, cellular & molecular therapy* 1997; **3**(4)**:** 257-266.

48. Özdemir E, St. John LS, Gillespie G, Rowland-Jones S, Champlin RE, Molldrem JJ *et al.* Cytomegalovirus reactivation following allogeneic stem cell transplantation is associated with the presence of dysfunctional antigen-specific CD8+ T cells. *Blood* 2002; **100**(10)**:** 3690-3697. doi: 10.1182/blood-2002-05-1387

49. Ljungman P. CMV infections after hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2008; **42**(S1)**:** S70-S72.

50. Ljungman P, Hakki M, Boeckh M. Cytomegalovirus in Hematopoietic Stem Cell Transplant Recipients. *Infectious Disease Clinics of North America* 2010; **24**(2)**:** 319-337. doi: http://dx.doi.org/10.1016/j.idc.2010.01.008

51. Loewendorf A, Benedict CA. Modulation of host innate and adaptive immune defenses by cytomegalovirus: timing is everything. *J Intern Med* 2010; **267**(5)**:** 483-501. e-pub ahead of print 2010/05/04; doi: JIM2220 [pii] 10.1111/j.1365-2796.2010.02220.x

52. Boeckh M, Ljungman P. How we treat cytomegalovirus in hematopoietic cell transplant recipients. *Blood* 2009; **113**(23)**:** 5711-5719. doi: 10.1182/blood-2008-10-143560

53. McCarthy AL, Peiris JSM, Taylor CE, Green MA, Sviland L, Pearson ADJ *et al.* Increase in severity of graft versus host disease by cytomegalovirus. *J Clin Pathol* 1992; **45:** 542-544.

54. Arvin AM. *Human herpesviruses : biology, therapy, and immunoprophylaxis*, Cambridge University Press: Cambridge ; New York, 2007.

55. Gautheret-Dejean A, Aubin JT, Poirel L, Huraux JM, Nicolas JC, Rozenbaum W *et al.* Detection of human Betaherpesvirinae in saliva and urine from immunocompromised and immunocompetent subjects. *Journal of Clinical Microbiology* 1997; **35**(6)**:** 1600-1603.

56. Plotkin SA. Vaccines for varicella-zoster virus and cytomegalovirus: recent progress. *Science* 1994; **265**(5177)**:** 1383-1385.

57. Craig JM, Macauley JC, Weller TH, Wirth P. Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion

disease. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine* 1957; **94**(1)**:** 4-12.

58. Biron KK. Antiviral drugs for cytomegalovirus diseases. *Antiviral Research* 2006; **71**(2–3)**:** 154-163. doi: http://dx.doi.org/10.1016/j.antiviral.2006.05.002

59. Boeckh M, Bowden RA, Gooley T, Myerson D, Corey L. Successful Modification of a pp65 Antigenemia-Based Early Treatment Strategy for Prevention of Cytomegalovirus Disease in Allogeneic Marrow Transplant Recipients. *Blood* 1999; **93**(5)**:** 1781-1782.

60. Ljungman P, Aschan J, Lewensohn-Fuchs I, Carlens S, Larsson K, Lonnqvist B *et al.* Results of different strategies for reducing cytomegalovirus-associated mortality in allogeneic stem cell transplant recipients. *Transplantation* 1998; **66**(10)**:** 1330-1334.

61. Ljungman P, Engelhard D, Link H, Biron P, Brandt L, Brunet S *et al.* Treatment of Interstitial Pneumonitis Due to Cytomegalovirus with Ganciclovir and Intravenous Immune Globulin: Experience of European Bone Marrow Transplant Group. *Clinical Infectious Diseases* 1992; **14**(4)**:** 831-835. doi: 10.1093/clinids/14.4.831

62. Zaia JA. Prevention of cytomegalovirus disease in hematopoietic stem cell transplantation. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2002; **35**(8)**:** 999-1004. doi: 10.1086/342883

63. Plotkin SA, Higgins R, Kurtz JB, Morris PJ, Campbell DA, Jr., Shope TC *et al.* Multicenter trial of Towne strain attenuated virus vaccine in seronegative renal transplant recipients. *Transplantation* 1994; **58**(11)**:** 1176-1178.

64. Odendahl M, Grigoleit GU, Bönig H, Neuenhahn M, Albrecht J, Anderl F *et al.* Clinical-scale isolation of ‘minimally manipulated’ cytomegalovirus-specific donor lymphocytes for the treatment of refractory cytomegalovirus disease. *Cytotherapy* 2014; **16**(9)**:** 1245-1256. doi: http://dx.doi.org/10.1016/j.jcyt.2014.05.023

65. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED *et al.* Reconstitution of Cellular Immunity against Cytomegalovirus in Recipients of Allogeneic Bone Marrow by Transfer of T-Cell Clones from the Donor. *New England Journal of Medicine* 1995; **333**(16)**:** 1038-1044. doi: doi:10.1056/NEJM199510193331603

66. Schmitt A, Tonn T, Busch DH, Grigoleit GU, Einsele H, Odendahl M *et al.* Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. *Transfusion* 2011; **51**(3)**:** 591-599. doi: 10.1111/j.1537-2995.2010.02940.x

67. Boeckh M, Murphy WJ, Peggs KS. Reprint of: Recent Advances in Cytomegalovirus: An Update on Pharmacologic and Cellular Therapies. *Biology of Blood and Marrow Transplantation* 2015; **21**(2, Supplement)**:** S19-S24. doi: http://dx.doi.org/10.1016/j.bbmt.2014.12.034

68. Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H *et al.* Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA–peptide tetramers. *The Journal of Experimental Medicine* 2005; **202**(3)**:** 379-386. doi: 10.1084/jem.20040613

69. Kumaresan P, Figliola M, Moyes JS, Huls MH, Tewari P, Shpall EJ *et al.* Automated Cell Enrichment of Cytomegalovirus-specific T cells for Clinical Applications using the Cytokine-capture System. 2015; (104)**:** e52808. doi: doi:10.3791/52808

70. Neuenhahn M, Albrecht J, Odendahl M, Schlott F, Dössinger G, Schiemann M *et al.* Transfer of minimally manipulated CMV-specific T cells from stem cell or third-party donors to treat CMV infection after allo-HSCT. *Leukemia* 2017; **31:** 2161. doi: 10.1038/leu.2017.16 https://www.nature.com/articles/leu201716#supplementary-information

71. Ozdemir E, Saliba RM, Champlin RE, Couriel DR, Giralt SA, de Lima M *et al.* Risk factors associated with late cytomegalovirus reactivation after allogeneic stem cell transplantation for hematological malignancies. *Bone Marrow Transplant* 2007; **40**(2)**:** 125-136.

72. Ljungman P, Perez-Bercoff L, Jonsson J, Avetisyan G, Sparrelid E, Aschan J *et al.* *Risk factors for the development of cytomegalovirus disease after allogeneic stem cell transplantation*, vol. 91. 2006.

73. Wu J-L, Ma H-Y, Lu C-Y, Chen J-M, Lee P-I, Jou S-T *et al.* Risk factors and outcomes of cytomegalovirus viremia in pediatric hematopoietic stem cell transplantation patients. *Journal of Microbiology, Immunology and Infection*. doi: http://dx.doi.org/10.1016/j.jmii.2015.07.011

74. Walker CM, van Burik J-AH, De For TE, Weisdorf DJ. Cytomegalovirus Infection after Allogeneic Transplantation: Comparison of Cord Blood with Peripheral Blood and Marrow Graft Sources. *Biology of Blood and Marrow Transplantation* 2007; **13**(9)**:** 1106-1115. doi: http://dx.doi.org/10.1016/j.bbmt.2007.06.006

75. Martino R, Rovira M, Carreras E, Solano C, Jorge S, De La Rubia J *et al.* Severe infections after allogeneic peripheral blood stem cell transplantation: a matched-pair comparison of unmanipulated and CD34+ cell-selected transplantation. *Haematologica* 2001; **86**(10)**:** 1075-1086.

76. Marty FM, Bryar J, Browne SK, Schwarzberg T, Ho VT, Bassett IV *et al.* Sirolimus-based graft-versus-host disease prophylaxis protects against cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation: a cohort analysis. *Blood* 2007; **110**(2)**:** 490-500. doi: 10.1182/blood-2007-01-069294

77. Miller W, Flynn P, McCullough J, Balfour HH, Jr., Goldman A, Haake R *et al.* Cytomegalovirus infection after bone marrow transplantation: an association with acute graft-v-host disease. *Blood* 1986; **67**(4)**:** 1162-1167.

78. Einsele H, Hebart H, Kauffmann-Schneider C, Sinzger C, Jahn G, Bader P *et al.* Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for

CMV infection. *Bone Marrow Transplant* 2000; **25**(7)**:** 757-763. doi: 10.1038/sj.bmt.1702226

79. Jaskula E, Bochenska J, Kocwin E, Tarnowska A, Lange A. CMV Serostatus of Donor-Recipient Pairs Influences the Risk of CMV Infection/Reactivation in HSCT Patients. *Bone Marrow Research* 2012; **2012:** 375075. doi: 10.1155/2012/375075

80. Acar K, Akı ŞZ, Özkurt ZN, Bozdayı G, Rota S, Türköz Sucak G. Factors Associated with Cytomegalovirus Reactivation Following Allogeneic Hematopoietic Stem Cell Transplantation: Human Leukocyte Antigens Might Be Among the Risk Factors. *Turkish Journal of Hematology* 2014; **31**(3)**:** 276-285. doi: 10.4274/Tjh.2013.0244

81. Ljungman P, Brand R, Einsele H, Frassoni F, Niederwieser D, Cordonnier C. *Donor CMV serologic status and outcome of CMV-seropositive recipients after unrelated donor stem cell transplantation: an EBMT megafile analysis*, vol. 102. 2003.

82. Boeckh M, Nichols WG. The impact of cytomegalovirus serostatus of donor and recipient before hematopoietic stem cell transplantation in the era of antiviral prophylaxis and preemptive therapy. *Blood* 2004; **103**(6)**:** 2003-2008. doi: 10.1182/blood-2003-10-3616

83. La Rosa C, Diamond DJ. The immune response to human CMV. *Future virology* 2012; **7**(3)**:** 279-293. doi: 10.2217/fvl.12.8

84. Lacey SF, Diamond DJ, Zaia JA. Assessment of cellular immunity to human cytomegalovirus in recipients of allogeneic stem cell transplants. *Biology of Blood and Marrow Transplantation* 2004; **10**(7)**:** 433-447. doi: http://dx.doi.org/10.1016/j.bbmt.2003.12.004

85. Borchers S, Ogonek J, Varanasi PR, Tischer S, Bremm M, Eiz-Vesper B *et al.* Multimer monitoring of CMV-specific T cells in research and in clinical applications. *Diagnostic Microbiology and Infectious Disease* 2014; **78**(3)**:** 201-212. doi: http://dx.doi.org/10.1016/j.diagmicrobio.2013.11.007

86. Altman JD, Moss PAH, Goulder PJR, Barouch DH, McHeyzer-Williams MG, Bell JI *et al.* Phenotypic Analysis of Antigen-Specific T Lymphocytes. *Science* 1996; **274**(5284)**:** 94-96. doi: 10.1126/science.274.5284.94

87. Borchers S, Luther S, Lips U, Hahn N, Kontsendorn J, Stadler M *et al.* Tetramer monitoring to assess risk factors for recurrent cytomegalovirus reactivation and reconstitution of antiviral immunity post allogeneic hematopoietic stem cell transplantation. *Transplant Infectious Disease* 2011; **13**(3)**:** 222-236. doi: 10.1111/j.1399-3062.2011.00626.x

88. Koehl U, Dirkwinkel E, Koenig M, Erben S, Soerensen J, Bader P *et al.* Reconstitution of Cytomegalovirus Specific T Cells after Pediatric Allogeneic Stem Cell Transplantation: Results from a Pilot Study Using a Multi-Allele CMV Tetramer Group. *Klin Padiatr* 2008; **220**(06)**:** 348-352. doi: 10.1055/s-0028-1086029

89. Gratama JW, Boeckh M, Nakamura R, Cornelissen JJ, Brooimans RA, Zaia JA *et al.* Immune monitoring with iTAg MHC Tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study. *Blood* 2010; **116**(10)**:** 1655-1662. doi: 10.1182/blood-2010-03-273508

90. Borchers S, Bremm M, Lehrnbecher T, Dammann E, Pabst B, Wölk B *et al.* Sequential Anti-Cytomegalovirus Response Monitoring May Allow Prediction of Cytomegalovirus Reactivation after Allogeneic Stem Cell Transplantation. *PLoS ONE* 2012; **7**(12)**:** e50248. doi: 10.1371/journal.pone.0050248

91. Bhutani D, Dyson G, Manasa R, Deol A, Ratanatharathorn V, Ayash L *et al.* Incidence, Risk Factors, and Outcome of Cytomegalovirus Viremia and Gastroenteritis in Patients with Gastrointestinal Graft-versus-Host Disease. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2015; **21**(1)**:** 159-164. doi: 10.1016/j.bbmt.2014.10.004

92. Henden AS, Hill GR. Cytokines in Graft-versus-Host Disease. *The Journal of Immunology* 2015; **194**(10)**:** 4604-4612. doi: 10.4049/jimmunol.1500117

93. van de Berg PJ, Heutinck KM, Raabe R, Minnee RC, Young SL, van Donselaar-van der Pant KA *et al.* Human Cytomegalovirus Induces Systemic Immune Activation Characterized by a Type 1 Cytokine Signature. *Journal of Infectious Diseases* 2010; **202**(5)**:** 690-699. doi: 10.1086/655472

94. Castiello L, Sabatino M, Jin P, Clayberger C, Marincola FM, Krensky AM *et al.* Monocyte-derived DC maturation strategies and related pathways: a transcriptional view. *Cancer immunology, immunotherapy : CII* 2011; **60**(4)**:** 457-466. doi: 10.1007/s00262-010-0954-6

95. Jonuleit H, Kühn U, Müller G, Steinbrink K, Paragnik L, Schmitt E *et al.* Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *European Journal of Immunology* 1997; **27**(12)**:** 3135-3142. doi: 10.1002/eji.1830271209

96. Riddell, Watanabe K, Goodrich J, Li C, Agha M, Greenberg P. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science* 1992; **257**(5067)**:** 238-241. doi: 10.1126/science.1352912

97. Riddell SR, Reusser P, Greenberg PD. Cytotoxic T cells specific for cytomegalovirus: a potential therapy for immunocompromised patients. *Reviews of infectious diseases* 1991; **13 Suppl 11:** S966-973.

98. Bunse CE, Borchers S, Varanasi PR, Tischer S, Figueiredo C, Immenschuh S *et al.* Impaired Functionality of Antiviral T Cells in G-CSF Mobilized Stem Cell Donors: Implications for the Selection of CTL Donor. *PLOS ONE* 2013; **8**(12)**:** e77925. doi: 10.1371/journal.pone.0077925

99. Szmania S, Galloway A, Bruorton M, Musk P, Aubert G, Arthur A *et al.* Isolation and expansion of cytomegalovirus-specific cytotoxic T lymphocytes to clinical scale from

a single blood draw using dendritic cells and HLA-tetramers. *Blood* 2001; **98**(3)**:** 505-512. doi: 10.1182/blood.V98.3.505

100. Sili U, Huls MH, Davis AR, Gottschalk S, Brenner MK, Heslop HE *et al.* Large-Scale Expansion of Dendritic Cell-Primed Polyclonal Human Cytotoxic T-Lymphocyte Lines Using Lymphoblastoid Cell Lines for Adoptive Immunotherapy. *Journal of Immunotherapy May/June* 2003; **26**(3)**:** 241-256.

101. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF *et al.* Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat Biotech* 2004; **22**(5)**:** 589-594. doi: http://www.nature.com/nbt/journal/v22/n5/suppinfo/nbt957_S1.html

102. Pincha M, Sundarasetty BS, Stripecke R. Lentiviral vectors for immunization: an inflammatory field. *Expert Review of Vaccines* 2010; **9**(3)**:** 309-321. doi: 10.1586/erv.10.9

103. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ *et al.* Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* 1990; **75**(3)**:** 555-562. e-pub ahead of print 1990/02/01;

104. Walter RB, Gyurkocza B, Storer BE, Godwin CD, Pagel JM, Buckley SA *et al.* Comparison of minimal residual disease as outcome predictor for AML patients in first complete remission undergoing myeloablative or nonmyeloablative allogeneic hematopoietic cell transplantation. *Leukemia* 2015; **29**(1)**:** 137-144. e-pub ahead of print 2014/06/04; doi: 10.1038/leu.2014.173

105. Cho BS, Lee SE, Song HH, Lee JH, Yahng SA, Eom KS *et al.* Graft-versus-tumor effect according to type of graft-versus-host disease defined by National Institutes of Health consensus criteria and associated outcomes. *Biol Blood Marrow Transplant* 2012; **18**(7)**:** 1136-1143. e-pub ahead of print 2012/01/21; doi: 10.1016/j.bbmt.2012.01.010

106. Yoon JH, Kim HJ, Shin SH, Lee SE, Cho BS, Eom KS *et al.* Stratification of de novo adult acute myelogenous leukemia with adverse-risk karyotype: can we overcome the worse prognosis of adverse-risk group acute myelogenous leukemia with hematopoietic stem cell transplantation? *Biol Blood Marrow Transplant* 2014; **20**(1)**:** 80-88. e-pub ahead of print 2013/10/24; doi: 10.1016/j.bbmt.2013.10.015

107. Heuser M, Gabdoulline R, Loffeld P, Dobbernack V, Kreimeyer H, Pankratz M *et al.* Individual outcome prediction for myelodysplastic syndrome (MDS) and secondary acute myeloid leukemia from MDS after allogeneic hematopoietic cell transplantation. *Annals of hematology* 2017; **96**(8)**:** 1361-1372. e-pub ahead of print 2017/06/15; doi: 10.1007/s00277-017-3027-5

108. Diaconescu R, Flowers CR, Storer B, Sorror ML, Maris MB, Maloney DG *et al.* Morbidity and mortality with nonmyeloablative compared with myeloablative conditioning before hematopoietic cell transplantation from HLA-matched related donors. *Blood* 2004; **104**(5)**:** 1550-1558. e-pub ahead of print 2004/05/20; doi: 10.1182/blood-2004-03-0804

109. Storb R, Gyurkocza B, Storer BE, Sorror ML, Blume K, Niederwieser D *et al.* Graft-versus-host disease and graft-versus-tumor effects after allogeneic hematopoietic cell transplantation. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2013; **31**(12)**:** 1530-1538. e-pub ahead of print 2013/03/13; doi: 10.1200/jco.2012.45.0247

110. Hiramoto N, Kurosawa S, Tajima K, Okinaka K, Tada K, Kobayashi Y *et al.* Positive impact of chronic graft-versus-host disease on the outcome of patients with de novo myelodysplastic syndrome after allogeneic hematopoietic cell transplantation: a single-center analysis of 115 patients. *European journal of haematology* 2014; **92**(2)**:** 137-146. e-pub ahead of print 2013/10/17; doi: 10.1111/ejh.12214

111. Suessmuth Y, Mukherjee R, Watkins B, Koura DT, Finstermeier K, Desmarais C *et al.* CMV reactivation drives posttransplant T-cell reconstitution and results in defects in the underlying TCRβ repertoire. *Blood* 2015; **125**(25)**:** 3835-3850. doi: 10.1182/blood-2015-03-631853

112. Lugthart G, van Ostaijen-ten Dam MM, Jol - van der Zijde CM, van Holten TC, Kester MGD, Heemskerk MHM *et al.* Early Cytomegalovirus Reactivation Leaves a Specific and Dynamic Imprint on the Reconstituting T Cell Compartment Long-Term after Hematopoietic Stem Cell Transplantation. *Biology of Blood and Marrow Transplantation* 2014; **20**(5)**:** 655-661. doi: 10.1016/j.bbmt.2014.01.018

113. Cantoni N, Hirsch HH, Khanna N, Gerull S, Buser A, Bucher C *et al.* Evidence for a Bidirectional Relationship between Cytomegalovirus Replication and acute Graft-versus-Host Disease. *Biology of Blood and Marrow Transplant* 2010; **16**(9)**:** 1309-1314. doi: 10.1016/j.bbmt.2010.03.020

114. Kalra A, Williamson T, Daly A, Savoie ML, Stewart DA, Khan F *et al.* Impact of Donor and Recipient Cytomegalovirus Serostatus on Outcomes of Antithymocyte Globulin 2013;Conditioned Hematopoietic Cell Transplantation. *Biology of Blood and Marrow Transplantation* 2016; **22**(9)**:** 1654-1663. doi: 10.1016/j.bbmt.2016.05.020

115. Elmaagacli AH, Steckel NK, Koldehoff M, Hegerfeldt Y, Trenschel R, Ditschkowski M *et al.* Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood* 2011; **118**(5)**:** 1402-1412. doi: 10.1182/blood-2010-08-304121

116. Green ML, Leisenring WM, Xie H, Walter RB, Mielcarek M, Sandmaier BM *et al.* CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood* 2013; **122**(7)**:** 1316-1324. doi: 10.1182/blood-2013-02-487074

117. Inagaki J, Noguchi M, Kurauchi K, Tanioka S, Fukano R, Okamura J. Effect of Cytomegalovirus Reactivation on Relapse after Allogeneic Hematopoietic Stem Cell Transplantation in Pediatric Acute Leukemia. *Biology of Blood and Marrow*

Transplantation 2016; **22**(2)**:** 300-306. doi: http://dx.doi.org/10.1016/j.bbmt.2015.09.006

118. Teira P, Battiwalla M, Ramanathan M, Barrett AJ, Ahn KW, Chen M *et al.* Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: a CIBMTR analysis. *Blood* 2016; **127**(20)**:** 2427-2438. e-pub ahead of print 2016/02/18; doi: 10.1182/blood-2015-11-679639

119. Jeljeli M, Guerin-El Khourouj V, Porcher R, Fahd M, Leveille S, Yakouben K *et al.* Relationship between cytomegalovirus (CMV) reactivation, CMV-driven immunity, overall immune recovery and graft-versus-leukaemia effect in children. *Br J Haematol* 2014; **166**(2)**:** 229-239. e-pub ahead of print 2014/04/08; doi: 10.1111/bjh.12875

120. Verduyn Lunel FM, Raymakers R, van Dijk A, van der Wagen L, Minnema MC, Kuball J. Cytomegalovirus Status and the Outcome of T Cell-Replete Reduced-Intensity Allogeneic Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* 2016; **22**(10)**:** 1883-1887. e-pub ahead of print 2016/07/30; doi: 10.1016/j.bbmt.2016.07.009

121. Sellar RS, Vargas FA, Henry JY, Verfuerth S, Charrot S, Beaton B *et al.* CMV promotes recipient T-cell immunity following reduced-intensity T-cell-depleted HSCT, significantly modulating chimerism status. *Blood* 2015; **125**(4)**:** 731-739. e-pub ahead of print 2014/12/17; doi: 10.1182/blood-2014-07-589150

122. Ogonek J, Varanasi P, Luther S, Schweier P, Kuhnau W, Gohring G *et al.* Possible Impact of Cytomegalovirus-Specific CD8+ T Cells on Immune Reconstitution and Conversion to Complete Donor Chimerism after Allogeneic Stem Cell Transplantation. *Biol Blood Marrow Transplant* 2017; **23**(7)**:** 1046-1053. e-pub ahead of print 2017/03/28; doi: 10.1016/j.bbmt.2017.03.027

123. Khan F, Agarwal A, Agrawal S. Significance of chimerism in hematopoietic stem cell transplantation: new variations on an old theme. *Bone Marrow Transplant* 2004; **34**(1)**:** 1-12.

124. Huisman C, de Weger RA, de Vries L, Tilanus MGJ, Verdonck LF. Chimerism analysis within 6 months of allogeneic stem cell transplantation predicts relapse in acute myeloid leukemia. *Bone Marrow Transplant* 2007; **39**(5)**:** 285-291.

125. van der Torren CR, van Hensbergen Y, Luther S, Aghai Z, Rychnavska ZS, Slot M *et al.* Possible role of minor h antigens in the persistence of donor chimerism after stem cell transplantation; relevance for sustained leukemia remission. *PLoS One* 2015; **10**(3)**:** e0119595. e-pub ahead of print 2015/03/17; doi: 10.1371/journal.pone.0119595

126. Schneidawind D, Federmann B, Faul C, Vogel W, Kanz L, Bethge WA. Allogeneic hematopoietic cell transplantation with reduced-intensity conditioning following FLAMSA for primary refractory or relapsed acute myeloid leukemia. *Annals of hematology* 2013; **92**(10)**:** 1389-1395. doi: 10.1007/s00277-013-1774-5

127. Buchholz S, Dammann E, Stadler M, Krauter J, Beutel G, Trummer A *et al.* Cytoreductive treatment with clofarabine/ara-C combined with reduced-intensity conditioning and allogeneic stem cell transplantation in patients with high-risk, relapsed, or refractory acute myeloid leukemia and advanced myelodysplastic syndrome. *European journal of haematology* 2012; **88**(1)**:** 52-60. doi: 10.1111/j.1600-0609.2011.01703.x

128. Bodinier M, Peyrat MA, Tournay C, Davodeau F, Romagne F, Bonneville M *et al.* Efficient detection and immunomagnetic sorting of specific T cells using multimers of MHC class I and peptide with reduced CD8 binding. *Nat Med* 2000; **6**(6)**:** 707-710. doi: 10.1038/76292

129. Dohner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Buchner T *et al.* Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* 2017; **129**(4)**:** 424-447. e-pub ahead of print 2016/11/30; doi: 10.1182/blood-2016-08-733196

130. Shulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE *et al.* Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *The American journal of medicine* 1980; **69**(2)**:** 204-217. e-pub ahead of print 1980/08/01;

131. Kaplan EL, Meier P. Nonparametric Estimation from Incomplete Observations. *Journal of the American Statistical Association* 1958; **53**(282)**:** 457-481. doi: 10.2307/2281868

132. Gray RJ. A Class of K-Sample Tests for Comparing the Cumulative Incidence of a Competing Risk. *The Annals of Statistics* 1988; **16**(3)**:** 1141-1154.

133. Prentice RL. Introduction to Cox (1972) Regression Models and Life-Tables. In: Kotz S, Johnson NL (eds). *Breakthroughs in Statistics: Methodology and Distribution*. Springer New York: New York, NY, 1992, pp 519-526.

134. Fine JP, Gray RJ. A Proportional Hazards Model for the Subdistribution of a Competing Risk. *Journal of the American Statistical Association* 1999; **94**(446)**:** 496-509. doi: 10.1080/01621459.1999.10474144

135. Kanda Y. Investigation of the freely available easy-to-use software /`EZR/' for medical statistics. *Bone Marrow Transplant* 2013; **48**(3)**:** 452-458.

136. Rubio MT, Savani BN, Labopin M, Polge E, Niederwieser D, Ganser A *et al.* The impact of HLA-matching on reduced intensity conditioning regimen unrelated donor allogeneic stem cell transplantation for acute myeloid leukemia in patients above 50 years-a report from the EBMT acute leukemia working party. *Journal of hematology & oncology* 2016; **9**(1)**:** 65. e-pub ahead of print 2016/08/05; doi: 10.1186/s13045-016-0295-9

137. Peggs KS, Verfuerth S, Pizzey A, Khan N, Guiver M, Moss PA *et al.* Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell

transplantation with virus-specific T-cell lines. *The Lancet* 2003; **362**(9393)**:** 1375-1377. doi: 10.1016/S0140-6736(03)14634-X

138. Parkman R, Cohen G, Carter SL, Weinberg KI, Masinsin B, Guinan E *et al.* Successful Immune Reconstitution Decreases Leukemic Relapse and Improves Survival in Recipients of Unrelated Cord Blood Transplantation. *Biology of Blood and Marrow Transplantation* 2006; **12**(9)**:** 919-927. doi: http://dx.doi.org/10.1016/j.bbmt.2006.05.008

139. Hoegh-Petersen M, Sy S, Ugarte-Torres A, Williamson TS, Eliasziw M, Mansoor A *et al.* High Epstein-Barr virus-specific T-cell counts are associated with near-zero likelihood of acute myeloid leukemia relapse after hematopoietic cell transplantation. *Leukemia* 2012; **26**(2)**:** 359-362. doi: http://www.nature.com/leu/journal/v26/n2/suppinfo/leu2011195s1.html

140. Melenhorst JJ, Castillo P, Hanley PJ, Keller MD, Krance RA, Margolin J *et al.* Graft versus leukemia response without graft-versus-host disease elicited by adoptively transferred multivirus-specific T-cells. *Molecular therapy : the journal of the American Society of Gene Therapy* 2015; **23**(1)**:** 179-183. e-pub ahead of print 2014/10/01; doi: 10.1038/mt.2014.192

141. Seckert CK, Grießl M, Büttner JK, Scheller S, Simon CO, Kropp KA *et al.* Viral latency drives ‘memory inflation’: a unifying hypothesis linking two hallmarks of cytomegalovirus infection. *Medical Microbiology and Immunology* 2012; **201**(4)**:** 551-566. doi: 10.1007/s00430-012-0273-y

142. Tey S-K, Goodrum F, Khanna R. CD8(+) T-cell recognition of human cytomegalovirus latency-associated determinant pUL138. *The Journal of General Virology* 2010; **91**(Pt 8)**:** 2040-2048. doi: 10.1099/vir.0.020982-0

143. Keskinen P, Ronni T, Matikainen S, Lehtonen A, Julkunen I. Regulation of HLA class I and II expression by interferons and influenza A virus in human peripheral blood mononuclear cells. *Immunology* 1997; **91**(3)**:** 421-429.

144. Litjens NHR, van der Wagen L, Kuball J, Kwekkeboom J. Potential Beneficial Effects of Cytomegalovirus Infection after Transplantation. *Frontiers in Immunology* 2018; **9**(389). doi: 10.3389/fimmu.2018.00389

145. Reusser P, Riddell S, Meyers J, Greenberg P. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. *Blood* 1991; **78**(5)**:** 1373-1380.

146. Luo X-H, Huang X-J, Liu K-Y, Xu L-P, Liu D-H. Protective Immunity Transferred by Infusion of Cytomegalovirus-Specific CD8+ T Cells within Donor Grafts: Its Associations with Cytomegalovirus Reactivation Following Unmanipulated Allogeneic Hematopoietic Stem Cell Transplantation. *Biology of Blood and Marrow Transplantation* 2010; **16**(7)**:** 994-1004. doi: http://dx.doi.org/10.1016/j.bbmt.2010.02.007

147. Körbling M, Freireich EJ. Twenty-five years of peripheral blood stem cell transplantation. *Blood* 2011; **117**(24)**:** 6411-6416. doi: 10.1182/blood-2010-12-322214

148. Smith MS, Goldman DC, Bailey AS, Pfaffle DL, Kreklywich CN, Spencer DB *et al.* Granulocyte-Colony Stimulating Factor Reactivates Human Cytomegalovirus in a Latently Infected Humanized Mouse Model. *Cell Host & Microbe* 2010; **8**(3)**:** 284-291. doi: http://doi.org/10.1016/j.chom.2010.08.001

149. Daenthanasanmak A, Salguero G, Sundarasetty BS, Waskow C, Cosgun KN, Guzman CA *et al.* Engineered dendritic cells from cord blood and adult blood accelerate effector T cell immune reconstitution against HCMV. *Molecular Therapy. Methods & Clinical Development* 2015; **1:** 14060. doi: 10.1038/mtm.2014.60

150. Sundarasetty BS, Kloess S, Oberschmidt O, Naundorf S, Kuehlcke K, Daenthanasanmak A *et al.* Generation of lentivirus-induced dendritic cells under GMP-compliant conditions for adaptive immune reconstitution against cytomegalovirus after stem cell transplantation. *Journal of Translational Medicine* 2015; **13:** 240. doi: 10.1186/s12967-015-0599-5

151. Gilboa E. DC-based cancer vaccines. *Journal of Clinical Investigation* 2007; **117**(5)**:** 1195-1203. doi: 10.1172/JCI31205

152. Ribas A, Butterfield LH, Glaspy JA, Economou JS. Current Developments in Cancer Vaccines and Cellular Immunotherapy. *Journal of Clinical Oncology* 2003; **21**(12)**:** 2415-2432. doi: 10.1200/jco.2003.06.041

153. Stripecke R. Lentiviral Vector-Mediated Genetic Programming of Mouse and Human Dendritic Cells. *Methods in Molecular Biology, Methods and Protocols* 2009; **506:** 139-158.

154. Daenthanasanmak A, Salguero G, Borchers S, Figueiredo C, Jacobs R, Sundarasetty BS *et al.* Integrase-defective lentiviral vectors encoding cytokines induce differentiation of human dendritic cells and stimulate multivalent immune responses in vitro and in vivo. *Vaccine* 2012; **30**(34)**:** 5118-5131. doi: http://dx.doi.org/10.1016/j.vaccine.2012.05.063

155. Pincha M, Sai Sundarasetty B, Salguero G, Gutzmer R, Garritsen H, Macke L *et al.* Identity, Potency, In Vivo Viability, and Scaling Up Production of Lentiviral Vector-Induced Dendritic Cells for Melanoma Immunotherapy. *Human Gene Therapy Methods* 2012; **23**(1)**:** 38-55. doi: 10.1089/hgtb.2011.170

156. Sundarasetty BS, Singh VK, Salguero G, Geffers R, Rickmann M, Macke L *et al.* Lentivirus-Induced Dendritic Cells for Immunization Against High-Risk WT1(+) Acute Myeloid Leukemia. *Human Gene Therapy* 2013; **24**(2)**:** 220-237. doi: 10.1089/hum.2012.128

157. Salguero G, Sundarasetty BS, Borchers S, Wedekind D, Eiz-Vesper B, Velaga S *et al.* Preconditioning Therapy with Lentiviral Vector-Programmed Dendritic Cells Accelerates the Homeostatic Expansion of Antigen-Reactive Human T Cells in

NOD.Rag1(−/−).IL-2rγc(−/−) mice. *Human Gene Therapy* 2011; **22**(10)**:** 1209-1224. doi: 10.1089/hum.2010.215

158. Janelle V, Carli C, Taillefer J, Orio J, Delisle J-S. Defining novel parameters for the optimal priming and expansion of minor histocompatibility antigen-specific T cells in culture. *Journal of Translational Medicine* 2015; **13:** 123. doi: 10.1186/s12967-015-0495-z

159. Focosi D, Bestagno M, Burrone O, Petrini M. CD57+ T lymphocytes and functional immune deficiency. *Journal of Leukocyte Biology* 2010; **87**(1)**:** 107-116. doi: 10.1189/jlb.0809566

160. Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 2010; **129**(4)**:** 474-481. doi: 10.1111/j.1365-2567.2010.03255.x

161. Fife BT, Pauken KE. The role of the PD-1 pathway in autoimmunity and peripheral tolerance. *Annals of the New York Academy of Sciences* 2011; **1217**(1)**:** 45-59. doi: 10.1111/j.1749-6632.2010.05919.x

162. Sester U, Presser D, Dirks J, Gärtner BC, Köhler H, Sester M. PD-1 Expression and IL-2 Loss of Cytomegalovirus- Specific T Cells Correlates with Viremia and Reversible Functional Anergy. *American Journal of Transplantation* 2008; **8**(7)**:** 1486-1497. doi: 10.1111/j.1600-6143.2008.02279.x

163. Derhovanessian E, Larbi A, Pawelec G. Biomarkers of human immunosenescence: impact of Cytomegalovirus infection. *Current Opinion in Immunology* 2009; **21**(4)**:** 440-445.

164. Yao S, Wang S, Zhu Y, Luo L, Zhu G, Flies S *et al.* PD-1 on dendritic cells impedes innate immunity against bacterial infection. *Blood* 2009; **113**(23)**:** 5811-5818. doi: 10.1182/blood-2009-02-203141

165. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA *et al.* Tissue expression of PD-L1 mediates peripheral T cell tolerance. *The Journal of Experimental Medicine* 2006; **203**(4)**:** 883-895. doi: 10.1084/jem.20051776

166. Yao S, Chen L. Reviving exhausted T lymphocytes during chronic virus infection by B7-H1 blockade. *Trends in Molecular Medicine* 2006; **12**(6)**:** 244-246. doi: http://dx.doi.org/10.1016/j.molmed.2006.04.007

167. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJD, Suresh M, Altman JD *et al.* Viral Immune Evasion Due to Persistence of Activated T Cells Without Effector Function. *The Journal of Experimental Medicine* 1998; **188**(12)**:** 2205-2213. doi: 10.1084/jem.188.12.2205

168. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. *Nat Med* 2002; **8**(8)**:** 793-800. doi: http://www.nature.com/nm/journal/v8/n8/suppinfo/nm730_S1.html

169. Humar A, St. Louis P, Mazzulli T, McGeer A, Lipton J, Messner H *et al.* Elevated Serum Cytokines Are Associated with Cytomegalovirus Infection and Disease in Bone Marrow Transplant Recipients. *Journal of Infectious Diseases* 1999; **179**(2)**:** 484-488. doi: 10.1086/314602

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PUBLICATIONS

Varanasi P, Ogonek J et al. *(2019)* Cytomegalovirus-specific CD8+ T-cells are associated with a reduced incidence of early relapse after allogenic stem cell transplantation. *PLoS One. 2019 Mar 19;14(3):e0213739. doi: 10.1371/journal.pone.0213739. eCollection 2019.*

Ogonek J, Verma K, Schultze-Florey C, Varanasi P, Luther S, Schweier P et al. (2017) Characterization of High- Avidity Cytomegalovirus-Specific T-Cells with Differential Tetramer Binding Coappearing after Allogeneic Stem Cell Transplantation. *The Journal of Immunology; 199(2): 792-805. doi: 10.4049/jimmunol.1601992*

Verma K, Ogonek J, Varanasi PR, Luther S, Bünting I, Thomay K, et al. (2017) Human CD8+ CD57- TEMRA cells: Too young to be called "old". *PLoS ONE 12(5): e0177405. https://doi.org/10.1371/journal.pone.0177405.*

Ogonek J, Varanasi PR, Luther S, Schweier P, Kühnau W, et al. (2017) Possible Impact of Cytomegalovirus-Specific CD8+ T-Cells on Immune Reconstitution and Conversion to Complete Donor Chimerism after Allogeneic Stem Cell Transplantation, *Biology of Blood and Marrow Transplantation.*

Tomić A, Varanasi PR, Golemac M, Malić S, Riese P, Borst EM, et al. (2016) Activation of Innate and Adaptive Immunity by a Recombinant Human Cytomegalovirus Strain Expressing an NKG2D Ligand. *PLoS Pathog 12(12): e1006015. doi:10.1371/journal.ppat.1006015*

Ogonek J, Kralj Juric M, Ghimire S, Varanasi PR, Holler E, Greinix H and Weissinger E (2016) Immune Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation. *Front. Immunol. 7:507. doi: 10.3389/fimmu.2016.00507*

Sylvia Borchers, Justyna Ogonek, Pavankumar R. Varanasi, Sabine Tischer, Melanie Bremm, Britta Eiz-Vesper, Ulrike Koehl, Eva M. Weissinger,(2014) Multimer monitoring of CMV-specific T-cells in research and in clinical applications, *Diagnostic Microbiology and Infectious Disease, Volume 78, Issue 3, Pages 201-212, ISSN 0732-8893, http://dx.doi.org/10.1016/j.diagmicrobio.2013.11.007.*

Bunse CE, Borchers S, Varanasi PR, Tischer S, Figueiredo C, Immenschuh S, et al. (2013) Impaired Functionality of Antiviral T Cells in G-CSF Mobilized Stem Cell Donors: Implications for the Selection of CTL Donor. *PLoS ONE 8(12): e77925. doi:10.1371/journal.pone.0077925*

ORAL PRESENTATIONS/TALKS

European Society for Blood and Marrow Transplantation **(2015)**
41st Annual Meeting, Istanbul, Turkey
Oral Session: Infection Biology
Title: Early Cytomegalovirus reactivation – a potential factor for early robust T-cell reconstitution and possibly a prognostic factor for early robust T-cell reconstitution and prognostic factor for GvHD after hematopoietic stem cell transplantation. *(Varanasi PR, Ogonek J et al.)*

Signal Transduction Society **(2013)**
17th Joint Meeting, Germany
Title: In depth phenotyping of T-cells after expansion in the presence of CMV antigen in vitro with different cytokine cocktails and in vivo. *(Varanasi PR, Borchers S et al)*

POSTER PRESENTATIONS

Joint Annual Meeting: German Society of Infectious Diseases (DGI) (2017)
& German Center of Infectious Research (DZIF), Hamburg, Germany
(Nominated for Best Poster Award)
Cytomegalovirus reactivation prior to acute graft-versus-host disease leads to fast immune reconstitution and overall survival. *(Varanasi PR, Ogonek J et al.)*

European Hematology Association (2016)
Copenhagen, Denmark
Bidirectional Relationship between CMV-R and aGvHD, *(Varanasi PR, Ogonek J et al.)*

European Society for Blood and Marrow Transplantation (2014)
Milan, Italy
Phenotyping and Functional comparison of CMV-CTLs in vitro expanded with conventional and smart dendritic cells. *(Varanasi PR, Borchers S et al.)*

Spring School of Immunology (2014)
Bavaria, Germany
Phenotyping of in vitro expanded CMV-CTLs with conventional and smart DCs. *(Varanasi PR, Borchers S et al.)*

Autumn School of Immunology (2014)
Merseburg, Germany
Optimization of Characterisation and in vitro production strategies for CMV-CTLs for adoptive transfer. *(Varanasi PR, Borchers S et al.)*

OTHER PROFESSIONAL TRAINING

Medizinische Hochschule Hannover, Germany (11.04 - 30.05.2013)
Institute of Biometry
Basic and Advanced Course in Medical Biometry.

Medizinische Hochschule Hannover, Germany (11.09 - 12.09.2012)
Gene Technology, Biosafety and Biosecurity.

REFERENCES

Available on request

Place / Ort Date / Datum Signature / Unterschrift

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10. Erklärung

Hiermit erkläre ich, dass ich die Dissertation „***Immune Response against human Cytomegalovirus in the Context of Hematopoietic Stem Cell Transplantation (HSCT)***“ selbstständig verfasst habe. Ich habe keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder anderer Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Ich habe die Dissertation an folgenden Institutionen angefertigt: Klinik für Hämatologie, Hämostaseologie, Onkologie und Stammzelltransplantation, MHH. Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion* oder für einen ähnlichen Zweck zur Beurteilung bei einer anderen Institution als der MHH eingereicht. Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

***Anmerkung:** Dies ist die **erneute Einreichung der korrigierten und aktualisierten Dissertation**, die vorab unter gleichem Titel und bei der gleichen Institution eingereicht wurde.

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