DISSERTATION

HHV-6-specific T-cell immune reconstitution among children and adolescents after allogeneic stem cell transplantation

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz“.

Christine Maria Schwarz

Graz, 07th of October 2019

Disclosures

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Abbreviations and Definitions

ACTS	Actin
ADV	Adenovirus
AF350	Alexa Fluor 350
aGvHD	Acute Graft-versus-Host Disease
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AMSA	Amsacrine
ATG	Anti-thymocyte globulin
BKV	BK polyomavirus
BM	Bone marrow
BU	Busulfan
CD	Cluster of Differentiation
cGvHD	Chronic Graft-versus-Host Disease
ciHHV-6	Chromosomally integrated HHV-6
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CNS	Central nervous system
CY	Cyclophosphamide
CyA	Cyclosporine A
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EBV	Epstein–Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISPOT	Enzyme Linked Immuno Spot Assay
FACS	Fluorescence-activated cell sorting
FK506	Tacrolimus
FLAMSA	Fludarabine/Amsacrine
FLU	Fludarabine
FMO	Fluorescence minus one
HCT	Haematopoietic cell transplantation
HHV-6	Human herpes virus 6

HLA	Human leucocyte antigen
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
IFN-γ	Interferon gamma
IL	Interleukin
MAC	Myeloablative conditioning
MEDOCS	Medical Documentation and Communication System
MEL	Melphalan
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MRD	HLA-matched related donor
MSD	HLA-matched sibling donor
MTX	Methotrexate
MUD	HLA-matched unrelated donor
NK cell	natural killer cells
PBSC	Peripheral blood stem cells
PCR	Polymerase Chain Reaction
pp65	Phosphoprotein 65
rcf	Relative centrifugal force
RIC	Reduced-intensity conditioning
RSV	Respiratory Syncytial Virus
SEB	Staphylococcal enterotoxin B
SIR	Sirolimus
TBI	Total body irritation
TCR	T-cell receptor
THT	Thiotepa
TNF-α	Tumor necrosis factor
TREO	Treosulfan
U54	Tegument protein
VP16	Etopside
VZV	Varicella Zoster Virus

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Abstract in German

Hintergrund

Das menschliche Herpesvirus 6 (HHV-6) verursacht bei gesunden Individuen nur geringe Symptome, bei immunsupprimierten PatientInnen, zum Beispiel nach humaner Stammzelltransplantation (HSZT), können HHV-6-Reaktivierungen zu Erkrankungen in verschiedenen Organsystemen führen. HHV-6 Reaktivierungen sind oftmals Ursache eines verzögerten Engraftments, Auslöser einer Graft-versus Host Erkrankung oder Trigger für andere Virusreaktivierungen. T-Lymphozyten spielen eine wichtige Rolle bei der Unterdrückung von Virusreaktivierungen. Die Inzidenz der viralen Reaktivierung nach HSZT und deren assoziierte Erkrankungen ist hoch - häufig aufgrund eines Fehlens einer virusspezifischen T-Zell Immunantwort. Über die Entwicklung HHV-6 spezifischer T-Zellen nach allogener HSZT ist nur wenig bekannt. Aus diesem Grund ist das Ziel dieses Projektes die Beschreibung des zeitlichen Ablaufs der HHV-6 spezifischen zellulären Immunrekonstitution nach allogener HSZT bei Kindern und Jugendlichen in Abhängigkeit der Zeit und dem klinischen Verlauf nach HSZT.

Material und Methoden

3, 6, 9, 12, 18 und 24 Monate nach allogener HSZT wurden von PatientInnen periphere mononukleäre Blutzellen isoliert, mit HHV-6-spezifischem Antigen (U54) stimuliert und für 10 Tage in Zellkultur kultiviert. Am Tag 10 wurden die Zellen für 6 Stunden mit dem Virusantigen re-stimuliert und danach mit fluoreszenz-markierten Antikörpern gegen Oberflächenmarker (CD3, CD4, CD8, CD56) und intrazytoplasmatische Aktivierungsmarker (IL-2, IFN-γ, TNF-α) gefärbt. Anschließend wurden die virusspezifischen T-Zellen mittels Durchflusszytometrie gemessen. T-Zellen mit intrazytoplasmatischer Expression dieser Aktivierungsmarker nach Stimulation mit HHV-6-spezifischem Antigen sind nach Definition HHV-6 spezifische T-Zellen. Ihr Nachweis weist somit auf eine HHV-6 spezifische zelluläre Immunität hin. Die HHV-6 spezifische Immunität der Patientengruppe wurde mit jener von Kindern und Jugendlichen einer Kontrollgruppe verglichen.

Ergebnisse

In der Kontrollgruppe wurden signifikant mehr HHV-6-spezifischen TNF-α-produzierende CD8+ T-Zellen bei Individuen nachgewiesen, die älter als 10 Jahre waren [<10 Jahre, Durchschnittsalter 6,6 (3,1%–9,2%) und 10–18 Jahre, Durchschnittsalter 17,2 (10,5%–18,3%)] (p = 0,033) und die Anzahl an HHV-6-spezifischen TNF-α produzierenden CD8+ T-Zellen korrelierte positiv mit dem Alter der Individuen (r = 0,465).

In der PatientInnengruppe gab es signifikant höhere Frequenzen an dreifach positiven CD8+ T-Zellen (IL-2/TNF-α /IFN-γ) [1,86% (0,38-4,5) vs. 0,93% (0-8,75), p = 0,034] und doppelt positiven CD8+ T-Zellen (IL-2 / TNF-α) [1,89% (0,21-5) vs. 1% (0-8,75); p = 0,040] bei PatientInnen im zweiten Jahr nach HSZT im Vergleich zu PatientInnen im ersten Jahr nach HSZT. Darüber hinaus wiesen PatientInnen im ersten Jahr nach HSZT signifikant niedrigere Frequenzen an CD8+ T-Zellen (TNF-α) im Vergleich zur Kontrollgruppe auf [1,34% (0,03-13,29) vs. 2,1% (0,72-9,83); p = 0,022]. Es wurden hingegen keine Unterschiede in der Frequenz an HHV-6 spezifischen CD8+ T-Zellen zwischen den PatientInnen im zweiten Jahr nach HSZT und der Kontrollgruppe gefunden.

Im ersten Jahr nach HSZT hatten PatientInnen ohne HHV-6 Reaktivierung signifikant höhere Frequenzen an HHV-6 spezifischen CD4+ T-Zellen (IL-2/TNF-α/IFN-γ und IFN-γ/IL-2) im Vergleich zu PatientInnen mit HHV-6 Reaktivierung im ersten Jahr nach HSZT [1,44% (0,2-19,33) vs. 0,98% (0-3,54); p = 0,048 und 2% (0,17-7,11) vs. 0,99% (0-3,54); p = 0,022]. Im zweiten Jahr nach HSZT gab es keine signifikanten Unterschiede in der Frequenz an HHV-6-spezifischen T-Zellen, weder für CD4+ noch für CD8+ zwischen den beiden Gruppen (HHV-6 nicht reaktiviert/reaktiviert). Signifikant höhere Frequenzen an HHV-6 spezifischen CD4+ T-Zellen (IL-2/TNF-α und IFN-γ/IL-2/TNF-α) bei PatientInnen ohne Virusreaktivierung bis zu 2 Jahre nach HSZT im Vergleich zu PatientInnen mit HHV-6 Reaktivierung wurden im 1. Jahr nach HSZT beobachtet [1,69% (0,17-7,11) vs. 0,99 % (0-3,54); p = 0,021 und 1,63% (0,38-10,17) vs.1,55% (0-4,22); p = 0,047].

Schließlich wurden in der Kontrollgruppe im Vergleich zu der Patientengruppe mit HHV-6-Reaktivierung im ersten Jahr nach HSZT signifikant höhere Frequenzen an HHV-6 spezifischen CD8+ T-Zellen (IL-2 / TNF-α / IFN-γ) beobachtet [1,29 % (0,6-7) vs. 0,61% (0,02-2,8); p = 0,048].

Schlussfolgerungen

Virusspezifische T-Zellen sind wichtige Effektorzellen bei der Bekämpfung von Virusinfektionen. Die Ergebnisse zeigen Unterschiede in der T-Zell Immunantwort gegen HHV-6 bei Individuen der Kontrollgruppe und PatientInnen mit und ohne HHV-6-Reaktivierung bis zu 2 Jahren nach allogener HSZT.

Abstract in English

Purpose

Human herpesvirus 6 (HHV-6) causes only minor symptoms in healthy individuals but in immunosuppressed patients, e.g. patients after allogeneic stem cell transplantation (HSCT), HHV-6 reactivations can lead to diseases in different organ systems. HHV-6 reactivations have also been reported to be a cause for delayed engraftment, a trigger of graft-versus-host disease and a co-factor for other virus reactivations. T-lymphocytes play an important role in the control of virus reactivations. The incidence of viral reactivations after HSCT is high, often due to a lack of virus-specific T-cell immune responses. Little is known about the development of virus-specific T-cells after allogeneic HSCT. The aim of this project is the description of the HHV-6 specific cellular immunity in children and adolescents after allogeneic HSCT in the context of the clinical course.

Material and Methods

3, 6, 9, 12, 18 and 24 months after allogeneic HSCT peripheral blood mononuclear cells were isolated from patient blood, stimulated with HHV-6-specific antigen (U54) and cultured for 10 days. On day 10, peripheral blood mononuclear cells were re-stimulated with the virus antigen U54 for 6 hours and, thereafter, stained for surface markers (CD3, CD4, CD8, CD56) and intracytoplasmatic activation markers (IL-2, IFN-γ, TNF-α) for flow cytometric detection of virus-specific T-cells. T-cells with intracytoplasmic expression of activation markers after stimulation with the virus antigen are HHV-6-specific T-cells. This indicated HHV-6 specific cellular immunity. The virus-specific immunity of patients to HHV-6 was compared to the virus-specific immunity of children and adolescents of a control group.

Results

In the control group significantly higher frequencies of HHV-6-specific TNF-α producing CD8+ T-cells were detected in individuals older than 10 years of age [<10 years, median age 6.6 (range 3.1–9.2) and 10–18 years, median age 17.2 (range 10.5–18.3)] ($p = 0.033$) and the frequencies of HHV-6 specific TNF-α producing CD8+ T-cells positively correlated with the age of the individuals ($r= 0.465$).

In the patient group, there were significant higher frequencies of triple positive CD8+ T-cells (IL-2/TNF-α/IFN-γ) [1.86% (0.38-4.5) vs. 0.93% (0-8.75), p= 0.034] and double positive CD8+ T-cells (IL-2/TNF-α) [1.89% (0.21-5) vs. 1% (0-8.75); p= 0.040] in patients in the second year after HSCT compared to patients in the first year after HSCT. Furthermore, patients during the first year after HSCT had significant lower frequencies of CD8+ T-cells (TNF-α) compared to the control group [1.34% (0.03-13.29) vs. 2.1% (0.72-9.83); p= 0.022]. No differences in the frequencies of HHV-6 specific CD8+ T-cells between the patients in the second year after HSCT and the control group were observed.

In the first year after HSCT patients without reactivation had significant higher frequencies of CD4+ specific HHV-6 T-cells (IL-2/TNF-α/IFN-γ and IFN-γ/IL-2) compared to patients with HHV-6 reactivation during the first year after HSCT [1.44% (0.2-19.33) vs. 0.98% (0-3.54); p= 0.048 and 2% (0.17-7.11) vs. 0.99% (0-3.54); p= 0.022]. In the second year after HSCT there were no significant differences in the frequencies of HHV-6 specific T-cells neither for CD4+ nor for CD8+ HHV-6 specific T-cells between the two groups (non-reactivated / reactivated). Significantly higher frequencies of HHV-6 specific CD4+ T-cells (IL-2/TNF-α and IFN-γ/IL-2/TNF-α) in patients without virus reactivation up to 2 years after HSCT in comparison to patients with HHV-6 reactivation during the first year after HSCT were observed [1.69% (0.17-7.11) vs. 0.99% (0-3.54); p= 0.021 and 1.63% (0.38-10.17) vs. 1.55% (0-4.22); p= 0.047].

Finally, significantly higher frequencies of HHV-6 specific CD8+ T-cells (IL-2/TNF-α/IFN-γ) were observed in the control group in comparison to patients with HHV-6 reactivation in the first year after HSCT [1.29% (0.6-7) vs. 0.61% (0.02-2.8); p= 0.048].

Conclusion

Virus-specific T-cells are important effector cells in the control of viral infections. Our results indicate differences in the T-cell immune response against HHV-6 in controls and patients with and without HHV-6 reactivation up to 2 years after allogeneic HSCT.

1. Background

Introduction

Human herpes virus 6 (HHV-6) is a very common virus pathogen that only causes minor symptoms in immunocompetent individuals but is linked to a variety of severe diseases in immunocompromised hosts. After hematopoietic stem cell transplantation (HSCT) herpes virus reactivations have been described to be associated with acute Graft-versus-Host Disease (aGvHD) and allograft rejections (1). HHV-6 as well as other herpesviruses have the ability for persistence in host cells after primary infection and are able to re-activate, especially in patients with immunodeficiencies (2). For example, HHV-6 reactivation has been reported in patients with haematological diseases such as Hodgkin's lymphoma, non-Hodgkin's and Burkitt's lymphoma, acute lymphoblastic leukaemia (ALL), chronic myeloid leukaemia (CML), and acute myeloid leukaemia (AML) (3).

The frequency of viral reactivations after allogeneic HSCT is high and indicates insufficient specific immunological responses and impaired reconstitution of virus-specific T-cell responses in these patient group that are very necessary for the protection against herpes virus reactivations after HSCT.

1.1 Stem cell transplantation

The transplantation of allogeneic haematopoietic stem cells is a potentially curative treatment for a variety of disorders like immunodeficiencies, aplastic anaemia and other haematopoietic malignancies (4). Many factors contribute to the success of a haematopoietic cell transplantation (HCT) as the type of underlying disease, the stem cell source, the HLA (human leucocyte antigen) matching of the donor and the recipient, the stage of the disease, the incidence and severity of a GvHD, the pre-transplant conditioning, post-transplant infections and many more (5).

While these factors influence the outcome of the transplantation, it must be assumed that rather the combination of all these factors has an impact on the success of the transplantation and on a rapid immune reconstitution after HSCT. The immediate phase after transplantation of stem cells is characterized by a severe immune deficiency of the patient that leads to a high susceptibility for viral, bacterial and fungal infections (6). Deficiencies of granulocytes and other mononuclear subsets promote infections in the first months after engraftment of

hematopoietic stem cells (HSC). Prolonged infections result from a combination of immune deficiency, ineffective T- and B- cell reconstitution as well as immunosuppressive therapy (7).

The first transfer of HSCs was conducted in 1968 in two patients with a severe congenital immune defect. Since then, there was an ongoing expansion of indications for HSCT (8,9).

HSCs originate from the bone marrow, from foetal liver and spleen. Morphically HSCs cannot be distinguished from white blood cells, except the expression of the surface antigen CD34+ (10). HSCs replicate themselves to obtain pluripotency and are able to develop into erythroid, myeloid, megakaryocytic and lymphoid lines. Mature HSCs are characterized by a gradual loss of their differentiation possibilities to other cell types (11). The proportion of CD34+ cells in the bone marrow is 1-2%, in umbilical cord blood 0.8-1.2% and in the peripheral blood only 0.1-0.2% (12).

During the process of an HSCT the HSCs can be self-donated what is called *autologous stem cell transplantation* or donated from another individual called *allogeneic stem cell transplantation*. An allogeneic stem cell transplantation is defined as the total replacement of the body´s haemato-lymphopoietic system by exogenous donor cells as siblings or other family members and foreign donors. Conditioning is a pre-treatment of the recipient before HSCT (13). A radio-chemotherapy or combined chemotherapy has the goal to finally eliminate the malignant HSCs from the patient and to create space for the transplant.

Bone marrow (BM) is aspirated from the posterior iliac crests under local or general anaesthesia, then filtered and directly infused in the recipient. In contrast, peripheral blood stem cells (PBSCs) are collected during apheresis, after the mobilization with growth factors (14). The possibility to mobilize HSCs from the BM into the peripheral blood and thus increasing the stem cell yield, led to a steady elevation in the number of PBSC transplants since the 1990s. One advantage of PBSC transplantations is the elimination of general anaesthesia for the stem cell donor, however, disadvantageous for the donor may be thrombocytopenia. Furthermore, the increase in the number of lymphocytes in the transplant seems to increase the risk of aGvHD and cGvHD for the recipient after allogeneic HSCT (15).

A GvHD is a mismatch of histocompatibility antigens between donor and recipient. An aGvHD occurs within the first 60 to 100 days after transplantation. For HLA-incompatible transplantations, first manifestations are observed already earlier. A GvHD appearance

significantly depends on the degree of the HLA-match of recipient and donor. Mainly skin, liver and gastrointestinal tract are affected by an aGvHD (16,17).

Most commonly, there is a rash that - depending on the severity – may manifest as a slight redness but can also appear in the form of a severe inflammatory disease. Some of the patients also experience impaired liver function. Fever, tachycardia, body weakness and weight loss can arise during a GvHD (18). Depending on the affected organs and the severity of the disease, four different stages of a GvHD are distinguished (I-IV) (19). The most common cause of death in patients with GvHD are emerging infections, especially re-activations of viruses as cytomegalovirus (CMV) or other herpes viruses (20). A cGvHD earliest appears on day 100 after transplantation and usually follows an aGvHD (21).

Immunosuppressive therapy (immunosuppression) is generally used to suppress the graft rejections in patients after allogeneic HSCT (15). There are currently several substances used for GvHD prophylaxis such as cyclosporine A (CyA), anti-thymocyte globulin (ATG) and methotrexate (MTX). However, first-line therapy against an aGvHD is the administration of cortisone, which causes most reactions to subside (22).

The application of allogeneic HSCT has expanded rapidly over the past decades. Advances in supportive care, in conditioning and management of complications (infections, GvHDs) will widen the applicability in the future in this broad and diverse field. In the following chapter the donor characteristics for HSCTs are briefly described.

1.1.1 HSC donors

For the selection of an appropriate donor for allogeneic HSCT, the histocompatibility antigens (MHC, major histocompatibility complex or HLA, human leukocyte antigens) are of importance. Through the MHC complexes the immune system can distinguish between different body tissues. MHC antigens are located on chromosome 6 and are characterized by a very wide variety of alleles. The alleles are inherited, so that a child receives the haploid genetic information from each parent, that is also so-called haplotype (23). Siblings are therefore HLA-identical with a probability of 0.25%, parents and children can usually only be haploidentical.

The MHC class antigens are classified in three groups, however, only MHC class I and MHC class II antigens are important for transplantations because they require extensive matching of between the donor and recipient to prevent an GvHD (24).

1.1.2 HLA-identical sibling donors

If a healthy HLA-matched sibling donor is available for a recipient before allogeneic HSCT, then this is the donor of choice, since the greatest possible HLA identity can be achieved in these cases. Transplant-related mortality is lower in these patients than in patients with HLA-identical foreign donors (25).

1.1.3 HLA-identical third-party donors

Due to the decreasing number of siblings in families and therefore potential suitable donors, larger donor files of HLA-identical foreign donor worldwide has gained significantly in importance (26).

1.1.4 Haploidentical family donors

If it is not possible to find a suitable HLA-identical donor, it is nowadays possible, to transplant HLA-haploidentical stem cells from a relative. This has been made possible due to advances in the field of CD34+ enrichment of the donor graft. Due to the indirect T-cell depletion associated with CD34+ enrichment, the expected GvHD in HLA-mismatches is largely prevented (27).

1.1.5 Manipulation of stem cells

Over the years, strategies have been developed to eradicate the negative effects of HLA mismatch transplantations through pharmacological immunosuppression and T-cell depletion of the donor graft. Most T-cells in peripheral blood express the $\alpha\beta$ T-cell receptor. $\alpha\beta$ T-cells, as parts of the adaptive immune response, are triggers for a GvHD and a depletion of these cells from the graft reduces the risk for GvHD after HSCT (28). The $\alpha\beta$ T-cell depletion approaches are mainly performed in the pediatric population (29,30).

Furthermore, to avoid a poor graft function and relapse it had been observed that the infusion of highly enriched CD34+ stem cells can additionally reduce the risk for GvHD (27,31).

1.2 Immunological Background

The immune system is a diverse network of cells and soluble factors that eliminates pathogens and can be divided into two parts: the innate immune system including dendritic cells (DCs),

natural killer cells (NK), macrophages and granulocytes as well as complement factors and the adaptive immune system including T- and B-cells (32).

1.2.1 Innate immune response

The innate immune response consists of cellular and biochemical defence mechanisms that become effective before or shortly after the infection. Thus, physical and chemical barriers such as antimicrobial substances on the skin surface form the first protection barrier against microorganisms (33). Phagocytes (granulocytes, macrophages) and NK cells interact with the complement system to eliminate pathogens. Soluble substances such as cytokines and chemokines coordinate the interaction of the different immune cells. The effector mechanisms of the innate immune system are directed against microbial structures. This early immune reaction is short and not pathogen specific but represents an important first and rapid barrier against various pathogens (34).

1.2.2 Adaptive immune response

In contrast to the innate immune response, the adaptive immune response ensures a permanent immune reaction. Lymphocytes with highly specific receptors detect and eliminate infected cells in the body. The enormous number of receptors is based on genetic level (35). Naive, non-activated lymphocytes circulate through the lymphatic system until their first antigen contact. Once a foreign protein or protein fragment (“epitope”) is recognized, antigen-specific lymphocytes get activated. After the infection, antigen-specific cells remain in the body and form the so-called “immunological memory”. These antigen-specific lymphocytes can react rapidly upon a renewed stimulation and ensure an even more efficient immune reaction to specific antigens (36). The adaptive immune system consists of the humoral and cellular immunity. The humoral immune response eliminates extracellular microorganisms and is mediated by antibody producing B-lymphocytes. Antigens are recognized by lymphocytes. The binding of the antibody to the antigen leads to an uptake and degradation by scavenger cells, such as macrophages (37).

Cellular immunity is mediated by T-lymphocytes and directed against intracellular microbes, such as viruses and some bacteria, that are not accessible to circulating antibodies (Figure 1). According to their respective function in the immune reaction, cytotoxic T-cells (CD8+ T -cells), T-helper cells (CD4+ T-cells) and regulatory T-cells (CD4+ CD25+ T-cells) and some other minor immune cell subtypes can be distinguished. In contrast to B-cells, T-lymphocytes

are not able to recognize specific antigens in soluble form. They require the presentation of epitopes in protein complexes such as the MHC (36).

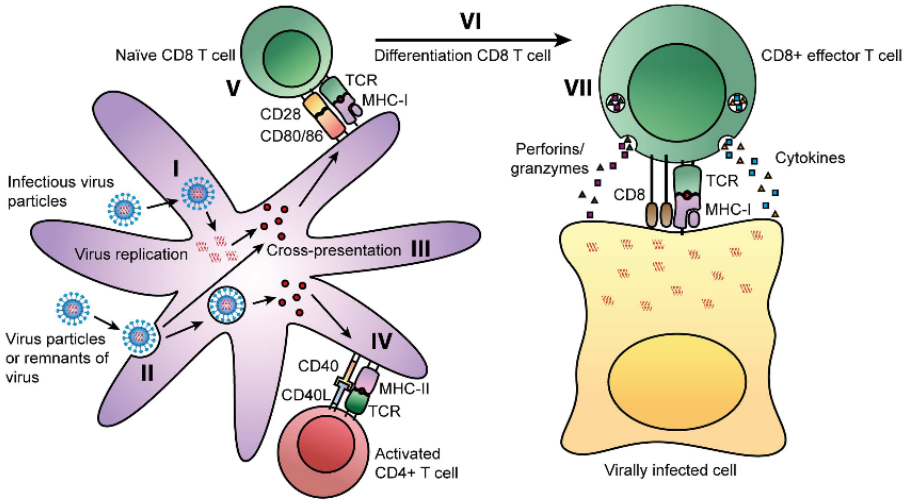

FIGURE 1 | Routes of presentation of viral peptides on DCs. Viruses can enter cells by two ways: some viruses can infect cells directly, leading to replication of virus inside the cells. During this process, some of the viral proteins will be degraded into peptide fragments, which will be presented on MHC class I molecules to $CD8^+$ T cells (I). APCs, such as DCs can also take up viral particles or remnants of virally infected cells (II). During processing by professional APCs, viral peptides can be presented on MHC class I molecules via the cross-presentation pathway (III). In parallel, these extracellular-derived peptides will be presented on MHC class II molecules. The TCR of virus-specific $CD4^+$ T can recognize MHC class II-peptide complexes on professional APCs. Next to the interaction of the MHC class II-peptide complex with the TCR, $CD4^+$ T cells can activate DCs by interaction of CD40 with CD40 ligand on the DC (IV). This interaction activates DCs and results in upregulation of maturation markers CD80/CD86. CD80 and CD86 interact with CD28 on naïve $CD8^+$ T cells (V). Together with the recognition of the MHC class I-peptide complex by the TCR, CD28 signaling will result in the activation of the $CD8^+$ T cell (VI). These activated $CD8^+$ T cells will differentiate into effector T cells that can recognize the MHC class I-peptide complex on virally infected cells. Binding of the TCR to the MHC class I-peptide complex leads to activation of the $CD8^+$ T cell and the release of cytotoxic granules containing perforins and granzymes, and the production of cytokines such as TNF-α and IFN-γ (VII).

Figure 1: Routes of presentation of viral peptides on DCs. Figure reproduced from Rosendahl, Huber et al. (38) from FRONTIERS in Immunology, CC BY 3.0 http://creativecommons.org/licenses/by/3.0/

1.2.3 MHC complexes

The genes of the MHC are located on chromosome 6 and were identified in the beginning of the 1950s (39). These genes encode proteins that are responsible for immunological individuality of all humans and tissues and are important for compatibility in solid organ transplantations as well as HSCTs. The MHC complexes are expressed on the cell surface of almost all nucleated human cells and are important for the differentiation between endogenous and exogenous antigens (40).

The MHC system is characterized by a strong polymorphism and different HLA phenotypes. In order of their discovery class I and class II antigens are distinguished. First, class I antigens are glycoproteins that form a complex of three neighbouring genes: HLA-A, -B and -C. These antigens are expressed on almost all human body cells. MHC-I antigens represent strong transplant antigens that interact primarily with cytotoxic T-cells. Activated cytotoxic T-cells

can lysate cells via the secretion of lytic enzymes (41). Second, MHC class II antigens HLA-D form a complex out of HLA-DP, -DQ and -DR antigens. Class II antigens are expressed only on antigen-presenting cells as B-cells, dendritic cells and stimulated macrophages and interact with T-helper cells during an immune reaction. Exogenous antigens fuse with MHC-II molecules and appear on the cell surface. During this process, T-helper cells get activated for cytokine secretion (e.g. IFN-γ, TNF-α, IL-2) and B-cells get activated for antibody production (38).

HLA compatibility between donor and recipient is one crucial factor for a successful transplantation. A mismatch of donor and recipient (HLA incompatibility) is an indispensable risk factor for the occurrence of a GvHD and for an elevated susceptibility to infections after HSCT as well as graft failure (8).

1.3 Immune recovery after HSCT

Radiotherapy and chemotherapy destroy the recipient's cellular and humoral immunity. The grade of immunosuppression after allogeneic HSCT depends on the primary disease, the HLA consistency, the kind and toxicity of the pre-treatment before allogeneic HSCT (conditioning) as well as T-cell content of the transplant (42). The age and thymic function of the recipient, the occurrence of a GvHD as well as infections (viral, bacterial, fungal) can also influence immune reconstitution after allogeneic HSCT. For at least one year after allogeneic HSCT most patients suffer from a severe immunodeficiency syndrome (43,44).

After the total depletion of the innate and adaptive host immune system via cytotoxic chemotherapy (conditioning) the gradual immune recovery of allogeneic immune cells is important, however, the time dynamics of the immune reconstitution and differentiation of immune populations is only partially understood. At first, the innate immunity is rapidly restored within the 1st month after HSCT together with monocytes, NK cells and granulocytes. After allogeneic HSCT, the recovery of T- and B-lymphocyte populations is much slower than those of myelomonocytic populations (43).

Up to four months after HSCT CD8+ T-cell numbers return to control values, but a skewed repertoire of memory T-cells remains. In young individuals after HSCT the recovery of CD4+ T-cells mainly depends on residual thymus function (45). In older patients however, the total CD4+ T-cell count can remain low for years and constitutes a potential risk factor. In young individuals, naïve CD4+ T-cells predominate over a half year after HSCT. B-cells normally

recover up to day 30 post- HSCT but many of their functions (e.g. antigen-specific antibody production) remain lowered for months after HSCT (46). Because of these circumstances, herpesvirus reactivations [mostly HHV-6, CMV, Herpes simplex virus (HSV) and Eppstein-Barr Virus (EBV)] occur 2-3 months post-HSCT. These viral reactivations are mostly a consequence of CD4+ and CD8+ functional immune deficiency as well as B-cell immaturity and leading to a suboptimal elimination of herpesvirus- affected leucocytes (20).

1.4 T-cell reconstitution after HSCT

As mentioned above, the adaptive immunity recovers slowly after allogeneic HSCT (47). Koning *et al*. (45) evaluated leucocyte subpopulations after allogeneic HSCT and found that CD8+ T-cell levels increase earlier than CD4+ T-cell levels post HSCT. Several T-cell related immune parameters were evaluated in a study by Klyuchnikov *et al*. (31). The immune reconstitution in AML patients after allogeneic HSCT was examined and it was found that an impaired CD3+CD8+ (day +90 after HSCT) as well as CD19+ (day +90 after HSCT) immune recovery increased the incidence for post-transplant sepsis or other severe infections. Furthermore, an elevated occurrence of an aGvHD (II-IV) was accompanied by reduced levels of CD3+CD4+ and CD19+ cells. Deficiencies of major immune populations result in severe post-transplant complications. An increase of thymus volume as well as a recovery of naïve CD45RA+CD45RO- CD4+ T-cells occurs within the first six months in paediatric patients, suggesting the importance for thymus dependent T-cell production for peripheral CD4+ T-cell reconstitution in these patients (48).

Regarding polyclonal T-cell reconstitution for specific viral pathogens only a small fraction of virus-specific T-cells is produced in patients after HSCT, thus, strategies for expansion and enrichment of virus-specific T-cells enhanced special research interest over the last 10-15 years. In the first months after transplantation, however, a narrow and altered repertoire of T-lymphocytes with memory-like phenotype is monitored in HSCT patients (49,50). Adoptive T-cell transfer involves induction of a virus-specific T-cell response in patients by direct infusion of virus-specific T-cells. Many research groups have investigated adoptive T-cell transfer for the establishment of early virus-specific T-cell populations in patients after allogeneic HSCT for the prevention of viral reactivations post HSCT (51).

Antigen-specific cytotoxic T-cells are important effector cells for the control of viral infections. The first pool of virus-specific T-cells (effector, memory or naïve T-cells) forms the donor graft (depending on type of graft- BM vs. PBSC) as well as resident antigen-

specific T-cells of the recipient (52,53). An early reconstitution of virus-specific T-cells is important for the control of viral reactivations after allogeneic HSCT. Thus, CD4+ as well as CD8+ virus-specific T-cells are markers for viral protection against viral infections (54).

1.5 Infections after HSCT

The risk of infections in the early post-transplantation phase (<day +30 after HSCT) is mainly caused by neutropenia, that increases the susceptibility for bacterial and fungal infections. Other main risk factors in patients after allogeneic HSCT for developing infections are the presence of central accesses, previous bacterial, fungal or viral infections, severe skin and mucosal damages and immunosuppression during and after the conditioning treatment (44) (Figure 2).

In the middle post-transplant phase (to day +100 after HSCT) patients are more likely to develop viral infections because of a combined quantitative and functional defect of B- and T-lymphocytes. Clinically important viral infections after allogeneic HSCT belong mainly to the group of human herpesviruses as CMV, EBV, HHV-6, HSV, Varicella Zoster Virus (VZV) as well as BK polyomavirus (BKV), Respiratory Syncytial Virus (RSV), Adenovirus (ADV) (55).

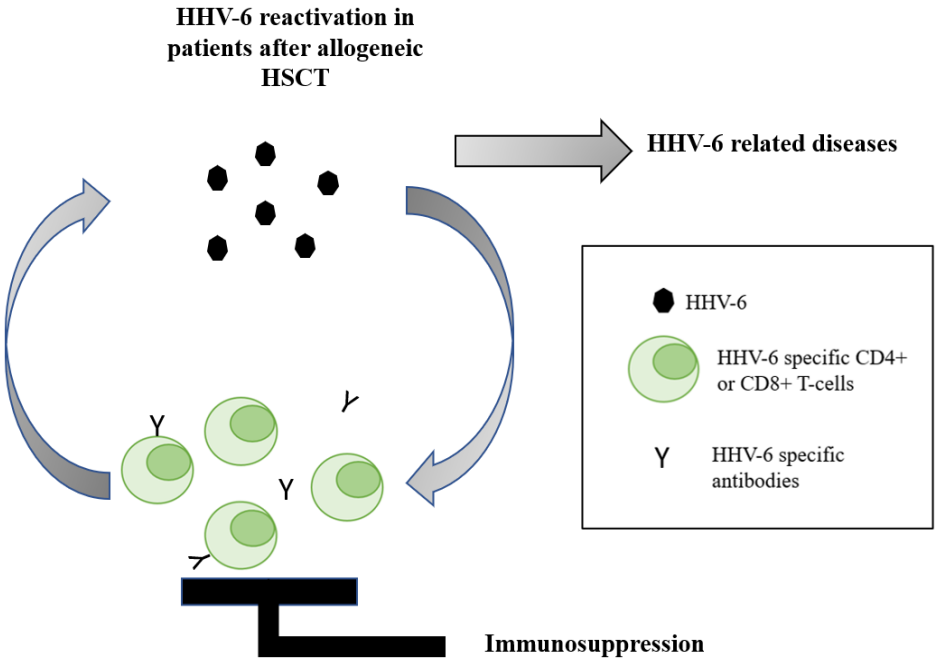

Figure 2: HHV-6 reactivation in patients after allogeneic HSCT. HHV-6 reactivates under conditions of deficient cell-mediated immunity. Schematic representation.

1.5.1 History and Epidemiology

HHV-6 was first isolated 1986 from the peripheral blood mononuclear cells of patients with lymphoproliferative disorders which was later referred to HHV-6 (56). Two closely related species, HHV-6A and HHV-6B, were then discovered. Based on both biological properties and genetic analyses, both HHV-6 species are members of the *Herpesvirales* order, *Herpesviridae* family, *Betaherpesvirinae* subfamily and *Roseolovirus* genus (57). HHV-7 and CMV are two other viruses that belong to the human beta-herpesviruses.

A primary infection with HHV-6B occurs in early childhood (6-9 months after birth) and causes exanthema subitum (58,59). The disease is characterized by high fever and the development of skin rashes. However, asymptomatic forms of this primary infection with an undifferentiated brief period of fever without skin rashes are also possible. After primary infection the virus establishes a latent infection and persistence in different host cells and it´s reactivation in healthy hosts is controlled by the adaptive immune response (Figure 3).

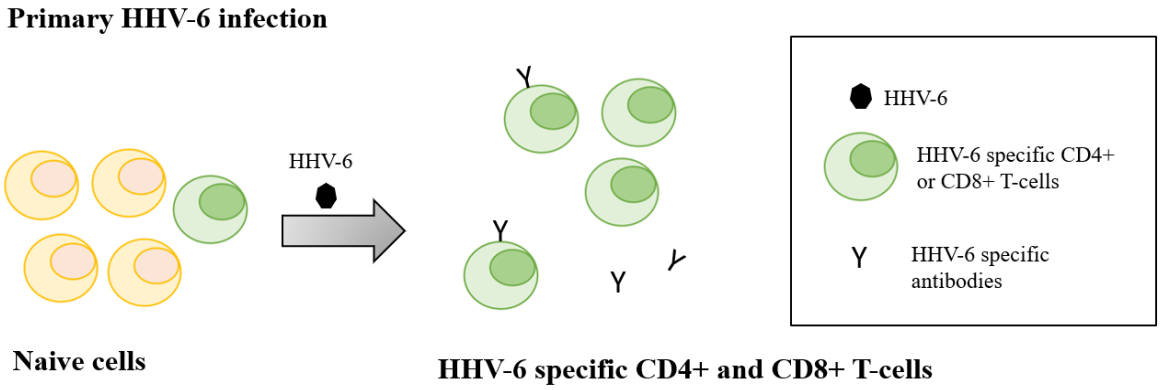

Figure 3: Primary HHV-6 infection in early childhood. Primary infection with HHV-6 elicits antibody and T-cell responses in healthy individuals. Schematic representation.

However, there is the possibility of virus reactivation later in life. HHV-6 reactivation occurs periodically in healthy carriers sub-clinically. Most individuals at the age of 2 years (about 95%) worldwide are HHV-6B positive (60).

In patients with deficient cell-meditated immunity and without proliferative T-cell responses (e.g. patients after allogeneic HSCT) the virus can rapidly reactivate (61). Additionally, an inherited chromosomal integration of HHV-6 DNA (ciHHV-6) into the human genome (prevalence of 0.2–5%) has been described for both HHV-6 subtypes but the clinical impact of this phenomenon remains still unclear (55,62). An infection with HHV-6 in most cases is not a problem for healthy carriers but in patients with an impaired immune status a

reactivation of HHV-6 can lead to serious complications like CNS (central nervous system) morbidity, pneumonitis, transplant rejection and delayed engraftment (58,60,61).

1.5.2 Clinical significance of HHV-6 after HSCT

HHV-6 reactivates in nearly 40-50% of HSCT patients (58) usually 2-6 weeks after transplantation in the time period of lowest neutrophil and lymphocyte counts (60,63,64). Interestingly, in more than 98% of HHV-6 viral detections after HSCT the HHV-6 B subtype could be found that subtype that is the main cause for encephalitis after allogeneic HSCT (65,66).

Foscarnet, Ganciclovir and Cidofovir are antiviral agents with activity against both virus subtypes HHV-6A and HHV-6B (67). Moreover, the use of virus-specific T-cells are a novel and promising therapeutic approach to treat HHV-6 disease. Some studies have shown that donor-derived virus-specific T-cells were administered with good clinical results (51,68–70). In future, HHV-6 specific T-cells may be more cost-effective and even more effective than common antiviral agents against HHV-6 in patients after allogeneic HSCT. However, a detailed understanding of virus-specific T-cells is required to generate these high affinity virus specific T-cell lines for adoptive immunotherapeutic strategies.

1.5.3 HHV-6 and the interaction with the immune system

During evolution, HHV-6, as well as other (herpes) viruses, developed various methods to undergo the immune response of the infected host organism for its benefits. The immunosuppressant properties of HHV-6 are primarily due to the impairment of the T-cell response. NK cells, immune cells of the innate immune response, form the first barrier against HHV-6 (71) which show cytotoxicity against HHV-6 infected cells (72) in an interleukin-15-dependent manner (73). Interestingly, in comparison to other herpesviruses, the virus-specific cell-mediated response is delayed in hosts during primary HHV-6 infection (74) because of the associated HHV-6 lymphotropism (75). HHV-6 has several immunosuppressive mechanisms regarding T-cell function (76) and immunomodulatory strategies for its life-long persistence in the host´s body (77).

The human glycoprotein CD46 was described as the main receptor for both HHV-6A and B (75). HHV-6 replicates in CD4+ T-cells, various cytotoxic effector cells like CD8 + T-cells and NK cells (54) and thus evades antiviral immune defence mechanisms. Moreover, HHV-6

also infects BM cells and causes suppressive effects on the growth and maturation of precursor cells. The binding of HHV-6 surface proteins to CD46 leads to several fundamental changes in the physiology of immune cells as the suppression of IL-12 production by antigen presenting cells (78). In parallel, regulatory T-cell response and complement activation are induced during HHV-6 infection. During an HHV-6 infection the expression of important surface receptors changes. The transcriptional suppression of CD3 on T-cells as well as increased CD4 expression on the surface of T-cells are some examples for the virus´ immunomodulatory strategies in the host (77).

Furthermore, down-regulation of the surface molecules like CD14, CD64 and HLA-DR during infection of primary human monocytes interferes with the activation of antigen-presenting cells. Moreover, HHV-6 affects the cytokine and chemokine production through increased production of inflammatory cytokines such as IFN-α, TNF-α, IL-8, IL-1β as well as IL-15 (79). The infection of CD4+ T-cells by HHV-6 induces apoptosis (80), TCR (T-cell receptor) and MHC-I downregulation (76), inhibition of IL-2 synthesis (81) as well as cell cycle arrest (82). Moreover, the virus has the ability to reduce the presentation of antigens in antigen-presenting cells (76).

1.6 The impact of HHV-6 specific T-cells

Despite these strong immune-evasive strategies, the immune system manages to control the virus that, in immunocompetent individuals, normally no HHV-6-associated diseases occur during life-long persistence. The high incidence and severity of HHV-6 infections in patients with impaired cellular immunity show the importance of the cellular immune response in controlling HHV-6. For other herpesviruses, such as EBV or CMV, it is known that a strong CD4+ and CD8+ antiviral T-cell response provides life-long protection against EBV- or CMV-associated diseases (83). The same would be assumed for HHV-6 infections. However, the T-cell response to HHV-6 has hardly been studied.

The T-cell response to HHV-6 has been mostly characterized by using peripheral blood from healthy adults. In the early 1990s, a Japanese research group was able to demonstrate that CD4+ T-cells within peripheral blood cells of healthy HHV-6 positive donors can be accumulated by stimulation with the lysate of HHV-6-infected cells.

Yakushijin *et al*. generated CD4+ virus specific T-cells from HHV-6-stimulated PBMCs that proliferated upon stimulation with HHV-6 in the presence of autologous antigen-presenting

cells and Yasukawa *et al*. investigated on the antigenic specificity of CD4+ T-cell clones against different herpesviruses as HHV-6, HHV-7 and HCMV (84,85). We recently investigated the HHV-6 specific T-cell response in healthy children and adolescents and found that individuals older than 10 years had significantly higher frequencies of HHV-6 specific CD8+ TNF-α T-cells than those younger than 10 years of age and data analysis showed a positive correlation between age of the individuals and frequency of these virus specific T-cells (86). Out of our results we observed that immunity against HHV-6 seems to evolve over time in healthy individuals.

However, HHV-6 specific T-cells are present at low frequency (<0.2%) (87–89) which contrasts with stronger responses for HCMV that are up to 4% (90) and makes it hard to study this immune cell population. In a study of de Pagter *et al*. it was shown that T-cell responses against HHV-6 were higher in HSCT patients (1), however, up to now, only few studies have focused on the HHV-6-specific immune response in this group of patients. In spite of the low frequencies of HHV-6 specific T-cells strong CD4+ and CD8+ T-cell proliferative responses of mainly memory cells are observed (91–93). Proliferation of these low-frequency HHV-6 specific T-cell populations *in vitro* are necessary for detailed study. Most of the expanded effector CD4+ and CD8+ populations secrete different cytokines as IFN-γ, TNF-α and IL-2 and have several cytolytic functions as the secretion of perforin (CD4+ T-cells) or granzyme B (CD8+ T-cells) (94). These cytotoxic T-cells can rapidly lyse virus infected cells and reacquire their cytotoxic activity when they are exposed to the antigen (84).

In a study of de Pagter *et al*. in paediatric HSCT patients, increased proportions of perforin-expressing CD8+ T-cells were associated with HHV-6 clearance (95). It has been reported that T-cells in patients with proliferate capacity were readily apparent after viral reactivation but this was not the case in patients without proliferate capacity (1). Nevertheless, as mentioned before, HHV-6 can bypass the cytolytic functions of CD8+ T-cells by downregulating class I MHC molecules (96), which may complicate the detection of HHV-6-specific CD8+ T-cells. CD4+ T-cells are important for their antiviral effector functions and in controlling herpesvirus infections (97,98), although less is known about their role in HHV-6 clearance. An infection with HHV-6B induces surface expression of MHC class II molecules that could enhance the detection of infected cells by CD4+ T-cells (99). Appropriate methods for the detection of these rare virus-specific T-cells are important to elucidate the role of HHV-6 specific T-cells in terms of viral clearance.

1.7 Detection of antigen specific T-cells

Despite the development of several technologies for the detection and analysis, the low frequencies of antigen-specific lymphocytes and the complexity of T-cell antigen recognition remains a critical issue in the research for HHV-6 immunity. Recent developments, however, increased the sensitivity for cytometric detection of antigen-specific T-cells to analyse the full CD8+ and CD4+ T-cell repertoire against HHV-6 antigens. Cytokine detection allows the identification of functional virus-specific T-cell subsets. Many research groups investigate the *in vitro* expansion of virus-specific T-cells for detailed epitope identification and characterization of preferred populations (69,84–86,89,100,101).

The possibility to simultaneously analyse T-cell responses in the naive, effector as well as regulatory T-cell compartment will help to define their important role within the complex immune system and will significantly improve diagnostic as well as therapeutic intervention strategies (102).

The effector functions of T-cells after contact with specific antigens can be analysed with different methods (103). Cytokine production of T-cells after antigen contact can be detected by ELISPOT (Enzyme Linked Immuno Spot Assay) (104) as well as intracellular and extracellular cytokine staining (105). Peptide major histocompatibility tetramer staining assays are very useful to measure antigen-specific T-cell responses ex *vivo*, however they are restricted to certain HLA- types (78,97). The determination of the frequencies of antigen-specific T-cells is limited with these methods, only the intracellular cytokine staining offers the possibility by the detection of multiple cytokines in combination with other (activation) markers. Intracellular staining is a highly sensitive method to determine the frequencies of antigen-specific T-cells and allows the functional characterization of these cells at the same time.

1.7.1 Flow cytometry – FACS

Fluorescence-activated cell sorting (FACS) is a frequently used method in immunology. Fluorochrome labelled antibodies label cell surface or intracellular proteins (antibody staining) and are detected through FACS analysis. FACS is mostly used to define and enumerate diverse cell types. The basis for FACS is a (coloured) single-cell suspension, which passes through a focused laser beam. The generated scatter and fluorescent lights are detected separately (32).

The forward scatter detects the light in forward direction when the laser light strikes the cell and captures the size of the cell. The side scatter detector obtains information about the cell granularity or complexity for different cell types. Finally, multiple detector arrangements with mirrors detect the fluorescence emissions of different cells. Therefore, diverse cells can be sorted by their size, granularity and fluorescence which makes it possible to identify a multitude of cell subtypes (32,102).

2. Synopsis

In recent decades allogeneic HSCTs has been described as standard procedure in the therapy of various malignant and non-malignant paediatric diseases. However, there is also the risk for transplantation-related morbidity and mortality due to delayed immune reconstitution by T-cell depletion, non-engraftment or graft rejection. Furthermore, the frequency of viral reactivations after allogeneic HSCT is high, indicating insufficient specific immunological responses and impaired timely reconstitution of virus-specific T-cell responses that are necessary for the protection against herpes virus reactivations after allogeneic HSCT.

3. Hypothesis

During the first 24 months after allogeneic HSCT a measurable, specific HHV-6 T-cell immunity develops with different cytokine patterns in the context of the clinical course.

In this study, the following specific issues were investigated:

- Analysis of HHV-6 specific T-cell immune response in a control group.
- Comparison of frequencies of HHV-6 specific T-cells in healthy subjects to frequencies of HHV-6 specific T-cells in patients after allogeneic HSCT
- Analysis of frequencies of HHV-6 specific T-cells in patients after allogeneic HSCT at different time points after allogeneic HSCT (3, 6, 9, 12, 18, 24 months)
- Comparison of frequencies of HHV-6 specific T-cells in patients with HHV-6 reactivation after allogeneic HSCT to frequencies of HHV-6 specific T-cells in patients without (measurable) HHV-6 reactivation after allogeneic HSCT.
- Comparison of frequencies of HHV-6 specific T-cells in different patient groups (patients with/without reactivation, 1^{st} year/ 2^{nd} year after allogeneic HSCT) to frequencies of the control group.
- Influence of specific transplantation characteristics of patients with allogeneic HSCT on the frequencies of HHV-6 specific T-cells after allogeneic HSCT.

4. Materials and Methods

Scientific research and experiments related to this thesis were mainly conducted at the routine laboratories at the Division of Pediatric Hematology/Oncology and flow cytometry measurements were conducted in the Imaging Core Facility at the Center for Medical Research (ZMF), Medical University of Graz.

4.1 Materials

Materials that were used for the experiments were categorised and listed in the following Table 1 – Table 4 below.

Table 1: Materials for PBMC isolation

Reagents	**Manufacturer**
S-Monovette®	Sarstedt, Nümbrecht, Germany
Heparin Sodium Ampules (5ml, 5.000 I.U./ml)	Wockhardt UK Ltd, Wrexham, UK
Falcon Tubes 50ml, 30x115mm style	Becton Dickinson, San Diego, CA, USA
Ficoll ® Paque Plus	GE Healthcare, Buckinghamshire, UK
DPBS	Gibco Life Sciences, Thermo Fisher Scientific, Waltham, USA
Trypan Blue 0.4%	STEMCELL Technologies, Cologne, Germany
Neubauer Chamber improved	BRAND GMBH + CO KG, Wertheim, Germany

Table 2: Materials for cell culture experiments

Reagents	**Manufacturer**
TexMACS medium	Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany
48-well polystyrene plate, flat bottom wells	Corning- Costar, New York, USA
PepMix U54	JPT, Berlin, Germany
PepMix Human Actin	JPT, Berlin, Germany
PepMix HCMVA pp65	JPT, Berlin, Germany
Interleukin-2 (IL-2)	Cellgenix, Freiburg, Germany
Interleukin-7 (IL-7)	Cellgenix, Freiburg, Germany
Staphylococcal enterotoxin B (SEB), 5mg lyophilisate	Sigma, Taufkirchen, Germany
Brefeldin A, 5mg lyophilisate	Sigma, Taufkirchen, Germany

Table 3: Materials for flow cytometry staining

Reagents	**Manufacturer**
Antibody CD3 PerCP	Bio Legend, San Diego, USA
Antibody CD4 Alexa Flour 700	Becton Dickinson, San Diego, CA, USA
Antibody CD8 APC-H7	Becton Dickinson, San Diego, CA, USA
Antibody CD56 FITC	Becton Dickinson, San Diego, CA, USA
Alexa Fluor 350 carboxylic acid, succinimidyl ester	Invitrogen, Carlsbad, CA, USA
Fix and perm kit	An Der Grub Bio Research GmbH, Vienna, Austria
Antibody IFN-γ- BV605	Becton Dickinson, San Diego, CA, USA
Antibody IL-2- BV510	Becton Dickinson, San Diego, CA, USA
Antibody TNF-α BV421	Becton Dickinson, San Diego, CA, USA
Brilliant Stain Buffer 5ml	Becton Dickinson, San Diego, CA, USA
Albunorm 200g/l	Octapharma Pharmaceuticals, Vienna, Austria
EDTA 0.5 M pH 8.0	Invitrogen, Carlsbad, CA, USA
Falcon Tubes 5ml, polystyrene round-bottom, 12x75mm style	Corning- Costar, New York, USA

Table 4: Further reagents/materials

Reagents	**Manufacturer**
Pipetus	Hirschmann-Laborgeräte, Eberstadt, Germany
Reax top shaker	Heidolph Instruments, Schwabach, Germany
CKX41 microscope	Olympus Life Sciences, Vienna, Austria
Eppendorf Centrifuge 5810R	Eppendorf, Hamburg, Germany
HeraSafe Bench	Heraeus, Hanau, Germany
HeraCell Incubator (37°C)	Heraeus, Hanau, Germany
epT.I.P.S. Pipette tips dualfilter 0.1-10 µL	Eppendorf, Hamburg, Germany
epT.I.P.S. Pipette tips dualfilter 2-100 µL	Eppendorf, Hamburg, Germany
epT.I.P.S. Pipette tips dualfilter 50-1,000 µL	Eppendorf, Hamburg, Germany
Eppendorf Reference pipettes, Volume: 0.5-10µl; 10-100µl; 100-1000µl	Eppendorf, Hamburg, Germany
Dispenser, Seripettor	BRAND GMBH + CO KG, Wertheim, Germany
Parafilm M	BRAND GMBH + CO KG, Wertheim, Germany
Primo tuberculin syringes 1ml	Büttner-Frank GmbH, Erlangen, Germany
Stripettes 5ml, 10ml, 25ml	Corning- Costar, New York, USA
Flow Cytometer LSR II	Becton Dickinson, San Diego, CA, USA
FACSDIVA acquisition/analysis software	Becton Dickinson, San Diego, CA, USA
SPSS version 25 for Windows	SPSS, Inc., Chicago, IL, USA

4.2 Methods

4.2.1 Clinical samples

For this prospective, cross-sectional study peripheral venous blood was drawn from 28 children and adolescents 3, 6, 9, 12, 18, and 24 months after allogeneic HSCT. Furthermore, a blood sample was taken from 25 age- and sex-matched healthy controls without any inflammatory, immunological or infectious diseases scheduled for elective surgery for orthopaedic pathologies, implant removals, herniotomies, circumcisions and others from the Department of Pediatric and Adolescent Surgery, Medical University of Graz. Information on clinical data of the patients was collected from medical records and the electronic documentation program MEDOCS (Medical Documentation and Communication System, SAP Germany). For patients after allogeneic HSCT, detailed medical history was collected, including:

- Indication for HSCT
- Conditioning
- Type of transplant
- Type of donor (family/foreign donor)
- GvHD prophylaxis and treatment
- Development of GvHD
- HHV-6 reactivation after HSCT
- CMV seropositivity of the donor
- CMV seropositivity of the recipient
- CMV infection after allogeneic HSCT, CD34+ stem cell count
- CD3+ T-cell count of the transplant

4.2.2 HHV-6 PCR

Routine HHV-6 virus screening of patient blood samples was performed by real-time quantitative PCR (Polymerase Chain Reaction). A positive viral reactivation was defined as a viral DNA load of at least 2.5E+02 copies/ml EDTA whole blood. The routine HHV-6 PCR results were taken out of MEDOCS.

4.2.3 Peripheral blood mononuclear cells isolation

S-Monovette tubes were used for venous blood sampling. The tubes contained granules coated with the coagulation activator silicate. The granules were drained under the sterile bench and then 1ml of heparin was injected with a syringe and the tube was screwed tightly.

After blood drawing (one tube ~9ml), the PBMCs were isolated from peripheral venous blood. Ficoll Paque Plus medium was used as a separation medium during the centrifugation. PBMCs were isolated in 50 ml Falcon tubes by Ficoll density gradient centrifugation and were washed twice with DPBS. Cells with lower density (lymphocytes, monocytes) cannot pass the Ficoll Paque Plus medium and thus form a thin layer between erythrocytes, granulocytes and the blood plasma (Figure 4).

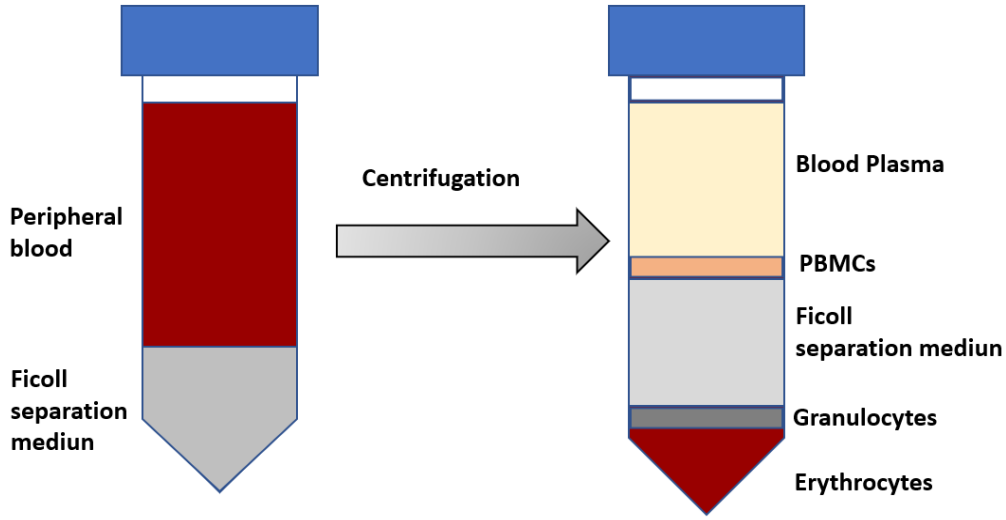

Figure 4: Isolation of PBMCs. Schematic representation of the isolation of PBMCs from peripheral blood.

In detail, 15 ml of Ficoll per 15 ml of peripheral blood were prepared in a 50 ml Falcon tube. The blood was diluted in proportion 1: 2 with DPBS and slowly covered with a pipette over the prepared Ficoll Paque Plus solution. The centrifugation was performed for 25 minutes at 400 rcf without brake function. Thereafter, the interphase with the PBMCs was carefully taken off with a Pasteur pipette and pooled in a 50 ml Falcon tube, refilled with DPBS to 50ml and centrifuged at 500 rcf for 10 min at room temperature. After centrifugation, the supernatant was discarded, and the cell pellet resuspended in 15ml DPBS, an aliquot was taken for cell number determination and the Falcon with the dissolved pellet was filled up to 50ml with DPBS and centrifuged once again at 400 rcf for 10 min at room temperature. Then the cell count was determined (see next section). After centrifugation, the supernatant was

discarded, the cell pellet resuspended in the previously determined amount of warm TexMACS medium (37°C).

4.2.4 Determination of cell number and vitality of cells

Trypan Blue (0.4%) staining was used for the discrimination between viable and non-viable cells and cell numbers were determined with a Neubauer chamber (86). The cell suspension was vortexed and an aliquot of 10 µl was taken out of the suspension. The cell sample was mixed with 10 µl trypan blue solution and pipetted into the Neubauer counting chamber and the cells were counted under the light microscope. The cover glass was fixed on the counting chamber by moistening the side surfaces. Vital cells were not stained by the dye, dead cells were stained with trypan blue and appeared blue under the light microscope. A counting grid of this chamber consists of 4 large squares, each square has 16 small squares. The volume of a large square corresponds to 0.1 mm^3. The cell number was calculated according to the following formula:

Cell number / ml = (counted cells / per grand square) x 2 x 10^4

After the determination of cell number, the cell count was adjusted to $5x10^6$ cells/ml in TexMACS medium.

4.2.5 Cell culture and stimulation assays

In this project we have analysed secretion of IL-2, IFN-γ as well as TNF-α in both CD4+ and CD8+ T-cells after stimulation of PBMCs with the HHV-6 specific antigen U54. U54, which is a tegument protein of HHV-6 and forms a cluster of proteins between the envelope and nucleocapsid and is the analogue of the CMV lower matrix phosphoprotein 65 (pp65) was used for cell culture experiments (86). To test overnight stimulation we used on the one hand U54 and on the other hand pp65 protein that is one of the most dominant CD4+ and CD8+ T-cell antigens of CMV and is widely used for the evaluation of CMV-specific immunity (90). An unstimulated well for the fluorescence minus one (FMO) control was also included. We established two different attempts for cell stimulation (overnight and 10 days stimulation) to evaluate if we can detect an appropriate HHV-6 specific immunity after overnight stimulation (86).

HHV-6 specific antigen U54

For the stimulation we used a peptide mix (PepMix U54, JPT Berlin, Germany) containing 15 nmol (25 µg) of the antigenic protein U54 (Swiss-Prot ID: Q9QJ29) for T-cell assays (antigen specific T-cell stimulation). The peptide mix contains 15meres with an overlap of 11 amino acids.

Short term (overnight) stimulation

For the overnight stimulation, PBMCs were suspended in TexMACS medium. Per well, $1.25x10^6$ cells were suspended in 250 µl TexMACS medium in a 48-well plate and were stimulated with U54, pp65 and Actin (negative control, ACTS) at a final concentration of 10 µg/ml. On the next day, staphylococcal enterotoxin B (positive control, SEB) was added at a final concentration of 2.5 µg/ml to a previously unstimulated well. Brefeldin A was added as a Golgi stop with a final concentration of 2.5 µg/ml to every well for the last 4 h of incubation before extracellular and intracellular staining (86).

Long-term stimulation (10 days)

Per well, $1.25x10^6$ cells were suspended in 250 µl TexMACS medium in a 48-well plate and were stimulated with U54 and ACTS at a final concentration of 10 µg/ml. Cells were expanded for 10 days in cell culture with interleukin-2 (IL-2; 10 U/mL) and interleukin-7 (IL-7; 10 ng/mL). Medium was changed every day or every second day (86).

On day 9, cytokines were washed out by changing medium (without interleukins) and on day 10, cells were restimulated for 6 h with the same antigens as on day 1. In addition, a previously unstimulated well was stimulated with SEB at a final concentration of 2.5 µg/ml. Before staining, Brefeldin A was added to all wells as a Golgi stop for the last 4 h of incubation with a final concentration of 2.5 µg/ml (86) .

4.2.6 Flow cytometry staining

After stimulation with Brefeldin A all wells were washed with 500µl cold DPBS and cells were harvested through "scraping" at the bottom with the pipette tip, then transferred to a FACS tube and filled up with DPBS. The cells were centrifuged at 20°C, 450 rcf for 3 minutes.

In the meantime, the Master Mix for surface staining was prepared. PBMCs were surface stained with monoclonal antibodies specific for CD3, CD4, CD8 and CD56. After centrifugation, the supernatant was discarded from the FACS tubes and all tubes were vortexed at low speed. Then, 7µl of the Master Mix (antibodies for surface staining) was added to each tube and carefully vortexed. The cells were incubated for 10 minutes in the dark at room temperature.

Next, antibodies were washed out with 4 ml of buffer solution (DPBS, Albunorm 20%, EDTA 0.25 mol/L) and cells in each tube were stained with 900µl AF350 premix for live/dead discrimination (Alexa Fluor 350 carboxylic acid, succinimidyl ester). After 10 min of incubation at room temperature the tubes were filled up with DPBS again and were centrifuged at 20°C for 3 minutes at 450 rcf.

Next, the cells were fixed and permeabilized using a Fix and Perm kit (86). The kit contains the fixation medium (Solution A) and the permeabilization medium (Solution B). First, cells in each tube were fixed with 100µl Solution A and incubated for 15 minutes at room temperature in the dark and then washed with DPBS. Second, the cell membranes were permeabilized with solution B. This enabled antibodies to have access to intracellular structures. Solution B and staining buffer were added to the antibodies for intracellular staining. Intracellular staining was conducted with antibodies against IFN-γ, IL-2 and TNF-α (Table 5).

50 µl staining buffer and 100 µl Solution B were added to the antibodies. Then, 158µl of the Master Mix for intracellular staining (including Solution B) was pipetted into each FACS tube, except the FMO control tube. To the FMO tube only 100µl of Solution B was added. After 30 min of incubation in the dark at room temperature, DPBS was added and the cells were centrifuged for 10 minutes at 300 rcf.

Finally, the stained cells were analysed by flow cytometry. For each measurement, 500,000 events were counted at each acquisition. Measurements were carried out on a LSR II flow cytometer and FACSDIVA acquisition/analysis software was used for data analysis (86) (Figure 5).

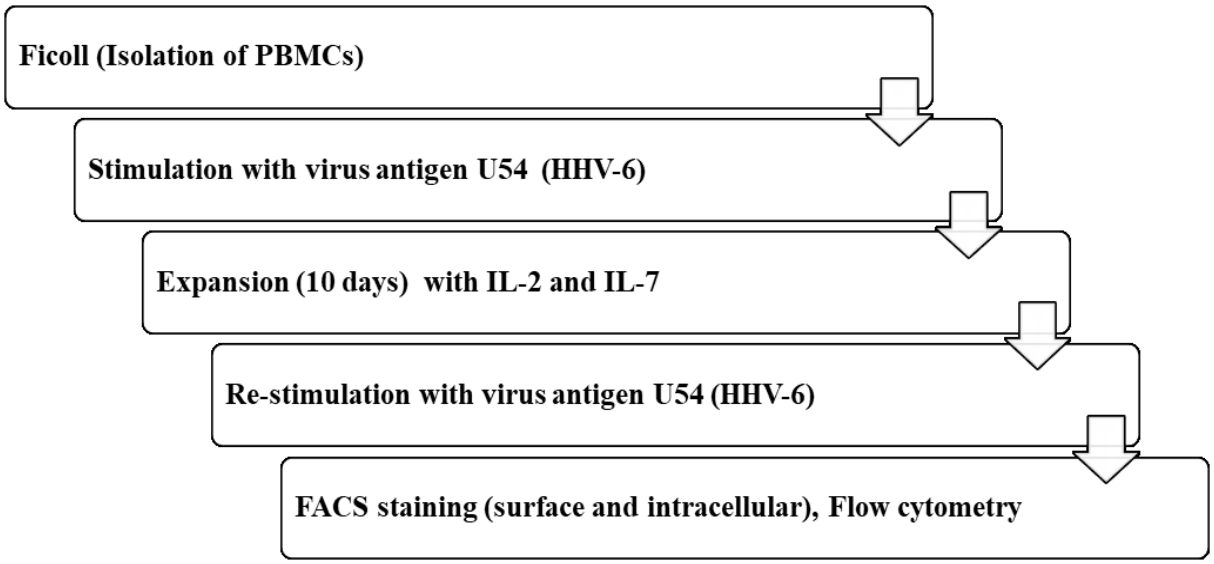

Figure 5: Flow diagram. Isolation, proliferation, expansion and flow cytometric measurement of HHV-6 specific T-cells.

Table 5: Antibodies for surface and intracellular staining of PBMCs

Antibodies	**Company**	**Order Nr.**	**Colour**	**Volume per well [µl]**
Surface- staining				
CD3	BioLegend	344814	PerCP	1.5
CD4	Becton Dickinson	560836	Alexa Fluor	1.5
CD8	Becton Dickinson	560179	APC H7	1.5
CD56	Becton Dickinson	562794	FITC	2.5
Intracellular staining				
IFN-γ	Becton Dickinson	562974	BV605	5
TNF-α	Becton Dickinson	562783	BV421	1.5
IL-2	Becton Dickinson	563265	BV510	1.5

4.2.7 Statistical analysis

In the control group, Mann Whitney-U test and the Fisher's exact test were used to describe the differences in the antigen-specific responses between the two age groups (<10 years and 10–18 years). Spearman rank correlation was used for describing the degree of association between age and frequency of HHV-6 specific T-cells.

For patients in the HSCT group, frequencies of HHV-6 specific T cells and rate of patients with detectable HHV-6 specific T cell response (defined as indicated below) were analysed for different time points after HSCT using descriptive statistical methods. For the description of the differences in the lymphocyte numbers between different patient groups and controls the non-parametric Mann Whitney-U test was used. *P*-values < 0.05 were considered statistically significant. In all analyses, data are presented as median values and ranges, if not otherwise indicated. All statistical evaluations, linear regression, and correlation analyses were performed by SPSS version 25 for Windows (SPSS Inc., Chicago, IL, USA).

4.2.8 Definition of HHV-6 specific T-cells

To define the HHV-6 (U54) specific T-cell response, the following two criteria had to be fulfilled:

1. The frequency of cytokine-secreting cells after U54 stimulation for CD4+ or CD8+ T-cells was at least 0.1 percentage points higher than in unstimulated cells of the negative control (ACTS).

2. The frequency of cytokine-secreting cells after U54 stimulation was at least two times higher than in the negative control (ACTS) (Figure 6).

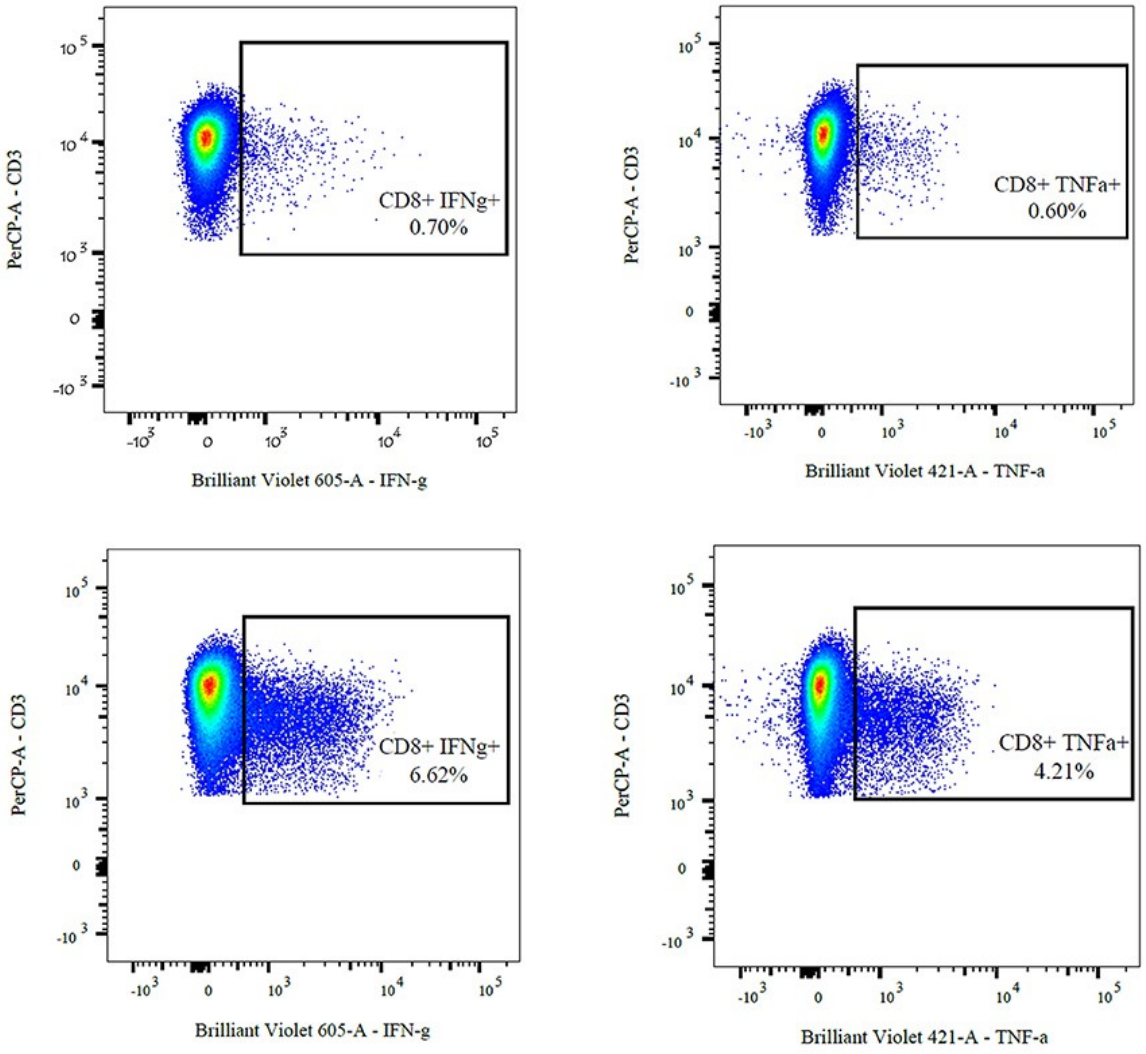

Figure 6: Cytokine secreting antigen specific CD8+ T-cells. FACS analysis of cytokine-producing CD8+ T-cells after stimulation with U54 and 10 days of expansion in cell culture. The upper two images show the negative controls (ACTS) compared to the U54 stimulated cells (left: IFN-γ, right: TNF-α). Figure reproduced from Schwarz et al. (86) from FRONTIERS in Pediatrics (Pediatric Infectious Diseases), CC BY 4.0 http://creativecommons.org/licenses/by/4.0/

5. Study Protocol

In the following Table 6 the details of this present study are summarized.

Table 6: Protocol synopsis

Title **Short title**	**HHV-6-specific T-cell immune reconstitution among children and adolescents after allogeneic stem cell transplantation** **CIRAST-HHV-6**
Study design	Multicenter, prospective obervational study
Centers	→ Medical University of Graz, Department of Pediatrics and Adolescent Medicine, Division of Pediatric Hematology/Oncology, → University Children's Hospital Tübingen, Department of Oncology/Haematology
Primary target values	• HHV-6 specific T-cell immunity in control group • HHV-6-specific T-cell immunity at different time points after allogeneic HSCT (3,6,9,12,18,24 months) in patients with or without (measurable) virus-reactivation
Number of study participants: **Number individuals of control group:**	N=28 N=25

5.1 Inclusion and exclusion criteria

Inclusion criteria

- Patients that underwent an allogeneic HSCT at one of the participating centers in the last 24 months or who underwent follow-up support at one of the participating centers during the study (up to 3 months before the end of the study).
- Age >1 year
- Written declaration of consent of the patient or of the parents / legal guardians

Exclusion criteria

- Severe transplantation-associated complications during the study period
- Rejection of the graft ("graft failure")
- Severe GvHD (grade 4)
- Relapse of the (malignant) disease or other complications (e.g. secondary malignancy) during the study period.
- Denied or withdrawal of consent

5.2 Study participant recruitment

Patients after allogeneic HSCT who received follow-up support at the respective centers were included. Patients were enrolled up to 24 months after allogeneic HSCT. After explaining the study procedures, patients were asked if they want to participate in the study. The study protocol was approved by the institutional review board and written informed consent was obtained from all subjects. Blood obtained in the collaborating institution (University Children's Hospital Tübingen, Department of Oncology/Haematology) was sent by overnight express to the laboratory at the Division of Pediatric Hematology/Oncology in Graz, where the analyses were performed.

5.3 Ethical Approval

The study was approved by the Institutional Review Board of the Medical University Graz and patients, parents or legal guardians of patients gave written informed consent in accordance with the Declaration of Helsinki. The project was first presented to the Ethics Committee in Graz in 2014. A positive vote was issued. A follow-up vote (26-178 ex 13/14) has been renewed annually and was valid until march 2019 (see suplplementary material). A positive vote was also issued from the Ethics Comitee in Tübingen.

6. Results

6.1 Control group

6.1.1 Short Term Stimulation with pp65 and U54

After a short overnight stimulation in three individuals (5.7, 8.3, and 9.8 years) with pp65 a CMV-specific immune response in all 3 individuals (Table 7) was detected. In contrast, no HHV-6-specific T-cell immune response was detected after overnight stimulation with U54 in none of the tested individuals. For this reason, we extended stimulation and expansion of the cells for 10 days for further analyses of HHV-6 specific T-cell immunity in the control group and patients (86).

Table 7: Short term stimulation with pp65 and U54 antigens. Table shows frequencies of CD4+ and CD8+ IFN-γ, IL-2 and TNF-α specific T-cells after stimulation with U54 and pp65 in 3 individuals.

		Frequency (%) of U54-specific CD4+ and CD8+ T-cells	**Frequency (%) of pp65-specific CD4+ and CD8+ T-cells**
CD4+	INF-γ response	1.29, 1.09, 0.70	1.55, 1.59, 3.21
	IL-2 response	1.71, 0.69, 0.83	1.22, 1.71, 3.41
	TNF-α response	1.89, 1.60, 0.28	3.11, 3.61, 3.75
CD8+	INF-γ response	1.96, 0.72, 0.90	1.91, 2.03, 3.44
	IL-2 response	1.63, 1.27, 0.75	1.0, 1.54, 1.93
	TNF-α response	1.18, 1.50, 1.00	2.6, 3.44, 3.69

6.1.2 Long Term Stimulation for ten days with pp65 and U54 in individuals of the control group

In these, 25 children and adolescents were included: 72% male, 28% female, median age 8.2 (range 3.1–18.3) years. All individuals showed an U54-specific response for at least one cytokine in either CD4+ or CD8+ T-cells. Twenty-four percentages of all individuals had a positive response in both cell types (86). In the following Table 8 percentages of individuals with virus-specific T-cell response, frequency of U54-specific T-cells and correlation coefficients between age and frequency of HHV-6 specific T-cells are listed.

Table 8: Number (percentages) of individuals with U54-specific T-cell response. Frequencies of U54-specific CD4+ and CD8+ T-cells and correlation coefficients between age and frequency of HHV-6 specific T-cells (Spearman Rho) are shown. Table adapted from Schwarz et al. (86) from FRONTIERS in Pediatrics (Pediatric Infectious Diseases), CC BY 4.0 http://creativecommons.org/licenses/by/4.0/ (IFN-γ, interferon-γ; IL-2, interleukin-2; TNF-α - tumour necrosis factor-α)

		Positive/tested individuals (%)	**Frequency (%) of U54-specific CD4+ T-cells, median (range)**	**Correlation coefficient (between age and frequency of HHV-6 specific T-cells) *p< 0.05**
CD4+	Positive INF-γ response	8/25 (32%)	1.31 (0.63-10.54)	-0.037
	Positive IL-2 response	3/25 (12%)	1.38 (0.10-11.48)	-0.002
	Positive TNF-α response	12/25 (48%)	2.29 (0.50-24.91)	0.145
	Positive response (any cytokine)	14/25 (56%)	1.48 (0.12-8.26)	0.168
	Positive IFN- γ/IL-2 response	2/25 (8%)	0.05 (0.01-0.29)	0.112
	Positive IFN-γ/TNF-α response	8/25 (32%)	0.15 (0.01-1.13)	0.077
	Positive IL-2/TNF- α response	3/25 (12%)	0.10 (0-0.30)	-0.079
	Positive IL-2/TNF-α/IFN-γ response	2/25 (8%)	0.05 (0.01-0.25)	-0.018
CD8+	Positive INF-γ response	10/25 (40%)	1.69 (0.80-10.21)	0.088
	Positive IL-2 response	3/25 (12%)	1.01 (0.60-4.67)	0.225
	Positive TNF-α response	14/25 (56%)	2.10 (0.72-9.83)	**0.465***
	Positive response (any cytokine)	17/25 (68%)	1.86 (0.69-8.04)	0.286
	Positive IFN-γ/IL-2 response	2/25 (8%)	0.07 (0-0.20)	0.146
	Positive IFN-γ/TNF-α response	11/25 (44%)	0.23 (0.02-2.92)	0.142
	Positive IL-2/TNF-α response	1/25 (4%)	0.07 (0-0.34)	0.141
	Positive IL-2/TNF-α/IFN-γ	1/25 (4%)	0.05 (0-0.2)	0.083

6.1.3 Comparison of frequencies of HHV-6 specific T-cells in individuals younger and older than 10 years

Comparing the age groups (<10 years, *n* = 13, 3.1–9.2, median age 6.6 years old and 10–18 years, *n* = 12, 10.5–18.3, median 17.2 years old), significantly higher frequencies of HHV-6-specific TNF-α producing CD8+ T-cells in the older age group (p = 0.033) were detected (86) (Figure 7).

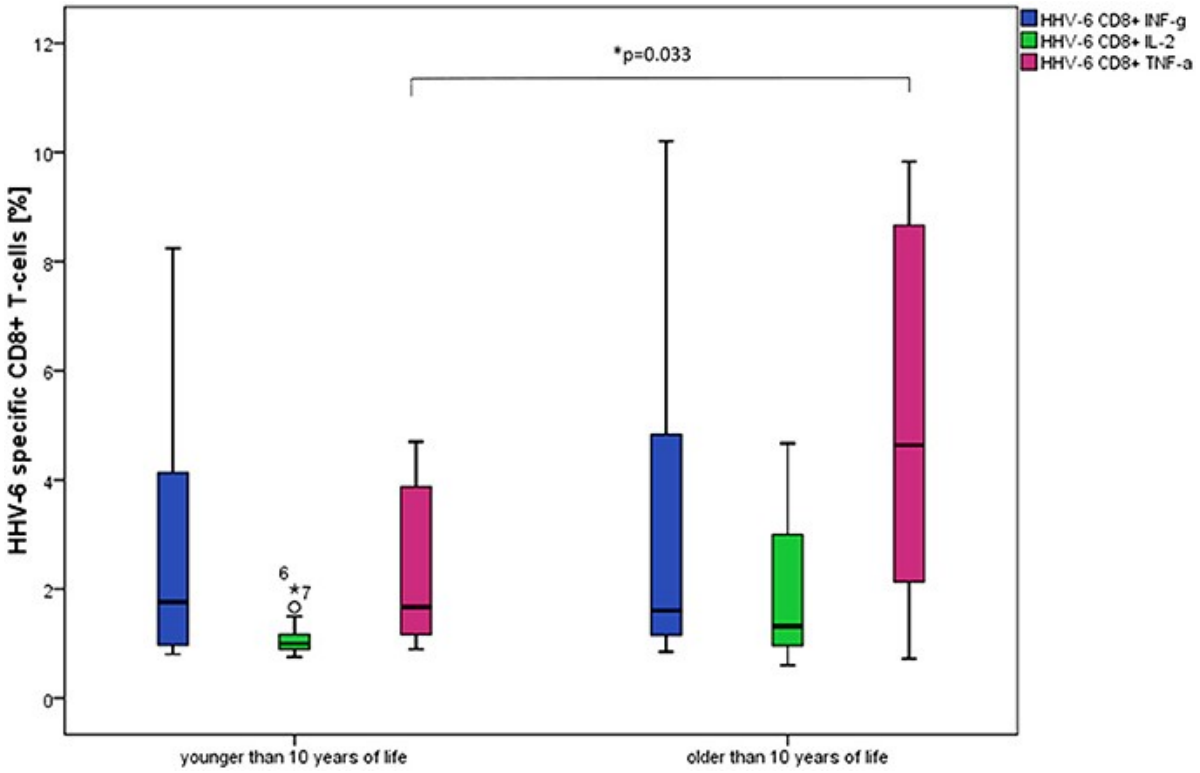

Figure 7: HHV-6 specific CD8+ T-cells in healthy individuals. Box plots show significantly higher frequencies of TNF-α producing CD8+ T-cells in individuals older than 10 years after stimulation with HHV-6 specific antigen U54 *p = 0.033. Figure from Schwarz et al. (86) from FRONTIERS in Pediatrics (Pediatric Infectious Diseases), CC BY 4.0 http://creativecommons.org/licenses/by/4.0/

Frequencies of TNF-α producing CD8+ T-cells positively correlated with the age of the individuals r = 0.465 (Spearman-Rho) on a two-sided significance level of p= 0.019. Linear regression analysis showed a positive relation between age and frequency of HHV-6-specific TNF-α producing CD8+ T-cells with a regression coefficient of r = 0.490 (Figure 8). No positive correlations were found for the other cytokines (86).

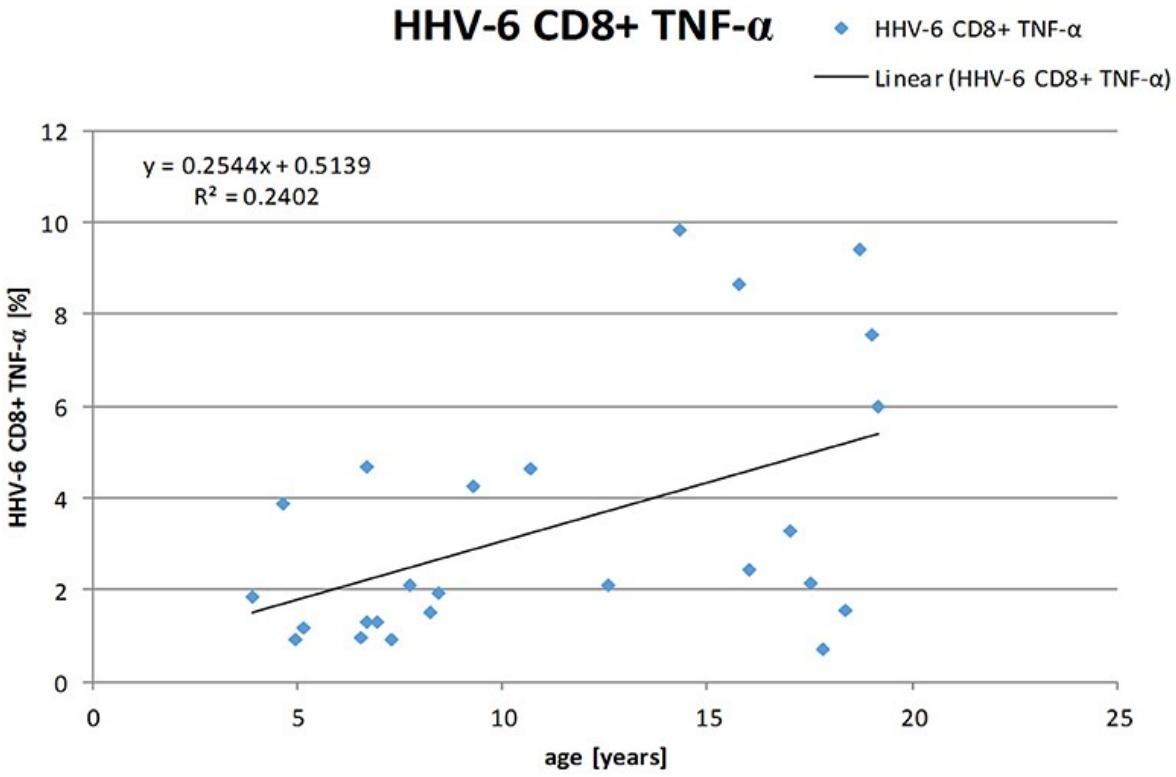

Figure 8: Linear regression analysis of HHV-6 specific CD8+ T-cells TNF-α. Results show a positive correlation between age and frequencies of HHV-6-specific TNF-α producing CD8+ T-cells. (Spearman-Rho $r = 0.490$). Figure from Schwarz et al. (86) from FRONTIERS in Pediatrics (Pediatric Infectious Diseases), CC BY 4.0 http://creativecommons.org/licenses/by/4.0/

6.2 Patient group

6.2.1 Patients, donors and disease characteristics

For analyses, all clinical data were recorded in an Excel spreadsheet.

Age and sex characteristic:

28 patients [median age at HSCT 7.87 years (range 1.50-22.04), 64% male (n=18), 34% female (n=10)] who received an allogeneic HSCT at the Division of Pediatric Hematology/Oncology, Medical University of Graz (n=22) or at the Department of Oncology/Haematology, University Children's Hospital Tübingen (n=6) between December 2013 and December 2017 were included in this study (Table 9).

Table 9: Patients´ and control group age and sex characteristics

	Patient group (n=28)	**Control group (n=25)**	***p-value***
Age: years, median (range)	10.12 (2.0-25.8)	8.2 (3.1–18.3)	0.432
Sex (%):			0.572
Male	n=18, 64%	n=18, 72%	
Female	n=10, 36%	n=7, 28%	

Indications for stem cell transplantation:

The indications for allogeneic HSCT comprised different malignant and non-malignant underlying diseases such as ALL (n=5, 18%), AML (n=4, 14%), primary immunodeficiencies (n=4, 14%), ß-Thalassamia (n=3, 11%), Sickle cell disease (n=3, 11%), Severe Aplastic Anaemia (n=2, 7%), Metachromatic leukodystrophy (n=2, 7%), Non-Hodgkin lymphoma (n=1, 3%), Juvenile myelomonocytic leukemia (n=1, 3%), Krabbe disease (n=1, 3%), Rhabdomyosarcoma (n=1, 3%), Ependymoma (n=1, 3%).

Stem cell source:

A total of 6 (21%) BM transplantations and 22 (79%) PBSC transplantations were performed.

Donor characteristics:

Depending on the availability of a suitable donor, patients received HSCs from HLA-identical sibling donors (n=6), or HLA-identical third-party donors (n=15). If no HLA-identical donor could be found, a haploidentical family donor, (usually a 'parent') became the donor (n=7).

Manipulation of donor cells:

CD19 depletion and T-cell receptor (TCR) αβ depletion and enrichment of CD34+ HSCs were performed in some cases. In Table 12 the manipulation of donor cells is summarized.

CD34+ and CD3+ stem cell count:

20 of 28 patients (71%) received a CD34+ stem cell count in the transplant with more than $6x10^6$ cells/kg bodyweight of the recipient and 7 of 28 (25%) received a transplant with a CD3+ cell count above $3x10^8$ cells/kg body weight of the recipient.

Type of conditioning:

We evaluated the type of conditioning (reduced intensity or myeloablative). Reduced-intensity conditioning (RIC) is a regime that uses less radiation and chemotherapy in comparison to the myeloablative conditioning regimen (MAC) that results in destruction of BM cells. In 24 cases (86%) a MAC and in 3 cases (11%) a RIC and in one case (3%) no condition was conducted.

GvHD prophylaxis:

Pharmacologic interventions to prevent GvHD for patients after allogeneic HSCT. In the following Table 10**,** the GvHD prophylaxis is summarized.

Table 10: GvHD prophylaxis of patients after allogeneic HSCT.

GvHD prophylaxis	**Number of patients (n)**
Mycophenolate mofetil (MMF)	15
Cyclosporine A (Cy A) and Methotrexate (MTX)	6
MMF and MTX	1
MMF and Sirolimus (SIR)	1
Cy A	1
Tacrolimus (FK506), MMF and Cy A	1
Cy A and MMF	1
Prograf and MTX	1
Methylprednisolon	1

aGvHD:

8 of 28 (29%) patients developed an aGvHD after allogenic HSCT, in 7 cases at grade 2, in 4 cases at grade 3 and in 3 cases grade 2 and 1 at grade 0. The affected organs were skin (9 cases), intestine (3 cases) and liver (2 cases).

CMV serostatus and reactivation of recipients and donors:

We have evaluated the CMV serostatus of the donors and recipients and CMV reactivations after allogeneic HSCT. In the following

Table 11 the serostatus of donors and recipients is listed. Among the patients, 5 patients developed a CMV reactivation (with positive PCR).

Table 11: CMV serostatus of donors and recipients and reactivations.

	CMV serostatus of donors (D) and recipients (R), Number of Individuals (n)	**CMV reactivations (n) of donors (D) and recipients (R), Number of Individuals (n)**
D+/R+	n= 8	n=5
D-/R-	n= 11	n=0
D+/R-	n= 3	n=0
D-/R+	n= 4	n=0
Not known	n= 2	n=0

D= donors; R= recipients; + = seropositive; - =seronegative

HHV-6 reactivation during follow-up:

In total, 14 of 28 (50%) of patients reactivated HHV-6 during follow-up defined as positive HHV-6 PCR. Patient characteristics are shown in the following Table 12.

Table 12: Patient characteristics.

PATIENT	SOURCE	DONOR	MANIPULATION	COND. TYPE	CONDITIONING CHEMOTHERAPY	GVHD PROPHYLAXIS
1	PBSC	MUD	α/β/CD19 depl // CD34+	MAC	BU/CY/MEL + FLAMSA	MMF /MTX 4x
2	PBSC	MSD	α/β/CD19 depl // CD34+	MAC	BU/FLU/THT/ATG	MMF / SIR
3	PBSC	haplo (6/10)	α/β/CD19 depl	MAC	TREO/FLU/THT/ATG	MMF
4	PBSC	MUD	α/β/CD19 depl // CD34+	MAC	TREO/FLU/THT/ATG	MMF
5	PBSC	haplo	α/β/CD19 depl // CD34+	MAC	TREO/FLU/THT/ATG	MMF
6	PBSC	MSD	α/β/CD19 depl // CD34+	RIC	FLU/CY/THT/ATG	MMF
7	PBSC	MUD (9/10)	unmanip.// α/β/CD19 depl // CD34+	MAC	TREO/CY/MEL/ATG	CyA / MTX 3x //MMF
8	PBSC	MUD	α/β/CD19 depl // CD34+	MAC	TREO/FLU/THT/ATG	MMF
9	PBSC	MSD	α/β/CD19 depl // CD34+	MAC	TREO/FLU/THT	MMF
10	PBSC	MUD (10/12)	α/β/CD19 depl // CD34+	MAC	TREO/FLU/THT/ATG	MMF
11	PBSC	MUD	unmanip.// α/β/CD19 depl // CD34+	MAC	TREO/CY/MEL/ATG	MMF
12	PBSC	MUD (11/12)	α/β/CD19 depl // CD34+	MAC	TREO/FLU/THT/ATG	MMF
13	PBSC	haplo	CD34+	0	0	MMF
14	PBSC	MUD	unmanip.	MAC	TBI/VP16/ATG	CyA / MTX 3x
15	BM	MSD	unmanip.	MAC	BU/CY/MEL	CyA

PATIENT	SOURCE	DONOR	MANIPULATION	COND. TYPE	CONDITIONING CHEMOTHERAPY	GVHD PROPHYLAXIS
16	BM	MUD	unmanip.	MAC	TREO/FLU/THT/ATG	CyA / MTX 3x
17	BM	MSD	unmanip.	MAC	TREO/FLU/THT/ATG	CyA / MTX 1x // MMF
18	PBSC	MRD (HLA-identical mother)	α/β/CD19 depl // CD34+	MAC	TREO/MEL/ATG	MMF
19	BM	MSD	unmanip.	MAC	TREO/FLU/THT/ATG	FK506/ MMF / CyA
20	PBSC	MUD	α/β/CD19 depl // CD34+	MAC	TREO/FLU/THT/ATG	MMF
21	PBSC	MUD	unmanip.	MAC	FLU/AMSA/CY // TREO/CY/ATG	CyA/MMF
22	PBSC	MUD	unmanip.	MAC	TBI/VP16/ATG	CyA / MTX 3x
23	PBSC	haplo (6/10)	unmanip	RIC	ATG/TBI/CY/FLU	0
24	BM	MUD 11/12	unmanip	MAC	FLU/BU/ATG/CY/THT	Prograf/MTX
25	PBSC	haplo (6/10)	unmanip	MAC	MEL/FLU/ATG/THT	MMF
26	PBSC	MUD	unmanip	RIC	TBI/CY/FLU/ATG	Methylprednisolon
27	PBSC	haplo (6/10)	unmanip	MAC	MEL/FLU/ATG/THT	0
28	BM	MUD 11/12	unmanip	MAC	FLU/BU/ATG//CY/THT	CyA/MTX

Abbreviations: MAC= myeloablative conditioning, RIC=reduced intensity conditioning, BU= Busulfan, CY= Cyclophosphamide, MEL= Melphalan, AMSA= Amsacrine, FLAMSA= Fludarabine/Amsacrine, FLU= Fludarabine, THT= Thiotepa, ATG= Anti-thymocyte globulin, TREO= Treosulfan, TBI= total body irritation, VP16= Etopside, MUD= HLA-matched unrelated donor, MSD= HLA-matched sibling donor, MRD= HLA-matched related donor, PBSC= peripheral blood stem cells, BM= bone marrow, MMF= Mycophenolate Mofetil, MTX= Methotrexate, SIR= Sirolimus, CyA= Cyclosporin-A, FK506=Tacrolimus, depl.= depleted, unmanip.= unmanipulated

6.2.2 Measurements of HHV-6 specific T-cell immunity

In total, 51 analyses of HHV-6 specific immunity during follow-up of patients were carried out at 3 months after HSCT (n=14), at 6 months after HSCT (n=11), at 9 months after HSCT (n=10), at 18 months after HSCT (n=4) and at 24 months after HSCT (n=3).

We have evaluated HHV-6 specific immunity in patients up to 24 months after allogeneic HSCT. In Table 13, number (percentages) of patients with HHV-6-specific T-cell responses at different time points are shown.

Table 13: Number (percentages) of patients with U54-specific T-cell response with different cytokines (and cytokine combinations) in CD4+ and CD8+ T-cells at different time points after HSCT

		Number of individuals with positive response, **3 months** after HSCT (N=14)	Number of individuals with positive response **6 months** after HSCT (N=11)	Number of individuals with positive response **9 months** after HSCT (N=10)	Number of individuals with positive response **12 months** after HSCT (N=9)	Number of individuals with positive response **18 months** after HSCT (N=4)	Number of individuals with positive response **24 months** after HSCT (N=3)
CD4+	Positive INF-γ response	3/14 (21%)	3/11 (27%)	4/10 (40%)	5/9 (55%)	2/4 (50%)	0/3 (0%)
	Positive IL-2 response	1/14 (7%)	4/11 (36%)	4/10 (40%)	0/9 (0%)	1/4 (25%)	0/3(0%)
	Positive TNF-α response	3/14 (21%)	4/11(36%)	7/10 (70%)	4/9 (44%)	3/4 (75%)	2/3 (66%)
	Positive IL-2/TNF-α/IFN-γ response	2/14 (14%)	2/11 (18%)	4/10 (40%)	0/9 (0%)	2/4 (50%)	0/3(0%)
	Positive IFN- γ/IL-2 response	5/14 (36%)	6/11(54%)	6/10 (60%)	4/9 (44%)	2/4 (50%)	1/3 (33%)
	Positive IFN-γ/TNF-α response	3/14 (21%)	2/11 (18%)	5/10 (50%)	0/9 (0%)	2/4 (50%)	1/3 (33%)
	Positive IL-2/TNF- α response	2/14 (14%)	1/11 (9%)	4/10 (40%)	0/9 (0%)	2/4 (50%)	0/3 (0%)
	Positive response (any cytokine)	3/14 (21%)	6/11 (54%)	3/10 (30%)	3/9 (33%)	3/4 (75%)	0/3(0%)

		Number of individuals with positive response, **3 months** after HSCT (N=14)	Number of individuals with positive response **6 months** after HSCT (N=11)	Number of individuals with positive response **9 months** after HSCT (N=10)	Number of individuals with positive response **12 months** after HSCT (N=9)	Number of individuals with positive response **18 months** after HSCT (N=4)	Number of individuals with positive response **24 months** after HSCT (N=3)
CD8+	Positive INF-γ response	5/14 (36%)	4/11 (36%)	3/10 (30%)	5/9 (55%)	2/4 (50%)	1/3 (33%)
	Positive IL-2 response	1/14 (7%)	3/11 (27%)	4/10 (40%)	3/9 (33%)	1/4 (25%)	0/3 (0%)
	Positive TNF-α response	4/14 (28%)	4/11 (36%)	3/10 (30%)	3/9 (33%)	1/4 (25%)	2/3 (66%)
	Positive IL-2/TNF-α/IFN-γ	0/14 (0%)	1/11 (9%)	3/10 (30%)	4/9 (44%)	1/4(25%)	0/3 (0%)
	Positive IFN-γ/IL-2 response	2/14 (14%)	4/11 (36%)	5/10 (50%)	4/9 (44%)	3/4(75%)	2/3 (66%)
	Positive IFN-γ/TNF-α response	1/14 (7%)	3/11 (27%)	3/10 (30%)	4/9 (44%)	1/4(25%)	1/3 (33%)
	Positive IL-2/TNF-α response	1/14 (7%)	2/11 (18%)	3/10 (30%)	2/9 (22%)	1/4(25%)	1/3 (33%)
	Positive response (any cytokine)	3/14 (21%)	3/11 (27%)	2/10 (20%)	4/9 (44%)	2/4 (50%)	1/3 (33%)

6.2.3 Frequencies of HHV-6 specific T-cells at different time points after allogeneic HSCT

In order to describe the course of the HHV-6 specific T-cell immunity we have analysed frequencies (%) of HHV-6 specific T-cells at 3, 6, 9, 12, 18 and 24 months after allogeneic HSCT.

Medians and range of HHV-6 specific T-cells at different time points are presented in Table 14. The frequencies (%) of HHV-6 specific T-cells (CD4+ and CD8+ for any cytokine) at different time points after allogeneic HSCT are shown as boxplots in the following Figure 9.

Table 14: Frequencies (%) of HHV-6 specific T-cells after stimulation with antigen U54 at different points after allogeneic HSCT. Table shows frequencies (%) as median (range) of CD4+ and CD8+ HHV-6 specific T-cells.

		Frequencies (%), median (range) of HHV-6 specific T-cells **3 months** after HSCT (N=14)	Frequencies (%), median (range) of HHV-6 specific T-cells **6 months** after HSCT (N=11)	Frequencies (%), median (range) of HHV-6 specific T-cells **9 months** after HSCT (N=10)	Frequencies (%), median (range) of HHV-6 specific T-cells **12 months** after HSCT (N=9)	Frequencies (%), median (range) of HHV-6 specific T-cells **18 months** after HSCT (N=4)	Frequencies (%), median (range) of HHV-6 specific T-cells **24 months** after HSCT (N=3)
CD4+	INF-γ	1.26 (0.34-4.4)	1.56 (0.62-6.51)	1.59 (0.81-20.11)	2.2 (1.04-8.67)	4.21 (0.96-9.12)	1 (0.98-1.47)
	IL-2	1.31 (0.33-3.3)	1.37 (0.42-4.5)	2.16 (0.66-4.03)	1.17 (0.9-6)	1.67 (1.58-2.02)	1 (0.98-1.47)
	TNF-α	1.74 (0.22-7.79)	1.62 (0.61-14.74)	2.08 (0.98-15.79)	1.84 (0.67-9.46)	3.19 (1.02-6.87)	2.69 (1.24-3.06)
	INF-γ/IL-2	0.9 (0-7)	1.56 (0.28-4.33)	2.18 (0.69-7.11)	1.49 (0.94-5.5)	2.16 (0.86-2.3)	1.4 (1-2.2)
	INF-γ/TNF-α	1.07 (0-9.57)	3 (0.44-12.75)	2.33 (1.01-10.68)	2.5 (0.75-25.25)	4.03 (0.86-14.52)	1.7 (1-5.35)
	IL-2/TNF-α	1.29 (0.18-3.4)	1.5 (0.5-8.27)	3.17 (0.81-7)	1.24 (0.65-3.67)	2.33 (1.58-2.71)	1.24 (0.5-2.36)
	INF-γ/IL-2/TNF-α	0.79 (0-6.25)	1.32 (0.24-3.54)	2.89 (0.64-19.33)	1.49 (0.64-5.5)	2 (0.83-2.43)	1.5 (1-9)
	INF-γ OR IL-2 OR TNF-α (any cytokine)	1.34 (0.38-3.55)	2.21 (0.63-4.22)	1.65 (0-10.17)	1.61 (0.96-5.92)	2.93 (1.04-5.73)	1.4 (1.25-1.59)

		Frequencies (%), median (range) of HHV-6 specific T-cells **3 months** after HSCT (N=14)	Frequencies (%), median (range) of HHV-6 specific T-cells **6 months** after HSCT (N=11)	Frequencies (%), median (range) of HHV-6 specific T-cells **9 months** after HSCT (N=10)	Frequencies (%), median (range) of HHV-6 specific T-cells **12 months** after HSCT (N=9)	Frequencies (%), median (range) of HHV-6 specific T-cells **18 months** after HSCT (N=4)	Frequencies (%), median (range) of HHV-6 specific T-cells **24 months** after HSCT (N=3)
CD8+	INF-γ	1.03 (0-14.75)	1.82 (0-14.43)	1.47 (0-12.73)	2.77 (0.41-17.77)	2.7 (1.17-5.88)	1.15 (0.83-11.86)
	IL-2	0.9 (0-2.37)	1 (0.42-7.11)	1.26 (0.11-10.32)	1.37 (0.61-3.24)	1.5 (1.02-2.53)	1.33 (1.01-1.78)
	TNF-α	1.08 (0.21-13.29)	1.31 (0.51-7.62)	1.58 (0.03-5.46)	1.55 (0.23-5.85)	1.55 (0.96-3.52)	3.09 (1.16-10)
	INF-γ/IL-2	0.95 (0-2.66)	1 (0.11-3.8)	1.25 (0-7.25)	2.27 (0.53-4.4)	1.29 (0.88-2.16)	1.2 (1-1.8)
	INF-γ/TNF-α	1 (0.16-20.28)	1.79 (0-11.54)	1.7 (0-10.95)	2.36 (0.41-13.66)	2.21 (1.34-3.67)	2.22 (2-18)
	IL-2/TNF-α	1 (0-2.33)	1.09 (0.1-8.75)	1.38 (0-8.25)	2.06 (0.21-5)	1.635 (0.93-2.95)	1.59 (1.5-3)
	INF-γ/IL-2/TNF-α	0.9 (0.02-2.37)	1.07 (0.15-8.75)	0.6 (0-7.25)	2.23 (0.38-4.5)	1.05 (0.75-2.68)	1.5 (1-3.6)
	INF-γ OR IL-2 OR TNF-α (any cytokine)	1.22 (0-11.13)	1.67 (0.55-10.2)	1.44 (0-7.48)	2.15 (0.45-9.11)	2.1 (1.13-4.03)	1.54 (1.01-7.75)

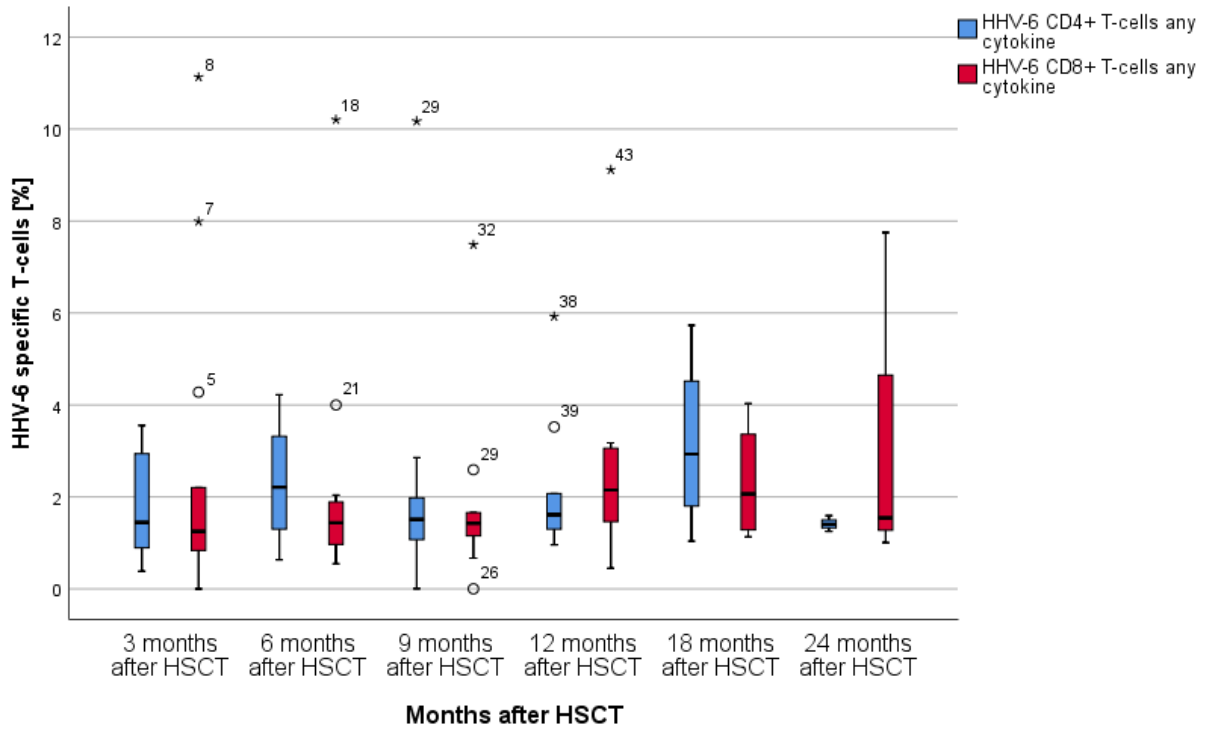

Figure 9: Frequencies (%) of HHV-6 specific T-cells (CD4+ and CD8+ for at least one cytokine) at different time points after allogeneic HSCT. The box plots display range, interquartile range and median.

6.2.4 Frequencies of HHV-6 specific T-cells between patient group in the first and second year after allogeneic HSCT and control group

In order to describe the HHV-6 specific immunity after HSCT and to find out if there are differences in the frequencies of HHV-6 specific CD4+ and CD8+ T-cells, we compared the frequencies of HHV-6 specific T-cells (for single cytokines and in different combinations) between the first and second year after allogeneic HSCT and also with those of the control group.

There were significant higher frequencies of triple positive HHV-6 specific CD8+ T-cells (IL-2/TNF-α/IFN-γ) and double positive HHV-6 specific CD8+ T-cells (IL-2/TNF-α) in patients in the second year after allogeneic HSCT when compared to patients in the first year after HSCT, see Table 15 and Figure 10.

Table 15: Frequencies (%) of HHV-6 specific CD8+ T-cells in patients in the first and second year after allogeneic HSCT. Table shows frequencies (%) as median (range) of CD8+ HHV-6-specific T-cells.

	Patients **in the first year after HSCT**	Patients **in the second year after HSCT**	p-value
HHV-6 CD8+ T-cells (IL-2/TNF-α/IFN-γ) (%), median (range)	0.93 (0-8.75)	1.86 (0.38-4.5)	0.034
HHV-6 CD8+ T-cells (IL-2/TNF-α) (%), median (range)	1 (0-8.75)	1.89 (0.21-5)	0.040

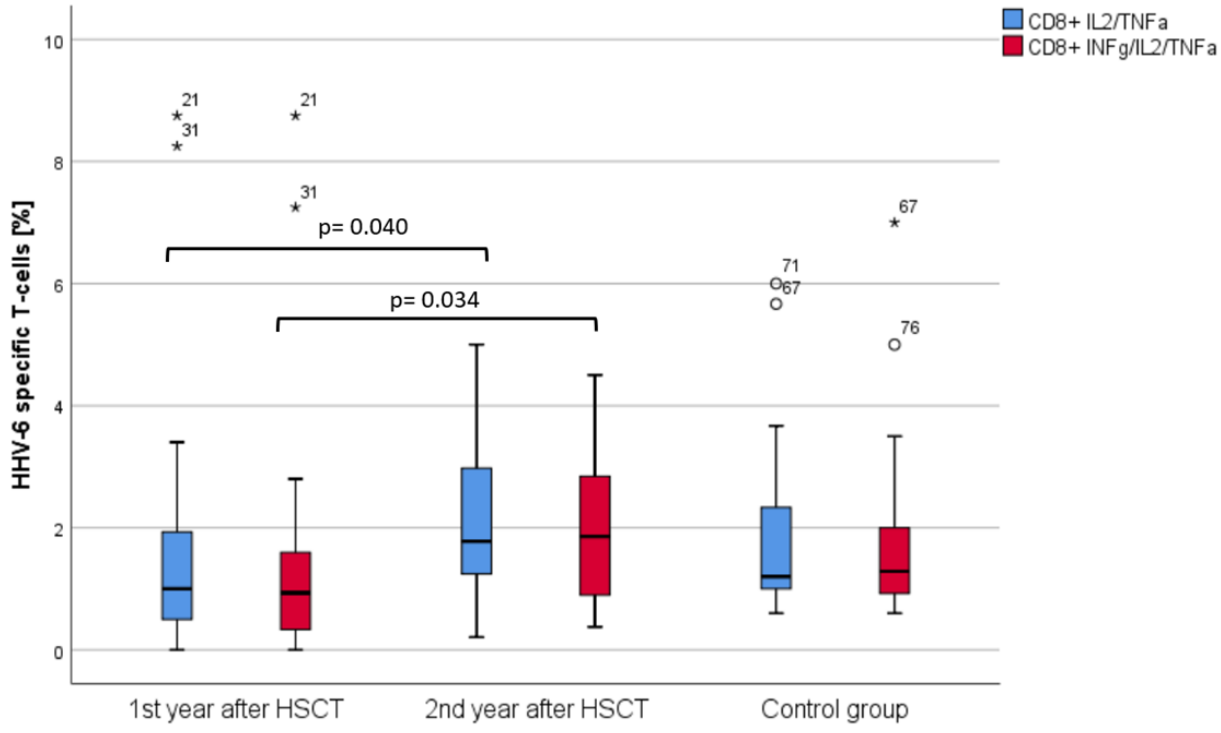

Figure 10: Frequencies (%) of HHV-6 specific CD8+ T-cells (IL-2/TNF-α and IFN-γ/ IL-2/TNF-α) in patients in the first and second year after HSCT and in the control group. Box plots show significantly higher frequencies of HHV-6 specific CD8+ T-cells in patients in the second year after allogeneic HSCT. The box plots display range, interquartile range and median.

Furthermore, patients during the first year after allogeneic HSCT had significant lower frequencies of HHV-6 specific CD8+ T-cells (TNF-α) compared to the control group, see Table 16 and Figure 11**.** No differences in the frequencies of HHV-6 specific CD8+ T-cells between the patients in the second year after HSCT and the control group were observed.

Table 16: Frequencies (%) of HHV-6 specific CD8+ T-cells in patients in the first year after allogeneic HSCT and control group. Table shows frequencies (%) as median (range) of CD8+ HHV-6-specific T-cells.

	Patients **in the first year after HSCT**	Control group	p-value
HHV-6 CD8+ T-cells (TNF-α) (%), median (range)	1.34 (0.03-13.29)	2.1 (0.72-9.83)	0.022

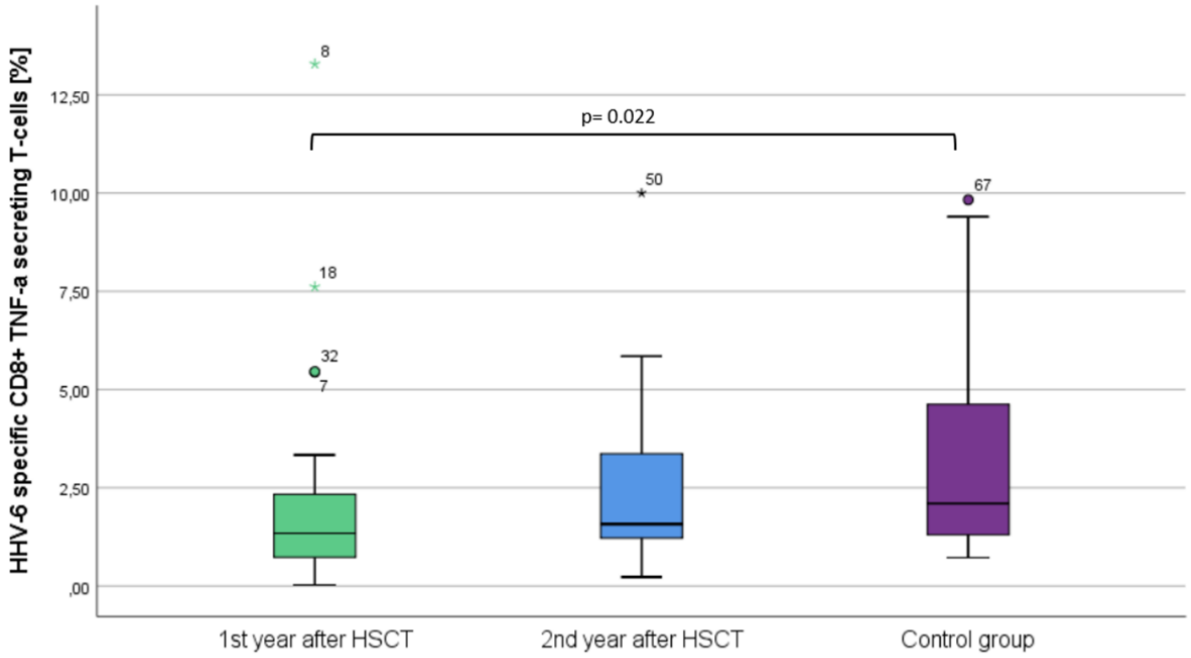

Figure 11: Frequencies (%) of HHV-6 specific CD8+ T-cells (TNF-α) in patients in the first and second year after HSCT and control group. Box plots show significantly higher frequencies of HHV-6 specific CD8+ T-cells (TNF-α) in individuals of the control group compared to patients in the first year after allogeneic HSCT. The box plots display range, interquartile range and median.

6.2.5 Comparison of frequencies of HHV-6 specific T-cells in patients with different stem cell sources, conditioning regimes and manipulations of donor cells

Furthermore, we have analysed if there are differences between frequencies of HHV6-specific T-cells and parameters such as stem cell source (PBSC or BM), conditioning type (RIC or MAC) and manipulation of the donor cells (unmanipulated vs. α/β/CD19+ depleted T-cells and CD34+ enriched graft). We have observed significant higher frequencies of HHV-6-specific CD8+ T-cells secreting IL-2 in patients with unmanipulated donor cells compared to patients with administered α/β/CD19+ depleted T-cells and CD34+ enriched donor graft in the first year after HSCT (Table 17 and Figure 12). No significant differences in relation to stem cell source, conditioning regimes and manipulation of stem cells were observed in the second year after allogeneic HSCT and for HHV-6 specific CD4+ T-cells.

Table 17: Frequencies (%) of HHV-6 specific CD8+ T-cells in patients with unmanipulated and manipulated donor graft in the 1st year after allogeneic HSCT. Table shows frequencies (%) as median (range) of CD8+ HHV-6-specific T-cells.

	Patients during the first year after HSCT, **unmanipulated** graft	Patients during the first year after HSCT, **α/β/CD19+ depleted T-cells and CD34+ enriched graft**	p-value
HHV-6 CD8+ T-cells (IL-2) (%), median (range)	1.61 (0.43-10.32)	0.9 (0-2.38)	0.032

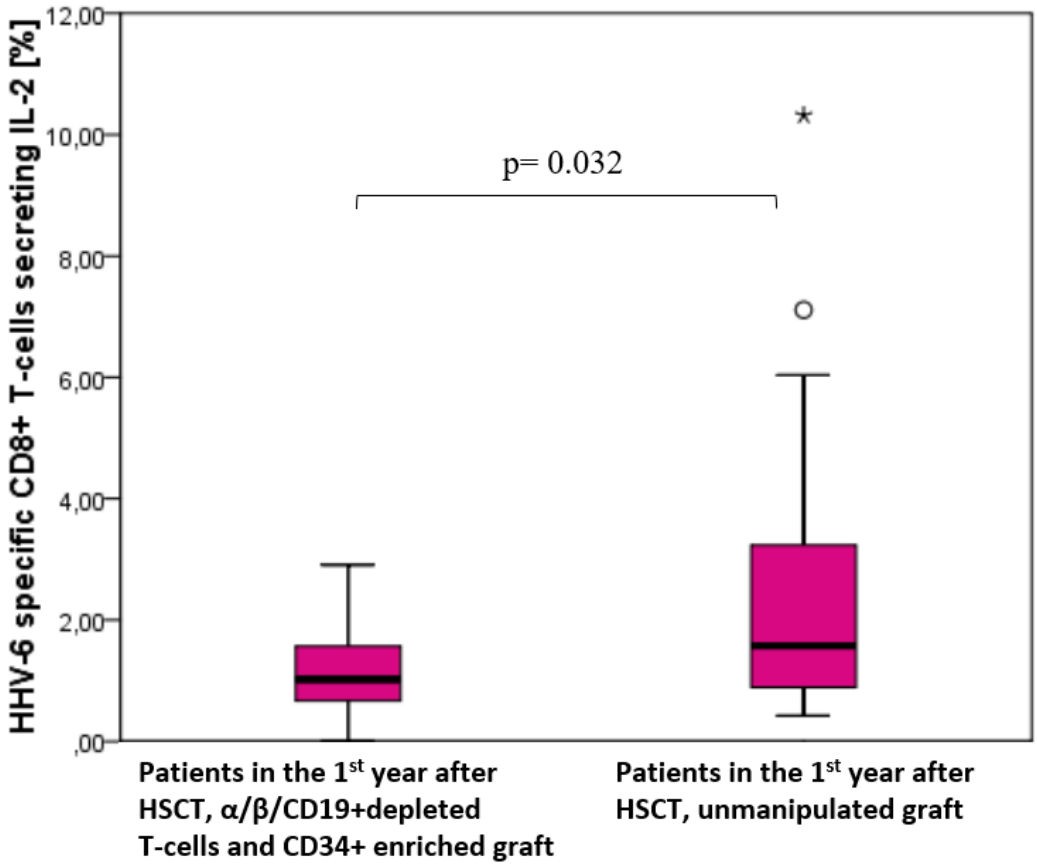

Figure 12: Frequencies of HHV-6 specific CD4+ T-cells (IFN-γ/IL-2 and IFN-γ/IL-2/TNF-α) in patients with manipulated and unmanipulated graft during the first year after HSCT. Box plots show significantly higher frequencies of HHV-6 specific CD8+ T-cells secreting IL-2 in patients with administered unmanipulated graft in comparison to patients with administered manipulated graft, p = 0.032. The box plots display range, interquartile range and median.

6.2.6 Frequencies of HHV-6 specific T-cells in patients with and without HHV-6 reactivation

The next step was to investigate whether there are differences in the frequencies of HHV-6-specific T-cells in patients with or without HHV-6 reactivation.

First, the frequencies of HHV-6 specific T-cells in the first year after HSCT (3, 6 and 9 months after HSCT taken together) in patients with and without viral reactivation and then the frequencies of HHV-6 specific T-cells in the second year (12, 18 and 24 months after HSCT taken together) in patients with and without virus reactivation were compared.

Second, the frequencies of HHV-6 specific T-cells in patients with viral reactivations during the first year after allogeneic HSCT were compared with those of patients without HHV-6 reactivation during the two years after allogeneic HSCT.

Finally, the frequencies of HHV-6 specific T-cells of the control group was compared with different patient groups: first year, second year after HSCT, with and without HHV-6 reactivation.

Comparison of frequencies of HHV-6 specific T-cells in patients with and without HHV-6 reactivation in the first and the second year after allogeneic HSCT

In the first year after allogeneic HSCT patients without reactivation had significant higher frequencies of CD4+ specific HHV-6 T-cells (IL-2/TNF-α/IFN-γ and IFN-γ/IL-2) compared to patients with HHV-6 reactivation during the first year after HSCT (Table 18 and Figure 13**)**. For HHV-6 specific CD8+ T-cells, no significant differences were found between the two groups (with/without reactivation) in the first year after HSCT. Furthermore, in the second year after HSCT no significant differences in the frequencies of HHV-6 specific cells neither for CD4+ nor for CD8+ HHV-6 specific T-cells between the two groups (non-reactivated / reactivated) were observed.

Table 18: Frequencies (%) of HHV-6 specific CD8+ T-cells in patients with and without HHV-6 reactivation in the first year after HSCT. Table shows frequencies (%) as median (range) of CD4+ HHV-6-specific T-cells.

	Patients **in the first year after HSCT without HHV-6 reactivation**	Patients **in the first year after HSCT with HHV-6 reactivation**	p-value
HHV-6 CD4+ T-cells (IL-2/TNF-α/IFN-γ) (%), median (range)	1.44 (0.2-19.33)	0.98 (0-3.54)	0.048
HHV-6 CD4+ T-cells (IFN- γ/IL-2) (%), median (range)	2 (0.17-7.11)	0.99 (0-3.54)	0.022

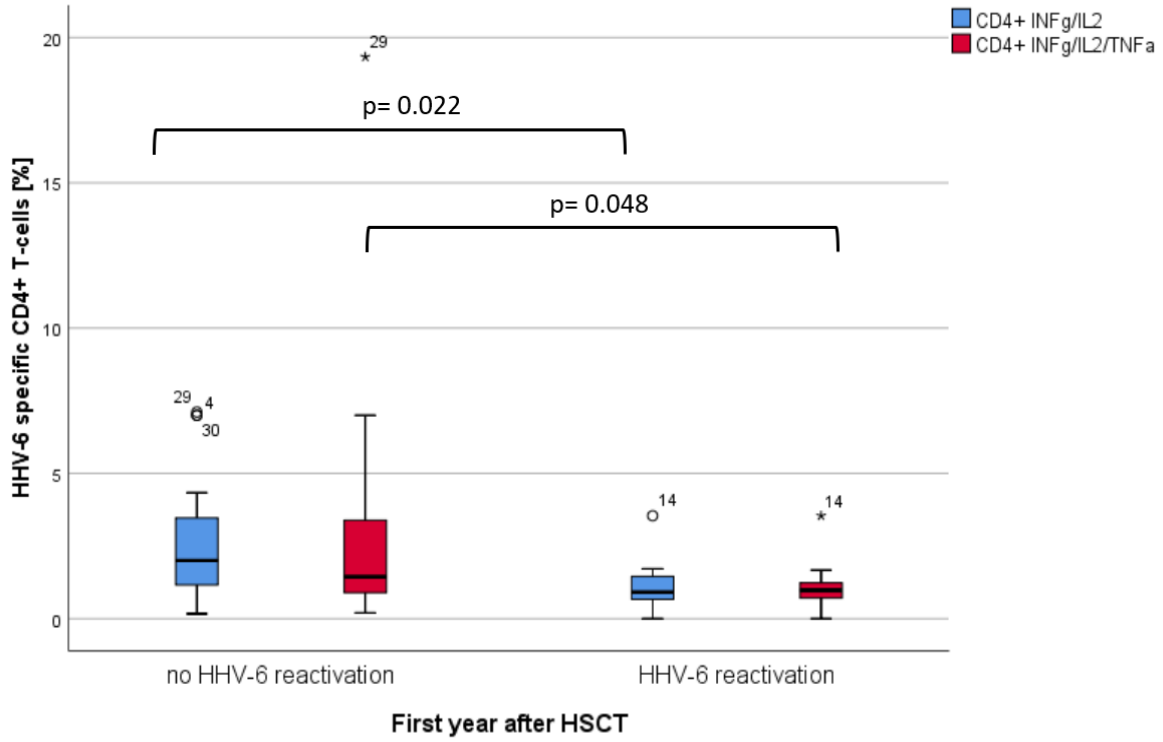

Figure 13: Frequencies of HHV-6 specific CD4+ T-cells (IFN-γ/IL-2 and IFN-γ/IL-2/TNF-α) in patients with and without viral reactivation during the first year after HSCT. Box plots show significantly higher frequencies of HHV-6 specific double (IFN-γ/IL-2) and triple (IFN-γ/IL-2/TNF-α) positive CD4+ T-cells in patients without HHV-6 reactivation in comparison to patients with HHV-6 reactivation, p= 0.022 and p= 0.048. The box plots display range, interquartile range and median.

Frequencies of HHV-6 specific T-cells in patients with HHV-6 reactivation during the first year after allogeneic HSCT compared to patients without HHV-6 reactivation during the 2 years after allogeneic HSCT

In order to find out if there are differences in the frequencies of HHV-6-specific T-cells within the two years after HSCT, we compared the frequencies of HHV-6-specific T-cells of patients with HHV-6 reactivation in the first year HSCT to the frequencies of those patients who did not reactivate HHV-6 within two years after HSCT.

Significantly higher frequencies of HHV-6 specific CD4+ T-cells (IL-2/TNF-α and IFN-γ/IL-2/TNF-α) in patients without virus reactivation two years after HSCT in comparison to frequencies of HHV-6 specific CD4+ T-cells (IL-2/TNF-α and IFN-γ/IL-2/TNF-α) of patients with HHV-6 reactivation during the first year after HSCT were observed (Table 19 and Figure 14**)**. For HHV-6 specific CD8+ T-cells, no significant differences were found between the two groups.

Table 19: Frequencies (%) of HHV-6 specific CD4+ T-cells in patients with HHV-6 reactivation during the first year after HSCT and patients without HHV-6 reactivation up to 2 years after HSCT. Table shows frequencies (%) as median (range) of CD4+ HHV-6-specific T-cells

	Patients with HHV-6 reactivation in the 1st year after HSCT	**Patients without HHV-6 reactivation up to two years after HSCT**	**p-value**
HHV-6 CD4+ T-cells (IFN-γ/IL-2) (%), median (range)	0.99 (0-3.54)	1.69 (0.17-7.11)	0.021
HHV-6 CD4+ T-cells (IL-2/TNF-α/IFN-γ) (%), median (range)	1.55 (0-4.22)	1.63 (0.38-10.17)	0.047

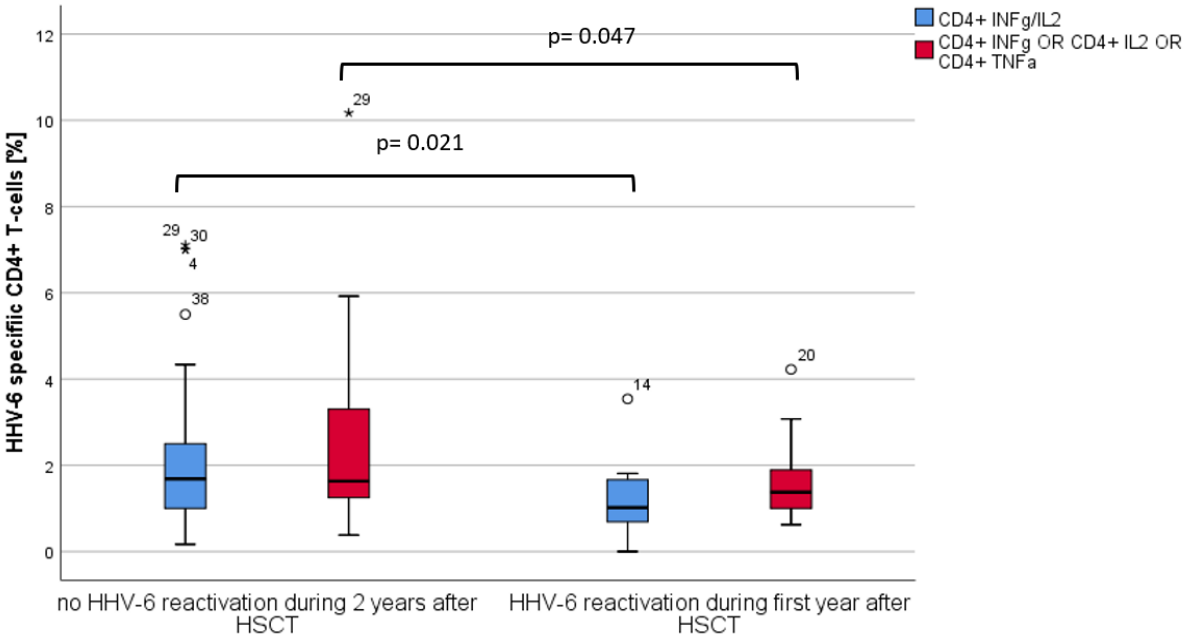

Figure 14: Frequencies of HHV-6 specific CD4+ T-cells in patients without HHV-6 reactivation up to 2 years after HSCT and frequencies of HHV-6 specific CD4+ T-cells in patients with HHV-6 reactivation during the first year after HSCT. Box plots show significantly higher frequencies of HHV-6 specific CD4+ T-cells (IL-2/TNF-α and IFN-γ/IL-2/TNF-α) in patients without virus reactivation after HSCT in comparison to frequencies of HHV-6 specific CD4+ T-cells (IL-2/TNF-α and IFN-γ/IL-2/TNF-α) of patients with HHV-6 reactivation during the first year after HSCT ($p = 0.021$; $p = 0.047$). The box plots display range, interquartile range and median.

6.2.7 Frequencies of HHV-6 specific T-cells in patients with HHV-6 reactivation during the 1st year after allogeneic HSCT compared to frequencies of HHV-6 specific T-cells of the control group

The frequencies of HHV-6 specific T-cells of the control group was compared to the different patient groups.

There was a significant difference in the frequencies of CD8+ specific HHV-6 T-cells (IL-2/TNF-α/IFN-γ) between patients with HHV-6 reactivation in the first year after HSCT and the control group (Table 20 and Figure 15).

For HHV-6 specific CD4+ T-cells, no significant differences were found between the patient groups and controls and no significant differences in the frequencies of HHV-6 specific T-cells neither for CD4+ nor for CD8+ HHV-6 specific T-cells between patients in the second year after HSCT and controls were observed.

Table 20: Frequencies (%) of HHV-6 specific CD8+ T-cells in patients with HHV-6 reactivation during the first year after HSCT and control group. Table shows frequencies (%) as median (range) of CD8+ HHV-6 specific T-cells.

	Patients with HHV-6 reactivation in the 1st year after HSCT	**Control group**	**p-value**
HHV-6 CD8+ T-cells (IFN-γ/IL-2/TNF-α) (%), median (range)	0.61 (0.02-2.8)	1.29 (0.6-7)	0.048

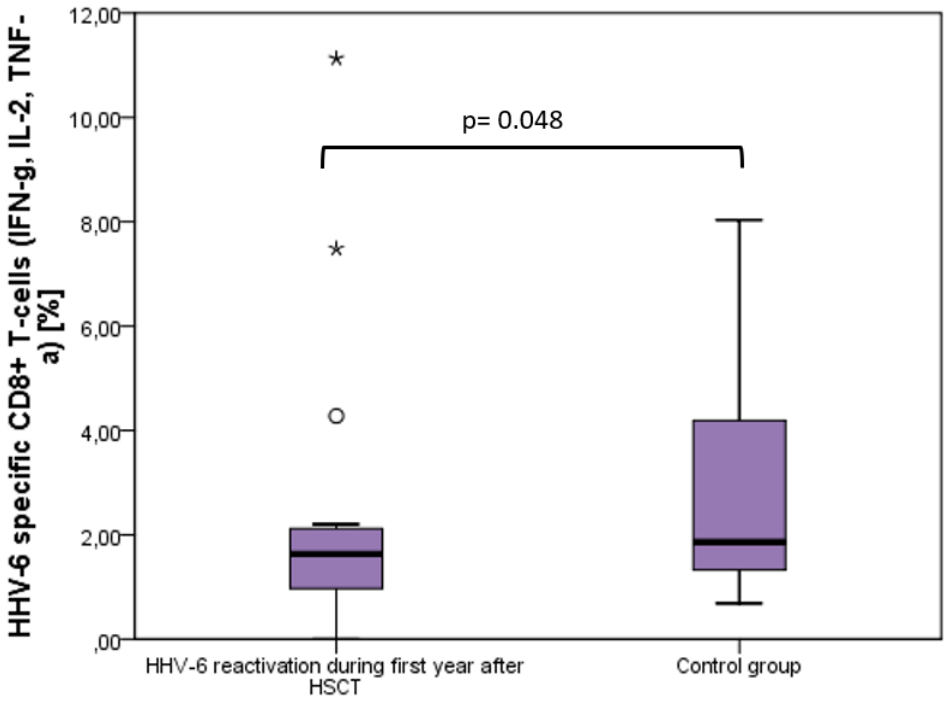

Figure 15: Frequencies of HHV-6 specific CD8+ T-cells (INF-γ/TNF-α/IL-2) in patients with HHV-6 reactivation during the first year after HSCT and the control group. Box plots show significantly higher frequencies of HHV-6 specific CD8+ T-cells (INF-γ/TNF-α/IL-2) in controls in comparison to patients with HHV-6 reactivation in the first year after allogeneic HSCT (p = 0.048). The box plots display range, interquartile range and median.

7. Discussion

T-cells, especially virus-specific T-cells constitute important effector cells in the control of viral infections.

In this project we have analysed secretion of IL-2, IFN-γ as well as TNF-α (single cytokines and in combination) in both CD4+ and CD8+ T-cells after stimulation with the HHV-6 specific antigen U54 in controls and patients after allogeneic HSCT.

We tested a short overnight stimulation of PBMCs with U54 antigen in the control group but could not detect an adequate antigen-specific response. Furthermore, we tested overnight stimulation with CMV lower matrix phosphoprotein 65 pp65, which is an analogue of U54 and could measure a CMV specific T-cell response. This protein is one of the most dominant CD4+ and CD8+ T-cell antigen of CMV and is commonly used for the evaluation of CMV-specific T-cell immunity (90). Fastenackels *et al*. showed that *ex vivo* T-cell reactivity in peripheral blood is detectable for HHV-6 (after stimulation of PBMCs for 40h), but at very low frequency and moreover found that HHV-6A- and HHV-6B-specific CD4+ T-cells are less differentiated than HCMV-specific T-cells. Despite strong virologic similarity of HHV-6 and HCMV they observed immunological differences in terms of the frequency and phenotype of effector and memory as well as regulatory virus-specific T-cells (106). Other studies also demonstrated that frequencies of T-cells recognizing HHV-6 are low and making identification and detection of these HHV-6 specific T-cell responses challenging (87).

Therefore, in order to obtain an adequate antigen-specific immune response we have cultured the cells in medium in the presence of interleukins (IL-7 and IL-2) for 10 days (107). After 10 days of cultivation we could observe an U54-specific response in individuals of the control group. These results proof the *in vivo* presence of HHV-6 specific T-cells that are capable of expansion after adequate *in vitro* stimulation and cultivation for a longer time which might be important for an adequate immune response. The role of IL-2 as a proliferation promoting factor (also called “T-cell growth factor”) for antigen-specific T-cells is important and IL-7 appears to be crucial for the formation and survival of memory T-cells. There is evidence that IL-7 contributes particularly to the proliferation of CD4+ memory T-cells (108,109).

A possible polyfunctionality of these virus-specific T-cells was determined by intracellular FACS analysis to detect IFN-γ, TNF-α and IL-2 production after stimulation with the HHV-6 specific antigen U54. The production of the typical TH1 cytokines such as IFN-γ, TNF-α and

IL-2 is a feature of highly active T-cells, which, in contrast to less active T-cells, are more potent in the control of virus-infected cells (110).

In first experiments, we analysed frequencies of virus-specific T-cells in individuals of the control group and found that individuals older than 10 years had significantly higher frequencies of HHV-6 specific CD8+ TNF-α secreting T-cells than those younger than 10 years of age. Accordingly, data analysis showed a positive correlation between the age of the individuals and frequencies of HHV-6 specific CD8+ TNF-α producing T-cells (86).

The results of the control group may demonstrate that immunity to HHV-6 could evolve over time. A primary infection occurs before the second year of life leading to a latent infection with HHV-6. In most cases antibodies persist throughout life (111). In parallel, the development of the HHV-6-specific T-cell response after primary infection permanently suppressing reactivation of latent virus infection must be regarded as a development of this response throughout life. This might be reflected by the observed higher frequencies of HHV-6 specific CD8+ T-cells secreting TNF-α in older children.

In the next step, we have analysed HHV6-specific T-cell immunity in patients up to 24 months after allogeneic HSCT. We, however, did not measure HHV-6 specific immunity at each time point (3, 6, 9, 12, 18 and 24 months after allogeneic HSCT) in all patients. As far as the sample size allows (51 measurements of HHV-6 specific CD4+ and CD8+ T-cell immunity in 28 patients), we made important observations: When analysing the HHV-6-specific T-cell immunity defined by positivity of at least one of the cytokines IFN-γ, TNF-α or IL-2 (“any cytokine”), we observed differences in the CD8+ and CD4+ T-cell immunity: the number of patients with HHV-6-specific CD4+ T-cell responses was higher than the number of patients with HHV-6-specific CD8+ T-cell responses. We also observed that most patients had no measurable HHV-6 specific T-cell immunity three months after allogeneic HSCT. The delayed memory T-cell response at 6 months or even 9 months after HSCT does not reflect exclusively the process of ongoing immune reconstitution but might probably be in part due to the impaired capacity of T-cells to proliferate due to the immunosuppressive therapy in the majority of patients after allogeneic HSCT, as was also observed for EBV and HCMV specific T-cells (112,113). In another study of Quintela *et al.* similar results in terms of general T-cell recovery were found. In this study a significantly slower CD8+ T-cell than CD4+ T-cell recovery during the first 6 months after allogeneic HSCT was observed (114). Another study reported a significant lower overall CD8+ T-cell count at 6 months after

allogeneic HSCT in 29 HHV-6-infected patients when compared with non-infected patients (115).

Furthermore, we have analysed if there are differences between frequencies of HHV6-specific T-cells and parameters such as stem cell source (PBSC or BM), conditioning type (RIC or MAC) and manipulation of the donor cells (unmanipulated vs. α/β/CD19+ depleted T-cells and CD34+ enriched graft). We have only observed significant higher frequencies in the HHV6-specific response for CD8+ T-cells secreting IL-2 in patients with unmanipulated donor cells compared to patients with administered α/β/CD19+depleted T-cells and CD34+ enriched donor graft in the first year after HSCT. This is in line with early studies comparing immune reconstitution in modified and unmodified grafts showing a significantly delayed CD3+, CD4+ and CD8+ T-cell reconstitution in recipients of T-cell depleted grafts, increasing the risk of infections (116,117). However, it is also important to note that multiple factors, as the presence of an acute or chronic GvHD as well as immunosuppressive drugs can also influence immune reconstitution in patients after allogeneic HSCT. Due to the rather small cohort, a multivariate analysis of our data seemed not feasible.

After comparing different clinical parameters and frequencies of HHV-6 specific T-cells we investigated differences in frequencies of HHV-6 specific T-cells in patients in the 1st and 2nd year after allogeneic HSCT in order to compare frequencies in the earlier and the later phase after allogenic HSCT. Furthermore, we wanted to investigate if there are differences in the frequencies of HHV-6 specific T-cells in patients with or without (detectable) HHV-6 reactivation after allogeneic HSCT and finally, we compared the frequencies of the patient groups (with/without HHV-6 reactivation and 1st and 2nd year after allogeneic HSCT) with those of the control group.

First, we identified significant higher frequencies of triple positive CD8+ T-cells (IL-2/TNF-α/IFN-γ) and double positive CD8+ T-cells (IL-2/TNF-α) in patients in the 2nd year after HSCT compared to patients in the 1st year after HSCT. However, we could only observe significant differences for CD8+ HHV-6 specific T-cells, not for CD4+ HHV-6 specific T-cells. As mentioned before, this might be additionally caused by immunosuppressive therapy during the first months after HSCT. (118). Lymphopenia leads to reduced T-cell protection against HHV-6 and other (herpes-) viruses and can be causative for viral reactivation (119).

When comparing the patient groups and the control group we observed that patients during the first year after HSCT had also significant lower frequencies of CD8+ T-cells (TNF-α)

compared to the control group while no differences in the frequencies of HHV-6 specific T-cells (neither CD4+ nor CD8+) between the patients in the second year after HSCT and the control group were observed. These results might show that patients after allogeneic HSCT slowly develop an HHV-6 specific T-cell immune response over time and regain a comparable immune response to HHV-6 as the individuals in the control group.

This is also in accordance with the development of the HHV-6-specific T-cell response throughout life and latent HHV-6 infection. The interplay between latent HHV-6 infection and immunological response to the virus promotes the development of HHV-6 specific T-cell immunity. Particularly, virus-specific CD8+ T-cells play an important role as they get activated after contact with virus antigens and differentiate into effector T-cells that are necessary to fight against virus-infected cells (89).

Most patients after allogenic HSCT still have a weak T-cell immunity and lower frequencies of HHV-6 specific T-cells than patients in a later phase after allogeneic HSCT. In the first months after allogeneic HSCT viral reactivations are common and virus-specific T-cell reconstitution and expansion depends on the transfer of virus-specific T-cells within the donor graft and the resident antigen-specific T-cells (44).

To evaluate possible associations between HHV-6 specific T-cell immunity and HHV-6 reactivation, we also compared frequencies of patients with/without HHV-6 reactivation and frequencies of the control group.

We found that in the 1st year after allogeneic HSCT patients without (detectable) reactivation had significant higher frequencies of CD4+ specific HHV-6 T-cells (IL-2/TNF-α/IFN-γ and IFN-γ/IL-2) compared to patients with HHV-6 reactivation during the 1st year after HSCT. For HHV-6 specific CD8+ T-cells, no significant differences were found between patients with/without HHV-6 reactivation in the 1st year after HSCT.

Moreover, when we compared the frequencies of HHV-6 specific T-cells in patients with HHV-6 reactivation during the 1st year after allogeneic HSCT to all patients without HHV-6 reactivation up to 2 years after allogeneic HSCT we also found significantly higher frequencies of HHV-6 specific CD4+ T-cells (IL-2/TNF-α and IFN-γ/IL-2/TNF-α) in patients that did not reactivate HHV-6 during follow-up.

The observed differences between patients with and without (proven) HHV-6 reactivation in the 1st year after HSCT may reflect a well-developed HHV-6 specific CD4+ T-cell immunity

(in terms of higher frequencies and immunologically active CD4+ T-cells) that protects against (clinically relevant) HHV-6 reactivations.

Interestingly, in the 2nd year after HSCT there were no significant differences observed in the frequencies of HHV-6 specific T-cells neither for CD4+ nor for CD8+ between patients with and without HHV-6 reactivation. As we could not observe any significant differences between patients with and without HHV-6 reactivation in the second year after allogeneic HSCT we assume that at a later phase after allogeneic HSCT the cellular immunity against HHV-6 has developed in most patients although few patients reactivated HHV-6 during the 2nd year after allogeneic HSCT.

Finally, we also wanted to investigate if there are significant differences between the patients (with and without HHV-6 reactivation) and the controls. We compared the frequencies of HHV-6 specific T-cells of the control group and different patient groups and found significant lower frequencies of HHV-6 specific CD8+ T-cells (IL-2/TNF-α/IFN-γ) in patients with HHV-6 reactivation in the 1st year after HSCT than in individuals of the control group while patients without HHV-6 reactivation did not show any significant differences to the control group. An HHV-6 reactivation due to immunosuppression therapy during the first months after allogeneic HSCT might lead to a dysregulation of the early T-cell immune reconstitution.

The knowledge about HHV-6 specific T-cell immunity and about the role of specific viral antigens has not been exhaustively researched. The first HHV-6 antigens were identified by Nastke *et al*. in 2012 (87) and a short time later, HHV-6 specific CD8+ T-cells, as an important part of the immune repertoire, were analysed (88). Further studies also demonstrated the presence and function of HHV-6 specific CD8+ T-cells (100,120). Today, there are a few research groups addressing HHV-6 specific CD8+ T-cell response. In a previous published work of Martin *et al.* a virus-wide analysis of CD8+ T-cell responses to HHV-6 was undertaken (121). Despite a few interesting studies, HHV-6 immunity in HSCT patients and healthy individuals is still poorly understood. However, the fact that HHV6-specific T-cells can be cultured and expanded is promising for future immunotherapy in patients after allogeneic HSCT (122).

Cellular immunotherapy provides a way to support the patient´s immune system. In adoptive T-cell transfer, pathogen-specific T-cells of the stem cell donor (or a third-party donor) are isolated from peripheral blood and re-infused into the patient (68). Donor-derived virus-

specific T-cells against different viral antigens in patients after HSCT were administered against different viral reactivations with remarkable clinical results (122–125). Understanding the immune response to HHV-6 is important for the development of immunotherapies in patients after allogeneic HSCT or other immunocompromised patient groups. Virus-specific T-cells may constitute an alternative to antiviral agents for allogeneic HSCT recipients (126). Until the patient´s immune response against HHV-6 has been reconstituted during the first 2 years after allogeneic HSCT (as shown by our data), adoptive transfer of virus-specific T-cells may be a strategy to restore the antiviral T-cell immunity of the recipient (70).

There are some limitations to this study: We did not measure HHV-6-specific immunity at every time point during follow-up in each patient, making it difficult to assess the development of HHV-6 T-cell immunity in each patient after allogenic HSCT. Unfortunately, the sample size of patients that were included is rather small. However, if possible, we tried to include every suitable patient after allogeneic HSCT in this study.

Another methodical limitation is the 10-day expansion of PBMCs in cell culture and the addition of IL-2 and IL-7 which promotes homeostatic expansion of memory T-cells and in parallel decreases the size of the overall T-cell pool. Furthermore, we have only characterized the T-cell responses to the viral tegument protein U54.

Despite a rather heterogenic cohort in terms of age, underlying diseases, conditioning regimes, stem cell source and manipulation, a multivariate analysis of our data considering all these factors seemed not feasible due to the rather small size of the cohort.

In the future, it would also be interesting to use cytotoxicity assays in advanced cell culture experiments in order to proof the function of HHV-6 specific T-cells.

In conclusion, this present work contributes to characterization of HHV6-specific T-cell immune reconstitution in patients (with and without HHV-6 reactivation) after allogeneic HSCT and in healthy individuals. We could show significant differences in frequencies of HHV-6 specific T-cells in patients in the 1st year after HSCT compared to patients in the 2nd year after HSCT, and significant lower frequencies of CD8+ T-cells (TNF-α) compared to the control group were identified in the 1st year after allogeneic HSCT. Patients without (detectable) HHV-6 reactivation in the 1st after HSCT had significant higher frequencies of HHV-6 specific CD4+ T-cells compared to patients with HHV-6 reactivation during the 1st year after allogeneic HSCT.

A better understanding of the functions of different virus-specific T-cells recognizing HHV-6 antigens might allow the development of new methods for generating these virus-specific T-cells. However, beside these methodological advances, it is also important to investigate and support the patient´s own immune reconstitution.

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10. Supplemental material

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FOLGEVOTUM

gültig bis 14.03.2019

EK-Nummer:	26-178 ex 13/14
Studientitel:	Cellular Immune Reconstitution against Human Herpesvirus Type 6 (HHV-6) in Children and Adolescent after allogeneic Stem Cell Transplantation
Prüfer:	Doz.Dr. Volker Strenger Univ.Klinik für Kinder- und Jugendheilkunde
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Die o.a. Studie wurde von der Ethikkommission erstmals im 'expedited Review' am 04.02.2014 behandelt. Die Ethikkommission ist zu folgendem Schluss gekommen:

Es besteht kein Einwand gegen die Durchführung der Studie in der vorliegenden Form.

Kommissionsmitglieder, die für diesen Tagesordnungspunkt als befangen anzusehen waren und daher gemäß Geschäftsordnung an der Entscheidungsfindung und Abstimmung nicht teilgenommen haben: keine

Zur Beurteilung vorliegende Dokumente:

Dokumente eingegangen am 14.01.2014, begutachtet im 'expedited Review' am 04.02.2014	
✓ Antragsformular ECS	14.01.2014
✓ Originalprotokoll HHV-6 nach SCT Protokoll 1.0	09.01.2014
Dokumente eingegangen am 14.01.2014, begutachtet im 'expedited Review' am 04.02.2014	
Informed Consent Form Kinder 8 - 14 Jahre 1.0	09.01.2014
Informed Consent Form Eltern	10.01.2014
Informed Consent Form Jugendliche 14 - 18 Jahre 1.1, 12/2013	
✓ CV Prüfer September 2013	
Dokumente eingegangen am 20.01.2014, begutachtet im 'expedited Review' am 05.02.2014	
✓ Antragsformular ECS Unterschriftenseiten	16.01.2014
Dokumente eingegangen am 28.02.2014 (in der nächsten Begutachtung mitbegutachtet)	
✓ Cover Letter	27.02.2014
✓ Antragsformular undatiert	
✓ Informed Consent Form 1.2	27.02.2014
✓ Informed Consent Form ICON Jugendliche 14 - 18 Jahre 1.2	27.02.2014
✓ Informed Consent Form Eltern 1.2	27.02.2014
✓ Sonstiges: Stellungnahme Änderungen Antragsformular undatiert	
Dokumente eingegangen am 06.03.2014 (in der nächsten Begutachtung mitbegutachtet)	
✓ Sonstiges: Beschreibung Aufgabengebiet der Dissertantin	03.03.2014
Dokumente eingegangen am 11.03.2014, begutachtet im 'expedited Review' am 14.03.2014	
✓ Informed Consent Form Kinder 8-14 Jahre 1.2	11.03.2014

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✓ Originalprotokoll 1.1	22.04.2015
✓ Protokoll Amendment	22.04.2015
✓ Zwischenbericht	24.04.2015
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✓ Zwischenbericht	06.04.2016
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✓ Zwischenbericht	13.12.2017
Dokumente eingegangen am 27.03.2018, begutachtet im 'expedited Review' am 04.04.2018	
✓ Zwischenbericht	21.03.2018

Datum Erstvotum: 14.03.2014

Die Ethikkommission geht - rechtlich unverbindlich - davon aus, dass es sich um keine klinische Prüfung nach AMG bzw. MPG handelt.

Es handelt sich um eine Studie im Rahmen einer Dissertation.

Das Votum der Ethikkommission berührt in keiner Weise die alleinige Verantwortung der Prüferin / des Prüfers / der Prüfer für die ordnungsgemäße Durchführung der Studie unter Einhaltung aller einschlägiger gesetzlicher Bestimmungen und Richtlinien.

Weiters machen wir darauf aufmerksam, dass der Kommission unverzüglich zu melden sind:

- Abweichungen vom Protokoll aus Sicherheitsgründen oder Protokolländerungen

- Änderungen, die das Risiko der Teilnehmer/-innen erhöhen oder die Durchführung der Studie wesentlich beeinflussen

- Mutmaßliche unerwartete schwerwiegende Nebenwirkungen - SUSARs (AMG-Studien ab 1.5.2004) oder schwerwiegende unerwünschte Ereignisse - SAEs (andere Studien)

- Jegliche Information über sonstige Umstände, die die Sicherheit der Teilnehmer/-innen oder die Durchführung der Studie beeinträchtigen können

Graz, 04. April 2018